The Genetic Basis of Human Athletic Performance

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Thesis presented in partial fulfilment of the requirements for the degree of Master of Exercise Science at the University of Cape Town.

Supervisor: Prof. L.J. Greenberg
Date: 13/08/2003

DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part, submitted it at any University for a degree.

Signature:.... Signed by candidate

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ABSTRACT

Research has suggested an association between the angiotensin-I converting enzyme (ACE) insertion/deletion (I/D) polymorphism and endurance performance, skeletal and cardiac muscle hypertrophy and performance in power associated sporting events. The nitric oxide synthase (ecNOS) G894T polymorphism is associated with endurance training induced decreased submaximal diastolic blood pressure and nitric oxide is a direct modulator of ACE activity.

The present study investigated the possible association between the ACE I/D and ecNOS G894T polymorphism and elite South African (SA) rugby players and controls as well as elite SA roadrunners and controls. During the course of this study various DNA extraction techniques was tested and the use of the polymerase chain reaction was introduced into the laboratory.

The present study indicated an association between ethnic origin and running status, personal best running times and the ACE I/D genotype distribution in a population of SA roadrunners. An association between ethnic origin and running status, personal best running times and the ecNOS G894T genotype distribution was also shown. Neither the ACE I/D nor the ecNOS G894T genotype was associated with elite status in SA rugby players.

Although these findings may suggest that the ACE I/D and ecNOS G894T polymorphisms may be useful markers for elite athletic performance, the effect of gene-environment interaction and differences in the ethnic distribution of the polymorphisms may have had an influence on the outcome of the present study.

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I would like to express my sincerest gratitude to:

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THESIS CONTENT

LIST OF ABBREVIATIONS

° C	Degree Celcius
$\cdot O_2^-$	Superoxide anion
A	Adenine or adenosine
A260	Absorbance at 260 nm
ABP	Arterial blood pressure
ACE	Angiotensin-I converting enzyme
ACTN2	α-actinin-2
ACTN3	α-actinin-3
ADH	Antidiuretic hormone
ADP	Adenosine diphosphate
AK1	Adenylate kinase
ANG I	Angiotensin I
ANOVA	Analysis of variance
ApoB	Apolipoprotein B
ATP	Adenosine triphosphate
a-v O_2 diff.	Artrio-ventricular oxygen difference
bis	N,N' methylene bis acrylamide
BK	Bradykinin
BMI	Body-mass index
bp	Base pair(s)
BP	Blood pressure
C	Cytosine or cytidine
Ca^{2+}	Calcium ions
cDNA	Complementary DNA
$CHCl_3$	Chloroform
CKM	Muscle-specific creatine kinase
Cl^-	Chloride ion
ConR	Control roadrunner
CR	Control rugby player
dATP	Deoxyadenosine triphosphate
DBP	Diastolic blood pressure
DBP50	Diastolic blood pressure measured during submaximal exercise at 50W
DCP 1	Dipeptidyl carboxypeptidase I
dCTP	Deoxycytoosine triphosphate
dGTP	Deoxyguanosine triphosphate
dH_2O	Distilled water

DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
dTTP	Deoxythymidine triphosphate
ecNOS	Endothelial nitric oxide synthase
EDHF	Endothelium derived hyperpolarizing factor
EDTA	Ethylenediamine tetraacetic acid
EIR	Elite roadrunner
ER	Elite rugby player
ET-1	Endothelin-1
EtBr	Ethidium bromide
EtOH	Ethanol
FGF	Fibroblast growth factor
g	Gram
G	Guanine or guanosine
× G	Gravitational constant
HCl	Hydrochloric acid
HLA	Human leukocyte antigen
HMW	High molecular weight
HR	Heart rate
HSD	Honestly significant difference (Tukey HSD)
IEF	Isoelectricfocusing
IGF	Insulin-like growth factor
JGA	Juxtaglomerular apparatus
kb	Kilobase(s); kilobase pairs
L	Liter
LHRH	Luteinizing hormone-releasing hormone
M	Molar
m	Meter
mA	Milli-ampere
MAP kinase	Mitogen activated protein kinase
$MgCl_2$	Magnesium chloride
MI	Myocardial infarction
ml	Milli-litre
mm	Milli-metre
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
mtND2	Subunit 2 NADH dehydrogenase
mtND5	Subunit 5 NADH dehydrogenase
mtTT	Threonine tRNA
MW	Molecular weight

