

**HAPTOGLOBIN GENOTYPIC POLYMORPHISMS AND
LONGEVITY AMONG THE GHANAIAN POPULATION IN THE
GREATER ACCRA REGION**

SELIKEM ABLA NUWORMEGBE

(10357524)



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DECLARATION

I, Selikem Abla Nuwormegbe, of the Department of Medical Biochemistry, University of Ghana Medical School, do hereby declare that, with the exception of references to other works, this thesis is the outcome of my own research work under the supervision of Dr Bartholomew Dzudzor of the Department of Medical Biochemistry, University of Ghana Medical School and Dr Charles Brown of the Department of Medical Laboratory Sciences, School of Allied Health of the University of Ghana.

Signature.....

SELIKEM ABLA NUWORMEGBE

(Student)



Date.....

Signature.....

DR. BARTHOLOMEW DZUDZOR

(Supervisor)

Date.....

Signature.....

DR CHARLES BROWN

(Supervisor)

Date.....

DEDICATION

Even the mystery which had been hid from ages and from generations, but now is made manifest to his saints; which is Christ in you, the hope of glory.

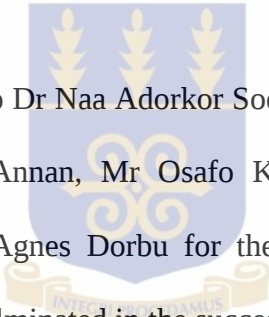
To the Almighty God for His grace that is always sufficient for me, my mother Grace Tornyeviadzi, my late father Stephen Awuku Nuwormegbe and my sibling Kwesi, Seyram and Seyenam for the love and support.



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The logo of the University of Ghana is a shield-shaped emblem. It features three golden wheat stalks at the top, a central golden sun-like symbol with rays, and a banner at the bottom with the motto 'IN DEUS NOBIS' in Latin. The shield is blue with golden elements.

I am also extremely grateful to Dr Naa Adorkor Sodzi Tetteh, Mr Emmanuel Allotey, Mr Hope Azasu, Miss Dorcas Annan, Mr Osafo Kantanka, Miss Dorothy Honny, Mr Christian Adabor and Miss Agnes Dorbu for the diverse and enormous support and encouragement which have culminated in the successful completion of this work.

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Last but not at all least, my appreciation also goes to the entire staff of the Medical Biochemistry Department, UGMS, for their support.

LIST OF ABBREVIATIONS

Bp	Base pair
COX	cyclooxygenase
Datp	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymidine triphosphate
dNTPs	Deoxynucleotide triphosphates
DNA	Deoxyribonucleic acid
DMSO	Dimethylsulfoxide
EDTA	Ethylenediaminetetra acetic acid
HCl	Hydrochloric acid
H ₂ O ₂	Hydrogen peroxide
LDL	low density lipoprotein
•OH	hydroxyl radical
Hp	Haptoglobin
Hb	Hemoglobin
HN	Hydroxynonenal
IL	Interleukin
8-oxodG,	8-oxoguanosine
ETC	Electron transport chain
MgCl ₂	Magnesium chloride



MDA	Malondialdehyde
MSLP	Maximum lifespan potential
MtDNA	Mitochondrial DNA
nDNA	Nuclear DNA
NO•	Nitric oxide radicals
O ₂ ⁻	Superoxide
OR	Odds ratio
PBS	Phosphate buffered saline
PGE	Prostaglandins
PCR	Polymerase chain reaction
Rpm	Revolutions per minute
RBC	Red blood cells
ROS	Reactive oxygen species
SD	Standard deviation
SPSS	Statistical Package for Social Sciences
SOD	Super oxide dismutase
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
T _H	T-helper cells
Taq	Thermus aquaticus
μl	Microlitre
WBC	White blood cells

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ABSTRACT

Background: Haptoglobin (Hp), an acute phase glycoprotein with antioxidant, anti-inflammatory and immune-modulatory functions may be an excellent candidate gene to investigate human longevity. Human Hp is polymorphic and these polymorphisms have been associated with different functional capacities of the proteins expressed as a result of their distinct biochemical and biophysical properties.

Aim: To determine the possible role of haptoglobin genotypes as genetic markers for longevity.

Methods: The study which was of cross-sectional design recruited 133 individuals aged 50 years and above. Blood samples were collected from participants for haematological analysis, haptoglobin genotyping by allele specific PCR and determination of oxidative stress by determining levels of reactive oxygen species by superoxide dismutase assay and oxidative DNA damage by comet assay.

Results: A significant positive correlation between SOD activity and age in the study population (p -value of 0.002) was observed while a significant negative correlation was observed between age and total white blood cells, neutrophil and platelet counts (p -values of 0.020, 0.028 and 0.006 respectively). The HP1 and HP2 allelic frequencies were found to be 49.5 % and 50.5% respectively while the genotypic frequencies were 38%, 25% and 37% for HP 2-2, HP 2-1 and HP 1-1 respectively and showed a departure from the Hardy-Weinberg equilibrium. Comparison of the distribution of haptoglobin genotypes among age categories showed no significant association ($p = 0.700$). SOD activity in the

study population was not significantly different when compared among the genotypes ($p = 0.877$). Extensive DNA damage/fragmentation was observed in the study population. The degree of damage among the genotypes however was similar.

Conclusion: The HP1/2 genotypic polymorphisms did not influence longevity in the Ghanaian population and no genotype conferred a survival advantage.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Aging in humans is characterized by a decline of multiple physiological functions in various cells and organs leading to an increased propensity to death. It is a major risk factor in several human diseases such as cancer, diabetes, osteoporosis, cardiovascular diseases, neurodegenerating conditions such as Alzheimer's and Parkinson's disease, etc. (Martin, 2011). The decline of physiological functions with age is attributed to molecular and cellular changes over time as well as a decrease in cell numbers which lead to less effective homeostatic mechanisms, and a decrease in the ability of cells to step up activity when challenged or stressed, thus increasing the vulnerability of the organism to its environment (Perez and Sierra, 2009).

Longevity, defined as age at death is a complex phenotype and substantial evidence suggest that it is influenced by a complex interplay of both genetic and non genetic (environmental) factors (Perls *et al.*, 2002). Studies of human populations with optimal environments and health-related behaviours suggest the average set of genetic variations should facilitate the average person's ability to live to about eighty five (85) year. However, average life expectancies are lower than this because individuals generally fight survival advantage with bad health habits that can lead to premature aging, chronic

illness, and death at a significantly younger age (Perls *et al.*, 2002). The successful study of the genetic mechanisms influencing lifespan in lower organisms laid the foundation for studies into corresponding genes in humans. Research conducted on *Caenorhabditis elegans*, *Saccharomyces cerevisiae* and *Drosophila melanogaster* revealed genetic variants that lead to lifespan extension in these lower animals (Kenyon *et al.*, 1993; Guarente and Kenyon, 2000; Patridge and Gem, 2002). One such gene identified in *Saccharomyces cerevisiae* is Sir2, (Kaeberlein *et al.*, 1999) and its homolog the SIRTUIN gene has been identified in humans to be associated with physiological roles such as stress response, aging and regulation of metabolism (Haigis and Guarente, 2006; Finkel *et al.*, 2009).

Research into the genetic contribution to longevity in human was done using twin registries and population-based samples. Results from twin registries range between 20% and 30% whereas those from population-based samples are slightly lower, ranging between 15% and 25% (Murabito *et al.*, 2012). The genetic influences on human lifespan become particularly important for survival after age sixty (Hjelmborg *et al.*, 2006). Studies comparing centenarians to average-aged individuals found polymorphisms in genes that are associated with long life such as *APOE* gene that codes for apolipoprotein E, an anti-atherogenic protein (Christensen *et al.*, 2006) and *FOXO3A* gene (Wilcox *et al.*, 2008) which regulates metabolism, cellular proliferation, stress tolerance and lifespan (Flaschbart *et al.*, 2009). These results have been replicated many times in other populations (Murabito *et al.*, 2012). However, the associations found in humans account for only a small percentage of the variation in lifespan therefore aging is most likely a

highly polygenic trait with each gene having a moderate effect on the longevity phenotype (Herskind *et al.*, 1996).

Many other candidate gene associations with longevity studied have not been replicated in additional populations (Christensen *et al.*, 2006; Murabito *et al.*, 2012) necessitating the need for further research into other candidate genes. Haptoglobin (Hp), an acute phase protein produced mainly by the hepatocytes of the liver, is a non-enzymatic antioxidant which binds free, cell-toxic haemoglobin (Hb) due to intravascular haemolysis thus preventing iron stimulated formation of reactive oxygen species or free radicals (McCormick and Atassi, 1990; Gutteridge, 1987). The free radical theory of aging suggested by Denham Harman in 1956 is the most popular aging theory which proposes one of the causes of aging as oxidative stress caused by reactive oxygen species (ROS). The targets of these free radicals are lipids, nucleic acids and proteins, leading to autoxidation of lipids, cross-linking of proteins and nucleic acids, altered cell membrane properties and peptide fragmentation (Harman, 1956). Hp also has anti-inflammatory (Jue *et al.*, 1983; Komoriya *et al.*, 1980), angiogenic (Cid *et al.*, 1993) and immune-modulatory (Arredouani *et al.*, 2003; Guetta *et al.*, 2007) functions in extravascular tissues and body fluids and may thus represent a candidate gene to investigate lifespan expectancy. Furthermore, haptoglobin has been documented to interact with other longevity genes such as APOA1 (Spagnuolo *et al.*, 2005) and APOE (Cigliano *et al.*, 2009). Genetic polymorphism within the human haptoglobin gene has been associated with a variety of different functional capacities, susceptibility to and outcome of several human pathologies due to their distinct biochemical and biophysical properties (Langlois

and Delanghe 1996; Sadrzadeh and Bozorgmehr 2004). Studies carried out in the central Italian population concerning Haptoglobin genotypes and longevity revealed that the Hp1-1 genotype is associated with increased probability of young subjects to attain increased lifespan. On the other side, carriers of Hp2 -2 and Hp2-1 alleles displayed an overall significant disadvantage in reaching old age (Napolioni *et al.*, 2011).

Demographic details indicate that the rate of growth of the aged population has greatly exceeded the rate of growth of the population as a whole with estimates that the number of people aged sixty and above globally will increase from 1 in 10 currently to 1 in 5 by 2050 (United Nations, 2001). With this increase in aged populations is the increased incidence of age-associated diseases and its attendant economic burden. The need to preserve the health of the aged is therefore much greater now so as to make them healthier, happier and more productive.

Leonard Hayflick, a respected authority in the field of aging studies at the University of California School of Medicine, San Francisco, in his article – ‘Commentary: The Future of Aging’, stated that “*Biogerontologists have an obligation to emphasize that the goal of research on aging is not to increase human longevity regardless of the consequences, but to increase active longevity free from disability and functional dependence*” (Hayflick, 2000).

1.2 Problem statement

The aging process is accompanied by a decline in functionality which imposes a huge impact on society, both socially and economically. According to the United Nations, the number of people aged 60 years or older will increase globally from 1 in 10 currently, to 1 in 5 by 2050, with the ratio of people aged 65 and above to those aged between 15 to 64 tripling in developing countries (United Nations, 2001).

Intrinsic biological aging is the major risk factor for virtually all of the major diseases including Alzheimer's disease, Parkinson's disease, Lewy body dementia, frontotemporal dementias, strokes, peripheral neuropathies, age-related macular degeneration, ocular cataracts, presbycusis, type 2 diabetes mellitus, osteoporosis, osteoarthritis, sarcopenia, all forms of arteriosclerosis, benign prostatic hyperplasia, and most types of cancer (Martin, 2011). The increasing aged population will thus be accompanied by increasing incidence of these age-associated diseases decreasing the quality of life of the aged, and its attendant strain on the national budget for the health sector. Family members taking care of their aged and sick relatives become less productive impacting negatively on the general growth of the economy. The emotional and psychological effects on care givers can also be very devastating especially in our part of the world where facilities and trained staff are not available to cater for the aged.

Despite increasing efforts, the precise mechanisms responsible for the aetiology of the aging phenomenon are not fully understood (Singh and Newman, 2011). Results from several longevity genes studied in populations of different origins have been conflicting

(Christensen *et al.*, 2006) thus necessitating the need for research into other candidate genes.

There is little or no information on the aged in Ghana much less genetic data on the possible inheritance of longevity in the Ghanaian population and no work has been done so far on the genetic polymorphism of the haptoglobin gene in the aged population of Ghana.

1.3 Justification of studies

Aging is a natural concomitant of life thus there is the need to properly understand the aging phenomenon so as to be able to provide the needed interventions to make the aged live a more healthy and productive life i.e. age successfully.

The aged have accumulated experience which is of immense benefit to the young. Furthermore, they should be able to look forward to a future, regardless of their age. They should also be able to continue to work and give to the community benefits of their skills and expertise. The more the aged employ their physical and intellectual capabilities, the greater likelihood they have of living even longer. The best way of intervening to help the aged medically lies in understanding the mechanisms of aging in both general and specific terms. Such knowledge will help in identifying individuals who are likely to develop certain age-associated diseases so as to put them on early preventive mechanisms or develop interventions which can delay the onset of these conditions. Unavailability of

such data in Ghana thus leaves the aged population with interventions developed from researches from other parts of the world which may not be suitable or effective.

Current demographic details contained in the International Population Reports of 2008 titled 'An Aging World' indicate that the number of aged in the world continues to increase in both absolute and relative terms and will for the first time outnumber children in less than ten year. The estimated change in the total size of the world's older population between July 2007 and July 2008 was found to be more than 10.8 million making an average of 870,000 each month with developing countries contributing 81% to this monthly average increase. Figures in the report on Ghana indicate the aged population 65 years and above range between of 3.0% - 4.9% of the entire population and is projected to increase to 5.0% – 10.9% by the year 2040. Aging research is therefore of much greater importance now since knowledge acquired will help boost the probability that the increased lifespan will be years of activity, free from disability.

1.4 General aim

The study aimed at determining the possible role of Hp genotypes as genetic markers for longevity.

1.5 Specific objectives

These are to

- Determine the proportions of Hp alleles and the distribution of the genotypes among the aged population in the Greater Accra region.
- Determine the levels of ROS in the erythrocytes of the study population.
- Determine the relationships between the Hp genotypes and levels of the ROS in the study population.
- Determine and compare oxidative DNA damage using comet assay in the white blood cells of the study population in relation to haptoglobin genotypes.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Aging in humans

Aging and longevity vary among humans. While some humans live to age hundred years and beyond, most others do not. The rate of aging also varies such that people become increasingly different from each other with age. Thus, while some individuals look frail, others appear fit at 70 years old. Again, different organs and systems age at different rates and in different ways in humans (Wheeler and Kim, 2010). Chronological age, which is age from the time of birth to the point of reference, thus fails to provide an accurate indicator of the aging process. Physiological age which is a gauging of the degree of growth or deterioration of an individual with regards to functional norms for multiple different body systems rather serves as an indicator of an individual's general health status and may also serve as an indicator of remaining healthy lifespan (Borkan and Norris, 1980). Measuring the changes that occur in different tissues with chronological age in a population results in the identification of biomarkers that can be used as indexes of physiological age. Such biomarker can determine if an individual is physiologically younger or older than his or her chronological age (Karasik *et al.*, 2005). Determining genes and pathways associated with the measurement of physiological age can reveal molecular processes which are important for the aging of particular tissues. For example, transcriptional and genetic association studies of different tissues have revealed common and unique pathways involved in human aging (Wheeler and Kim, 2010).

