Influence of Antioxidant Genotype and Antioxidant Status on Progression of Chronic Kidney Disease

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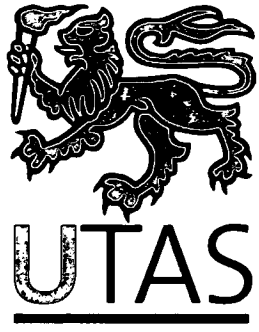
Submitted in fulfilment of the requirements for the
Degree of Doctorate in Philosophy

School of Human Life Sciences

University of Tasmania

October 2010

UNIVERSITY OF TASMANIA



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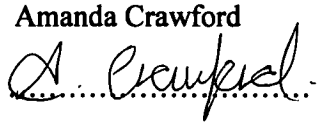
Signed 

Date 2010

Acknowledgements

I dedicate this thesis to my loving partner, Kevin Lyall, who donated one of his kidneys to me, so that I'd be able to avoid dialysis and live my life to the full. I thank Kevin for all his love and support on the journey of my PhD, he is my best friend. In addition, to my mother who died due to chronic kidney disease when I was young and who has been my driving force in my adult life and my inspiration to carry out research with a hope to help people with similar kidney problems.

I wish to express my sincere gratitude and appreciation to everyone who has been involved in the underlying work of this thesis, with special thanks to:

Professor Rob Fassett, co-supervisor, who recruited all patients for the trial along with Marianne Smith and Lisa Anderson. Without Rob's faith in me, I would not have become involved in this project that has a special place in my heart. He understood me when I said that I wanted to continue my studies, and that it had to be something that I was passionate about. If I am able to improve the life of one person who suffers from CKD, then I have succeeded in my goal.

Dale Kunde, my research consultant, who often had to deal with my research-related questions and also panics. Dale was my rock all the way through this process and a great friend.

Associate Professor Dominic Geraghty, my main supervisor and Graduate Research Coordinator. Dominic was a positive influence on me and kept my panics to a minimum.

Associate Professor Jeff Coombes, co-supervisor, who helped me develop my writing skills and has the patience of a saint.

Professor Madeleine Ball, co-supervisor, for her guidance and giving me the opportunity to study in the School of Human Life Sciences.

Dr Iain Robertson, biostatistician, who took the time to explain the confusing and sometimes very frustrating tricks of the statistical trade and also the program STATA.

Dr Kiran Ahuja, research fellow, who helped with many aspects of my PhD including statistical analysis, writing, positive encouragement and being a friend.

This research was possible thanks to the Clifford Craig Medical Research Trust, Renal Research Tasmania, and the University of Tasmania who provided generous funding and cofunded my scholarship. I would also like to take this opportunity to express thanks to all the participants involved in the study, without whom this research would not have been possible.

Although all the data collection, laboratory analysis and statistical analysis were all performed by myself, a number of people were instrumental in assisting with my PhD laboratory work, especially Jeff Becket, and the university technicians Hetty Binns and Barbara Parish. Special thanks to Merrilyn Johnson, who also kept me sane by taking me for long walks when I was very stressed; thank you so much.

I would also like to thank family, friends and colleagues, who were always available to help, support, and lend a hand and an ear during the years of my PhD candidature. Special thanks to my father and Rose for their support with everything I have strived for in life. In addition, thanks to my cousins, Connie and Alan and both their families, for supporting me and encouraging me to strive for my goals. I would also like to thank Jim and Jo Lyall, Rain and Beck Lyall-Elms for their support and Deborah Kunde for all the encouragement and entertainment through my PhD years.

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Abbreviations

CKD	Chronic kidney disease
GPx	Glutathione peroxidase
SOD	Superoxide dismutase
SNP	Single nucleotide polymorphism
eGFR	Estimated glomerular filtration rate
CVD	Cardiovascular disease
ESRD	End stage renal disease
MDRD	Modification of diet in renal disease
RBC	Red blood corpuscles
ROS	Reactive oxygen species
H_2O_2	Hydrogen peroxide
O_2	Oxygen
$O_2^{\bullet}$	Superoxide
NO	Nitric oxide
ONOO	Peroxynitrite
-OH	Hydroxyl radical
LOOH	Lipid hydroperoxide
LDL	Low-density lipids
AOPP	Advanced oxidation protein products
PD	Peritoneal dialysis
HD	Haemodialysis
DNA	Deoxyribonucleic acid
Zn	Zinc
Cu	Copper
Mn	Manganese
GSSG	Oxidised glutathione
GSH	Glutathione
Ala	Alanine
Val	Valine
Pro	Proline
Leu	Leucine
C	Cytosine
G	Guanine
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
NASH	Non-alcoholic steatohepatitis
ALD	Alcoholic liver disease
BMI	Body mass index

Abstract

Chronic kidney disease (CKD) is a significant public health issue that affects an estimated 11 percent of adults over the age of 25 years in Australia. Slowing the progression of kidney disease is a major therapeutic challenge for nephrologists. Oxidative stress is an imbalance between reactive oxygen species (ROS) and antioxidants levels in cells or tissues. It has been linked to a number of diseases, including cardiovascular disease (CVD) and CKD. Decreased antioxidant levels can be caused by a number of factors, such as, genetic mutations that reduce the effectiveness of the antioxidants, or toxins that deplete the concentration of antioxidant enzymes. Antioxidants may be separated into two distinct groups, exogenous and endogenous compounds. Important endogenous antioxidant enzymes include superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase which together form the primary defence system against ROS.

In recent years, the relationship between single nucleotide polymorphisms (SNPs) of antioxidant enzymes and diseases associated with oxidative stress has been a topic of significant interest. SNPs of the antioxidant enzymes SOD2 Ala16Val, GPx1 Pro197Leu and catalase C-262T have been associated with the pathogenesis of cancer, cardiovascular disease and diabetes. However, links between SNPs of these enzymes and CKD have yet to be investigated. Thus, the overall aim of the present study was to determine associations between antioxidant enzyme SNPs, antioxidant activities and renal function at baseline and changes thereof over a year in CKD patients.

Two major studies will be presented. The first uses a case-control design and baseline data from the CKD patient cohort (n=230) and control subjects (n=224) to determine (a) the frequency of specific SNPs, present in GPx, SOD and catalase genes and whether

antioxidant enzyme activities are significantly different between CKD patients (n=230) and control subjects (n=224), and (b) associations between antioxidant genotypes and renal function. The second study uses baseline and one year data from 185 CKD patients from the cohort and investigated whether the progression of kidney disease (i.e, decline in eGFR) was (a) associated with specific genotypes resulting from the aforementioned GPx, SOD and/or catalase SNPs, and (b) associated with altered plasma GPx, RBC GPx, RBC SOD and/or RBC catalase activities.

In the case-control analysis, significantly ($p=0.023$) more CKD patients had the GPx Leu/Leu genotype (n=5) compared to controls (n=0). Although not statistically significant, patients with the GPx1 Leu/Leu or SOD2 Ala/Ala genotypes had reduced eGFR compared with the GPx1 Pro/Leu and SOD2 Val/Val genotypes. CKD patients had significantly lower plasma GPx and RBC catalase activities compared to controls ($p<0.0001$). In contrast, both RBC GPx and RBC SOD activities were significantly higher in CKD patients ($p<0.0001$). In addition, in CKD patients, a significant positive association was found between eGFR and plasma GPx activity ($p<0.0001$). Plasma GPx increased ($p<0.0001$) and RBC GPx decrease ($p<0.05$) with disease progression from Stages 1 to 5. Interestingly, when the controls were stratified according to eGFR (and hence, Stage of CKD), 13% (30 subjects) were found to be in Stage 3 according to Kidney Disease Outcomes Initiative (KDOQI) guidelines.

The second study showed that eGFR declined over 12 months in both SOD2 Ala/Val and Val/Val patients, indicating a more rapid progression of kidney disease compared to patients with the Ala/Ala genotype (Ala/Val compared with Ala/Ala: $p=0.001$; Val/Val compared with Ala/Ala: $p=0.005$). The progression of CKD did not appear to be influenced by SNPs of GPx1 or catalase. There was a direct relationship between the rate in change of plasma GPx activity and the rate of change of eGFR over the 12 month period ($p=0.025$).

In summary, this is one of the largest case-control studies comparing antioxidant enzyme genotypes and activities in CKD patients conducted to date. In addition, this study is the first (cohort) study to investigate the role of antioxidant enzyme SNPs in the progression of CKD. The data suggest that CKD is associated with impaired plasma GPx and catalase activities and enhanced RBC GPx and SOD activities when compared to controls. Secondly, although genotype frequencies were similar for patients and controls, lower eGFR was associated with the GPx1 Leu/Leu genotype. Thirdly, CKD patients with the SOD2 Ala/Val or Val/Val genotype had a greater decline in kidney function over the 12 month study period when compared to patients with SOD2 Ala/Ala genotype. These findings suggest that SOD and GPx therapies that enhance the activities of these antioxidants may slow the progression of CKD. In addition, as 13% of the control participants were found to have impaired kidney function (eGFR $<60mL/min/1.73m^2$), there appears to be significant undiagnosed CKD in Northern Tasmania, that may warrant routine assessment of kidney function in the general population.

CHAPTER 1: GENERAL INTRODUCTION

1.1 Chronic Kidney Disease (CKD)

CKD is a debilitating and potentially fatal disease which affects an estimated one in seven of adults over the age of 25 years in Australia.[1] In 2008, there were 10,062 individuals receiving dialysis treatment in Australia with 2,476 newly diagnosed. Of these, 177 were recorded in Tasmania and 52 were recently diagnosed.[2] Treatment for end stage renal disease (ESRD) costs approximately $80,000 per patient per year.[3] However, the combined economic, social and personal cost is impossible to measure. To date, 177 Tasmanians have received kidney transplants with the follow-up care increasing the financial burden.[2] There is considerable social and personal cost involved which varies between patients due to their response to treatment and level of supportive care required for everyday activities. Therefore, tests that can identify individuals who may have a faster progression of CKD, may help with slowing the progression of this potentially fatal disease.

1.1.1 Stages

Renal function is monitored by measuring estimated glomerular filtration rate (eGFR). The clinical definition of CKD is eGFR of less than 60mL/min/1.73m^2 body surface area for longer than three months or demonstrable kidney damage.[4, 5] To aid in the development of specific treatment plans for each patient and to evaluate the disease progression, CKD has been classified by Kidney Health Australia into 5 different stages.[6] The scale starts at the early stages of CKD, which require no medical treatment, to renal failure, which requires either dialysis or kidney transplant for the survival of the patient (Table 1-1).

Table 1-1: Stages of Chronic Kidney Disease

Stages	Description	eGFR ($mL/min/1.73m^2$)
1	Kidney damage with normal/increased GFR. If the eGFR is >60mL/min/1.73m^2 for more than 3 months or more then this is classed as CKD.Defined when there has been kidney damage for more than 3 months, then this is classified as CKD. This will be determined by kidney biopsy or the presence of markers such as albumin/creatinine ratio or total protein/creatinine ratio which indicate kidney damage.	≥ 90
2	Kidney damage with small reduction in eGFR and classified as mild CKD.	60-89
3	This measurement of the eGFR has to have occurred for 3 months or more (with/without the kidney damage markers), classified as moderate CKD.	30-59
4	This measurement of the eGFR has to have occurred for 3 months or more (with/without the kidney damage markers), classified as severe CKD.	15-29
5	ESRD. The patient should be considered for renal replacement therapy, i.e. transplant or dialysis.	<15

Table Modified from Kidney Health Australia[6]

1.1.2 Progression / Monitoring

Monitoring the progression of renal disease is important for ensuring the efficacy of the treatment and also estimating the time until ESRD. The rate at which CKD progresses varies considerably and this makes it difficult to accurately determine the rate at which a patient's kidney function will deteriorate. In addition, it has been well documented and reviewed that the progression of kidney function is associated with the ageing process due to number of factors including the reduction in renal mass and changes in glomerular structure.[7] Diagnostic tests used to monitor the progression of CKD include creatinine clearance, serum creatinine concentration and plasma urea[5, 8]. Creatinine clearance is calculated using urine creatinine concentration (μmol/L) (measured from 24

hour urine samples), urine flow rate (mL/min)[5, 8] and the serum creatinine concentration (μmol/L) to give an estimate of true glomerular filtration rate (GFR) (healthy adults have a GFR of ~120mL/min).[5]

Serum creatinine concentration is a convenient way to estimate glomerular filtration (i.e., eGFR). There are two different ways by which this can be determined the Cockcroft-Gault, and Modification of Diet in Renal Disease (MDRD) equations. Both formulae use the age and gender of the patients. However, in the Cockcroft-Gault equation, the patient's weight is used as a regulator within the calculation, whereas the MDRD uses ethnicity within the equation.[4, 5] Serum creatinine concentration depends on muscle bulk and can change independently of renal function due to an increase or decrease in the patients' muscle mass. Therefore, due to variations in body composition with age, an athletic young man may have a similar serum creatinine concentration to an elderly woman with a renal problem. The reference range for an adult is 60-120μmol/L, although an individual's results will not vary by that proportion on a daily basis. There may be some discrepancies when measuring eGFR in the early stages of renal disease, as small increases in serum creatinine may result in major discrepancies in eGFR. There is also a problem when measuring eGFR in patients in the advanced stages of CKD, where small changes in eGFR do not reflect the large changes in serum creatinine.[9] MDRD was considered the standard calculation for determining eGFR in 2005, when this study was designed and still forms the basis for the current NKF KDOQI CKD classification. However, currently there is controversy regarding the use a new and maybe more accurate method of calculating eGFR, the Chronic Kidney Disease Epidemiology Collaboration (CKD-Epi),[10, 11] hence in future studies this measurement maybe replace MDRD.

It is important for the nephrologist to monitor the decline in renal function regularly, as this can help determine the effect that treatment is having on the kidney function as well

as help with estimating the time at which the patient will require dialysis or renal replacement.

1.1.3 Causes

There are a number of underlying causes of CKD. Some of these causes are outlined in Table 1-2.

Table 1-2: Causes of Chronic Kidney Disease

Cause of Disorder	Examples
Immunologic	Glomerulonephritis
Infection	Pyelonephritis, tuberculosis
Urinary Obstruction	Neoplasm, urethral constriction
Metabolic	Diabetes mellitus, gout, amyloidosis
Vascular	Hypertension, infarction, sickle cell anaemia
Hereditary Kidney Diseases	Polycystic Kidney disease, Bartter's syndrome, Alports syndrome, renal hypoplasia
Nephrotoxin-induced	Analgesic nephropathy, drugs, heavy metal poisoning, industrial solvents, radiation nephritis
Other	Multiple myeloma, hypocalcaemia

Table modified from Patho Physiology[12]

1.1.4 Incidences in Australia

In 2008, for the fifth consecutive year, diabetic nephropathy (34%), including type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM), was the main of cause of ESRD in Australia. Diabetes surpassed glomerulonephritis as the main cause of ESRD due to the increased numbers of individuals with T2DM, that make up 91% of diabetic nephropathy patients in Australia, who progressed to ESRD.[2] Glomerulonephritis, or inflammation of the glomeruli in the kidney, was the next major cause of ESRD in 2008 (22%). In addition, hypertension or vascular disease, contributes to 15% of CKD cases; reflux nephropathy, 3%; polycystic kidney disease, 6%; and analgesic nephropathy, 2%. These six are the main causes of CKD and the rest fall under the title of miscellaneous causes, which accounts for approximately 10% of CKD cases.[2] In Australia in 1998, there were 296 per million of the population receiving dialysis, and this increased in 2008 to 471 per million population.[2] The detection of

CKD is difficult in the early stages due to the lack of symptoms. However, in the later stages, uraemic syndrome is the main symptom of extreme renal failure, resulting from the increase in waste products (e.g., urea) in the blood and the reduced removal of nitrogen from the blood stream.[13] The symptoms of uraemic syndrome are discussed in more detail in section 1.1.5.

1.1.5 Symptoms

Most symptoms are identified under the heading of uraemic syndrome, resulting in systemic renal failure that affects all the body systems[12] (Table 1-3). The neurological system is affected by an increase in neurotoxins (mainly in the later stages of CKD), deficiency of ionized calcium in the spinal cord, retention of potassium and phosphates and altered fluid balance.[12, 14] As specific pathways gradually deteriorate, a number of symptoms occur ranging from drowsiness, an inability to concentrate and poor memory, to more severe symptoms including hallucinations and seizures that may eventually lead to coma.[14] When the gastrointestinal system is affected, the patient can have nausea, vomiting and/or anorexia. However, it is speculated that it could be due to the gastric ulcers that occur in a high percentage of CKD patients.[12, 121] Gastric bleeding also affects the haematologic system by causing anaemia. Anaemia can be caused by a number of other factors including the shortened life span of red blood corpuscles (RBC) and a decrease in erythropoietin (due to the reduction in renal production and secretion).[15, 16] Anaemia also occurs due to a deficiency in folate, iron and an increased production of parathyroid hormone. Parathyroid hormone leads to fibrous tissues taken up bone marrow space, which affects the overall musculoskeletal system.[12] In addition, the musculoskeletal system is affected by a calcium imbalance which results from a reduction in the bones' ability to absorb calcium, a condition known as osteodystrophy. This imbalance can also cause a number of bone lesion conditions.[12] However, the major effect of uraemic syndrome is on the cardiovascular system, with fifty percent of

CKD patients dying prematurely from cardiovascular complications.[17-19] The reasons for the deterioration in cardiovascular function are vascular changes and increased secretion of renin which leads to hypertension, pericarditis and narrowing of arteries. With numerous studies reporting links between CVD, inflammation and oxidative stress, this study focuses specifically on oxidative stress in CKD patients. Although, inflammation may play an important role in the oxidative stress along with progression of CKD, however this is beyond the scope of this thesis.[20]

Table 1-3: Clinical Features of Chronic Kidney Disease

Clinical Features	Description of symptoms
Cardiovascular	Hypertension, anaemia and pericarditis
Gastrointestinal	Anorexia, nausea, vomiting and gastrointestinal bleeding
Muscular and skeletal	Disorders of calcium and phosphorus, bone pain, myopathy and growth failure
Neuropath	Lethargy, peripheral neuropathy
Well being	Depression
Dermatological	Pruritus, pallor, ecchymoses and hyperpigmentation
Hematologic	Anaemia

Table modified from Patho Physiology[12]

1.2 Interactions between Reactive Oxygen Species and Antioxidants

1.2.1 Oxidative Stress and Antioxidants

The term oxidative stress is used to describe a relative increase in the amount of reactive oxygen species (ROS) and includes not only oxygen radicals but also non-radical derivatives such as, nitrogen and chlorine.[21] Oxygen free radicals and hydrogen peroxide (H_2O_2) are produced and reused during normal metabolism by cells. The ROS have an unpaired electron giving them the capability to cause damage by reacting with lipids, proteins and deoxyribonucleic acid (DNA).[19] Oxidative stress is an imbalance between antioxidants and oxidants. The progression of many diseases has been linked to oxidative stress, including CVD,[22] cancers,[23] diabetes,[24] CKD,[25] Alzheimer's disease and other neurodegenerative diseases.[26] ROS are unstable and cause damage to macromolecules via oxidation. Oxidative stress can occur due to an increased

concentration of reactive species and/or a reduction in antioxidant capacity. This can be caused by a number of factors such as a genetic mutation that alters the effectiveness of the antioxidants,[27] toxins that diminish the antioxidant enzyme[28] or disease processes that can deplete the concentration of antioxidants.[29] Antioxidants are the body's defence against ROS and may be exogenous or endogenous compounds. Exogenous antioxidants are consumed in the diet and consist largely of carotenoids (e.g. β-carotene), tocopherols (e.g. vitamin E), ascorbic acid (vitamin C) and selenium. Endogenous antioxidants include thiols such as glutathione and the enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase. These antioxidant enzymes are an important defence against ROS and work together by scavenging ROS and thereby reducing oxidative stress.

1.2.2 Superoxide Dismutase (SOD)

SOD contains an active site that has transition metals for rapid electron exchange. The major role of SOD is to scavenge the free radical, superoxide ($O^{2\bullet}$) and convert it to H_2O_2 (Figure 1-1).[30] Although there are many factors that affect the activity of these antioxidants, it has been reported that high levels of H_2O_2 inhibit SOD and activate catalase.[31] Three isoforms of SOD have been identified. SOD1 contains copper (Cu) and zinc (Zn) within the active site (also known as CuZn SOD), and is mainly found in the cytoplasm of cells. The second isoform is SOD2 or manganese (Mn) SOD which contains manganese in the active site. SOD2 is the only antioxidant enzyme known to be present within mitochondria. This has important implications due to mitochondria being a major site for the production of ROS during normal cellular metabolism.[32] SOD3, or extracellular (EC) SOD, also has Cu and Zn within the active site and is the least studied of the three SOD isoforms.[33]

1.2.3 Glutathione peroxidase (GPx)

GPx is a selenioprotein, which is responsible for the breakdown of H_2O_2 and other organic peroxides, including oxidised membrane phospholipids, as well as cholesterol and long chain fatty acids (Figure 1-1). GPx metabolises these substances and converts them to water and oxidised glutathione (GSSG) using glutathione as a reducing agent.[34] In the structure of GPx, there is a single seleno-cysteine residue present in the active site which is essential for the activity of GPx and is present in all five isoforms of the enzyme that have been identified to date.[35-37] GPx1 (cGPx; RBC GPx) is found in cytoplasm and red blood cells (RBC). It is synthesised in the kidney and also produced in smaller amounts by the liver.[38] GPx2 is a cytosolic form found in liver and colon, whereas GPx3 (plasma GPx) is the only extracellular form of the GPx family that is found in plasma. GPx3 is primarily produced within the proximal tubule cells in the kidney where decreased activity correlates with a decrease in renal function.[39-41] GPx4 (PHGPx) is a phospholipid hydroperoxide and a monomer in structure, whereas the structure of the other GPx's are tetramers. GPx4 is found in most tissues, although it has been found in high levels in the testis and is a structural protein in the heads of sperm.[42] Finally, there are few details on GPx5 available.[43, 44] It has been thought that GPx is the main antioxidant enzyme involved in the breakdown of H_2O_2. However, more recent studies suggest that GPx and catalase activity share this role equally.[45, 46]

1.2.4 Catalase

Catalase is a tetrameric haemoprotein that converts H_2O_2 to H_2O and O_2 (Figure 1-1). The reaction requires two H_2O_2 molecules; therefore it has a high conversion rate.[47] Catalase is present in all aerobic cells, with the highest levels in the liver, kidney and erythrocytes. Acatalasaemia is an inherited autosomal recessive disease caused by an abnormality of the catalase gene. This disease is characterised by severely reduced catalase activity within erythrocytes and the main clinical symptom is persistent oral

gangrene.[48] In addition, it has been proposed that impaired catalase activity has a direct relationship to the oxidant sensitivity of glucose-6-phosphate dehydrogenase (G6PD)-deficiency and impaired catalase activity. G6PD maintains levels of glutathione, which is intimately involved in the conversion of H_2O_2 to water and oxygen by GPx.[49]

1.2.5 Pathways for Reactive Oxygen Species and Antioxidants

A number of biochemical reactions produce $O_2^{\bullet}$, a very reactive substance due to the extra outer shell electron. In crossing biological membranes, it can either promote free radical signals or induce oxidative damage. Antioxidants protect cells and tissues from damage by ROS and are able to recycle the ROS present in the cell or tissue (Figure 1-1).[50, 51] SOD is responsible for the conversion of superoxide to H_2O_2 through a catalytic reaction. However, superoxide may also react with nitric oxide (NO), a vasodilator released by endothelial cells, producing peroxynitrite ($ONOO^{\bullet}$), which is also a very reactive and permeable oxidant. H_2O_2 can react or interact with a number of chemicals and follow several different pathways. One of these pathways involves the interaction of H_2O_2 with iron or copper to produce the hydroxyl radical ($^{\bullet}OH$) – another ROS. However, when an imbalance occurs, due to either a decrease in the antioxidants (e.g., SOD, GPx or catalase) or an increase in ROS levels, the cell succumbs to oxidative stress. A reduction in either enzyme function or over-production of ROS can lead to oxidative stress, and this has been linked to the progression of kidney disease.[13, 44, 52-54]

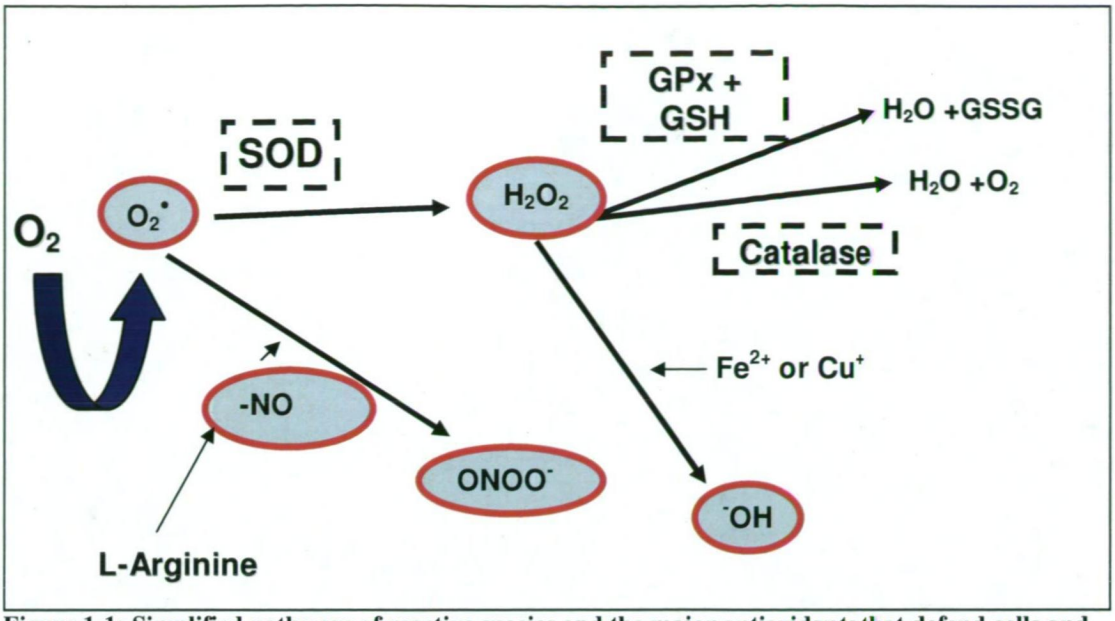

Figure 1-1: Simplified pathways of reactive species and the major antioxidants that defend cells and tissue from damage.
Diagram modified from Morena et al. and Tylicki et al.[50, 51]
Superoxide ($O_2^{\bullet}$); oxygen (O_2); superoxide dismutase (SOD); glutathione peroxidase (GPx); hydrogen peroxide (H_2O_2); peroxynitrite ($ONOO^{\bullet}$); nitric oxide (NO); hydroxyl radicals ($^{\bullet}OH$); iron (Fe^{2+}); copper (Cu^{+}); hydrogen (H_2O); oxidised glutathione (GSSG); glutathione (GSH).