N	Number of samples
NaCl	Sodium chloride
$NaClO_4$	Sodium perchlorate
NADH	Nicotinamide adenine dinucleotide
nm	Nano meter
NO	Nitric oxide
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PKC	Protein kinase C
Q	Cardiac output
QTL	Quantitative-trait loci
RAU	Randse Afrikaanse Universiteit
RBC	Red blood cell
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RPP	Rate pressure product
RPP50	Rate pressure product measured during sub maximal exercise at 50W
SA	South Africa
SBP	Systolic blood pressure
SBP50	Systolic blood pressure measured during sub maximal exercise at 50W
sd	Standard deviation
SDS	Sodium dodecyl sulphate, sodium lauryl sulphate
SINE	Short interspersed elements
ssDNA	Single-stranded DNA
SSISA	Sport Science Institute of South Africa
T	Thymine or thymidine
TE	Tris EDTA (buffer)
TKM1	Low salt buffer (MODIFIED LAHIRI)
TKM2	High salt buffer (MODIFIED LAHIRI)
T_m	Melting temperature
UV	Ultraviolet
V	Volt
V/cm	Volt per centimeter
v/v	Volume:volume ratio
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VO_{2max}	Maximal oxygen uptake
X^2	Chi-Square statistical test

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CHAPTER 1

INTRODUCTION

1.1 The Hereditary Nature of Performance.

The frequently raised question concerning the contribution of an athlete's genotype (genetic constitution) to his ultimate exercise performance capacity is one of the more complex research questions in exercise science.

People recognize Kenyan athletes as the strongest distance runners the world over. Most of the top runners come from four of Kenya's 40 tribes, and the majority from the Kalenjin, which constitutes less than 10 % of the Kenyan population (Noakes TD 1992). Although it is clear that a connection does exist, definite genetic linkage between these runners' endurance ability and their genetic heritage is still to be established. A recent study by Nurok *et al.* (2001) supports the hypothesis of superior distance running ability among the Kalenjin.

The same holds true for West African slaves taken to North and Central America in the 1700's. The descendants of these people are today the world's fastest sprinters. We know that slave traders selected only the strongest, healthiest people who could work the hardest and that the weaker among them died on the slave ships while crossing the Atlantic. In so doing, they unknowingly selected a phenotype (appearance of an individual) for superior sprinting.

Although it is well documented that there are individual differences in the trainability of maximal oxygen uptake (VO_{2max}), the cardiovascular system, energy substrate utilization and other physiological systems, the genetic factors that underlie them are poorly understood (Rivera *et al.* 1997, Bouchard C 1992). Sensitivity and responsiveness to training seems to be at least in part genetically determined (Rivera *et al.* 1997, Bouchard C 1992, McArdle *et al.* 1996).