2.2 Biomarkers of aging

These are physical and chemical properties which manifest in the human body to indicate that the body is aging. A true biomarker should satisfy the criteria by the High Tech Bio-Medical Technologies for disease treatment and life extension experimental and clinical data (www.anti-aging-guide.com). These criteria are

- It must predict the rate of aging and be a better predictor of life span than chronological age.
- It must be able to be tested on a regular basis.
- It must work both for humans and other species, such as laboratory animals.
- There is support from human clinical assessment and complementary research studies.
- The studies are based on a significant representative sample.
- The result has a clear association with aging.
- A relatively narrow standard deviation is present.

The best markers are not susceptible to influence from the external environment. Currently about twenty four (24) factors meet the criteria and include 17-ketosteroid/17-hydroxycortio steroid ratio(male), handgrip strength, ascorbic acid, hemoglobin A1C, basal metabolic rate, lung capacity- FEV1, blood pressure- pulse, lung capacity- FVC, blood pressure- systolic, maximum oxygen uptake (male), body Mass Index (female), near vision, caries index, noradrenaline- plasma (male), creatinine clearance, periodontal index, PSA total (male), fibrinogen, skin elasticity, hair baldness (male), testosterone free

(male), hair grayness and serum zinc (www.anti-aging-guide.com). In addition, there are a number of other factors which may be partially considered biomarkers of aging because their reliability has not been confirmed through a sufficient amount of clinical and experimental data. They include body flexibility, blood urea nitrogen, LDL cholesterol, melatonin levels, static balance, serotonin levels etc (www.anti-aging-guide.com).

2.3 Demographics

Globally, age sixty is regarded as the lower chronological age threshold for the elderly. However, age fifty is increasingly being used by African gerontologists (WHO, 2000) who argue that life expectancy at birth in Sub Saharan Africa is typically ten or more years lower than in the developed regions, and that the social construction of old age are set at a younger age (Kinsella and Phillips, 2005).

Current demographic details indicate that the rate of growth of the elderly population has greatly exceeded the rate of growth of the population as a whole. According to the United Nations, the number of people worldwide aged sixty and above will increase from 1 in 10 currently to 1 in 5 by 2050 (United Nations, 2001). The International Population Reports of 2008 titled 'An Aging World' indicates that the population of the aged will for the first time outnumber children in less than ten year. The estimated change in the total size of the world's older population between July 2007 and July 2008 in the report was found to be more than 10.8 million making an average of 870,000 each month. Figures in the report on Ghana indicate the aged population who are 65 years and above are between

3.0% - 4.9% of the entire population and is projected to increase to 5.0% – 10.9% by the year 2040.

2.4 Theories of aging

Aging is a complex phenotype hence several theories have emerged to explain the aging process due to the absence of firmly established primary causes. These theories are classified based on the level at which the aging mechanism is targeted (Jayanthi *et al.*, 2010). They are:

- The evolutionary theories which comprise the mutation accumulation theory, the antagonistic pleiotropy theory, the programmed death theory and the disposable soma theory.
- The systemic theories which consist of the neuroendocrine theory, the rate-of-living theory and the immunologic theory
- The cellular/molecular theories comprising the error catastrophic theory, the free radical theory, the waste product theory, the telomere theory, and the somatic mutation theory.

The free radical theory which was suggested by Denham Harman in 1956 is however the most popular aging theory because of the plausible explanations it provides for the aging phenomenon.

2.4.1 The free radical theory

This theory proposes one of the causes of aging as oxidative stress caused by oxygen free radicals or reactive oxygen species (ROS) produced mainly in the mitochondria during aerobic respiration (Harman, 1972). The theory is based on the chemical nature of these free radical reactions and their ubiquitous prominent presence in living systems. It predicts that lifespan can be increased by slowing down the rate of initiation of random free radical reactions by dietary restriction and decreasing body temperature and/or decreasing their chain lengths by increasing the concentrations of free radical reaction inhibitors in the body, or by increasing the resistance of body constituents to free radical attack (Harman, 1991). He enumerated the following works which further support his free radical theory of aging:

- Studies of the origin and evolution of life,
- Studies of the effect of ionizing radiation on living things,
- Dietary manipulations of endogenous free radical reactions,
- The growing number of studies that implicate free radical reactions in the pathogenesis of specific diseases e.g. cancer and atherosclerosis.

Several studies into Mitochondrial DNA (mtDNA) and aging reviewed by (Alexeyev *et al.*, 2004) and specifically, the detection of 8-oxodG (7,8-dihydro-8-oxoguanine), a mutagenic DNA base lesion caused by oxidative stress in mtDNA isolated from the livers of both rats and mice (Sohal and Dubey, 1994; Hamilton *et al.*, 2001) further confirms

that progressive accumulation of oxidative DNA damage contributes to age related diseases and the aging process.

Genetic studies in worm, fly, and mouse have also linked stress resistance or reduced free radical production with increased lifespan. Research conducted on mutant strains of *C. elegans* that are resistant to oxidative stress revealed they have extended lifespan, whereas those more susceptible to free radicals have shortened lifespan (Larsen, 1993; Ishii, 2000). Another study in mice showed that those lacking the antioxidant enzyme superoxide dismutase 1 (SOD1) exhibit a 30% decrease in life expectancy (Elchuri *et al.*, 2005) while treatment of antioxidant drugs in mice increases the median lifespan up to 25% (Schriner *et al.*, 2005). Other studies in *Drosophila melanogaster* also showed that the simultaneous overexpression of SOD1 and catalase both antioxidant enzyme, extended their lifespan (Orr and Sohal, 1994). Further study in mice lacking Ogg1 and Mth, two enzymes of the base excision repair pathway that repair oxidative DNA damage, show a 50% reduction in life expectancy (Xie *et al.*, 2004).

2.4.1.1 Sources of reactive oxygen species

Free radicals are chemical species containing one or more unpaired electrons with an example being oxygen which has two unpaired electrons. The unpaired electrons of oxygen react to form partially reduced, highly reactive species called reactive oxygen species (ROS) which include superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($\bullet OH$) and nitric oxide (NO) (Oliveira *et al.*, 2010). These reactive oxygen species through different molecular pathways cause oxygen toxicity hence are also called

oxidants (Beckman and Ames, 1998). Reactive oxygen species are produced both endogenously and exogenously. Various enzyme systems generate ROS as by-products and these include the mitochondrial electron transport chain, cytochrome P-450, peroxisomes, lipoxygenase, cyclooxygenase, NADPH oxidase complex, and xanthine oxidase (Oliveira *et al.*, 2010) in response to different environmental stimuli such as growth factors, inflammatory cytokines, ionizing radiation, hyperoxia, ultra violet radiations, chemical oxidants, toxins, chemotherapeutics and transition metals (Cui *et al.*, 2012).

The mitochondrial electron transport chain (ETC) (fig. 2-0) is the main generator of ROS as by-products of aerobic respiration (Harman, 1972). Electron transport involves a coordinated four-electron reduction of O_2 to H_2O . The electrons are donated by NADH or succinate to complexes I (NADH dehydrogenase) and II (succinate dehydrogenase) respectively of the mitochondrial ETC. Ubiquinone (coenzyme Q or UQ), which accepts electrons from complexes I and II undergoes two sequential one-electron reductions to ubi-semiquinone and ubiquinol (the Q cycle) and ultimately transferring reducing equivalents to the remainder of the electron transport chain i.e. complex III (UQ-cytochrome *c* reductase), cytochrome *c*, complex IV (cytochrome-*c* oxidase), and finally to oxygen(O_2) (Beckman and Ames, 1998). However, a one-electron reduction of molecular oxygen to form superoxide anion radical due to electron leakage may occur. Complex I and especially complex III are the prime sites for electron leakage mediated by the reduced form of ubiquinone, ubiquinol (Oliveira *et al.*, 2010). The superoxide anion radical can undergo a spontaneous enzymatic dismutation by superoxide dismutase

(SOD2) to yields hydrogen peroxide (H_2O_2) which diffuses out of the mitochondria or is inactivated by reaction with glutathione catalyzed by glutathione peroxidase. If the quantity of ROS produced exceeds the capacity of these two enzymes, cellular superoxide anion and H_2O_2 levels rise. In the presence of free transition metals, in particular iron and copper, the superoxide anion radical and hydrogen peroxide together generate the extremely reactive hydroxyl radical ($\cdot\text{OH}$) which is assumed to be the species responsible for initiating the oxidative destruction of biomolecules (Beckman and Ames, 1998)

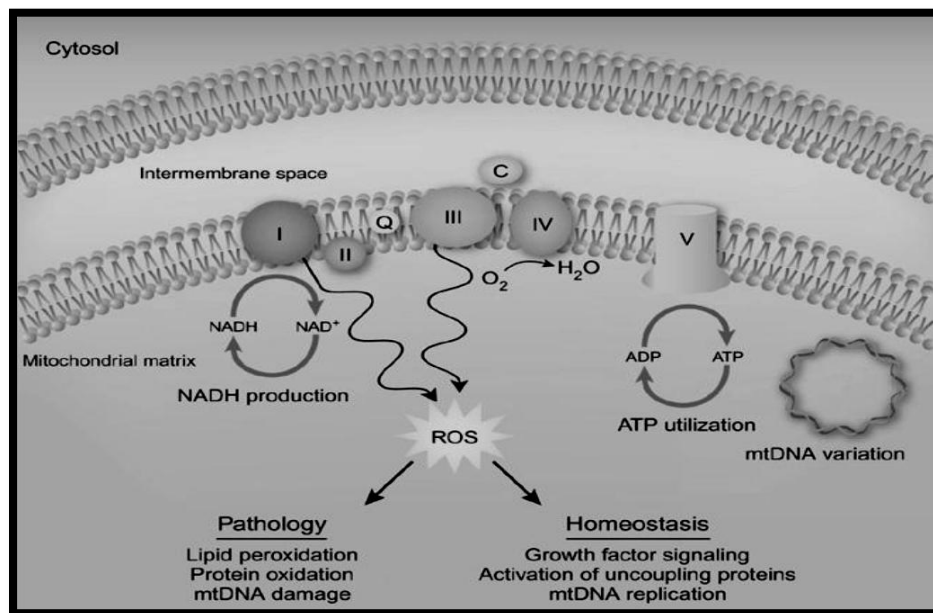


Fig. 2-0: Mitochondrial electron transport chain ROS production

(Adapted from Oliveira *et al.*, 2010).

Nitric oxide radicals (NO•) are generated in biological tissues by specific nitric oxide synthases (NOSs), which metabolize arginine to citrulline.

2.4.1.2 Importance of ROS

Intracellular ROS levels are normally maintained low and within a narrow range regulated by the balance between the rate of production and the rate of removal by various enzymatic and non enzymatic antioxidants such as tocopherols, flavonoids, carotenoids, urates, ubiquinol, ascorbates, glutathione, catalase, glutathione peroxidase, thioredoxin peroxidase and superoxide dismutase (SOD) (Sies, 1993; Beckman and Ames, 1998).

ROS at low levels and under normal conditions act as signalling molecules in many physiological processes including redox homeostasis and cellular signal transduction by activating proteins such as tyrosine kinases, mitogen activated protein kinases and Ras proteins (Droge, 2002). ROS thus serve as important mediators of signal transduction pathways as signalling molecules in cell survival, cell cycle control, apoptosis, differentiation and several stress responses depending on the type of cell. Hydroxyl radicals for example stimulate the activation of guanylate cyclases which catalyze the conversion of guanosine triphosphate (GTP) to the second messenger cyclic guanosine monophosphate (cGMP) (Mittal and Murad, 1977). cGMP-mediated signalling cascades play a central role in the regulation of diverse physiological processes, including vascular smooth muscle motility, intestinal fluid and electrolyte homeostasis and retinal

phototransduction (Lucas *et al.*, 2000). The diverse roles of ROS in many cellular processes suggest that they are not merely detrimental by-products of oxidative phosphorylation but also generated purposefully to modulate signalling pathways thus regulating gene expression (Cui *et al.*, 2012). The regulative effects of ROS are exerted through their reduction-oxidation (redox) potential (Weinberg and Chandel, 2009).

NO• is also an important oxidative signalling biomolecule involved in many physiological processes including neurotransmission, blood pressure regulation, defence mechanisms, smooth muscle relaxation, and immune regulation (Oliveira *et al.*, 2010)

2.4.1.3 Oxidative stress and aging

Under normal physiological conditions cellular ROS exist in balance with antioxidant molecules. Oxidative stress occurs when this critical balance is disrupted due to depletion of antioxidants or accumulation of ROS i.e. increase generation of ROS beyond the scavenging capacity of the cellular antioxidants or both (Barouki, 2006) . When oxidative stress occurs, cells counteract the oxidant effects to restore redox balance by resetting critical homeostatic parameters. Such cellular activity leads to activation or silencing of genes encoding defensive enzymes, transcription factors and structural proteins. When these defensive (repair) mechanisms of the body become weak, ROS accumulate in the cells leading to the oxidative damage to biomolecules such as lipids, nucleic acids and proteins and a flawed modulation of the signal transduction pathways. These disturbances

cause organelle damage, changes in gene expression followed by altered cellular responses which ultimately result in aging related diseases and aging (Cui *et al.*, 2012) . For example, lipid peroxidation and oxidative modifications of plasma membrane ion-motive ATPases, glucose transporters and G protein-coupled receptor signalling are implicated in the pathogenesis of Alzheimer's disease (Hyun *et al.*, 2006).

