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University of Cape Town

Genetic and Biochemical Analysis of ACE Inhibitor-Induced Angioedema in Black and Coloured South Africans

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Declaration

I, **Retsilisitsoe Moholisa**, declare that this thesis is my own, unaided work (except where acknowledgements indicate otherwise). Neither the whole work nor part thereof has been, is being, or is to be submitted for any degree or examination at any other university.

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Although this thesis presents my original and unaided work, it would not have been possible to complete it without the following people:

My main Supervisor for this thesis Professor Edward Sturrock, to you I'm eternally grateful for your mentorship, encouragement, support, especially in tough times and your brilliant scientific insightful knowledge. Thank you for taking me into your lab and nurturing me.

Dr. Patricia Owen, I'm extremely grateful for your help and support in PCR work for this thesis. I won't forget the time in your lab and your guidance.

Professor Brian Rayner, thank you very much for your guidance and mentorship in helping me to recruit patients at the hypertension clinic. Thank you also for your insight in helping me to complete this thesis.

Doctors Clint Cupido, Erica Jones and Joalice Stark, thank very much for your contributions and tireless work in recruiting patients. To Joalice, thank you for the initial work you did in the ACE I/D polymorphism and C-2399A SNP analysis.

Ms Sylva Schwager, to you no words can describe how grateful I am for your unwavering support. Thank very for the technical support in the lab with the enzyme assays.

Dr. Badri Motassium, thank for all your help and support with statistical analysis. This added so much value in completing this thesis

The Zinc Metalloprotease Group past and present; Ross Douglas, Dr. Ayesha Parker, Colin Anthony, Tony Chang, Dr. Trudi O'Neill, Dr. Adele Thomas, Nailah Conrad, Riyad Domingo, Dr. Wendy Kroger, Kerry Gordon, Christopher Yates and Kate Larmuth. You guys are the best bunch ever and I will never forget your support in my journey. It has been a pleasure working with you guys.

Sia, thank you for your wise words, I will never forget them.

My parents Tsae and Mathabo Moholisa, for your love, support and encouragement. Thank you for believing in me.

My siblings; Thabo, Polotso and Manchenchele Moholisa, I thank God that I have you as family, I love your guys.

Lastly to my funders; National Research Foundation, the Stella and Paul Lowesntein Educational Scholarship and University of Cape Town.

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^{o}C- Degrees Celsius

M- Molar

min- Minute

mA- Milliampere

mg- Milligrams

ml- Milliliters

mM- Millimolar

mU- Milliunits

pmol- Picomoles

nM- Nanomolar

U-Units

µl- Microlitre

V- Volts

A- Adenine

AAE- Acquired angioedema

ACE- Angiotensin coverting enzyme

ACEi- Angiotensin coverting enzyme inhibitors

ACEi-AE- Angiotensin converting enzyme inhibitor induced angioedema

ACEi-cough- Angiotensin converting enzyme inhibitor induced cough

ACE2- Angiotensin coverting enzyme 2

Ac-SDKP- N-acetyl-seryl-aspartyl-lysyl-proline

AD- Adenyl cyclase

ADH- Anti-diuretic hormone

AE- Angioedema

AngI- Angiotensin I

AngII- Angiotensin II

Ang1-7- Angiotensin 1-7

Ang1-9- Angiotensin 1-9

ANOVA- Analysis of variance

AGT- Angiotensinogen

AMPS- Ammonium persullfate

APP- Aminopeptidase P

ARBs- Angiotensin type 2 receptor blockers

ATP- Adenosine tri-phosphate

AT1R- Angiotensin type 1 receptor

AT2R- Angiotensin type 2 receptor

AUC- Area under curve

BK- Bradykinin

BKRs- Bradykinin receptors

BKR-1- Bradykinin type 1 receptor

BKR-2- Bradykinin type 2 receptor

C- Cytosine

Ca- Calcium

cAMP- Cyclic adenosine monophosphate

CCB- Calcium channel blocker

cGMP- Cyclic guanosine monophosphate

CHF- Congestive heart failure

CINH- Complement 1 inhibitor esterase

DAG- Diacylglycerol

DNA- Deoxyribonucleic acid

DNTPS- Deoxyribonucleotides

des-arg^{9}-BK- des-arginine^{9}-Bradykinin

des-arg^{9}-kallidin- des-arginine^{9}-kallidin

DPPIV- Dipeptidyl peptidase IV

EDHF- Epithelial derived-hyperpolarazing factor

eNOS- Endothelial nitric oxide synthase

ER- Endoplasmic reticulum

fXI- factor XI

fXIa- Activated factor XI

fXII- factor XII

fXIIa- Activated factor XII

GPI- Glycosylphosphatidylinositol

GTP- Guanine tri-phosphate

HAE-I- Hereditary angioedema type I

HAE-II- Hereditary angioedema type II

HAE-III- Hereditary angioedema type III

HHL- Hippuryl-histidine-leucine

HL- Histidine-leucine

HMWK- High molecular weight kininogen

IP_3- Inositol tri-phoshate

JAK- Janus activated kinase

KKS- Kinin-Kallikrein System

LMWK- Low molecular weight kininogen

NEP- Neprilysin

NO- Nitric oxide

PA-I- Plasminogen activator inhibitor

PBS- Phosphate buffered saline

PCR- Polymerase chain reaction

PGH2- Prostaglandin

PGI2- Prostacyclin

PK- Plasma kallikrein

PLC- Phospholipase C

PPGK- Proline-Proline-Glycine-Leucine

PRCP- Prolylcarboxypeptidase

RAAS- Renin-Angiotensin-Aldosterone-System

sGC- Guanine cyclase synthase

SNP- Single nucleotide polymorphism

T-Thymine

TEMED- Tetramethylethylenediamine

TF- Tissue factor

TK- Tissue kallikrien

ZFHL- Z-phenylalanine-histidine-leucine

Angiotensin converting enzyme inhibitors (ACEi) are routinely used as first line treatment for hypertensive patients because of their protective effects against heart and kidney disease. Despite their clinical benefits, ACEi used is associated with adverse side effects such as life threatening angioedema (ACEi-AE) and persistent dry cough (ACEi-cough). The aetiology of ACEi-AE remains unresolved. Currently there is no marker to predict which patients using ACEi will develop ACEi-AE. Advances in ACEi-AE research have proposed that increased plasma bradykinin (BK) levels are associated with ACEi-AE. BK acts on widely expressed bradykinin type 2 receptors (BKR-2) resulting in the activation of proinflammatory responses and nitric oxide generation. Furthermore, it has been shown that ACEi-AE is associated with significantly low plasma aminopeptidase P, an enzyme that plays a crucial role in BK degradation during ACE inhibition. It was also shown that C2399A single nucleotide polymorphism (SNP) at XPNPEP2 was a significant predictor of APP activity in Caucasians.

In this study the analysis of the association between ACE insertion (I)/deletion (D) polymorphism, XPNPEP2 C2399A SNP, BKR-2 +9/-9 polymorphism and C-58T SNP were examined in 77 controls, 52 ACEi-AE patients and 36 ACEi-cough patients. Furthermore activities of ACE and APP were determined in serum of 29 controls, 52 ACEi-AE patients and 36 ACEi-cough patients and correlated with genotype.

The results revealed no significant association between ACE-II genotype and ACEi-AE and ACEi-cough. Similarly, the ACEi-AE and ACEi-cough patients showed significantly lower A allele incidence compared to controls. The BKR-2 -9 allele was significantly associated with ACEi-AE and not ACEi-cough. The -58T allele displayed no significant association with both ACEi-AE and ACEi-cough.

The ACE activity in serum was significantly decreased in cough and AE subjects compared to controls. Surprisingly, the ACE activity was similar in all patients when correlated with genotype (II, ID, DD) contrary to the expected increase in ACE activity usually observed in patients with DD genotype.

APP activity was measured in serum using a fluorimetric enzyme assay. The APP activity was significantly decreased in patients with ACEi-AE and ACEi-cough compared to controls.

However, when APP activity was correlated with the genotype (A, AC, C), there was no significant difference in APP activity in all groups. These data suggest that other genetic and biochemical differences in our population might explain the phenotypic variability of the ACE and APP activities.

Literature Review

1. Introduction

Angiotensin I-converting enzyme inhibitors (ACEi) are widely used in the treatment hypertension, myocardial infarction, congestive heart failure and diabetic nephropathy. These drugs improve patient clinical outcomes; and reduce morbidity and mortality. Hence they are often used as first line of treatment. Although clinically effective, ACEi are associated with side adverse effects such as persistent dry cough (ACEi-cough) and a potentially life-threatening angioedema (ACEi-AE) [1]. Given that 35-40 million people worldwide are currently taking ACEi, the number of people at risk for ACEi-AE is substantial [2].

1.1 ACE Protein and Role in Renin-Angiotensin-Aldosterone System

Angiotensin I converting enzyme (ACE) is a zinc dipeptidyl caboxypeptidase widely distributed on the surface of epithelial and endothelial cells. Furthermore it plays a crucial role in blood pressure regulation through its action in the renin-angiotensin-system (RAAS) (figure1.1) and kinin-kallikrein-system (KKS). Conditions such as reduced renal perfusion, sympathetic activation, and increased loss of salt stimulate the juxtaglomerular cells of the kidney to produce the aspatyl protease, renin (**figure 1.1**). The precursor peptide, angiotensinogen (AGT) produced by the liver is converted to vasoinactive peptide angiotensin I (AngI) by renin [3]. AngI is then cleaved by ACE to produce a potent vasoconstrictor angiotensin II (AngII) (figure1.1). AngII then exerts its important physiological actions at several sites including the vascular cells, smooth muscle cells, the adrenal cortex, kidney and brain through its type 1 (AT1R) and type 2 (AT2R) receptors. AngII plays a key role in fluid and electrolyte balance as well as maintenance of arterial blood pressure [4]. Other physiologic responses of AngII include increased release of epinephrine and norepinephrine as well as stimulation of thirst and increased secretion of anti-diuretic hormone and adrenocorticotrophic hormone. AngII further acts on the zona glomerulosa of the adrenal cortex to stimulate the release of aldosterone, which in turn activates the epithelium sodium channel in the collecting duct of the kidney, resulting in sodium retention, potassium excretion and systemic volume increase[5]. Furthermore AngII stimulates various

cytokines and growth factors and thus promotes cell growth and proliferation. Thus AngII plays a crucial role in the development of atherosclerosis and vessel wall change due its growth effects.

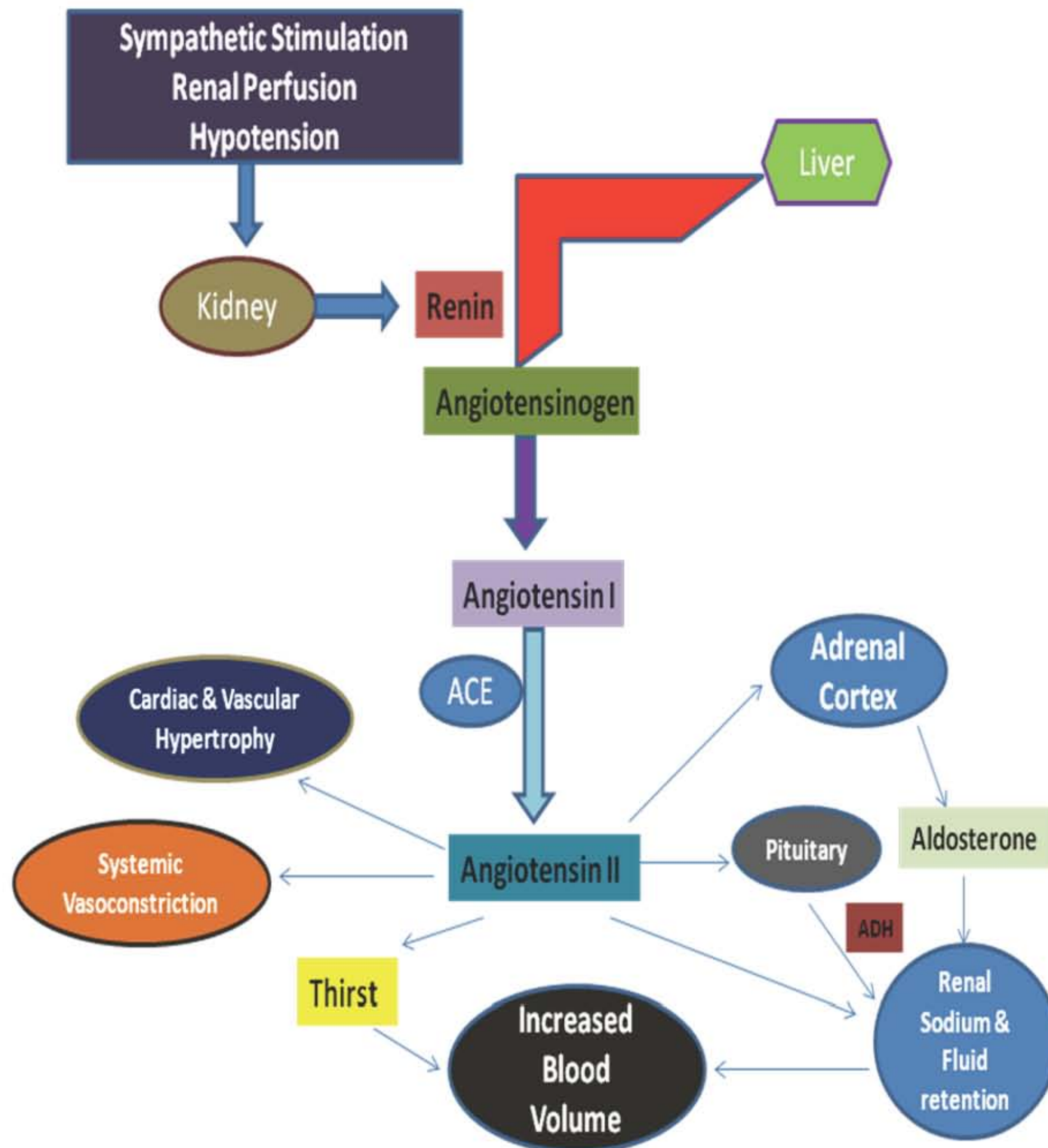


Figure 1.1: Schematic presentation of the renin-angiotensin-aldosterone system. ACE,angiotensin converting enzyme; ADH, anti-diuretic hormone.

Literature Review

1.2 The Kinin-Kallikrein System

The KKS is a complex system composed of pharmacologically active low-molecular-weight vasoactive kinins such bradykinin (BK), kallidin and methionyl-lysl-bradykinin. Kinins are released into body fluids and tissues due to enzymatic cleavage of precursor kininogens by serine proteases known as kallikreins. These peptides play a key role in inflammation due to their ability to activate endothelial cells and consequently lead to vasodilation, increased vascular permeability, nitric oxide production and arachidonic acid mobilization. Kinins have been shown to stimulate nerve endings and thus leading to burning dysethesia(6). Hence classical parameters of inflammation can be attributed to kinin formation[6].

1.2.1 Bradykinin Formation

Two distinct biological pathways are responsible for the generation of BK (**figure 1.2**). The first has of two components namely: an enzyme tissue kallikrein (TK) and a plasma substrate low-molecular-weight-kininogen (LMWK)[7]. TK has wide tissue distribution and is processed intracellularly from precursor prokallikrein. It digests LMWK to yield a decapeptide, Lys-bradykinin (kallidin). Plasma aminopeptidases then cleave the N-terminal Lys resulting in the formation of nonapeptide BK[6].

The second pathway is a complex biological system that forms part of the initiating mechanism for the activation of the intrinsic coagulation pathway[7]. The initiating protein, factor XII (fXII) binds to negatively charged macromolecular surfaces and autoactivates to form factor XIIa (fXIIa)[8]. Plasma substrates for fXIIa are prekallikrein and factor XI (fXI) that circulate complexed with high-molecular-weight- kininogen (HMWK). These complexes also attach to the initiating surfaces with the major attachment sites being on the two domains of HMWK[9]. This then places both prekallikrein and fXI in optimal conformation for cleavage to plasma kallikrein (PK) and fXIa. It is worth mentioning that TK and PK are separate gene products and have little sequence homology though they have related functions. TK has a high affinity for LMWK but can also cleave HMWK, whereas PK exclusively

cleaves HMWK. The two kininogens are homologous starting at the N-terminus and continuing to twelve amino acids beyond the BK moiety only differing at the C-terminus due to alternative splicing[10]. BK is a potent vasodilator that is converted to an inactive metabolites primarily by ACE[11] and secondarily aminopeptidase P [12]. A small fraction of BK or kallidin (3.5%) is converted into a potent proinflammatory peptide, des-Arg^{9}-BK or des-Arg^{10}kallidin by carboxypeptidase N, which are then inactivated by ACE and aminopeptidase P (APP) [12]. BK and kallidin act on constitutively expressed B2 receptors whereas des-Arg^{9}-BK or des-Arg^{10}kallidin act on B1 receptors, only induced as result of inflammation by the presence of cytokines such as Interleukin-1 and tissue necrosis factor α [13].

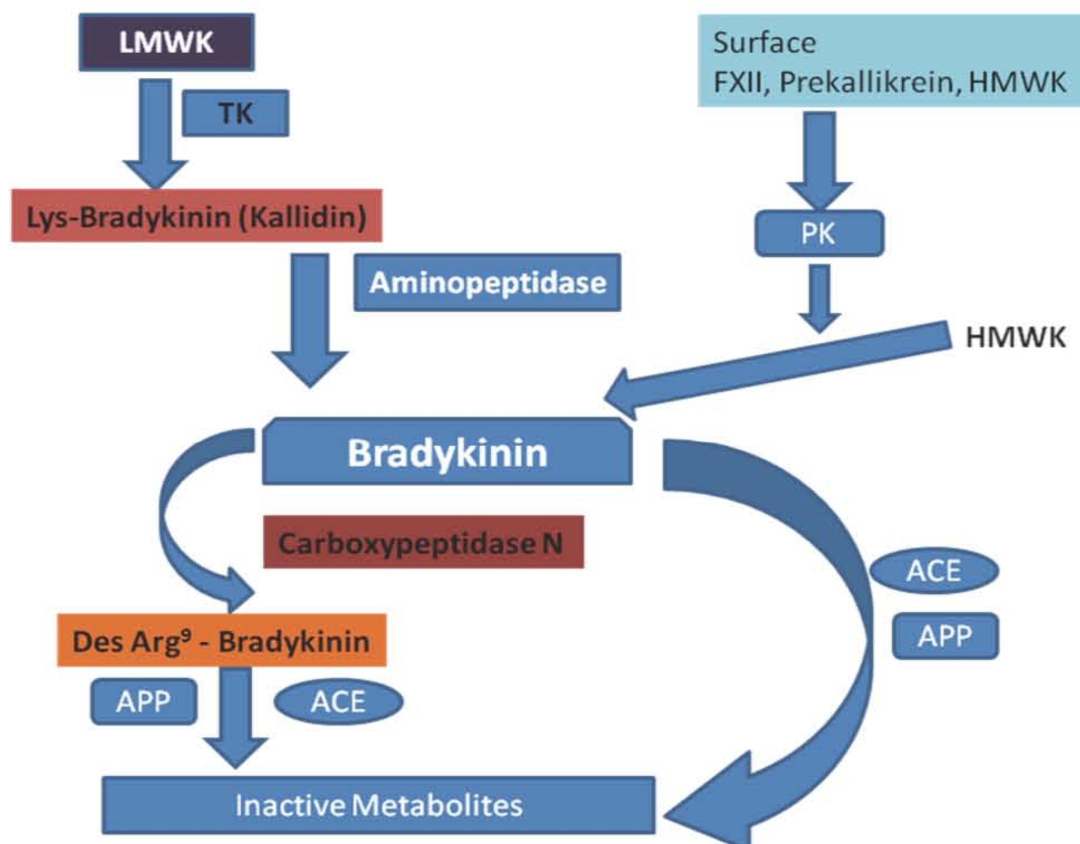


Figure1.2: Pathways for formation and degradation of bradykinin. LMWK, low molecular weight kininogen; TK, tissue kallikrein; HMWK, high molecular weight kininogen; PK, plasma kallikrein; APP, aminopeptidase P; ACE, angiotensin converting enzyme.

1.2.2 Bradykinin Receptors, Signalling and Physiologic Actions of BK

Bradykinin receptors (BKRs) are G protein-coupled receptors belonging to the seven transmembrane domain family. Two BKRs have been identified, namely type 1 (BKR-1) and type 2 (BKR-2), based on their pharmacological properties and on expression cloning[14]. The BKR-1 gene is located on chromosome 14q32.1-q32.2 whilst BKR-2 gene has been mapped to chromosome 1432q. The two receptors share 36% amino acid sequence homology [15]. BKR-1 is synthesized de novo in various tissues following injury, whereas BKR-2 is constitutively expressed in many tissues including neurons of the brain stem, basal nuclei, cerebral cortex, thalamus and hypothalamus. Though BKR-1 is induced under inflammatory states, it has been recently shown that these receptors centrally mediate nociception suggesting their constitutive presence in the brain and/or spinal cord[16].

BK is a potent vasodilator that leads to the liberation of vasodilatory mediators such as nitric oxide (NO), prostacyclin (PGI2) and endothelium-derived hyperpolarizing factor (EDHF)[17]. BKR-2, expressed on the endothelial cells leads to activation of phospholipase C gamma via transient tyrosine phosphorylation causing increased liberation of inositol 1,4,5-triphosphate (IP_3) and diacylglycerol[18]. Increased IP3 then raises intracellular calcium (Ca) by liberation from internal stores or by an increased Ca influx and thus leading to the activation of the Ca sensitive endothelial nitric oxide synthase (eNOS)[19]. Additionally, elevated Ca activates Ca sensitive phospholipase A_2, which hydrolyzes membrane phospholipids to liberate arachidonic acid[20]. Furthermore, BK signalling also activates tyrosine kinase 2 (Tyk2) of the Janus-activated kinase (JAK) family resulting in phosphorylation and nuclear translocation of STAT3[21]. It was also shown that BK activates protein kinase A thus leading to acute increase of NO due to phosphorylation of eNOS at Ser^{179} [22].

Cardiovascular actions of BK such as release of NO, PGI2 and EDHF as well as release of tissue plasminogen activator (tPA) are mediated via the BKR-2 (**figure 1.3**). BK also displays cardio-protective effects by preconditioning tissue for protection against myocardial ischemia/reperfusion injury[23]. In addition BK reduces infarct size area and has inhibitory

effects on cardiomyocytes[24]. Moreover BK has been shown to enhance insulin-dependent increase from pancreatic β cells through increase of intracellular calcium in response to hyperglycemia[25].

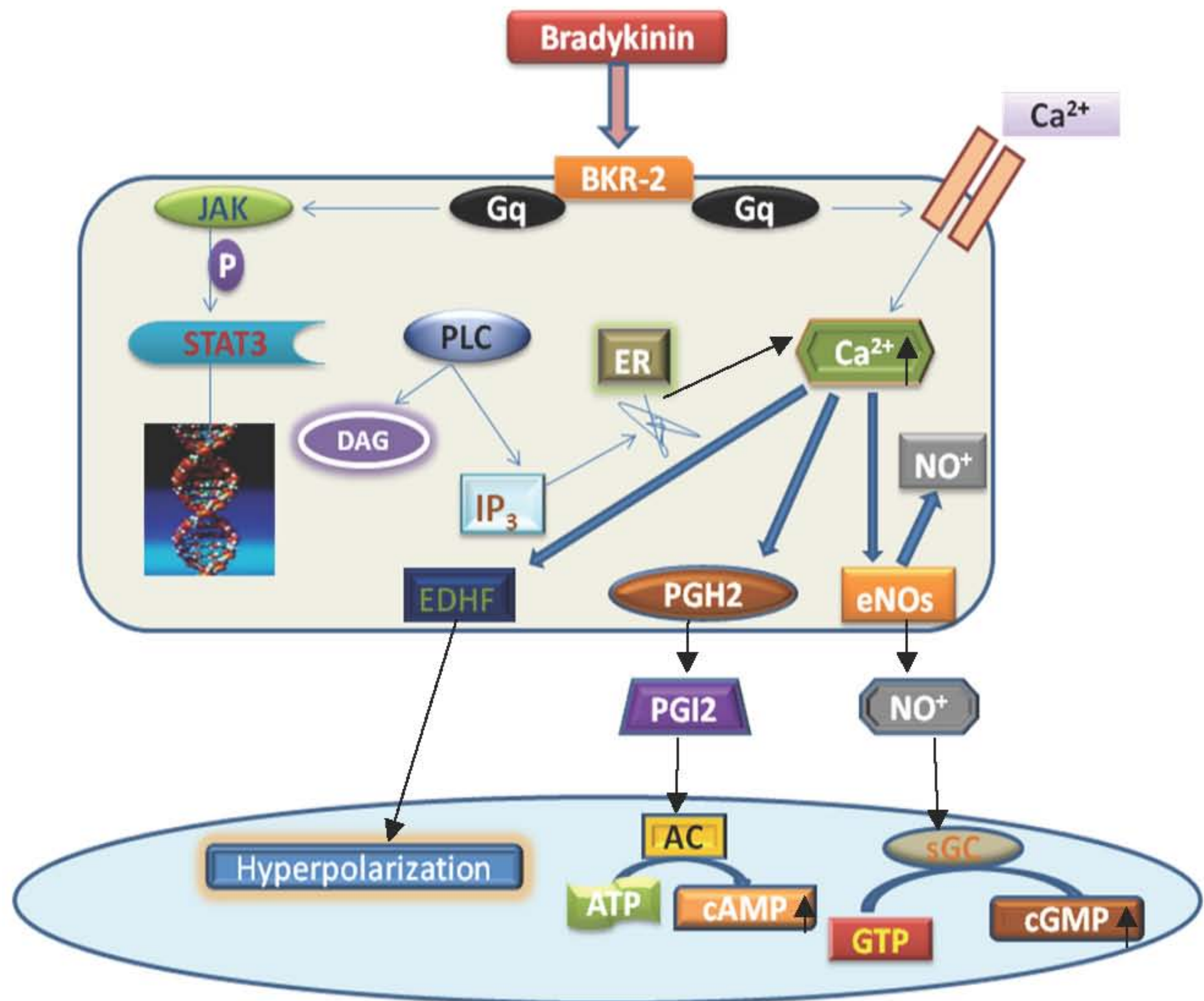


Figure 1.3: Mechanism of Bradykinin induced activation on vascular endothelial cells. Nitric oxide and prostacyclin act in an autocrine and paracrine manner. BKR-2, bradykinin type 2 receptors; JAK, Janus activated kinase; PLC, phospholipase C; DAG, diacylglycerol; IP_3, Inositol triphosphate; ER, endoplasmic reticulum; Ca^{2+}, calcium; NO^+, nitric oxide; eNOS, epithelial nitric oxide synthase; EDHF, epithelial dihyperpolarizing factor;PGH2, prostaglandin;PGI2, prostacyclin; AC, adenyl cyclase; ATP, adenine tri-phosphate; cAMP, cyclic adenine monophosphate; sGC, guanine cyclase synthase; GTP, guanine tri-phosphate; cGMP, cyclic guanosine monophosphate. Adapted from Bas *et al* 2010.