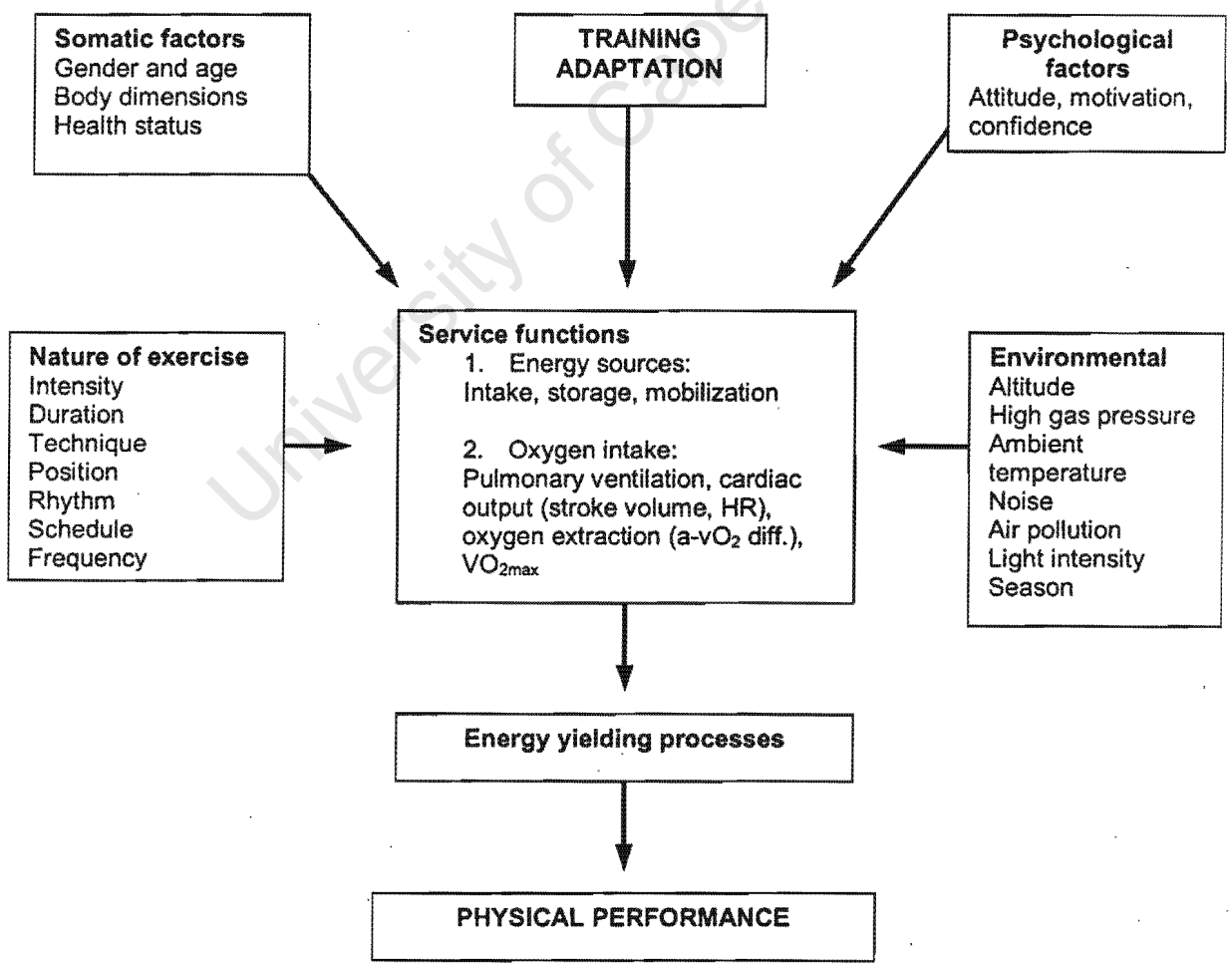
Individuals on the same training program show a highly variable response to training, which is largely mediated by genetic variation and environmental interaction (Bray MS 2000). Studies have shown that there are non-, low- and super-responder genotypes for ΔVO_{2max} to regular standardized training, and that these differences in individual sensitivity are inherited (Bouchard C 1992, Dionne *et al.* 1991, Gagnon *et al.* 1997, Bouchard *et al.* 1989). Results from the HERITAGE (HEalth, RIsk factors, exercise Training And GEnetics) Family Study

have confirmed this with certain families showing faster and greater response to training than others with a maximum heritability of VO_{2max} being around 47-51% (Bouchard *et al.* 2000). In addition, it was shown that the heritability of VO_{2max} in the untrained state has a significant genetic component (Dionne *et al.* 1991). The HERITAGE Family Study is a multicentre project involving five universities in the United States and Canada. Its aim is to elucidate the effect of genotype on the response of the endocrine, metabolic and cardiovascular system to aerobic exercise (Bouchard *et al.* 1995).