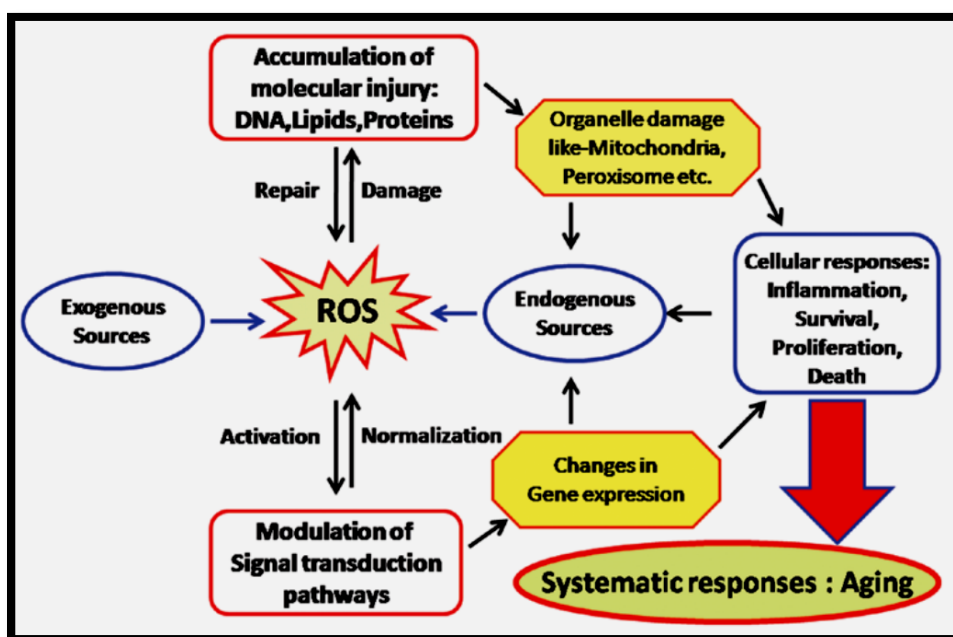


Fig. 2-1: ROS and their effect on biomolecules and the signal transduction pathways when defensive mechanisms of body become weak (Adapted from Pandey and Rizvi, 2010)

A modification of the free radical theory is that even under normal physiological conditions, a chronic state of oxidative stress exists in cells of aerobic organisms because of an imbalance between oxidants and antioxidants. The imbalance leads to a steady-state

accumulation of oxidative damage in biomolecules that increases with age, resulting in a progressive loss in the functional efficiency of various cellular processes. An example of these oxidative end products is lipofuscin formed by lipid peroxidation in cells and is seen to accumulate with age (Beckman and Ames, 1998). The ultimate outcome of oxidative stress is therefore a function of oxidant generation, antioxidant defences, and repair of oxidative damage.

2.4.1.3.1 Oxidative damage to proteins

Proteins are the most abundant macromolecules making up approximately 70% of the dry mass of most cells. They carry out very important biological functions which include structural, hormonal, enzymatic and immunological (Shi *et al.*, 2010). Protein peroxidation leads to fragmentation of polypeptide chains, oxidation of amino acid side chains, and generation of protein–protein cross-links. These result in modifications in protein conformation, enzymatic activity, binding affinity and recognition sites for other interacting proteins leading to functional loss (Stadtman, 2006). Among the protein oxidation modifications, the oxidation of amino acid residues to generate carbonyl moieties is greatest in magnitude (Dean *et al.*, 1997). The carbonyl content of proteins is therefore a general biomarker for estimating oxidative stress mediated proteins peroxidation. Studies using animal models have shown that cellular content of protein carbonyls increases with age (Chaudhuri *et al.*, 2006) in the plasma of mice and rats (Jana *et al.*, 2002). This age dependent increase in protein carbonyls is evident of the fact that oxidative stress increases with age as the antioxidant capacity of the body declines.

Severe oxidative stress leading to a decrease in the proteolytic degradation and accumulation of mis-folded proteins may be the cause and/or effect of many disorders and aging. The measurement of protein oxidation is a clinically important predictor of aging and age-related diseases. It is estimated that almost a third of proteins in cells of older animals are dysfunctional as enzymes or structural proteins due to oxidative damage (Pandey and Rizvi, 2010). The alterations of protein conformation reflected by changes in hydrophobicity and the formation of aggregates have also been used as a biomarker of the age-related increase in the oxidation of protein (Chao *et al.*, 1997).

2.4.1.3.2 Oxidative damage of lipids

Lipids are the primary component of biological membranes and also function as steroid hormones, retinoic acids and prostaglandins thus influencing many biological processes that affect cell survival (Barouki, 2006). Lipids are highly sensitive to oxidative damage either by direct reaction with ROS, or by indirect interaction with reactive aldehydes due to the high number of double bonds in unsaturated fatty acids resulting in the formation of unstable carbon radicals. Lipid peroxidation is a free-radical chain reaction accelerated by ROS. It leads to the formation of hydroperoxides and endoperoxides, which also produce reactive aldehyde and carbonyl intermediates, including alkanals, alkenals, hydroxyalkenals, malondialdehyde (MDA) and hydroxynonenal (HNE) (Shi *et al.*, 2010). These secondary oxidation products are highly unstable and can attack nucleophilic

groups in proteins leading to permanent modifications in biomembranes and membrane-bound proteins. Cell membranes are phospholipid bilayers with extrinsic proteins and are thus direct targets of lipid oxidation (Pandey and Rizvi, 2010).

These changes can alter membrane fluidity, integrity and permeability resulting in functional loss (Shi *et al.*, 2010). They can also influence gene expression and protein synthesis which can lead to further damage by cross linking proteins. One important biomarker of oxidative damage to lipids is the level of MDA. Studies showed a significant positive correlation between the erythrocyte MDA level and human age (Rizvi and Maurya, 2007). An increase in MDA levels also correlated significantly with a decrease in the antioxidant capacity of plasma during human aging (Rizvi and Maurya, 2007). MDA can also react with the free amino group of proteins, phospholipids and nucleic acids leading to structural modification, which can lead to a dysfunction of the immune system (Pandey and Rizvi, 2010).

Studies have revealed that lipid peroxidation modifies low density lipoprotein (LDL) to proatherogenic and proinflammatory forms and also generates products which are mutagenic and carcinogenic. It has also been implicated as the underlying mechanisms in several diseases such as cardiovascular diseases, cancer, neurological disorders and also aging (Greenberg *et al.*, 2008; West and Marnett, 2006). Other findings have also emphasized the importance of lipid peroxidation in relation to caloric restriction and longevity (Sanz *et al.*, 2006). Long-lived mammals and birds were seen to possess low degrees of unsaturation in their cellular membranes resulting in lower levels of lipid peroxidation and lipid oxidation-derived protein modifications (Sanz *et al.*, 2006).

2.4.1.3.3 Oxidative damage of DNA

Deoxyribonucleic acid (DNA) is the molecule that carries all the genetic information of an organism (except for some RNA viruses) in a cell. It can be thought of as a blueprint containing the instructions that govern the production of proteins and other biomolecules essential for cell function. The collection of these instructions is the genome and the informational units of the genome are the genes. Genes are translated into protein via the genetic code which defines the protein sequence and this translation process is central to life. Oxidative damage to DNA can therefore impact significantly on cell survival through loss or modification of genetic information especially in post mitotic cells (Chaudhary *et al.*, 2011). Genomic instability i.e. the time-dependent loss or corruption of information in DNA was proposed as a mechanism of aging in the 1950s with studies on mice exposed to radiation showing a reduction in lifespan (Hemplemann and Hoffman, 1953). Studies indicate oxidative damage by reactive oxygen species is one of the initial causes of endogenous genomic damage and instability (Hasty *et al.*, 2003). Research on oxidative damage to DNA causing mutations and cancer have revealed at least hundred different types of oxidative DNA lesions including base modifications such as 8-hydroxyguanosine (8-oxodG), thymidine glycol and 8-hydroxycytosine, single and double-strand breaks, and interstrand cross-links (Cadet *et al.*, 1997). Other studies in invertebrates also showed reactive oxygen species cause point mutations in both nuclear and mitochondrial DNA as well as large-scale genomic rearrangements (Hartman *et al.*, 2004). Studies into DNA repair mechanisms in species with various maximum lifespan potential (MSLP) showed long-lived animals have superior capacities for DNA repair compared to short-lived ones (Shi *et al.*, 2010).

Mitochondria play a central role in the free radical theory of aging (Harman, 1972). The mitochondrial theory of aging feeds into the free radical theory and proposes that mtDNA mutations accumulate progressively in life resulting in deficiencies in cellular oxidative phosphorylation. This leads to enhanced ROS production which in turn increases the rate of mtDNA damage and mutation. A vicious cycle of exponentially increasing oxidative damage and dysfunction thus results which ultimately culminates in death (Alexeyev *et al.*, 2004). Studies show that mitochondrial DNA (mtDNA) is more vulnerable to oxidative damage than nuclear DNA (nDNA) because of its proximity to mitochondrial ROS production resulting in a fifteen fold higher levels of DNA oxidative damage (Chaudhary *et al.*, 2011). mtDNA makes up only 1–3% of cellular genetic material but evidence suggest that its contribution to cellular physiology could be much greater due to its high mutation rate as a result of the close proximity to the ETC and the lack of protective histones (Alexeyev *et al.*, 2004). It encodes proteins of the ETC or components required for their synthesis thus any coding mutations will affect the ETC. This in turn could affect both the assembly and function of nuclear genes proteins in the ETC complexes. Defects in the ETC will thus have pleiotropic effects due to effects on oxidative phosphorylation to generate energy in cells (Alexeyev *et al.*, 2004).

A number of studies have linked mutations in mtDNA to aging and age related disorders. Deletions and point mutations in mtDNA have been seen to increase dramatically with age. Deletions in mtDNA were undetected in young individuals but increased to levels as high as 2% in old individuals. This age-related increase in mtDNA deletions was found in organisms as diverse as worms, mice, and humans (Muller *et al.*, 2007). A point mutation

and overexpression of the heart specific PolG, a DNA polymerase gamma which is the nucleus-encoded catalytic subunit of mtDNA polymerase responsible for proofreading newly synthesized mtDNA, results in the lack of the proofreading ability causing massive cardiomyopathy in mice and leading to death within weeks after birth. Mutations in human polG have also been found to be profoundly pathogenic. In addition to causing progressive external ophthalmoplegia, they also increase the risk for Parkinson's disease and decrease menopausal age to below 35 years (Muller *et al.*, 2007). On the other hand, studies show that low generation of mitochondrial ROS in long-lived mammals and birds is associated significantly with lower levels of 8-oxodG in the mtDNA in their brain and heart cells. However, no such association was found between 8-oxodG in nDNA and maximum lifespan potential (MLSP) (Herrero and Barja, 1999; Barja and Herrero, 2000).

Studies have also implicated mtDNA in human longevity. The Framingham Longevity Study of Coronary Heart Disease indicated that longevity is more strongly associated to maternal age at death than paternal age at death, suggesting that mtDNA inheritance might be involved (Brand *et al.*, 1992). Other studies found mtDNA polymorphisms in high frequency in centenarians suggesting a protective effect during aging (Zhang *et al.*, 2003) and these protective effects have been reported for the age-related neurodegenerative Parkinson's disease (Van der Walt *et al.*, 2003).

Many different techniques have been used to assess in vivo, ex vivo and in vitro the oxidative damage to DNA and these include alkaline filter elution (Doerjer *et al.* 1988),

chromosomal aberrations (Manikantan *et al.*, 2010) measurement of micronuclei in cells (Fenech and Morley, 1985), measurement of 8-hydroxydeoxyguanosine (8-OHDG) (Wu *et al.*, 2004) and the comet assay (Singh *et al.*, 1988, Manikantan *et al.*, 2010).

2.5 Haptoglobin (Hp): Structure and Genetics

Haptoglobin is an acute phase α_2 -sialoglycoprotein discovered by Polonovski and Jayle in 1938 (Sadrzadeh and Bozorgmehr 2004). The protein is present in the serum of all mammals, but polymorphism is found only in humans (Bowman, 1993). Hp is mainly produced by the hepatocytes of the liver and found at levels of 30–300 mg/dL in normal human serum and increases up to three to eight fold during the acute phase reaction (Asleh and Levy, 2005). The normal serum concentration is age dependent and is measurable from three months with a gradual increase until adult concentrations are reached at age twenty (Wobeto *et al.*, 2008). Its expression is regulated at three different levels. These are developmental control for lack of expression in foetal liver, tissue-specific control for selective expression in hepatocytes and modulation of expression during the acute phase reaction in response to injury (Dobryszczycka, 1977). Small quantities are expressed in other tissues, such as lung, skin, spleen, kidney, and adipocytes and its levels are increased similar to that observed in hepatocytes after inflammation (Sadrzadeh and Bozorgmehr, 2004; Asleh and Levy, 2005). Its synthesis is increased by growth hormone, insulin, bacterial endotoxin, prostaglandin, and cytokines such as IL-6, IL-1 and IL-8, and tumour necrosis factor (Sadrzadeh and Bozorgmehr, 2004; Raynes *et al.*, 1991). The acute phase elevation of the hp gene transcription rate in

the liver results from an increase in the binding affinity of hepatocyte regulatory DNA-binding proteins. These include the family of nuclear factors and the β Isoform of CCAAT-enhancer binding protein identified as a ligand to the hormone response elements of haptoglobin (Dobryszczyka, 1977). Hp is also highly expressed in arteries after sustained flow changes induced by shear stress and nitric oxide which influence IL-6 expression. Here, it plays a role in cell migration and arterial restructuring (Smeets *et al.*, 2002).

In humans, the HP locus is located on the long arm of chromosome 16 (16q22.1) and is polymorphic with two main alleles, HP1 and HP2, resulting in three distinct genotypes; HP1-1, HP 2-2 and HP 2-1 (Bowman, 1993; Smithies, 1955). The Hp protein is made up of two types of polypeptide chains, a heavy beta (β) chain made up of 245 amino acids with molecular weight of 40 kd and two light alpha (α) chains, α_1 and α_2 , made up of 83 and 142 amino acids with molecular weight of 8.9 kd and 16 Kd respectively (Sadrzadeh and Bozorgmehr, 2004). The genetic polymorphism in humans arises from differences in the α -chains i.e. α_1 and α_2 chains, (Napolioni *et al.*, 2011). The alpha and beta chains are encoded by a single gene and synthesized as a single polypeptide chain that is proteolytically cleaved into a short α -chain and a long β -chain that remain connected through a disulfide bond. The α - β units are also linked to each other by disulphide bonds (Wejman *et al.*, 1984; Malchy *et al.*, 1973).

The HP1 (fig. 2-2) allele consists of five exons with the first 4 exons encoding for the α_1 subunit and the fifth exon encoding for the beta subunit. Two types of α_1 chains have also

been identified by gel electrophoresis namely α 1F chain (fast) and α 1S chain (slow) (Smithies *et al.*, 1962) with the difference between them being the presence of amino acids Asp-Lys in α 1F and Asn-Glu in α 1S at positions fifty two and fifty three in their α_1 -chains respectively (Napolioni *et al.*, 2011). The HP2 (fig. 2-2) allele which seems to originate from an intragenic duplication of exons 3 and 4 in the HP1 allele caused by an unequal crossover between the HP1F and HP1S alleles, consists of seven exons. The first six exons encode for the α_2 subunit and the seventh exon encodes for the beta subunit (Galiccia and Ceuppens, 2011; Wobeto *et al.*, 2008). The main difference between α_1 and α_2 is therefore the presence of a duplicated approximately 1.7 Kb DNA segment in α_2 (Napolioni *et al.*, 2011). Consequently, the Hp1-1 phenotype consists of homodimers of two α - β units whiles the Hp2-1 and Hp2-2 consist of polymers due to the duplication in Hp2 of the cysteine that forms the disulfide bond between the α -subunits (Galiccia and Ceuppens, 2011). These phenotypes have different biologic activities and have been associated with different functional capacities, susceptibility to and outcome of several human pathologies due to their distinct biochemical and biophysical properties (Langlois and Delanghe, 1996).