1.3 Interaction between the RAAS and the KKS

The first recognized important link between the RAAS and the KKS was the previously mentioned bifunctional activity of ACE to cleave BK into inactive peptides and convert AngI to the biologically active octapeptide AngII (**figure 1.4**). Moreover BK has been shown to be a preferred substrate of ACE over AngI with a K_m of 0.18μM and a catalytic efficiency ~20 times higher([26;27]. Angiotensin converting enzyme 2 (ACE2), is an ACE homologue that has recently been discovered and was shown to have different substrate specificity than ACE [28;29]. Located mainly in the heart, kidney and testis, ACE2 is a caborxypeptidase that degrades AngI to angiotensin 1-9 (Ang1-9) and AngII to a heptapeptide angiotensin 1-7 (Ang1-7) [30]. Ang1-9 enhances arachidonic acid release by BK and resensitizes BKR-2 [28;31]. Before ACE2 was discovered, angiotensinase C or prolylcarboxypeptidase (PRCP), a serine protease had been known to degrade AngII with K_m of 2mM [32], however ACE2 has been shown degrade AngII more efficiently [32;33]. Ang1-7 counteracts vasoconstrictor effects of angiotensin II by stimulating NO and PGI2 release thereby causing vasodilation. ACE and ACE2 have different specifities for BK, whereas ACE degrades BK, ACE2 does not cleave BK but can degrade des-Arg^9-BK[34] The physiological implication of this finding remains to be elucidated.

1.3.1 The Role of PRCP, TK and PK

Recently it has been shown that PRCP is an endothelial activator of PK where PK assembles to be active and thus indicating another interaction between the RAAS and the KKS[35]. PRCP the first identified endothelial cell activator of PK activates PK independent of fXIIa[35]. PRCP activates PK at K_m 7-17nM suggesting that PRCP is better activator than an angiotensin degrading enzyme[35;36]. Furthermore activation of PK leads to BK formation. Over 20 years ago both PK and TK were proposed to be proteolytic activators of plasma prorenin[37]. Prorenin is the inactive precursor of renin composed of a 43 amino acid N-terminus prosegment that covers the enzymatic cleft and obstructs substrate access. However this hypothesis fell out of favour when it was demonstrated that activation of prorenin by

PK required acid inactivation of plasma protease inhibitors and contact activation of fXII[38]. However, prorenin can be activated by PK at neutral pH though some mechanism of plasma protease inhibitor inactivation is required[38]. Furthermore, it was shown that a patient with decreased total kininogen had reduced plasma renin elevation when assuming the upright position post salt loading[39]. Thus leading to the notion that PK participates in prorenin expression. Though TK has been proposed as a prorenin activator, there is little evidence on how tissue prokallikrein is activated. However, porcine pancreatic kallikrein activates prorenin at pH 8.5[40]. Mouse tissue kallikriens mK1, mK9, mK13 and mK22 have been shown to activate prorenin and additionally human hK1 activates prorenin[41;42]. Whilst TK and PK along with cathespin B and PC5 proteolytically activate prorenin, it is still not clear as to which enzyme converts prorenin to renin[43;44]. Interestingly, it was shown that TK knockout mouse had reduced renal renin mRNA compared with wild type and on the other hand BKR-2 knockout mice had increased renin mRNA[45]. The meaning of these data, however remains unclear although it is possible that TK could be a promoter of renin gene expression whereas PK may have a role in renin activation[30]. Additionally prorenin has been recently shown to be activated non-proteolycally in experimental vascular smooth muscle cells. This mechanism leads to prorenin binding to the recently identified prorenin/renin receptor. This enhances the efficiency of AGT cleavage by renin by unmasking prorenin catalytic activity. Prorenin bound to the receptor cleaves AGT with kinetics similar to that of the fully active renin in solution without the proteolytic removal of its prosegment.

1.3.2. Interaction between Ang 1-7 and BK

Another area of interaction between the two systems is the influence of BK and Ang1-7 on the vasculature. Ang1-7 is breakdown product of AngII by ACE2, PRCP, neprilysin (NEP) and endopeptidase 24.15[32;33;46;47]. Each of the enzymes also plays a role in BK degradation, whether direct or indirect. Ang1-7 exerts its biological effects by binding to AT1R to block AngII or by stimulating AT2R. It has been recently shown that a G protein-coupled receptor MAS is the receptor site of Ang1-7 action[48]. Ang1-7 is degraded by ACE

to angiotensin 1-5. Biologically, Ang1-7 leads to BK potentiation and mediation of vascular activity of BK[49]. Moreover Ang1-7 augments BK by locally acting as a synergistic regulator of kinin induced vasodilation by inhibiting ACE and releasing NO. Thus Ang1-7 is both a substrate and inhibitor of ACE[50]. Both Ang1-7 and Ang1-9 potentiate BK effects by inducing crosstalk between ACE and BKR-2[31].

1.3.3 Interactions between the RAAS and KKS at Receptor Level.

Both AT1 and AT2R have been implicated in the crosstalk between the RAAS and the KKS. The RAS has been shown to stimulate renal BK production and cGMP formation via the AT2R[51]. During salt depletion, renin inhibition and not the AT2R inhibition results in reduced renal BK levels, thus leading to the notion that stimulation of the AT2R releases BK and NO[51]. Liu et al showed in experimental heart failure that ACE inhibition and AT1R blockade have cardioprotective effects that are due to direct effects on the heart as well as secondary haemodynamics mediated by BK formation via activation of the AT2R[52]. How the AT2R system leads to increased BK formation remains unclear. However, it might be mediated by increased TK expression or alternatively by increased PRCP, PK or HMWK expression[30;53]. Interestingly, the AT1R has been shown to interact with BKR-2[54;55]. This interaction leads to formation of stable heterodimers causing increased activation of Gαq and Gαi proteins[54]. Heterodimerization also leads to the alteration in the endocytic pathways of both receptors[54]. Interestingly, it has been shown that AT2R and BKR-2 heterodimerization leads to a 4-5 fold increase in BKR-2 protein levels in pre-eclamptic women.[55] Additionally, the AT2R bind to AT1R to form heterodimers to antagonize AT1R function[55].

1.3.4 Role of the Interactions between the RAAS and the KKS in Thrombosis, Fibrinolysis and Angiogenesis.

Already mentioned as an important modulator of vascular biology and blood pressure homeostasis, the KKS and the RAAS play a crucial role in thrombosis, fibrinolysis and angiogenesis. The RAS has been postulated to be a prothrombotic system[56]. AngII has been shown to increase plasminogen activator inhibitor-1 (PAI-1) mRNA, antigen and active protein levels in both cultured astrocytes and endothelial cells[57]. Furthermore AngII also increases tissue factor (TF) mRNA and activity without affecting tissue factor protease inhibitor (tPA) in rat aortic endothelial cells[58]. Interestingly, artrial natriuretic peptide abolishes AngII-induced expression of TF and PAI-1.[59] Additionally adrenomedullin, a vasodilating peptide also blocks AngII upregulation of TF and PAI-1 mRNA[57]. Activation of MEK/ERK and Rho-kinase pathways in cultured smooth muscle cells contributes to AngII –induced PAI-1 mRNA increase[60]. Moreover, AngII increases TF mRNA and antigen but not PAI-1 in cultured human monocytes[61].

In contrast to the RAS, the KKS has been known to be fibrinolytic[30]. Deficiency of fXII was shown to cause defective fibrinolysis[62]. PK, fXIIa, and fXIa have the ability to activate plasminogen to plasmin, although much less efficiently than tissue-type plasminogen activator and two chain urokinase plasminogen activator[8;63;64]. Nonetheless, PK has been shown to activate single chain urokinase[40]. Activation of PK bound HWMK on endothelial cells leads to kinetically favorable single chain urokinase formation with subsequent formation of plasmin[40;65]. In addition to stimulating fibrinolysis through single chain urokinase activation, PK induced BK formation influences fibrinolysis by stimulating tPA release from the venous endothelium[66;67]. This mechanism is mediated via BKR-2 and is independent of NO and PGI2 liberation induced by BK[68]. BK also stimulates cyclooxygenase 2 (COX2) together with PGI2 thereby contributing to the anticoagulant state[69;70]. COX2 stimulation also results in increased renal medullary blood flow, increased urinary output, and reduced pressor effects of AngII and thus contributing to the antithrombotic state[70].

Furthermore, the KKS contributes to the antithrombotic state via the activity of HMWK and LWMK. Both HMWK and LMWK were shown inhibit thrombin-induced platelet aggregation[71]. Furthermore, it was shown that a patient with plasma that is deficient with total kininogen had lower threshold of gamma thrombin-induced platelet activation than normal[72]. ACE has been shown to degrade BK to BK-(1-5) and this breakdown product also inhibits thrombin[73].

The role of the RAS and the KKS in angiogenesis has recently been established. It has been known for a while that BK may have some proangiogenic effects with BK having being shown to stimulate a neovascular response in implanted sponges in experimental rat model with the new vessel formation being blocked by [Leu^8]des-Arg^9-BK[74]. Moreover, intact HMWK and LMWK proteins are also proangiogenic[75]. Recently HMWK-induced angiogenesis was blocked by targeting its 5th domain with monoclonal antibody and thus interfering with PK cleavage of HMWK to liberate BK[76;77].Furthermore, gene transfer of human TK to BKR-2 deficient mice augmented capillary density and restoration of the physiologic angiogenic response required for wound healing in this animal[78]. Moreover, gene transfer of TK also resulted in increased urinary kinin cGMP and cAMP and showed a protective effect on neointima formation[78]. Therefore either upregulating the KKS or interfering with the RAS influences angiogenesis. Interaction between BK and AngII is essential for normal cardiac development. BKR-2 deficient mice treated with AT1R antagonist from birth were shown to have reduced left ventricular mass, chamber volume, wall thickness, and myocardial fibrosis than untreated mice suggesting the intimate involvement of AngII in cardiac development[79;80]. The RAS components are closely involved in reparative angiogenesis. AT1R was shown to be necessary for reparative angiogenesis in limb ischaemia models[81]. In addition to AngII and BKR-2, other components of the RAS and the KKS also contribute to angiogenesis. BKR-1 also participates in reparative angiogenic process[30]. Cultured cells stimulated with BKR-1 initiate endothelial cell proliferation and survival, while antagonizing the BKR-1 results in apoptosis[82]. Lastly AGT and its breakdown products interfere with angiogenesis[83]. AGT is a serpin and an antithrombin that has strong antiangiogenic activity. Investigations showed that AGT and its derivatives

[reactive loop cleaved form] and des [AngI] AGT have antiangiogenic activity[83]. In summary, degradation protein products of the RAS and the KKS are antiangiogenic whereas intact proteins and defined biologically active peptides are proangiogenic.

Figure 1.4: Interactions of the RAS and the KKS. HMWK; high molecular weight kininogen; HMWKa, plasma cleaved high molecular weight kininogen; PK, plasma kallikrein; PRCP, prolylcarboxypeptidase; ACE, angiotensin converting enzyme; ACE2, angiotensin converting enzyme 2; TF, tissue factor; PAI-1, plasmonogen activator inhibitor 1; tPA, tissue plasminogen activator; NO, nitric oxide; PGI2, prostacyclin; AT1R, angiotensin type 1 receptor; AT2R, angiotensin type 2 receptor; BKR-2, bradykinin type 2 receptor. Adapted from Schmaier *et al* 2003

1.4 Clinical Indications of ACEi

Inhibition of ACE by ACEi impacts on both the RAAS and the KKS. Circulating AngII is potent regulator of blood pressure and inhibition of the RAAS results in a fall in blood pressure [84]. Captopril was the first ACEi introduced in 1981 with other ACEi enalapril and lisinopril being approved later. Currently there about 17 ACEi approved for clinical use. ACEi are recommended for the treatment of hypertension, especially in the presence of left ventricular hypertrophy and congestive heart failure [85-87]. Together with aspirin, β-adrenergic antagonists and statins, ACEi are now considered to be routine therapy to prevent secondary cardiovascular diseases due to their long-acting protective cardiovascular effects[88-90]. According to the American Heart Association, the prevalence of heart failure in the United State is estimated to be 2.2%. Patients with congestive heart failure (CHF) that were stabilized with diuretics should be further considered for ACEi therapy, unless there are specific contraindications (such as aortic stenosis) [91]. Moreover, ACEi slow the progression of atherosclerotic vascular disease, a benefit attributed to their direct vascular protective and anti-atherogenic effects. In addition to their antihypertensive and cardiovascular protective effects, ACEi have shown renal protective effects [92].

Many individuals with hypertension or CHF are insulin resistant and at the higher risk of developing type II diabetes mellitus. A meta-analysis of a randomized controlled trials has shown that ACEi consistently and significantly reduced the incidence of type II diabetes mellitus in individuals with hypertension or CHF and prevents the progression to nephropathy [93]. This antiproteinuric effect is independent of changes in blood pressure [94]. In patients with type I diabetes mellitus ACEi have been shown to prevent diabetic nephropathy [95]. Other beneficial effects of ACEi therapy include antithrombosis, increased adiponectin levels and increased insulin sensitivity[96-98]. Furthermore ACEi also mediate angiogenesis. ACE inhibition results in delays in postichemic reparative process and increase vessel density and capillary numbers. Many of the beneficial effects of ACEi are to the inhibition of BK breakdown and are mainly mediated through the BKR-2. However increased BK half life is also associated with negative effects of ACEi (**figure 1.5**). Importantly, ACEi

have also been shown to have be antiproliferative and antifibrotic effects[99]. A rat model study revealed that N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP), a natural inhibitor of hematopoietic stem cell proliferation and natural substrate of ACE, inhibited rat cardiac fibroblast proliferation and collagen synthesis and mesangial cell proliferation[100]. Ac-SDKP also prevents left ventricular and renal fibrosis in hypertensive rats. Thus the antifibrotic and antiproliferative of ACEi are mediated by preventing endogenous Ac-SDKP degradation, thereby increaseing plasma and tissue Ac-SDKP levels[99;99]. Furthermore increased Ac-SDKP levels suppress cardiac collagen deposition by decreasing both expression of transforming growth factor-β and Smad2 phosphorylation, which are essential for collagen synthesis[100]. It is noteworthy that antifibrotic effects of ACEi in angiotensin II induced hypertension are not associated with hemodynamic changes.

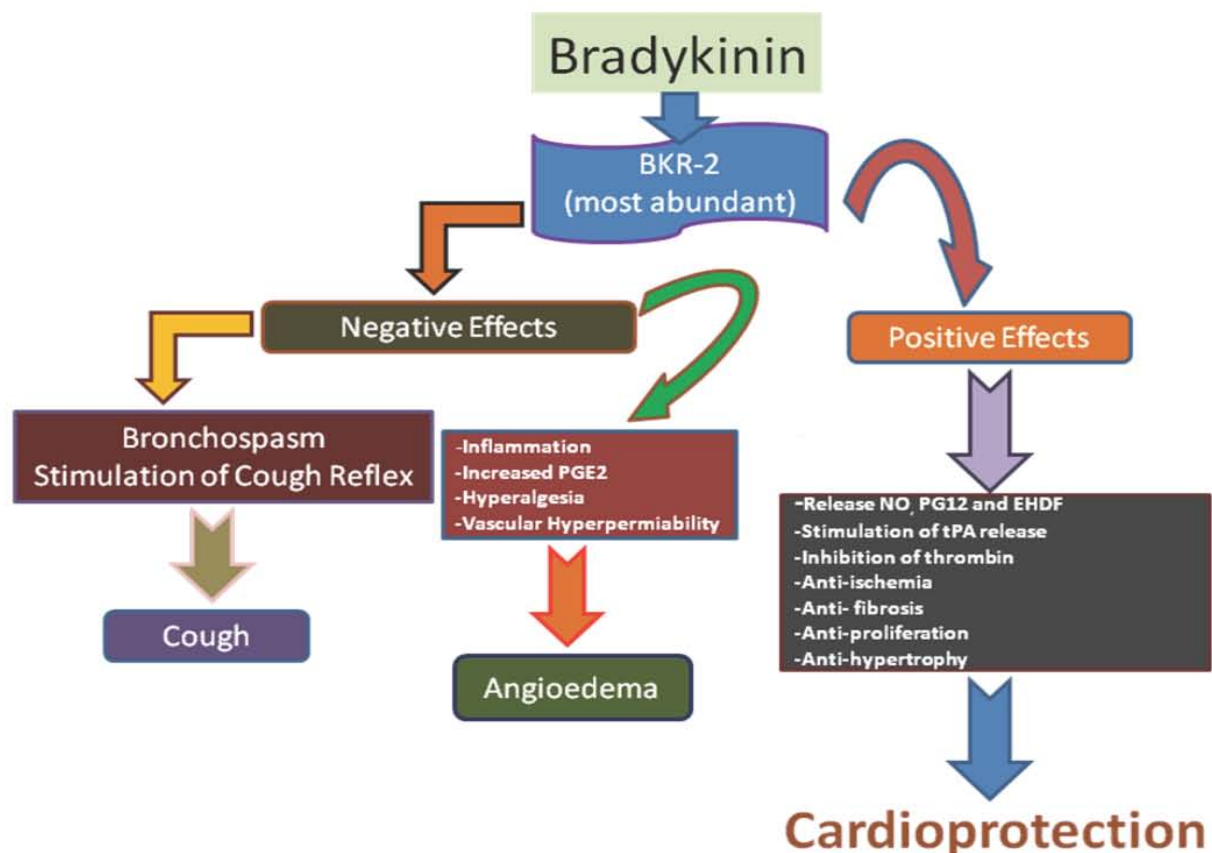


Figure 1.5: Detrimental and beneficial effects of bradykinin via its type 2 receptor. BKR-2, bradykinin type receptor; PGH2, prostaglandin; NO, nitric oxide; PGI2, prostacyclin; EDHF, epithelial dihyperpolarizing factor. Adapted from Bas *et al* 2010

1.5 Clinical Presentation of ACEi Associated AE

Angioedema (AE) is characterized by self-limited, nonpitting edema of vascular origin. The most common sites of ACEi-AE are the lips, tongue and face [101-103] (**figure 1.6**). Involvement of the glottis obstructs the airway and causes death by asphyxiation. Rarely ACEi-AE may involve the bowel wall [104;105] and patients present with nausea, vomiting, abdominal pain, diarrhea or ascites. In such cases, diagnosis may be confirmed by the identification of bowel edema on CT scan. Discontinuation of ACEi is used to resolve symptoms, although failure to suspect ACEi-AE might necessitate surgical intervention (fig1.5b). ACEi-AE incidence is highest during the first month of treatment [2]. A 14-fold increase of AE during the first week of treatment compared with subsequent exposure was observed in one study, and another study observed a 9 fold incidence of AE during the first month of ACEi use. Although the risk of AE per exposure is greatest in the first month of treatment with ACEi, the majority of cases occur after 1 month of treatment with as many as 27% cases reported after more than six months occurring after initiation of ACEi therapy [105;106]. AE onset in one series ranged from 1 day to 10 years of treatment [107]. Early recognition and discontinuation of ACEi remains a primary therapy for ACEi-AE [2]. However, it has been reported that administering complement 1 esterase inhibitor (CINH) concentrate decreased symptoms of AE in a patient who had ACEi-AE [108]. Also, the kallikrein inhibitor ecallantide, which was approved by the FDA in 2009 [109]. Administration of fresh frozen plasma has also been effectively used to treat ACEi- AE [110].

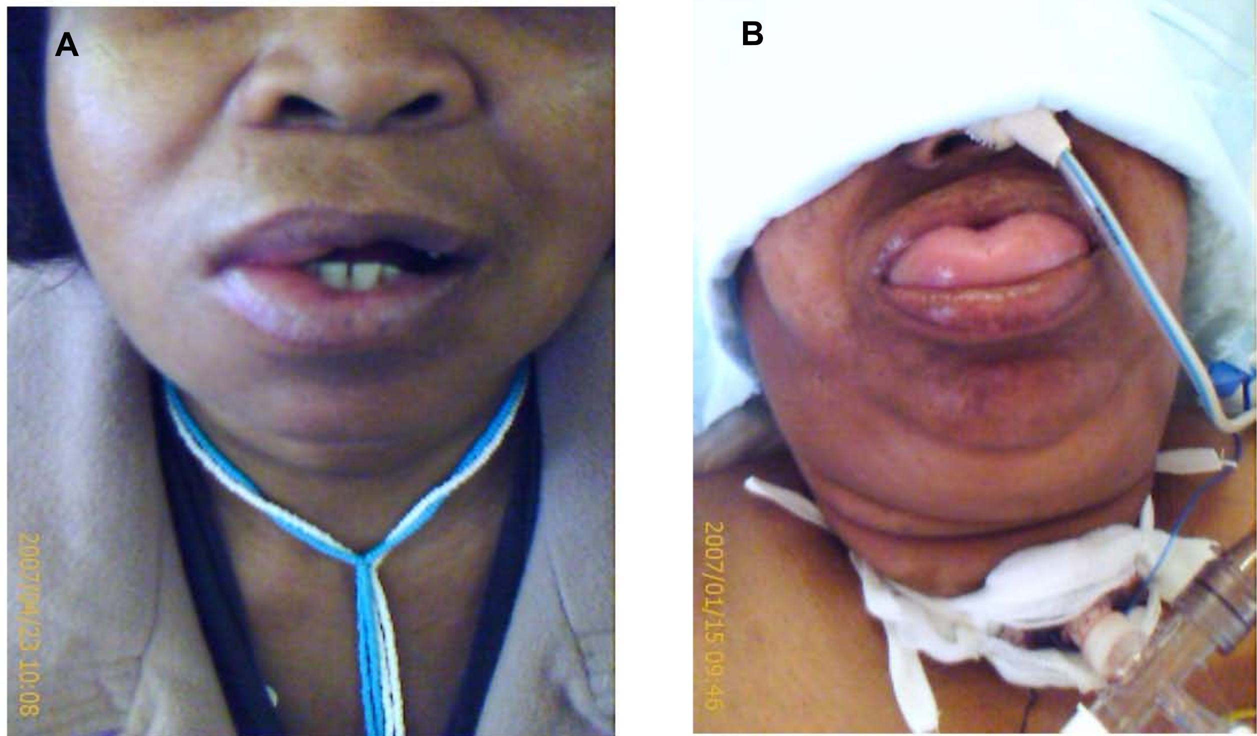


Figure 1.6: A patient with swollen upper and bottom lip. B patient with more severe angioedema and patient had to undergo tracheostomy. Pictures taken at Victoria Hospital Wynberg, Cape Town by Dr Clint Cupido.

1.6 Other Forms of Angioedema

Hereditary angioedema (HAE) is a rare autosomal dominant disorder characterised by recurrent attacks of AE as a result of CINH deficiency[111]. It consists of three variants namely type I (HAE-I), type II (HAE-II) and type III (HAE-II)[112-114]. HAE-I and HAE-II occur as a result of mutations in the C1 inhibitor gene located on q12-q13.1 in the subregion of chromosome 11[115]. Mutations result in either no activity of CINH (HAE-I) or dysfunctional CINH (HAE-II)[115;116]. The complement system is composed of 20 proteins that circulate as inactive precursors. Classical or alternative activation of this system results in opsonization, lysis, chemotaxis, histamine release and immune complex clearance. The enzymes in these cascades are identified by numbers C1-C9. C1 the first component of

the classical pathway is composed of three subunits C1q, C1r and C1s. C1 binds to immunoglobin-bound to antigens triggering activation of other components of the cascade. CINH normally limits the amount of complement activation, however in HAE-I and HAE-II due to CINH deficiency the complement system is activated leading to release of biologically active peptides such as bradykinin. This results in increased vascular permeability and edema of the airway, trunk, limbs, genital tract and gastrointestinal tract. Both types of HAE are associated with low C4 levels and normal levels of C3. C1q levels are also normal except during AE attacks[117]. HAE-I is composed of approximately 85% of CIHN-associated HAE cases characterised by defective expression of 1 allele leading to both low antigenic and functional C1-INH[118]. On the other hand HAE-II comprises 15 % of all CINH-associated HAE cases[118]. It is characterised by functionally low C1-INH levels with either increased or normal C1-INH antigenic levels with the presence of a dysfunctional mutant protein. For both HAE types, C1-INH is usually 5-30% of the normal instead of the 50% expected if the single allele was fully expressed. Thus the difference leads to permanent C1 and contact phase activation subsequently leading to peripheral C1-INH consumption[119;120].

Type III HAE (HAE-III) manifests only in women and its symptoms resemble those of CINH-associated HAE, but occurs in the presence of normal C1-INH levels[121]. It is associated with increased levels of estrogen. It has been reported that HAE-III is associated with the gain of function mutation in the gene encoding fXII[122]. The Thr328Lys substation in exon 9 results in increased fXII activity but not protein levels[122]. Earlier studies showed that an estrogen response element in promoter of fXII gene leads to increased gene expression during increased estrogen states[121]. Thus both increased estrogen levels and Thr328Lys substitution leads to high fXII activity resulting in increased BK production.

Acquired AE (AAE) usually results from lymphoproliferative disorders, autoimmune responses, neoplastic and infectious diseases[123]. AAE is characterised by low functional C1-INH, low C4 levels and normal C3 levels. Contrast to HAE, AAE is also characterised by large numbers of idiotype-anti-idiotype immune complexes consuming C1q molecules and afterward C1-INH[124].

1.7 Epidemiology of ACEi Associated AE

The reported incidence of ACEi-AE varies from 0.1-0.7%, calculated from post-marketing surveillance or epidemiologic studies, to as great as 2.8-6.0% when ascertained prospectively in clinical trials [103;106;125]. Black Americans have increased incidence of ACEi-associated AE that is 4-5 times more that of white Americans[106;125;126]. In Cape Town black and coloured South Africans, the incidence of ACEi-associated AE has also been reported to be high [127]. A history of ACEi-cough increases the risk of AE [128]. Nevertheless, patterns of racial differences in the incidence of ACEi-induced cough and ACEi-AE do not suggest a tight link between cough and AE. These are exemplified by the frequent development of cough in Asian patients who are not at an increased risk of AE [128;129]. Furthermore, a high prevalence of cough has been observed in the South African Indian Durban population on ACEi perindopril and no reported ACEi-AE [130].