Since an athlete's maximum aerobic power is not the only determinant of performance as seen in Figure 1.1, we can assume that genetic factors will also influence the various other performance-associated traits shown in Table 1.1 (p5).

Figure 1.1: Factors affecting aerobic muscle performance.

Adapted from: Åstrand PO, Rodahl K. Textbook of Work Physiology. McGraw-Hill; New York:1986.



Historical Perspective.

During the past two decades, exercise scientists started using the new powerful molecular techniques, including Southern and Northern blotting and the polymerase chain reaction (PCR), to probe the human genome for answers related to the genetic basis of athletic performance. As performance prediction is very important, it seems a logical step to use these techniques to study the human genome for genetic predictors of performance. However, to this date, true markers of athletic potential have not been identified.

Initial studies focused on protein charge variation in red blood cell (RBC) proteins, human leukocyte antigen (HLA) and skeletal muscle enzymes associated with aerobic performance (Chagnon *et al.* 1986, Marcotte *et al.* 1987, Bouchard *et al.* 1988). RBC protein variants were not associated with endurance performance. Furthermore, none of the Krebs-cycle enzymes showed any charge variants. Protein variants identified for muscle-specific creatine kinase (CKM) and adenylate kinase (myokinase, AK1M) were not associated with high-untrained VO_{2max} values or endurance performance (Rivera *et al.* 1999). Adenylate kinase catalyses the transfer of phosphate and may be important for maintaining optimal cellular ATP and ADP levels during exercise.

Early Exercise Genetics.

The first studies on genes and their effect on exercise performance had mixed success. Researchers considered two types of genetic variation or polymorphism. The first is where variations are found in the coding (exon) regions of DNA. A variation in the exon may translate into a change in the amino acid composition of the protein. Such a change may affect the biological activity and/or the interactions of the particular protein with other molecules and cell components. The second is where DNA variations are found in the non-coding DNA (intron or untranslated flanking regions, gene promoters etc.) of a gene. These DNA variations do not affect the amino acid composition of the protein directly, but may increase or decrease the expression of the gene.

Genetic Polymorphisms Affecting VO_{2max}

Rivera and co-workers (1997) indicated a significantly lower ΔVO_{2max} in response to 20 weeks of endurance training in homozygotes for the *Nco I* polymorphism in the 3' untranslated region of the muscle-specific creatine kinase (CKM) gene. However, the *Taq I* and *Nco I* restriction-fragment-length polymorphisms or RFLPs did not contribute to the classification of elite endurance athletes. Rivera *et al.* (1999) provided further support for the notion that the *Nco I* CKM polymorphism or some gene in close linkage disequilibrium with it,

may contribute to individual differences in the response of VO_{2max} to endurance exercise. Sib-pair linkage analysis on 277 sib-pairs showed significant linkage between the CKM locus and ΔVO_{2max} in response to a standardized training protocol in the HERITAGE Family Study. In a study of selected genes of the respiratory chain encoded for by mitochondrial DNA (mtDNA), it was reported that carriers of three mutations of subunit 5 of the NADH dehydrogenase gene (mtND5) and one in the threonine tRNA (mtTT) had a relative VO_{2max} significantly higher in the untrained state than in non-carriers (Rivera *et al.* 1998). In contrast, carriers of a particular base mutation in subunit 2 of NADH dehydrogenase (mtND2) had a lower relative VO_{2max} (Rivera *et al.* 1998). Only 3% of the mitochondrial genome was screened and further research is clearly warranted.