1.2.6 Markers of Oxidative Stress

CKD patients are monitored for an increased risk of developing CVD and this is normally predicted by the Framingham risk factors.[55] However, in more recent times there has been an increased use of less traditional methods of measuring the risk of CVD, *viz*, the measurement of specific chemical biomarkers present in the body that signal the increase in oxidative stress and/or inflammation.[17, 19, 44, 54] The biomarkers that are currently used to indicate oxidative stress and inflammation in humans are listed in Table 1-4.

Table 1-4: Common Biomarkers for Oxidative Stress and Inflammation

BioMarkers	Marker for	References
Lipid Oxidation	Oxidative	17, 44, 54, 56-59
LOOH	stress	
LDL		
TBA		
TBARS		
Arachidonic Acid	Oxidative	17, 25, 54, 58
Derivatives	stress	
F2-isoprostanes		
Carbohydrate		17, 58
AGE		
Amino Acids	Inflammation	17, 18, 25, 54
Cysteine		
Homocystiene	Oxidative	17, 56, 57, 60
	stress	
Cytokines	Inflammation	17, 18, 25, 54
(IL)-6		
TNF-a		
Proteins	Inflammation	17, 18, 25, 54, 57, 60
CRP		
Thiol oxidation	Oxidative	17, 25
Carbonyl formation	stress	
AOPP	Oxidative	17, 18, 58
	stress	
Deoxyribonucleic acid	Oxidative	58
8-OHdG	stress	

LOOH, lipid hydroperoxide; LDL, low-density lipids; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substance; AGE, advanced glycosylation end products; (IL)-6, interleukin; CRP, C-reactive protein; AOPP, advanced oxidation protein products; 8-OHdG, 8 hydroxy 2' deoxyguanine. Table modified from Himmelafarb.[17]

High-density lipoprotein (HDL) is involved in reducing oxidized low density lipids (LDL). As renal function decreases, so does the concentration of HDL, and an increase in oxidized LDL will occur due to oxidative stress.[44] It has been noted that lipid peroxidation is increased in haemodialysis (HD) and ESRD (creatinine 666 ± 91μmol/L) patients compared with healthy controls, thus demonstrating that both HD and ESRD patients are subject to oxidative stress.[19] Thiobarbituric acid reactive substances (TBARS) are another measure of oxidative stress. TBARS measure aldehydes, such as malondialdehyde (MDA). However, this test is not very specific and can be quite inaccurate.[58] F2-isoprostanes are markers for oxidative stress and measure

the levels of free radicals produced by oxidation of arachidonic acid. Free F2-isoprostanes have been shown to increase in CKD patients.[25] This biomarker test is relatively new and has been found to be more reliable for determining levels of oxidative stress, although it is a more complex test that requires expensive equipment.[58] Inflammatory cytokines and proteins such as C-reactive protein (CRP) and fibrinogen are also believed to be good markers.[18] The levels of CRP have been found to predict mortality in HD patients.[57] It has also been shown to be associated with the development of CVD complications in CKD patients.[18]

Oxidative proteins, such as advanced oxidation protein products (AOPP), are more stable markers than lipids. The increase in AOPP in CKD patients has been associated with an increased risk of developing CVD complications.[18] However, it has been demonstrated that an increase in triglyceride levels in both CKD patients and healthy controls can increase the measurement of AOPP.[61] Another protein marker for oxidative stress is carbonyl formation which indicates the amount of reactive aldehyde in plasma.[25] Oxidative stress has also been linked to damaged DNA, by mutated bases or breaks on strands caused by ROS for which the biomarker is 8 hydroxy 2' deoxyguanine (8-OHdG).[58] Other factors that have been found to be associated with CVD and are related to oxidative stress are malnutrition and endothelial dysfunction.[17] Endothelial cell dysfunction has been shown to be important in the early stages of cardiovascular complications.[59] Although the antioxidant enzymes, GPx, SOD and catalase, have been suggested as markers for oxidative stress, there have been conflicting data published in relation to CKD. However, only plasma GPx has shown potential as a marker of oxidative stress in CKD patients.[13, 35, 53, 62-67]

1.2.7 Antioxidant Single Nucleotide Polymorphisms (SNPs)

In recent years, there has been increased interest in the relationship between mutations of the genes encoding antioxidant enzymes and the diseases associated with oxidative

stress. The most common mutation studied has been the SNP which occurs when single bases are changed or deleted. This mutation may result in a change in amino acid and hence, a change in phenotype. During the course of the present study, a number of large population studies emerged in the literature that aimed to expand the database of SNPs and haplotypes present in the human genome. The International HapMap Project[68], is a recent database set up to develop a haplotype map, to be used for identify common genetic variations in of the human genome. In addition, the Human Variome Project[69]and 1000 Genomes Project[70] were also both set up to help in the determining the effect of genetic variation on human health. The SOD, GPx and catalase SNPs selected for this study constitute the majority of the published research on antioxidant SNPs. The SOD2 rs4880 Ala16Val SNP is a substitution of alanine (Ala) to valine (Val) at amino acid position 16. The GPx1 rs1050450 Pro197Leu SNP, a substitution of proline (Pro) to leucine (Leu) at amino acid position 197. Catalase rs1001179 C-262T SNP (C: base cytosine; G: base guanine) have not been examined to the same extent as the SOD2 SNP, although both appear to have significant associations with disease. An extensive review of the literature regarding these three SNPs and disease will be covered in section 2.3. The aforementioned SNPs are reported to be associated with altered progression and/or risk of several diseases, including CVD,[71-73] breast cancer,[74] lung cancer,[75] diabetic neuropathy[76] and acute renal failure.[77] Based on recent literature, there is potentially a link between diseases associated with oxidative stress, antioxidant genotypes and activities. However, these SNPs have yet to be investigated in relation to CKD. Therefore, a primary aim of this thesis is to determine if there is an association between CKD and the antioxidant gene SNPs, SOD2 Ala16Val, GPx1 Pro197Leu and catalase C-262T.

CHAPTER 2: LITERATURE REVIEW

2.1 Relationship Between Oxidative Stress and CKD

Oxidative stress has been shown to be particularly prevalent in CKD patients.[19, 25, 56, 59, 78, 79] As early as 1994,[19] it was shown that there was a correlation between HD and oxidative stress. Increased lipid peroxidation has been linked with the increase in mortality of HD patients.[57] Oberg, McMenamin-Crow et al.[80] concluded that even before renal replacement is required, patients with CKD at stages 3-5 show signs of oxidative stress.[25] Antioxidant activities (SOD, GPx and catalase) have been used as markers in HD and CKD at stage 4 and 5 patients.[19, 59, 79, 81] Other studies have shown that when a patient receives a kidney transplant, there is a notable decrease in the levels of oxidative stress markers compared with patients on HD, and antioxidant levels in transplant recipients may return to normal levels when they have normal renal clearance.[78] Oxidative stress in CKD patients has been linked to endothelial-dysfunction which has also been associated with the increase in the mortality of CKD patients from premature CVD.[59] In addition, endothelial-dysfunction has been reported to be present in all stages of CKD and has a strong correlation with the decline in eGFR.[82] Impaired endothelium-dependent vasodilation and oxidative stress is apparent in stage 5 CKD patients. In a study involving 37 patients with chronic renal failure that were thought to require renal replacement therapy in the near future, oxidised glutathione (GSSG) positively correlated with serum creatinine concentration.[59, 79] A study by Fumeron, et al,[83] reported that in HD patients, GSSG and glutathione (GSH) increased during short term treatment with vitamin C supplements. However, GSSG and GSH returned to pre-supplementation levels soon after treatment.[83] This shows that although GSSG and GSH are affected by the decrease in the kidney function, the addition of vitamin C supplementation short term, does not increase GSSG and GSH sufficiently to have a significant benefit on HD.[83] Finally, it has been shown that patients on HD with

increased iron storage also have DNA injury due to oxidative stress.[84] In conclusion, there is some evidence that oxidative stress is prevalent in CKD and dialysis patients which may contribute to the progression of the disease and associated co-morbidities.

2.2 Antioxidant Activities in Relation to CKD Disease

The relationship between antioxidant enzymes and CKD has mainly revolved around glutathione peroxidase (GPx). Indeed, eight studies have examined GPx activity in RBC lysate or plasma in non-dialysis patients[13, 35, 53, 62-66] and of these, two also investigated SOD activity.[53, 63] However, there have been no studies that have examined the activities of all three antioxidants in CKD patients to date.

Although, Zwolisska and co-workers[85] examined RBC GPx, SOD and catalase activities in child/young adult (aged 5-18 years) dialysis patients (peritoneal dialysis (PD), 10; HD, 21), and found that both RBC GPx, RBC SOD and catalase enzyme activity were significantly reduced compared to healthy age matched controls. However, this result did not correlate with the patient's serum creatinine levels.[85]

Studies examining the relationship between GPx enzyme activity and CKD have resulted in conflicting reports, depending on whether activity was measured in plasma or RBC. Zachara et al.[62] examined 150 CKD patients at different stages of the disease and found CKD patients had decreased RBC GPx. However, there was no association with the antioxidant activity and stage of the disease.[62] In addition, three smaller studies all supporting this finding in CKD patients. Richard and co-workers examining 14 CKD patients with high blood creatinine levels (~442μmol/l) found that RBC GPx was significantly reduced compared to a healthy control group.[65] Another study that involved renal patients at different stages of impairment, including 12 patients diagnosed with nephrotic syndrome, 48 CKD, 50 HD patients, and 50 healthy controls, also reported RBC GPx activity was significantly decreased in the nephrotic syndrome

group and CKD patients when compared with the control group.[35] Bulucu et al.[64] also reported reduced RBC GPx activity compared with control populations. In addition, both studies reported that there was no correlation between RBC GPx activity and serum creatinine.[35, 64] In contrast, Ceballos-picot et al.[53] conducted a large case-control study involving 185 non-dialysis CKD patients (divided into groups from mild to severe CKD, based on creatinine clearance), and found that RBC GPx activity was increased in CKD patients compared with 31 healthy controls.[53] Furthermore, this finding was supported by a smaller case-control study with 36 CKD patients compared to 13 healthy controls.[63] However, this study also reported that RBC GPx activity increased with the severity of CKD.[63]

Plasma GPx activity has been reported to be significantly decreased compared to healthy subjects and the decline correlates with the decline in the kidney function.[13, 35, 53, 62, 63, 66] Ceballos-picot et al.[53] reported that in 185 non-dialysis patients (with mild to advanced renal failure) and 48 patients receiving dialysis (HD and PD), plasma GPx activity declined gradually as the kidney disease progressed. In addition, dialysis patients had significantly decreased plasma GPx activity compared with the control group. In a smaller study of only 18 CKD patients and 78 dialysis patients (20 HD and 58 PD), plasma GPx activity in CKD was lower than in 118 healthy controls and inversely related to serum creatinine.[66] Plasma GPx activity was also significantly reduced in the HD and PD patients compared to controls, and plasma GPx activity was significantly higher in HD patients compared with the PD patients. The lowest plasma activity was found in females that were receiving PD. However, within the control group, females had higher plasma GPx activity compared with male controls.[66] In addition, this study reported that RBC GPx activity in non-dialysis and dialysis patients was significantly higher than in controls.[66]

El-far et al.[35] reported CKD patients had significantly reduced plasma GPx activity compared with the control group. However, this was not the case in patients with nephrotic syndrome. In addition, in CKD patients, there was a correlation between plasma GPx activity and serum creatinine and blood urea nitrogen levels, whereas RBC GPx activity showed no correlation with these two parameters. Plasma GPx activity was significantly increased pre-dialysis compared with post-dialysis but when compared with the control group, both were significantly decreased.[35] Another large study that examined plasma GPx enzyme activity involved 150 patients between the ages of 18-80 at different stages of CKD. The patients were divided into four groups (incipient, 55; moderate, 40; advanced, 40 and ESRD, 15), with the controls consisting of 30 healthy subjects aged between 23-64. Plasma GPx activity was significantly different between CKD patients and healthy controls. This study also found that as kidney function declined that there was a corresponding decrease in plasma GPx activity and patients with ESRD had one third the plasma GPx activity of healthy controls.[62]

Two smaller studies also found plasma GPx activity was significantly reduced compared to controls. Bulucu et al.[64] examined 20 nephrotic syndrome patients and Richards et al.[65] investigated two groups, 14 non-dialyzed uremic patients and 17 CKD patients. Schiavon et al.[13] investigated only plasma GPx enzyme activity in 130 individuals at various stages of CKD and subdivided them into groups according to their diagnosis, and compared them with 20 controls. They found that the plasma GPx activity was significantly reduced in patients with ESRD, prerenal ureamia and Bartter's syndrome, and renal transplant recipients. It was also noted that when plasma GPx activity decreased, this coincided with the increase in serum creatinine.[13] In addition, it has been well documented that plasma GPx is primarily produced within the proximal tubules of the kidney. Hence, when kidney function declines, the production of plasma GPx is also reduced.[39-41]

Finally, only two studies examined both GPx and RBC SOD activity in CKD patients. Ceballos-picot and co-workers[53] found that RBC SOD activity in CKD patients was similar to that of the control population, whereas RBC SOD activity was significantly reduced in HD patients compared with controls.[53] In contrast, Mimic-Oka and co-workers[63] found that RBC SOD activity was significantly increased in 36 non-dialysis patients compared with 13 controls and 10 HD patients. However, they also reported that HD patients had a decreased RBC SOD activity compared to both the control group and the non-dialysis CKD patients.[63] It should be noted that this study was small and the age range was limited to 21-56 years old.

In conclusion, studies of RBC GPx and SOD activities in CKD patients have been contradictory and catalase activity has yet to be investigated. Moreover, plasma GPx activity, although extensively investigated, has not been examined in CKD patients over an extended time period. To resolve the previous contradictory reports, the present study used large CKD and control populations and studied a cohort of the CKD patients over a 12 month period. Furthermore, as the influence of antioxidant enzyme SNPs in CKD patients' aetiology was unknown, the antioxidant genotypes of all participants were determined.

2.3 Relationships Between SNPs of Antioxidant Enzymes and Disease

2.3.1 Abstract

The presence and progression of numerous diseases have been linked to deficiencies in antioxidant systems. The relationships between abnormal genotypes arising from specific antioxidant enzyme single nucleotide polymorphisms (SNPs) and diseases associated with elevated oxidative stress have been widely studied. The purpose of this review is to analyse evidence from these studies. The antioxidant enzyme SNPs selected for analysis in this review are based on those most frequently investigated in relation to diseases in humans: superoxide dismutase (SOD)2 Ala16Val (79 studies), glutathione peroxidise (GPx)1 Pro197Leu (20 studies) and catalase C-262T (18 studies). Although the majority of evidence supports associations between the SOD2 Ala16Val SNP and diseases such as breast, prostate and lung cancer, diabetes and cardiovascular disease, the presence of the SOD2 Ala16Val SNP confers only a small, clinically insignificant reduction in the risk of these diseases. Other diseases such as bladder cancer, liver disease, nervous system pathologies and asthma have not been consistently related to SOD SNP genotypes. The GPx1 Pro197Leu and catalase C-262T SNP genotypes have been associated with breast cancer, but only in a small number of studies. Thus, currently available evidence suggests antioxidant enzyme SNP genotypes are not useful for screening for several specific diseases in humans.

2.3.2 Introduction

The most common mutation in DNA studied has been the SNP. A SNP occurs when single bases in genes are changed or deleted, and may result in an amino acid change at a specific position and therefore may produce a change in phenotype. The purpose of this section is to review and analyse studies that have investigated the relationships between SNPs arising from GPx, SOD and catalase antioxidant enzyme and diseases associated with elevated oxidative stress. After a general discussion of their many polymorphisms, one specific SNP and resulting genotypes will be examined in more detail. The antioxidant SNPs selected for this review and the present study were based on those most frequently studied in relation to diseases in humans. Indeed, the SNPs chosen in this review constitute the majority of the research in this area, i.e., of the 90 articles reporting on SOD SNPs and disease, 79 addressed the SOD2 Ala16Val SNP, 20 of 27 GPx studies investigated GPx Pro197Leu, and 18 of 25 catalase articles centred on the C-262T SNP. The National Centre for Biotechnology Information (NCBI) database was used to identify antioxidant enzyme SNPs and the PubMed (Medline) database was searched to locate original studies that investigated the relationships between the genotypes involved with the SNP and disease. The searches were completed on 1st June 2009.

2.3.3 Superoxide Dismutase (SOD) Isoforms and SNPs

The various isoforms of SOD have been discussed in section 1.2.2., with 111 SNPs identified for SOD1, 190 for SOD2 and 100 for SOD3 (NCBI) (1st June 2009) (see Appendix A5.2. for a link to SOD SNPs).

Two SNPs of SOD1 have been studied in relation to disease and referenced in the NCBI database. The rs7277748 SNP is present in exon 1, in the TATA box promoter region, and changes A>G. No association was found between this SNP and familial

amyotrophic lateral sclerosis.[86] The SNP, rs1788180, is present in intron 1 and changes C>T. This SNP was found to be associated with an increased risk of nephropathy in patients with T1DM.[87]

There have been nine SNPs reported for SOD2 that are present in exons and therefore change the amino acid, and may affect the enzyme produced. Only two of these SNPs have been investigated in relation to disease occurrence. The most investigated SOD SNP, rs4880, is present in exon 2 and substitutes a C>T at position 2734. This changes the amino acid from alanine (Ala) to valine (Val) at position 16 (SOD2 Ala16Val genotype, although it has been referred to as Ala-9Val due to the SNP being at position 9-amino acid upstream of cleavage site).[88] This SNP has been the most widely studied because the Val allele of this SNP results in reduced expression and production of an unstable mRNA, which affects the import of SOD2 into the mitochondrion.[89] Seventy-nine studies have investigated the relationship between the Ala16Val mutation and disease (see later 2.3.4). Another SOD2 SNP that is located in an exon is rs5746136 that produces an A>G substitution, and has been associated with an increased risk of Alzheimer's disease.[90] A recently described mutation in the promoter region changes C>T at position -102, and therefore does not code for amino acids.[91-93] This SNP is not yet referenced in the NCBI database but the resulting SNP genotype has been associated with a positive outcome in breast cancer patients receiving radiotherapy.[92]

Five SNPs have been identified in the 5' end in the SOD3 gene, none of which code for amino acid production, although all have been investigated in relation to disease. Two SNPs, rs8192288 and rs8192287, are both mutations that change G>T and both have been investigated in relation to lung function (in normal individuals) and found to be associated with reduced forced vital capacity.[94] One study compared six SNPs present in SOD3 in hypertensive subjects and normotensive controls and found no differences in genotype frequency. Of the six SNPs, only rs13306703 and rs2536512 were

significantly associated with the presence of essential hypertension.[95] Two of the SNPs present in exon 2, rs1799895 and rs2536512, are A>G missense mutations. One of the other SNPs was present in intron 1, rs17881426, and substitutes A>T. The other three were identified in the 5' region: rs13306703, which is a C>T substitution; rs699474, which is an A>C/G substitution, and rs699473, which is also a C>T substitution. The latter mutation was found to have no association with the occurrence of brain tumours.[96]

2.3.4 SOD2 (MnSOD) Ala16Val and Disease

There have been 79 studies investigating the relationship between the SOD2 Ala16Val genotype and disease, approximately half of which reported a relationship (Table 2-1). Associations with breast cancer,[97-108] bladder cancer,[109] Behcet's disease,[110] prostate cancer,[111-117] lung cancer,[118, 119] cardiovascular disease,[71, 72] acute myeloid leukaemia,[120] cardiomyopathy,[73] T1DM and T2DM,[121-126] hypersensitivity to para-phenylene diamine,[127] sclerosis,[128] non-alcoholic steatohepatitis,[129] malignant pleural mesothelioma,[130] motor neuron disease,[131] brain tumours,[96] and schizophrenia[132, 133] have been reported.

No association between SOD2 Ala16Val genotypes and breast cancer,[104, 134-136] bladder cancer,[112, 137] chronic obstructive pulmonary disease,[138-140] ovarian cancer,[141] skin cancer,[142] oesophageal cancer,[143-145] gastric cancer,[93] pancreatic cancer,[146] asthma,[147, 148] non-Hodgkin lymphoma,[149, 150] lung cancer,[75, 151, 152] distal colorectal adenomas,[153] gastric cancer,[93] T2DM,[154] cirrhosis,[155] alcohol liver disease,[156, 157] progressive massive fibrosis,[158] macular degeneration,[159] alcoholic liver disease,[156] mood disorders,[160] hypersensitivity to para-phenylene diamine,[127] ankylosing spondylitis,[161] rheumatoid arthritis[162, 163] or preeclampsia[164] have been reported. These studies will now be discussed in more detail using disease sub-headings.

2.3.4.1 Breast Cancer

Eighteen original investigations[97-108, 134-136, 165-167] have studied the association between SOD2 Ala16Val SNP genotypes and breast cancer risk. Two reviews have also been published along with a recent meta-analysis examining cancer and the SOD Ala16Val genotype.[168-170] Twelve investigations reported an increased risk of breast cancer with specific genotypes using multivariate analysis techniques.[97-108] The largest study involved 1,265 Caucasian and 760 African American women with breast cancer and 1,812 controls.[101] The investigators found that women with the Ala/Ala genotype who had either: 1) smoked for longer than 20 years (OR 2.3, 95% CI 1.3-4.1); 2) had chest exposure to high doses of radiation due to medical procedures other than breast cancer treatment or diagnosis (OR 1.5, 95% CI 1.0-2.2); or 3) had been exposed to ionizing radiation at work (OR 1.6, 95% CI 0.8-3.1), had an increased risk of the disease. However, the combined effects were weak. The frequency of the Ala/Ala genotype was 25% in Caucasians and 18% in African Americans.[101] A similar result was also reported by Tamimi et al.[106] in a study of 2,173 participants, 968 of which had breast cancer. The study found a non-significant association (OR 1.41, 95% CI 0.77-2.60) between the Ala/Ala genotype and breast cancer in current smokers compared with the Val/Val genotype and non-smokers.

A large study involving 1,125 Chinese women with breast cancer reported that those with the Ala/Ala genotype had a slightly increased, but not statistically significant, risk of the disease, if they had high levels of oxidative stress (OR 2.4, 95% CI 0.6-9.5), a low intake of antioxidants (OR 2.6, 95% CI 0.7-10.0) or a low intake of fruits and vegetables (OR 2.4, 95% CI 0.7-8.0).[98] However, the frequency of this genotype was only 2%, in keeping with the low prevalence of this genotype across Asian populations.[98] Interestingly, Asian countries such as China and Japan have a reduced incidence of breast cancer compared with Europe and North America.[171]

Other studies that examined pre and postmenopausal women included Egan et al.[107] with a cohort of 470 American women, Ambrosone et al.[97] with 263 Caucasian women with breast cancer and a study involving 965 Finnish Caucasians, 483 of whom had breast cancer.[105] All studies reported minor relationships between breast cancer and the Ala/Ala genotype or Ala allele. Ambrosone et al.[97] reported an OR of 4.3 (95%CI 1.7-10.8) and a larger association amongst those who consumed low amounts of fruits and vegetables and other sources of dietary antioxidants (OR 6.0, 95%CI 2.0-18.2).[97] Egan et al.[107] reported an OR of 1.27 (95%CI 0.91-1.77) for an association between SOD2 SNPs and breast cancer, providing limited support for an association between SOD2 SNPs and breast cancer. The Finnish study reported that the Ala allele frequency was significantly greater in breast cancer patients (OR 1.5, 95% CI 1.1-2.0). The significance was increased in: 1) postmenopausal women who used oestrogen replacement therapy (OR 2.5, 95% CI, 1.3-4.8), and 2) women who smoked for longer than 15 years.[105]

The Ala allele was associated with an increased risk of breast cancer when alcohol consumption and body mass index (BMI) where considered.[99, 104] Slanger et al.[99] found that women with the Ala allele (25%) had a significantly increased risk of breast cancer when their alcohol consumption was >19g per day, compared with those that consumed no alcohol. A small case-control study involving 187 women, 84 of whom had breast cancer, found no association between the disease and the Ala16Val SNP of SOD2 (22%). However, after further analysis, those with the SOD2 Ala allele had a significantly increased risk of breast cancer if their BMI was greater than 24kg/m^2 and they had other SNPs (catechol O-methyltransferase, COMT-L, involved in the metabolism of catecholamines and catecholestrogens, and CYP1B1*1 which produces cytochrome P450) (OR 1.42, 95%CI 1.04–1.93).[104] In male (n=11) and female (n=89)

breast cancer patients, the Ala/Ala genotype frequency was significantly greater in those with cancer (OR 2.5, 95% CI 1.39-4.54).[108]

A study examining breast cancer survival after treatment in 279 patients found no significant association between survival and SOD2 genotype. However, the Ala/Ala genotype did endow individuals with a slightly reduced hazard of all cause mortality.[166]

A study involving 118 females <36 years of age reported that those with the Val allele genotype (28%) had an increased risk of breast cancer compared to women with the Ala/Ala genotype (OR 2.90, 95% CI 1.39-6.14).[102]

The largest study that reported no association between the SOD2 Ala16Val genotype and breast cancer was performed in 2,118 American women, 1,034 of whom had breast cancer.[135] The investigators found no significant relationship, even when risk factors for breast cancer, such as high BMI, smoking, alcohol and menopausal status were taken into account.[135] A study in Caucasian women found no association between breast cancer and the SOD2 Ala16Val genotype, although the only variables taken into account were family history and menopausal status.[134] The risk of radiotoxicity in breast cancer patients was not related to the SOD2 SNP genotype.[165] Additionally, a small population study of 80 breast cancer patients found no significant association between the SOD2 genotype Ala16Val and postoperative tissue changes, such as breast size and/or shape.[167] Cox et al.[100] also investigated the GPx1 Pro197Leu genotype in addition to SOD2 Ala16Val genotype and found no association between SOD2 SNP and breast cancer when the SOD2 data was analysed alone. However, when both genes were studied, there was a significant association between breast cancer risk and the Ala/Ala genotype for SOD2 and the Leu/Leu genotype for GPx1 (OR 1.87, 95% CI 1.09-3.19).