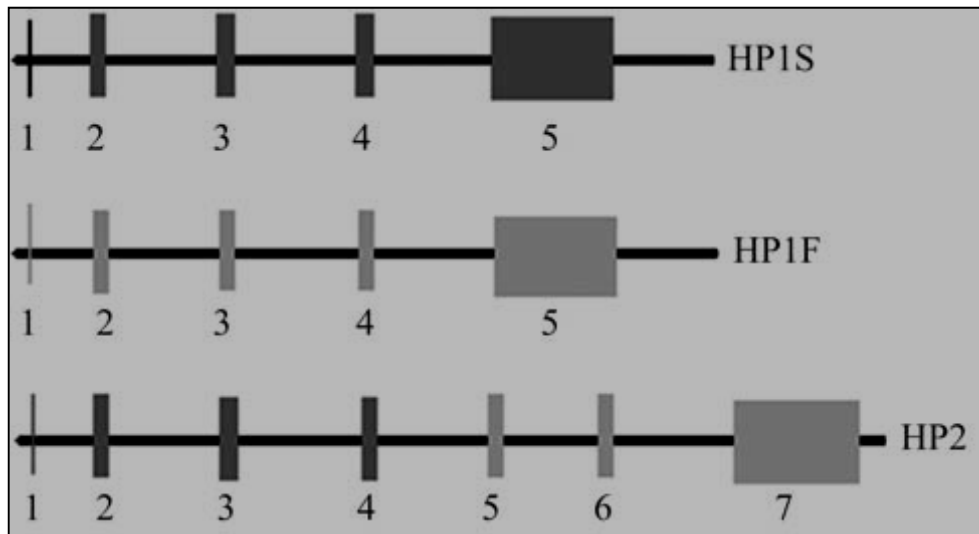


Fig.2-2: Schematic representation of the organization of the Hp gene.

The boxes indicate exon (adapted from Yano *et al.*, 1998).

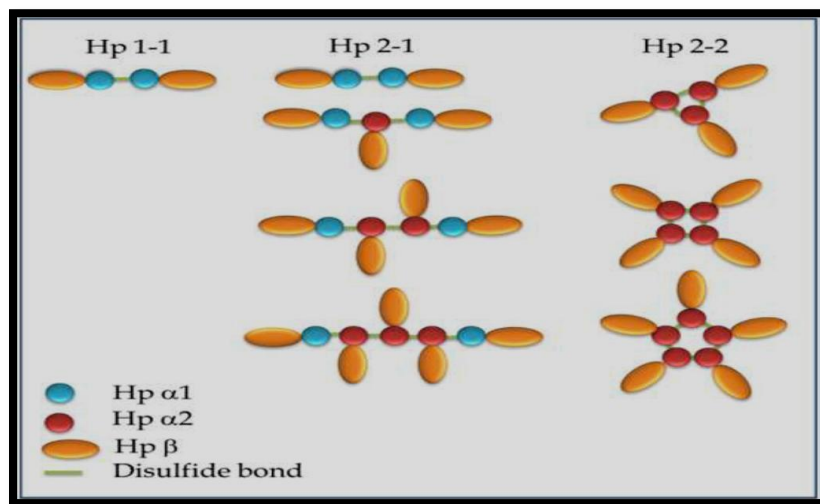


Fig. 2-3: Schematic representation of the structure of the different Hp polymers determined by phenotype (Adapted from Galicia and Ceuppens, 2011).

Other rare genetic variants of haptoglobin phenotypes have been reported. These include haptoglobin Carlberg which is a mixture of Hp 2-2 and Hp 2-1 polymers with structurally normal chains, haptoglobin Johnson with α chain abnormalities and haptoglobin Marburg and Bellevue with β chain abnormalities (Sadrzadeh and Bozorgmehr, 2004). Another rare phenotype, Hp2-1 modified (Hp2-1M), has been seen in Africans and African-Americans and results from an A-61C mutation in the promoter region of the Hp gene (Maeda, 1991). The mutation is also seen in the Hp0 phenotype which is characterized by a significantly reduced expression of the protein termed hypohaptoglobinaemia or a null expression of the protein termed ahaptoglobinaemia (Grant and Maeda, 1993; Teye *et al.*, 2003). In East Asian populations, a 28 kb deletion from the HP gene promoter region referred to as Hp^{Del} was shown to result in hypohaptoglobinemia while the homozygous genotype Hp^{Del}/Hp^{Del} corresponded to ahaptoglobinaemia (Wobeto *et al.*, 2008).

2.6 Geographic Distribution

The distribution of HP1 and HP2 alleles differ globally according to racial origins. The highest frequency of HP1 allele is observed in South America and Africa with Ghana having a frequency of 0.52 while Southeast Asia has the lowest frequency of 0.2 (Wobeto *et al.*, 2008; Langlois and Delanghe, 1996). There is however an increasing prevalence of the HP 2 allele in Africa largely attributed to selective pressure from malaria (Teye *et al.*, 2006). A study relating malaria, anaemia and HP genotype showed that the Hp2-2 phenotype was associated with asymptomatic malaria at the end of the malaria season (Atkinson *et al.*, 2006). The European population has an HP1 allele frequency of 0.4 with

a phenotypic distribution of approximately 15% Hp1-1, 50% Hp2-1, and 35% Hp2-2 (Carter and Worwood, 2007). North America, Europe, Asia and Australia have a predominant HP2 allele (Langlois and Delanghe, 1996).

2.7 Functions

Haptoglobin has several physiological functions with the main one being binding and clearing free cell-toxic haemoglobin from circulation after intravascular haemolysis. This function results in its antioxidant and antibacterial activities (McCormick *et al.*, 1990; Gutteridge, 1987; Sadrzadeh and Bozorgmehr, 2004). Haptoglobin, by binding haemoglobin and removing it from circulation make iron which is an essential element for bacterial growth unavailable thus inhibiting bacterial growth. Haptoglobin is synthesized locally in the lungs and is a major source of antimicrobial activity in the mucous layer and alveolar fluid thus protecting against infection (Eaton *et al.*, 1982; Barclay, 1985). Additionally, it prevents nitric oxide depletion which is an important toxic defence molecule against infectious organisms (Galicía and Ceuppens, 2011).

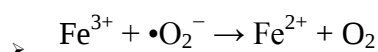
The haptoglobin-haemoglobin complex is removed by binding to the CD163 receptors expressed on the surface of macrophages and monocytes (Graversen *et al.*, 2002). CD163 expression is increased by interleukin-6, interleukin-10 and glucocorticoids (Langlois and Delanghe, 1996). When unbound to Hb, Hp is cleared from the plasma between three and half to five days. However when bound to Hb, the average time for removal of the Hp-Hb complex mainly by hepatocytes is approximately twenty minutes (Wobeto *et al.*, 2008). It

also has anti-inflammatory (Jue *et al.*, 1983; Komoriya *et al.*, 1980), angiogenic (Cid *et al.*, 1993) and immune-modulatory (Guetta *et al.*, 2007; Arredouani *et al.*, 2003; Kim *et al.*, 1995) functions in extravascular tissues and body fluids.

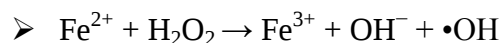
2.7.1 Antioxidant Function

Haemoglobin (Hb) is the oxygen-transporting molecule in blood, but also a potentially cytotoxic compound due to its highly reactive heme groups. The iron in heme catalyzes the conversion of superoxide anion radical and hydrogen peroxide to generate the extremely reactive hydroxyl radical ($\bullet\text{OH}$) in the Haber-Weiss and fenton reactions which occur in steps one and two respectively as indicated below.

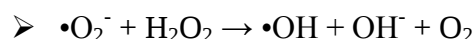
The first step involves the reduction of ferric ion to ferrous ions by superoxide anion radicals



The second step involves the oxidation of ferrous ions to ferric ions by hydrogen peroxide yielding hydroxyl radicals.



The net reaction is



•OH is highly reactive and together with the other reactive oxygen species is responsible for initiating the oxidative destruction of biomolecules leading to several human disorders and aging (Cui *et al.*, 2012).

Cell free Hb also acts as a scavenger of nitric oxide (NO), an endogenous antioxidant and vascular homeostasis regulator. NO depletion by Hb results in severe consequences such as blood vessel constriction, thrombosis, pulmonary and systemic hypertension, platelet activation and smooth muscle responses (Alayash, 2011).

During intravascular haemolysis, such as in malaria and haemoglobinopathies, cell free haemoglobin is captured by haptoglobin leading to the formation of the haptoglobin-haemoglobin complex, an irreversible non-covalent protein-protein interaction. The complex is formed by haptoglobin dimerizing through a β -strand swap between two complement control protein (CCP) domains. The haptoglobin serine protease domain then interacts with both the α and β subunits of haemoglobin forming a highly overlapping interface (Andersen *et al.*, 2012). The Hp-Hb complex is removed from circulation by binding to the 130-kDa transmembrane scavenger receptor CD163 expressed in circulating blood monocytes and tissue macrophages (Graversen *et al.*, 2002). The complex binds to the amino terminal cysteine-rich domain region of the receptor and the cytoplasmic tail of the receptor then conveys the internalization of the Hp-Hb complex by receptor-mediated endocytosis (Graversen *et al.*, 2002). After the CD163-mediated internalization of Hp-Hb, the globin moieties are degraded in the lysosome, whereas the heme is converted by the cytosolic heme oxygenase-1 (HO-1) into

free iron (Fe^{+2}), carbon monoxide (CO) and biliverdin. Biliverdin is subsequently converted into bilirubin by the biliverdin reductase and is excreted in bile and urine (Nielsen and Moestrup, 2009).

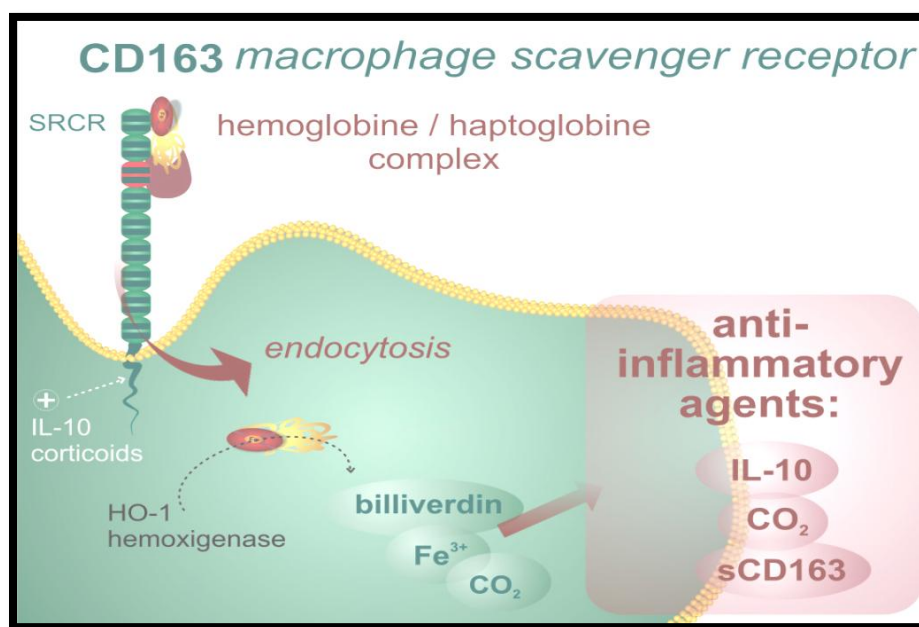


Fig. 2-4: Schematic representation of free haemoglobin elimination by macrophages via CD163 scavenger receptor (Adapted from Onofre *et al.*, 2009).

Hp 2-2 binds with a 10-fold higher affinity to CD163 compared with Hp 1-1 due to clustering of several binding sites in the multimeric ligand complex (Kristiansen *et al.*, 2001). Therefore the Hb and multimeric Hp 2-2 complexes have a higher affinity for CD163 than the complexes of Hb and dimeric Hp 1-1 while the Hp2-1-Hb complex has intermediate binding affinity. However Hp1-1-Hb complexes are more rapidly cleared than the Hp 2-2-Hb complexes, resulting in significantly higher antioxidant function

(Asleh *et al.*, 2003). This is because Hp allelic variants differ significantly in shape and size and consequently in their sieving ability to enter the subendothelial space from serum to bind free Hb released at sites of vascular injury (Melamed-Frank *et al.*, 2001). Hp1-1 is a homodimer with a molecular size of 86kd, Hp2-1 is a linear polymer with molecular size between 90-300kd and Hp2-2 is a cyclic polymer with molecular size between 170-900kd (Asleh *et al.*, 2003). Another reason for the lower antioxidant capacity of Hp 2-2 compared to Hp 1-1 is Hp 2-2 has lower haemoglobin binding affinity (Langlois and Delanghe, 1996). Haptoglobin, by binding haemoglobin and removing it from circulation, prevents iron-stimulated formation of reactive oxygen species which may result in peroxidation of polyunsaturated fatty acids within cell membranes causing damage to cells, oxidation of low density lipoprotein leading to vascular endothelial cell damage and atherosclerosis and oxidative damage in renal tissues causing renal damage (Gutteridge, 1987; Sadrzadeh and Bozorgmehr, 2004).

Besides preventing the toxic effects of cell free Hb, Hp also prevents peroxidative modification of Hb. Exposure of Hb to H_2O_2 causes structural modifications which include altered heme protein product formation, extensive crosslinking of α -globin chains, and irreversible oxidative modifications of specific amino acids within the CD163 binding region of the Hb β -globin chain. These alterations lead to reduced CD163 binding, severely impairing endocytosis of the oxidized Hb and further damaging tissues (Buehler *et al.*, 2009).

Hp also acts as a molecular chaperone inhibiting the inappropriate self association of proteins induced by oxidation or heat. Hp thus protects against protein misfolding to preserve cellular function (Yerbury *et al.*, 2005).

2.8 Haptoglobin Genotypes and Human Disorders

Several human disorders have been associated with the haptoglobin phenotypes and this has been attributed to their ability to bind haemoglobin resulting in several functional differences (Langlois and Delanghe, 1996).

A lesser degree of clearance of free haemoglobin by Hp 2-2 may result in some retention of iron in individuals with this genotype. Oxidative stress, induced by excess iron or other causes, is associated with ascorbic acid depletion and as such ascorbic acid concentrations have been found to be lower in healthy individuals with Hp2-2 compared to those with the other genotypes (Langlois *et al.*, 1997). Hp2-2 has therefore been implicated in most studies as showing susceptibility to several human pathologies. These include cardiovascular disorders, complications of diabetes, haematological disorders e.g. retinal haemorrhage (detachment), chronic renal failure due to hypertension or diabetes mellitus, pregnancy-induced hypertension (preeclampsia), neurological disorders, inflammatory bowel diseases, lupus and infectious diseases such as tuberculosis, HIV and hepatitis B (Galicía and Ceuppens, 2011; Sadrzadeh and Bozorgmehr, 2004). Some other studies, however suggest that the Hp2-2 phenotype provides protection against severe

Plasmodium falciparum infection and placental parasite burden (Cox *et al.*, 2007; Quaye *et al.*, 2000). The persistent haemolysis associated with the parasite burden in individuals with the Hp2-2 phenotype (Atkinson *et al.*, 2006) could increase ROS burden and decrease antioxidant levels. The increased ROS burden and decreased antioxidant levels result in oxidative stress to which the *Plasmodium* species is vulnerable, leading to a reduction in parasite burden (Quaye, 2008). Immunological tolerance in individuals in malaria-endemic areas also results due to an accelerated processing of parasite antigens for presentation to naive T cells (Quaye, 2008).