Researchers examined human chromosome 22 using seven microsattelite markers spanning the entire chromosome (Gagnon *et al.* 1997). Chromosome 22 is the second smallest human chromosomes and was the first chromosome to be sequenced (Dunham *et al.* 1999). While standardized training increased base VO_{2max} significantly, no linkage was found between any of the markers and VO_{2max} or its response to training. Data from the same cohort suggest that genetic variation at the Na^+-K^+-ATPase α2 locus influences the trainability of VO_{2max} and maximal power output (W_{max}) determined on a cycle ergometer in previously sedentary individuals (Rankinen *et al.* 2000a). Homozygotes for the variant allele in the α2 exon 1 had a decreased VO_{2max} response, while the variant allele of the α2 exon 21-22 had increased VO_{2max} responsiveness to regular endurance exercise. The authors state that these markers explain only 1.5-2.4% of the variation in ΔVO_{2max} and that this is in line with the polygenic nature of the cardiorespiratory fitness phenotype (Rankinen *et al.* 2000a).

Genetic Polymorphisms Affecting Muscle Phenotype

North *et al.* (1999) focused on the ACTN2 and ACTN3 genes. These genes encode the skeletal muscle proteins, α-actinin-2 and α-actinin-3, which maintain the spatial relationship between myofilaments. Deficiency of α-actinin-3 is associated with muscular dystrophy. The expression of ACTN3 is limited to type 2 fibers and will have an influence predominately in power sports. North predicted that 16% of the world population is α-actinin-3 deficient, which could result in muscular weakness (North *et al.* 1999).

Genetic Polymorphisms Affecting Receptors

Wolfarth and associates (2000) examined the allelic frequencies and genotype distribution of two RFLPs in the alpha-2A-adrenoceptor gene (ADRA2A) and beta-2-adrenoceptor gene

(ADRB2) in elite endurance athletes and controls. *Dra I* restriction digestion suggested variability in the ADRA2A gene was weakly associated with elite endurance athlete status while the *Ban I* ADRB2 polymorphism showed no statistical significant difference between groups. These results indicate that the genetic variation in the ADRA2A gene or a gene locus in close proximity may be important in attaining athletic excellence in aerobic endurance dominated sports.

Genetic Polymorphisms Affecting Growth Factors

Ciliary neurotrophic factor (CNTF) has trophic effects in muscle and neuronal tissue. The role of CNTF genotype on muscular strength and quality were examined in the Baltimore Longitudinal Study of Ageing. Data showed that individuals with the CNTF G/A genotype had significantly greater muscular strength and quality at high contraction speeds than subjects homozygous for the G allele (G/G) (Roth *et al.* 2001).

Table 1.1: Summary of evidence for familial resemblance, maternal/paternal effect and heritability level in endurance related phenotypes and determinants.
From: Bouchard C. Genetic determinants of endurance performance. In: Shepherd and Åstrand, editors. Endurance in Sport. Oxford: Blackwell Scientific Publications; 1992. p. 149-159.

PHENOTYPE	FAMILIAL CONCENTRATION	HERITABILITY	MATERNAL OR PATERNAL EFFECT
Submaximal power output	+	No	No
90-min performance	++	++	Unknown
VO_{2max}	+	+	Slight maternal
Heart size	++	+	No
Stroke volume*	++	++	Unknown
Muscle fiber composition	++	+	Unknown
Muscle oxidative potential	++	+	Unknown
Lipid substrate oxidation	++	+	Unknown
Lipid mobilization	++	+	Unknown

* Estimated from maximal oxygen pulse

\+ Significant heritability

++ Highly significant heritability

This is just a short introduction to a rapidly growing body of work directed at elucidating the genetic determinants of athletic ability. The present study will focus on the angiotensin-converting enzyme (ACE) I/D and nitric oxide synthase (ecNOS) G894T polymorphisms and their usefulness as markers for elite athletic performance.

1.2 The ACE I/D Polymorphism and Exercise Performance.

The Renin-Angiotensin System (RAS): Basic Physiology

The renin-angiotensin system plays a key role in the regulation of cardiac and vascular physiology and is an important determinant of cardiovascular homeostasis (Dostal *et al.* 1992).