Two studies have examined the SOD2 Ala16Val SNP along with SNPs of estrogen-metabolising genes. Silva et al.[103] reported that Portuguese women with the Ala allele

genotype who never breast fed, had a non-significant decrease in the risk of breast cancer (OR 0.575, 95% CI 0.327-1.011). Cheng et al.[136] reported no association between breast cancer and catechol estrogen metabolising genes (e.g., COMT) and the SOD2 Ala16Val SNP.

Figure 2-1 summarises the relationship between SOD2 genotype and breast cancer. This quantitative overview is for illustrative purposes, to combine the statistical analysis of the major studies. Analysing the total population of cases and controls from each study reviewed, the Mantel-Haenszel OR and 95% CI were determined in relation to the Ala/Ala genotype compared with Ala/Val and Val/Val genotypes. The summary shows that there is a non-significant decrease in the risk of breast cancer in those with the Ala/Ala genotype compared with the Ala/Val and Val/Val genotypes (OR 0.97, 95% CI 0.90-1.05). Figure 2-1 also illustrates the significant heterogeneity across the studies, possibly due to true variation in genetic expression. However, a more likely explanation relates to the limitations of observational methodology (e.g., differences in unrecorded confounding factors, or publication bias) and our limited secondary analysis of the published reports.

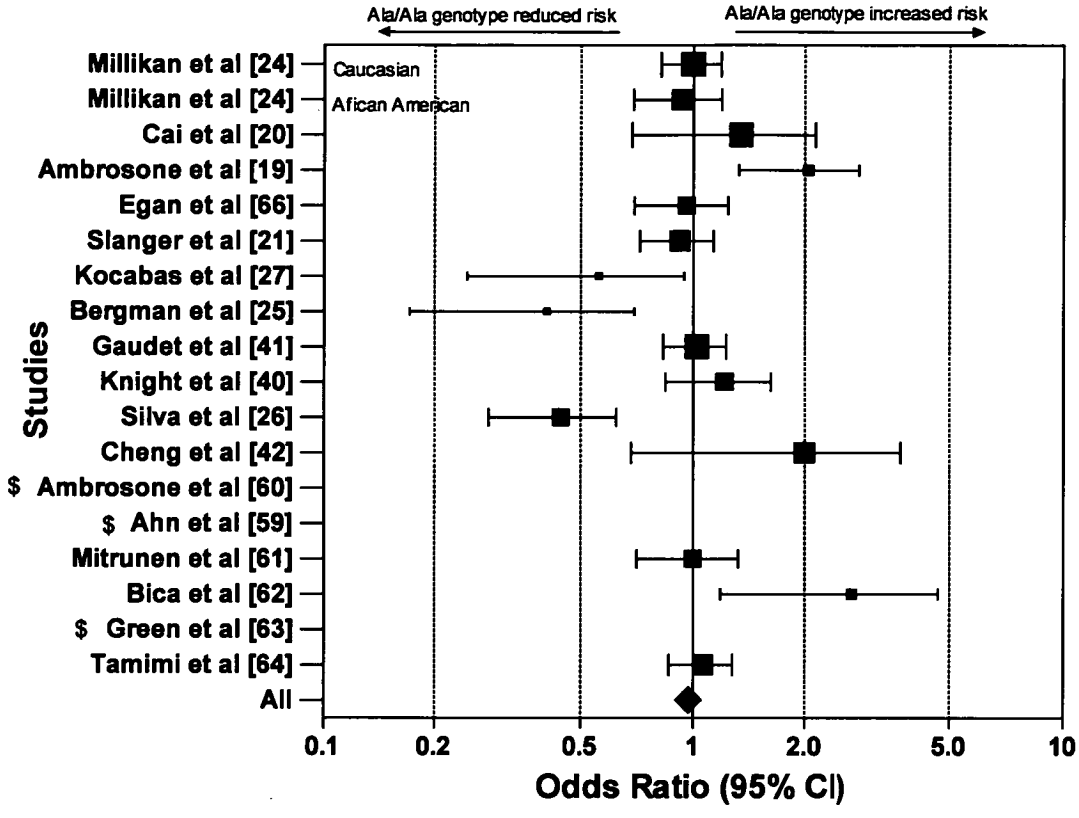

Figure 2-1: Studies investigating the relationship between the Ala/Ala genotype of the SOD2 Ala9Val and occurrence of breast cancer: a quantitative overview of results

$ The appropriate data was not published

■ The size of the square indicates the population size in the study

♦ The result of all the studies with the top and bottom points are an estimate of effect size and the left and right points show the 95% CI

Combined Mantel-Haenszel Odds Ratio: 0.97 (0.90 to1.05). Chi-squared test for homogeneity: 56.25; $p<0.001$

2.3.4.2 Prostate Cancer

Nine studies examined prostate cancer in relation to the SOD2 Ala16Val SNP. In the largest, Kang et al.[114] examined SNPs in SOD1, SOD2 and SOD3 in 3,162 Americans, including 1,213 Caucasians and 107 African Americans diagnosed with prostate cancer. This study found that Caucasians with the Ala/Ala (OR 1.28, 95% CI 1.03-1.60) and Val/Ala genotype (non-significant) had an increased risk of prostate cancer when compared with patients with Val/Val genotype. This association was not found in African Americans, but the patient population studied was smaller. This study also found in Caucasians that there was an association between the incidence of prostate cancer and a low dietary intake of vitamin E (OR 1.56, 95% CI 1.11-2.20). Seven other studies found that prostate cancer was significantly associated with the Ala/Ala or Ala allele genotype when the population was stratified into either disease stages of prostate cancer or other factors (e.g., alcohol consumption, age and antioxidant levels). Three studies reported that the Ala/Ala or Ala allele polymorphism were associated with high-grade tumours or an aggressive form of prostate cancer.[111, 112, 116] One study found an association with the Ala/Ala genotype and high grade tumours, but only when the high selenium levels were taken into account (OR 1.89, 95% CI 1.01-3.56).[112] Woodson et al.[111] also reported that high-grade prostate tumours were significantly associated with the Ala allele (OR 2.72, 95% CI 1.15-6.40). An aggressive form of prostate cancer was reported to be significantly associated with Ala/Ala genotype, although only when the men had low levels of the antioxidant, lycopene (RR 3.10, 95% CI 1.37-7.02).[116] Ergen et al.[117] reported in a study involving 100 participants, of whom 50 had prostate cancer that the Ala/Ala genotype frequency was significantly increased in patients compared with controls (OR 1.14, 95% CI 1.02-1.25). In fact, none of the 50 controls possessed the Ala/Ala genotype. The fifth study found that Ala/Ala was associated with a greater risk of prostate cancer if diagnosed under the age of 65 (OR 5.20, 95% CI 0.92-

29.26).[113] The sixth study, involving 187, mainly Caucasian patients, reported that the Ala/Ala genotype frequency was increased significantly in those with prostate cancer (OR 1.65, 95% CI 1.03-2.66). The significance of the association was increased in patients that smoked and had a SNP in the *N*-acetyltransferase 1 (NAT1 isoform) gene.[115] A small study reported that the frequency of the Ala/Ala genotype was significantly increased in all prostate cancer patients compared with controls (OR 2.5, 95% CI 1.39-4.54).[108]

A larger contradictory study involving 724 men with prostate cancer, found that there was no association between prostate cancer and the SOD2 genotype, even in patients under the age of 65, those that took vitamin supplements and those with other risk factors for prostate cancer.[172] Wang et al.[173] performed a meta-analysis of 34 studies that examined the association between SOD2 Ala16Val polymorphism and cancer. This meta-analysis failed to find an association between the Ala16Val SNP and the risk of all cancers. However, there was a significant association between Ala/Ala and Ala/Val genotypes and prostate cancer compared with Val/Val (OR1.2, 95% CI 1.0-1.3).

In summary, a number of studies have shown prostate cancer is associated with the Ala/Ala genotype. However, each study stratified the population into specific groups in order to reveal this relationship. Figure 2-2 provides a summary of the results of these studies and demonstrates a significant association between prostate cancer and the SOD2 Ala/Ala and Val/Ala genotypes (OR 1.16, 95%CI 1.03-1.32).

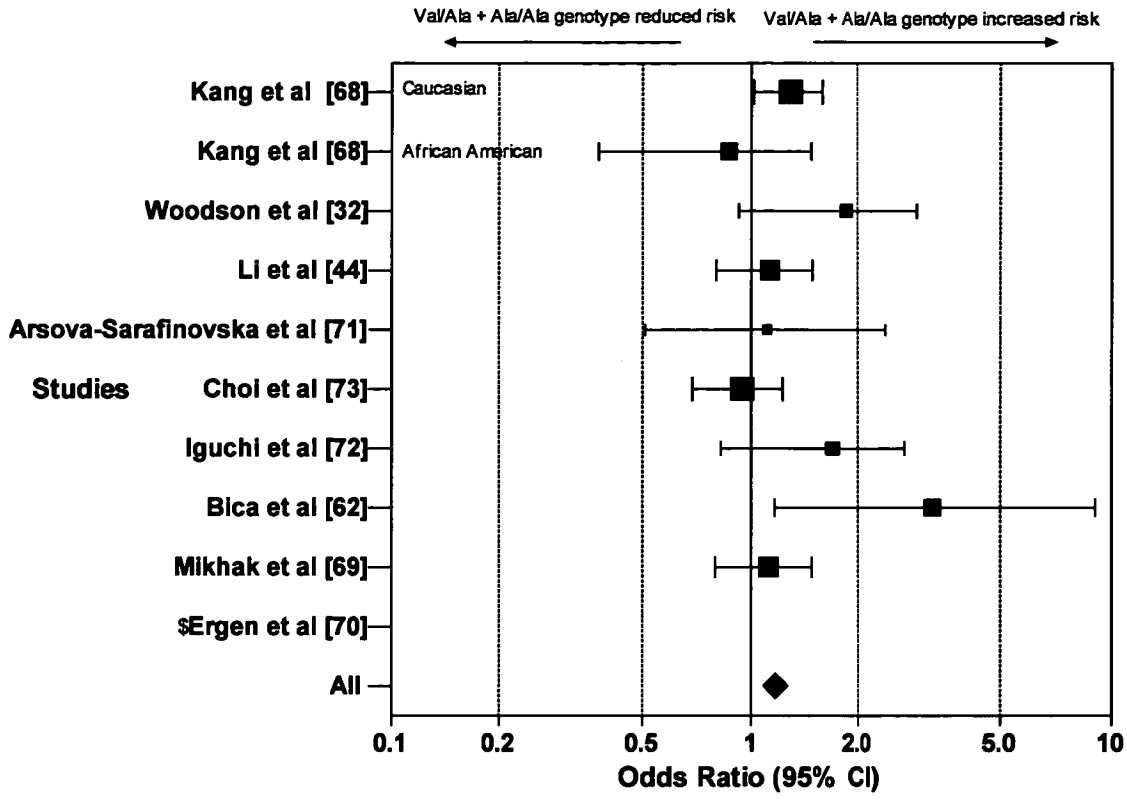

Figure 2-2: Studies investigating relationships between the Val/Ala and Ala/Ala genotype compared with Ala/Ala of the SOD2 Ala9Val SNP and occurrence of prostate cancer: a quantitative overview of the results

$ The appropriate data was not published

■ The size of the square indicates the population size in the study

♦ The result of all the studies with the top and bottom points are an estimate of effect size and the left and right points showing the 95% CI. Combined Mantel-Haenszel Odds Ratio: 1.16 (1.03 to1.32). Chi-squared test for homogeneity: 18.77; $p<0.0272$

2.3.4.3 Other Cancers

Twenty-one studies have investigated the relationship between the SOD2 Ala16Val SNP and other cancers.[75, 93, 109, 118-120, 130, 137-146, 149-152] Wang et al.[151] found that individuals with the Val allele had an increased risk of lung cancer if there was low or no asbestos exposure compared to high exposure (OR 2.14, 95% CI 1.52-3.01), although the small number in the high asbestos exposure group limited the findings. The largest case-control study involved 2,168 participants, of whom 935 had lung cancer. There was an increased risk of lung cancer in individuals with the Val/Val genotype when they had two other polymorphisms, *viz*, in p53 and in the X-ray repair cross-complementing group 1 (XRCC1) gene that encodes a protein involved in DNA repair (OR 2.54, 95% CI 1.38-4.68). These two SNPs had previously been linked to an increased risk of lung cancer.[119] A similar result was reported in a study of 150 participants, including 100 with lung cancer. This study examined lung cancer cell types and found that the Val/Val genotype frequency was significantly greater than the Ala/Ala and Ala/Val genotypes in those with squamous cell cancer.[118] Three studies of chronic obstructive pulmonary disease reported no association with the SOD2 genotype.[138-140] A study by Landi et al.[130] also investigated 80 malignant pleural mesothelioma patients and 349 controls and found an increased risk of the disease in patients that carried the Ala/Ala genotype (OR 3.07, 95% CI 1.55-6.05). Although this result is in disagreement with those of the larger studies (1,746 cases when combined), Landi and co-workers[130] examined a specific form of cancer, malignant pleural mesothelioma that is mainly caused by asbestos exposure.

There have been two smaller studies, in specific ethnic groups, *viz*, Taiwanese[152] and Chinese[75], the largest involving 243 cases, which found no association between lung cancer and the SOD2 Ala16Val SNP.[75, 152]

An increased risk of bladder cancer has also been associated with the Ala16Val SNP in SOD2. The relationship was found in heavy smokers with the Val/Val genotype (OR 2.24, 95% CI 1.15-4.36).[109] However, Inchimura et al.[137] performed a similar study in Japanese subjects who had a very low frequency of the Ala/Ala genotype and found no significant association between this SNP and the risk of bladder cancer.

A large case-control study involving 1,172 patients, investigated the associations between thirteen SNPs, including the SOD2 Ala16Val SNP and the risk of non-Hodgkin lymphoma. The total cohort was 2,154 and the authors reported no significant association with non-Hodgkin lymphoma. However, a significant association was observed between B-cell lymphomas and individuals with the Ala/Ala SOD2 genotype (OR 1.3, 95% CI 1.0-1.6).[149] A similar sized case-control study (928 cases and 1,446 controls) recruited from the USA and UK, found no association between SOD2 Ala16Val SNP genotypes and non-Hodgkin lymphoma.[150]

Eighty-nine patients with acute myeloid leukaemia were investigated to determine whether there was an association between the SOD2 Ala16Val SNP and survival after treatment. This non case-control study found that patients with the Ala/Val genotype had a greater chance of survival compared to those with the Ala/Ala genotype (95% CI 19.6-52.4).[120] The investigators also reported that patients with the Ala/Ala genotype had significantly worse outcomes after treatment compared to patients with Val/Val genotype (OR 2.0, 95% CI 1.1-3.5). Finally, other forms of cancers have been studied including gastric, oesophageal, pancreatic, ovarian and skin cancer, with all studies reporting no association with SOD2 genotypes.[93, 141-146]

2.3.4.4 Diabetes

Seven studies have investigated the relationship between the SOD2 genotypes and co-morbidities of T1DM or T2DM. A study conducted within a Korean population reported an association with diabetic macular edema and the Ala allele in those with

diabetic retinopathy (n=130) (OR 1.59, 95% CI 1.02-2.02).[122] However, there was no association with the development of diabetes itself.[122] In the same cohort, the authors reported that the Ala allele was significantly different between T2DM patients with albuminuria, compared with those without ($p<0.05$), but there was no difference between the SOD2 genotypes of T2DM patients and controls.[124] In contrast to these two investigations, another study showed that the Val/Val genotype in T2DM patients was associated with diabetic retinopathy (OR 2.1, 95% CI 1.2-3.4).[125] Two other studies reported that the Val/Val homozygote genotype or the Val allele was associated with diabetic nephropathy.[121, 123] One study examined T1DM and found an increased risk of diabetic nephropathy with the Val/Val genotype (OR 1.32, 95% CI 1.00-1.74).[121] The second study examined patients with T2DM and reported that the Val/Val genotype was significantly higher in those with nephropathy ($p=0.0057$).[121, 123] A smaller study, that examined patients with either T1DM or T2DM, reported a greater frequency of the Val/Val genotype in these patient groups compared with a controls ($p<0.05$).[126] Finally, a study in 91 T2DM patients found no association between SOD2 genotype and coronary artery calcium score (used to determine the presence of vascular calcification seen in atherosclerotic cardiovascular disease).[154] Although all of these studies examined patients with diabetes, they investigated different co-morbidities and hence, the results are difficult to compare.

2.3.4.5 Cardiovascular Disease (CVD)

Three studies have examined the relationships between SOD2 Ala16Val SNP genotypes and biological measurements associated with CVD. The largest, involving 989 participants, found a significant relationship between carotid artery intima-media thickness (CIMT), a measure of atherosclerotic burden, and the Val allele in women that had high levels of low-density lipoprotein (LDL) cholesterol ($p=0.03$).[72] The second study examined 217 patients with haemochromatosis and 212 controls and reported that

those with haemochromatosis and the Val allele had an increased risk of cardiomyopathy compared with those with the Ala allele. However, only 27 patients had cardiomyopathy, and eleven (30%) of these had the Val/Val genotype (p=0.0006).[73] The third study involved 252 participants and examined oxidised LDL, a marker of oxidative stress and a biomarker for the development of atherosclerosis. The investigators found that in patients with oxidised LDL <0.5nmol/mg, the Ala/Val and Val/Val genotypes were associated with increased levels of oxidised LDL compared with Ala/Ala genotype in the same group (OR 3.61, 95% CI 1.42-9.17).[71]

2.3.4.6 Liver Disease

The SOD2 Val/Val genotype was found more frequently in 63 patients diagnosed with non-alcoholic steatohepatitis (NASH) when compared with 150 controls (OR 2.49, 95% CI 1.17-5.32).[129] A study that examined hereditary haemochromatosis and chronic hepatitis C to determine the development of cirrhosis in these groups, concluded that SOD2 genotypes were not markers for the development of cirrhosis.[155] A study of 176 heavy drinkers, of whom 100 had alcoholic liver disease (ALD) and 76 controls, reported that there was no association with the SOD2 Ala16Val SNP and risk of ALD.[156] Another study involving 218 patients with ALD and 218 heavy drinkers without ADL also reported no association between SOD2 SNP and the development of ALD.[157]

2.3.4.7 Neurological and Mental Health

There have been two studies investigating whether the SOD2 Ala16Val SNP could be used to predict the occurrence of tardive dyskinesia in schizophrenic patients on antipsychotic therapy. There was no association seen when this SNP was examined on its own. However, when both studies included either, 1) a dopamine D3 receptor SNP[132] or 2) a NAD(P)H dehydrogenase quinone oxidoreductase 1 (NQO1) SNP, involved in

the reduction of quinones,[133] a significant association with the Val/Val genotype was found in patients with and without tardive dyskinesia ((1) x^2=8.09, degrees of freedom =3, p=0.04) and ((2) x^2=8.00, degrees of freedom =3, p=0.042).[132, 133] In a study examining 80 patients with a major depressive disorder, 61 with a bipolar disorder and 106 controls, it was found that there was no association between any of these mood disorders and the SOD2 Ala16Val SNP.[160]

2.3.4.8 Other conditions

A study examining hypersensitivity to the skin irritant dye, para-phenylene diamine, found that older females with the Ala/Ala genotype had an increased risk of dermatitis (OR 1.5, 95% CI 0.7-2.34).[127] In a small study (35 females and 37 males), the Ala allele was found to be associated with an increased risk of being diagnosed with sporadic motor neurone disease, particularly in females (OR 5.5, 95% CI 1.5-19.9).[131] A case-control study of patients with brain tumours (glioma, meningioma and acoustic neuroma), found that the Ala allele was associated with an increased risk of acoustic neuroma (OR 2.0, 95% CI 1.0-4.2).[96] The Ala allele was also found to be increased in black South African patients with diffuse cutaneous systemic sclerosis (OR 2.11, 95% CI 1.1-4.05).[128] Another study reported that the Val/Val genotype was significantly higher in patients with Behcet's disease (blood vessel inflammatory disease) compared with controls (OR 2.41, 95% CI 1.09-5.32).[110] Seven studies found no association with SOD2 Ala16Val SNP genotypes and diseases including preeclampsia,[164] distal colorectal adenomas,[153] progressive massive fibrosis,[158] ankylosing spondylitis[161] and rheumatoid artritis.[162, 163] However, one of these studies found that the autoimmune disease, Sjogren's syndrome, was associated with the Val/Val genotype.[163]

2.3.4.9 Summary

The majority of studies investigating the relationships between the SOD2 Ala16Val genotypes and disease have been conducted in breast cancer with most showing an association with the Ala/Ala genotype. A combined quantitative overview indicates that this confers a slightly reduced risk of breast cancer in people with this genotype. Although the Ala/Ala genotype has been shown to be associated with an increased risk of prostate cancer, a quantitative overview showed no such association. In a smaller number of studies, the Ala allele or the Ala/Ala genotype have been associated with non-Hodgkin lymphoma, brain tumours, motor neurone disease, malignant pleural mesothelioma and systemic sclerosis. Although individuals with the Val/Val genotype or the Val allele have been shown to have an increased risk of bladder cancer, NASH, leukaemia and co-morbidities in T1DM and T2DM, other studies do not support these relationships. In the three studies examining relationships with CVD, all have found a strong positive association with the Val allele and markers of CVD risk.

Table 2-1: Studies investigating relationships between genotypes of the SOD2 Ala16Val SNP and disease

Condition	Reference	Participants	Genotype			Finding
		Cases/Controls	Val/Val Cases (n [%])/ Controls (n [%])	Val/Ala Cases (n [%])/ Controls (n [%])	Ala/Ala Cases (n [%])/ Controls (n [%])	
Breast Cancer	Millikan, et al.[101]	Caucasians 760/1,135	273 [21]/266 [23]	681[54]/586[52]	311[25]/283[25]	Ala/Ala had a weak association with breast cancer with specific environmental factors (smoking, radiation)
		African American 760/677	259[34]/196 [29]	372[49]/357[53]	129[17]/124[18]	
Breast Cancer	Cox, et al.[100]	1,262/1,533	Data not given	Data not given	Data not given	Ala/Ala associated with breast cancer when combined with another SNP in GPx1 (OR 1.87, 95% CI 1.09-3.19)
Breast Cancer	Cai, et al.[98]	1,125/1,197	831[74]/884[74]	266[24] /290[24]	28[2]/23[2]	Ala/Ala associated with breast cancer in patients with elevated oxidative stress and a low intake of antioxidants (OR 2.4, 95% CI 0.7-8.0)
Breast Cancer	Gaudet, et al.[135]	1,034/1,084	253[25]/264[24]	511[49]/539[50]	270[26]/281[26]	No significant association.
Breast Cancer	Tamimi et al.[106]	968/1,205	255[26]/297[25]	468[49/612[51]	245[25]/296[24]	Ala/Ala genotype was associated with a significant increase risk of breast cancer in current smokers compared with Val/Val genotype and non-smokers (OR 1.41, 95% CI 0.77-2.60)
Breast Cancer	Slanger, et al.[99]	614/1,080	144[23]/263[24]	318[52]/528[49]	152[25]/289[27]	Ala allele significantly associated with breast cancer when patients consumed high quantities of alcohol
Breast Cancer	Cheng, et al.[136]	469/740	343[73]/545[74]	115[25]/183[25]	11[2]/11[1]	No significant association.
Breast Cancer	Egan, et al.[107]	470/497	120[21]/130[26]	250[53]/240[49]	118[25]/127[26]	Val/Ala had a minor association with breast cancer (OR1.8, 95%CI 1.0-3.1) in premenopausal women
Breast Cancer	Mitrunen, et. al.[105]	483/482	153[32]/124[26]	231[48]/255[53]	98[20]/100[21]	Ala allele to be associated with increase risk of breast cancer (OR 1.5, 95% CI 1.1-2.0)
Breast Cancer	Silva, et al.[103]	241/636	59[24]/109[22]	146[61]/339[60]	36[15]/188[18]	Ala allele had minor non significant association with decreased risk of breast cancer in patients that had never breast fed (OR 0.575, 95% CI 0.327-1.011, p=0.054).
Breast Cancer	Knight, et al.[134]	372/399	107 [27]/ 90[24]	187 [47]/ 195 [52]	105 [26]/ 87 [23]	No significant association.