Hp 1-1 is associated with hematological disorders such as sickle cell disease in African Americans, acute and chronic myeloid leukemia and acute lymphoid leukemia, malignant neoplasms such as breast and cervical carcinoma, postmenopausal osteoporosis and cirrhosis (Sadrzadeh and Bozorgmehr, 2004). Studies indicate some levels of protection of the Hp 1-1 phenotype against infections. Patients infected with HIV-1 with Hp2-2 phenotype were seen to have a poor prognosis which is related to a more rapid rate of viral replication than HIV-1 infected patients with the Hp1-1 phenotype probably due to the less effective heme scavenging role of Hp2-2 resulting in iron retention and reduced antioxidant levels (Delanghe *et al.*, 1998).

A high frequency of Hp 2-1 has been associated with a family history of ovarian carcinoma and high frequency of celiac disease (Sadrzadeh and Bozorgmehr, 2004; Galicia and Ceuppens, 2011).

CHAPTER THREE

3.0 METHODOLOGY

3.1 Study design

The study was cross-sectional. Information relating to the demographic characteristics and health status of study participants were documented using a standard questionnaire.

3.2 Study site

The study was conducted in the Greater Accra Region of Ghana due to its cosmopolitan nature. Study participants were the aged who attended the HELP-AGE Ghana centre at La in the Kpeshi sub-metropolitan area. The centre serves as a counselling, recreational and screening site for the aged and membership consists of aged people from Osu, La, Kaajaano and their environs.

3.3 Study subjects

Using the WHO classification of the aged in Africa, the subjects for the study were randomly selected male and female Ghanaians aged 50 years and above with their prior consent.

3.4 Inclusion and Exclusion criteria

Ghanaians aged 50 and above whose ages could be confirmed were enrolled into the study while those whose ages and nationalities could not be confirmed were excluded.

3.5 Sample size determination

The minimum sample size was obtained using the formula:

$$N \geq \{(Z_{1-\alpha/2})/m\}^2.$$

Where Z is the coefficient of significance (1.96 for level of significance of $\alpha = 0.05$)

N is the minimum sample

m is the margin of allowable error which determines the power of the study ($1-\beta$)

Since the variance is unknown the minimum sample size required such that there is 95% chance of absolute error estimate of the mean within $\frac{1}{2}$ standard deviation of the mean would be:

$$N = \{1.96/0.5\}^2 = 16$$

Samples were put into age brackets as follows: 50-59, 60-69, 70-79, 80-89, 90-100.

Therefore minimum sample size for each category was 16, making a minimum total of 80.

However, a total of one hundred and thirty three (133) samples were obtained for the study.

3.6 Sample collection and Preparation

Five (5) ml of venous blood samples was collected into EDTA tubes. Samples were centrifuged at 4000rpm for 10mins at 4°C to separate into buffy coat, plasma and red cells. The buffy coat and serum were stored at -20°C and the red cells stored at 4°C until required for use.

3.7 Haematological measurements

Haematological parameters which include, white blood cells (WBC), haemoglobin (Hb), platelets (PLT), lymphocytes (LYM) and neutrophils (NEU) were measured for each sample with an auto haematological analyser (Sysmex K21, Japan).

3.8 Genomic DNA extraction

Genomic DNA was extracted from the buffy coat using QIAGEN DNeasy tissue kit (QIAGEN Co., Germany) according to the manufacturers' protocol. The extracted DNA was stored at -20°C until required.

3.9 Genotyping of Haptoglobin Polymorphisms

Haptoglobin genotyping was performed by allele specific PCR using the method described by Yano *et al.* (1998). Three PCRs were performed on each sample to genotype the HP1S, HP1F, and HP 2 alleles using forward primers C51, (5' -GCA ATG ATG TCA

CGG ATATC-3') and F3,(5'-CAG GAG TAT ACA CCT TAA ATG-3') and reverse primers C42, (5'-TTA CAC TGG TAG CGA ACCGA-3'), C72, (5'-AAT TTA AAA TTG GCA TTT CGCC-3') and S2, (5'-TTA TCC ACT GCT TCT CAT TG-3'). The F3 and C42 primer pair identified the HP2 allele, C51 and S2 primers the HP1S allele, and F3 and C72 primers the HP1F allele. The amplifications were performed in a 25 µl reaction mix containing 5 µl template DNA, 0.125 µl of the *Taq* polymerase enzyme (5U/µl) (Sigma Missouri, USA), 1.0 µl of each of the oligonucleotide primers at 10 µM, 0.5 µl of each of the four deoxyribonucleotide phosphates (dNTPs) at 10 mM and 3 µl of 10× PCR buffer (with MgCl₂). The amplification conditions were; an initial denaturation at 94°C for 15 minutes, thirty five (35) cycles of denaturation at 94°C for 40 seconds, primer annealing at 52°C for 1 minute for the C51-S2 and F3-C72 primer reactions and 58°C for 1 minute for the F3-C42 primer reactions and strand extension by the *Taq* polymerase at 72°C for 2 minutes. A final strand extension at 72°C for 5 minutes was then performed to complete the reaction. The amplification reaction was performed using Techgene PCR machine. After the reaction, 10 µl of the PCR product was run by electrophoresis at 120 volts (Labnet International, Power station 300) in 2% agarose gel (Biopioneer Co, USA) stained with 0.5 µg/ml ethidium bromide (Life Technologies Co, USA) in 1X Tris acetate EDTA (TAE) running buffer (Biopioneer Co, USA) using 2 µl of blue/orange DNA loading dye (6X) (Promega Co, USA). A hundred base pair nucleotide sequence molecular size marker (Sigma Mo, USA) was run alongside the PCR products. The gel was photographed using UV-illumination (UVIsave gel documentation system, model GAS9200/1/2/3, Version 12) and analyzed. Product sizes of 1,400, 1,200, and 935 base pairs were obtained for the HP1F, HP1S, and HP2 alleles respectively.

3.10 Analysis of oxidative DNA damage in white blood cells by Comet assay

3.10.1 Sample preparation and Assay

Glacial acetic acid was introduced into the buffy coat to get rid of all traces of red blood cells that were present as a result of the separation process. The buffy coat samples containing the white blood cells were then diluted with phosphate buffered saline (PBS) and counted to obtain a working concentration of 1×10^5 cells /ml. A DNA Comet AssayTM (Trevigen Inc., Gaithersburg, MD, USA) was carried out as described by the manufacturer. In this assay, WBCs were immobilized in a bed of low melting agarose on a Trevigen Comet slide. After a gentle cell lyses, samples were treated with alkali to unwind and denature the DNA and hydrolyze sites of damage. The samples were then subjected to Tris borate EDTA (TBE) electrophoresis. The Comet AssayTM SYBR® Green I nucleic acid gel staining kit (Trevigen Inc., Gaithersburg, MD, USA) was used to stain the processed cells from the comet assay as instructed by the manufacturer.

3.10.2 Photomicrography and Evaluation of DNA damage

The slides were viewed by an epifluorescence microscope using the fluorescein filter. SYBR® Green I maximum excitation and emission are respectively at 494 nm and 521 nm. Photomicrographs obtained from the various slides were taken immediately using a Konica Minolta 100VX film (Konica Minolta Co. Ltd., Japan). The comet tails were scored according to DNA content (intensity).

3.11 Superoxide dismutase activity as a measure of oxidative stress

The SOD activity levels were determined by a colorimetric method using SOD Assay kit (Cayman Chemicals, Michigan- USA). 250 µl each of the red cells was lysed with ice-cold (4°C) deionized distilled water (4°C) and centrifuged at 6000 rpm for 20 minutes. 1000 µl of the supernatant (erythrocyte lysate) was collected for assay and stored on ice. The supernatant fluid was then diluted by a factor of 1:100 with sample buffer, and 10 µl of the diluted solution used to assay Cu/ZnSOD (Copper/Zinc Superoxide dismutase) activities as described by the manufacturer.

3.12 Ethics

The study was reviewed and approved by the Research and Protocol Review Committee of the University of Ghana Medical School (U.G.M.S.).

3.13 Statistical analysis

Demographic and laboratory data of patients were recorded on a data entry form, and entered into MS-Excel database. Data was analyzed with PASW Statistics Student Version 18 and summarized into tables and histograms. Frequencies were represented by percentages. Differences between categorical variables were assessed using chi-square test for proportions. Comparison of measures of centrality was done by analysis of variance (ANOVA), unpaired t-test or Kruskal-Wallis test as appropriate. Pearson's correlation test was used to assess the correlation between continuous variables. A *p*-value of less than 0.05 was considered significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Description of Study population

The study comprised 37 (28 %) and 96 (72 %) healthy aged males and females with mean ages of 70.9 years and 75.7 years respectively. The minimum age was 62 years for males and 53 years for females while the maximum ages were 88 years and 91 years respectively. The modal ages were 68 years and 75 years for the males and females respectively. The entire study population had a minimum age of 53 years and a maximum of 91 years. The median age was 73 years, the mean age was 71.8 years and the modal age was 75 years as shown on the histogram (fig. 4-1).

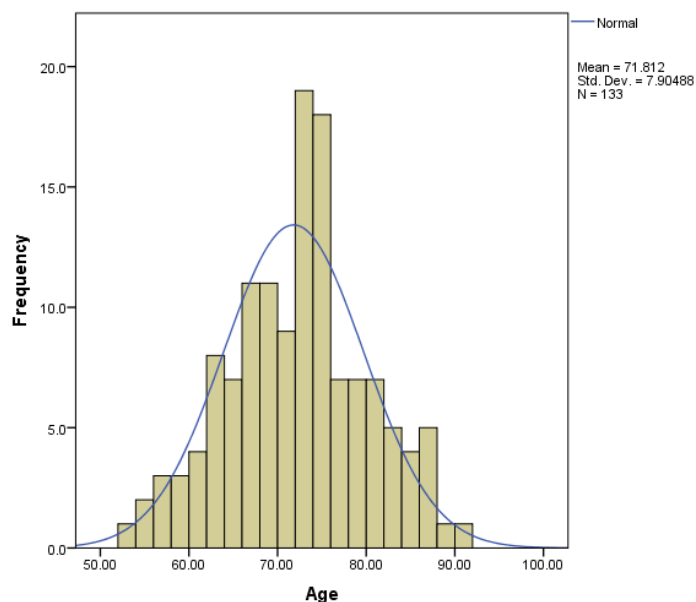


Fig. 4-1: Age distribution of the study population

4.2 Relationship between age and SOD activity and haematological parameters

A positive significant linear relationship (*p-value* of 0.002) was recorded between age and SOD activity among the study population (table 4-1). Total white blood cells, neutrophil and platelet counts also showed a significant reciprocal relationship ($r = -0.206$, -0.194 , and -0.241 respectively) with age. No relationship was observed between age and lymphocyte counts and Hb levels (*p-values* are 0.122 and 0.575 respectively).

Table 4-1: Correlations between age and SOD Activity, WBC, LYM, NEUT, PLT and HGB among the study population

		SOD Activity	WBC	LYM	NEUT	PLT	HGB
Age	Pearson Correlation	0.345*	-0.206*	-0.137	-0.194*	-0.241**	-0.500
	<i>P-value</i>	0.002	0.020	0.122	0.028	0.006	0.575

*. Correlation is significant at the 0.05 level (2-tailed).

**.Correlation is significant at the 0.01 level (2-tailed).

4.3 Relationship between Gender and SOD activity

Comparison of SOD activity between genders showed males had a higher mean value than females. The difference was however not statistically significant (*p-value* is 0.525) (table 4-2).

Table 4-2: Comparison of SOD activity and Gender

	Gender	N	Mean	p-value
SOD Activity	Male	38	224.49 ± 139.11	0.525
	Female	95	207.62 ± 133.28	

N is sample number

4.4 Haptoglobin genotyping

Haptoglobin genotyping showed visible and distinct bands from 100 out of the 133 people sampled (fig. 4-2).

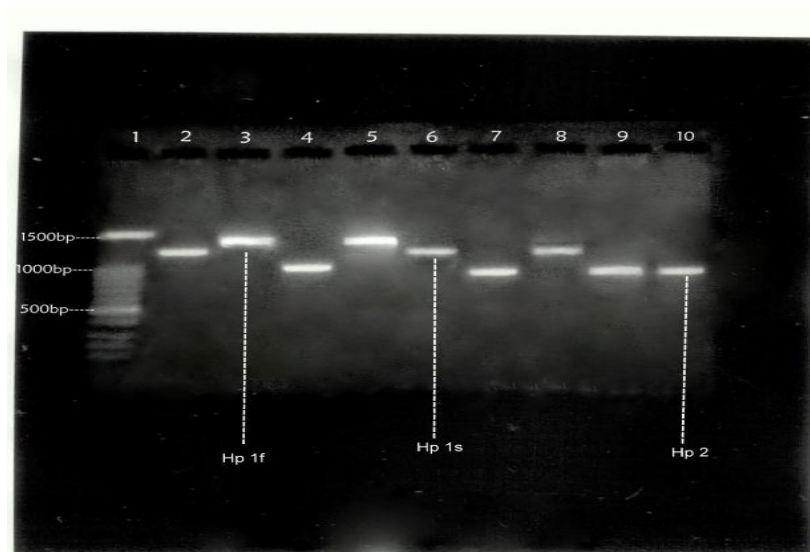


Fig. 4-2: An electrophoregram of HP allele PCR product

Lane 1 shows a 100bp molecular size marker.

Lanes 2, 6 and 8 show the HP1s allele with an amplicon size of 1200bp.

Lanes 3 and 5 show the HP1f allele with an amplicon size of 1400bp.

Lanes 4, 7, 9 and 10 show the HP2 allele with an amplicon size of 935bp

4.5 Distribution of Haptoglobin alleles among the study population

The allelic frequencies for HP1 and HP2 were found to be 49.5 % and 50.5 % respectively with a 95% confidence interval (table 4-3)

Table 4-3: Haptoglobin allelic frequencies for the study population

Haptoglobin Allele	Population studied	N	Allelic frequency % (95 % C.I.)
HP1	Aged (53-90 years)	100	49.5 (39.7 – 59.3)
HP2			50.5 (40.7 – 60.3)

N is sample Size

4.6 Comparison of haptoglobin genotypes among the study population

HP 2-2, HP 2-1 and HP 1-1 constitute 38%, 25% and 37% respectively of the total frequency (table 4-4). Out of a total of 27 males the proportion of HP 1-1, HP 2-1, HP 2-2 were 44.4 % (12), 18.5 % (5) and 37.1 % (10) respectively and of a total of 73 females the proportions of HP 1-1, HP 2-1 and HP 2-2 were 34.2 % (25), 27.4 % (20) and 38.4 % (28) respectively (table 4-5).