The juxtaglomerular apparatus (JGA) (shown in Figure 1.2, p7) encircling the afferent arterioles of the kidney is responsible for the synthesis of renal renin (Meyer BJ 1988, Kem and Brown 1990). The JGA is made up of:

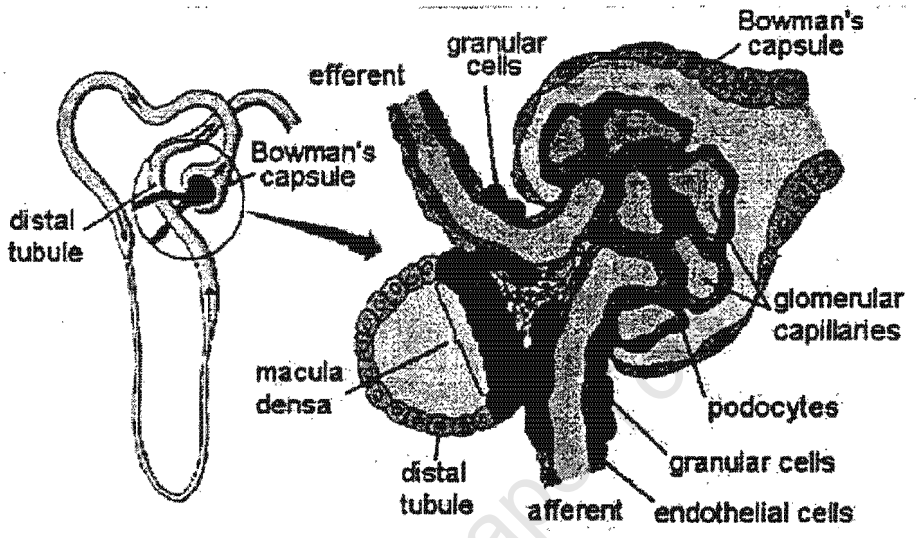
1). eight to 12 modified smooth muscle cells (granular cells) in the wall of each afferent arteriole in close proximity to the glomerulus,

2). macula-densa cells in the distal convoluted tube and

3). mesangeal cells (special connective tissue cells) in close contact with both the granular- and macula-densa cells. The granular cells produce renin.

Renin-like enzymes (iso-renin) are produced by the uterus, placenta, cerebrum, hypothalamus, pineal gland, hypophysis, and adrenal glands and in the endothelium of large arteries and veins (Meyer BJ 1988, Kem and Brown 1990). Uterine and placental renin increases with pregnancy. Amniotic fluid also has a high renin activity. The renin originates from the chorion and myometrium and may help in the regulation of uterine blood flow during pregnancy. Renin in the brain (cerebrum, hypothalamus, pineal gland and hypophysis) is synthesized locally because renin is a large peptide and does not pass the blood-brain-barrier. The RAS helps to maintain brain fluid homeostasis (Meyer BJ 1988, Kem and Brown 1990).

Figure 1.2: The juxtaglomerular apparatus. Renin is secreted by granular (juxtaglomerular) cells. The macula densa is involved in the regulation of afferent arteriolar resistance (tubulo-glomerular feedback).



Indications are that the endothelium has its own RAS, which works independently from the intra-renal system. This system works as a functional connection between the vascular endothelium (tunica intima) and smooth muscle tissue (tunica media) (Meyer BJ 1988, Kem and Brown 1990).

Three mechanisms regulate renin secretion by the JGA. These are:

1) The granular cells of the afferent renal arteriole function as baro- (pressure-, stretch) receptors. A decrease in arterial blood pressure in the afferent arteriole stimulates the JGA to release renin. An increase in blood pressure inhibits the release of renin.