Condition	Reference	Participants	Genotype			Finding
		Cases/Controls	Val/Val Cases (n [%])/ Controls (n [%])	Val/Ala Cases (n [%])/ Controls (n [%])	Ala/Ala Cases (n [%])/ Controls (n [%])	
Breast Cancer	Ambrosone, et al.[97]	266/295	39[14]/63[21]	137[52]/169[57]	90[34]/63[21]	Ala/Ala associated with breast cancer in premenopausal patients (OR=4.3, 95%CI 1.7-10.8) and larger in those consuming low amounts of fruit and vegetables (OR = 6.0, 95%CI 2.0-18.2).
Breast Cancer	Bica, et. al.[108]	100/372 Male 11/155 Female 89/217	M 5[46]/42[27] F 24[27]/52[24]	M 5[45]/105[68] F 51[57]/147[68]	M 1[9]/8[5] F 14[16]/18[8]	Ala/Ala genotype was associated with a significant increase in breast and prostate cancer when all patients analysed together (OR 2.5, 95% CI 1.39-4.54, p=0.002).
Breast Cancer	Ahn, et al.[165]	(cases) 446	113[26]	204[48]	111[26]	No significant association.
Breast Cancer	Ambrosone, et al.[166]	279	67[25]	137[51]	63[24]	No significant association.
Breast Cancer	Bergman, et al.[102]	118/174	33[28]/43[25]	73[62]/88[50]	12[10]/43[25]	Val allele associated with increased risk of breast cancer in females <36 years old (OR 2.90, 95% CI 1.39-6.14, p=0.002)
Breast Cancer	Kocabas, et al.[104]	84/103	28[33]/28[27]	38[45]/40[39]	18[22]/38[37]	Ala allele significantly associated with breast cancer in patients with and increased BMI and other genotypes (OR 1.42, 95%CI 1.04–1.93)
Breast Cancer	Green, et al.[167]	80 Tissue damage 39/ No tissue damage 36	13[33]/8[22]	17[44]/22[61]	9[23]/6[17]	No significant association
Prostate Cancer	Kang, et al.[114]	1,320/1,842 (Caucasians:1,213 /1,433; African Americans:107/409)	275[24]/376[27] 31[30]/122[31]	578[50]/686[50] 57[55]/194[49]	297[26]/320[23] 15[15]/79[20]	Ala/Ala and Ala/Val is associated with prostate cancer in Caucasians (OR 1.28, 95% CI 1.03-1.60; 1.28, 95% CI 0.97-1.42, Ptrend=0.028)
Prostate Cancer	Choi, et al.[172]	724/1,474	128[25]/356[25]	258[51]/683[49]	116[23]/349[25]	No significant association.
Prostate Cancer	Li, et al.[112]	567/764	132[23]/190[24]	288[51]/379[50]	147[26]/195[26]	Ala/Ala associated with more aggressive form of prostate cancer (RR 1.89, 95% CI 1.01-3.56, p=0.01)

Condition	Reference	Participants	Genotype			Finding
		Cases/Controls	Val/Val Cases (n [%])/ Controls (n [%])	Val/Ala Cases (n [%])/ Controls (n [%])	Ala/Ala Cases (n [%])/ Controls (n [%])	
Prostate Cancer	Mikhak, et al.[116]	612/612	156[24]/162[25]	320[50]/331[51]	166[26]/159[24]	Ala/Ala was associated with aggressive prostate cancer in men with low lycopene status (RR 3.10, 95% CI 1.37-7.02, p=0.02)
Prostate Cancer	Bica, et al.[108]	51/372	10[19]/94[25]	32[63]/252[68]	9[18]/26[7]	Ala/Ala genotype was associated with significant increase in prostate and breast cancer when all patients analysed together (OR 2.5, 95% CI 1.39-4.54, p=0.002).
Prostate Cancer	Woodson, et al.[111]	197/190	43[22]/49[26]	98[49]/102[53]	58[29]/40[21]	Ala/Ala associated with high grade tumour in prostate cancer (OR 2.72, 95% CI 1.15-6.40, p=0.02)
Prostate Cancer	Iguchi et al.[115]	187/175	41[22]/40[23]	86[46]/96[55]	60[32]/39[22]	Ala/Ala was significantly associated with prostate cancer (OR 1.65, 95% CI 1.03-2.66)
Prostate Cancer	Arsova-Sarafinovska, et al.[113]	85/151	19[22]/41[27]	46[54]/73[48]	20[24]/37[25]	Ala/Ala genotype showed an increased risk of early onset prostrate cancer if diagnosed under 65 years (OR 5.20, 95% CI 0.92-29.26, p=0.05)
Prostate Cancer	Ergen, et al.[117]	50/50	19[38]/32[64]	25[50]/18[36]	6[12]/0[0]	Ala/Ala frequency significantly higher in prostate cancer patients (OR 1.14, 95% CI 1.02-1.25, p=0.0012)
Lung Cancer	Liu, et al.[119]	935/1,233	208[27]/322[23]	472[51]/626[51]	255[22]/285[26]	Val/Val associated with lung cancer in the presence of two other SNPs (OR 2.54, 95% CI 1.36-4.68)
Lung Cancer	Wang, et al.[151]	911/957	173[21]/255[27]	513[51]/482[50]	225[28]/220[13]	No significant association, although the Val allele showed an association with increased risk of lung cancer with low or no asbestoses exposure (OR 2.14, 95% CI 1.52-3.01)
Lung Cancer	Lin, et al.[152]	198/332	139[70]/233[70]	59[30]/81[30]	Val/ Ala and Ala/Ala Data was pooled together	No significant association.
Lung Cancer	Ho, et al.[75]	234/239	176[75]/180[75]	58[25]/52[22]	0[0]/7[3]	No significant association.

Condition	Reference	Participants	Genotype			Finding
		Cases/Controls	Val/Val Cases (n [%])/ Controls (n [%])	Val/Ala Cases (n [%])/ Controls (n [%])	Ala/Ala Cases (n [%])/ Controls (n [%])	
Lung Cancer	Zejinilovic et al.[118]	100/50	54[54]/34[68]	42[42]/3[6]	4[4]/13[26]	Val/Val genotype frequency was significantly higher in squamos cell type lung cancer (OR 7.00, 95% CI 2.28-21.48, P<0.001)
Malignant Pleural Mesothelioma (MPM)	Landi, et al.[130]	80/349	16[20]/98[28]	27[34]/170[49]	37[46]/81[23]	Ala/Ala genotype showed an increased risk of MPM (OR 3.07, 95% CI 1.55-6.05, p=0.001)
Chronic Obstructive Pulmonary Disease	Young, et al.[138]	230/210	53[23]/46[22]	120[52]/114[54]	57[25]/50[24]	No significant association.
Chronic Obstructive Pulmonary Disease	Mak et al.[139]	165/165	119[73]/131[80]	44[26]/32[19]	1[1]/1[1]	No significant association
Pulmonary Disease	Hirvonen et al.[140]	Asbestos insulators 124 With 63/ Without 61	17[28]/15[24]	29[47]/36[57]	15[25]/12[19]	No significant association.
Bladder Cancer	Ichimura, et al.[137]	213/209	169[79]/157[75]	41[19]/48[23]	3[2]/4[2]	No significant association.
Bladder Cancer	Hung, et al.[109]	201/214	63[34]/45[21]	89[44]115[/54]	44[22]/54[25]	Val/Val genotype associated with an increased risk of bladder cancer particularly among heavy smokers (OR 2.24, 95% CI 1.15-4.36)
Non-Hodgkin lymphoma (NHL)	Lightfoot, et al.[150]	928/1,446	211[24]/358[25]	463[51]/713[49]	229[25]/317[26]	No significant association.
Non-Hodgkin lymphoma (NHL)	Wang, et al.[149]	1,172/982	285[25]/240[26]	545[49]/486[52]	290[26]/211[23]	No association was observed with NHL. However the Ala/Ala genotype was associated with B-cell lymphomas (OR 1.3, 95% CI 1.0-1.6, p=0.01)

Condition	Reference	Participants	Genotype			Finding
		Cases/Controls	Val/Val Cases (n [%])/ Controls (n [%])	Val/Ala Cases (n [%])/ Controls (n [%])	Ala/Ala Cases (n [%])/ Controls (n [%])	
Ovarian Cancer	Johnatty, et al.[141]	543/1,130	123[23]/276[25]	273[50]/546[48]	147[27]/308[27]	No significant association
Skin Cancer	Han, et al.[142]	805/873	184[24]/196[24]	402[52]/425[51]	187[24]/212[25]	No significant association
Oesophageal Cancer	Sun et al.[143]	Cancer patients 170	35[20] 43[27]	88[52] 80[50]	47[28] 37[23]	No significant association.
		Heavy drinkers 160	139[35]	211[53]	50[12]	
		Healthy controls 400				
Oesophageal Cancer	Di Martino et al. [144]	490/94	128[26]/20[21]	234[48]/39[42]	122[25]/34[37]	No significant association
Oesophageal Cancer	Murphy, et al.[145]	396/223	93[23]/60[27]	196[49]/113[51]	107[27]/48[22]	No significant association
Gastric Cancer	Martin, et al.[93]	274/361	72[26]/99[27]	153[56]/209[58]	49[18]/53[15]	No significant association.
Acute Myeloid Leukaemia	Koistinen, et al.[120]	89	20[22]	49[56]	20[22]	Ala/Val patients found to have a greater chance of survival (95% CI 19.6-52.4, p=0.02).
Pancreatic Cancer	Li, et al.[146]	24/23	10[42]/8[35]	11[46]/10[43]	3[12]/5[22]	No significant association
T1DM (with/without diabetic nephropathy)	Mollsten, et al.[121]	1,510 955/555	255[27]/130[24]	457[48]/284[51]	239[25]/140[25]	Val/Val had an increased risk of diabetic nephropathy in smokers (OR 1.32, 95% CI 1.00-1.74, p=0.049)
T2DM Diabetic Nephropathy	Nomiyama, et al.[123]	478/261	206[84]/158[70]	Val/ Ala and Val/Val (Data pooled)	85[15]/29[29]	Val/Val genotype was significantly higher in diabetic nephropathy (p=0.0057)
T1DM and T2DM	Flekac, et al.[126]	T1DM 120	79[66]	36[30]	5[4]	
		T2DM 306	220[72]	80[26]	6[2]	Val/Val genotype significantly different in both T1DM and T2DM compared with controls (p<0.05)
		Control 180	52[29]	90[50]	38[21]	

Condition	Reference	Participants	Genotype			Finding
		Cases/Controls	Val/Val Cases (n [%])/ Controls (n [%])	Val/Ala Cases (n [%])/ Controls (n [%])	Ala/Ala Cases (n [%])/ Controls (n [%])	
T2DM (Albuminuria)	Lee, et al.[124]	371/178	314[82]/146[84]	57[18]/32[15]	Val/ Ala and Ala/Ala (Data pooled)	An association was found in frequency with Ala allele between albuminuria +/- ($p<0.05$). However there was no difference between T2DM and controls
T2DM (diabetic macular edema)	Lee, et al.[122]	304/192	243[79]/152[79]	61[20]/40[20]	Val/ Ala and Ala/Ala (Data pooled)	Ala allele associated with diabetic macular edema. However not associated with the development of T2DM (OR 1.59, 95% CI 1.02-2.02, $p=0.03$)
T2DM (with/without diabetic retinopathy)	Petrovic, et al.[125]	426 283/143	63[22]/51[35]	140[49]/69[48]	80[28]/23[16]	Val/Val genotype was found to be an risk factor for diabetic retinopathy (OR 2.1, 95% CI 1.2-3.4, $p=0.006$)
T2DM (coronary artery calcium score)	Nemoto et al.[154]	91	No data given	No data given	No data given	No significant association.
Carotid Athersclerosis	Kakko, et al.[72]	989	265[27]	492[50]	232[23]	Val allele was associated with higher IMT in females with high low-density lipoproteins ($p=0.03$)
Cardiomyopathy (cases=hereditary haemochromatosis)	Valenti, et al.[73]	217/212	37[17]/51[24]	132[60]/118[56]	48[23]/43[20]	Val allele showed an increased risk of cardiomyopathy ($p=0.0006$)
CVD (cases=LDL≥ 0.5nmol/mg control= LDL< 0.5nmol/mg)	Gottlieb, et al.[71]	82/170	49[60]/84[49]	26[32]/44[26]	7[8]/42[25]	Val/Val and Ala/Val genotypes had higher LDL in the control group (OR 3.61, 95% CI 1.42-9.17)
Alcoholic Liver Disease (ALD)	Stewart, et al.[157]	ALD 281 Non- ALD 218 Controls 244	72[25] 58[27] 64[26]	156[56] 109[50] 125[51]	53[19] 51[23] 55[23]	No significant association.
Non-Alcoholic Steatohepatits (NASH)	Namikawa, et al.[129]	63/150	[84]/[68]	[11]/[29]	[5]/[3]	Val/Val frequency higher in NASH patients compared with controls (OR 2.49, 95% CI 1.17-5.32, $p=0.016$)

Condition	Reference	Participants	Genotype			Finding
		Cases/Controls	Val/Val Cases (n [%])/ Controls (n [%])	Val/Ala Cases (n [%])/ Controls (n [%])	Ala/Ala Cases (n [%])/ Controls (n [%])	
ALD	Martins, et al.[156]	100/75	23[23]/19[25]	55[55]/37[50]	22[22]/19[25]	No significant association.
Cirrhosis	Stickel, et al.[155]	Hereditary Haemochromatosis 157 Chronic Hepatitis-C 285 Controls 160	32[20] 69[28] 36[22]	94[60] 136[48] 81[51]	31[20] 80[24] 43[27]	No significant association found in any of the groups
Schizophrenia (with and without Tardive Dyskinesia (TD))	Pae et al.[133]	107/106	73[68]/72[68]	34[32]/34[32]	0/0	Val/Val genotype frequency was significantly different in with schizophrenia patients with TD (x2=8.0, degrees of freedom =3, p=0.042)
Schizophrenia (with and without TD)	Zhang, et al.[132]	42/52	30[73]/33[63]	12[29]/19[37]	0/0	The presence of another polymorphism and the Val allele was associated with TD in schizophrenia patients (x2=8.09, degrees of freedom =3, p=0.04)
Mood Disorders	Pae, et al.[160]	141/106	80[57]/68[64]	60[42]/38[36]	1[1]/0	No significant association.
Asthma	Holla, et al.[147]	299/327	78[23]/78[26]	150[51]/168[50]	71[24]/81[23]	No significant association.
Asthma	Mak, et al.[148]	251/316	192[77]/233[76]	59[23]/74[24]	Val/ Ala and Ala/Ala (Data was pooled together)	No significant association.
Brain Tumour	Rajaraman, et al.[96]	565/494	129[25]/122[27]	162[51]/220[49]	123[24]/109[24]	Ala allele showed an increase of acoustic neuroma (OR 2.0, 95% CI 1.0-4.2, p<0.05)
Distal Colorectal Adenomas	Levine, et al.[153]	456/495	139[27]/140[25]	209[40]/234[42]	108[21]/121[22]	No significant association.
Hypersensitized to Para-phenylene	Brans, et al.[127]	157/201	43[26]/46[25]	73[47]/104[52]	41[27]/51[23]	Association found with Ala allele in women over the age of 45yrs (OR 1.5, 95% CI 0.7-2.34)
Rheumatoid Arthritis	Mattey, et al.[162]	153/218	42[24]/61[28]	73[48]/112[51]	38[28]/45[21]	No significant association.

Condition	Reference	Participants	Genotype			Finding
		Cases/Controls	Val/Val Cases (n [%])/ Controls (n [%])	Val/Ala Cases (n [%])/ Controls (n [%])	Ala/Ala Cases (n [%])/ Controls (n [%])	
Rheumatoid Arthritis	Yen, et al.[163]	112/96	71[63]/61[63]	34[30]/32[33]	7[6]/3[3]	No significant association.
Risk of Preeclampsia	Kim, et al.[164]	106/115	78[74]/83[72]	26[24]/30[26]	2[2]/2[2]	No significant association.
Motor Neuron Disease (MND)	Van Landeghem, et al.[131]	72/136	16[22]/46[34]	35[49]/69[51]	21[29]/21[15]	Ala allele associated with a higher risk of MND, especially in females (OR 5.5, 95% CI 1.5-19.9)
Macular Degeneration	Esfandiary, et al.[159]	94/95	44[47]/47[50]	44[47]/41[43]	6[6]/7[7]	No significant association.
Behcet Disease	Nakao, et al.[110]	78/107	68[87]/79[74]	9[12]/27[25]	1[1]/1[1]	Val/Val genotype was found to have an association with Behcet disease (OR 2.41, 95% CI 1.09-5.32, p=0.03)
Ankylosing Spondylits	Yen, et al.[161]	70/93	43[62]/60[65]	26[37]/30[32]	1[1]/3[3]	No significant association.
Progressive Massive Fibrosis (in coal miners)	Yucesoy, et al [158]	350/350	90[30]/88[27]	138[47]/168[51]	69[23]/72[22]	No significant association.
Systemic Sclerosis (Scleroderma)	Tikly, et al.[128]	51/61	16[31]/28[45]	22[43]/23[37]	14[27]/11[18]	A trend of Ala allele frequency increased in diffuse cutaneous systemic sclerosis patients (OR 2.11, 95% CI 1.1-4.05)

2.3.5 Glutathione Peroxidase (GPx) Isoforms and SNPs

Various isoforms of GPx are discussed in section 1.2.3. However, a search of NCBI found the following numbers of GPx isoform SNPs in humans: 46 in GPx1, 73 in GPx2, 120 in GPx3, 88 in GPx4 and 93 in GPx5 (see Appendix A5.1. for a link to GPx SNPs). The GPx1 isoform has four SNPs that change the amino acid produced and only one has been studied in relation to diseases in humans, rs1050450. This is a C>T mutation that changes the amino acid at position 197 (noted in the NCBI database as position 200 and it has also been recorded to be at position 198) the amino acid changes from proline (Pro) to leucine (Leu). The GPx1 Pro197Leu SNP has been the most investigated SNP in the GPx enzymes in relation to disease involving twenty studies. Therefore, this SNP will be the main focus of this part of the review.

However, another SNP, which does not code for an amino acid in GPx1, is rs1800668, and is present in exon 1 in the 5' URT section. This SNP, a C>T substitution, has been investigated in relation to oesophageal cancer along with 61 other SNPs in specific enzymes involved in DNA repair. It was found that specific genotypes were associated with an increased risk of cancer.[174] A study that examined 325 SNPs included the GPx1 SNP, rs3448. This SNP is located in the 3' end of the gene and is a C>T mutation that does not code for an amino acid. The study examined all the SNPs in relation to non-pathological cognitive ageing and reported that only one specific SNP, present in the amyloid precursor protein gene, had a significant association with cognitive ageing.[175]

Three SNPs of the GPx2 isoform have been identified in the amino acid coding region of exon 2. However, none of these have been studied in relation to disease. The GPx3 isoform has three SNPs that are present in amino acid coding regions of exons 1 and 4, although they also have not been investigated in relation to disease. However, two

studies that focussed on eight GPx3 SNPs in the promoter region found that the SNPs were associated with an increased risk of stroke and thrombosis.[176, 177]

The GPx4 isoform has the largest number of SNPs identified within exon regions - three in exon 1, two which are silent, and the third that is a missense mutation with a A>G substitution changing the amino acid at position two from asparagine (Asn) to serine (Ser). However, this mutation has not yet been studied in relation to any disease. Similarly, two other missense mutations in exons 3 and 7, have not been studied in relation to disease. Other silent mutations are also found in exons 2, 4, 5 and 7. The silent mutation identified in exon 4, rs1042863, is a T>C substitution that changes the amino acid at position 122, with glycine (Gly) being unchanged. This SNP was reported to be associated with all cause mortality in patients diagnosed with breast cancer.[178] However, another study of breast cancer survivors examined 54 polymorphisms in different antioxidant genes including GPx4. This GPx4 SNP is a silent mutation in exon 7, rs713041, that changes a C>T at position 220 and a C>G mutation in the 5' region, rs757229. The study reported that both SNPs were associated with mortality in breast cancer patients.[178] A SNP that has yet to be cited in the NCBI database, a C>T mutation at position 718 of GPx4, has been linked with colorectal cancer.[179] This SNP has also been associated with increased leukotriene biosynthesis with C/C genotype compared with T/C and T/T genotypes.[180] In colorectal adenoma, there were no relationships between six SNPs in GPx isoforms 1-4.[181] In GPx5, only four of the 66 identified SNPs are in amino acid coding regions and none of these mutations have been studied in relation to disease.

2.3.6 GPx1 Pro197Leu and Disease

Twenty studies have investigated the relationship between the GPx1 Pro197Leu SNP and diseases including cancer, diabetes and vascular disease (Table 2-2).

2.3.6.1 Breast Cancer

Hu and Diamond[182] examined the GPx1 SNP in African American women, 79 with confirmed breast cancer and 517 controls. They found a greater incidence of the Leu/Leu genotype in women with breast cancer (23%) compared to the control group (12%) (OR 1.9, 95% CI 1.02-3.58). This study also examined whether the specific alleles affected the function of GPx1 using the human breast carcinoma cell line MCF-7. The Leu allele was less responsive to an increase in selenium, indicating that this genotype has less GPx activity and presents an increased risk of breast cancer.[182] A larger study involving 372 cases and 399 controls that also took into account the female menopausal state found no relationship between breast cancer and the GPx1 SNP.[134] This finding was supported by two of the largest studies both involving above 3,000 subjects.[183, 184] The females in both studies were almost all (94%) Caucasian, suggesting that any association found may be race-dependent. In a study by Raven-Haren et al.[74] involving 754 women, of whom 377 were diagnosed with breast cancer, a slightly higher risk of breast cancer was reported for participants that carried the Leu allele compared with Pro/Pro genotype (OR 1.43, 95% CI 1.07-1.92, p=0.05). In addition, the Leu/Leu genotype was associated with an increased risk of breast cancer in a study that examined two SNPs, GPx1 Pro197Leu and SOD2 Ala16Val. An increased risk of breast cancer was only uncovered when both GPx1 Leu/Leu and SOD2 Ala/Ala genotypes were found in patients (OR 1.87, 95% CI 1.09-3.19) when compared with patients carrying both GPx1 Pro/Pro and SOD2 Val/Val genotypes.[100] In summary, three studies have reported that both the GPx1 Leu/Leu genotype and Leu allele are associated with an increased risk of breast cancer.

2.3.6.2 Other cancers

Ratnasinghe et al.[185] reported that the incidence of lung cancer was increased by 80% in individuals with the GPx1 Pro197Leu SNP compared to patients with the Pro/Pro genotype. Furthermore, the incidence was 130% higher if the patient had the Leu/Leu genotype (OR 2.3, 95% CI 1.3-3.8). In a study that compared 213 Japanese bladder cancer patients with 209 healthy native Japanese controls, the authors reported an increased incidence of bladder cancer, especially high grade tumours, if the patient had the Pro/Leu genotype compared to Pro/Pro genotype (OR 2.58, 95% CI 1.07-6.18).[137] There were no patients or controls with the Leu/Leu genotype present in this Japanese population group. Two other studies report conflicting results, concluding that the Leu allele was associated with reduced risk of lung cancer.[186, 187] Rosenberger et al.[187] examined individuals between the ages of 24-50 and found that the Leu allele played a protective role against lung cancer within this age group (OR 0.6, 95% CI 0.4-0.8). The larger study, with 1,230 participants and 432 patients with lung cancer, also reported that the Leu allele was associated with a lower risk of lung cancer among a younger age group (50-60 years) (OR 0.35, 95% CI 0.15-0.82). In addition, this study reported a non-significant trend in older age groups with the Leu allele towards a reduced risk of lung cancer.[186] The Leu allele was significantly associated with the risk of developing non-Hodgkin lymphoma in a study that involved 928 patients and 1,446 controls from the UK and USA (OR 1.25, 95% CI 1.05-1.48).[150] Finally, there have been two studies that reported no association with the GPx1 Pro197Leu SNP when investigating prostate cancer in smokers and survival after treatment for acute myeloid leukaemia.[120, 172]

2.3.6.3 Diabetes and Other Diseases

A study involving 184 T2DM Japanese patients investigated the relationships between the GPx1 Pro197Leu SNP genotypes and degree of macrovascular disease.[37] They reported that CIMT and other measures of macrovascular disease were significantly greater in patients with the Pro/Leu compared to those with the Pro/Pro genotype (P=0.003, n=33 and 151, respectively). Peripheral vascular disease was also found to be higher in individuals with the Pro/Leu genotype than those with the Pro/Pro genotype (p=0.027, n=5 and 7, respectively).[37] A similar study examined the coronary artery calcium score in 91 T2DM patients and also found that the Pro/Leu genotype frequency was significantly higher in patients with scores higher than 1000 HU, (OR 3.61, CI 0.97-13.42).[154] A study involving 180 T1DM patients reported a higher frequency of the Pro/Leu genotype and less Pro/Pro in the 86 patients diagnosed with diabetic polyneuropathy compared to 94 patients without polyneuropathy, although the difference in the frequencies was not statistically significant.[38] The same group also reported in a study involving 466 patients with T1DM, found that the frequencies of GPx1 genotypes between those with and without diabetic neuropathy were not significantly different.[76] It should be noted that the studies that examined diabetic neuropathy did not include a healthy (non-diabetic) control group. Sergeeva et al.[188] also compared the GPx1 SNP in T2DM patients who had sustained a myocardial infarction or stroke and found no genotype differences between the two groups and they were not different from control (non-diabetic) participants.

Finally, Forsberg et al.[189] also compared the genotype of stroke patients with healthy controls, and found no association. In addition, none of the ten SNPs present in numerous antioxidants, including GPx1 Pro197Leu, were associated with GPx1 genotype and the occurrence of brain tumours.[96]

2.3.6.4 Summary

Genotypes of the GPx1 Pro197Leu SNP have been associated with an increased risk of various diseases. The GPx1 Pro/Leu genotype has been linked to lung cancer, bladder cancer and complications in T2DM. The Leu allele and Leu/Leu genotype have been shown to increase the risk of breast cancer. Studies that have investigated the potential association of the GPx1 Pro197Leu SNP genotypes and diabetes, stroke, brain tumours and prostrate cancer are inconclusive.

Table 2-2: Studies investigating the relationships between genotypes of the GPx1 Pro197Leu SNP and disease

Condition	Reference	Participants Cases /Controls	Genotype			Findings
			Pro/Pro Cases n [%]/ Controls n [%]	Pro/Leu Cases n [%]/ Controls n [%]	Leu/Leu Cases n [%]/ Controls n [%]	
Breast Cancer	Cebrain et al. 2006[184]	2,271/2,280 (>94% Caucasians)	Data not given	Data not given	Data not given	No significant association
Breast Cancer	Cox et al[100]	1,262/1,533	Data not given	Data not given	Data not given	No significant association with GPx1 SNP on its own. However when stratified with another SNP in SOD2, the Leu/Leu genotype was associated with breast cancer (OR 1.87, 95% CI 1.09-3.19, p=0.03)
Breast Cancer	Ahn et al.[183]	1,0.38/1,088 (>94% Caucasians)	472[45]/523[48]	456[44]/453[42]	110[11]/112[10]	No significant association.
Breast Cancer	Knight, et al.[134]	372/399	192[48]/169[45]	171[43]/164[44]	34[9]/39[10]	No significant association.
Breast Cancer	Ravn-Haren, et al.[74]	377/377	176[47]/205[54]	168[44]/136[36]	33[9]/36[10]	Leu (T) allele associated with a slightly higher risk of breast cancer (OR 1.43, 95% CI 1.07-1.92, p=0.05)
Breast Cancer	Hu et al.[182]	79/517	36[46]/244[47]	25[32]/209[40]	18[23]/64[13]	Leu/Leu genotype significantly higher in African American breast cancer patients (OR 1.9, 95% CI 1.02-3.58, p=0.045)
Prostate Cancer	Choi, et al.[172]	724/1,474	249[49]/704[50]	213[42]/578[41]	38[7]/109[7]	No significant association.
Lung Cancer	Raaschou-Nielsen et al.[186]	432/798	209[48]/348[44]	184[43]/358[45]	39[9]/92[11]	Leu allele was associated with a lower risk of lung cancer specifically among 50-60 years old (IRR 0.35, 95% CI 0.15-0.82, p=0.02)

Condition	Reference	Participants Cases /Controls	Genotype			Findings
			Pro/Pro Cases n [%]/ Controls n [%]	Pro/Leu Cases n [%]/ Controls n [%]	Leu/Leu Cases n [%]/ Controls n [%]	
Lung Cancer	Ratnasinghe, et al.[185]	315/313	91[29]/132[42]	157[50]/135[43]	67[21]/46[15]	Leu allele genotype frequency higher in lung cancer (OR 2.3, 95% CI 1.3-3.8, p<0.001)
Lung Cancer	Rosenberger et al.[187]	186/207	114 [61]/97[47]	63[34]/89[43]	9[5]/21[10]	Leu allele was reported in patients people below the age of 50 to have a protective role in the early onset of lung cancer (OR 0.6, 95% CI 0.4-0.8,p=0.002)
Bladder Cancer	Ichimura, et al.[137]	213/209 (Japanese)	166[77]/187[89]	47[22]/22[10]	0[0]/0[0]	Pro/Leu genotype significantly associated with advanced tumour stare in Japanese bladder cancer patients (OR 2.58, 95% CI 1.07-6.18, p=0.034)
Non-Hodgkin's lymphoma (NHL)	Lightfoot, et al.[150]	928/1,446	453[49]/773[54]	387[42]/551[38]	81[9]/120[8]	A significant association between Leu allele and increase risk of NHL (OR 1.25, 95% CI 1.05-1.48, p=0.01)
Acute Myeloid Leukaemia	Koistinen, et al.[120]	89	21[24]	39[44]	29[33]	No significant association.
T1DM (diabetic neuropathy)	Chistiakov, et al.[76]	216 (DN+) 250 (DN-)	151[70]/167[67]	60[28]/77[31]	5[2]/6[2]	No significant association.
T2DM	Hamanishi, et al.[37]	Participants total 184 (Japanese)	151[82]	33[18]	0[0]	Pro/Leu genotype associated with intima-media thickness in Japanese population (p=0.003, n=33/151 respectively)

Condition	Reference	Participants Cases /Controls	Genotype			Findings
			Pro/Pro Cases n [%]/ Controls n [%]	Pro/Leu Cases n [%]/ Controls n [%]	Leu/Leu Cases n [%]/ Controls n [%]	
T2DM (with and without complications)	Sergeeva, et al.[188]	103/52 53 MI cases 50 Stroke cases	27[52] 28[53] 28[56]	19[37] 21[40] 20[40]	6[11] 4[7] 2[4]	No significant association.
T1DM (diabetic polyneuropathy)	Zotova, et al.[38]	86 (DNP+) 94 (DNP-)	60[70]/64[67]	24[28]/28[31]	2[2]/2[2]	No significant association.
T2DM (coronary artery calcium score)	Nemoto et al.[154]	91	71[78]	20[22]	Data not given	Pro/Leu genotype had significant higher coronary artery calcium score than patients with Pro/Pro genotype (p=0.006)
Brain Tumour	Rajaraman, et al.[96]	565/494	250[47]/236[51]	226[43]/178[39]	52[10]/46[10]	No significant association.
Stroke	Forsberg, et al.[189]	101/214	56[53]/113[55]	38[38]/85[38]	7[7]/16[7]	No significant association.