Table 4-4: Distribution of haptoglobin genotypes among gender in the study Population

Gender	HP1-1			Total	HP2-1		Total	HP2-2	Total
	1s-1f	1s-1s	1f-1f		2-1f	2-1s			
Male	5	3	4	12	3	2	5	10	27
Female	4	5	16	25	14	6	20	28	73
Total				37			25	38	100

Table 4-5: Distribution of haptoglobin genotypes (proportions) among the study population

Gender	HP 1-1	HP 2-1	HP 2-2	Total
	(95 % C.I)	(95 % C.I)	(95 % C.I)	(95 % C.I)
Male	44.4	18.5	37.1	27.0
Female	34.2	27.4	38.4	73.0

4.7 Hardy-Weinberg Equilibrium

The difference between the observed and expected haptoglobin genotypic frequency (Table 4-6) in the aged population was found to be statistically significant representing a departure from the Hardy-Weinberg equilibrium.

Table 4-6: Observed and expected HP genotypic frequencies in the study Population

HP genotype	Observed number	Expected number	p-value (χ^2)
Hp 1-1	37	24.5	< 0.000001
HP 2-2	38	25.5	
HP 2-1	25	50.5	
<i>p-value</i> < 0.05 is significant			

4.8 Comparison of haptoglobin genotypes among the age brackets in the study population

Analysis of the distribution of haptoglobin genotypes among the age categories of the study population showed that no age category had a significant association with any of the haptoglobin genotypes (table 4-7).

Table 4-7: Distribution of haptoglobin genotypes among the age categories of the study population

Age groups (Years)	HP Genotypes			Total
	1-1	2-1	2-2	
50-59	3	1	4	8
60-69	15	9	10	34
70-79	14	11	18	33
80-89	5	3	6	14
90-99	0	1	0	1
Total	37	25	38	100

Pearson Chi-square (χ^2) 5.527, Asymptotic sig. (2-sided) *p-value* = 0.700

4.9 Comparison of SOD activity among haptoglobin genotypes

Levels of SOD activity measured among the genotypes as indicated in table 4-8 showed no significant association between the various haptoglobin genotypes and SOD activity (*p-value* of 0.488) be it low, normal or increased.

Table 4-8: Distribution of SOD activity among the haptoglobin genotypes

Haptoglobin genotypes	SOD Activity			Total
	Low	Normal	Increased	
HP 1-1	1	14	22	37
HP 2-1	2	8	15	25
HP 2-2	0	14	24	38
Total	3	36	61	100

Assuming reference limits of 30.49 U/ml – 129.57 U/ml (Mean $\pm$ 2 S.D, mean = 80.03, S.D = 24.77) being SOD activity measured in young people, SOD activity in the study subjects was categorized as low (<30.49 U/ml), normal (30.49 – 129.57) and high (> 129.57 U/ml).

Pearson Chi-square (χ^2) 3.485, *p value* is 0.488.

4.10 Comparison of age and SOD activity among the haptoglobin genotypes

The distribution of SOD activity and age in terms of the mean and median values (table 4-9) was found not to be significantly different among the haptoglobin genotypes (*p-values* of 0.877 and 0.790 respectively).

Table 4-9: Comparison of age and SOD activity among haptoglobin genotypes

HP genotypes	Age	<i>p-value</i>	SOD Activity	<i>p-value</i>
	Mean $\pm$ S.D		Median (Range)	
HP 1-1	70.43 $\pm$ 7.79	0.790	213.20 (23.8 – 428.33)	0.877
HP 2-1	71.68 $\pm$ 8.09		199.50 (20.78 – 485.10)	
HP 2-2	71.53 $\pm$ 8.54		210.55 (31.44 – 499.38)	

4.11 Comparison of DNA damage among Hp genotypes

Single cell gel electrophoresis of white blood cells of selected study samples based on HP genotypes showed same degrees of DNA damage represented as average percentage DNA in comet tail indicated in table 4-10 as shown by photomicrograph of samples in figures 4-3 to 4-8

Table 4-10: Visual Classification of DNA damage in genotypes

Sample number	Age	HP genotype	Visual score	Average % DNA in comet tail
32	80	HP 1-1	3	70%
21	88	HP 2-1	3	80%
2	84	HP 1-1	3	80%
24	85	HP 2-2	3	80%
36	84	HP 2-2	3	80%
44	90	HP 2-1	4	85%

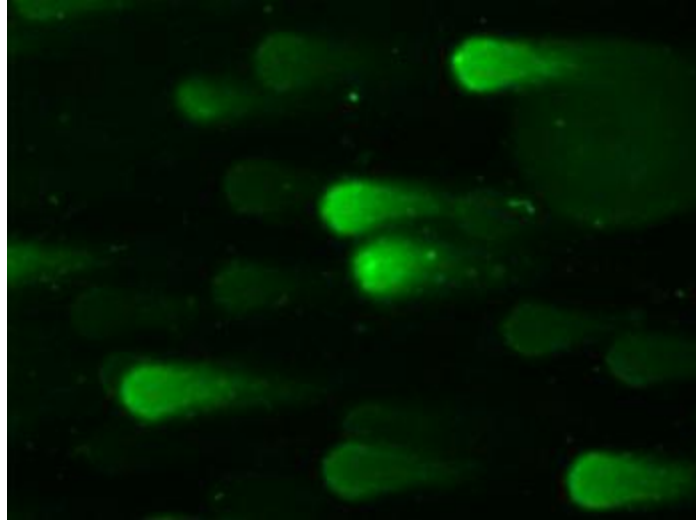


Fig. 4-3: Photomicrograph of sample 32 ($\times 100$)

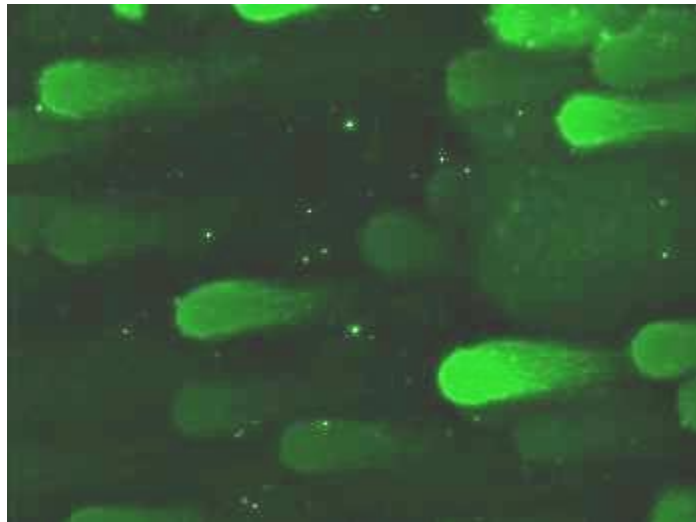


Fig. 4-4: Photomicrograph of sample 21 ($\times 100$)

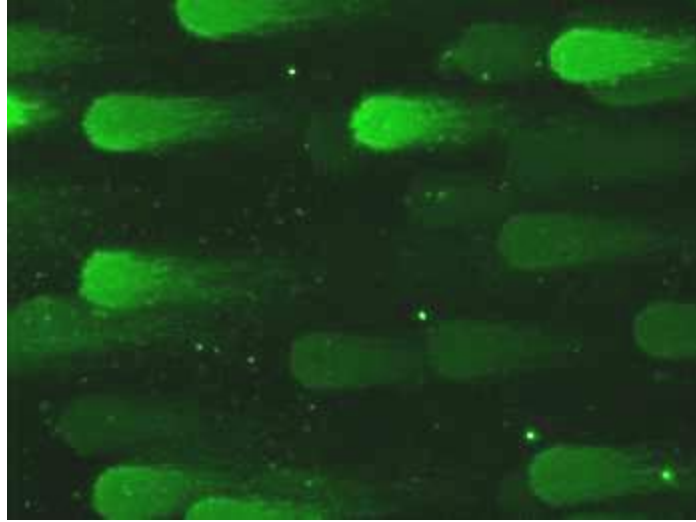


Fig. 4-5: Photomicrograph of sample 2 ($\times 100$)

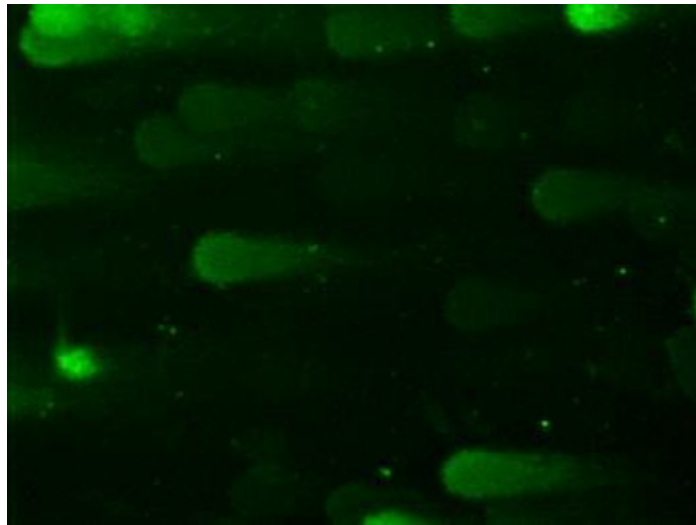


Fig. 4-6: Photomicrograph of sample 24 ($\times 100$)

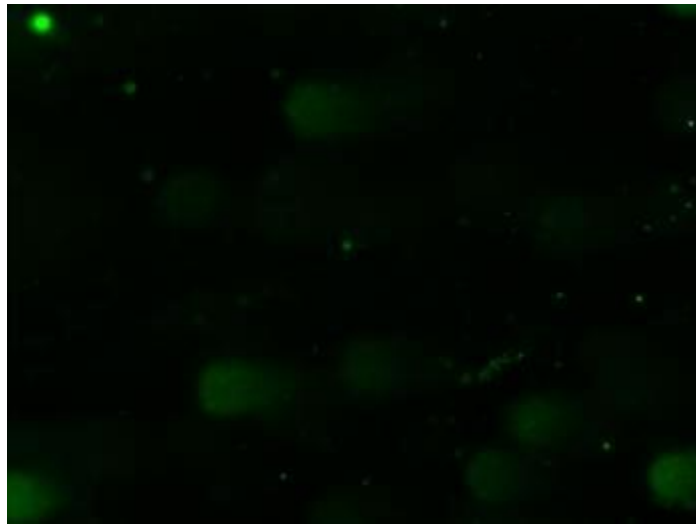


Fig. 4-7: Photomicrograph of sample 36 ($\times 100$)

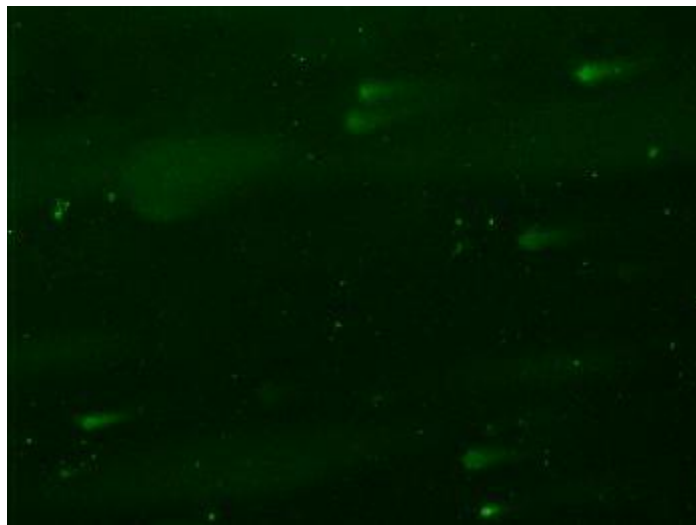


Fig. 4-8: Photomicrograph of sample 44 ($\times 100$)

CHAPTER FIVE

5.0 DISCUSSION AND CONCLUSION

5.1 Discussion

One hundred and thirty three (133) aged people were recruited in the study with females predominating with 72% and also having a higher mean age of 75.7 compared to 70.9 in males. These observations are consistent with studies which indicate that women live longer than men all over the world (Austad, 2006). Several reasons have been assigned to this phenomenon. Recent studies in the Japanese population reported age-related changes in various immunological parameters which differ between men and women. A decline in the number of T cells, certain subpopulations of T cells (CD8⁺ T cells, CD4⁺CDRA⁺ T cells, and CD8⁺CD28⁺ T cells), B cells, and in the proliferative capacity of T cells was seen to be greater in men than in women (Hirokawa *et al.*, 2013). This is consistent with studies which indicate that aging is associated with a decline in the normal function of the immune system, leading to increased susceptibility to various diseases and shortened life span (Chung *et al.*, 2009). In the same study of the Japanese population, an age-related increasing trend in the number of CD4⁺ T cells, CD4⁺ CDRO⁺ T cells, and natural killer cells (CD56⁺CD16⁺) and CD4⁺ T cell/CD8⁺ T cell ratio was seen to be greater in women than in men (Hirokawa *et al.*, 2013). Another study reported higher mitochondrial oxidative stress in men than women due to higher levels of oestrogens in women which protect them against aging by upregulating the expression of antioxidants (Vina *et al.*, 2011). It was demonstrated that the hormone upregulates the expression of

antioxidant enzymes through the oestrogen receptor and mitogen-activated protein kinase (MAPK) activation. This in turn activates the nuclear factor kappa B (NF-Kb) signalling pathway, resulting also in the upregulation of longevity-related gene expression. The role of gender in regulating longevity may thus be linked to gender specific genetic differences (Hirokawa *et al.*, 2013).

A significant negative relationship was found between age and total white blood cells, neutrophil and platelet counts (*p-values* of 0.020, 0.028 and 0.006 respectively). This implies that total cell counts decreased with increasing age and is consistent with studies that showed blood cells decreased with age (Hirokawa *et al.*, 2013; Santimone *et al.*, 2011; Kubota *et al.*, 1991). Various reasons attributed to this decrease in these studies include decreasing half-life of the cells with age such that the rate of production then falls short of the rate of apoptosis causing a deficit in total counts with age. The phenomenon is also suggested may be due to a reduction in the hematopoietic stem-cell reserve with age, a reduced ingestion and or digestion of protein and or absorption of the end products of protein digestion with age. In the case of platelet, a survival advantage with decreased count is also proposed.