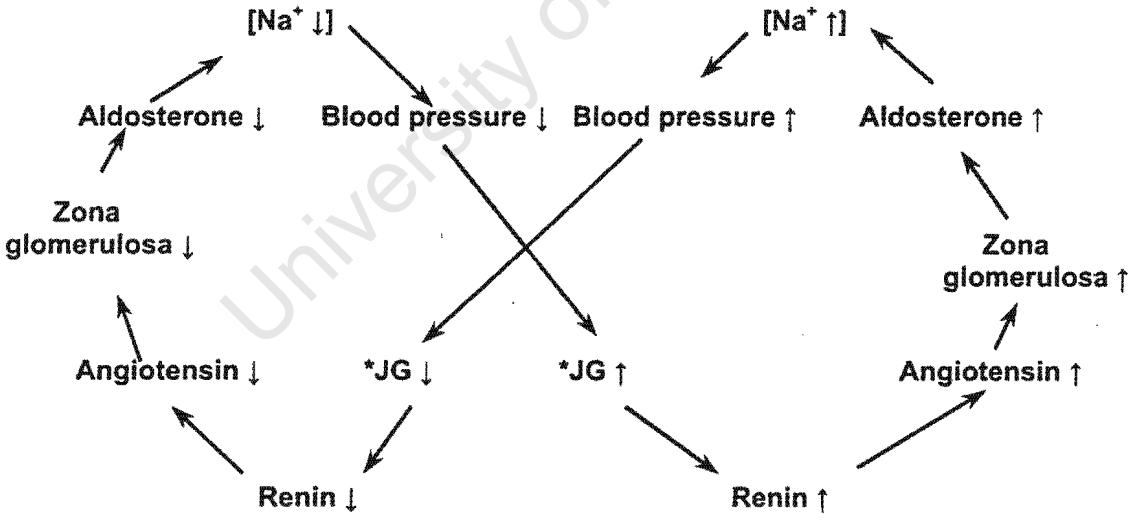
2) A decreased [Na^+] in the renal tubules will stimulate the JGA to increase renin secretion through intra-renal chemoreceptors. Aldosterone also modulates renin production not only through Na^+, but also via the kinins and prostaglandins (illustrated in Figure 1.3, p8).

3) Adrenoreceptors are sensitive to extra-renal blood pressure changes. The JGA is stimulated, via sympathetic nerves, to release renin in response to a decrease in blood volume (↓ blood pressure) and *visa versa*. Thus, renin secretion increases with blood loss and dehydration and the extra-renal adrenoreceptors can be thought of as extra-renal baroreceptors (stretch receptors) (Meyer BJ 1988, Kem and Brown 1990).

Renin is an angiotensinogen specific proteolytic enzyme. It cleaves a 10 amino acid fragment off angiotensinogen. The deca-peptide fragment is known as angiotensin I (ANG I) (see Figure 1.3 for detail).

The substrate for renin (the α_2-globulin, angiotensinogen) is synthesized in the liver, myocardium, head, skin, skeletal muscle, and kidney and released in the blood and lymphatic system (Kem and Brown 1990, Danser *et al.* 1992). ANG I is the precursor (prehormone) of angiotensin II (ANG II). ANG I stimulates the release of catecholamines from the adrenal gland (Ca^{2+} dependant) and noradrenaline release by peripheral sympathetic nerves. ANG I decreases blood flow through the cortical zone and the medulla of the kidney via angiotensin III (ANG III, ± 7% of ANG I in blood is converted to ANG III in the kidneys). Angiotensin I also decreases renin secretion in the kidney (negative feedback mechanism) and stimulates the thirst sensation and release of anti-diuretic hormone (ADH) by the hypothalamus.

Figure 1.3: The physiological relation between [Na^+], renin and aldosterone secretion and angiotensin production. * Juxtaglomerular apparatus. Adapted from: Meyer BJ 1988. ↑ stimulates; ↓ decreases



What is ACE?