2.3.7 Catalase and SNPs

The role of catalase is discussed in detail in section 1.2.4. A search of the NCBI database identified 245 catalase SNPs, with most of the studies focusing on the relationship between diseases in humans and rs1001179, a C>T substitution at position -262 from the transcription start site. This SNP is found within the GC-rich area of the promoter region and the mutation resides in the 5'-untranslated promoter sequence that increases expression, and has been correlated with blood catalase levels. [190-192] This SNP will be reviewed in depth in section 2.3.8, with details of published studies shown in Table 2-3.

Ten other catalase SNPs have been identified in the exon regions and only one has been investigated in disease states. This silent mutation, rs769217, found in exon 9, changes T>C and leaves the amino acid at position 389 as aspartic acid (Asp). It has been shown to have no association with the prevalence of T1DM,[193] no evidence of increased susceptibility to systemic lupus erythematosus (SLE)[194] and no association with non-pathological cognitive ageing.[175] This SNP was also studied in relation to breast cancer along with over 50 other SNPs, including three other catalase SNPs, rs511895, rs7104301 and rs1049982. It was, however, reported that specific SNPs found in the antioxidant enzyme, thioredoxin, were associated with an increased risk of breast cancer.[195]

Another thirteen catalase SNPs present in the promoter region of the gene have been identified, of which three have been studied in relation to disease. The SNPs are: rs 7943316, an A>T substitution at position -21 (A-21T); rs769214, an A>G substitution at position -844 (A-844G); and rs1001179, a C>T SNP at position -262 (C-262T). The A-21T SNP is not associated with either T1DM or T2DM.[126] One study examined the

association between all three silent SNPs along with another 45 SNPs and the occurrence of post cardiac surgery myocardial infarction.[196] A-844G SNP genotypes appeared to protect against postoperative myocardial infarction. As the C-262T SNP has been the most frequently investigated in disease states, it will be discussed in more detail (see Appendix A5.3. for a link to catalase SNPs).

2.3.8 Catalase C-262T and Disease

There have been eighteen studies that have investigated the catalase SNP C-262T in relation to disease (Table 2-3).

2.3.8.1 Breast Cancer

An increased risk of breast cancer has been linked to the catalase C-262T SNP in a number of case-control studies. A large study investigated 2,088 women of whom 1,017 were diagnosed with breast cancer. The authors found that women carrying the C/C genotype and who consumed more that 10 pieces of fruit per week had a reduced risk of breast cancer compared with those than had the C/T or T/T genotype (OR 0.59, 95% CI 0.38-0.89). This study also reported that women with C/C genotype had higher catalase red blood cell activity.[197] Another study conducted by the same group in 446 breast cancer patients found that there was no association with this catalase SNP and the development of radiotoxicity from treatment.[165] A smaller study in 279 breast cancer patients after either radiation therapy and/or chemotherapy found that the T/T genotype was weakly associated with a reduced risk of death in women that had received cancer treatment: this was not statistically significant (p=0.150).[166] The two largest studies, in which over 50 SNPs were examined, including catalase C-262T, found no associations with breast cancer risk when the SNPs were examined alone.[184, 195] However, one study

reported an association between C-262T and breast cancer when a two-way analysis was performed that included another catalase SNP, rs511895 (p=0.0085).[195]

2.3.8.2 Other Cancers

Three studies examining non-Hodgkin lymphoma, lung cancer and survival in acute myeloid leukaemia found no association with the C-262T catalase genotype.[75, 120, 150] However, when investigating the relationship with prostate cancer, it was noted that in patients diagnosed under 65 years of age, the T/T genotype was weakly associated with an increased risk (OR 2.0, 95% CI 0.97-3.95).[172]

2.3.8.3 Diabetes

Three studies have investigated the relationship between T1DM and catalase C-262T SNP genotypes.[38, 76, 193] The largest of the three studies involved two separate groups: one consisting of 1,642 individuals from large families, and the second, a large case-control study involving 7,460 participants of which 3,530 had T1DM. No association between catalase C-262T genotypes and the risk of T1DM was found.[193] In contrast to these findings, the other two studies (466 and 180 patients, respectively) reported that T1DM patients with the catalase C allele had an increased risk of developing diabetic neuropathy.[38, 76]

2.3.8.4 Other Diseases

A case-control study involving 100 patients diagnosed with hypertension and 93 normotensive subjects investigated the relationship between blood pressure and two SNPs within the promoter region of the catalase gene, C-262T and A-844G. There was a significant association between hypertension and the catalase genotype combination -844 A/A and -262 C/T and T/T.[198] A study investigating the risk of asthma in

association with catalase C-262T SNP, involved 567 participants, of whom 251 had asthma, reported that the C allele frequency was significantly higher in patients compared with controls (p=0.033). It was also found that non-smokers carrying the T allele were protected against the development of asthma (OR 0.35, 95% CI 0.15-0.85).[148] A later study performed by the same group found that there was no association between catalase genotype and chronic obstructive pulmonary disease.[139] A case-control study involving 267 participants, of which 137 had been diagnosed with Alzheimer's disease, reported no association with the C-262T polymorphism.[199] There have also been no associations found between the catalase genotype and the autoimmune disease, systemic lupus erythematosus,[194] or acute renal failure.[77]

2.3.8.5 Summary

A small number of studies have shown equivocal results when investigating the associations between genotypes from the catalase C-262T SNP and disease in humans. The T allele in the catalase C-262T SNP has been reported to protect against the development of asthma, and the development of diabetic nephropathy in T1DM patients. In contrast, the C/C genotype was found to be associated with a reduced risk of developing breast cancer. However, other studies have reported that individuals with the T/T genotype have an increased risk of hypertension and prostate cancer. The remainder of the studies (67%) reported no association between disease and the catalase C-262T SNP.

Table 2-3: Studies investigating the relationships between genotypes of the catalase C-262T SNP and disease

Condition	Reference	Participants	Genotype			Findings
		Cases/Controls	CC Cases n [%]/ Controls n [%]	CT Cases n [%]/ Controls n [%]	TT Cases n [%]/ Controls n [%]	
Breast Cancer	Cebrain et. al. 2006[184]	4,474/4,580	Data not given	Data not given	Data not given	No significant association
Breast Cancer	Oestergaard et. al. 2006[195]	2,271/2,280	Data not given	Data not given	Data not given	No significant association
Breast Cancer	Ahn, et al.[197]	1,008/1,056	614[61]/679[64]	349[35]/335[32]	45[5]/42[4]	C/C genotype showed a reduced risk of breast cancer in women that consumed >10 pieces of fruit per week (OR 0.59, 95% CI 0.38-0.89, p=0.02)
Breast Cancer	Ahn, et al[165]	446	233[56]	162[39]	22[5]	No significant association.
Breast Cancer	Ambrosone, et al.[166]	279	171[62]	93[33]	15[5]	No significant association. However T/T genotype was slightly associated with decreased hazard of death (p=0.150).
Prostrate Cancer	Choi, et al.[172]	724/1,474	317[62]/885[63]	165[32]/461[32]	26[5]/57[4]	T/T genotype associated with an increased risk in men diagnosed with prostate cancer before the age of 65 (OR 2.0, 95% CI 0.97-3.95, p=0.079)
Lung Cancer	Ho, et al.[75]	240/240	209[90]/217[91]	19[10]/23[8]	0[0]/2[1]	No significant association.
Non-Hodgkin lymphoma (NHL)	Lightfoot, et al.[150]	928/1,446	554[61]/867[60]	298[33]/498[35]	57[6]/72[5]	No significant association.
Acute Myeloid Leukaemia	Koistinen, et al.[120]	89	38[43]	39[44]	12[13]	No significant association.
T1DM	Pask, et al.[193]	3,530/3,930	2,189[62]/2,397[61]	1,165[33]/1,376[35]	176[5]/157[5]	No significant association.
		As well as in large families 1,642	985[60]/1,018[62]	575[35]/542[33]	82[5]/82[5]	

Condition	Reference	Participants	Genotype			Findings
		Cases/Controls	CC Cases n [%]/ Controls n [%]	CT Cases n [%]/ Controls n [%]	TT Cases n [%]/ Controls n [%]	
T1DM (diabetic neuropathy)	Chistiakov, et al.[76]	216 (DN+) 250 (DN-)	83[38]/80[32]	80[37]/74[30]	53[25]/96[38]	T allele shown to protect against the development of diabetic neuropathy (OR 1.98, 95% CI 1.53-2.58, p=0.002)
T1DM (diabetic neuropathy)	Zotova, et al.[38]	86 (DPN+) 94 (DPN-)	56[65]/50[53]	26[30]/42[45]	4[5]/2[2]	T allele showed a reduced risk of diabetic neuropathy (OR 1.6, 95% CI 1.06-2.42, p=0.027)
Asthma	Mak, et al.[148]	251/316	237[95]/279[91]	12[5]/29[9]	CT and TT Data pooled	T allele was found to protect against asthma in non - smokers
Chronic Obstructive Pulmonary Disease	Mak et al.[139]	165/165	149[91]/153[93]	13[8]/11[7]	2[1]/0[0]	No significant association.
Hypertension	Zhou, et al.[198]	220/191	6[5]/2[1]	34[30]/37[28]	86[68]/91[72]	C/T and T/T genotype showed an association with hypertension when patients had another SNP present
Alzheimer's Disease	Goulas, et al.[199]	137/130	84[61]/74[57]	44[32]/49[38]	9[9]/7[7]	No significant association.
Systemic Lupus Erythematosus (SLE)	D'Souza, et al.[194]	100/113	97[87]/102[91]	14[13]/10[9]	0[0]/0[0]	No significant association.
Acute Renal Failure	Perianayagam, et al.[77]	200	112[56]	74[37]	14[7]	No significant association.

2.3.9 Conclusions

There is weak evidence that the most investigated SNPs of the SOD2, GPx1 and catalase genes have been associated with a number of diseases. The SOD2 Ala16Val SNP has been linked with cancer, in 51% of all studies investigated (67% for breast cancer) and the Ala/Ala genotype or Ala allele are particularly indicative of this relationship. The same genotypes have also been associated with an increased risk of motor neurone disease, brain tumours, systemic sclerosis and schizophrenic subjects developing tardive dyskinesia during antipsychotic therapy. The Val/Val or Val allele has been positivity associated with CVD risk and with co-morbidities in 57% of studies in T1DM and T2DM patients. The GPx1 Pro197Leu SNP provides the genotype Leu/Leu or Leu allele and has been associated with breast cancer in 50% of studies. However, with other cancers, the results are inconclusive, with only 29% of studies showing that the Leu allele plays a protective role against cancer, 29% showing that there is an increased risk of cancers and 29% of studies showing no association with GPx1 genotype. The catalase C-262T SNP was reported to be associated with the reduced risk of breast cancer in patients with the C/C genotype, and in 80% of studies, to have no association with breast cancer. With other cancers, only one study found an association between the T/T genotype and prostate cancer and the rest found no association. In T1DM, there were 4,176 cases examined and in only 15% of these cases was there an association found with the T allele. The T allele genotype has also been linked to asthma and hypertension. In summary, this review has demonstrated, at best, weak evidence that specific diseases are linked to selected SNPs of SOD2, GPx1 and catalase genes. However, to the best of our knowledge, there have been no studies examining CKD with any of the three antioxidant enzyme SNPs. Hence, the overall aim of the present study is to examine a CKD population and determine any specific genotypes have any affect on the aetiology CKD.

2.3.10 Aims of this study

Variations in antioxidant enzyme activities may be the result of polymorphisms present in the genes regulating these antioxidants in CKD patients. Therefore, the aims of the present study were to determine whether:

(a) the frequency of specific SNPs, present in GPx1, SOD2 and catalase genes and whether antioxidant enzyme activities are significantly different between CKD patients (n=230) and control subjects (n=224),

(b) there are associations between antioxidant genotypes and renal function.

Additionally, a cohort study was employed to determine whether the progression of kidney disease (i.e., decline in eGFR) over approximately one year was associated:

(c) with specific genotypes resulting from the aforementioned GPx1, SOD2 and/or catalase SNPs, and

(d) altered plasma GPx, RBC GPx, RBC SOD and/or RBC catalase activities.

Chapter 3 describes and discusses the results obtained from the case-control study (Research Aims a and b).

Chapter 4 describes and discusses the results obtained from the cohort study (Research Aims c and d).

Chapter 5 provides a general discussion and provides suggestions for future research.

There is some repetition as chapters 2, 3 and 4 were written for publication in various peer reviewed journals.

CHAPTER 3: ANTIOXIDANT GENOTYPE AND ACTIVITY IN RELATION TO KIDNEY FUNCTION: A CASE-CONTROL STUDY

3.1 Abstract

Background and Objectives: Oxidative stress is associated with the progression of chronic kidney disease (CKD). Single nucleotide polymorphisms (SNPs) of the antioxidant enzymes superoxide dismutase (SOD)2 Ala16Val, glutathione peroxidase (GPx)1 Pro197Leu and catalase C-262T have been associated with the pathogenesis of cancer and diabetes. However, links between SNPs of these enzymes and CKD have yet to be investigated. The aims of this study were to compare antioxidant genotypes and activities of CKD patients and controls, and to determine their relationship to kidney function. ***Methods:*** CKD patients (n=230) and controls (n=224) were screened for the SNPs, GPx1, SOD2 and catalase. In addition, plasma and RBC GPx, RBC SOD and RBC catalase activities were measured. ***Results:*** Significantly more CKD patients (n=5) had the GPx1 Leu/Leu genotype compared to controls (n=0). Although not significant, patients with the GPx1 Leu/Leu or SOD2 Ala/Ala genotypes had reduced eGFR compared with the GPx1 Pro/Leu and SOD2 Val/Val genotypes. CKD patients had significantly lower plasma GPx and RBC catalase activities compared to controls. In contrast, both RBC GPx and RBC SOD activities were significantly higher in CKD patients ($p<0.001$). ***Conclusion:*** CKD is associated with impaired plasma GPx and catalase activities and enhanced RBC GPx and SOD activities. While genotype frequencies were similar for both groups, lower eGFR was associated with the GPx1 Leu/Leu genotype.

3.2 Introduction

Variations in antioxidant enzyme activities of GPx, SOD and catalase may be the result of polymorphisms present in the genes regulating antioxidants in CKD patients. Therefore, the aims of the present study were to determine whether: (a) the frequency of specific SNPs, present in GPx1 Pro197Leu, SOD2 Ala16Val and catalase C-262T genes and antioxidant activities are significantly different between CKD patients and control subjects, and (b) there is an association between antioxidant genotypes and CKD.

3.3 Methods

3.3.1 Study Subjects

The study was approved by the Human Research Ethics Committee (Tasmania) Network (H08618). A total of 269 CKD patients were recruited in Tasmania, Australia, (population ~0.5 million), this included greater than 90% of the Northern Tasmania's diagnosed CKD population (physicians estimate) between March 2006 and May 2008, from the Launceston General Hospital, private clinical practices in the Launceston area, and the North West Satellite Renal Unit (Burnie). Of the patients initially recruited and who provided written consent, 39 did not provide a blood sample; hence, data from 230 were included in this study. Inclusion criteria for the CKD subjects were $\geq$ 18 years of age, diagnosed with CKD using the modification of diet in renal disease (MDRD) formula to estimate glomerular filtration rate (eGFR) <60mL/min/1.73m^2. The primary causes of CKD, according to the classification system used by the Australian and New Zealand Dialysis Transplant Registry,[200] were vascular disease (n=77), glomerulonephritis (n=43), diabetic nephropathy (type 1, n=9; type 2, n=40), reflux nephropathy (n=11), polycystic kidney disease (n=7), analgesic nephropathy (n=2), other (n=36) and uncertain diagnosis (n=5). Control subjects were recruited from the

general population in North and North West Tasmania and through the Australian Electoral Commission roll. Letters were mailed to over 2,000 individuals. Approximately 500 people provided written consent to take part in the study with 41 reporting they had a prior diagnosis of kidney disease. The 459 participants that were left they were age and sex matched to the CKD patients as closely as possible as they responded, leaving 224 participants for inclusion into this study as general population controls. Inclusion criteria for the control subjects were no known history of kidney disease or impairment. Some control subjects had existing high blood pressure (n=70), T2DM (n=13), angina or other cardiac conditions (n=33). The control participants were grouped by age and gender to match the CKD patients as closely as possible. However, the study was limited by the difficultly of recruiting controls >80 years, which resulted in a significant difference in the mean ages of the two groups.

3.3.2 Blood Sampling and Biochemical Analysis

Venous blood, collected in anticoagulant-free and lithium heparin tubes, was centrifuged, serum and plasma separated, and stored at -80°C for later analysis of serum creatinine and plasma GPx activity. RBC lysate was prepared from lithium heparin samples that were washed with 0.9% saline and lysed with cold distilled H_2O and stored at -80°C for measurement of RBC GPx, SOD and catalase activities.[201] Both plasma and RBC GPx activities were measured on a DataPro Clinical Analyzer (Thermo-Electron Corporation Melbourne, Australia), RBC SOD and catalase activities measured on a Cobas Mira Clinical Analyzer (Roche Laboratories, USA). Commercially available kits (Ransel, Randox Laboratories Ltd, Crumlin, Ireland) were used to measure plasma, RBC GPx and RBC SOD activities. The inter- and intra-batch coefficients of variation (CV) were: plasma GPx 6% and 10.2%; RBC GPx activity, 5.2% and 6.7%, and SOD activity 4.6% and 5.7%, respectively. RBC catalase activity was measured using

Slaughter and co- workers[202] methodology and inter- and intra-batch CV's were 1.8% and 5.3%, respectively.

Serum creatinine was measured using an Architect C8000 biochemical analyser (Abbott Diagnostics, Abbott Park, USA, Calibrator MC Cal Traceable to NIST SRM 909b-2), within 3 months of blood collection, and used to calculate eGFR by the MDRD equation.

3.3.3 Genotyping Method

Genomic DNA was isolated from whole blood within two weeks of blood collection using a Viogene Blood and Tissue Genomic DNA Extraction Miniprep System (Diagnostic Technology, Belrose, Australia). GPx1 Pro197Leu and SOD2 Ala16Val SNPs were determined using a RFLP-PCR assay and catalase C-262T SNP was determined through sequencing by Australian Genome Research Facility Ltd (AGRF),

3.3.3.1 Glutathione Peroxidase

The GPx1 Pro197Leu SNP was genotyped using an RFLP-PCR assay based on the methods from Forsberg, et al.[203] Primers were designed to produce a 460bp amplicon containing the SNP site, using the NM 000581 sequence from NCIB and Primer3. PCR primers synthesized by Geneworks (Adelaide, Australia). DNA was amplified using Qiagen Hotstar Taq MasterMix (Qiagen, Australia) and 0.25 uM of each primer in a 10 uL volume on a PTC-200 (MJ Research) with the initial temperature at 94°C for 15 minutes, then 94°C for 45 s, then 56°C for 45 s, then 72°C for 30 s repeated 34 times and then 72°C for 10minutes (Table 3-1). Ten units of *ApaI* which cuts at the sequence GGGCCC site was added directly to the PCR mix followed by incubation at 37°C for 60 min. Digestion fragments were visualized by electrophoresis in a 3% agarose gel in TBE buffer (see Appendix 1). Expected fragment sizes are as follows:

- homozygous proline/proline- 252, 120, and 88bp,
- heterozygous proline /leucine – 252, 208, 120, 88bp, and
- homozygous leucine/leucine – 252 and 208bp

Each PCR assay included a NTC, homozygous proline/proline and homozygous leucine/leucine DNA sample as positive controls. Also 10% of the samples were sequenced to ensure the accuracy of the genotyping.

3.3.3.2 Superoxide Dismutase

The SOD2 Ala16Val was genotyped using an RFLP-PCR assay based on the method of Li, et al.[112] Primers were designed to produce a 492bp amplicon containing the SNP site, using the NM 001024466 sequence from NCBI Genebank and Primer3 [112]. PCR primers weresynthesized by Geneworks (Adelaide, Australia) (Table 3-1). The isolated DNA was amplified using Qiagen Hotstar Taq MasterMix (Qiagen, Australia) incorportating the 'Q solution' at 1X concentration in the final reaction mix according to manufacturers instructions and 0.25 uM of each primer in a 10 uL volume on a PTC-200 (MJ Research) with the initial temperature at 94°C for 15 minutes, then 94°C for 45 s, then 56°C for 45 s, then 72°C for 30 s repeated 34 times and then 72°C for 10minutes. Ten units of *BsaWI* which cuts at the sequence WCCGGW (where W = A/T) site was added directly to the PCR mix followed by incubation at 60°C for 60 min. Digestion fragments were visualized by electrophoresis in a 2% agarose gel in TBE buffer (see Appendix 1). Expected fragment sizes are as follows:

- homozygous alanine/alanine- 492 bp,
- heterozygous alanine/valine – 492, 339 and 153 bp, and
- homozygous valine/valine – 339 and 153 bp

Each PCR assay included a NTC, homozygous alanine/alanine and homozygous valine/valine DNA sample. Also 10% of the samples were sequenced to ensure the accuracy of the genotyping.

3.3.3.3 Catalase

The catalase SNP C-262T, the genotype was carried out be sequencing a 260bp fragment. The PCR primers were designed by Forsberg, et al.[191] and were synthesized by Geneworks (Adelaide, Australia). DNA was amplified using Qiagen Hotstar Taq MasterMix (Qiagen, Australia) and 0.25uM of each primer in a 10uL volume on a TC-200 (MJ Research) with the initial temperature at 94°C for 15 minutes, then 94°C for 30s, then 55°C for 30 s, then 72°C for 30 s repeated 35 times (Table 3-1). The PCR product was diluted one in two, sequencing carried out by Australian Genome Research Facility Ltd (AGRF), with the reverse primer (See Appendix 1 for sequencing)

Table 3-1: Methodology for determining antioxidant SNPs

SNP	Primers	PCR Protocol	Restriction Digest
GPx1 Pro197Leu	F=5'-CGCCAAGAACGAAGAGATTC-3'	Denature: 94°C for 15 mins	*Apa1* incubates at 37°C for 60 mins
	R=5'-CAGGTGTTCCTCCCTCGTAG-3'	followed by 94°C for 45 s	Pro/Pro-252, 120 and 88 bp
		Annealing: 56°C for 45 s	Pro/Leu-252, 208, 120, 88 bp
		Extension: 72°C for 30 s	Leu/Leu-252 and 208 bp
		#Cycles:30	
SOD2 Ala16Val	F=5'-CACCAGCACTAGCAGCATGT-3'	Denature: 94°C for 15 mins	*BsaWI* incubation at 60°C 60 min
	R=5'-GGTGACGTTCAGGTTGTTCA-3'	followed by 94°Cfor 30 s	Ala/Ala-492 bp,
		Annealing: 56°C for 30 s	Ala/Val-492, 339 and 153 bp
		Extension: 72°C for 30 s	Val/Val-339 and 153 bp
		#Cycles: 37	
Catalase C-262T	F=5'-TGAAGGATGCTGATAACC-3'	Denature: 94°C for 15 mins	
	R=5'-ATCAGCACCACCCCCTCGTC-3'	followed by 94°C for 30 s	
		Annealing: 55°C for 30 s	N/A
		Extension: 72°C for 30 s	
		#Cycles: 35	

F = forward primer; R = reverse primer

3.3.4 Statistical Analysis

Hardy-Weinberg analysis was performed on all three genotype population groups. SOD2 and catalase genotypes for both controls and patients were within the Hardy-Weinberg equilibrium. However, deviation from the Hardy-Weinberg equilibrium was observed in GPx1 genotypes for both patients and controls; both having reduced Leu/Leu genotypes and higher Pro/Leu genotypes. Therefore, the comparison of patient and control genotype frequencies were analysed using the Chi squared test. Sample size calculations predicted a minimum requirement of 239 patients and 239 controls (refer Appendix 2).[189]

The associations between outcome measures, primary exposure measures and other covariates were estimated using general linear modelling (GLM). Ordinal logistic regression was used as a non-parametric alternative to GLM, when skewness and heteroskedasticity of residuals indicated that assumptions of simple linear regression were violated, and comparisons reported as odds ratio (OR) with all analysis being adjusted for age. .Selection of covariates to include in the multivariate models was performed using backward stepwise regression with P-value criteria being 0.1. When determining associations with either eGFR or antioxidant enzyme activities in patients and controls separately, the age, gender, smoking status, all antioxidant activities, antioxidant genotypes, primary causes of CKD (patients), co-morbidities (controls) and medications were examined. P-values were corrected for multiple comparisons using the Holm method. Whilst R2 values would have assisted in testing 'goodness of fit' to the regression model, these were all <0.1, and hence, deemed irrelevant and not included All statistical analyses were performed using Stata 10.1/IC (StataCorp LP, College Station, TX, USA).