1.8 Pathophysiological Mechanism of ACEi Associated AE

The pathophysiology of ACEi-AE remains to be elucidated. Nonetheless, it appears to be a biochemically, rather than immunologically mediated phenomenon [131]. ACE inactivates a potent vasodilator BK and converts AngI to a potent vasoconstrictor AngII. The action of ACEi depends mainly on blocking ACE in RAS to decrease AngII production and cause vasodilation by increased BK levels in the KKS. The long term benefit of ACEi in patients with heart failure results from increased plasma BK half-life [132]. Hence it has been suggested that BK accumulates in patients on ACEi because of the inhibition of ACE responsible for its metabolism[133;134]. This causes vasodilation and edema in susceptible individuals[135]. ACEi increase circulating levels of BK and also local levels of BK responsible for vasodilation[136]. This explains why local swelling in ACEi related AE is triggered by minor trauma and not generalized swelling. Trauma may also activate the KKS leading to AE. The levels of CINH, C4 and IgE are normal in ACEi-AE patients. Additionally, half of the patients presenting with ACEi-AE may also have a defect in the enzyme involved in the metabolism of des-Arg^{9}BK, leading to its accumulation[137]. It has

been suggested that an enzyme defect rather than circulating inhibitor could be responsible for abnormal metabolism of des-Arg^9BK when ACE is inhibited. Abnormal endogenous des-Arg^9BK degradation was found in plasma of patients presenting with ACEi-AE[138]. This evidence suggests that one possible pathogenic mechanism of AE lies in the catabolic site of kinin metabolism. Although less frequent, occurrence of AE due to angiotensin type II receptor blockers (ARBs) suggests that BK is not the only mechanism involved in ACEi induced AE. Whilst the levels of CINH are usually normal in ACEi-AE patients, it has been found that ACEi caused AE in CINH deficient subjects[139]. Furthermore ACEi lead to resensitization of BKR-2, further compounding to ACEi-AE pathophysiology in susceptible individuals. ACEi-AE is also associated with increased plasma concentrations of acute phase proteins fibrinogen and C-reactive protein suggesting that these acute phase reactants might be involved in the pathogenesis of ACEi-AE[140;141]. Interestingly, the levels of these acute phase proteins returned to normal after ACEi discontinuation and AE resolution. Furthermore, the levels of these proteins were normal in patients treated with ACEi who had no AE. This result suggests a contribution of the inflammatory process in the pathophysiology of AE. However, it remains to be seen as whether or not acute phase proteins are truly triggering AE attacks.

1.9 Aminopeptidase P Deficiency and Adverse Events Associated With ACE Inhibition

APP degradation of BK increases during ACE inhibition[142;143]. Thus during ACE inhibition, deficient APP activity leads to enhanced conversion of kallidin by PK and BK by CPN thus leading to accumulation of BK and des-Arg^9BK[2]. Adam *et al* reported a 30% decreased activity in serum APP in 39 patients that had a history of ACEi-AE compared to 39 hypertensive controls[144]. Furthermore metabolism of BK and des-Arg^9BK was characterized following stimulation of formation with ex vivo incubation of plasma with glass beads[145]. In the presence of ACEi (enalapril 130nM), the investigators showed a slight but significant (P=0.022) decrease in catabolism of BK in patients who had a history of AE. However, there was no significant difference in maximal BK concentration or area under curve

(AUC) in AE patients versus controls. In contrast, both maximal des-Arg^{9}BK and the AUC for des Arg^{9}BK were significantly increased in AE patients as well as the half-life of degradation. These data confirm increased formation of des-Arg^9 by CPN in sera of patients who had AE raising the possibility of decreased degradation of this peptide. Decreased APP activity does not account entirely for the pathophysiology of ACEi-AE despite this interesting data. This is because one third of individuals who had ACEi induced AE had normal levels of APP. Additionally, the occurrence of decreased APP activity in the population (18%) is much more than that of angioedema due to ACEi. Furthermore, decreased APP activity or des-Arg^{9}BK metabolism in black patients with a history of ACEi-AE has not been investigated. Taken together, these data suggests that other pathways other than APP play a role in ACEi- AE.

1.10 Pharmacogenetics of ACEi Induced AE

Contrary to hereditary angioedema, in which mutations in the CINH have been identified no specific mutations in ACEi-AE have been identified. However, an ethnic difference in the occurrence of AE provides some presumptive clues that genetic factors play a role. Research has primarily focused on enzymes involved in the degradation of BK. Based on the data that APP activity is reduced in patients with ACEi-AE, Duan *et al* performed a quantitative trait locus analysis to determine the genetic factors regulating APP activity in eight pedigrees in which at least one family member had developed an anaphylactoid reaction during hemodialysis (six families), AE (one family) or both (one family)[12]. Human APP exists in two forms, a glycosylphosphatidylinositol (GPI) anchored membrane form (hmAPP) encoded by XPNPEP2 gene and a cytosolic form (hcAPP) encoded by XPNPEPL gene. The gene encoding for hmAPP is localized on chromosome Xq26.1 whereas hcAPP gene is located on 10q25.1. Investigators found that variation in XPNPEP2 gene accounted for 34% of the heritability of plasma APP activity[12]. No linkage was found with hcAPP gene suggesting that it is hmAPP that primarily contributes to plasma APP activity. Interestingly the investigators identified a single nucleotide polymorphism *C-2399A* that associated with

plasma APP activity, such that the A allele was associated with lower APP activity. Moreover, in white unrelated ACEi associated cases and controls, an association between *C-2399A* genotype and APP was confirmed. Additionally the investigators identified a 175bp genomic deletion (*g.2953-3127*) that resulted in a truncated protein in one family with a proband of both AE and anaphylactoid reaction. The mutant transcript lacked both predicted sites and the GPI anchor. Although interesting, this finding that variation in gene encoding for hmAPP predicted APP activity in families with a history of anaphylactoid reactions, the applicability of these findings to those who are at risk for ACEi-AE is uncertain. Hypotension is typical characteristic of patients who have anaphylactoid reactions and AE; however it is not a feature of ACEi-AE. In addition, mutations in an X-linked gene are not likely to account for the majority of ACEi-AE as ACEi- AE affects more women than men.

The ACE gene is located on chromosome 17 in humans and the polymorphism is detected in intron 16 based on the insertion/deletion (*I/D*) of 287 bp. The average serum levels of DD genotype are higher than that of *ACE-II* genotype [146]. Polymorphisms in the ACE gene have been shown to correlate with hypertension[147], physical performance[148], left ventricular hypertrophy [149] and diabetic nephropathy[150] in different societies. The role of ACE gene polymorphism in Caucasians was proven to have no effect in ACEi or ARB induced angioedema subjects [146].

The BKR-2 has high affinity for both BK and kallidin, but not other BK metabolites. BK vasodilation is mediated primarily through these receptors. The *+9/+9* genotype of BKR-2 *+9/-9* exon 1 polymorphism has been shown to be associated with decreased vasodilation and tPA production in response to BK during ACE inhibition compared with *-9/-9* allele[151]. Furthermore, the *-9/-9* allele has been associated with increased severity of AE in cases of HAE-I[152]. Interestingly, the *C-58T* BKR-2 promoter polymorphism has been associated with increased risk of essential hypertension in African Americans and also with ACEi-cough in Chinese patients[153;154]. However both +9/-9 and *C-58T* have never been investigated and associated with ACEi-induced AE.

Literature Review

1.11 Hypothesis

Given that most studies were conducted elsewhere, it was hypothesized that genetic polymorphisms and biochemical mechanisms play a key role in ACEi induced-AE in black and coloured South Africans.

Aim and Objectives of this Study

The aim of the study was to investigate whether the correlation between serum ACE and mAPP activity activities are associated with C-2399A SNP in the XPNPEP2 gene and ACE I/D polymorphism in the ACE gene in ACEi induced cough and ACEi associated-AE patients. Furthermore the roles of BKR-2 +9/-9 and -C58T polymorphisms in ACEi-AE and ACEi-cough patients were investigated. To achieve these, the following objectives were established:

1. Recruiting controls (patients on treatment with ACEi but free of side effects), ACEi-AE patients as well as ACEi induced cough patients
2. PCR amplification and analysis of ACE I/D polymorphism; XPNPEP2 gene C2399A SNP; BKR-2 +9/-9 and C-58T polymorphisms and determination of their association with ACEi-AE and ACEi-cough.
3. Analysing serum ACE activity in controls, cough and AE patients and determine the significance in levels between the three cohorts
4. Serum APP activity determination in controls, ACEi-AE and ACEi induced cough patients and show whether there is a significant difference in APP levels between the three cohorts
5. Correlating between activities and genotypic data in all three cohorts and show whether there is an association with ACEi-AE and ACEi induced cough.

Participant Recruitment and Sampling

2.1 Participants Recruitment

All cohorts were recruited at the Hypertension Clinic at Groote Schuur Hospital and Victoria Hospital Wynberg. Three different cohorts were recruited as part of the study. Cohort 1 was composed of hypertensive patients currently on treatment with ACEi enalapril for more than two years and with no apparent side effects. These participants were used as controls for the study. These participants were also asked to provide details of the dosage of ACEi. Cohort 2 was patients that previously had or presented to the hospital with ACEi-AE. Cohort 3 was composed of patients who had previously presented/reported to the hospital with ACEi-cough whilst on treatment and were subsequently discontinued from ACEi. All participants underwent preparation to explain explicitly the rationale and significance of the study. Once a participant agreed to be part of the study, a questionnaire (Appendix I) was then filled with participant details such as name; hospital no; age; gender and race. Participants were also asked to provide whether they currently smoked or had a history of smoking. Seasonal allergies and co-morbidities such as diabetes, congestive heart failure and kidney failure were also recorded. All participants were requested to give details of other antihypertensive drugs they were taking. The date of the episode, frequency, localization and severity of AE were recorded. AE was then divided in to three categories depending on the severity as follows:

Mild: Patients presenting with AE and ACEi being discontinued and the swelling returning to normal after 2-3 hours.

Moderate: Patients presenting with massive swelling, ACEi discontinuation and antihistamine or adrenalin administration to reduce the swelling.

Severe: Patients presenting with massive swelling, airway obstruction and hospitalized overnight as well as patients presenting with airway obstruction requiring either intubation or tracheostomy.

2.2 Sample Collection and Processing

All selected participants were requested to donate blood samples after completing the questionnaire and DNA ethics forms respectively. For controls, samples were taken when they were taking enalapril and when they were not taking enalapril for 48 hours. 5 milliliters (ml) of venous blood was collected from each participant by venipuncture of a forearm vein into each of EDTA, serum separator (SST) and heparin Vacutainer tubes. The tubes were immediately mixed by inversion to ensure adequate mixing with the anticoagulant. Heparin tubes were immediately placed on ice and EDTA and SST were kept at room temperature until centrifugation. For serum, SST tubes were centrifuged at 3000rpm for 10 min at 4^oC and within 30 min of centrifugation aliquots of the supernatant were transferred into pre-labeled eppendorf tubes and stored at -80^oC until further analysis. For DNA, EDTA tubes were centrifuged at 3000rpm at 4^oC for 10 min. Then the buffy coat (white layer between red blood cells and plasma) was extracted and transferred into pre-labeled eppendorf tubes and stored at -20^oC until further analysis. The buffy coat contains lymphocytes that are rich in DNA. For plasma, the heparin tubes were centrifuged at 3000rpm at 4^oC for 10min and the supernatant was transferred into pre-labeled eppendorf tubes and aliquots placed on ice immediately. The samples were stored at -80^oC until further analysis.

2.3 Summary

Participants' characteristics are shown in **Table 1**. A total of 165 participants were enrolled as part of the study. In cohort 1, a total of 77 participants were enrolled with coloureds being the majority with 71.4% compared to 25.9% blacks and 2.7% whites. There were more females (57.2%) than males (42.8%) in this cohort. The majority were non-diabetic with 70.1% versus 29.9% diabetics. All participants in cohort 1 were on treatment with 10mg ACEi enalapril taken twice daily specifically in the mornings and the evenings for more than 2 years and were free of ACEi side effects. With regards to cohort 2, a total 52 patients were recruited. Similarly, the majority were coloureds with 61.5% compared to 32.6% blacks and 5.9% whites. Also females were the majority with 89% compared to 11% males and a large amount were non-diabetics with 54% compared to 46% diabetics. With respect to cohort 3, a total of 36 patients were enrolled. Similar to both cohort 1 and 2, coloureds comprised the majority with 72% compared to 14% each of both blacks and whites. There were also more females (78.8%) than males (21.2%) and also more non-diabetics (75%) than diabetics (25%). Enalapril an ACEi was the causative agent of both ACEi-AE and ACEi-cough. The time of onset of angioedema after drug introduction varied from 1 month to 10 years. All cohorts were non-smokers/had no history of smoking and were also free of seasonal allergies.

Table 1: Participants Characteristics

Characteristics	**Cohort 1 (Controls) n=77**	**Cohort2 (ACEi-AE) n=52**	**Cohort3 (ACEi-Cough) n=36**
Race	n(%)	n(%)	n(%)
Coloureds:	55(71.4)	32(61.5)	27(72)
Blacks:	20(25.9)	17(32.6)	5(14)
Whites:	2(2.7)	3(5.9)	5(14)
Gender			
Male:	33(42.8)	4(11)	11(21.2)
Female:	44(57.2)	33(89)	41(78.8)
Comorbidities			
Diabetic:	23(29.9)	17(46)	13(25)
Non-Diabetic:	54(70.1)	20(54)	39(75)

AE was graded as mild, moderate and severe (**See Table 2**). A large amount of patients had mild AE (57.6%) with the common sites of AE being the lips, tongue and face. Similar to earlier observations, coloureds comprised the majority of the mild cases with 60% with blacks and whites comprising of 37% and 3% respectively. There also were more females (76.7%) than males (23.3%) in mild AE cases. Moderate AE patients constituted 13.5% of all AE cases. Similarly, coloureds made-up the majority of moderate AE cases with 57% compared to 29% blacks and 14% whites. There also were more females (57.2%) than males (42.8%) with moderate AE. Severe AE patients made up 28.9% of all AE cases. There were also more coloureds (67%) than blacks (27%) and whites (6%) in severe AE cases as with the mild and the moderate cases. There were also more females (93.3%) than males (6.7%) with severe AE. Of the severe cases three patients required intubation and two required tracheostomy.

Table 2: Angioedema Grades

	Mild n=30 (57.6)	Moderate n=7(13.5)	Severe n=15(28.9)
Race	n(%)	n(%)	n(%)
Coloureds:	18(60)	4(57)	10(67)
Blacks:	11(37)	2(29)	4(27)
Whites:	1(3)	1(14)	1(6)
Gender			
Male:	7(23.3)	3(42.8)	1(6.7)
Female:	23(76.7)	4(57.2)	14(93.3)

Table 3 presents the most common additional antihypertensive medications concomitantly used by all participants in the study. Diuretics were used by 80.5% controls; 82.7% ACEi-AE patients and 75% ACEi-cough patients. Hydrochlorothiazide was the most commonly used diuretic. Calcium channel blockers (CCB) were used by 84.4% controls; 63.5% ACEi-AE patients and 94.4% ACEi-cough patients. Amlodipine was the most commonly used CCB. β adrenergic receptor blocker Atenolol was taken by 67.5% controls; 34.6 ACEi-AE patients and 63.8% ACEi-cough patients. Cardura, an α adrenergic Blocker, was taken by 18.2% controls; 3.8 ACEi-AE patients and 13.8% ACEi-cough patients. Combined α β adrenergic receptor blocker Carvedilol used by 5.2% controls and 1.9% ACEi-AE patients. Potassium sparing diuretics were used by 25.9% controls; 9.6% ACEi-AE patients and 27.7% ACEi-cough patients. Spironolactone was the most commonly used. Hyralazine, a direct-acting smooth muscle relaxant was used by 36.4 % of controls; 11.5% ACEi-AE patients and 25% ACEi-cough patients. Angiotensin receptor blocker (ARB) Telmisartan was used by about 13.5% ACEi-AE patients and 19.4% ACEi-cough. These patients did not develop AE and cough associated with ARB use. Lastly simvastatin, a cholesterol lowering drug was used by 44.2% controls; 15.4% ACEi-AE patients and 47.2% ACEi-cough patients.

Table 3: Other Antihypertensive Medications

	Cohort 1 (Control) n=77	**Cohort2 (ACEi-AE)** n=52	**Cohort 3 (ACEi-Cough)** n=36
Drug	**n(%)**	**n(%)**	**n(%)**
Diuretics (Hydrochlorothiazide, Furosimide)	62(80.5)	43(82.7)	27(75)
Calcium Channel Blockers (Verapimil, Amlodipine)	65(84.4)	33(63.5)	34(94.4)
βAdrenergic Receptor Blockers (Atenolol)	52(67.5)	18(34.6)	23(63.8)
αAdrenergic Receptor Blockers (Cardura)	14(18.2)	2(3.8)	5(13.8)
Combined Adrenergic Receptor Blockers (Carvedilol)	4(5.2)	1(1.9)	-------------
Potassium Sparing Diuretics (Spironolactone, Amiloride)	20(25.9)	5(9.6)	10(27.7)
Hydralazine	28(36.4)	6(11.5)	9(25)
Angiotensin Receptor blockers(Telmisartan)	-------------------------	7(13.5)	7(19.4)
Simvastatin	34(44.2)	8(15.4)	17(47.2)

2.5 Samples

DNA samples were collected from all 165 participants in the study. The total serum and plasma sample collected was 117. Serum and Plasma samples were collected from 29 controls when they were taking enalapril and not taking enalapril. The difficulty was getting samples from the rest of the control participants when they were not taking enalapril as they did not come back to the hospital after the specified time period. Serum and plasma samples were collected from 52 ACEi-AE and 36 ACEi-cough patients. .

2.5 Ethics Approval

The study protocol was approved by the University of Cape Town Human Research Ethics Committee (Appendix II). All participants filled and signed an informed DNA consent form (Appendix III).

Analysis of genetic polymorphisms in XPNPEP2, ACE, and BKR-2

3.1 Introduction

Genetic Polymorphism is defined as the difference in DNA sequence among individuals, groups, or populations. Sources include single nucleotide polymorphisms, sequence repeats, insertions, deletions and recombination. (e.g. a genetic polymorphism might give rise to blue eyes versus brown eyes, or straight hair versus curly hair). Genetic polymorphisms may be the result of evolutionary processes, or may have been induced by external agents such as viruses or radiation. If a difference in DNA sequence among individuals has been shown to be associated with disease, it will usually be called a genetic mutation. Thus genetic differences between individuals can be used to study differences in drug responses to determine the therapeutic effect as well as adverse side effects. The ACE *I/D* and the BKR-*+9/-9* polymorphisms and the XPNPEP2 *C-2399A* and the BKR-2 SNPs have been associated with ACEi side effects in other populations.

Aim: To investigate the importance of known genetic polymorphisms in the XPNPEP2, ACE, and BKR-2 genes respectively in ACEi associated AE and ACEi induced cough patients in black and coloured South Africans.

The objectives of the present work were therefore to:

1. To investigate the role of *ACEI/D* polymorphism in ACEi-AE and ACEi -cough patients
2. To assess the association of *C-2399A* single nucleotide polymorphism (SNP) with ACEi-AE and ACEi-cough
3. To evaluate the role of BKR-2 *9bp* insertion/deletion polymorphism in ACEi-AE and ACEi-AE patients
4. To analyse the BKR-2 *C-58T* SNP and asses its association with ACEi-AE and ACEi-cough

Analysis of genetic polymorphisms in XPNPEP2, ACE, and BKR-2

3.2 Methods

3.2.1 DNA Extraction

Extraction of DNA was performed using QIAGEN QIAamp® DNA Blood Mini Kit (250) according to the manufacturer's instructions.

The QIAamp DNA purification procedure comprises of four steps and was carried using the QIAamp Spin Column in a standard microcentrifuge. The spin column procedure ensures that there is no sample-to-sample cross contamination and allow for safe handling of potentially infectious samples.

Twenty microlitres of QIAGEN Protease® or Proteinase K was pipetted into a 1.5ml micro-centrifuge tube and 200µl of patient's sample (buffy coat) was added. Protease or Proteinase K serves the function of degrading cellular proteins such as Histones. Smaller sample volumes were adjusted to 200µl final volume with PBS before loading. Then 200µl AL buffer was added to remove lipid membranes and disrupt the cell membrane and the nuclear envelope causing cells to burst open and release DNA. The mixture was mixed by pulse-vortexing for 15sec. The mixture was then incubated at 56°C for 2hours. 200µl ethanol was added to the mixture and pulse-vortex for 15sec to mix. The mixture was carefully added to a spin column placed in a 2ml collection tube and centrifuged at 13000rpm for 1min. The column was placed into another clean collection tube and the tube containing the resulting filtrate was discarded. 500µl AW1 buffer was added to the column and centrifuged for 1min. The column was then placed into a clean collection tube and collection tube containing the filtrate was discarded. To the column, 500µl of AW2 was added and centrifuged for 3min. After centrifugation the tube containing the filtrate was discarded and the column was placed on a clean eppendorf tube marked with the patient number. Finally 200µl TE buffer was added to the column, incubated at room temperature 1-5min to increase DNA yield and thereafter centrifuged for 1min. The DNA concentration in the tube was determined using a nano drop spectrophotometer. The tube was stored at stored at -20°C before further analysis.

3.2.2 PCR Amplification of the ACE Insertion/Deletion Polymorphism

PCR was employed to amplify the deletion (D) or insertion (I) allele fragments from intron 16 of the ACE gene located on chromosome 17. Readily available software Oligo®, was used to design primers. Primer pairs were checked for length, compatible melting temperatures and possible primer dimers. The primer pair was the blasted in using NCBI blast software to ensure primer specificity. **Figure 3.1** presents primer pair used for the analysis.

Forward Primer →

5’gcttgcagcatgtggccccaggccggggactctgtaagccactgctggagagccactcccatcctttctcccatttctctagacctgctgcc
tatacagtcactttttttttttttttttttgagacggagtctcgctctcgctctgtcgcccaggctggagtgcagttggcgggatctcggctcactg
caacgtvvgcctcccgggttcacgccattctcctgcctcagcctcccaagtagctgggaccacagcgcccgccactacgcccggctaattttt
tgtatttttagtagagacggggtttcaccgttttagccgggatggtctcgatctcctgacctcgtgatccgcccgcctcggcctcccaaagtgct
gggattacaggcgtgatacagtcacttttatgtggtttcgccaattttattccagctctgaaattctctgagctccccttacaagcagaggtgagc
taagggctggagctcaggcattcaaacccctaccagatctgacgaatgtgatggccacgtcccggaaatatgaagacctgttatgggcatggga
gggctggcgagacaaggcggggagagccatcctccagttttacccgaa 3’

← **Reverse Primer**

Figure 3.1 Primer positions on the intron 16 of the ACE gene. The 287bp insertion or deletion sequence is indicated by red coloured sequence.

The PCR reaction was performed in total volume of 15µl. The PCR reagent contained premixed master mix consisting of: 0.6µl 5mM dNTP with a final concentration of 0.2mM; 3µl 5x Green Go Taq® (Promega) flexi buffer pH 8.5 with a final concentration of 1x buffer; 0.9µl 5mM $MgCl_2$ at the final concentration of 1.5mM; 8.525µl nuclease free water; 0.15µl of 50pmol final concentration 0.01pmol of both the forward and reverse primers; 0.075µl Go Taq flexi DNA polymerase 5U/µl with a final concentration of 1U. The presence of the ACE I/D was amplified by adding 1µl DNA from each specimen to the mix. The amplification was performed using the GeneAmp® PCR System 2700 (Applied Biosystems). The DNA was denatured at 98°C for 2min, then underwent 30 cycles of 96°C for 30sec,

annealing at 65°C for 30sec and 45sec at 72°C for DNA extension. After the cycle, the reaction was completed at 72°C for 8min. The PCR products were then separated using 2% agarose gel run for 30 minutes at 120V and 250mA. The agarose gel was made up using commercially available agarose (LE, analytical grade, Promega) mixed in 1x Tris-acetate-EDTA buffer to make 2% gel solution. The agarose was dissolved by heating in the microwave and 5µl of 10mg/ml ethidium bromide was added before being set. After electrophoresis the flourChem 500 transilluminator was used to visualize *I* and *D* alleles (**figure 3.2**).

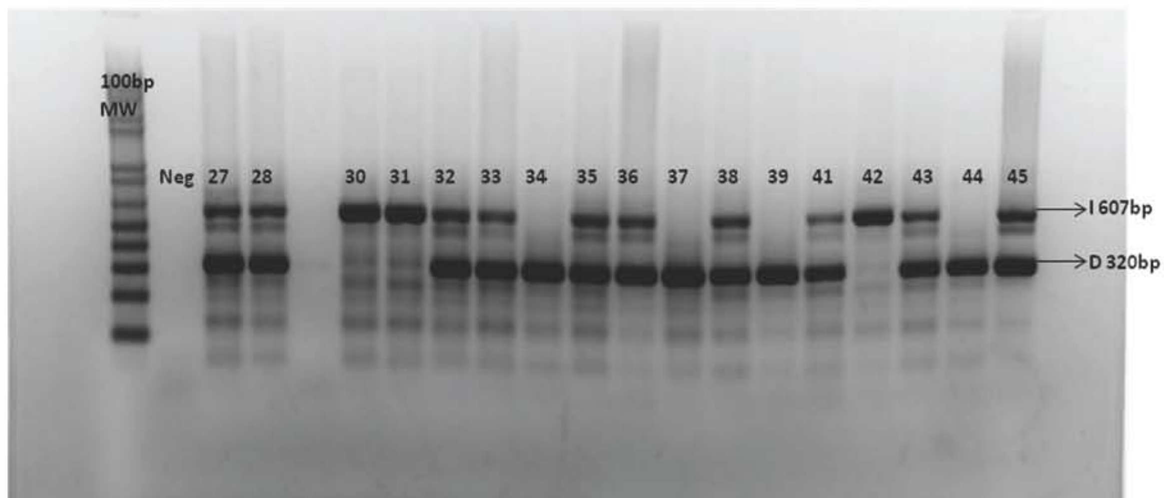


Figure 3.2 2% agarose gel after electrophoresis confirming the PCR product of the ACE *I/D* polymorphism. The presence of the *ACE-I* allele is indicated by a 607bp product whereas the *ACE-D* allele is indicated by a 320bp product. A negative control confirmed no contamination in the reagents or primers

3.2.3 XPNPEP2 Gene C-2399A SNP Detection

The *C-2399* (*-2399A C>A, rs3788853*) SNP in the promoter region of the XPNPEP2 gene was genotyped using allele specific primers described elsewhere (152). Oligonucleotides for PCR were designed from the genomic sequences for the human XPNPEP2 gene. This required three PCR primers for each polymorphic site. The allele specific primers differed at the position of the 3'-most nucleotide. Two standard PCRs were performed using common primer (Pr.F 5'-AACCTCCCCACGTTGAATCA-3') and either one of the allele-specific primers (Pr.R A 5'GCACTGCTGAAATAGCAGTTGTTAT-3') or (Pr.R C 5'-GCACTGCTGAAATAGCAGTTGTTAG-3').