There was also a significantly high positive correlation between SOD activity and age in the study population (*p* value of 0.002). This increase in enzyme activity seems to be a compensatory and adaptive effect to the increasing levels of ROS with aged. This is in accordance with other studies (Ceballos-Picot *et al.*, 1992; Rizvi and Maurya, 2007)

which are consistent with the free radical theory of aging (Harman, 1956). Studies into other antioxidative enzymes activities such as glutathione reductase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in erythrocytes also reported increased levels with age (Rodriguez-Martinez and Ruiz-Torres, 1992). This was suggested to be as a result of a positive feedback mechanism in response to rising lipid peroxidation. Other studies however discovered decreased SOD activity with age (Guemouri *et al.*, 1991; Adiga and Adiga, 2008; Andersen *et al.*, 1997). This may be due to a high degree of oxidative damage to biomolecules with age due to increasing oxidative stress resulting in decreased synthesis of the enzyme or mutations that lead to reduction in its activity. The increased or decreased SOD activity with age may thus reflect the strength of the individual's antioxidative system and DNA repair system to oxidative damage. In another study, the activity of erythrocyte SOD (Cu/Zn-SOD) was decreased in centenarians compared to the individuals below 80 years of age (Andersen *et al.*, 1998). The result was suggested might be due to reduced demand for SOD due to the lower metabolic rate and reduced oxygen consumption as a result of decreased caloric intake and less physical activity in centenarians.

Comparison of SOD activity between genders showed women had a lower mean value (207.62 U/ml) than men (224.49 U/ml) suggesting higher reactive oxygen species levels in men than women. This is consistent with studies that reported higher mitochondrial oxidative stress in men than women due to higher levels of oestrogens in women (Vina *et al.*, 2011).

Out of the 133 aged people sampled, 100 samples produced visible and distinct bands after genotyping. Samples that did not show bands might have had their DNA damaged/fragmented due to genomic instability that occurred with age (Hemplemann and Hoffman, 1953) as a result of oxidative stress (Harman, 1956). Genomic instability compromises the integrity of the DNA extracts which impacts negatively on accurate replication by polymerases (Lindahl, 1993). For instance, oxidative attack on the deoxyribose moiety results in the loss of a base leaving an apurinic/apyrimidinic (AP or abasic) sites. Polymerases stall at these AP sites disabling further replication. Extensive DNA oxidative damage resulting in many such AP sites will thus prevent amplification reactions. The breakdown of AP sites into nicks further compounds the problem because it leads to the fragmentation of the DNA compromising replication.

The allelic frequency of the haptoglobin gene was seen to be 49.5 % and 50.5% for HP1 and HP2 alleles respectively. The frequencies of occurrence of the various haptoglobin genotypes were found to be 38%, 25% and 37% for HP 2-2, HP 2-1, and HP 1-1 respectively. The HP1 allelic frequency obtained is close to the 52% reported in the general Ghanaian population (Teye *et al.*, 2006) which falls within the 95% confidence interval of this study. The difference between observed and expected HP genotypic frequencies in the study population was significantly different ($p < 0.00001$) indicating the study population was not in Hardy-Weinberg equilibrium at the HP locus. This suggests that the allelic and genotypic frequencies recorded in this study population are likely to change with other generations. Deviations from Hardy-Weinberg equilibrium are attributed to factors such as small population size, non-random mating, migration,

mutation and selection for particular alleles in a population (Tamarin, 2001; Nussbaum *et al.*, 2007). A bigger population size in further research will rule out the effect of small population size.

Analysis of the distribution of haptoglobin genotypes among age categories showed no significant association with any haptoglobin genotype (*p-value* of 0.700). The results obtained suggest that the HP1/2 genotypic polymorphisms did not influence longevity in the Ghanaian population and no genotype conferred a survival advantage. This is inconsistent with findings in the Italian and Polish populations (Napolioni *et al.*, 2011; Turowska *et al.*, 1991). In the Italian populations, the HP1-1 genotype and the HP1 allele were seen to be associated significantly with increased probability of young subjects to attain increased lifespan while carriers of HP2 allele displayed an overall significant disadvantage in reaching old age. In the Polish study, a considerable increase of HP1-1 genotype was evident in subjects aged 81–91 years. On the other hand, both HP2-1 and HP2-2 carriers display a significant deleterious effect compared to HP1-1 suggesting that possessing the HP1-1 genotype could confer a survival advantage for attaining longevity consistent with its superior functional capacities (Langlois and Delanghe, 1996). The conflicting results obtained from the Ghanaian population in this work may be partly explained by the large difference between the sizes of population sampled in these studies. The Italian studies sampled 1500 aged people while the Polish studies sampled 723 elderly people.

Photomicrographs of samples' comet slides showed high average percentage DNA in comet tail implying a high degree of DNA damage/fragmentation in the study population. This is consistent with the free radical theory of aging (Harman, 1956). The time-dependent loss or corruption of information in DNA was proposed as a mechanism of aging (Hemplemann and Hoffman, 1953) and studies indicate oxidative damage of DNA by ROS is one of the initial causes of this genomic instability (Hasty *et al.*, 2003). Reactions of $\bullet\text{OH}$ with the sugar moiety of DNA give rise to sugar modifications and strand breaks leading to DNA fragmentation which is the most common DNA damage by ROS (Cooke *et al.*, 2003). The degree of DNA damage among the various genotypes by visual scoring gave similar results. This is consistent with results obtained from correlation studies of SOD activity in the various genotypes where the median levels of SOD activity were found not to be statistically different in all three genotypes. The distribution of SOD activity and age in the study population was also not significantly different in all genotypes. All genotypes however recorded a higher number of subjects with increased SOD activity. This implies that the genotype that one possesses seem not to influence the level of free radicals and hence aging according the free radical theory (Harman, 1956). This result may however be partly due to ahaptoglobinaemia and hypohaptoglobinaemia which may be present among the population sampled. Teye *et al.*, in 2003 reported a 13.8% prevalence of these phenotypes in the Ghanaian population. Studies of the expression and level of expression of the haptoglobin protein in plasma in further research will rule out these factors.

5.2. Conclusion

The HP1 and HP2 allelic frequencies in the Ghanaian aged population are 49.5 % and 50.5% respectively whiles the genotypic frequencies are 38%, 25% and 37% for HP 2-2, HP 2-1 and HP 1-1 respectively. The HP1/2 genotypic polymorphisms did not seem to influence longevity in the Ghanaian population and no genotype confers a survival advantage.

RECOMMENDATIONS

It is recommended that, the association between haptoglobin genotypes and longevity in a larger sample size be studied in Ghana. Expression studies of the protein in plasma should also form part of the research to better elucidate its role in longevity in the Ghanaian population.

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APPENDICES

APPENDIX A

Genomic DNA extraction from buffy coat (Qiagen Co. Ltd., UL)

- **Principle**

DNeasy kits are advanced silica gel membrane technology for the rapid and efficient purification of total cellular DNA. The buffer system is optimized to allow direct cell lysis followed by selective binding of DNA to the DNeasy membrane. Simple purification processes completely remove contaminants and enzyme inhibitors such as proteins and divalent cations.

- **Materials and Reagents**

DNeasy Kit

Proteinase K

Microcentrifuge

Heating block

Microcentrifuge tube

Phosphate buffered saline

Vortex

Pipette and tips

Eppendorf tubes

Absolute ethanol

Collection tubes

- **Methodology**

1. 20µl of proteinase K was pipetted into 1.5 ml microcentrifuge tube
2. 100µl of buffy coat of the sample was added onto the proteinase K
3. 100µl of phosphate buffered saline (PBS) was added to adjust the volume to 220µl
4. 200µl buffer AL was added and mixed thoroughly by vortexing
5. The mixture was incubated for 10min at 56°C
6. 200µl of absolute ethanol was added to the sample and mixed thoroughly by vortexing
7. The mixture from step 6 was pipetted into DNeasy mini spin column and centrifuged at 8000rpm for 1min. The flow-through and collection tube were discarded.
8. The DNeasy mini spin column was placed into a new 2ml collection tube. 500µl buffer AW1 was added and centrifuged at 8000rpm for 1min. The flow-through and collection tube were discarded.
9. The DNeasy mini spin column was placed into a new 2ml collection tube. 500µl buffer AW2 was added and centrifuged at 14000rpm for 3min to dry the DNeasy membrane. The flow-through and collection tube were discarded.

10. The DNeasy mini spin column was placed into a new 1.5ml collection tube. 50µl buffer AE was added and incubated at room temperature for 1min and centrifuged at 8000rpm for 1min. The resulting DNA sample was divided into 2 aliquots of 25µl each and stored at -20°C.

APPENDIX B

Polymerase chain reaction and gel electrophoresis

- **Principle**

The Polymerase Chain Reaction (PCR) is a molecular biology technique that provides an extremely sensitive means for amplifying small quantities of DNA across several orders of magnitude to generate exponential copies of that particular DNA sequence. It consists of cycles of repeated heating and cooling (thermal cycling) of the reaction for DNA denaturation, primer annealing and enzymatic replication of the target DNA sequence. Key components to enable selective amplifications include the target DNA whose portion is to be amplified, primers with sequences complementary to the target DNA, deoxyribonucleotides (dNTPs) and a heat-stable DNA polymerase. PCR is self propagating in the sense that as the reaction progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified.

Gel electrophoresis is a method adopted in molecular biology to separate DNA by length, size and charge. Nucleic acid molecules are separated by applying an electric field to

move the negatively charged molecules through an agarose matrix with the smaller molecules migrating faster and farther than the larger molecules as a result of the ease with which they migrate through the pores of the gel. This phenomenon is referred to as sieving. Loading dyes such as Cresol Red and Orange Green are used to enable viewing of the post electrophoresis in natural light. The gel is treated with ethidium bromide which intercalates into the major groove of the DNA and fluoresces reddish-orange under UV light. Hence, by running a sample through an Ethidium bromide treated gel, any band containing more than 20ng DNA become distinctly visible.

- **Materials and reagents**

10x PCR buffer

1M HCl

70% ethanol

Aerosol Filter Pipette Tips

Agarose

Electrophoresis set up (Labnet International, Power station 300)

Ethidium Bromide

Gel photography system (UVIsave gel documentation system, model GAS9200/1/2/3, Version 12)

MgCl₂

Micro-centrifuge

Micro-centrifuge tube

Nuclease free water

PCR Machine (Techgene)

Pipette

Taq polymerase

Hundred base pair of DNA molecular size markers (Sigma Mo, USA) The DNA mole.

- **Reagents preparation**

Working solution of Hp genotype primers

Concentration of stock solution (C1) -100 μ M

Volume of stock solution (V1) - ?

Concentration of working solution (C2) -10 μ M

Volume of working stock needed (V2) - 200 μ l

Number of Moles of primer $\div$ Volume of primer = Concentration

Thus; Concentration x volume = Number of moles

Initial number of moles of the primer = final number of moles of the primer

$$C1 \times V1 = C2 \times V2$$

$$V1 = \frac{C2 \times V2}{C1}$$

$$C1$$

$$V1 = \frac{10 \mu\text{M} \times 200\mu\text{l}}{100 \mu\text{M}}$$

$$100 \mu\text{M}$$

$$V1 = 20\mu\text{l}$$

20µl of the primer stock solution was pipetted and added to 180µl of nuclease free water to make a 10µM working concentration, the resulting solutions was vortexed to mix and stored at -20 °C for future use.

➤ **Working solution of Deoxynucleotide triphosphates (dNTPs).**

Concentration of stock solution (C1) - 100 mM

Volume of the stock concentration used in making the working concentration (V1) - ?

Concentration of working solution (C2) -10 mM

Volume of working solution needed (V2) - 100µl

$$C1 \times V1 = C2 \times V2$$

$$V1 = \frac{C2 \times V2}{C1}$$

$$V1 = \frac{10 \text{ mM} \times 100\mu\text{l}}{100 \text{ mM}}$$

$$V1 = 10\mu\text{l}$$

10µl stock solution was added to 90µl of nuclease free water to make 10 mM working concentration, the resulting solutions was vortexed to mix and stored at -20 °C for future use.

➤ **1X Tris acetate (TAE) Buffer**

Stock concentrations of TAE buffer (C1) - 50X

Volume of the stock concentration used in making the working concentration (V1) - ?

Concentration of working solution needed (C2) - 1X

Volume of working solution needed (V2) = 500ml

$$C1 \times V1 = C2 \times V2$$

$$V1 = \frac{C2 \times V2}{C1}$$

$$V1 = \frac{1 \times 500\text{ml}}{50}$$

$$V1 = 10\text{ml}$$

10ml of the 50x stock solution at room temperature was taken into a 500ml volumetric flask and then topped up to the 500ml mark with distilled water, the resultant solution was mixed thoroughly and stored at room temperature for future use.

➤ **Two percent (2%) agarose gel preparation and casting**

Two grams (2g) of agarose was weighed into a heat resistant bottle and 100ml of 1x TAE added. The solution was heated to dissolve the agarose, cooled down to just above room temperature and mixed thoroughly with 5µl of ethidium bromide. The resultant solution was poured into a gel casting tray with combs to create the sample wells and allowed to set.

Human haptoglobin PCR reaction condition

Reagents	X 1
DNA Template	5µl
10 X Buffer + 15mM MgCl ₂	3µl
dATP (10mM)	0.5µl
dGTP (10mM)	0.5µl
dTTP (10mM)	0.5µl
dCTP (10mM)	0.5µl
Forward Primer	1 µl
Reverse primers	1 µl
Taq Polymerase	0.125µl
Nuclease free water	12.875µl
Total	25µl

DNA sequence detail of synthetic oligonucleotide Primers of haptoglobin genotyping

Primers for Hp	Sequence (5'-3')
C51(f)	GCA ATG ATG TCA CGG ATATC
F3 (f)	CAG GAG TAT ACA CCT TAA ATG
C42(r)	TTA CAC TGG TAG CGA ACC GA
C72(r)	AAT TTA AAA TTG GCA TTT CGCC
S2 (r)	TTA TCC ACT GCT TCT CAT TG

Key: *f*: forward; *r*: reverse (Yano *et al.*, 1998)

Appendix C**COMET ASSAY**

- Principle**

Comet Assay or single cell gel electrophoresis assay provides a simple and effective method for evaluating DNA damage in cells. The principle of the assay is based upon the ability of denatured, cleaved DNA fragments to migrate out of the cell under the influence of electric field. Undamaged DNA migrates slower and remains within the confines of the nucleoid when current is applied. Increase in DNA damage results in

smaller fragments which migrate faster in the electric field. Evaluation of the DNA “comet” tail shape and migration patterns allows for assessment of DNA damage.

- **Materials and Equipment**

Lysis Solution

Comet low melting agarose (LMA)

Trevigen CometSlide™

200 mM EDTA, pH 10

10X PBS, Ca⁺⁺ and Mg⁺⁺ free

NaOH Pellets

Dimethylsulfoxide (DMSO)

10X TBE Buffer

Silver staining kit

Glacial acetic acid

Methanol

Deionised water

Pipette and tips

Boiling water bath and 37° C water bath

Horizontal electrophoresis apparatus

Light transmission microscope

1 L graduated cylinder

Eppendorf tubes

Improved Neubauer counting chamber

- **REAGENT PREPARATION**

- 1. TBE (1X)**

100ml of TBE (10X) was added to 900mls of distilled water to obtain TBE (1X).

To prepare 10X TBE:

Tris Base = 108g

Boric acid = 55g

EDTA = 9.3g

Tris base was dissolved in 900ml of distilled water and the volume adjusted to 1litre.

The solution was stored at room temperature.

- 2. 5% Acetic acid v/v**

25ml of acetic acid added to 475ml distilled water to obtain the needed total volume of 500ml.