3.4 Results

The demographics of CKD patients and controls are presented in Table 3-2. There were no significant differences between patients and controls in gender distribution or smoking status. Patients were significantly (P<0.001) older than controls, and hence, all analyses were adjusted for age. Although, antioxidant activities were not influenced by age in CKD patients, age was associated with reduced plasma GPx in controls (OR 0.71, 95% CI 0.51 to 0.97, p=0.034). Interestingly, when the controls were stratified according to eGFR (and stages of CKD), 13% were found to be in stage 3 according to Kidney Disease Outcomes Initiative (KDOQI) guidelines[6] (Table 3-2).

Table 3-2: Demographics and clinical characteristics of CKD patients and controls

	Controls (n=224)	CKD patients (n=230)
Gender, Female/Male	111/113	106/124
Age, mean±SD (range)	63.6±10.7 (27-85)	68.6±12.9 (24-92)*
Smoking Status, n (%)		
Never Smoked	107 (48%)	80 (35%)
Given up	85 (38%)	53 (23%)
Current Smoker	15 (7%)	10 (4%)
Stages of CKD, n (%)		
Stage 1 (>90ml/min/1.73m^2)	28 (13%)	0 (0%)
Stage 2 (89-60ml/min/1.73m^2)	166 (74%)	9 (4%)
Stage 3 (59-30ml/min/1.73m^2)	30 (13%)	104 (45%)
Stage 4 (29-15ml/min/1.73m^2)	0 (0%)	91 (40%)
Stage 5 (<15ml/min/1.73m^2)	0 (0%)	26 (11%)
Medications, n (%)		
Erythropoietin	0 (0%)	115 (50%)
ACE inhibitor	36 (16%)	104 (45%)
Angiotensin receptor blocker	29 (13%)	74 (32%)
Statin	41 (18%)	83 (36%)
Anti-inflammatory	7 (3%)	17 (7%)

Significant difference in mean age was estimated by general linear modelling. *p<0.001

Antioxidant enzyme genotype frequencies for CKD patients and controls are shown in Table 3-3. Significantly (p=0.023) more patients had the GPx1 Leu/Leu genotype

compared to controls (5 vs 0), with the primary cause of CKD being diabetes for all five patients (T1DM, n=1; T2DM, n=4) and four patients in stage 4 of CKD. However, the precise odds ratio for this comparison could not be determined due to the zero count in the controls. There were no other statistically significant differences in genotype frequencies between patients and controls.

Table 3-3: Genotype frequency and the odds ratio (OR) for differences in genotypes between CKD patients and controls

	Controls n=224	CKD patients n=230	OR (95%CI)
GPx Pro197Leu			
Pro/Pro	112 (50%)	110 (48%)	0.92 (0.62-1.35)
Pro/Leu	112 (50%)	115 (50%)	1.0 (0.68-1.50)
Leu/Leu	0	5 (2%)	∞ (1.23-∞)*
SOD2 Ala16Val			
Ala/Ala	55 (25%)	54 (23%)	0.94 (0.60-1.48)
Ala/Val	119 (53%)	116 (51%)	0.90 (0.61-1.32)
Val/Val	50 (24%)	60 (26%)	1.23 (0.78-1.94)
Catalase C-262T			
C/C	138 (62%)	148 (64%)	0.77 (0.52-1.15)
C/T	73 (33%)	74 (32%)	0.94 (0.62-1.42)
T/T	13 (5%)	8 (4%)	1.62 (0.61-4.59)

Due to skewness and heteroskedasticity, the odds ratio (OR) and 95% confidence intervals (95%CI) were estimated using ordinal logistic regression and adjusted for age,*p=0.027. The precise estimate 95% CI cannot be determined due to the zero count in the controls with Leu/Leu genotype.
∞ = infinity.

As expected, eGFR was significantly ($p<0.001$) lower in patients than in the control group and this was consistent across the three-antioxidant genotypes (Table 3-4). There was a trend towards reduced eGFR in patients with the Leu/Leu GPx1 genotype when compared to patients with the Pro/Leu genotype ($p=0.031$) and eGFR was non-significantly lower in patients with the SOD2 Ala/Ala genotype compared with the Val/Val genotype ($p=0.074$). In addition, controls with the catalase C/T genotype had significantly lower eGFR compared to controls with the catalase T/T genotype ($p=0.028$) (Table 3-4).

Table 3-4: Comparison of eGFR according to antioxidant genotypes, in both CKD patients and controls separately. Additional comparisons were also made within the same genotype and with those that were statistically significant or approaching significance are reported below.

	Controls (n=224)		**CKD patients** (n=230)	
	Mean±SD	**OR (95% CI)**	**Mean±SD**	**OR (95% CI)**
All (eGFR ml/min/1.73m^2)	73.15±13.34		31.00±14.31	0.01(0.002-0.009)**
Genotypes				
GPx1 Pro197Leu				
Pro/Pro	73.53±12.56	Ref	30.67±14.84	Ref
Pro/Leu	72.77±14.11	1.25 (0.79-2.00)	31.60±14.05	1.17 (0.72-1.92)
Leu/Leu	———	———	24.40±5.31	0.63 (0.82-1.25)[a]
SOD2 Ala16Val				
Ala/Ala	73.89±14.70	Ref	28.85±13.62	Ref
Ala/Val	72.84±13.06	1.17 (0.59-2.36)	30.75±14.16	1.27 (0.74-2.14)
Val/Val	73.08±12.64	0.95 (0.53-1.70)	33.42±15.06	1.82 (0.94-3.49)[b]
Catalase C-262T				
C/C	73.20±13.36	Ref	31.45±14.07	Ref
C/T	71.89±13.14	1.25 (0.75-206)*	31.51±15.09	0.80 (0.48-1.36)
T/T	79.77±13.17	2.42 (0.87-6.72)	27.25±11.90	0.59 (0.21-1.65)

Mean eGFR in CKD patients compared with controls. The odds ratio (OR) and 95% confidence interval (95%CI) were estimated using ordinal logistic regression due to skewness and heteroskedasticity and adjusted for age.
** p<0.0001; * p=0.028 (OR 3.02, 95% CI 1.04 to 8.74) catalase C/T compared with T/T in controls;
[a] p=0.054 (OR 0.53, 95% CI 0.28 to 0.101) GPx1 Pro/Leu compared with Leu/Leu in CKD patients;
[b] p=0.074 (OR 1.82, 95% CI 0.94 to 3.49) SOD2 Ala/Ala compared with Val/Val in CKD patients.

Figure 3-1 A shows that CKD patients had significantly lower plasma GPx (OR 0.04, 95% CI 0.03 to 0.07) and catalase (OR 0.48, 95% CI 0.34 to 0.67; Figure 3-1 D) enzyme activities compared to controls. In contrast, both RBC GPx and SOD enzyme activities were significantly higher in patients than in controls (OR 2.77, 95% CI 1.96 to 3.9; OR 2.71 95% CI 1.91 to 3.84, respectively) (Figure 3-1 B, C).

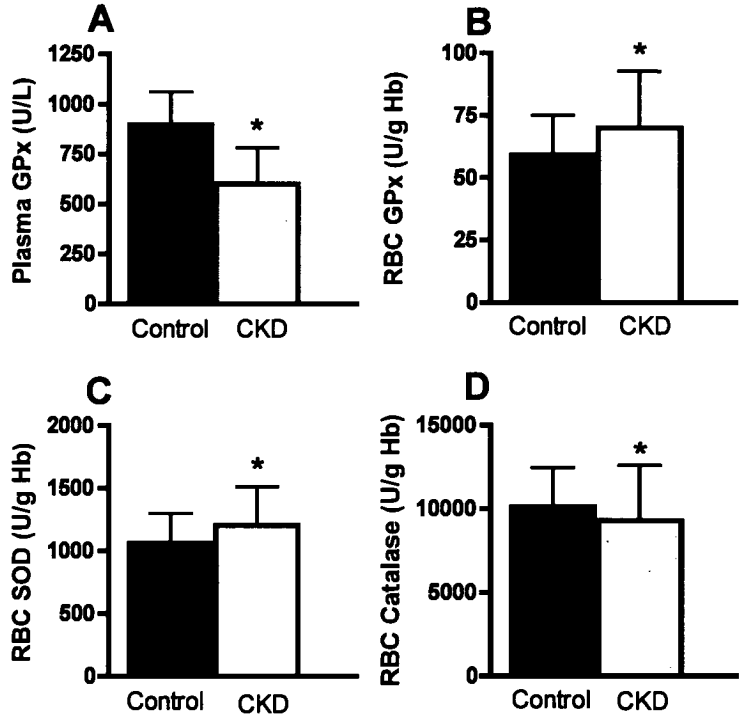

Figure 3-1: Antioxidant enzyme activities in CKD patients and controls.
(A) plasma GPx, (B) RBC GPx, (C) RBC SOD and (D) RBC catalase. Data analysed using general linear modelling and logistic regression and adjusted for age, * $p<0.0001$. Error bars show standard deviation.

The relationships between eGFR and antioxidant enzyme activities in patients and controls determined using univariate analysis are shown in Figure 3-2 A. There was a significant correlation between eGFR and plasma GPx activity in CKD patients (OR 4.29, 95% CI 2.52 to 7.31, $p<0.0001$) and this relationship persisted in multivariate

analysis ($p<0.0001$) (Table 3-5). This is our best estimate of an independent effect. There were no statistically significant associations between eGFR and RBC GPx, SOD and catalase enzyme activities in patients and controls (Figure 3-2 B, C and D). In controls, eGFR was lower in subjects taking anti-inflammatory and/or statin medications ($p=0.05$ and $p=0.02$, respectively, Table 3-5) and significantly higher in males ($p=0.001$). However, given that the MDRD formula is less accurate when eGFR is greater than 60ml/min/1.73m^2,[11] the latter observation should be viewed with some caution.

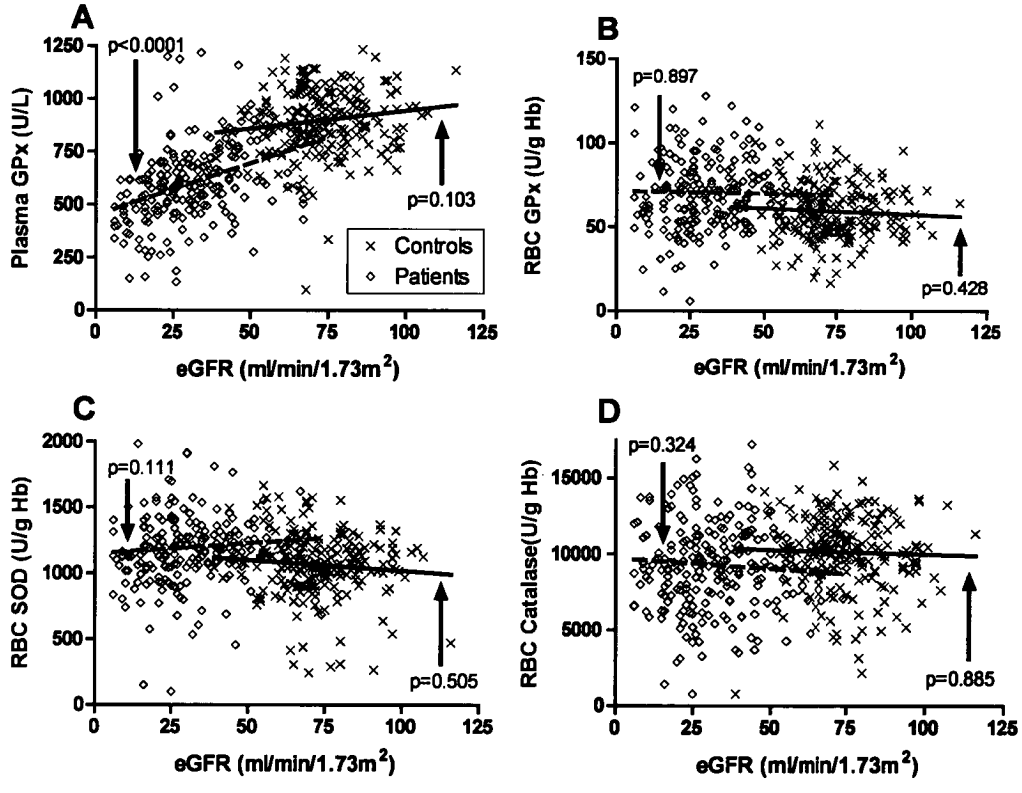

Figure 3-2: Relationships between eGFR and antioxidant enzyme activities in CKD patients and controls.
(A) plasma GPx, (B) RBC GPx, (C) RBC SOD and (D) RBC catalase. Lines represent linear regression and adjusted for age Dashed line = patients, solid line = controls..

Table 3-5: Multivariate analysis derived statistically significant associations with eGFR in CKD patients and controls with variables selected for inclusion determined through stepwise regression.

	Controls	CKD patients
GPx1 Pro197Leu		
Pro/Pro	———	———
Pro/Leu	———	1.08 (0.65-1.78)
Leu/Leu	———	0.65 (0.33-1.26)
Antioxidant Activities		
Plasma GPx Activity	1.33 (1.01 - 1.74)*	2.63 (1.80 - 3.84)**
RBC SOD Activity	0.74 (0.52 - 1.05)	———
Co-variates		
Age	0.36 (0.27-0.50)**	1.23 (0.96-1.58)
Gender (male)	1.53 (91.19-1.96)*	0.93 (0.72-1.20)
Still smoking	3.67 (1.43-9.37)*	
Causes of CKD		
T1DM	———	1.14 (0.38-3.38)
Medications		
Erythropoietin	———	0.29 (0.17-0.49)**
Statin	0.44 (0.22-0.90)$	———
Anti-inflammatory	0.11 (0.01-1.02)*	———
Constant	70.24 (67.86-72.62)	39.43 (36.26-42.61)

These associations were estimated using ordinal logistic regression due to skewness and heteroskedasticity. Blanks were data excluded from logistic regression analysis. Other exclusions: RBC GPx and catalase activities; SOD and catalase genotypes; *co-variants:* given up smoking; *causes of CKD*: glomerulonephritis, T2DM, vascular, polycystic kidney disease, uncertain, other and analgesic nephropathy; *co-morbidities*: T2DM *medications:* angiotensin II and ace inhibitors. The odds ratio (OR) and 95% confidence intervals (CI) co-variates shown in the model were selected by stepwise regression. Constant, is mean eGFR when all other confounders was minimum or zero. $p=0.05; *p<0.05; **p<0.0001

When data from patients and controls was combined, plasma GPx activity was significantly reduced in stages 3, 4 and 5 CKD compared with stage 1 (OR 0.14, 95% CI 0.07 to 0.29; OR 0.03, 95% CI 0.02 to 0.07; OR 0.01, 95% CI 0.04 to 0.02, respectively, Figure 3-3 A). RBC GPx activity was significantly higher in stages 3, 4 and 5 CKD compared with stage 1 (OR 2.55, 95% CI 1.37 to 4.73; OR 2.30, 95% CI 1.13 to 4.67; OR 3.05, 95% CI 1.14 to 8.13, respectively, Figure 3-3 B). Participants with stages 3 and 4 CKD had significantly higher SOD activity compared with stage 1 (OR 4.01, 95% CI 1.91 to 8.42; OR 2.69, 95% CI 1.21 to 6.00, respectively, Figure 3-3 C). SOD activity for stage 5 CKD was also higher but this was not statistically significant (OR 1.86, 95% CI 0.73 to 4.67). Catalase activity was similar at all stages of CKD (Figure 3-3 D).

Plasma GPx was significantly reduced in the total participant population that carried the GPx1 Leu/Leu genotype, compared with participants with the Pro/Pro and Pro/Leu genotypes (OR 0.23, 95% CI 0.10 to 0.51, $p<0.001$; OR 0.21, 95% CI 0.09 to 0.47, $p<0.001$, respectively). However, when patient and control plasma GPx data were examined separately, there were no significant differences in GPx activities between GPx1 genotypes. Table 3-6 shows that when confounding variables were taken into account, plasma GPx activity was only significantly reduced in patients with the catalase C/T genotype ($p=0.042$).

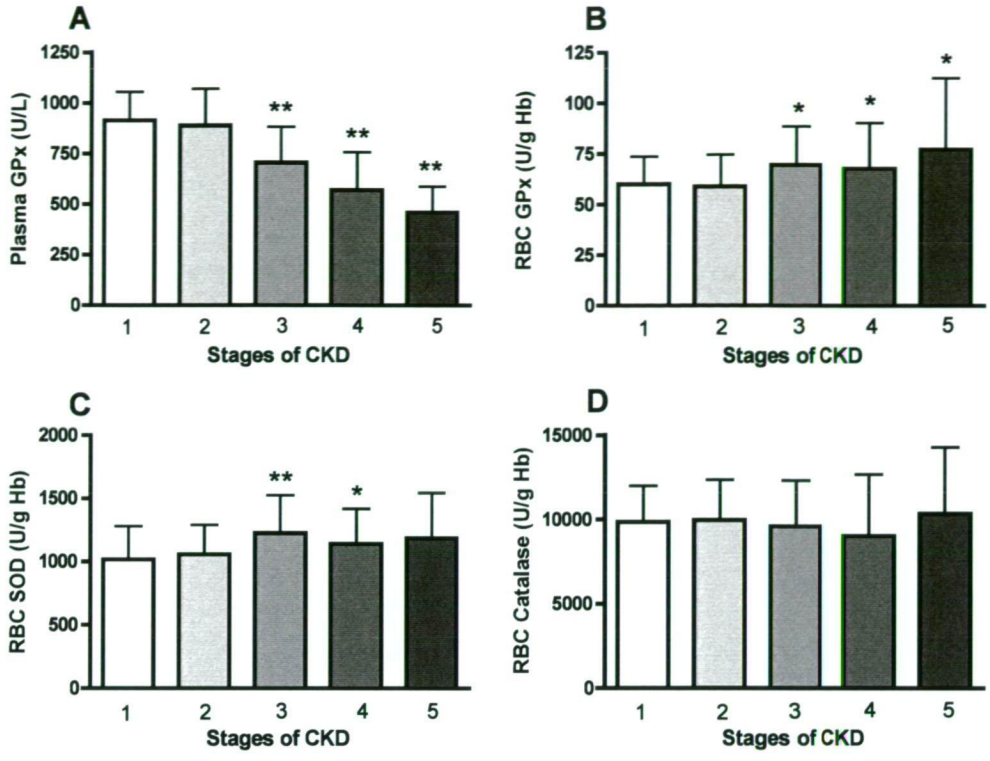

Figure 3-3: Antioxidant activities in all participants (n=454) in the different stages of CKD.
(A) plasma GPx, (B) RBC GPx, (C) RBC SOD and (D) RBC catalase.
Numbers of controls and patients in stages: stage 1, n=28 and n=0; stage 2, n=166 and n=9; stage 3, n=30 and n=104; stage 4, n=0 and n=91 and stage 5, n=0 and n=26, respectively. * $p<0.05$; ** $p<0.001$, significant difference from stage 1. Error bars show standard deviation.

Table 3-6: Multivariate analysis derived statistically significant associations between antioxidant enzyme activities in CKD patients and controls with variables selected for inclusion determined through stepwise regression.

	Plasma GPx		RBC GPx		RBC SOD		RBC Catalase	
	Control	CKD	Control	CKD	Control	CKD	Control	CKD
GPx1 Pro197Leu								
Pro/Pro	———	———	———	———	———	———	———	———
Pro/Leu	———	———	0.53 (0.32-0.85)*	———	0.55 (0.33-0.92)*	2.07 (1.27-3.36)*	1.42 (0.87-2.32)	———
Leu/Leu	———	———	———	———	———	11.52 (2.73-48.59)*	———	———
SOD2 Ala16Val								
Ala/Ala	———	———	———	———	———	———	———	———
Ala/Val	———	———	———	1.13 (0.43-2.97)	———	———	———	———
Val/Val	———	———	———	2.88 (1.06-7.82)*	———	———	———	———
Catalase C-262T								
C/C	———	-	———		———	———	-	-
C/T	———	0.44 (0.20-0.97)*	———	2.25 (0.98-5.14)	———	———	0.20 (0.11-0.36)**	0.40 (0.23-0.68)*
T/T	———	0.30 (0.02-3.09)	———	2.76 (1.07-7.11)*	———	———	0.13 (0.06 - 0.27)**	0.29 (0.13-0.66)*
Co-variants								
Age	0.74 (0.53-1.02)	0.93 (0.69-1.25)	1.01 (0.77-1.32)	1.25 (0.85-1.84)	0.82 (0.60-1.10)	0.93 (0.74-1.16)	1.26 (0.96-1.66)	0.87 (0.70-1.08)
Gender (male)	0.81 (0.50-1.30)	1.22 (0.88-1.71)	1.02 (0.80-1.30)	0.79 (0.57-1.09)	1.04 (0.82-1.31)	0.95 (0.74 - 1.21)	0.89 (0.69-1.14)	0.98 (0.76-1.26)
Given up smoking	———	0.44 (0.23-0.85)*	———	———	———	———	———	———
Still smoking	———	———	———	0.16 (0.02-1.17)	———	———	———	———
Co-morbidities								
High blood pressure	0.63 (0.36-1.12)	———	———	———	1.65 (1.01-2.69)*	———	———	———

Angina/Cardiac conditions	———	———	———	———	2.16 (1.11-4.19)*	———	———	———
Medication								
Erythopoietin	———	0.34 (0.18-0.63)*	———	1.47 (0.73-2.96)	———	0.74 (0.45-1.20)	———	———
Ace inhibitors	———	———	———	1.84 (0.91-3.75)	———	———	1.87 (1.00-1.00)*	———
Anti inflammatory	———	———	6.50 (2.42-17.41)**	0.25 (0.09-0.65)*	———	———	———	———
Constant	911 (886-937)	708 (658-758)	59.74 (56.44-63.04)	63.68 (54.00-73.37)	1037 (986-1089)	1133 (1061-1204)	10506 (9965-11047)	9012 (8420-9604)

The associations were estimated using ordinal logistic regression due to skewness and heteroskedasticity. Blanks were excluded from stepwise regression analysis other exclusions: causes of CKD: glomerulonephritis, T1DM, T2DM vascular, uncertain, other and analgesic nephropathy; co-morbidities: T2DM medications: angiotensin II and statins. The odds ratio (OR) and 95% confident intervals (CI) co-variates shown in the model were selected by stepwise regression. Constant, is mean of the antioxidant activity when all other confounders was minimum or zero.*$p<0.05$; **$p<0.0001$

Figure 3-4 A shows that RBC GPx activity was significantly reduced in controls with the Pro/Leu GPx1 genotype compared with Pro/Pro (OR 0.58, 95% CI 0.37 to 0.92, p=0.02). This effect persisted in control participants with the Pro/Leu genotype when adjusted for all confounding variables (p=0.009) (Table 3-6). Furthermore, multivariate analysis showed that RBC GPx activities in patients with the SOD2 Val/Val and catalase T/T genotypes were significantly higher when compared with their respective other heterozygote and homozygote genotypes (p=0.037 and p=0.035, respectively).

RBC SOD activity in patients with the GPx1 Pro/Leu genotype had significantly higher SOD activity compared with patients with the GPx1 Pro/Pro genotype (OR 2.18, 95% CI 1.36 to 3.50, p=0.001) (Figure 3-4 B). Patients with the GPx1 Leu/Leu genotype also had significantly higher SOD activity compared to those with either the Pro/Pro or Pro/Leu genotypes (OR 10.24, 95% CI 3.13 to 33.51, p<0.001 and OR 4.69, 95% CI 1.47 to 14.94, p=0.009, respectively). The opposite was found in controls with the GPx1 Pro/Leu genotype, who had significantly reduced SOD activity compared with the Pro/Pro genotype (OR 0.57, 95% CI 0.36 to 0.91, p=0.019). Table 3-6 shows that after multivariate analysis, the effects persisted in both patients and controls, with this being our best estimates of independent effects.

Applying univariate analysis, catalase activity was significantly reduced in all participants with both the catalase C/T and T/T genotypes, compared with catalase C/C genotype (OR 0.35, 95% CI 0.25 to 0.49, p<0.001; OR 0.32, 95% CI 0.21 to 0.49, p<0.001, respectively). Catalase activity was also significantly reduced in both patients and controls separately, with both catalase C/T and T/T genotypes compared with C/C genotype (patients: OR 0.45, 95% CI 0.28 to 0.72, p=0.001; OR 0.48, 95% CI 0.24 to 0.99, p=0.049, respectively; controls: OR 0.22, 95% CI 0.13 to 0.38, p<0.001; OR 0.15, 95% CI 0.08 to 0.029, p<0.001,

respectively) (Figure 3-4 C). Table 3-6 shows that this effect persisted after multivariate analysis, with both patients and controls with the catalase C/T and T/T genotypes having significantly reduced catalase activity compared with C/C genotype (patients: p=0.001; p=0.003; controls both: p<0.0001, respectively).

3.5 Discussion

This is the first study to examine and compare relationships between antioxidant enzyme genotypes and activities in CKD patients and a control population. The major findings were; 1) patients with CKD were more likely to have the GPx Leu/Leu genotype, 2) CKD patients with the GPx1 Leu/Leu genotype had reduced kidney function compared to those with Pro/Leu genotype, 3) kidney function was better in CKD patients with the SOD2 Val/Val genotype compared to those with the Ala/Ala genotype, and 4) there were significant differences between the antioxidant enzyme profiles of CKD patients compared with controls. These were independently related to kidney function and genotype.

The small number of individuals with the GPx1 Leu/Leu genotype limits the significance of the greater prevalence of this genotype in CKD and poorer kidney function in these patients. Previous studies also reported a low prevalence of this genotype. Studies investigating T2DM[37] and bladder cancer[137] reported no individuals (total of 606 Japanese patients and controls) with the Leu/Leu genotype. In contrast, the frequency of the GPx1 Leu/Leu genotype in Caucasians is higher, approximately 10% of control or disease groups.[183, 184, 189] In the present study, the frequency of the Leu/Leu genotype in both patients and controls was lower than predicted by Hardy-Weinberg analysis which may have been due to consanguinity, assortative mating and small sample size with the latter being the most likely cause of this genetic drift.