The total reaction volume for PCR was 15µl. The PCR reagent contained: 2µl 5mM dNTPS at final concentration of 0.2mM; 1.5µl 10X Taq buffer at final concentration of 1X; 0.3µl 5mM $MgCl_2$ at the final concentration of 1.5mM; 10.375µl nuclease free water; 0.15µl 50µM at the final concentration of 0.5µM each of the common, forward and reverse primers; 0.15µl 50µM at the final concentration of 0.5µM of the forward and reverse cystinosis gene primers. The cystinosis gene primers were included to check whether DNA has been added to the PCR tube and used as control and finally 0.075µl of 5U/µl Super Therm Gold DNA Taq polymerase with a final concentration of 1U was also added premixed master mix. The C2399A SNP was amplified by adding 1µl of each patient sample to the total reaction mixture. The GeneAmp® PCR System 2700 (Applied Biosystems) was used for amplification. The amplification program was set at the initial step of denaturation step of 95°C for 10min for 1 cycle, followed by 30sec of denaturation at 95°C for 30 cycles, annealing at 65 for 30sec and 50sec at 72°C for DNA extension, followed by final extension of 1 cycle for 8min. The PCR products were then separated using 2% agarose gel and A or C alleles was visualized using flourChem 500 transilluminator (**figure 3.3**).

Figure 3.3 Represents 2% agarose gel after electrophoresis of the *C-2399A* SNP in the XPNPEP2 gene. The presence of the PCR product for the cystinosis gene housekeeping control is confirmed by 681bp band/s. The presence of *C-2339A* SNP is demonstrated by 351 bp band.

3.2.4 BKR-2 +9/-9 bp Insertion/Deletion Polymorphism PCR Detection

The non-coding exon 1 9 bp insertion (*+9*) or deletion (*-9*) (*rs71103505, ggtggggac/-*) BKR-2 polymorphism located on chromosome14q32.1-q32.2 was determined and amplified using PCR. Readily available software Oligo®, was used to design primers. The primers were checked for length, compatible melting temperatures and possible primer dimers. The primer pair was also blasted using NCBI primer blast software to check that the primer pair binds to the BKR-2 *+9/-9* region and not in any other region in the human genome. The primer pair used for PCR detection is presented on **figure 3.4.**

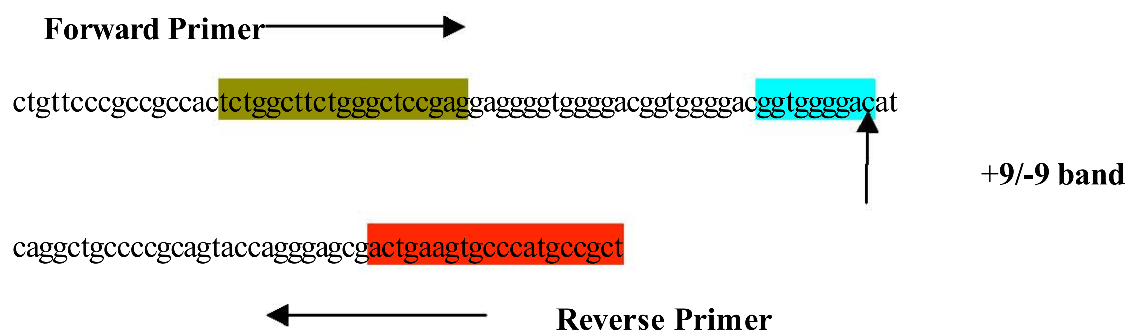


Figure 3.4 Primer position on the BKR-2 exon 1 +9/-9

The PCR reaction was performed in a total reaction volume of 15µl. The PCR reagent consisted of: 0.6µl 5mM dNTPS with a final concentration of 0.2mM; 1.5µl 10X Taq buffer with a final concentration of 1X; 0.18µl 25mM $MgCl_2$ with a final concentration of 0.03mM; 11.95µl nuclease free water; 0.15µl 50µM with a final concentration of 0.5µM of both the forward and reverse primers and 0.075µl of 5U/µl Super Therm Gold DNA Taq polymerase with a final concentration of 1U. The BKR-2 *+9/-9* bp was amplified by adding 1µl DNA of each patient to the PCR mix. The amplification was performed using the GeneAmp® PCR System 2700 (Applied Biosystems). The amplification program consisted of the initial denaturation step of 95°C for 10min, followed by 30sec of denaturation at 95°C for 30 cycles, annealing at 60.6 for 30sec and 30sec at 72°C for DNA extension, followed by final extension of 1 cycle for 8min. The PCR products were then separated on 7.5% polyacrylamide gel run for 60 minutes at 250V and 500mA. The polyacrylamide gel was made up using commercially available 30% Acrylamide/Bis (Bio-Rad Laboratories Inc) mixed in 10x Tris-Borate-EDTA buffer, 10% AMPS and 1% TEMED to make 7.5% gel solution. After electrophoresis polyacrylamide gel was immersed in ethidium bromide solution and the flourChem 500 transilluminator was used to visualize *+9* and *-9* alleles.

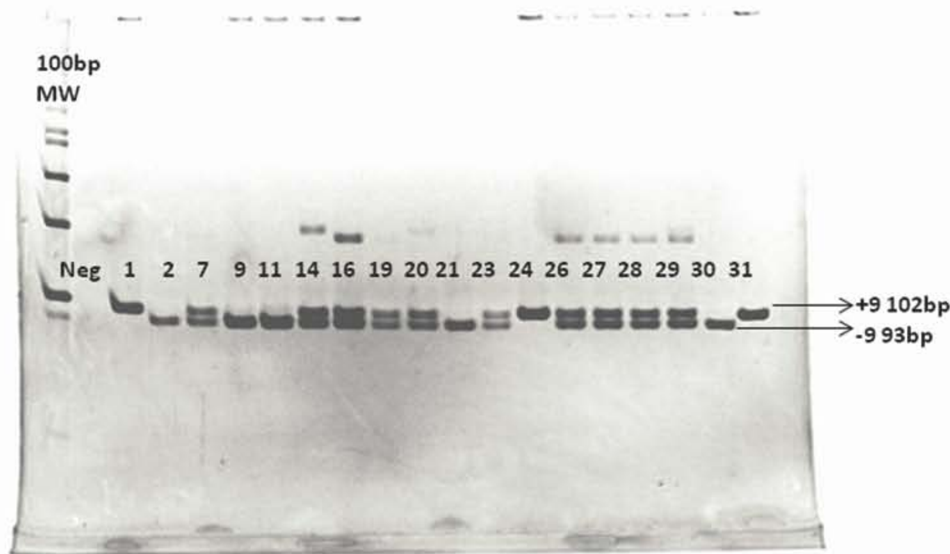


Figure 3.5 Demonstrates 7.5 %Polyacrylamide gel confirming the PCR product of the BKR-2 *+9/-9* polymorphism. The presence of the *+9/-9* is confirmed by the presence of 102 and 93bp bands. Negative control confirmed no contamination in the reagents or primers.

3.2.5 BKR-2 C-58T SNP PCR Detection

The BKR-2 *C-58T* (*-58C>T*, *rs1799722*) SNP in the promoter region of the BKR-2 gene located on chromosome14q32.1-q32.2 was detected using PCR. Readily available software Oligo®, was used to design primers, check for length, compatible melting temperatures and possible primer dimers. The primers were then blasted using NCBI primer blast software to check for primer specificity. The primer pair used for PCR detection is presented on **figure 3.6**. Because the *C-58T* substitution does not alter a recognition sequence for a restriction enzyme, a partial recognition site for *TSP45I* (Fermentas) was introduced by introduction of a single mismatch base in the forward primer (**figure 3.6)**.

Analysis of genetic polymorphisms in XPNPEP2, ACE, and BKR-2

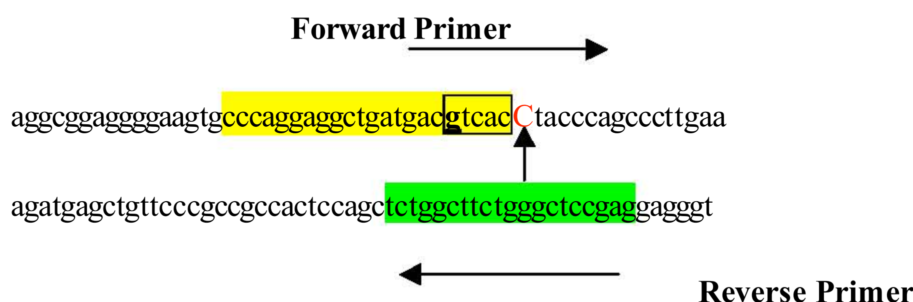


Figure 3.6 Primer position for BKR-2 *C-58T* (red). Forward primer had a single mismatch base (bold) to introduce a recognition site for *TSP45I* which is shown by the box. The *C-58T* SNP is indicated by the C.

The PCR reaction was performed in total volume of 15µl The PCR mix contained: 0.6µl 5mM dNTPS with a final concentration of 0.2mM; 1.5µl 10X Taq buffer with a final concentration of 1X; 0.18µl 25mM $MgCl_2$ with a final concentration of 0.03mM; 11.95 nuclease free water; 0.15µl 50µM made to a final concentration of 0.5µM of both the forward and reverse primers and 0.075µl 5U Super Therm Gold DNA Taq polymerase with a final concentration of 1U. The BKR-2 promoter *C-58T* SNP was amplified by adding 1µl DNA from each specimen. The amplification was performed using the G-STORM® PCR System (Vacutec). The amplification program consisted of the initial denaturation step of 95°C for 10min, followed by 30sec of denaturation at 95°C for 30 cycles, annealing at 60.6 for 30sec and 30sec at 72°C for DNA extension, followed by final extension of 1 cycle for 8min. (fig3.5). 10µl of the PCR product was digested with restriction enzyme *TSP45I* overnight at 37°C. *TSP45I* restriction is produced by *Neisseria mucosa C9-2* and recognizes DNA sequences 5'GTSAC'3 and 3'CASTG'5 respectively (**figure 3.6**). The resulting products were then separated on a 4% agarose gel run for 40 minutes at 120V and 250mA. 4g of the agarose powder was dissolved in 100ml 1X TE buffer and 5µl of 10mg/ml ethidium bromide was added before being set. After electrophoresis the flourChem 500 transilluminator was used to visualize T and C alleles.

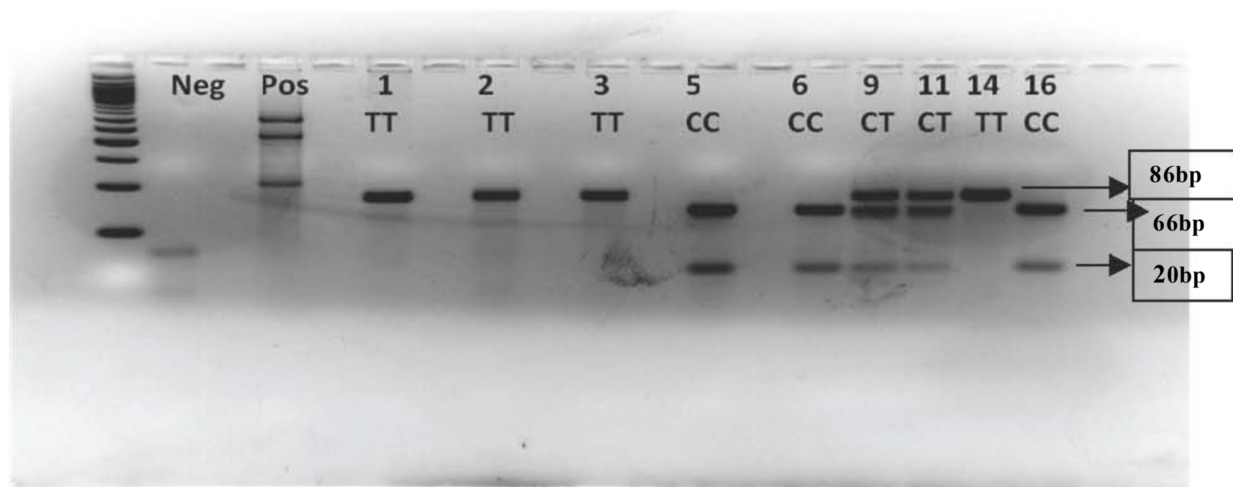


Figure 3.6 Presents 4% agarose gel after electrophoresis of the *C-58T* SNP after Restriction enzyme digestion with *TSP45I.* The presence of the *T* allele is confirmed by the presence of 86bp band whereas the *C* allele is confirmed by PCR product of 66 and 20bp bands. The negative control confirmed no contamination in reagents or primers. The *ACE-II* was used as a positive control and the PCR product was confirmed by the presence of 310bp, 200bp and 97bp bands respectively.

Statistical Analysis

The controls were tested for Hardy-Weinberg equilibrium (HWE) for all genotypes in this study. The Bonferroni correction test was done for all the genotypes and the P value of< 0.01 was considered significant for analysis. The chi squared test was used to test for significance between cases and controls. Statistical analysis was carried out using SPSS 18.0 (SPSS Inc., Chicago, Illinois, USA)

Analysis of genetic polymorphisms in XPNPEP2, ACE, and BKR-2

3.2 Results

3.2.1 ACE Insertion/Deletion Polymorphism Screen

The role *ACE I/D* polymorphism was assessed in unrelated 165 South African hypertensive patients with or without a history of ACEi-AE or ACEi-cough (**Table 4**). Genotypes were in the HWE (P=3.75) for all controls. The ACE *I/D* polymorphism displayed no significant difference overall between males and females (**P=0.910**) in the study. Amongst the South African blacks, coloureds and whites, no significant difference was shown in the study (**P=0.087**). Thus results are indicative of equal distribution of the *I/D* alleles in all patients. The ACE *I/D* polymorphism has been correlated with hypertension, physical performance, left ventricular hypertrophy and diabetic in different societies. Previous work has demonstrated ACEi-AE was higher in blacks and showed a link with the *ACE-II* genotype [155]. In another study no association was found between ACEi-AE and the ACE *I/D* polymorphism in Caucasians [146]. Since the majority of cases in the study with ACEi-AE and ACEi-cough were black and coloured South Africans, the role of *ACE-II* genotype was assessed. The study exhibited no significant association of the *ACE-II* genotype with both ACEi-AE (**P=0.816**) and ACEi-cough (**P=0.503**) when compared to controls.

Analysis of genetic polymorphisms in XPNPEP2, ACE, and BKR-2

Table 4: ACE I/D Polymorphism Analysis

Characteristic	II	ID	DD	Total	P value
Sex	**n (%)**	**n(%)**	**n(%)**	**n(%)**	
Male:	16(34.0)	22(46.8)	9(19.2)	47(100.0)	
Female:	40(33.9)	52(44.1)	26(22.0)	118(100.0)	
Total	56(33.9)	74(44.8)	35(21.3)	165(100.0)	**0.910**
Ethnicity					
Blacks:	17(42.5)	17(42.5)	6(15)	40(100.0)	
Coloureds:	33(28.7)	56(48.6)	26(22.7)	115(100.0)	
Whites:	6(60.0)	1(10.0)	3(30.0)	10(100.0)	
Total	56(33.9)	74(44.8)	35(21.3)	165(100.0)	**0.087**
All Cohorts					
Controls vs ACEi-AE	24(31)vs11(21)	30(40)vs21(40)	23(29)vs20(39)		**0.445**
Controls vs ACEi-cough	24 (31)vs9(25)	30(40)vs17(47)	23(29)vs16(44)		**0.597**

Analysis of genetic polymorphisms in XPNPEP2, ACE, and BKR-2

The relationship between ACE *I/D* polymorphism and ACEi-AE severity was sought (**Table 5**). To my knowledge this has never been done previously. The results revealed significantly lower *ACE-II* genotype compared to *ACE-ID* and *ACE-DD* genotypes (**P=0.01**). Furthermore, no significant difference was found between males and females ACEi-AE cases with respect ACE *I/D* polymorphism (**P=0.60).**

Table5: ACEi-AE Grades

Characteristic	II	ID	DD	Total	P Value
ACEi-AE Grade	**n(%)**	**n(%)**	**n(%)**	**n(%)**	
Mild:	5(18.5)	12(33.3)	13(63.4)	30(100.00	
Moderate:	3(42.9)	2(28.5)	4(57.3)	7(100.0)	
Severe:	3(20.0)	8(53.3)	4(26.7)	15(100.0)	
Total	11(21.1)	20(38.5)	21(40.4)	52(100.0)	**0.001**
Sex					
Male:	3(27.3)	5(45.4)	3(27.3)	11(100.0)	
Female:	8(19.5)	15(36.6)	18(43.9)	41(100.0)	
Total	11(21.1)	20(38.5)	21(40.4)	52(100.0	**0.60**

Analysis of genetic polymorphisms in XPNPEP2, ACE, and BKR-2

3.2.2 Screening of the C-2399A SNP Associated Within the XPNPEP2 Gene

To investigate the association of *C-2399A* SNP with ACEi-AE or ACEi-cough, DNA from 165 unrelated South Africans with or without the histories of ACEi-AE or ACEi-cough was screened. The genotyping results of the *C-2399A* SNP with regards different South African ethnic groups are shown in **Table 3.** The SNP revealed no significant difference between blacks, coloureds and whites (**P=0.91).** It should be noted that whites had low sample size compared to other groups.

Table 6 C2399A SNP Genotyping Results

Characteristic	A	AC	CC	Total	P Value
Ethnicity	**n(%)**	**n(%)**	**n(%)**	**n(%)**	
Black:	9(22.5)	14(35.0)	17(42.5)	40(100.0)	
Coloured:	11(9.6)	36(31.3	68(59.1)	115(100)	
White:	-------	3(30)	7(70)	10(100)	
Total	20(12.1)	53(32.1)	92(55.8)	165(100.0)	**0.91**

The relationship between *C-2399A* SNP was sought in males and females including ACEi-AE, and ACEi-cough (**figure 3.7**)**.** Genotypes were in the HWE (P=0.72) among female controls. **Figure 3.7A** presents the association of *C-2399A* SNP in all males including patients and controls. The results revealed that males had lower *-2399A* (30%) allele frequency compared to -2399C allele (70%, **P=0.0001**). Females also had a lower *-2399A* (5.04%,) allele frequency compared to *-2399AC* (44.54%, P=0.0001) and *-2399C* (50.42%, **P=0.0001**) (**figure 3.7B**). There was no significant difference with *-2399A* allele frequency between all males (30%) and

all females (27%, **P=0.64**) (**figure 3.7C**). There was also no significant difference in *-2399C* allele frequency in males (70%) and females (73%, **P=0.64).**

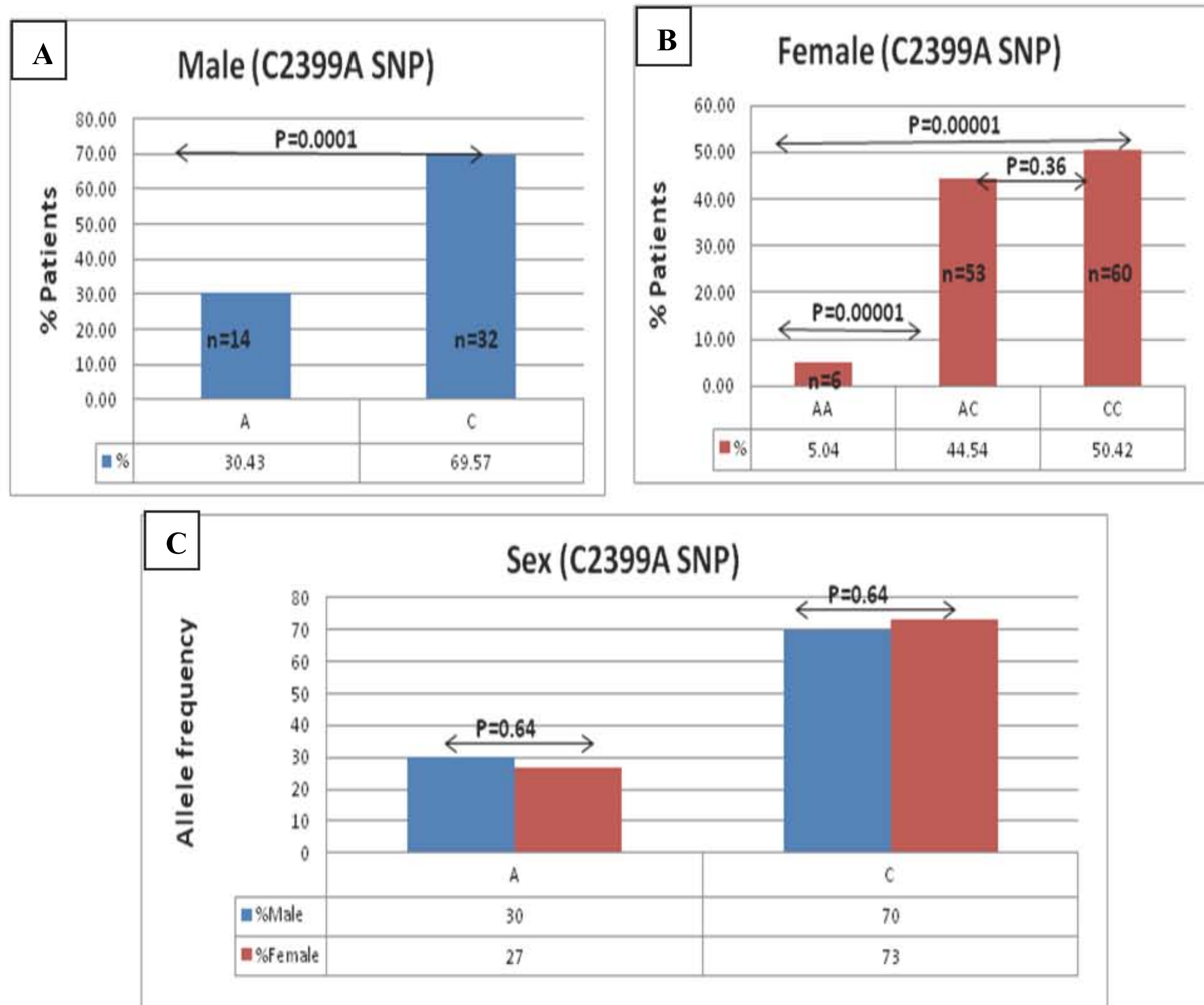
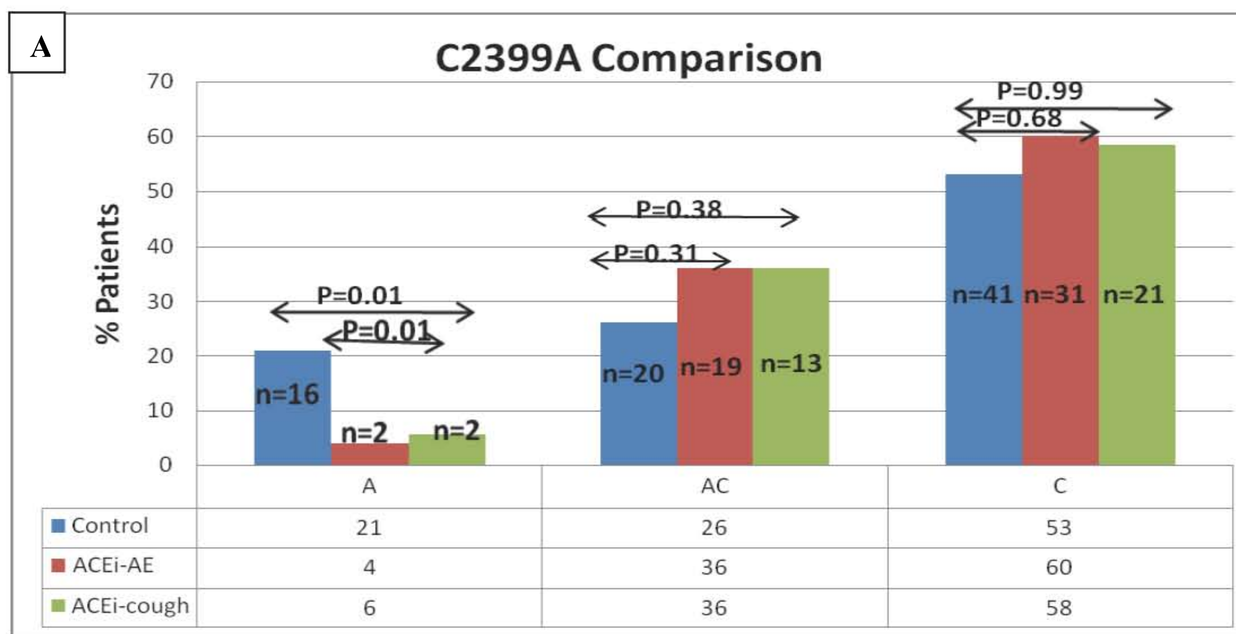


Figure 3.7 An Analysis of the *C-2399A* SNP in males. **B** Analysis of the *C-2399A* SNP in females. **C** *C-2399A* Allele frequency in males versus females

Analysis of genetic polymorphisms in XPNPEP2, ACE, and BKR-2

The frequency of the *C-2399A* alleles was compared in ACE-AE and ACEi-cough patients against controls. Controls exhibited significantly higher *-2399A* allele frequency compared to ACEi- AE patients (21% vs 4%, P=0.01) (**figure 3.8A**). The *-2399A* allele was also significantly lower in ACEi-cough compared to controls (8% vs 21%, **P=0.01**) (**figure 3.8A**). This was surprising because the -2399A allele has been shown to be associated with decreased enzyme activity and associated with ACEi-AE (Duan 2004). However, due to low sample size, the association between *C-2399A* SNP and ACEi-AE and ACEi-cough cannot be discounted. There was no significant association with *-2399AC* allele with ACEi-AE patients compared to controls (36% vs 26%, **P=0.31**). Also no significant association was observed with *-2399AC* in ACEi-cough patients compared to controls (26% vs 36%, **P=0.38**). When the *A* allele was treated as a dominant (*A+AC*) no significant difference was observed between cases and controls (**figure 3.8B**). There was also no significant difference in *C* allele in both ACEi-AE (60% v 53%, **P=0.68**) and ACEi-cough (58% vs 53%, **P=0.99**) patients against controls (**figure 3.8A**).