- 3. 70% ethanol**

280ml of ethanol was added to 120ml of distilled water to obtain the needed total volume of 400ml.

- 4. 1X PBS (Ca ++ and Mg ++ free)**

Used concentration w/v: weighed 9.55g of the PBS and dissolve in 1litre of the distilled water, Homogenized and autoclaved.

- 5. Lyses solution**

Add 40mls of lysis solution (from manufacturer) to 4mls of dimethylsulfoxide (DMSO). Chill at 4°C or in ice for at least 20mins before used. (Addition of DMSO is optional and is required only for samples containing heme, such as blood cells or tissue samples). Buffer formulation is proprietary.

- **Methodology**

1. Melt low melting agarose in a beaker or boiling water (100°C) for 5min. (loosened cap)
2. Transfer to 37°C water bath for at least 20mins to cool.
3. Add low melting agarose (37°C) 500µl + 50µl PBS + cells
4. 500µl + 50µl cells 1:10
5. 1:10 cells (PBS) + 50µl agarose
6. Pipette 75µl immediately unto comet slides and spread evenly. (When working with many samples place aliquots of molten agarose in a pre-warmed microcentrifuge tubes placed at 37°C to prevent hardening. If cells (sample) are not spreading evenly on the slide; warm the slide at 37°C before application).
7. Place slide flat at 4°C in the dark (refrigerator) for 10mins. A 0.5mm clear ring appears at the edge of CometSlide area. Increasing gelling to 30mins improves adherence of samples in the high humidity environments.

8. Prepare lysis solution 20mins after chilled on ice before use. For 10 slides prepare 40ml lysis + 4ml DMSO chilled at 20mins before use.
9. Immerse slide in prechilled lysis solution and leave on ice or at 4°C for 30min to 60mins.
10. Tap excess buffer from slide and immerse in freshly prepared Alkaline solution, pH>13.

(alkaline solution is prepared by dissolving 0.6g of NaOH pellets in a mixture of EDTA (200mM, 250µl) and distilled water (49.75ml). The solution warms during preparation, so should be allowed to cool to room temperature.
11. Leave CometSlide in alkaline solution for 20mins to 60mins at room temperature, in the dark.

- **TBE ELECTROPHORESIS**

12. Remove slide from alkaline solution; gently tap excess buffer from slide and wash by immersing in 1X TBE buffer for 5mins, twice.
13. Transfer slide from 1X TBE buffer to a horizontal electrophoresis apparatus.

Place slides flat onto a gel tray and align equidistant from the electrodes. Pour 1X TBE buffer until level just covers samples. Set power supply to 1 volt per cm (measured electrode to electrode). Apply voltage for 10mins.
14. Gently tap off excess TBE, and dip slide in 70% ethanol for 5mins.

15. Air-dry samples. Drying brings all the cells in a single plane to facilitate observation. At this stage, samples may be stored at room temperature, with dessicant. Sample must be well dried before staining.

- **EPIFLUORESCENCE STAINING PROCESS**

- **Preparation of SYBR Green Staining Solution**

Composition

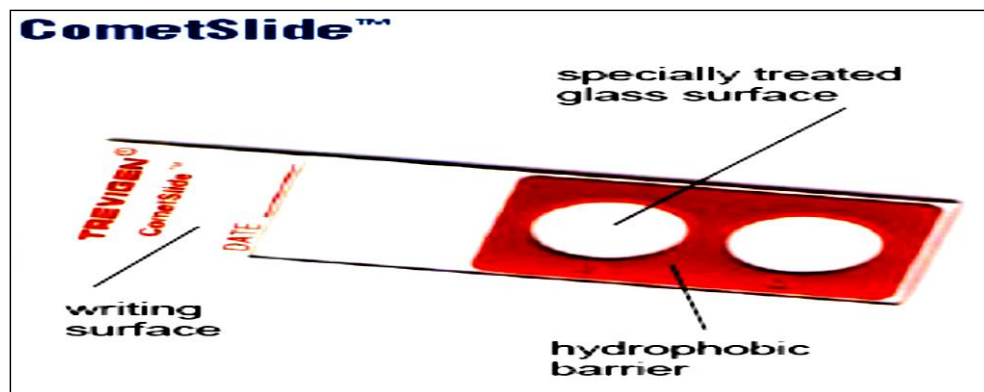
- | | |
|---|------|
| 1) SYBR Green I | 1µl |
| 2) 1×TE buffer, pH 7.5, (TE: 10mM Tris-Cl pH 7.5, 1mM EDTA) | 10µl |

Add 5ml of 1M Tris-HCl to 1ml 0.5M EDTA and add distilled H₂O to 500 ml

Prepare stain when ready to stain immediately.

- **Staining Reaction**

Cover sample area with 50µl of diluted staining solution (SYBR Green 1).



CometSlide

Analysis and evaluation table showing visual classification of DNA damage, according to the relative proportion of DNA in the tail (score 0-4), obtained by single-cell electrophoresis. Score 0 represents undamaged cells and score 4 represents the most heavily damaged cell.

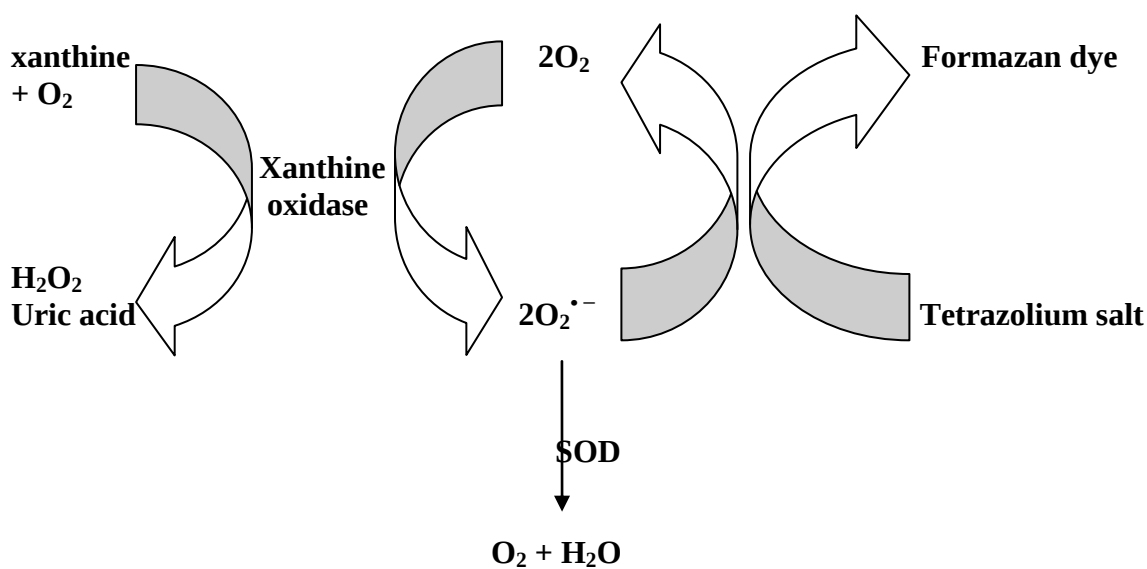
Scores	0	1	2	3	4
Percentage DNA in the tail	<5	5-20	20-40	40-80	>80
Average	2.5	12.5	30	60	90
	0%	6.8%	25%	55%	85%
	0%	12%	28%	65%	97%

Appendix D

Superoxide dismutase (SOD) assay

- **Principle**

The assay quantitatively measures SOD activity in a variety of samples. It utilizes a tetrazolium salt for the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. The superoxide radicals generated convert the tetrazolium salt into a yellow coloured formazan dye with absorbance at 450nm. The SOD in the sample competes with the salt for the superoxide radicals and thereby inhibits the production of the formazan dye. The degree of inhibition corresponds to the activity of SOD. High SOD activity causes a decrease in the superoxide radical concentration by conversion into molecular oxygen and hydrogen peroxide resulting in a decreased pigmentation of the formazan dye.



- **Materials and Reagents**

SOD Assay kit (*Cayman Chemicals, Michigan- USA*)

Deionized water

Centrifuge

Pipette and tips

Eppendorf tubes

96 well microplate

Plate reader

- **Reagent Preparation**

- 1. Assay Buffer (10x)**

Dilute 3mls of assay buffer concentrate with 27mls of HPLC-grade water (or de-ionised distilled water) for assaying 96 wells. This final assay buffer should be used to dilute the radical detector. Store at 4°C, this is stable for at least two months.

- 2. Sample Buffer (10x)**

Dilute 2mls of sample buffer concentrate with 1.8mls of HPLC-grade water (or de-ionised distilled water) for assaying 96 wells. This is used to prepare the SOD standard and dilute the xanthine oxidase and SOD samples prior to assaying. Store at 4°C, this is stable for at least two months.

3. Radical Detector

Prior to use, transfer 50 μ l of radical detector to another vial and dilute with 19.95mls of diluted assay buffer for 96 wells. Cover with foil. The diluted radical detector is stable for two hours. Store unused radical detector at -20°C .

4. SOD Standard

Dilute 20 μ l of the SOD Standard with 1.98ml of sample buffer (dilute) to obtain the SOD stock solution. Take seven clean glass test tubes and mark them A-G. Add the amount of SOD stock and sample buffer (dilute) to each tube as described in manufacturer's manual.

5. Xanthine Oxidase

Prior to use, thaw one vial and transfer 50 μ l of the supplied enzyme to another vial and dilute with 1.95ml of sample buffer (dilute) for 96 wells. Store on ice. Preparation is table for one hour.

- **Sample preparation**

Plasma and Erythrocyte Lysate

1. Collect blood using an anticoagulant such as heparin, citrate, or EDTA
2. Centrifuge the blood at 700- 1,000 x g for 10 minutes at 4°C . Pipette off the top yellow plasma layer without disturbing the white buffy layer. Store plasma on ice until assaying or freeze at -80°C . The plasma sample is stable for at least one

month. Plasma should be diluted 1:5 with Sample buffer before assaying for SOD activity.

3. Remove the white buffy layer (leucocytes) and store at -80°C .
4. Lyse the erythrocytes (red blood cells) in four times its volume of ice- cold HPLC- grade water (or de-ionised distilled water)
5. Centrifuge at $10,000 \times g$ for 15 minutes at 4°C .
6. Collect supernatant (erythrocyte lysate) for assaying and store on ice. Store at -80°C if not assaying same day. Sample is stable for at least one month. The erythrocyte lysate should be diluted 1:100 with sample buffer before assaying or SOD activity.

- **Performing the Assay**

1. **SOD Standard Wells:** Add 200 μl of the diluted Radical Detector and 10 μl of Standard (tubes A-G) per well in the designated wells on the plate.
2. **Sample Wells:** Add 200 μl of the diluted Radical Detector and 10 μl of Sample to the wells.
3. Initiate the reaction by adding 20 μl of diluted Xanthine Oxidase to all the wells you are using. Note: If assaying sample backgrounds, add 20 μl of Sample buffer instead of xanthine oxidase.

4. Carefully shake the 96- well plate for a few seconds to mix. Cover with the plate cover.
5. Incubate the plate on a shaker for 20 minutes at room temperature. Read the absorbance at 440- 460 nm using a plate reader.

- **Calculations**

1. Calculate the average absorbance of each standard and sample (if assay was done in duplicates). If assayed, subtract sample absorbance from the sample.
2. Divide standard A's absorbance by itself and divide standard A's absorbance by all the other standards and samples absorbances to yield the linearized rate (LR).
3. Plot the linearized SOD standard rate (LR) (from step 2 above) as a function of final SOD activity (U/ml).
4. Calculate the SOD activity of the samples using the equation obtained from the linear regression of the standard curve by substituting the linearized rate (LR) for each sample.

$$\text{SOD (U/ml)} \left[\left(\frac{\text{sample LR} - \text{y-intercept}}{\text{slope}} \right) \times \frac{0.23\text{ml}}{0.01\text{ml}} \right] \times \text{sample dilution}$$

Appendix E

Consent Form

Title: Haptoglobin genotypic polymorphisms and longevity among the Ghanaian
Population in the Greater Accra Region

Principal investigator: Selikem Abla Nuwormegbe

Address: Medical Biochemistry Department, UGMS, Korle-Bu.

Dear Volunteer,

As a participant in this research you are required to read and understand the information on this form and give your consent by signing or thumb printing it before a witness. Please ask for explanation and/or further information as required.

Purpose of study

This study aims at investigating aging in Ghana so as to understand the aging process better and be better positioned to make interventions to improve/maintain quality of life in old age.

General information and your part in the study

To qualify to be a part of this study you must be a Ghanaian and aged 50years and above. You will be required to answer a questionnaire. A sample of your blood will be taken for laboratory analysis. Sterile techniques and disposable material will be used for all blood sample collection. Your privacy will be respected at all times.

Possible benefits

You will not receive any direct benefits from this study. However your participation will help us acquire more knowledge about aging in Ghana to help in the development of necessary interventions for successful aging.

Possible risks

Little pain from the bruising at the bleeding site is expected. The total volume of blood which will be collected from you will not cause you any harm.

Withdrawal from study

Participation in this study is strictly voluntary. You can decide not to participate and your decision will be respected.

Confidentiality

All information gathered will be treated as confidential material. You will not be named in any report.

Contacts

You may contact the principal investigator Selikem Ablu Nuworgmegbe, Department of Medical Biochemistry, University of Ghana Medical School, on phone number 0243309389 with any questions, complaints or problems related to the study.

Your rights as a participant

This research has been reviewed and approved by the University of Ghana Ethical Review Committee. The Ethical Review committee reviews research studies in order to ensure that participants' rights are protected even before the research is started. If you have any questions about your rights as a research participant, you may contact the Chairman, Ethical Review Committee, University of Ghana Medical School.

Volunteer agreement

The above document describing the benefits, risks and procedures for the research has been read and explained to me. I have been given the opportunity to ask questions which have all been answered to my satisfaction. I agree to participate as a volunteer.

Date

Signature/Thumbprint of Volunteer

If a volunteer cannot read himself/herself a witness must sign below:

I was present while the benefits, risks and procedures were read to the volunteer. All questions were answered and the volunteer has agreed to participate in the research.

Date

Signature/Thumbprint of Witness

I certify that the nature, purpose, potential benefits and possible risks associated with participating in this research have been explained to the above individual.

Questionnaire

Name:

Demographic characteristics

1. Age/Date of birth.....

2. Sex M F

Health Status

3. “In general, how would you rate your health at present?

Very good Good Moderate Poor Very poor

4. Sight

How would you rate your vision now as compared to your middle years? (40 years)

The same Worse Better

5. Hearing

How will you rate your hearing now as compared to you middle years? (40 yrs)

The same Worse Better

6. Have you been diagnosed with any non-communicable diseases/chronic

illnesses/disorders? Yes / No

7. If yes please specify

8. Do you have health insurance? Yes /No

9. Have you assessed health care from any facility or person in last 30 days? Yes /
No

If yes from hospital/clinic pharmacy traditional healer

Other, Specify.....

10. Do you need daily care? Yes / No

If yes what for?