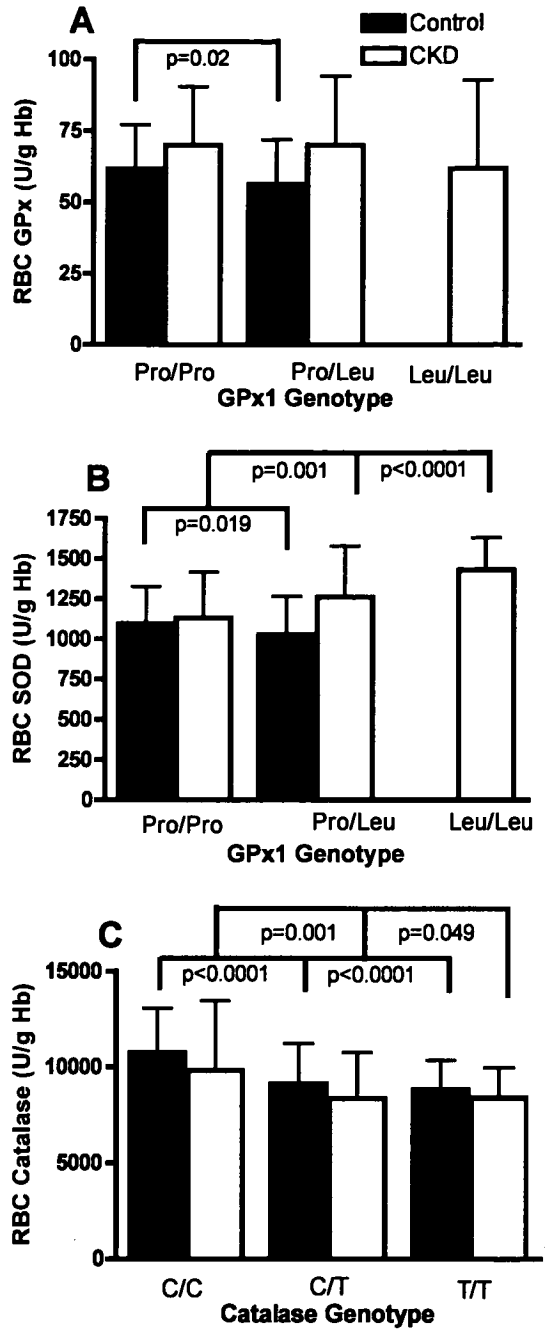

Figure 3-4: Comparison of antioxidant activities in different genotypes. (means with standard deviation bars). (A) RBC GPx in GPx1 genotype, (B) RBC SOD in GPx1 genotype and (C) RBC catalase in catalase genotype. Error bars show standard deviation.

The Leu/Leu GPx1 genotype was found to be associated, non-significantly, with reduced eGFR compared to Pro/Leu genotype in CKD patients. Although no studies have investigated the relationship between GPx1 Pro197Leu genotype and CKD, five studies have investigated the relationship between the GPx1 Pro197Leu genotype and diabetes. Two of these have reported associations between the GPx1 Pro/Leu genotype and cardiovascular disease in diabetes.[37, 154] In the current study, the five patients with the Leu/Leu genotype had diabetes as their cause of CKD and four patients were in stage 4 of CKD.

Kidney function in CKD patients with the SOD2 Val/Val genotype was increased ($p=0.074$) compared to those with the Ala/Ala genotype. Although no previous studies have investigated the relationship between the SOD2 Ala16Val genotype and CKD, two studies found that the Val/Val genotype or Val allele were associated with a significantly increased risk of developing diabetic nephropathy.[121, 123] Sixty CKD patients from the present study were carrying the Val/Val genotype, with only nine having diabetes as their primary cause, suggesting the current finding may not be linked to diabetes.

There were no differences in kidney function between the different catalase genotypes in CKD patients. Perianayagam and co-workers[77] reported similar lack of association between the catalase C-262T genotype and acute renal failure.

Plasma GPx activity was approximately 16% lower in CKD patients compared to controls and there was a strong positive association between plasma GPx activity and eGFR in CKD patients (but not in controls). The findings that plasma GPx activity was significantly reduced in the total participant population in stages 3, 4 and 5 CKD when compared with stage 1, is supported by previous studies reporting a parallel decline in

plasma GPx activity and kidney function in CKD patients.[35, 53, 62, 64-66, 204, 205] The majority of these case-control studies examined small CKD populations (around 50 or less).[35, 64-66, 204] Only two studies consisted of 150 or more CKD patients,[53, 62] and only 30 healthy participants as controls, compared with the present study where 224 controls were studied. The most likely explanation for the decline in plasma GPx activity is that the kidney is the main source of production of plasma GPx, and when kidney function decreases, there is a corresponding decline in plasma GPx activity.[39]

RBC GPx activity was significantly higher in CKD patients than controls. Previous studies examining RBC GPx activity in CKD have produced equivocal results with two studies reporting similar relationships[53, 63] but another study finding that RBC GPx activities were significantly reduced in CKD patients.[65] However, the present study involved both a much larger CKD patient population and a CKD control group. In addition, when the total study population (patients and controls) was stratified into stages of CKD, RBC GPx activity was significantly higher in stages 3-5 compared with stage 1. A possible explanation for the higher RBC GPx associated with lower kidney function could be the reduced life expectancy and more regular replacement of the RBC (hence RBC GPx) in subjects with declining kidney function.[15, 16]

RBC SOD activity was significantly increased in CKD patients compared to controls, and when examined in the total participant population was significantly higher in stages 3 and 4 CKD than stage 1. In contrast, Ceballos-Picot et al.[53] reported that RBC SOD activity was not significantly different in CKD patients and controls, although the study was limited by a small number of controls (30).

RBC catalase activity, in this study was found, to be significantly lower in CKD patients compared with the control group. In a previous study, catalase activity in

children with CKD (aged 5-18 years) also reported significantly reduced RBC catalase activity compared to age matched controls.[85] One potential explanation for the decrease in RBC catalase activity in CKD, compared to the increase in both RBC GPx and SOD activities, is that catalase may degrade in very high hydrogen peroxide environments.[206] In addition, catalase is preferentially exploited in a high hydrogen peroxide environment as it requires two hydrogen peroxide molecules when converting same to water and oxygen. Therefore, catalase becomes saturated more quickly compared with GPx and SOD antioxidants in the shortened RBC life span in CKD.[15, 16, 47]

The presence of SNPs in antioxidant enzymes had a significant effect on specific enzyme activities (genotype-phenotype association) in CKD patients and controls. There were no significant relationships between GPx1 genotypes and both plasma and RBC GPx activities in patients. In contrast, control participants' RBC GPx activity was reduced in GPx1 Pro/Leu genotype compared with Pro/Pro genotype. A similar finding was reported in breast cancer patients with RBC GPx activity reduced with the Leu allele compared with Pro allele.[74] Furthermore, the GPx1 genotype in both patients and controls was significantly associated with RBC SOD activity. However, in patients with the GPx1 Pro/Leu genotype, SOD activity increased compared with Pro/Pro genotype and SOD activity was reduced in control subjects with Pro/Leu genotype compared with Pro/Pro genotype. To our knowledge, this significant relationship has not been reported previously, but the reason for this association is unclear and requires further investigation.

Finally, we observed reduced catalase activity in CKD patients and controls with C/T and T/T genotypes compared with C/C. Perianayagam et al.[77] reported similar findings in acute kidney injury patients, with the T allele having decreased catalase enzyme

activity compared with C/C genotype. On the other hand, Forsberg and co-workers[191] reported catalase activity was significantly higher in Swedish men with the T allele compared with C allele. This variation in genotype-phenotype association may be due to the measurement of protein levels of catalase in whole blood by Forsberg and co-workers, whereas enzyme activity in RBC was measured in the current study.

In conclusion, antioxidant genotype frequencies were similar in CKD patients and controls, and a non-statistically significant trend towards lower eGFR was found in patients with the GPx1 Leu/Leu genotype and higher eGFR in patients with the SOD2 Val/Val genotype. In addition, CKD was associated with impaired plasma GPx and RBC catalase activities and enhanced RBC GPx and SOD activities compared with general population controls. The marked differences in antioxidant enzyme activities of CKD patients compared with controls suggests impaired kidney function has a significant effect on antioxidant status. Further studies of antioxidant genotypes and activities in a larger CKD population are warranted.

CHAPTER 4: GLUTATHIONE PEROXIDASE, SUPEROXIDE DISMUTASE AND CATALASE GENOTYPES AND ACTIVITIES ON THE PROGRESSION OF CKD

4.1 Abstract

Background and Objectives: Chronic kidney disease (CKD) is a significant public health issue that affects an estimated 11 percent of adults over the age of 25 years in Australia. Oxidative stress has been linked to the progression of a number of diseases, including CKD. The antioxidant enzymes, superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase, together form the primary defence system against reactive species and oxidative stress. ***Methods:*** We examined the relationship between antioxidant enzyme single nucleotide polymorphisms (GPx1 Pro197Leu, SOD2 Ala16Val and catalase C-262T), changes in antioxidant activities and the progression of CKD (decline in estimated glomerular filtration rate (eGFR)) in 185 patients over 12 months. ***Results:*** CKD patients with the SOD2 Ala/Val and Val/Val genotypes had a significantly greater decline in kidney function compared to those with the Ala/Ala genotype (Ala/Val compared with Ala/Ala OR 0.35, 95% CI 0.19 to 0.64, p=0.001; Val/Val compared with Ala/Ala OR 0.25, 95% CI 0.10 to 0.65, p=0.005). The progression of CKD was not influenced by SNPs of the GPx1 or catalase genes studied. There was a direct relationship (multivariate analysis), between the rate of change of plasma GPx activity and the rate of change of eGFR over the 12 month period (p=0.025). ***Conclusion:*** CKD patients with the SOD2 Ala/Val and Val/Val genotypes have a greater decline in kidney function than those with the Ala/Ala genotype.

4.2 Introduction

The antioxidant enzyme SNPs, SOD2 Ala16Val, GPx1 Pro197Leu and catalase C-262T, and changes in the activities of SOD, GPx and catalase, have been associated with altered progression and/or risk of several diseases, including breast cancer,[74] lung cancer,[75] diabetic neuropathy[76] and acute kidney injury.[77] Moreover, the potential links between the activities of antioxidant enzymes and CKD have been the subject of several case-control studies.[35, 53, 64-66, 207] However, to date, there have been no studies addressing the role of antioxidant enzyme SNPs and activities in the progression of CKD. Thus, the aims of the present study were to determine whether the progression of kidney disease, defined for this study as decline in eGFR over one year, was (a) linked to specific genotypes resulting from the aforementioned GPx1, SOD2 and/or catalase SNPs, and (b) associated with altered GPx, SOD and/or catalase activities.

4.3 Methods

The protocols used for obtaining blood samples and methods for measuring antioxidant activities, genotyping and statistical analysis are detailed in Chapter 3 (section 3.2.2–3.2.3).

4.3.1 Study Subjects

All CKD patients recruited (261) were asked to provide four blood samples over approximately 12 months. Hence, there was bias towards patients at later stages of CKD. The inclusion criterion was that they provided a minimum of three samples, more than 1 month apart over this period, in order to determine the rate of change of eGFR and antioxidant activities. (Of the 261 patients recruited, eight died, three commenced dialysis, two received transplants and 32 provided insufficient samples. Hence, the

study population consisted of 185 CKD patients. The demographics of this population are detailed in Table 4-1.

Table 4-1: Demographics and clinical characteristics of CKD patients. Smoking status was only available on 104 CKD patients (% expressed accordingly).

	(n=185)
Gender (Male)	95 (52%)
Age (years)	69.5±12.0 (range 30-80)
Primary causes of CKD	
Vascular	69 (37%)
Glomerulonephritis	33 (18%)
T2DM	32 (18%)
T1DM	8 (4%)
Reflux nephropathy	9 (5%)
Polycystic kidney disease	4 (2%)
Analgesic nephropathy	2 (1%)
Uncertain	4 (2%)
Other	24 (13%)
Medications	
Erythropoietin	80
ACE inhibitors	74
Statins	61
Angiotensin receptor blockers	52
Anti-inflammatory steroids	14
Smoking Status	
Never Smoked	57 (55%)
Given up	39 (37%)
Current Smoker	8 (8%)

4.3.2 Statistical Analysis

Hardy-Weinberg analysis was carried out on all genotype population groups. SOD2 and catalase genotypes for both controls and patients were within the Hardy-Weinberg equilibrium. Sample size calculation predicted that in order to detect a 2.4-fold increase in eGFR decline, 19 homozygous mutations and 231 patients of other genotypes were required. Whilst we aimed to recruit an even larger number of patients, 261 patients from North, North West and North East of Tasmania were recruited (see Appendix 2).

The majority of the statistical analysis has been described in section 3.3.4. When determining associations with rate of change of either eGFR or antioxidant enzyme activities, age, gender, smoking status, change over time of all antioxidant activities, initial eGFR, initial antioxidant activities, antioxidant genotypes, primary cause of CKD and medications were presented for selection by stepwise regression for inclusion in the multivariate model. P-values were corrected for multiple comparisons using the Holm method. All statistical analyses were performed using Stata 10.1/IC (StataCorp LP, College Station, TX, USA).

4.4 Results

Over the 12 month study period, eGFR declined in 61% of the patients, increased in 24% and was stable in 15%. Patients with the Ala/Val and Val/Val SOD2 genotypes had a more rapid decline in kidney function compared to those with the Ala/Ala genotype (Ala/Val compared with Ala/Ala OR 0.35, 95% CI 0.19 to 0.64; Val/Val compared with Ala/Ala OR 0.25, 95% CI 0.10 to 0.65) (Table 4-2, Figure 4-1 A). Furthermore, this effect persisted when adjusted for all confounding variables with Ala/Val and Val/Val genotypes linked to a faster rate of decline in kidney function (p=0.002; p=0.006, respectively) (Table 4-3). Furthermore, this effect persisted when

adjusted for all confounding variables with Ala/Val and Val/Val genotypes linked to a faster rate of decline in kidney function (p=0.002; p=0.006, respectively) Thirty seven percent of the study population had vascular disease as their primary cause of CKD, with 48% and 30% having the Ala/Val and Val/Val genotypes, respectively. However, the prevalence of these genotypes was similar for both CKD of vascular origin and the total study population (Ala/Val, 50% and Val/Val, 26%) (Table 4-2). Progression of CKD was not influenced by GPx1 or catalase SNPs (Figure 4-1 B and C, respectively).

Table 4-2: Antioxidant enzyme genotypes and changes in kidney function and antioxidant enzymes (mean±SD)

	GPx1 Pro197Leu Genotypes			SOD2 Ala16ValGenotypes			Catalase C-262TGenotypes		
	Pro/Pro (n=82)	**Pro/Leu (n=83)**	**Leu/Leu (n=4)**	**Ala/Ala (n=40)**	**Ala/Val (n=85)**	**Val/Val (n=44)**	**C/C (n=109)**	**C/T (n=53)**	**T/T (n=7)**
Age	69.6 (±11.8)	70.0 (±11.6)	55.7 (±15.8)*	70.8 (±12.1)	68.1 (±12.2)	70.8 (±11.1)	71.5 (±10.0)	66.5 (±14.0)	60.7 (±15.0)
Gender – male (%)	40	47	1	20	46	22	57	26	5
Baseline									
eGFR (ml/min/1.73m^2)	30.16 (±13.63)	31.54 (±13.94)	24.50 (±6.14)	28.29 (±11.85)	31.43 (±13.93)	31.40 (±14.62)	31.80 (±14.32)	27.69 (±11.21)	38.20 (±18.43)
Plasma GPx (U/L)	619.06 (±179.86)	600.79 (±175.03)	544.70 (±94.66)	580.31 (±165.92)	622.28 (±186.97)	609.67 (±164.14)	620.07 (±178.09)	587.75 (±170.22)	588.71 (±190.30)
RBC GPx (U/g Hb)	72.78 (±19.96)	73.67 (±25.34)	64.91 (±34.88)	73.58 (±26028)	72.47 (±24.51)	73.63 (±16.53)	74.01 (±23.31)	69.46 (±22.34)	84.39 (±20.74)
SOD (U/g Hb)	1153 (±262.89)	1266 (±331.06)	1408 (±223.27)	1299 (±332.19)	1200 (±312.31)	1165 (±241.03)	1232 (±319.30)	1173 (±281.61)	1256 (±152.02)
Catalase (U/g Hb)	9200 (±3650)	9444 (±3273)	9327 (±3861)	9267 (±2758)	9285 (±3835)	9448 (±3310)	9437 (±3536)	8956 (±3149)	10335 (±4525)
Change over year									
eGFR (ml/min/1.73m^2/year)	-3.41 (±10.86)	-2.90 (±9.43)	1.99 (±12.87)	1.68 (±6.06)	-3.61 (±7.91)**	-6.21 (±14.79)*	-2.94 (±10.49)	-3.26 (±10.17)	-2.77 (±5.41)
Plasma GPx (U/L/year)	-16.84 (±259.96)	-38.04 (±301.61)	-111.63 (±79.14)	-17.21 (±354.60)	-33.33 (±242.88)	-33.26 (±269.85)	-25.20 (±278.00)	-51.74 (±291.73)	72.08 (±144.73)
RBC GPx (U/g Hb/year)	-2.21 (±40.93)	-8.94 (±41.53)	-0.77 (±55.07)	-7.67 (±38.56)	0.61 (±42.80)	-15.25 (±40.09)	-8.35 (±39.79)	1.60 (±44.75)	-15.95 (±37.95)
RBC SOD (U/g Hb/year)	25.07 (±535.74)	15.77 (±612.66)	-283.78 (±546.56)	85.21 (±406.55)	27.88 (±640.44)	74.27 (±566.90)	-42.88 (±513.79)	163.36 (±669.50)	-250.74 (±490.83)
RBC Catalase (U/g Hb/year)	-2877 (±5138)	-3031 (±5783)	-2988 (±5287)	-3035 (±5553)	-3031 (±5783)	-3265 (±3554)	-3565 (±5597)	-2168 (±4445)	-2751 (±3569)

*** ($p<0.01$) compared with Pro/Pro; ** Significantly different ($p<0.001$) to Ala/Ala; * Significantly different ($p<0.01$) to Ala/Ala.

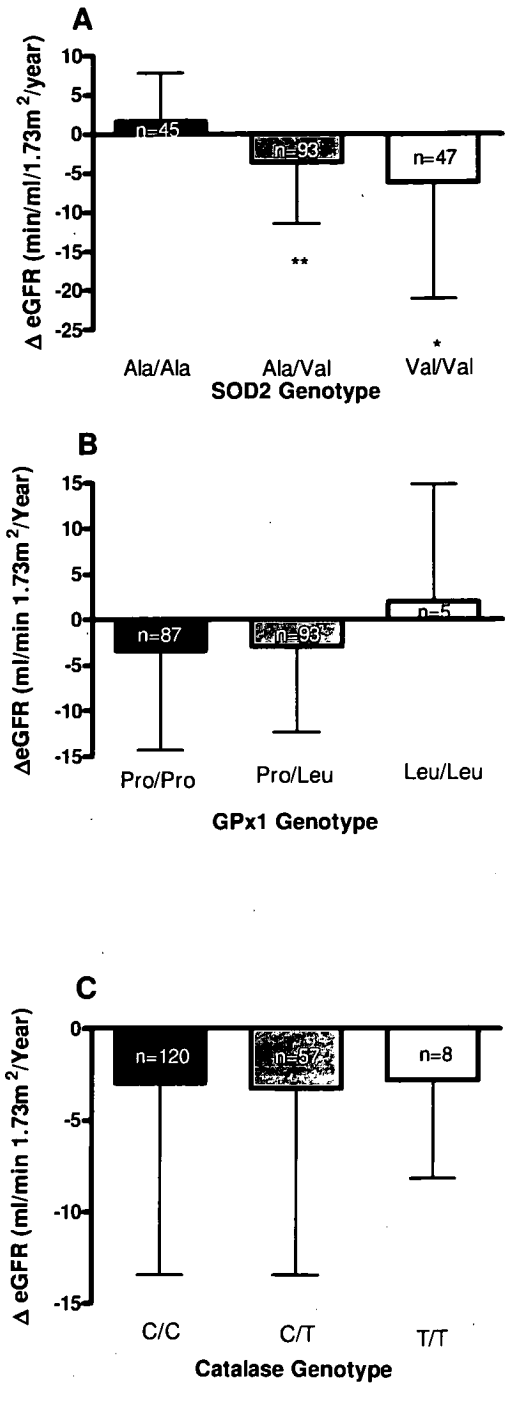

Figure 4-1: Rate of change (Δ) in eGFR in CKD patients (n=185) over 12 months for different genotypes.

A) SOD2 Ala16Val genotypes. B) GPx1 Pro197Leu genotypes and C) catalase C-262T genotypes. Data adjusted for age. Error bars show standard deviation. **p<0.001, *p=0.009

In patients with the GPx1 Leu/Leu genotype (n=5), the rate of change in plasma GPx activity was significantly reduced compared with GPx Pro/Pro genotype (OR 0.42, 95% CI 0.20 to 0.87, p=0.02). When adjusted for all confounding variables, the change in plasma GPx activity over the 12 month study period was significantly reduced in patients with the GPx1 Leu/Leu genotype (p=0.026) (Table 4-3). However, changes in RBC GPx, RBC SOD and RBC catalase activities over time were not influenced by any of the SNPs when either univariate or multivariate analysis was performed.

When simple univariate analysis was performed, a trend towards a relationship between change in plasma GPx activity and change in eGFR over the 12 month study period was apparent (p=0.069) (Figure 4-2 A). However, when all confounding variables were taken into account, there was a significant association between the decline in eGFR over time and the decline in plasma GPx activity (p=0.025) (Table 4-3), this being our best estimate of an independent effect. Conversely, the changes in RBC GPx, RBC SOD and RBC catalase activities were not associated with the change in eGFR over 12 months (Table 4-3). (Figure 4-2 B, C and D, respectively).

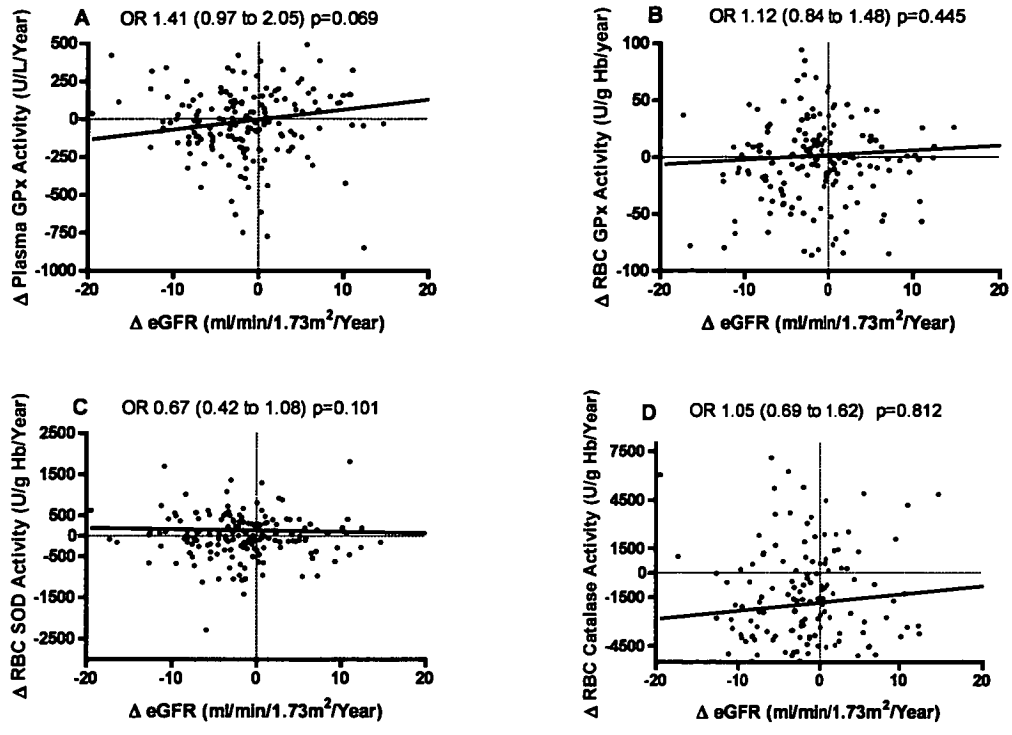

Figure 4-2: Relationship between rate of changes (Δ) of antioxidants and eGFR
A) plasma GPx, B) RBC GPx, C) RBC SOD and D) RBC catalase activities over 12 months and Δ in eGFR over the same period in CKD patients. Odds ratio (OR, with 95% confidence) and p values indicate significance of relationships.

Patients with higher eGFR on entry into study (baseline) had a faster decline in kidney function over 12 months (p=0.046) (Figure 4-3). However, baseline antioxidant activity did not appear to influence the rate of decline of eGFR over the 12 month study period. On the other hand, the rate of change of all four enzymes activities was inversely related to the activity of the individual enzyme at baseline (plasma GPx: p<0.0001; RBC GPx: p<0.0001; RBC SOD: p=0.004 and RBC catalase: p<0.0001) (Figure 4-4 A, B, C and D, respectively). This remained significant (p, all<0.0005) when adjusted for all confounding variables (Table 4-3).

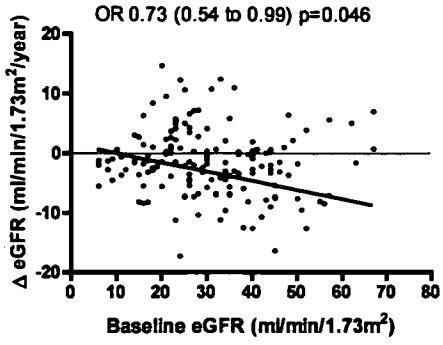

Figure 4-3: Relationship between rate of change (Δ) in eGFR over 12 months and eGFR on entry into the study (baseline measurement) for CKD patients (n=185).
Odds ratio (OR, with 95% confidence intervals brackets) and p values indicate significance of relationships.

Changes in individual antioxidant activities were influenced by the baseline activity of other antioxidant enzymes. Higher baseline catalase activity was associated with increasing plasma GPx (p=0.017) over 12 months (Figure 4-4 E). When multivariate analysis was performed, this association persisted (p=0.003) (Table 4-3). In addition, higher baseline RBC GPx activity was associated with declining RBC catalase activity over 12 months (p<0.0001) (Figure 4-4 F). Finally, when adjusted for all confounding variables, there was a positive association between baseline SOD activity and catalase activity over the 12 month study period (p=0.017) (Table 4-3).

Table 4-3: Multivariate analysis derived associations between rate of change (Δ) of eGFR and Δ of antioxidants activities compared with baseline measurements and Δ of antioxidant.