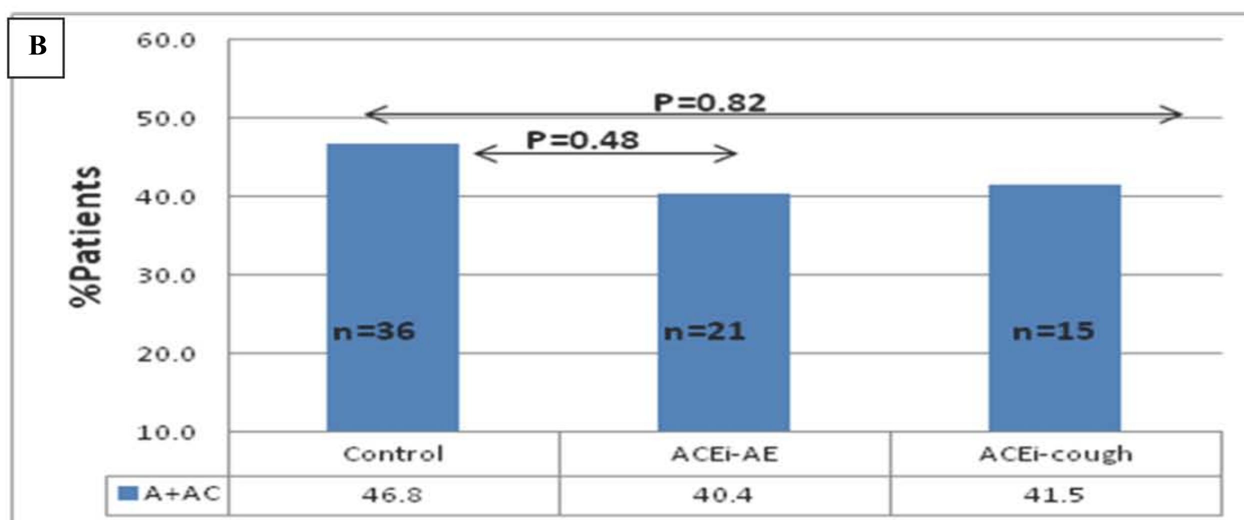


Figure 3.8 A Comparison of *C-2399A* SNP between ACEi-AE; ACEi-cough patients and controls. **B** Analysis of dominant *A* allele in ACEi-AE and ACEi-cough patients versus controls.

Analysis of genetic polymorphisms in XPNPEP2, ACE, and BKR-2

Table 7 presents the relationship between the *C-2399A* SNP and ACEi-AE severity. To my knowledge, there is no data in the literature regarding these. The results exhibited no significant association between the *-2399A* allele and ACEi-AE severity as both *-2399AC* and *-2399C* were significantly higher (**P=0.001**). Amongst ACEi-AE cases the *-2399A* allele was significantly higher in males compared to females (9.1% vs2.4%, P=0.01) (**Table 7**).It should noted that the sample size was small for males.

Table 7 Association of C-2399A SNP and ACEi-AE Grades

Characteristic	A	AC	C	Total	P Value
ACEi-AE Grade	**n(%)**	**n(%)**	**n(%)**	**n(%)**	
Mild:	1(3.3)	10(33.3)	19(63.4)	40(100.0)	
Moderate:	1(14.3)	2(31.3)	4(57.1)	7(100.0)	
Severe:	--------	8(53.3)	7(46.7)	15(100.0)	
Total	2(100.0)	20(100.0)	30(100.0)	52(100.0)	**0.001**
Sex					
Male:	1(9.1)	--------	10(90.9)	11(100.0)	
Female:	1(2.4)	20(48.8)	20(48.8))	41(100.0)	
Total	2(100.0)	20(100.0)	30(100.0)	52(100.0)	**0.01**

3.2.3 BKR-2 +9/-9 Insertion/Deletion Polymorphism

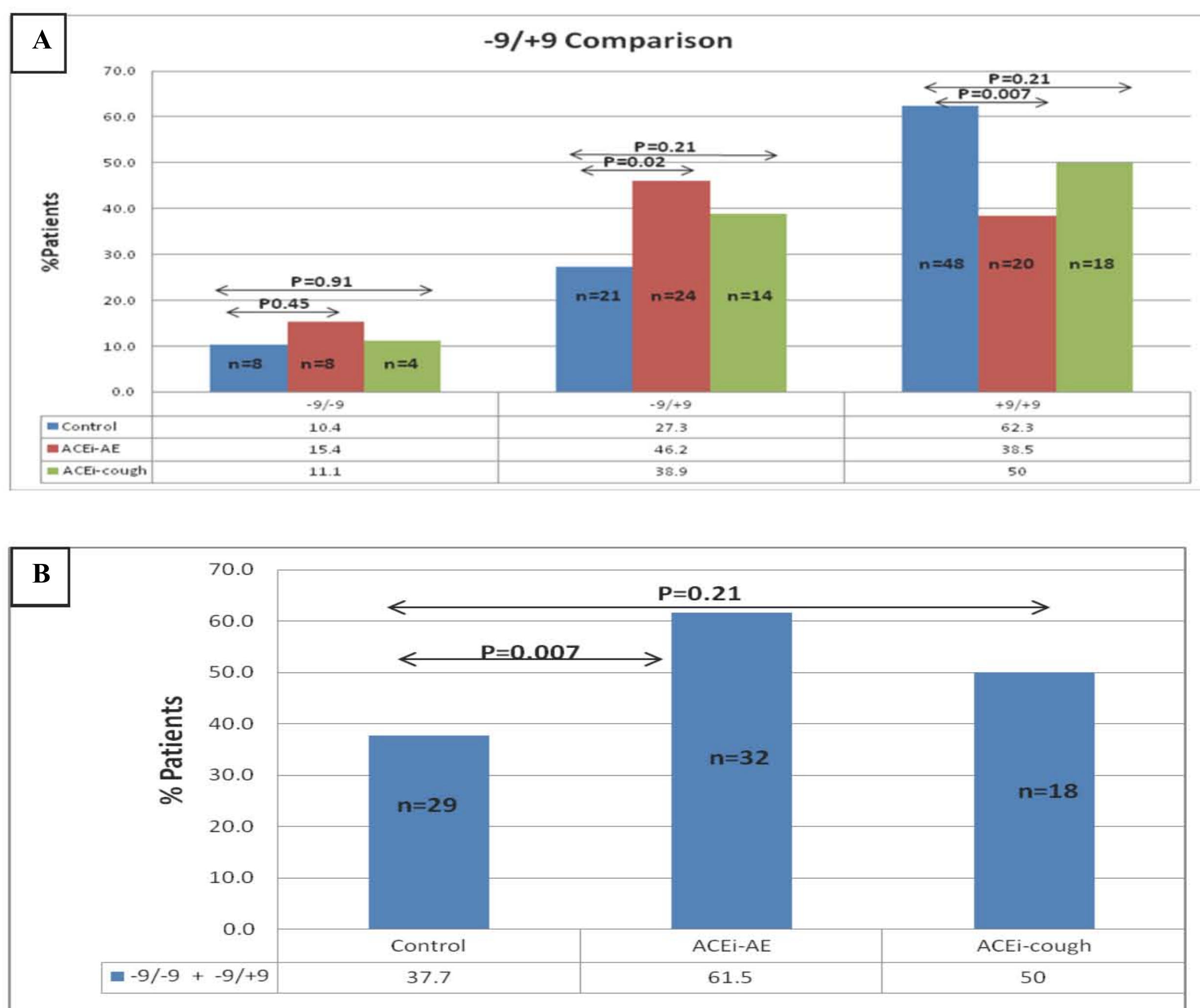
The BKR-2 *+9/-9* polymorphism was screened from the DNA of 165 unrelated South African hypertensive patients and its association with ACEi-AE or ACEi-cough was determined. Genotyping results for the BKR-2 *-9/+9* polymorphism results are presented in **Table 8.** Genotypes were in the HWE (**P=3.20**) among controls. The *+9/-9* genotype frequencies were similar between males and females (**P=0.52**) of all cohorts. There was no significant association between +9/-9 and blacks, coloureds and whites (**P=0.35**) of all cohorts.

Table 8: BKR-2 +9/-9 Polymorphism Genotyping Results

Characteristic	-9/-9	-9/+9	+9/+9	Total	P Value
Sex	**n(%)**	**n(%)**	**n(%)**	**n(%)**	
Male:	9(19.1)	21(44.7)	17(36.2)	47(100.0)	
Female:	15(12.7)	61(51.7)	42(35.6)	118(100.0)	
Total	24(14.5)	82(49.7)	59(35.8)	165(100.0)	**0.52**
Ethnicity					
Blacks:	7(17.5)	23(57.5)	10(25)	40(100.0)	
Coloureds:	17(14.8)	53(46.1)	45(39.1)	115(100.0)	
Whites:	-------	6(60)	6(60)	10(100.0)	
Total	24(14.5%)	82(49.7)	59(35.8)	165(100.0)	**0.35**

The *-9/-9* genotype was not significantly associated with ACE-AE in case-control study of patients (15.4% vs 10.4%, P= 0.45) (**figure 3.9A**). The *-9/-9* genotype was also not associated with ACEi-cough (11.1% vs 10.4%, P=0.91). There was no significant association

with *-9/+9* genotype with ACEi-AE (46.2% vs 27.3%, **P=0.02**) and not with ACEi-cough (38.9% vs 27.3%, P=0.21) against in case-control status (**figure 3.9A**). The *+9/+9* genotype was significantly lower in ACEi-AE patients controls to compared (38.5% vs 62.3%, P=0.007). The *+9/+9* was similar between ACEi-cough patients and controls (50% vs 62.3%, **P=0.21**). When *-9* allele was treated as dominant (*-9/-9* + *-9/+9*) in ACEi-AE patients against controls, the *-9* allele was significantly associated with ACEi induced AE (61.5% vs 37.7%, **P=0.007**). Similarly the *-9* was treated as dominant in ACEi-cough patients versus controls and the results displayed no significant association (50% vs 37.7%, **P=0.21**).



	-9/-9	-9/+9	+9/+9
Control	10.4	27.3	62.3
ACEi-AE	15.4	46.2	38.5
ACEi-cough	11.1	38.9	50

	Control	ACEi-AE	ACEi-cough
-9/-9 + -9/+9	37.7	61.5	50

Figure 3.9 A Comparison of the *-9/+9* BKR-2 polymorphism in controls against ACEi-AE and ACEi-cough patients**. B** Analysis of the dominant *-9* variant in controls against ACE-AE and ACEi-cough patients.

Analysis of genetic polymorphisms in XPNPEP2, ACE, and BKR-2

The relationship between *+9/-9* genotype and ACEi-AE severity was sought (**Table 9**). This was done because the *-9* variant has been associated with increased severity in HAE patients [152]. This has never been investigated in ACEi-AE cases. The results displayed no significant association with *+9/-9* genotype and ACEi-AE severity (**P=0.36**). There was also no significant association with the *+9/-9* genotype with male and female ACEi-AE cases (**P=0.45**).

Table 9: BKR-2 -9/+9 Polymorphism ACEi-AE Grading Analysis

Characteristic	-9/-9	-9/+9	+9/+9	Total	P Value
ACEi-AE Grade	n(%)	n(%)	n(%)	n(%)	
Mild:	5(16.7)	13(43.3)	12(40)	30(100.0)	
Moderate:	1(14.3)	2(28.6)	4(57.1)	7(100.0)	
Severe:	2(13.3)	9(60)	4(26.7)	15(100.0)	
Total:	8(15.4)	24(46.2)	20(38.4)	52(100.0)	**0.36**
Sex					
Male:	3(27.2)	4(36.4)	4(36.4)	11(100.0)	
Female:	5(12.2)	20(47.8)	16(39.0)	41(100.0)	
Total:	8(15.4)	24(46.2)	20(38.4)	52(100.0)	**0.45**

3.2.4 BKR-2 C-58T SNP Screen

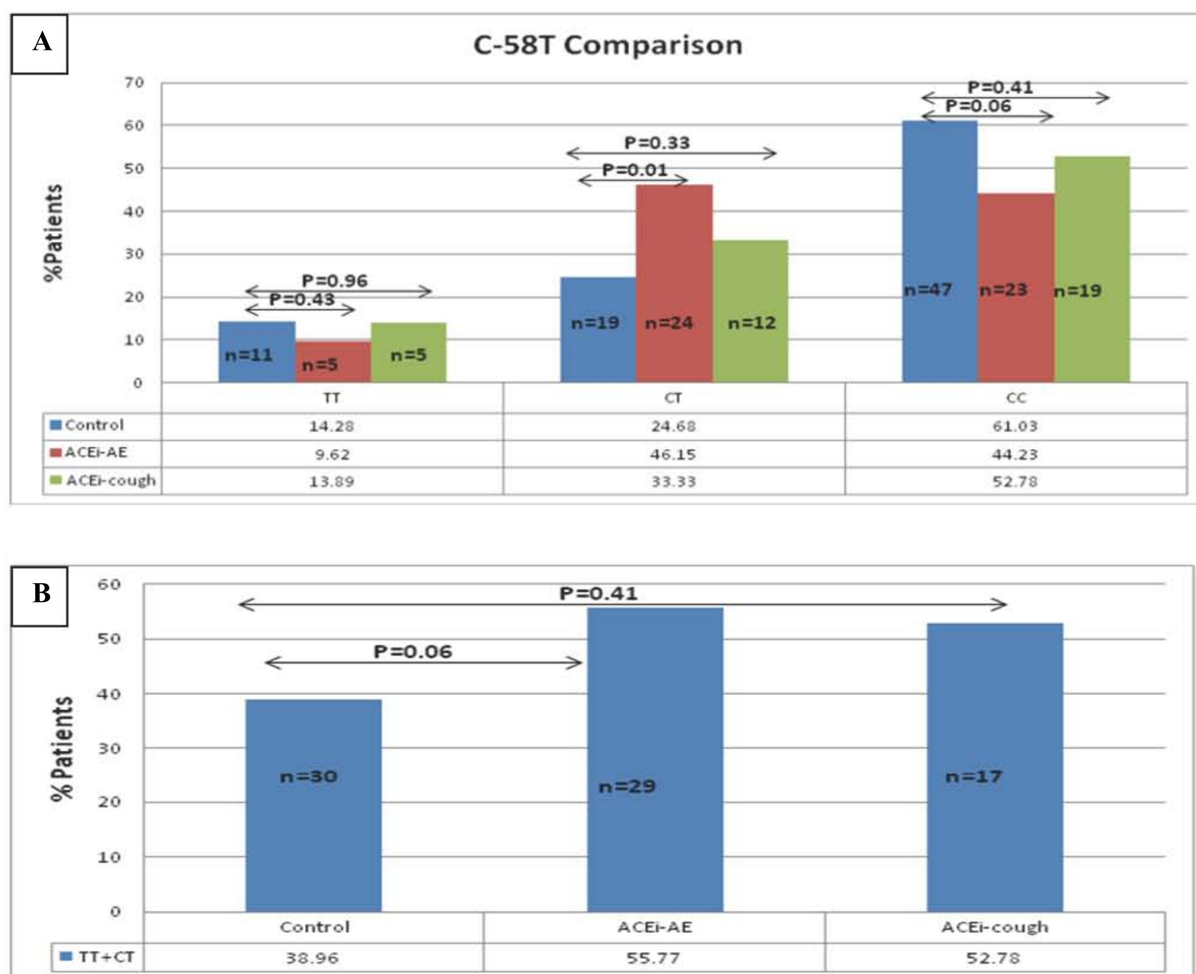
The *C-58T* SNP in the BKR-2 gene was screened in 165 unrelated South African hypertensive individuals and associated with ACEi-AE or ACEi-cough. **Table 10** shows genotyping results of the BKR-2 *C-58T*. Genotypes were in HWE (**P=6.20**) amongst controls. The *C-58T* SNP genotype frequencies displayed a significantly higher *CC* homozygotes and *CT* heterozygotes in both males and females (**P=0.0001**) compared to *TT* homozygote. The *CC* homozygote and *CT* heterozygote was also a significantly higher in blacks, coloureds and whites (**P=0.0001**) compared to the *TT* homozygote.

Table 10: BKR-2 C-58T SNP

Characteristic	**TT**	**CT**	**CC**	**Total**	**P Value**
Sex	n (%)	n (%)	n (%)	n (%)	
Male:	7(14.9)	17(36.2)	23(48.9)	47(100.0)	
Female:	16(13.5)	54(45.8)	48(40.7)	118(100.0)	
Total:	23(14.0)	71(43.0)	71(43.0)	165(100.0)	**0.0001**
Ethinicity					
Blacks:	5(12.5)	18(45)	17(42.5)	40(100.0)	
Coloureds:	17(14.8)	48(41.7)	50(43.5)	115(100.0)	
Whites:	1(10.0)	5(50.0)	4(40.0)	10(100.0)	
Total:	23(14.0)	71(43.0)	71(43.0)	165(100.0)	**0.0001**

The *TT* homozygote was similar in ACEi-AE patients compared to controls (9.62 % vs 14.28%, P=0.43). The *TT* homozygote was also similar in ACEi-cough patients versus

controls (13.89% vs 14.28%, P=0.96) (**figure 3.10A**). The *CT* heterozygote was significantly associated with ACEi-AE (46.15% vs 24.68%, **P=0.01**). There was no significant association between *CT* heterozygote and ACEi-cough (33.3% vs 24.68%, **P=0.33**). The *CC* genotype was similar in both ACEi-AE (44.23% vs 61.03, **P=0.06**) and ACEi-cough (52.78% vs 61.03%, **P=0.41**) against controls. When the *T* allele was treated as a dominant (i.e *TT* + *CT*), no significant association with the *-58T* allele was observed in both ACEi-AE (55.77 vs 38.96, P=0.06) and ACEi-cough (52.78 vs 38.96%, **P=0.41**) versus controls (**figure 3.10B**).



	TT	CT	CC
Control	14.28	24.68	61.03
ACEi-AE	9.62	46.15	44.23
ACEi-cough	13.89	33.33	52.78

	Control	ACEi-AE	ACEi-cough
TT+CT	38.96	55.77	52.78

Figure 3.10 A Evaluation of *C-58T* SNP in ACEi-AE and ACEi-cough patients against controls. **B** Analysis of dominant the of the *T* allele in ACEi-AE and ACEi-cough patients versus controls

Analysis of genetic polymorphisms in XPNPEP2, ACE, and BKR-2

The *C-58T* SNP was further associated with ACEi-AE severity and sex within ACEi-AE cases (**Table 11**). The results displayed a significant association *C-58T* genotype with ACEi-AE severity (**P=0.0001**). This was attributed to higher *-58C* allele in moderate and severe ACEi-AE. The *-58T* allele was significantly higher in female than in male AE cases (12.2% vs 0%, **P=0.0001**). This can however be attributed to low sample size in male ACEi-AE patients.

Table 11: BKR-2 C-58T SNP ACEi-AE Grades Analysis

Characteristic	TT	CT	CC	Total	P Value
ACEi-AE Grade	**n (%)**	**n (%)**	**n (%)**	**n (%)**	
Mild:	3(10)	16(53.3)	11(36.7)	30(100.0)	
Moderate:	---	2(28.6)	5(71.4)	7(100.0)	
Severe:	2(13.3)	6(40.0)	7(46.7)	15(100.0)	
Total:	5(9.6)	24(46.2)	23(44.2)	52(100.0)	**0.0001**
Sex					
Male:	------	5(45.4)	6(54.6)	11(100.0)	
Female:	5(12.2)	19(46.3)	17(41.5)	41(100.0)	
Total:	5(9.6)	24(46.1)	23(44.3)	52(100.0)	**0.0001**

Analysis of genetic polymorphisms in XPNPEP2, ACE, and BKR-2

Discussion

Clinically it is imperative to elucidate the pathogenic mechanisms of both ACEi-AE and ACEi-cough. Therefore, there is an urgent need to identify predictive markers for the development of these side effects. Interestingly, a higher risk for the development of ACEi-AE has been shown for black Americans suggesting that genetic predisposition might modulate the development of ACEi-AE[106;125]. This study examined the association of the ACE *I/D* and BKR-2 *+9/-9* polymorphisms and the XPNPEP2 *C-2399A* and the BKR-2 *C-58T* SNPs with ACEi-AE and ACEi-cough in black and coloured South Africans.

The ACE *I/D* polymorphism has been associated with cardiovascular and renal diseases such as myocardial infarction and idiopathic nephrotic syndrome. According to the results in this study, there appears to be no significant association between the ACE *I/D* polymorphism and ACEi associated AE or cough. The *ACE-II* genotype has been demonstrated to be dominant in black ACEi- and -ARB induced AE patients [155]. Results in this study revealed no significant association between the *ACE-II* genotype ACEi-AE or ACEi-cough. Similarly, the *ACE-II* genotype showed no significant association with ACEi-AE severity. Also there was sex-related genotype association in all ACEi-AE cases.

The *C-2399A* SNP in the XPNPEP2 gene that encodes aminopeptidase P was previously shown to be associated with ACEi-AE as well hypersensitivity reactions in case-controlled studies of white patients [12;156]. Moreover, the *C-2399A* SNP has been associated with ACEi-AE in black American males and not whites [157]. In this study, there was no significant association of the C-2399A SNP with sex or ethnicity in all cohorts. Furthermore, when cases were compared to controls no association was demonstrated between ACEi-AE and ACEi-induced cough cases and the C-2399A SNP genotype even when treating *-2399A* as a dominant allele (**figure 3.8B**). Moreover, the *C-2399A* genotype was not associated with ACEi-AE severity and sex within the ACEi-AE cases.

Analysis of genetic polymorphisms in XPNPEP2, ACE, and BKR-2

The BKR-2 *+9/-9* polymorphism has been associated with significantly higher risk of coronary risk attributed to hypertension [158]. Whilst the *-9/-9* genotype has been associated with severe hereditary AE- type I [152]. The *+9* allele is associated with decreased BKR-2 gene transcription and mRNA expression compared to the *-9* allele [152]. In the present study there was no association with *+9/-9* genotype with sex and ethnicity. There was also no association with ACEi-AE severity. There was no significant association between the *+9/-9* genotype and ACEi-AE and ACEi-cough cases when treating *+9/-9* as an intermediate allele. However when treating *-9* allele as dominant, there was a significant association of the *-9* allele with ACEi-AE but not ACEi-cough (**figure 3.9B**).

The BKR-2 *C-58T* SNP has been associated with ACEi-cough in Chinese patients [153]. Furthermore, the *-58C* allele has been associated with essential hypertension in black Americans [154]. Similar to the BKR-2 *-9/-9* genotype, the *-58T* allele is also associated with increased transcription rate of the BKR-2 gene when compared to the *-58C* allele. In the current study the protective *-58C* allele was significantly higher in males and female but there was no association with the defective *-58T* variant. The *-58C* allele was also significantly higher in black, coloured and white South Africans. The defective *-58T* allele was neither associated with ACEi-AE nor ACEi-cough in our local population (**figure 3.10B**). Furthermore the protective *-58C* allele was significantly higher within the ACEi-AE severity thus exhibiting no association between *-58T* and ACEi-AE severity. Moreover, there was sex-related genotype association with the *-58T* allele in females with ACEi-AE compared to males. However, it is difficult to conclude unequivocally due to limited sample size of males.

Analysis of ACE and APP Activities in ACEi Induced AE and Cough

4.1 Introduction

Angiotensin converting enzyme (ACE) and aminopeptidase P (APP) are enzymes that are responsible for the breakdown of bradykinin (BK). BK is a proinflammatory, vasodilatory nona-peptide that is increased in circulation in events of both ACE inhibitor induced AE and cough. During ACE inhibition APP is the main metabolizing enzyme of BK. Therefore defects in BK metabolism predispose patients who are taking ACEi to AE and cough. Serum ACE activity has been previously diagnostically measured in diseases such as sarcoidosis and myocardial infarction using tripeptide substrates hippuryl-histidine-leucine and z-phenylalanine-histidine-leucine in a spectroflourometric method. APP activity has been previously measured using a flourigenic substrate K(Dnp)PPGK(Abz).

AIM: To evaluate the role of APP and ACE activities in serum of patients and their association with ACEi-induced AE and cough. Furthermore the enzyme activities and genotypes were correlated.

The objectives of the present work were therefore to:

1. To determine serum ACE activity in patients with ACEi-induced AE and cough
2. To determine the correlation between serum ACE activity and ACE I/D genotype in ACEi-induced AE and cough patients
3. To analyse the serum levels of APP in patients with ACEi-induced AE and cough.
4. To assess the correlation between serum APP activity and C2399A SNP genotype in patients with ACEi-induced AE and cough.

Analysis of ACE and APP Activities in ACEi Induced AE and Cough

4.2 Methods

4.2.1. Histidine-Leucine Standard Curve

A 5mM (10x) histidine-leucine (HL) stock was generated by dissolving 13.41 mg HL (Sigma Aldrich, USA) powder in 10ml distilled water. A 0.5mM HL was made in 1x potassium phosphate ($1xK_2HPO_4$) buffer of 0.1M K_2HPO_4; 0.1M KH_2PO_4, pH 8.3 with 0.3 M NaCl. HL standards as set out in **Table 10** (Appendix IV) were made from 0.5mM HL in $1xK_2HPO_4$ buffer. A standard curve was generated by taking 120µl of each standard in duplicates in eppendorf tubes, adding 725µl 0.28M NaOH and adding 50µl opthaldialdehyde (20mg/ml in 100% methanol) to derivatise for 10min to form a fluorescent adduct. The reaction was terminated by adding 100µl 3N HCl and 250µl was transferred to 96 well plate and fluorescent intensities were read spectroflourimetrically. A plot of fluorescent intensity against nanomoles of HL generated using GraphPad Prism 5 software (Appendix IV).

4.2.2 Hippuryl-Histidine-Leucine Substrate Preparation

A 5.7mM Hippuryl-histidine-leucine (HHL) was prepared by dissolving 57.4mg HHL powder (Sigma Aldrich, USA) in 4.165 ml 0.025 M NaOH and heating at 37°C to ensure that HHL was completely dissolved. The mixture was allowed to equilibrate to room temperature before adding 4.0ml 5x Potassium Phosphate buffer stock solution (0 .5M K_2HPO_4; 0.5M KH_2PO_4, pH 8.3) and 2.0ml of 3M NaCl. Lastly 9.835 ml distilled water was added to make a 20ml working solution which was stored at -4°C prior the assay.

4.2.3 Z-Phenylalanine-Histidine-Leucine Substrate Preparation

A 20mM stock solution of z-phenylalanine-histidine-leucine (ZFHL) was made up by dissolving 110 mg of ZFHL powder (Bachem, Switzerland) in 1ml 0.28 M NaOH and 9ml distilled water was added to make a final volume of 10ml and aliquots of 2ml were stored at -20°C. The working stock solution of 2mM was prepared by taking 4 ml 5x stock buffer

solution (0.5M K_2HPO_4; 0.5M KH_2PO_4, pH 8.3; 1.5 M NaCl) and 14 ml distilled water to which 2ml ZFHL (20mM) stock solution was added to make 20ml.

4.2.4 Serum ACE Assay

Serum ACE activity was determined using a spectroflourimetric assay described by Danilov [159] adapted from the method originally developed by Friedland and Silverstein in 1976 [160].The assay employs tripeptide substrates HHL and ZFHL. The assay is based on the principle that ACE cleaves penultimate peptide bond to yield HL moiety that forms a fluorescent adduct upon addition of o-pthaldialdehyde**.** The amount of adduct produced is directly proportional to the amount of dipeptide cleaved by ACE and compared to fluorescent values obtained from HL standard samples of known product concentration. The ACE activity was calculated in mU/ml from the equation:

$$\text{ACE Activity} = \frac{\text{nmol of HL obtained from the sample} \times \text{total reaction volume}}{\text{incubation time} \times \text{HL std curve slope} \times \mu\text{l sample}}$$

Patients and control serum was diluted 1:5 in 1x phosphate buffered saline (PBS) and 10µl of the diluted serum was added to 1.5ml eppendorf tube containing 120µl 5.7 mM HHL (Sigma Aldrich Chemicals, USA) as well as 120µl 2 mM ZFHL substrate in duplicates on ice, mixed by vortexing and incubated at 37^oC for 30 minutes . A Blank Zero Time (BZT) sample was used as control and prepared according to the protocol apart from containing no serum aliquot before. The reaction was then terminated by adding 725µl 0.28 M NaOH and mixed. An equal amount of serum/plasma was added to BZT tubes, mixed by vortexing and used as controls when calculating the amount of non-enzymatic degradation of both HHL and ZFHL in NaOH {Friedland, 1976 360 /id}. Samples were allowed to reach room temperature before the addition of 50µl volume of the fluorimetric agent o-pthaldialdehyde (20mg/ml 100% methanol). Samples were thoroughly mixed by vortexing and the reaction was allowed to proceed for ten minutes at room temperature. The reaction was terminated by adding 100µl 3N HCl and mixed. The samples were centrifuged for two-five minutes in a microcentrifuge at 12000rpm at room temperature to precipitate an unidentified protein-o-pthaldialdehyde complex that might interfere with fluorescent analysis.

A 250µl aliquot of the supernatant was transferred to 96-well microtitre plate for adduct analysis. Samples fluorescent intensities were determined on the Cary ECLIPSE flourimeter (Varian, Walnut Creek, CA) at the excitation and emission wavelength of 360 and 486nm respectively. The ZFHL/HHL ratio was then calculated to determine presence of inhibitor in blood. A ratio > 1.10 indicates the presence of ACEi and that the patient is not compliant.

4.2.5 Serum Dilution and Determination of ACE Stability

It has been previously shown that ACE is inhibited by unidentified low molecular weight endogenous inhibitors in serum, urine and other tissues [159].It was therefore decided to dilute serum in 1x PBS in a dilution series as follows; 1:2, 1:4 and 1:8 simultaneously with undiluted serum so that the effect of these endogenous inhibitors can be observed and minimized with subsequent assays. ACE activity was then determined as described before in section 4.2.4.

Serum ACE stability was determined by using serum stored at -80°C diluted 1:5 in PBS, aliquoted and stored at room temperature (20–22°C) and +4°C for a two week period. At the indicated times, 20µl of ACE solutions were added to 120µl of substrate (5.7 mM HHL or 2 mM ZPHL), and ACE activity was determined as described in section 4.2.4.

4.2.6 ACE Inhibition

Clinically used ACEi lisinopril (10mg) was dissolved in distilled water to make a 1mM stock. Different concentrations of lisinopril (0, 0.1, 0.3, 1, 3, 10, 30 nM) were then made-up in 1x potassium phosphate buffer. Serum was diluted 1:5 in 1XPBS and 180µl was added to the eppendorf tube containing 20µl of each inhibitor concentration in triplicates. The mixture was mixed by vortexing and pre-incubated for 2 hours at room temperature so that the enzyme and inhibitor reaches the equilibrium. The residual ACE activity was determined with 5mM HHL and 2 mM ZFHL, as described earlier in section 4.2.4. The ZFHL/HHL ratio was then calculated to determine presence of inhibitor.

4.2.7 Serum APP Assay

Serum APP activity was assessed using the quenched fluorescent substrate K(Dnp)Pro-Pro-Gly-Leu(Abz) (PPGK), using a method developed by Molinaro in 2005 [161].The Abz (o-aminobenzoic acid) acts fluorescent donor group of the substrate whereas Dnp (2,4 dinitrophenyl) is the fluorescent acceptor group. The substrate mimics the NH_2 terminus of BK/ or des-AR^9BK and is highly specific for APP activity. APP cleaves the substrate between the Pro-Pro bond and thus results in increase in fluorescence over time. One unit of APP corresponds to 1 pmol of PPGK hydrolyzed.

The flourogenic substrate PPGK (a kind gift from A.K Carmona) was dissolved in 1ml 100% DMSO buffer to make 10 mM stock solution. Using the extinction coefficient of E_{365m}=17300 $m^{-1}cm^{-1}$. The concentration of 1/100 dilution in distilled water was determined and 1mM aliquots were made and stored at -20°C.

Serum was diluted 1:10 in 1mM HEPES buffer pH 7.5 at room temperature. The PPGK substrate was diluted 1:125 from 1mM stock in 1mM HEPES buffer pH 7.5 at room temperature. 50µl patient sample was added to 250µl substrate in duplicates in eppendorf tubes and mixed by vortexing. An aliquot of 300µl was transferred to 96 well microtitre plate. Fluorescent intensities were then were read on a Cary ECLIPSE flourimeter (Varian, Walnut Creek, CA) at the excitation and emission wavelength of 320 and 420nm in that order at 0min, 15min, 30min and 60min. A standard curve was generated using a series of standard samples to convert fluorescence to pmol of the product from total hydrolysis of the substrate (AppendixIV). Standard samples were primed in the same manner as the patient samples. The enzymatic activity was calculated as picomoles per minute per milliliter plasma (pmol/min/ml) calculated according to the formula:

$$\text{APP Activity} = \frac{\Delta \text{FU}}{\text{Slope} \times \text{incubation time} \times \mu\,\text{l sample}}$$

4.2.8 Serum APP Stability

For serum APP activity, fresh serum was diluted in 1mM Hepes buffer pH 7.5 to give a APP activity of pmol/min/ml and stored at -80°C, +4°C and room temperature for two weeks. At specific times 50µl of sample was added to 250µl PPGK substrate and APP activity was determined using the above-mentioned method.

4.2.9 Statistical Analysis

Enzymatic results were evaluated using STATISTICA version 9 software (Stasoft®, USA). Data was first evaluated for normal distribution using the Shapiro-Wilk's test ($p>0.05$ for normal distribution). Paired t-Test for dependent samples was used to analyze control patients when on and off treatment for normally distributed data (data reported as Mean±SD) whereas Wilcoxon's nonparametric t-Test was used analyze data that is not normally distributed (data presented as Median, 25-75% quartile with minimum and maximum value). One way ANOVA (Tukey's multiple comparison test) was used to compare between means of controls, cough and angioedema subjects for normally distributed data. Kruskal-Wallis nonparametric ANOVA test was used to compare differences treated controls, cough and angioedema subjects of data that was not normally distributed.

4.3 Results

4.3.1 Validation of the Serum ACE Activity Assay

Serum ACE levels were determined simultaneously in undiluted sera and compared to a dilution series to determine the effect of the endogenous inhibitor on ACE activity. **Figure 4.1** shows a pooled serum sample from three normotensive controls. This was done because a low molecular weight endogenous ACE inhibitor was shown to interfere with ACE activity level measurements in serum [159]. The results showed that with increasing dilution (1:2 through to 1:8) that there was a progressive increase in ACE activity with both HHL and ZFHL substrates compared to undiluted (**figure 4.1A**). The ACE activity was higher when using the ZFHL compared to HHL substrate from undiluted serum through to 1:8 dilution. There was a decrease in ZFHL/HHL ratio with increasing serum dilutions (**figure 4.1B**). The undiluted serum and the 1:2dilution showed a highest ZFHL/HHL ratio of 1.54 and 1.44 (normal <1.10) respectively indicating the presence of inhibitor. The 1:4 and 1:8 dilutions showed a lower ZFHL/HHL ratio of 1.10 and 1.09 respectively which meant that the effect of the endogenous inhibitor was minimized. It was then decided that a 1:5 dilution would be used for subsequent assays as further dilution would compromise fluorescent intensities.

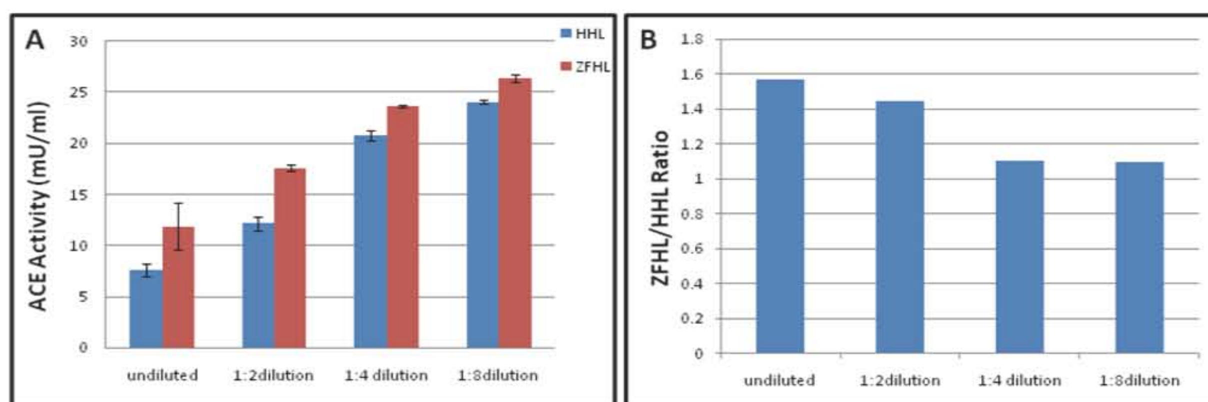


Figure 4.1: A ACE activity of serum serial dilutions of pooled serum using HHL and ZFHL substrates. Activity is multiplied by dilution factor for each dilution. A dilutional increase of ACE activity is shown indicating true inhibitory effect. **B** ZFHL/HHL ratio confirming the presence of inhibitor in undiluted sera and 1:2 dilution compared to 1:4 and 1:8 dilutions.

Due to prolonged storage of serum samples, ACE stability was assessed after storing the serum at different temperatures for two weeks. **Figure 4.2** describes ACE activity using samples from the same individual to achieve this objective. It was determined that ACE was very stable with no significant loss of activity over two weeks at +4°C (20.1 mU/ml) and room temperature (20.3mU/ml) when compared sample stored -80°C (20mU/ml).

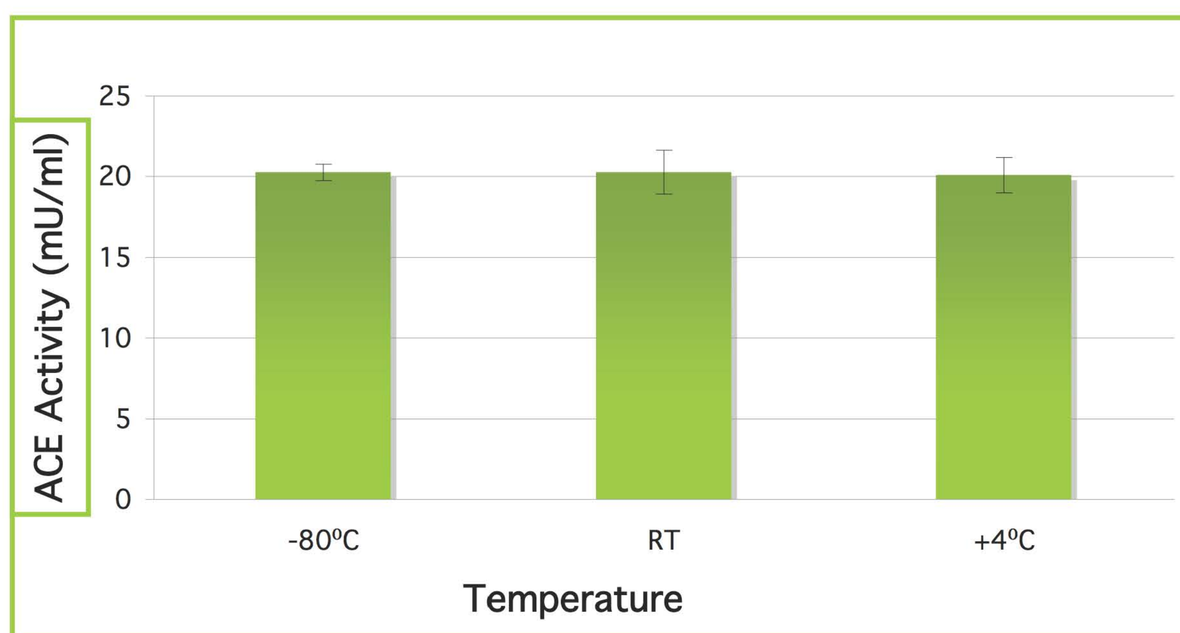


Figure 4.2: The effect of temperature on the stability of ACE. The assay was repeated three times and the intra-assay and inter-assay variability was less than 10%.

4.3.2 ACE Inhibition

To investigate the detection limit for the presence of ACEi in human blood, the ZFHL/HHL ratio was determined after incubating serum enzyme solution with varying concentrations of lisinopril. A significant increase of the ZFHL/HHL ratio was observed at 0.3nM lisinopril consistent with reports in the literature (**figure 4.3**) [159]. Bearing in mind that clinically ACEi are given to patients at a dose of 10mg per day, per kg, 2 times daily, it has been shown that at peak the concentration of ACEi is 50nM and 5nM after 24 hours in human blood (154). Thus this analysis is sensitive enough to detect the concentration of ACEi in human blood following their administration.

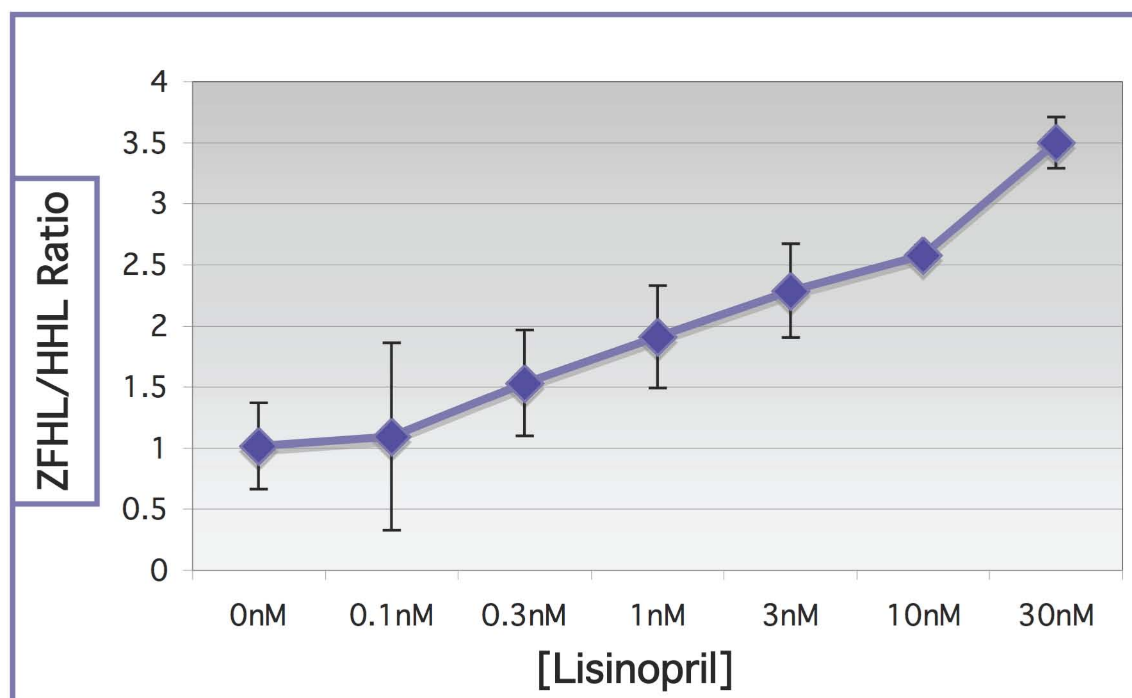


Figure 4.3: Effect of ACEi lisinopril on ZFHL and HHL substrate hydrolysis. The assay was repeated three times intra and inter- assay variation was less 10%.

4.3.3 Analysis of Serum ACE Activity in Patients with ACEi-associated AE and Cough

ACE activity was assayed in controls, ACEi-AE and ACEi-cough patients simultaneously using 5.7 mM HHL and 2 mM ZFHL substrates (**figure 4.4A**). Serum samples from a group of 117 patients comprising of 29 controls, 52 AE and 36 ACEi-induced cough patients were assayed for ACE activity. For controls ACE activity was determined they were taking enalapril and when they were not taking enalapril. **Figure 4.4B** shows the Shapiro-Wilk's test for normality of ACEi-AE and ACEi-cough patients including controls off enalapril. The data showed a Gaussian distribution (symmetrical) which meant that there was a normal distribution (P=0.817) and parametric tests were used for subsequent statistical analyses.

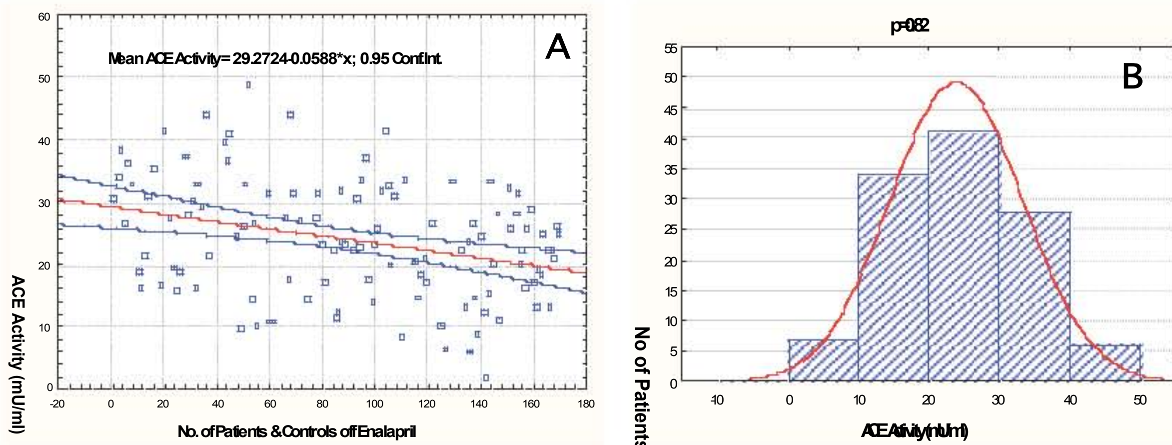


Figure 4.4: A Scatter plot of ACE activity of 117 patients off enalapril in the study. **B** Histogram of the Shapiro-Wilk's test showing normal distribution of 117 patient's ACE activity off enalapril.

There was a significant difference in the mean ACE activity of controls when they taking enalapril (9mU/ml) compared to when they stopped taking enalapril (34mU/ml) (**figure 4.5A**). To determine the compliance of the control cohort, the ZFHL/HHL ratio was determined for serum samples taken while on and off enalapril. The ZFHL/HHL ratio was significantly higher when controls were on enalapril compared to when they were off enalapril (3.25 vs 0.91, P=0.0001). This unequivocally proved that the controls were off treatment for the specified time period of 48 hours.

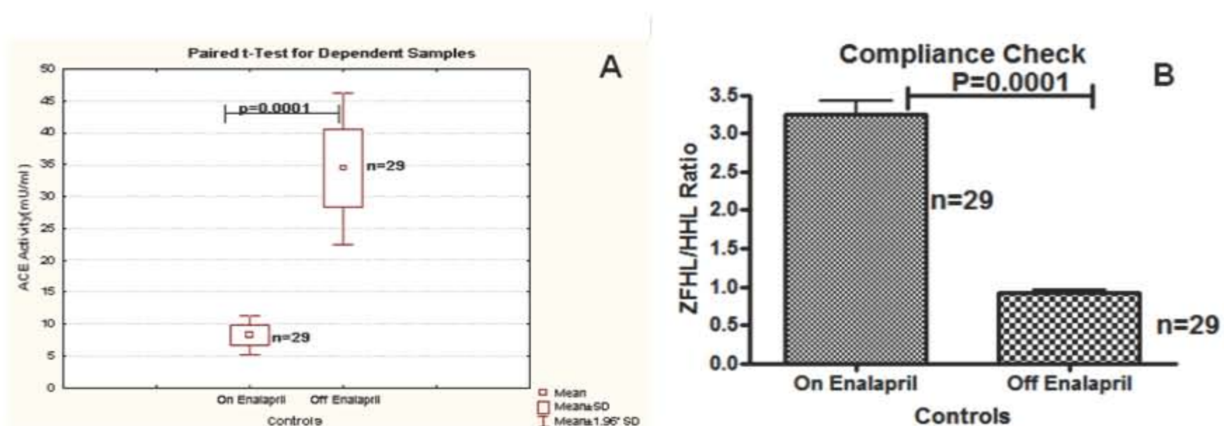


Figure 4.5: A A paired T-test was used to compare the mean ACE activity of patients on and off enalapril. **B** ZFHL/HHL ratio to check for control compliance.

Analysis of ACE and APP Activities in ACEi Induced AE and Cough

The mean ACE activities of controls, cough and angioedema patients were compared using Tukey's multiple comparison test. There was a significant difference in the mean ACE activity of treated patients against both cough (P=0.000114) and AE (P=0.000114) patients (fig4.6). There was also a significant difference in the mean ACE activity of cough patient versus AE (P=0.000298) patients. It should be noted that all patients were off enalapril.

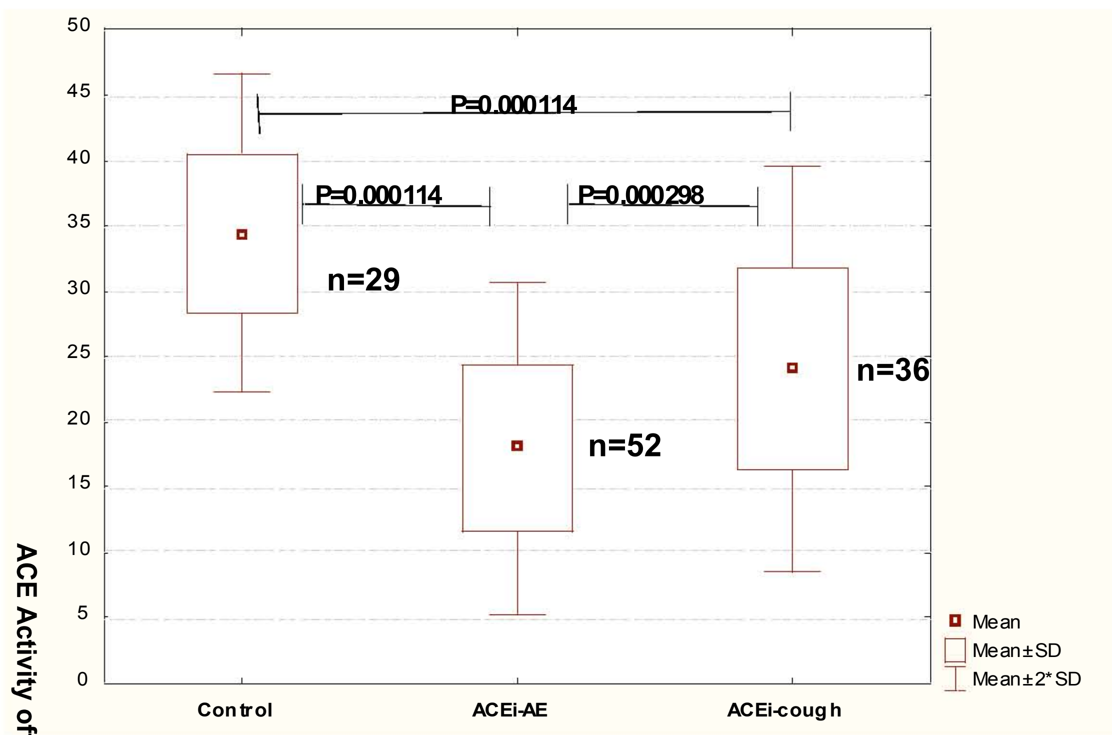


Figure 4.6: Box and Whisker plot of one way analysis of variance of serum ACE levels in controls against ACEi-AE and ACEi-cough patients.

4.3.6 Correlation between Serum ACE Activity and ACE Genotype in Patients

The ACE activity in serum was correlated with the ACE genotype previously determined in chapter 3 from all cohorts including controls using the Tukey's multiple comparison post hoc ANOVA test (**figure 4.7**). The mean activities of serum ACE was surprisingly similar for the ACE-II (ACE activity=23.94mU/ml), ACE-ID (ACE activity=24.49mU/ml) and ACE-DD (ACE activity=23.09mU/ml) in contrast to reports in the literature that ACE-II genotype individuals have lower ACE activity [146].