	Δ eGFR (ml/min/1.73m^2/year) OR (95% CI)	Δ Plasma GPx (U/L/year) OR (95% CI)	Δ RBC GPx (U/g Hb/year) OR (95% CI)	Δ RBC SOD (U/g Hb/year) OR (95% CI)	Δ RBC Catalase (U/g Hb/year) OR (95% CI)
GPx1 Pro197Leu					
Pro/Pro	—	—		—	—
Pro/Leu	—	0.66 (0.37-1.19)	0.73 (0.41-1.29)	—	—
Leu/Leu	—	0.15 (0.03-0.80)*	0.61 (0.22-1.67)	—	—
SOD2 Ala16Val					
Ala/Ala	—	—	—	—	—
Ala/Val	0.37 (0.20-0.70)**	—	—	—	—
Val/Val	0.26 (0.10-0.67)**	—	—	—	—
Catalase C-262T					
C/C	—	—	—	—	—
C/T	—	0.66 (0.35-1.25)	—	1.14 (0.63-2.06)	1.53 (0.87-2.70)
T/T	—	1.09 (0.36-10.04)	—	0.62 (0.12-3.22)	1.77 (0.60-5.25)
Δ eGFR (ml/min/1.73m^2/year)	—	1.49(1.05-2.11)*	1.39 (0.84-2.31)	0.66 (0.43-1.02)	
Δ Antioxidants					
Plasma GPx (U/L/year)	—	—	—	0.77 (0.51-1.15)	—
RBC GPx (U/g Hb/year)	—	—	—	1.53 (1.13-2.06)	1.49 (1.09-2.03)*
RBC SOD (U/g Hb/year)	0.68 (0.48-0.97)*	—	1.55 (1.05-2.29)*	—	—
RBC Catalase (U/g Hb/year)	—	—	1.60 (1.13-2.25)**	—	—
Baseline Measurements					
eGFR (ml/min/1.73m^2)	0.75 (0.53-1.04)	—	—	—	—
Plasma GPx (U/L)	—	0.45 (0.30-0.68)***	—	—	—
RBC GPx (U/g Hb)	—	—	0.20 (0.12-0.32)***	—	0.75 (0.52-1.09)
RBC SOD (U/g Hb)	—	—	—	0.63 (1.14-2.06)**	1.44 (1.07-1.94)*
RBC Catalase (U/g Hb)	—	1.49(1.12-1.74)**	—	—	0.34 (0.24-0.59)***
Causes of CKD					

Vascular	—	0.83 (0.46-1.52)	—	—	0.99 (0.54-1.85)
T1DM	0.46(0.16-1.29)		7.80 (1.97-30.90)**	0.87 (0.10-7.90)	—
T2DM	0.62(0.26-1.50)		0.90 (0.45-1.79)	0.46 (0.25-0.81)**	—
Medications					
Statins	1.35 (0.68-2.70)	—	—	—	—
Angiotensin II	—	—	—		—
Ace Inhibiter	1.27 (0.68-2.47)		—	0.97 (0.55-1.70)	1.17 (0.66-2.10)
Constant	0.49 (-1.85-2.83)	-18.60 (-41.84- 79.04)	3.41 (-5.81-12.62)	-51.35 (-91.88-194.59)	-3253 (-4184-2321)

These associations were estimated using ordinal logistic regression due to skewness and heteroskedasticity, with variables selected for inclusion by stepwise regression. Blanks and other variables that were not statistically significant in stepwise regression analysis: co-variants: age, gender, never smoked, given up smoking, still smoking; causes of CKD: uncertain, other, reflux nephropathy and analgesic nephropathy; medications: erythropoietin, anti-inflammatory and ace inhibitors. Constant, is mean change of eGFR and the antioxidant activities when all other confounders was minimum or zero

The odds ratio (OR) and 95% confident intervals (CI) for the associations with eGFR and antioxidants activities in each by separate mode are those that were selected by stepwise regression. *p<0.05, **p<0.01 and ***p<0.001.

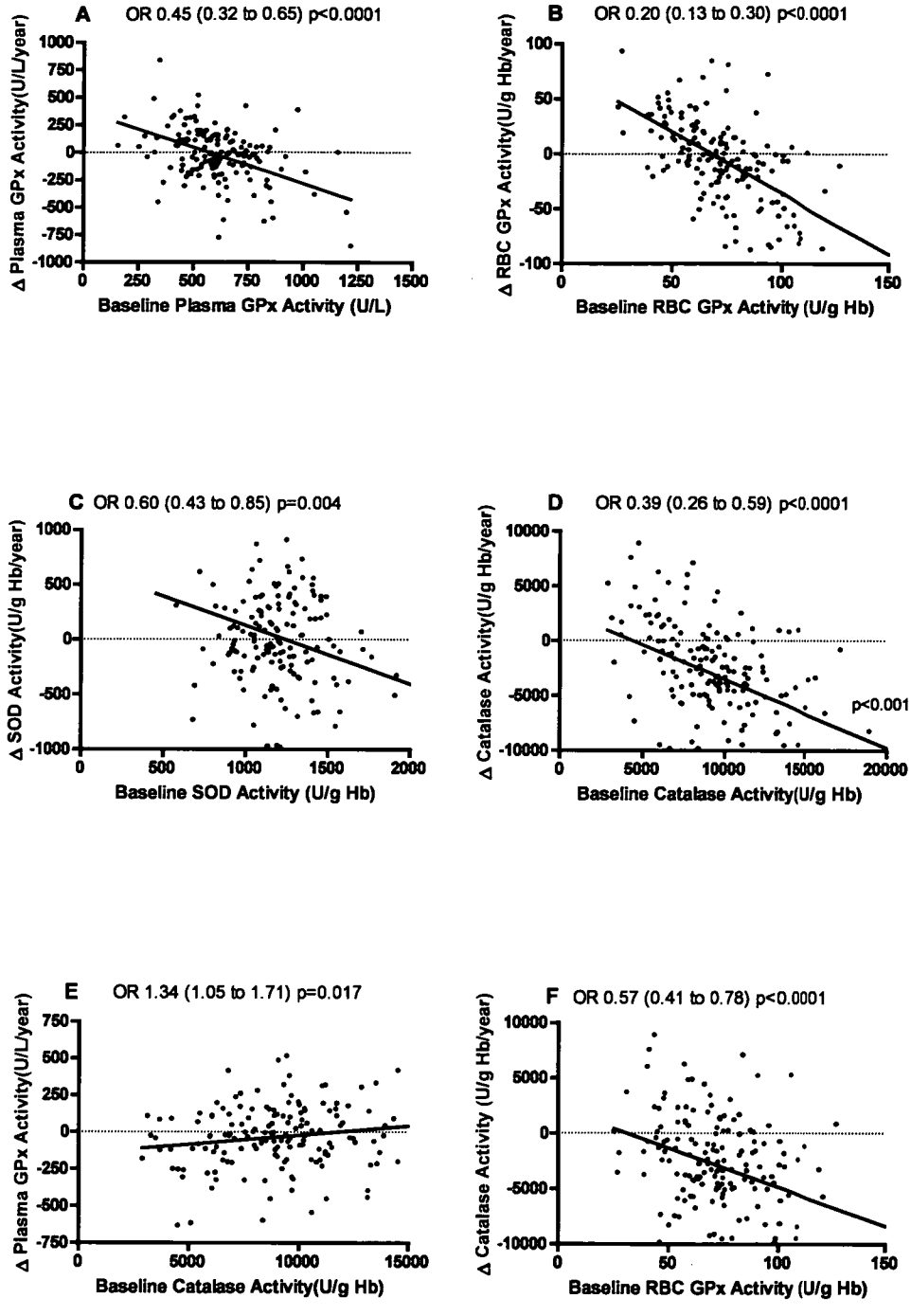

Figure 4-4: Relationships between rate of change (Δ) in antioxidant activities over 12 months and antioxidant activity measurements on entry into study (baseline) in CKD patients.
A) Δ and baseline plasma GPx activity; B) Δ and baseline RBC GPx activity; C) Δ and baseline SOD activity; D) Δ and baseline catalase activity; E) Δ plasma GPx activity and baseline catalase activity; F) Δ catalase activity and baseline RBC GPx activity. Odds ratio (OR) and 95% confidence intervals and p values indicate significance of relationships.

The rate of change in plasma GPx activity was inversely linked to the change in both RBC SOD and catalase activities over 12 months (p=0.025 and p=0.041, respectively) (Figure 4-5 A and B, respectively). However, when all confounding variables were taken into account, this relationship did not persist (Table 4-3). In addition, the change of RBC GPx activity displayed a positive association with change in both RBC SOD and RBC catalase activities (p=0.02; p<0.0001, respectively) (Figure 4-5 C and D, respectively), which persisted when the data was subjected to multivariate analysis (p=0.028; p=0.008, respectively; Table 4-3).

When diagnosed as the primary cause of CKD, T1DM was associated with an increase in RBC GPx activity over time (p=0.003, Table 4-3) whereas T2DM was associated with a faster decline in SOD activity over time (p=0.008, Table 4-3).

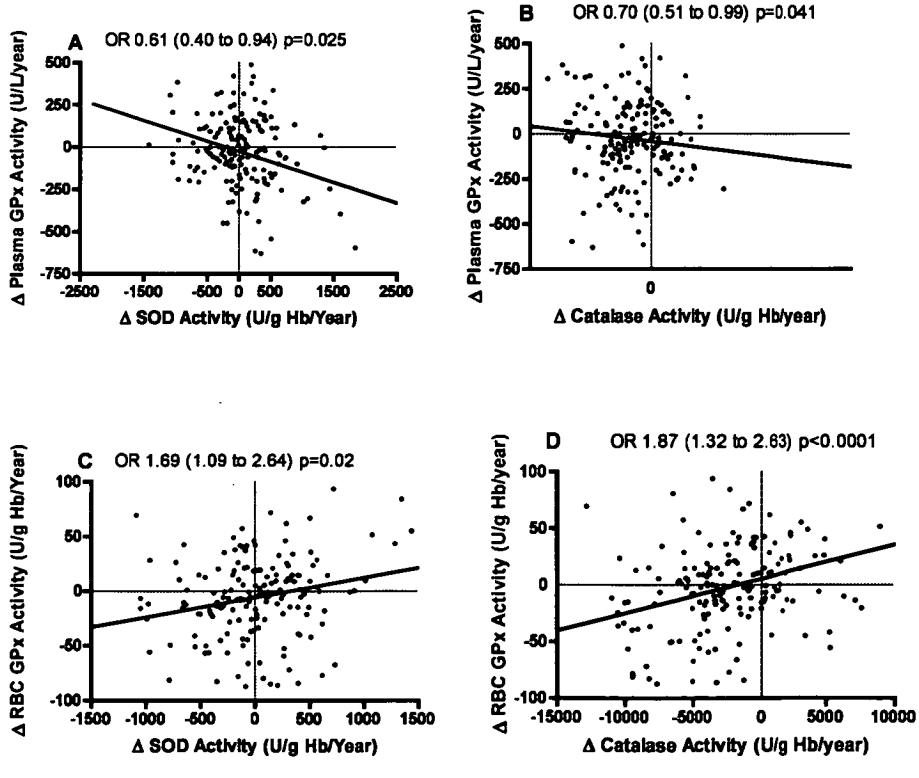

Figure 4-5: Relationships between rates of change (Δ) in antioxidant activities over 12 months in CKD patients.
A) Plasma GPx and RBC SOD; B) RBC GPx and RBC SOD; and C) RBC catalase and RBC GPx. Odds ratio (OR), and 95% confidence intervals brackets) and p values indicate significance of relationships.

4.5 Discussion

This is the first study to investigate antioxidant enzyme genotypes and activities in relation to the progression of kidney disease over an extended period (12 months). The major findings were that: 1) CKD patients with the SOD2 Ala/Val and Val/Val genotypes had a significantly greater decline in kidney function compared to patients with the Ala/Ala genotype, 2) declining plasma GPx activity was associated with a decline in kidney function, and 3) declining kidney function over 12 months was associated with increasing RBC SOD activity, when multivariate analysis was performed.

The most significant finding was the accelerated decline in kidney function in patients with either SOD2 Ala/Val or Val/Val genotypes. Why this polymorphism predisposes individuals to CKD (and other diseases) is not clear. It has been suggested that the amino acid change from Ala to Val may have an effect on the structure of SOD2, changing an alpha helix structure to a beta sheet.[208] Furthermore, the Ala allele has been associated with higher MnSOD mitochondrial activity. Therefore the Ala allele may be more protective against oxidative stress, [209] and against progression of diseases that have been linked to oxidative stress, including CKD. In addition, the Val allele may have a direct effect on the transport of MnSOD into the mitochondria.[208] In rats exposed to chronic hypoxia, expression of MnSOD in the kidney is increased which may protect the kidney from oxidative damage caused by superoxide.[67, 210] Hence, future studies should examine whether MnSOD, which is predominantly of mitochondrial origin, is up-regulated in the kidney and whether it is affected by the Ala16Val genotype MnSOD (SOD2). Although this study measured RBC SOD activity this does not contain MnSOD (SOD2),

An alternative explanation for this decline is co-existing vascular disease and diabetes (T1DM and T2DM), which were the primary cause of CKD in 22% and 37% of patients, respectively. In the present study, the Ala/Val and Val/Val SOD2 genotypes accounted for 78% of patients with CKD of vascular origin. A large number of studies (>70) have investigated the relationship between the SOD2 Ala16Val genotype and disease, including three relating to CVD,[71-73] and seven to T1DM and T2DM.[121-126] A large case-control study involving 989 participants (504 hypertensive; 485 controls) found a significant relationship between carotid artery intima-media thickness (CIMT), a measure of atherosclerotic burden, and the Val allele in women that had high levels of LDL cholesterol ($p=0.03$).[72] Whilst another study found that haemochromatosis patients (217) with the Val allele had an increased risk of cardiomyopathy compared with those with the Ala allele, only 27 patients in total had cardiomyopathy, and eleven (30%) of these had the Val/Val genotype.[73] A cohort study involving 252 participants, reported that the Ala/Val and Val/Val genotypes were associated with increased levels of oxidised LDL compared with Ala/Ala genotype in patients with oxidised LDL <0.5nmol/mg.[71] Finally, Mollesten and co-workers'[121] case-control study found an increased risk of diabetic nephropathy in T1DM patients with the SOD2 Val/Val genotype. In addition, another study reported the frequency of the Val/Val genotype was found to be higher in T2DM patients with diabetic nephropathy.[123] However, the present study provides evidence that links the SOD2 Ala/Val and Val/Val genotypes to both CVD and diabetes, in addition to CKD.

Declining plasma GPx was positively associated with declining eGFR over 12 months. Previous case-control studies involving a single measurement in time, found a direct relationship between reduced plasma GPx activity and decreased kidney function in CKD patients.[35, 53, 62, 64-66, 204] Most of these previous studies involved 50 or less CKD

patients,[35, 64-66, 204] and in the two studies involving 150 or more CKD patients, the control group was small (~30 healthy participants).[53, 62] The present study is the first, to our knowledge, to show a direct association between faster progression of kidney disease and lower plasma GPx over an extended timeframe. A likely explanation for the decline in plasma GPx activity is that the kidney is the main source of production of plasma GPx, and hence, when kidney function decreases, plasma GPx activity decreases.[39]

Interestingly, declining eGFR over 12 months was shown by the multivariate analysis to be associated with increasing RBC SOD activity. A case-control study by Ceballos-Picot et al.[53] previously reported that RBC SOD activity was unchanged in CKD, but there have been no previous cohort studies. One possible explanation for the current finding could be the reduced life expectancy and reduced deformability of RBCs in subjects with declining kidney function.[16, 211] As mature erythrocytes being anucleate, protein synthesises is thought to take place during erythropoiesis.[212] Consequently, RBC SOD is not depleted and therefore, high levels of the antioxidant are still present in RBC.

In univariate analysis, baseline eGFR was shown to be inversely related to change in eGFR over 12 months. This association disappeared when all confounding variables were taken into account. Our findings suggest that baseline antioxidant enzyme activities are associated with declining antioxidant activities over time. Nevertheless, these findings may simply be a regression to the mean. Furthermore, the interplay between baseline antioxidant activities and changes in other antioxidant activities over time demonstrates a strong link between the GPx and catalase activities. This conclusion is supported by previous studies that proposed a direct relationship to the oxidant sensitivity of glucose-

6-phosphate dehydrogenase (G6PD)-deficiency and impaired catalase activity. G6PD maintains levels of glutathione, which is intimately involved in the conversion of H_2O_2 to water and oxygen by GPx.[49]

Our overall findings regarding RBC GPx and SOD activities contradict those of Karajibani et al.[213] who reported that there was no significant correlation between RBC SOD and RBC GPx activities in 71 CVD patients and 63 healthy controls. Thus, the present study suggests a potential link between RBC antioxidant activities in CKD that has not been reported in CVD. However, these findings also suggest that there is a direct link between GPx activities and the activities of both SOD and catalase in oxidative stress.

There are a number of factors that may limit the findings of this study. A larger study population and an extended study time in subsequent studies are recommended to strengthen the antioxidant genotype findings (particularly as the number of GPx1 Leu/Leu genotypes was so low) and provide a better picture of kidney disease progression. In addition, the study population was Caucasian only. Future investigations should include other ethnic groups as there is some evidence that the GPx1 Leu/Leu genotype may be very limited in Asian populations. Therefore, the associations reported here in relation to the GPx1 genotype may not be relevant to Asian or African populations. Calculating eGFR using MDRD from serum creatinine also has its limitations as a measure of kidney function, compared with radio isotopic methods, which are impractical because of cost and logistics. Additionally, the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation is a new and accurate method of calculating eGFR and may in the future become the new gold standard.[10, 11] Finally, measurement of other enzymes and cofactors involved in the antioxidant enzyme

pathways (e.g., G6PD and glutathione) would provide a more complete picture of antioxidant status in CKD.

To conclude, we followed kidney function in 185 CKD patients over 12 months. Patients with the SOD2 Val/Val or Ala/Val genotypes had a greater decline in kidney function suggesting that SOD2 genotypes may be used to identify CKD patients at risk of more rapid disease progression. Being able to tailor a patient's treatment, which could include supplementation therapies, might be investigated to determine whether they may slow progression of CKD.[214] Similarly, a faster decline in kidney function is associated with decreasing plasma GPx activity. Hence, enhancing the activity of GPx may also slow the progression of kidney disease.

CHAPTER 5: THESIS SUMMARY AND CONCLUSION

5.1 General Discussion

Kidney disease is potentially fatal and the number of people being diagnosed with CKD is increasing every year. The social and personal cost involved varies considerably between patients due to their response to treatment and the level of supportive care required for everyday activities Furthermore, it costs the Australian government upwards of $80,000 per year for every patient that progresses to ESRD, requiring replacement therapy by HD, PD or a kidney transplant.[3] Hence, the ability to identify patients at risk of rapid disease progression, in addition to identifying people at risk of developing CKD, would reduce the strain of community support, enable nephrologists to improve treatment and potentially reduce cost to the government.

In recent years, the role of specific genotypes in the pathogenesis and progression of disease has become a major focus for research. This has centred mainly on SNPs, which involve a single base substitution, deletion or addition. Therefore, the overall aim of this research was to determine whether specific SNPs in antioxidant genes and associated changes in antioxidant enzyme activities are implicated in the pathogenesis of CKD and /or play a role in the progression of CKD to ESRD.

5.2 Chapter 3

The case-control study revealed a significant difference between the GPx1 Leu/Leu genotype frequency of CKD patients and controls, with all five subjects possessing the Leu/Leu genotype having CKD, and four of these patients in the later stage of CKD (eGFR <30mL/min/1.73m^2). However, a larger population study is required to reliably conclude that the Leu/Leu GPx1 genotype is overrepresented in the CKD population and that it may predispose such patients to progress more rapidly to ESRD. In contrast,

SOD2 and catalase genotype frequencies in CKD patients were not found to be significantly different from controls. However, GPx1 genotype was significantly associated with reduced RBC GPx activity in the control population participants with the Pro/Leu genotype. This may suggest that the GPx1 Pro/Leu genotype is associated with a change in the phenotype of RBC GPx when not in a state of oxidative stress. The catalase C-262T SNP was significantly associated with reduced catalase activity regardless of whether CKD was present. When a participant possessed either catalase C/T or T/T genotypes, they had significantly reduced catalase activity compared to all participants with C/C genotype.

Plasma GPx and RBC catalase activities were significantly lower in CKD patients compared with the control population, suggesting an elevated state of oxidative stress in the patients. On the other hand, RBC GPx and SOD antioxidant activities were increased in CKD patients, which may be due to the reduced viability of RBC observed in CKD patients. This seems paradoxical, but, it may be due to the reduced lifespan of RBC in CKD patients. The activity of RBC antioxidant enzymes is likely related to the lifespan of the RBC (i.e., the younger the RBC, the more activity). Therefore, as CKD patients would have overall "younger" RBCs, this may explain the higher antioxidant enzyme activity.

The current study showed that plasma GPx activity was (significantly) linked to the decline in kidney function. A possible explanation is that plasma GPx is primarily produced in proximal tubules in the kidney. Hence, when kidney function declines, so does the production of plasma GPx.[39] This has been reported in a number of previous studies,[13, 35, 53, 62, 63, 66] but the present study, to our knowledge, is the first to examine such a large CKD population (n=230) and control group (n=224). In contrast, reduced kidney function in CKD patients was not associated with changes in activities of the

other antioxidant enzymes. Interestingly, RBC SOD antioxidant activity was associated with GPx1 genotype in both patients and controls, but the reason for this association is unclear and warrants further investigation. Overall, these data suggest that small alterations in activity caused by either SNPs or oxidative stress may produce a major disturbance in the overall antioxidant status.

A most surprising finding, unrelated to the primary aim, was that 13% (approximately one in seven) of the general (control) population in the North and North West of Tasmania had undiagnosed CKD (indeed 30 were in stage 3, based on eGFR measurements). This data corroborates the finding of Chadban and co-workers in 2003[1] that 11% Australians over the age of 25 years are living with CKD stages 3-5 undiagnosed.

5.3 Chapter 4

This cohort study is the first, to our knowledge, to examine kidney function in CKD patients over 12 months in relation to antioxidant genotypes and antioxidant enzyme activities.

The major finding of this study was that kidney function was adversely associated with the presence of the SOD2 Ala/Val and Val/Val genotypes. However, neither GPx1 or catalase genotypes studied had a significant association with the progression of CKD. This suggests antioxidant genotype may play a role in the progression of CKD, as patients with SOD2 Ala/Val or Val/Val genotypes were progressing more rapidly towards ESRD. However, with recent large scale studies including HapMap and 1000 Genomes Project finding evidence that there are numerous Therefore, genotyping might help both nephrologists and the patients pursue a more aggressive course in their personalised treatment. This study also extends the findings of the case-control study, *viz*, that not only is plasma GPx associated with reduced kidney function, but that a

faster decline in plasma GPx activity over time correlates with accelerated progression of kidney function. The present study demonstrated significant associations between antioxidant genotypes and activities. However, whether altered antioxidant enzyme genotypes and/or activities contribute directly to CKD will require further research.

A dynamic interplay between the three antioxidant enzymes was found, with reduced activity of one enzyme (e.g., plasma GPx), leading to an increase in the activity of another antioxidant (e.g., RBC SOD or RBC catalase) over time. This may demonstrate a direct interaction of the GPx-SOD-catalase antioxidant system during both normal and oxidative stress status.

5.4 Future Research and Limitations

The present thesis examined a very specific and a limited population in the North and North West of Tasmania, Australia. Furthermore, all participants involved in the study were Caucasian. Therefore, to strengthen the principal findings of both the case-control and cohort studies, it is recommended that further studies should include other ethnic groups (e.g., Aboriginal, Torres Straight Island, Asian). Future case-control and cohort studies should be larger to strengthen the genetic findings. Although, the current case-control, investigation had 96% and 94% of the cases and controls, respectively, prescribed by the sample size calculation, the cohort study, had less than the required sample size. The cohort study population was only 80% (n=185) of the required CKD number predicted by the sample size calculation to determine a minimum 2-fold increase in the rate of decline of eGFR due to a specific genotype. However, the current thesis recruited greater than 90% of the Northern Tasmania's diagnosed CKD population (physicians estimate), so further recruitment would have required and requiring the involvement of other hospitals and nephrologists which was not accessible at the time. In addition, it well is documented and understood that there is a linear

decline of kidney function with age. Therefore, in the present study CKD patients were age and sex matched with the control population as closely as possible. However, there was still a significant age difference between the groups and so all analyses performed were adjusted for age.

With the current trend towards investigating the role of SNPs in disease, a major aim of this study was to determine whether knowledge of a patient's antioxidant enzyme genotype would assist in the treatment of CKD. This has been achieved, albeit with some limitations. Nevertheless, it must be noted that the clinical implications of finding a SNP that may affect the progress of a disease are limited as there are numerous other factors, including other SNPs, that affect the progression of all diseases. Hence, future studies should include the investigation of other SNPs and haplotypes. In recent years, studies of haplotype have become more common than those of single SNPs. Thus, the next logical step for the present study would be to examine haplotypes present in SOD2. This may help determine whether genetic variations affect the progression of CKD. The apparent lack of association between SOD activity and SOD2 genotypes in the present study may be due to the fact that SOD2 is found in mitochondria, and is quite difficult to measure. Whilst there are methods to measure SOD activity in mitochondria in kidney cells, it was not possible to conduct this in the present study. It would be useful to investigate other SNPs, including those present in plasma GPx genes [177], and to examine haplotypes identified in antioxidant genes. Slowing the progression of CKD is a major priority for patients, nephrologists and the health funders. Therefore, when patients are identified as having a greater risk of progressing faster to ESRD, intervention is required. For example, a patient identified with the SOD2 Ala/Val or Val/Val genotype might be monitored more frequently to ensure that more timely (and intensive) treatment in an effort to slow the decline in kidney function. Presently,

treatment is based on blood pressure control by angiotensin converting enzyme inhibitors and angiotensin receptor blockers, and dietary and lifestyle changes. The present study provides some evidence for antioxidant intervention in the CKD population which suggests antioxidant supplementation may be beneficial. Hence, well designed randomised control trials involving antioxidant supplementation, with, GliSODin, a SOD supplementation that has been shown to up regulate antioxidants and protect against oxidative stress apoptosis, are warranted.[215] Finally, the current research suggests that CKD patients with specific SOD2 genotypes may progress to ESRD more rapidly compared to other patients. Therefore, with this knowledge, we may be able to help to slow the progression of CKD and delay dialysis whilst maintaining the quality of life of patients. This would undoubtedly reduce costs and allow for improved treatment of more people.

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