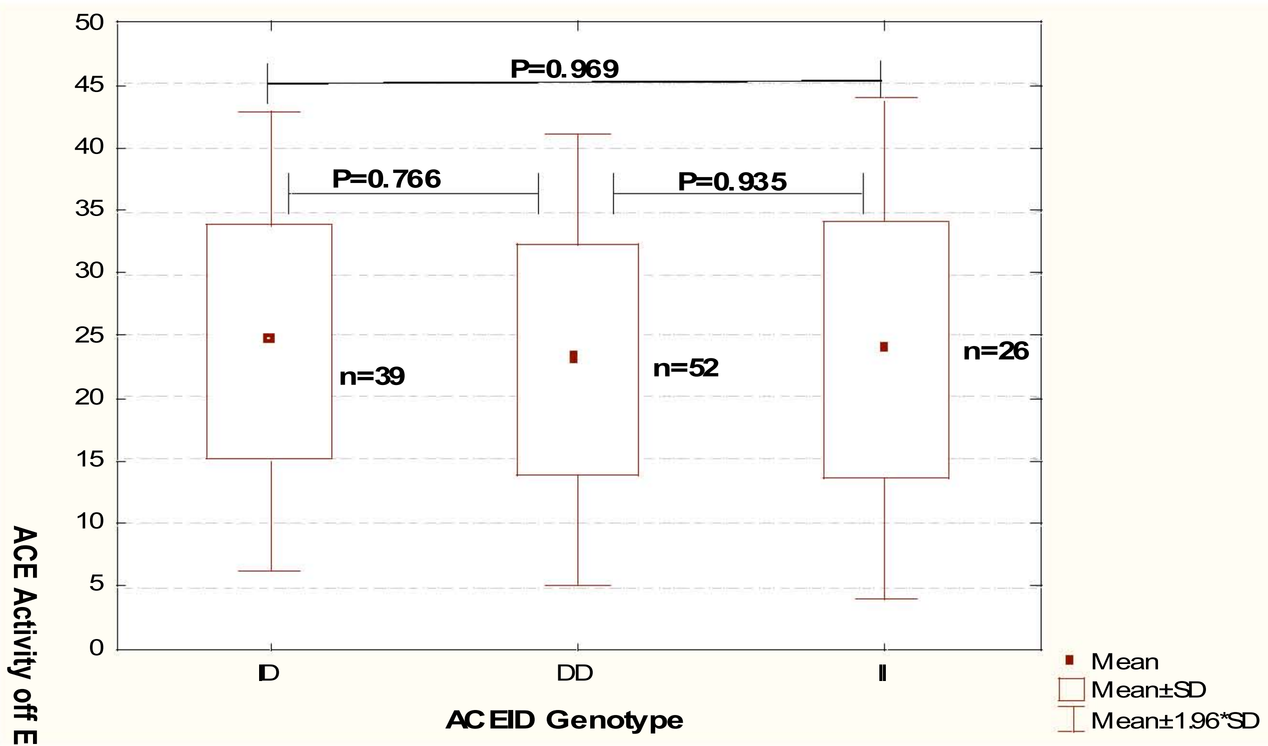


Figure 4.7: A box–and-whisker plot of one way analysis of variance of correlation between ACE phenotype and the ACE insertion/deletion genotype in all patients.

The activity of ACE in serum was then correlated with ACE genotype in controls, ACEi-AE and ACEi-cough patients (**figure 4.8**). It was found that ACE II homozygotes in controls had significantly higher mean ACE activity compared to both ACEi-AE (35.68mU/ml vs 17.05mU/ml, P=0.001) and cough (35.68mU/ml vs 23.05mU/ml, P=0.01) patients. There was no significant difference between mean ACE activity between ACEi-AE and ACEi-cough

patients (23.05mU/ml vs 17.05mU/ml, P=0.05).With regards to ACE ID heterozygote genotype, the ACE activity was significantly higher in controls compared to ACEi-AE (34.50mU/ml vs 17.61mU/ml, P= 0.001) and ACEi-cough (34.50mU/ml) vs 25.36mU/ml, P= 0.01) patients. No significant difference was observed between ACEi-AE and ACEi-cough patients (17.61mU/ml vs 25.36mU/ml, P= 0.05) ACE activity. Lastly ACE DD genotype showed significantly higher ACE activity in controls than in ACEi-AE (33.40mU/ml vs 18.74mU/ml, P=0.001) and ACEi-cough (33.40mU/ml vs 21.62mU/ml, P=0.01) patients. No significant difference was shown between ACEi-AE and ACEi-cough patients (18.74mU/ml vs 21.62mU/ml, P= 0.05). Overall there was no significant difference in ACE activity and ACE genotype correlation in all cohorts.

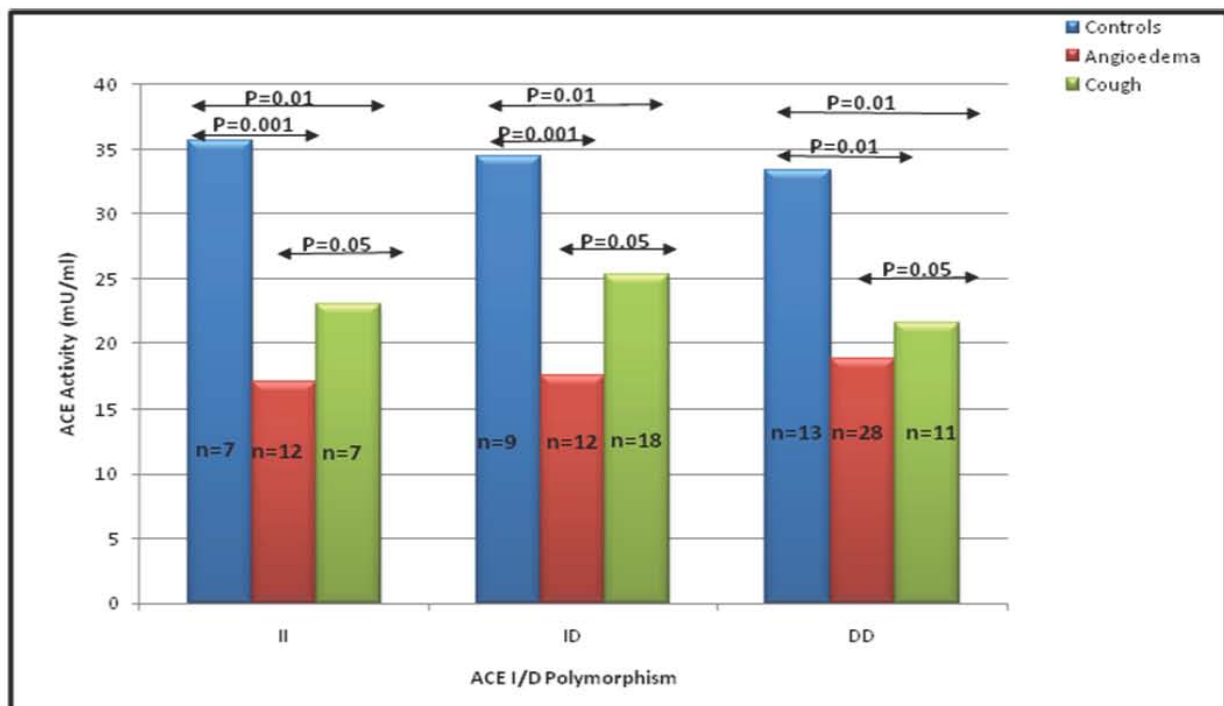


Figure 4.8: Bar chart of Correlation between serum ACE activity and ACE genotype in controls against ACEi-AE and ACEi-cough patients. Blue bars signify controls; red bars signify ACEi-AE patients and green bars signify ACEi-cough patients

4.3.5 The determination of Serum APP Activity

APP activity was checked for stability in serum by incubating serum samples at -80°C, room temperature and +4°C for two weeks. **Figure 4.9** shows that there was no significant reduction in APP activity at various temperatures. The APP activity was 410.9pmol/ml, 471.8pmol/min/ml and 474.4pmol/min/ml at +4°C, room temperature and -80°C respectively.

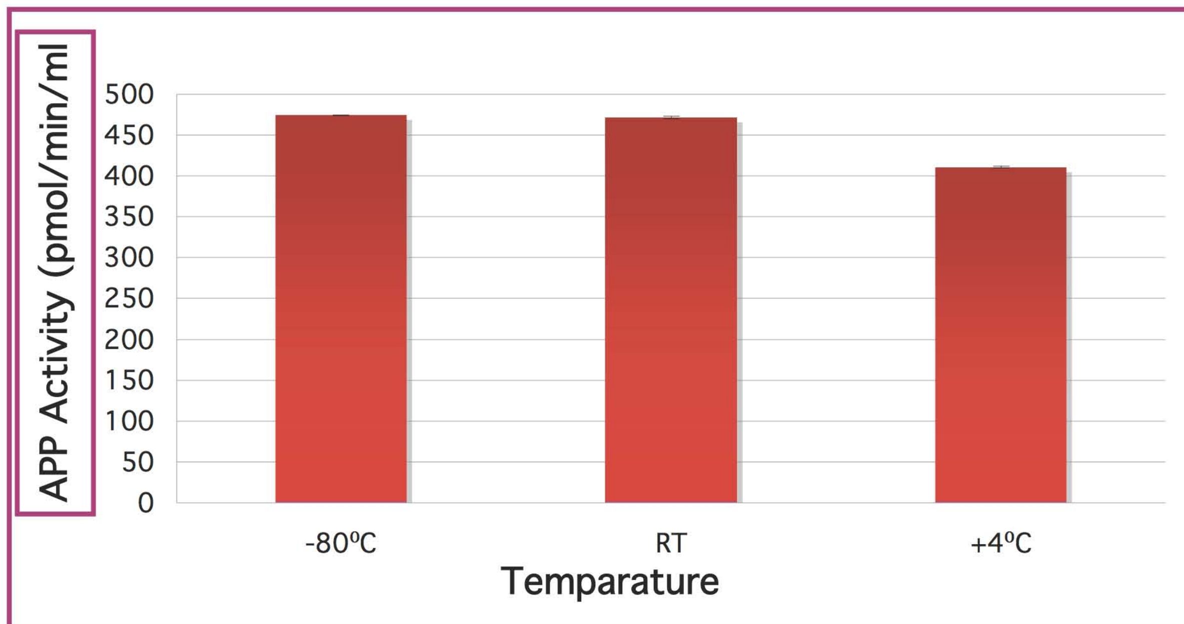


Figure 4.9: Determination of APP activity of serum samples stored at different conditions for period of two weeks. RT, room temperature.

APP activity was determined in serum samples from 117 patients comprising 29 treated controls, 36 ACEi cough patients and 52 angioedema patients. **Figure 4.10A** demonstrates an example of serum APP activity of angioedema patients. For treated controls, APP activity was measured when patients were on enalapril and off enalapril for 36 hours similar to what was done with the ACE activity determination. With regards to the APP activity, the data was not normally distributed with the Shapiro-Wilk's test because the data was skewed to the right instead of being symmetrical (p=0.0001) (**figure 4.10B**). Consequently, nonparametric tests were used for further statistical analysis.

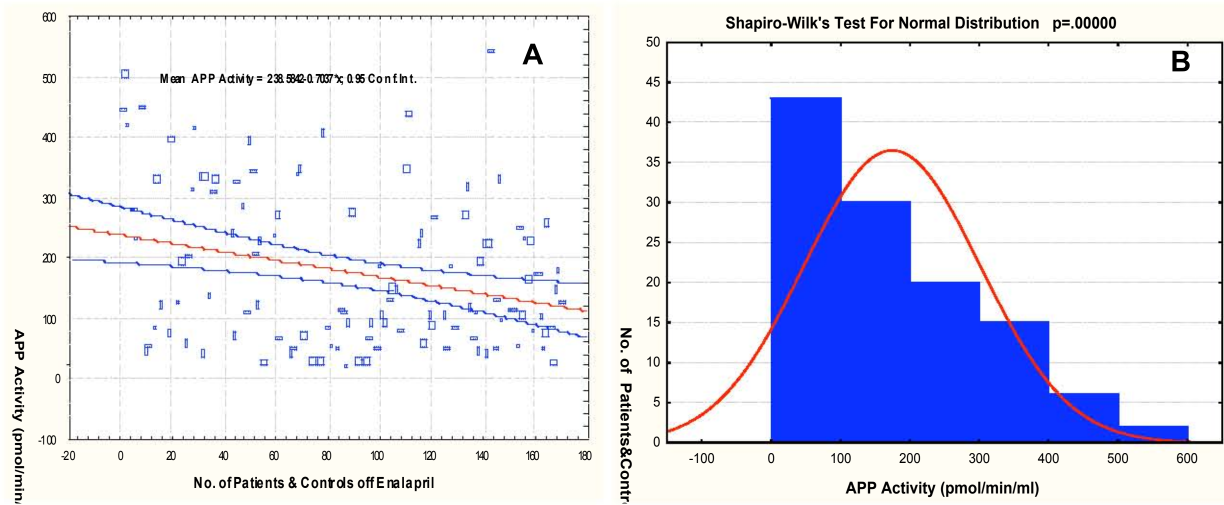


Figure 4.10: **A** Scatter plot of 117 patient's APP activity off enalapril. **B** Histogram of the Shapiro-Wilk's test for normal distribution of serum APP eznyme activity in all patients off enalapril treatment.

For controls, the APP activity was significantly higher when patients were off enalapril compared to when they were on enalapril (125.54pmol/min/ml vs (55.32pol/min/ml, P=0.000003) suggesting non-specific inhibition of APP by ACEi (**figure 4.11**).

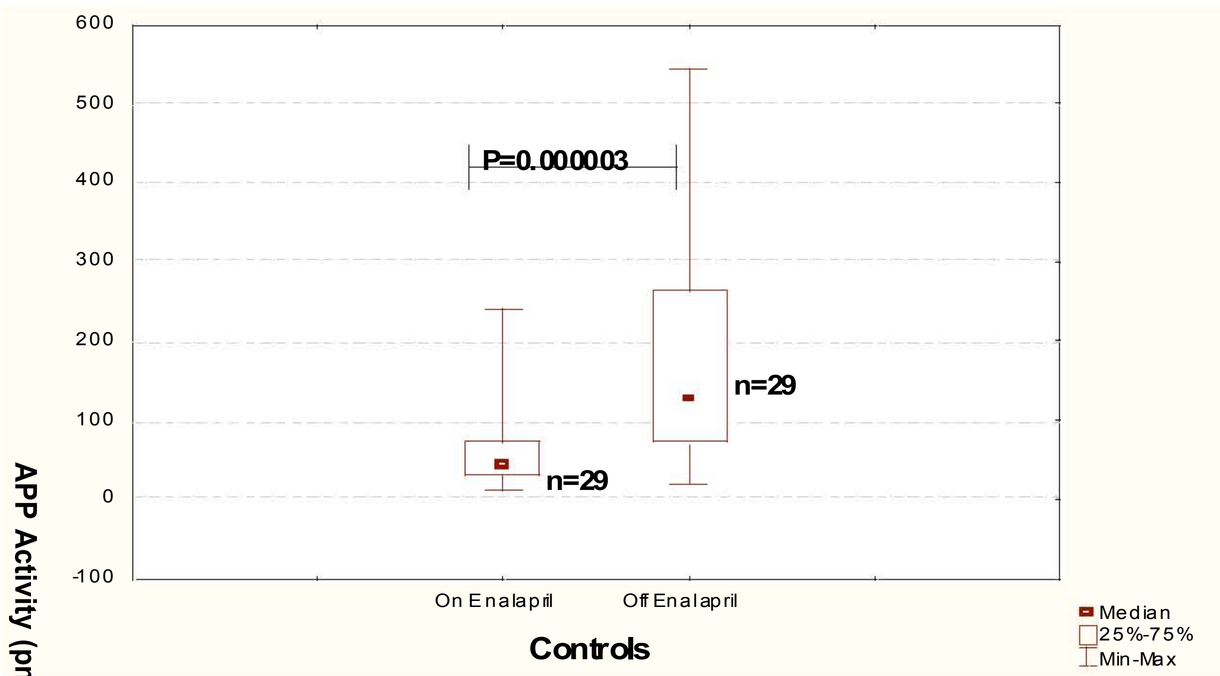


Figure4.11: Wilcoxon Non-parametric t-Test for dependent variables of treated control patients when on and off treatment with 10mg enalapril.

The APP activity was significantly lower in both ACEi-AE (320.46 pmol/min/ml vs 110.12 pmol/min/ml P=0.0007) and ACEi-cough (320.46 pmol/min/ml vs 100.25 pmol/min/ml P=0.0003) patients when compared to treated controls (**figure 4.12**). There was no significant difference in the APP activity of ACEi-AE against ACEi-cough (110.12 pmol/min/ml vs 100.25 pmol/min/ml, P=1.000).

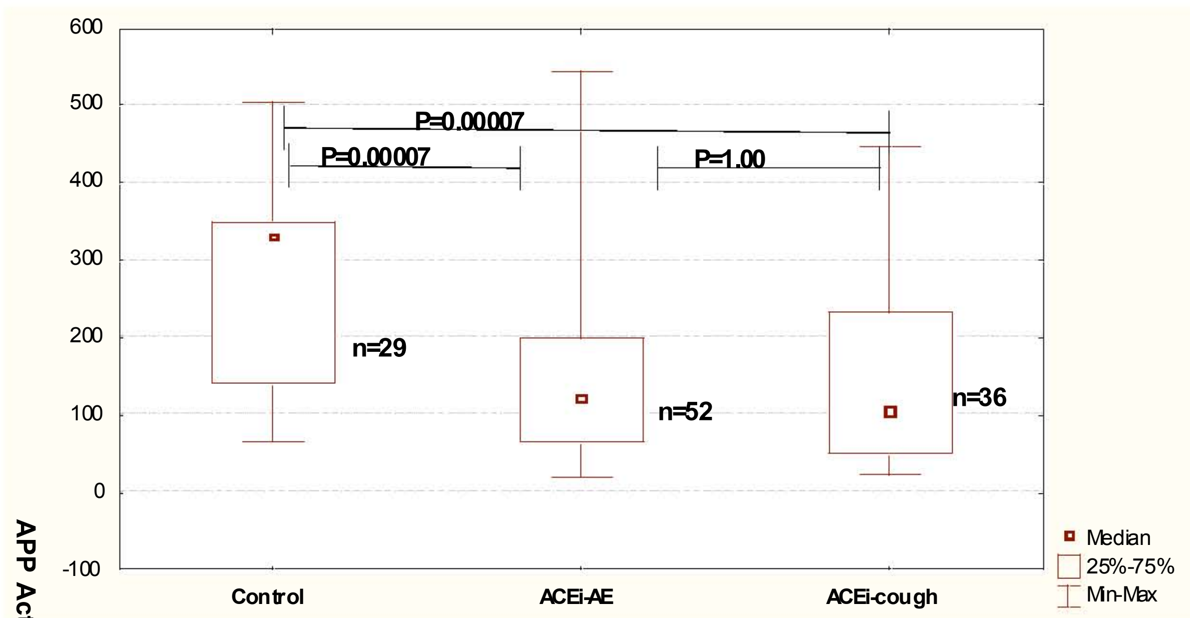


Figure 4.12: Box and Whisker plot of the comparison of APP activity between controls against ACEi-AE and ACEi-cough patients using Kruskal-Wallis nonparametric ANOVA test.

4.3.6 Correlation between APP Activity and APP Genotype in Patients

When APP activity was correlated with the APP genotype in all patients, there was no significant difference between activity 2399A and 2399C (266.05pmol/mon/ml vs 155.65pmol/min/ml, P=1.00) disputing reports in the literature that people with the A allele had a significantly lower APP activity (**figure 4.13**). There was no significant difference when 2399A was compared to 2399AC (266.05pmol/min/ml vs 100.23pmol/min/ml, P=0.232). The 2399C allele showed a significantly higher APP activity when compared to 2399AC (155.65pmol/min/ml vs 100.23pmol/min/ml, P=0.025)

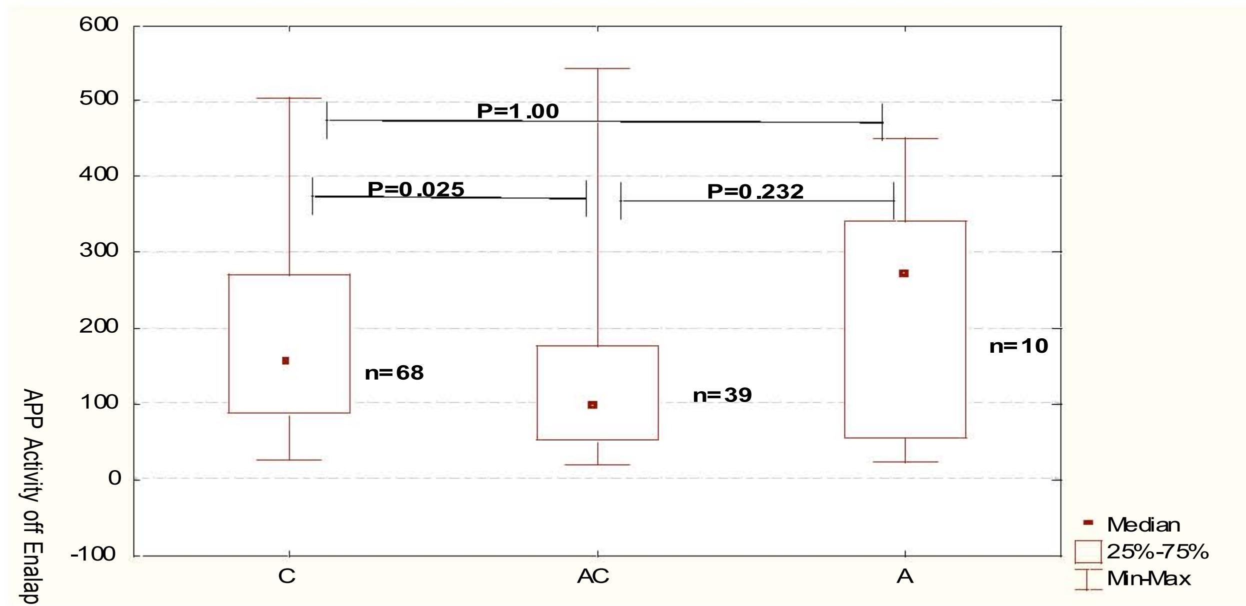


Figure 4.13: Box and Whisker plot of Correlation between APP activity and C2399A SNP of al cohorts using the Kruskal Wallis ANOVA Nonparametric test for three or more independent variables.

APP activity was correlated with APP genotype in controls, ACEi-AE and ACEi-cough patients. **Figure 4.14** presents comparison between C2339A SNP genotypes and APP activity of controls against ACEi-AE and ACEi-cough patients. The APP activity with regards to 2399A genotype was significantly higher in controls compared to ACEi-AE patients (344.16pmol/min/ml vs 57.25pmol/min/ml, P=0.0.1). The APP activity 2399A genotype was also significantly higher in cough against ACEi-AE patients (389.25pmol/min/ml vs 57.25pmol/min/ml, P=0.01). No significant difference in 2399A genotype APP activity was observed between controls and ACEi-cough patients (344.16 pmol/min/ml vs 389.25 pmol/min/ml, P=0.05). The 2399AC genotype showed significantly higher APP activity in controls compared to ACEi-AE (265.17pmol/min/ml vs 145.97pmol/min/ml, P=0.01) and ACEi-cough patients (265.17pmol/min/ml vs 109.63pmol/min/ml, P=0.01). No significant difference was observed between ACEi-AE and cough (145.97pmol/min/ml vs 109.63pmol/min/ml, P=0.05) 2399AC APP activity. The 2399C genotype APP activity was significantly higher in controls than in ACEi-AE

(258.89pmol/min/ml vs 144.57pmol/min/ml, P=0.01) and ACEi-cough (258.89pmol/min/ml vs 127.13 pmol/min/ml, P=0.01) patients. There was no significant difference in 2399C genotype APP activity between ACEi-AE and ACEi-cough patients (144.57pmol/min/ml v 127.13pmol/min/ml, P=0.05). Suprisingly the 2399A genotype APP activity was higher than 2399AC and 2399C genotypes in controls. The 2399A genotype APP activity was also significantly higher in cough patients than both 2399AC and 2399C. The 2399A genotype APP activity was significantly lower than both the 2399 AC and 2399 C in ACEi-AE patients.

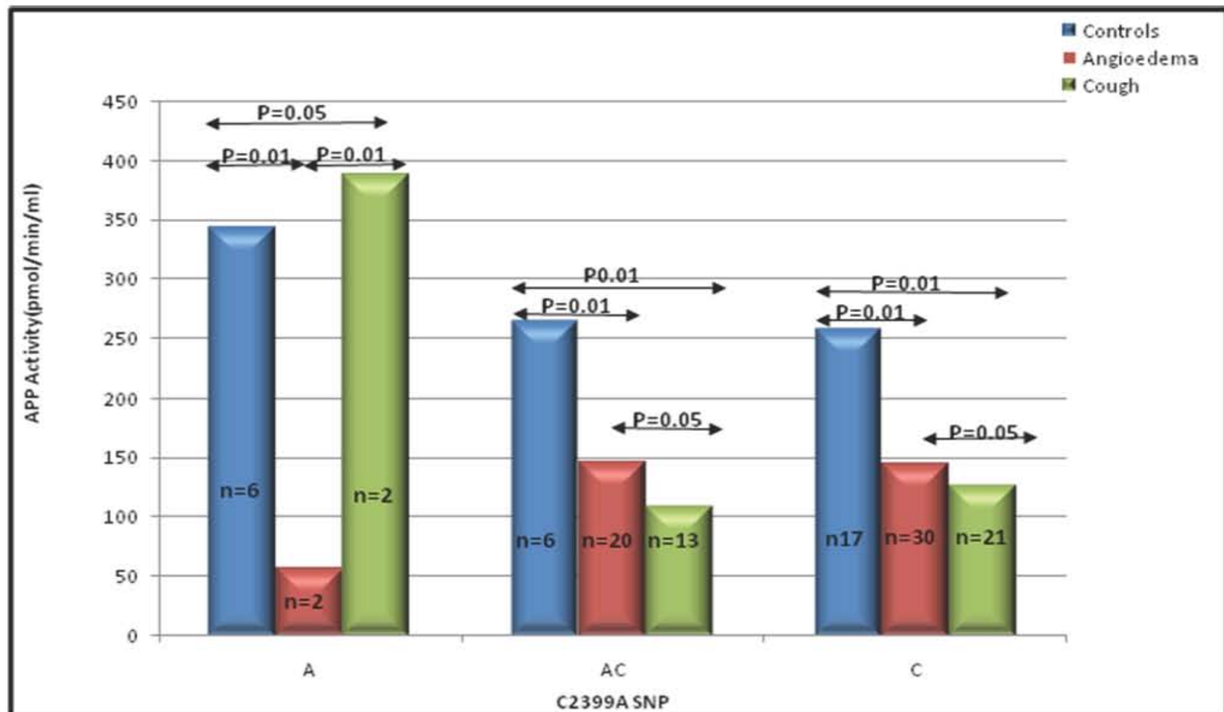


Figure 4.14: Bar Chart presenting correlation between APP activity and APP genotype in controls against ACEi-AE and ACEi-cough patients. Blue bars represent controls; red bars represent ACEi-AE patients and green bars represent ACEi-cough patients.

4.4 Discussion

About 40 000-280 000 patients that take ACE inhibitors are affected by a rare but potentially life threatening AE[157;162]. Blacks, coloureds , females, and people that smoke and suffer from seasonal allergies are at the greater risk of experiencing this adverse effect suggesting that genetic and environmental factors influence the development of ACEi-AE [103;125;127]. Earlier investigations postulate that defective degradation of the vasoactive peptide BK leads to ACEi-AE and ACEi-cough [134]. BK is primarily degraded by ACE and APP and during ACE inhibition APP assumes the primary role [12]. The relationship of correlation between ACE activity and ACE I/ polymorphism as well as the relationship between APP activity and the previously described functional polymorphism in the XPNPEP2 gene were assessed in ACEi-AE and ACEi-cough South African coloured and black patients.

A serum ACE activity assay was validated by checking the effect of endogenous inhibitors on ACE activity and by assessing the effect temperature has on ACE stability. The measurement of serum ACE activity has been shown to be affected by the presence of endogenous low molecular weight inhibitors [159]. A serial dilution was therefore performed to reduce this effect and it revealed that with increasing dilution ACE activity increases compared to undiluted sera when using both HHL and ZFHL as substrates (**figure 4.1A**). The increased ZFHL/HHL ratio in undiluted and a 1:2 dilution of serum, when compared to 1:4 and 1:8 dilutions, further confirmed the presence of inhibitor(s) (**figure 4.1B**). The activity of ACE was shown be stable at temperatures of -80 °C, 4 °C and 22 °C after two weeks (**figure 4.2**).

ACE inhibitors are routinely used as first line treatment for hypertension, congestive heart failure and diabetic nephropathy. However determination of these drugs in human blood remains tedious due to difficulty in extracting ACE inhibiting compounds, the technology that is required for their analysis, and their low concentration in serum [163-166]. Recently the use of the ratio of ACE activities using the substrates ZFHL and HHL has been employed to detect the presence of ACEi in human blood [159]. The fact that binding of ACEi dramatically increased the ZFHL/HHL ratio (**figure 4.3**) suggests that this simple and

inexpensive method is an effective way of detecting ACEi in sera. The sensitivity of the assay was as low as 0.3nM with the widely used ACEi lisinopril. Therefore this assay can be used to quantify the presence of ACEi in blood and the extent of inhibition. A big advantage of this approach is that the quantification of the degree of ACE inhibition via the ZFHL/HHL ratio does not need knowledge of the initial ACE activity of a given patient [159].

Serum ACE activity was investigated in controls when they were on and off ACEi enalapril. It was shown that ACE activity was significantly higher when patients were not taking enalapril compared to when they were taking enalapril indicating *in vivo* ACE inhibition (**figure 4.5A**). The lower ZFHL/HHL ratios further confirmed that these patients were compliant (**figure 4.5B**) thus emphasizing the clinical value of using the ZFHL/HHL ratio in determining ACE inhibition. Furthermore, ACE activity was assessed in ACEi-AE and ACEi-cough patients and found to be significantly lower activity in both cases than controls. Since ACEi-AE and ACEi-cough patients were no longer taking enalapril the reason for their lower ACE activity might be due to decreased induction of ACE synthesis in endothelial cells mediated by unknown transcriptional element/s. It has previously been shown that chronic ACE inhibition leads to increased serum ACE levels[167]. Importantly, the diuretic, hydrochlorothiazide has been shown to have ACE inhibitory capability[168] and thus might have an effect on ACE levels in ACEi-AE and ACEi-cough patients in this study. ACE activity was also considerably higher in ACEi-cough patients than ACEi-AE patients. The reason for this is unclear however it is possible that ACEi-cough patients have higher ACE expression than ACEi-AE patients leading to higher enzyme activity. Clearly, this requires further investigation.

Previous work has shown that levels of plasma and cellular ACE are strongly influenced by genetic factors [169-172]. Earlier investigations have showed that individuals with the *ACE-II* genotype have lower ACE activity than the *ACE-DD* genotype, with the *ACE-ID* individuals having intermediate ACE levels [171;172]. Correlation between ACE activity and the ACE *I/D* genotype has been investigated in other disease states [173;174]. In the present study correlation between ACE activity and the ACE *I/D* genotype was explored and overall there

was no significant difference in ACE activity between *II*, *ID* and *DD* genotypes (**figure 4.7**). This might, in part, be due to large inter-individual variability in ACE activity. Indeed other studies have proven that the ACE *I/D* is marker for ACE activity and accounts for 28-40% inter-individual variability in serum ACE activity or immunoreactive levels [170;171]. Moreover, the relationship between the activity of ACE in serum and the ACE genotypes was sought in both ACEi-AE and ACEi-cough patients. The correlation between the *ACE-II* homozygotes displayed significantly higher ACE activity in controls compared to ACEi-AE and ACEi-cough patients. Similarly, the *ACE-ID* genotype exhibited higher activity in controls versus ACEi-AE and ACEi-cough patients. The *ACE-DD* genotype also revealed higher ACE activity in controls compared to ACEi-AE and ACEi-cough patients.

The activity of APP was shown to be stable at varying temperatures. APP activity was significantly lower in controls when they were on enalapril compared to when patients were not taking enalapril. The activity of APP purified from pig renal cortex and mouse lung has been previously illustrated to be inhibited by enalapril at nanomolar concentrations [175;176]. The present work unambiguously proves that enalapril inhibits APP activity in human serum.

Previous work has demonstrated that APP activity is significantly lower in ACEi-AE patients in a case-control study [12]. A similar trend was observed in the present study whereby APP activity displayed significantly lower activity ACEi-AE patients compared to controls. The present study also further showed APP activity in ACEi-cough is significantly lower than that of controls. This unequivocally proves that both ACEi-AE and ACEi-AE are associated with decreased APP activity.

APP is a zinc metalloprotease attached to the membrane by a GPI anchor. It is encoded by the XPNPEP2 gene and the *C-2399A* SNP has been shown to be associated with APP altered activity [12]. The -2399A genotype is associated with decreased APP activity whereas both the *-2399AC* and the *-2399C* are associated with increased activity. The relationship between the *C-2399A* SNP was therefore examined in our local population. Overall there was no significant difference in *-2399A* and *-2399C* APP activities (**figure 4.13**). This could be

attributed to low sample size in patients that had *-2399A*. Furthermore, the XPNPEP2 gene accounts for 34% heritability of plasma APP activity [12]. This means other genetic loci might be controlling plasma APP activity in our population and needs further investigation. Nonetheless, the correlation between serum APP activity and the APP genotype was assessed in controls against ACEi-AE and ACEi-cough. The results revealed that *-2399A* genotype in both controls and ACEi-cough patients had significantly higher APP than ACEi-AE patients (**figure 4.14**).

Conclusions and Future Directions

Much research has been done on the clinical and molecular bases of the ACEi side effects. However the mechanism(s) that cause ACEi-AE and ACEi-cough are still not fully understood. The widely most accepted mechanism is that inhibition of ACE increases kinin levels resulting in the activation of a proinflammatory response and nitric oxide generation [137;177]. Nonetheless, relatively little is known about the genetic susceptibility of hypertensive patients to ACEi-induced AE and cough. Relatively few studies have been carried out to determine whether selected polymorphism in ACE, XPNPEP2 and BKR-2 predispose patients to ACEi-related side effects. Most of these studies have been carried out in Caucasian and Chinese/Korean/Japanese patients [153;178-181] .

The aim of this thesis was to assess the relationship between ACEi-AE and ACEi-cough and known genetic polymorphisms in the ACE, XPNPEP2 and the BKR-2 genes in black and coloured South Africans in the Western Cape. Furthermore, ACE and APP activities were measured in serum and correlated with genotypes in ACEi-AE and ACEi-cough patients.

Much work has been done on the ACE *I/D* polymorphism and its association with many diseases and physiological conditions. The *ACE-DD* genotype has been associated with asthma in one study [182]. However, in another study the *ACE-DD* genotype was demonstrated to have no meaningful effect on asthma[183]. The *ACE-II* genotype has been associated with greater risk of developing ACEi-cough [184]. In this study however, there was no significant association between the ACE *I/D* polymorphism and ACEi-AE or ACEi-cough. The ACE gene *I/D* polymorphism has been shown to affect the levels of serum ACE [171]. In this study, ACE activity in both ACEi-AE and ACEi-cough patients was lower than that of controls. When ACE activity was correlated with genotype, it was higher in controls compared to ACEi-AE and ACEi-cough patients with all genotypes. These data suggests that low ACE activity predisposes patients to ACEi-AE and ACEi-cough. However, the association of the ACE *I/D* polymorphism with ACEi-AE and ACEi-cough remains' unclear and needs further investigation with a larger sample size to make more meaningful conclusions.

In order to elucidate the further the molecular basis of ACEi related side effects the role of the *C-2399* SNP in the XPNPEP2 gene was investigated in this thesis. The *C-2399A* SNP has

been shown to be associated with serum APP activity and ACEi-AE [12;181]. The functional *-2399A* variant is associated with significantly lower APP activity compared to *-2399AC* and *-2399C* alleles. This makes patients with the *-2399A* allele more susceptible to developing ACEi associated AE and cough. In contrast, this study showed no clear association between the *C-2399A* genotype and ACEi-AE or ACEi-cough.

APP plays a pivotal role in degrading BK and des-Arg^{9}BK during ACE inhibition. Therefore decreased APP activity leads to high BK and des-Arg^{9}BK in the bloodstream leading to inflammation. Indeed studies showed that ACEi-AE is associated with slower degradation of both BK and des-Arg^{9}BK due decreased APP activity [138]. Additionally, earlier studies suggest that black Americans exhibit increased sensitivity to the effects of bradykinin-induced vascular permeability compared to their white counterparts [185]. In the present study APP activity was decreased in both ACEi-AE and ACEi-cough patients compared to controls. When APP activity was correlated with the *C-2399A* genotype, APP activity was lower in ACEi-AE patients, but not in ACEi-cough patients. However, it is difficult to conclude unambiguously that *C-2399A* is/not associated with both ACEi-AE and ACEi-cough. Furthermore APP activity has been shown to be decreased in men compared to women [157]. It can thus be postulated that sex hormones contribute to sex differences in enzyme activity. Indeed, it has been reported that progesterone increases APP activity [186]. The effect of estrogen on APP expression or activity has not being reported and thus needs further investigation. Androgen increases APP activity in patients with HAE-I giving rise to the notion that increased testosterone concentrations in men is unlikely to account for sex differences [187]. Importantly, it has been reported that XPNPEP2 escapes X-inactivation[188].

Defects in multiple pathways may contribute to the etiology of both ACEi-AE and ACEi-cough. BK increases vascular permeability and stimulates cough reflex in the lung by stimulating the BKR-2 receptor. Interestingly, it has been suggested that the therapeutic effect of ACEi may involve activation of the BKR-2 [31;31]. Indeed, it was demonstrated that BKR-2 antagonists prevents both ACEi-AE and ACEi-cough in animal models[189].

Conclusions and Future Directions

The BKR-2 *+9/-9* polymorphism has been shown to be clinically relevant with the *-9* variant showing increased vasodilation during ACE inhibition. Furthermore the *-9* allele is associated with symptomatic HAE in cases of CINH deficiency[152]. To my knowledge, this first study to associate both ACEi-AE and ACEi-cough with the *+9/-9* BKR-2 polymorphism. The results showed a significant association with *-9* variant with ACEi-AE but not ACEi-cough. Even though this data is interesting, further exploration is needed.

Some reports have analyzed the role of genetic variant *C-58T* in the BKR-2 in ACEi-AE and ACEi-cough and showed no association [180;190]. In contrast, two reports showed an association with *C-58T* with ACEi-cough [153;191]. In this study the analysis revealed no association between ACEi-AE and ACEi-cough and *C-58T* genotype.

BK stimulates the release of substance P from nerve ends and substances P increases vascular permeability through stimulation of the neurokinin type 1 receptor (NKR-1). In animal models blocking NKR-1 prevents ACEi-AE. Substance P has also been postulated to be increased in ACEi-AE and ACEi-cough events [192;193]. Thus variation in enzymes responsible for inactivation of both BK and substance P or BK and substance P receptors may contribute to both ACEi-AE and ACEi-cough. Interestingly, NKR-1 *Gly231Glu* polymorphism has been associated with ACEi-cough in Korean patients [143;194]. BK is degraded by ACE, APP and NEP whilst substance P is broken down by ACE, dipeptidyl peptidase IV (DPPIV) and NEP. It has been reported previously that DPPIV is decreased in some patients with ACEi-AE[193;195]. However no functional polymorphism has been found in the DPPIV gene and associated with ACEi-AE and ACEi-cough. Therefore next logical step would be to investigate functional polymorphism/s in the DPPIV gene and their association with DPPIV activity and therefore ACEi-AE and ACEi-cough.

Dual inhibition of ACE and NEP leads to three times higher risk of developing ACEi-AE compared with ACEi treatment alone. Interestingly, the *rs2016848* variant in the NEP has been associated with ACEi-cough in one study [177]. Therefore this needs further exploration in our local population and the association of this variant with serum NEP activity needs to be investigated.

BK also causes the secondary activation of prostaglandins especially PGE2 and PGI2 and the former acts on EP 1, 2, 3 and 4 receptors to exert its biological effects. EP receptors are encoded by PTGER1-PTGER4 genes and a recent report indicates that an *rs11209716* variant in the PTGER3 is associated with ACEi-cough. The relationship between prostaglandins and PGI2 with ACEi-AE and ACEi-cough has been less explored. Nonetheless, PGE2 has been shown to mediate cough via the EP3 receptor in asthmatic patients [196;197]. Moreover, PGE2 analog, beraprost is a novel therapeutic option for cough [197].

In summary this thesis explored the association between known genetic polymorphisms in the ACE, XPNPEP2 and BKR-2 genes with the pathogenesis of ACEi-AE and ACEi-cough in black and coloured South Africans in the Western Cape. Further studies are needed to confirm/disprove the findings of the current study. Furthermore, it is difficult rule out the possibility of false-negative/positive results due to limitations in sample size and the number of tests performed in this study. The difficulty in elucidating the genetic basis of complex diseases is entrenched in a lot of factors that have an effect in the development of a disease. Some of the genetic effects may interact in multifaceted ways that are not detected by single locus methodology used in the study. Therefore in future, analysis of gene-gene interactions in black and coloured South Africans are crucial for a better understanding of the contribution of genetic factors in ACEi-AE and ACEi-cough

Angioedema Project Patient Request form

SPECIMEN NUMBER: ☐☐☐☐☐☐☐☐☐☐☐

PATIENT DETAILS
Patient Surname:_______________________ First Name (s):________________________________

Date of Birth: ☐☐ ☐☐ ☐☐☐☐ (dd/mm/yyyy)

Sex: M ☐ F ☐

Hospital No: ☐☐☐☐☐☐☐☐☐☐☐

RACE: Black ☐ Coloured ☐ Caucasian ☐

SPECIMEN DETAILS

Date of collection: ☐☐ ☐☐ ☐☐☐☐ (dd/mm/yyyy)

Time: ☐

Heparin Blood: ☐ EDTA Blood: ☐ SST: ☐

Clinical History

Y N Angioedema: Date Of Episode: ______________

Y N Diabetes:

Drug History

ACE INHIBITORS: Enalapril: ☐ Dose ☐ Other: _____________ Dose: ______

Time on ACEi: ______________ Cough: _______________

ANTIHYPERTENSIVES: Diuretics: ☐ β Blockers: ☐ CCBs: ☐

α Blockers: ☐ Spironolactone: ☐ Hydralazine: ☐ Other: _____________

Aspirin/Dispirin: ☐ Dose: ________________

NSAIDs: ☐ Name: _____________ Dose: _____________________

Other: ☐ Specify: __________________________________

DESCRIPTION OF ANGIOEDEMA EPISODE:

Patient Consent ☐

UNIVERSITY OF CAPE TOWN

Health Sciences Faculty
Research Ethics Committee
Room E52-24 Groote Schuur Hospital
Old Main Building
Observatory
7925
Telephone [021] 406 6338 . **Facsimile** [021] 406 6411
e-mail:
sumayah.arietfdien@uct.ac.za

27 March 2009

REC REF: 031/2008

Prof BL Rayner Department of Medicine

Dear Prof Rayner

PROJECT TITLE: ANALYSIS OF A VARIANT IN XPNPEP2 IN BLACK AFRICAN AND COLOURED SOUTH AFRICANS PRESENTING WITH ANGIOEDEMA INDUCED BY ANGIOTENSIN I-CONVERTING ENZYME INHIBITORS.

Thank you for your letter to the Ethics Committee dated 26th March 2009

It is a pleasure to inform you that the Ethics Committee has granted **annual approval** for the study to continue for another year.

Approval is granted till 31st March 2010.

Approval is granted to approach the Hospital Pharmacy to obtain information about patients who are taking ARB:s. Given the potentially sensitive issue of undertaking research among patients in other specialist clinics, we recommend that you approach the physicians / heads of the particular clinics where you are most likely to source your participants and explain the nature of your research to them. Indeed, they may be able to assist you with recriIitment.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principaJ investigator.

Please quote the REC. REF in all your correspondence.

Yours Sincerely

PROFESSOR M BLOCKMAN

CHAIRPERSON. HSF HUMAN ETHICS

PATIENT INFORMATION AND INFORMED CONSENT:

Introduction: We are conducting research into the causes of angioedema, an important complication of ACE inhibitors, a commonly used blood pressure pill. We need to take 5 blood samples to help us analyse differences and activity of a certain gene that may cause angioedema. We are specifically analysing the XPNPEP2 gene, and the activity of certain enzymes (serum ACE and aminopeptidase activity) and inflammatory mediators (bradykinin) related to this gene while you are taking the ACE inhibitor and after stopping the medication. Your doctor will only stop the ACE inhibitor if it is safe for you to do this.

1. I, ____ , hereby consent that my blood cells be analysed for genetic changes in the XPNPEP2 gene.

2. I request that no portion of the sample be stored for later use. ☐ (MARK IF APPLICABLE)

Or

I request that a portion of the sample be stored indefinitely for (DELETE WHERE NOT APPLICABLE)

a) possible re-analysis
b) analysis for benefits of members of my immediate family
c) research purposes, subject to the approval of the University of Cape Town Research Ethics Committee, provided that any information from such research remain confidential.

3. The results of the analysis will be made known to me or my doctor if and when available. In addition I authorise that they be made known to the following: (DELETE WHERE NOT APPLICABLE)

a) other doctors involved in my care
b) the following family members ____________________________________
c) other ____

4. I have been informed that:

a) There are risks and benefits of genetic analysis of stored material
b) The analysis procedure is specific to this genetic condition mentioned above, and can not determine the complete genetic make up of an individual
c) The laboratory is under an obligation to maintain confidentiality
d) Genetic analysis may not be informative for myself or other family members
e) There may be no direct benefit to me
f) Even in the best of conditions, current technology is not perfect and there is a possibility of incorrect results

5. I understand I can withdraw my consent for any aspect of the above at any time without affecting my future medical care.

6. **ALL OF THE ABOVE HAS BEEN EXPLAINED TO ME IN A LANGUAGE THAT I CAN UNDERSTAND AND MY QUESTIONS HAVE BEEN ANSWERED**

Patient's Name: ________________________________(print)

Patient's Signature:______________________________Date:________

Buffers and solutions

Potassium Phosphate Buffer, pH 8.3 containing 3M NaCl.

A 5X stock was prepared: 0.5M KH_2PO_4; 0.5 MK_2HPO_4, pH 8.3 and 3M NaCl (M_w=58.5g).

3.42g KH_2PO_4 (M_w= 136.99); 4.35g K_2HPO_4 (M_w=174.18) and 8.766g NaCl (M_w=58.5) in 50ml of distilled water. Adjusted pH to 8.3.

Working solution:1X buffer.

Phosphate Buffered Saline

A 1X stock was prepared: 0.2M $Na_2HPO_4.2H_2O$; 0.8M KH_2PO_4; 0.3M KCl and $7x10^{-3}$M NaCl.

1.15g $Na_2HPO_4.2H_2O$ (M_w=177.99); 0.23g KH_2PO_4 (M_w=174.18); 0.23g KCl (M_w= 74.56); 8g NaCl (M_w=58.5) in 1000ml distilled water

0.28M NaOH

Weight 11.2g NaOH (M_w=40.00) pellets and made-up to 1000ml with distilled.

0.025M NaOH

Take 4.46ml 0.28M NaOH and made-up to 50ml with distilled water.

3N HCl

32% HCl=10N

15ml 32% HCl made up to 50ml with distilled water.

***o*-pthaldialdehyde (150 mM)**

20 mg *o*-pthaldialdehyde (M_w= 134.1)(Sigma) in 1.0 ml methanol.

10 % AMPS

Weight 0.1 g AMPS and made up to 1ml with distilled water

His-Leu standard curve

His-Leu (HL) standards

Stock solution of His-Leu (M_w=268.3) (Sigma):

13.41 mg dissolved in 10 ml sterile distilled water

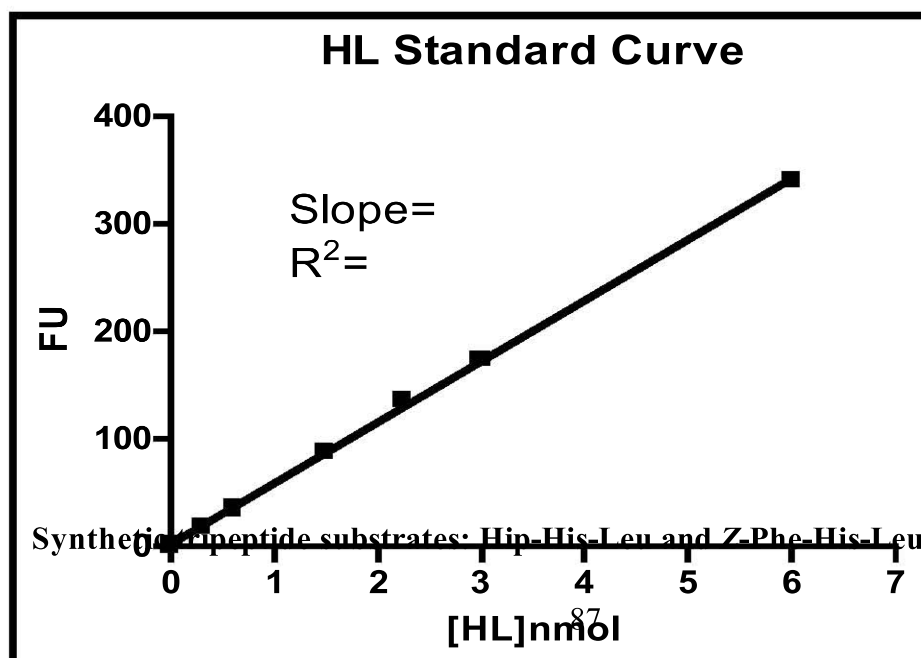
HL standards set out in **Table 10** were made in $1xK_2HPO_4$ buffer.

Fluorescence was measured at λ_{Ex}=360 and λ_{Em}=486 nm on a Cary Eclipse fluorometric plate reader (Varian).

A standard curve of fluorescent units vs nmoles HL was plotted using GraphPad Prism 4.01. HL. Slope was determined via linear regression analysis.

Table 10: HL Standards

	HL Concentration (nmol per 30µl)	HL in $1xK_2HPO_4$ buffer (ml)	$1xK_2HPO_4$(ml)
1	0	0	5.00
2	0.30	0.1	4.90
3	0.60	0.2	4.75
4	1.50	0.5	4.50
5	2.25	0.75	4.25
6	3.00	1.0	4.00
7	6.00	2.0	3.00



Synthetic tripeptide substrates: Hip-His-Leu and Z-Phe-His-Leu

5.7 mM Hippuryl-His-Leu

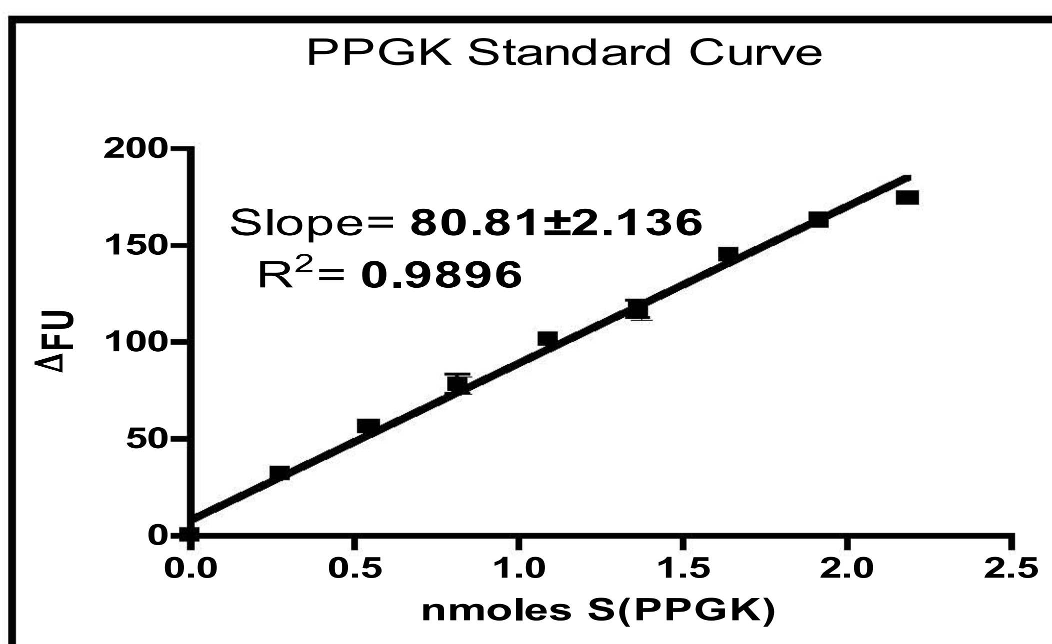
48.5 mg N-Hippuryl-His-Leu tetrahydrate (M_w= 501.5) (Sigma) dissolved in 4.165 ml heated 0.025 M NaOH. Then added: 4.0 ml 5 x Potassium Phosphate buffer, pH 8.3 (without NaCl) 2.0 ml 3M NaCl 9.835 ml sterile distilled water.

20 mM *Z*-Phe-His-Leu stock:

110 mg *Z*-Phe-His-Leu-OH (Bachem) (M_w= 549.63) dissolved in 1.0 ml 0.28 N NaOH. Then added: 9.0 ml sterile distilled water in a drop-wise fashion to prevent precipitation aliquoted into 1.0 ml Eppendorf tubes and stored aliquots at-20°C until use.

Standard Curve for K(Dnp)PPGK(Abz) Substrate

The change in fluorescence from the baseline levels (before addition of APP) to total hydrolysis of a range of substrate concentrations (0.-2.5moles) was measured at the excitation and emission wavelength of 320 and 420 nm respectively. A standard curve was generated by plotting this change in fluorescence versus nmoles of substrate hydrolysed. The slope was determining using a linear regression curve plotted with GraphPad Prism 4.01 software.



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