The angiotensin-I converting enzyme (ACE; EC 3.4.15.1) or dipeptidyl carboxypeptidase I (DCP 1, peptidyldipeptidase A, kininase II, pulmonary microvascular ectoenzyme) is a widely

distributed transmembrane or free zinc metallopeptidase expressed in various tissues (see Table 1.2 for tissue distribution) (Meyer BJ 1988, Linz *et al.* 1999). ACE is anchored by its hydrophobic C-terminal segment in the cell membrane (Costerousse *et al.* 1993).

Circulating ACE has been shown in plasma, amniotic- and seminal fluid (Meyer BJ 1988, Linz *et al.* 1999). Plasma ACE is currently thought to originate from the trans-membrane form of the vascular endothelial cell, and is generated by post-translational proteolytic cleavage of its C-terminal membrane anchor (Linz *et al.* 1999, Costerousse *et al.* 1993, Danser *et al.* 1995). The enzyme responsible for this cleavage has not been identified. Less than 10% of the ACE in the body is freely circulating and the remaining 90%+ is tissue bound (Fabre *et al.* 1999). It is not known whether ACE is present in skeletal muscle.

Some tissues have all the components of a complete RAS. *In vitro* studies have shown that brain-, kidney-, adrenal-, vascular tissue and neonatal cardiac myocytes possess all the components of the renin-angiotensin system (Danser *et al.* 1992, Liu *et al.* 1998). Dostal and associates (1992) showed that ventricular cardiomyocytes and fibroblasts from neonatal rat hearts produce ACE, ANG I and ANG II in tissue culture using immunocytochemical methods.

Table 1.2: Tissue sites of ACE production and occurrence. (Meyer BJ 1988, Linz *et al.* 1999)

MEMBRANE-BOUND ACE	CIRCULATING ACE
Vascular endothelial cells (very abundant in lung tissue)	Plasma
Absorptive endothelial cells	Amniotic fluid
Neurons	Seminal fluid
Monocytes, macrophages, T-lymphocytes	
Brain	
Hart	
Kidney	
Liver	
Uterine tissue	
Male germinal cells	
Adventitia and smooth muscle	

What does ACE do?

ACE is responsible for the cleavage of carboxyl-terminal (C-terminal) dipeptides and tripeptides from a number of biologically active oligopeptides (Dostal *et al.* 1992, Meyer BJ 1988, Linz *et al.* 1999). These include angiotensin I (ANG I), bradykinin (BK), luteinizing hormone-releasing hormone (LHRH), neurotensin and substance P (Linz *et al.* 1999, Jaspard *et al.* 1993). ACE may also be involved in the metabolism of various other biological peptides because of its wide distribution in the body and because of its broad specificity (Dostal *et al.* 1992).

ACE is unusual in that it can act as either a dipeptidyl carboxypeptidase or an endopeptidase, depending on its substrate *in vitro* (Jaspard *et al.* 1993). ACE cleaves blocked di- or tripeptides off oligopeptides like substance P and LHRH and a tripeptide from BK after the removal of the C-terminal arginine by carboxypeptidase N (Jaspard *et al.* 1993). ACE also cleaves the blocked N-terminal tripeptide of LHRH. ACE has two active sites (C-terminal and N-terminal) and both possess dipeptidyl carboxypeptidase and endopeptidase activity. Both sites are activated by chloride (Cl^-) and have a zinc atom as cofactor (Jaspard *et al.* 1993).

ACE and ANG II

Angiotensin converting enzyme converts the deca-peptide ANG I to either of two active products, namely:

1) des-Asp^1-angiotensin I (nona-peptide) or

2) angiotensin II (octa-peptide).

As the name implies, ACE is responsible for the enzymatic cleavage of a di-peptide off the inactive deca-peptide ANG I, to form the vaso-active (constrictor), proliferative (trophic) and aldosterone stimulating octa-peptide angiotensin II (Meyer BJ 1988). The blood vessel endothelial cells are an important source of ACE, where either circulating or locally produced ANG I can serve as substrate for enzymatic activation (Costerousse *et al.* 1993, Fabre *et al.* 1999). This function of ACE is central to the renin-angiotensin system.

It is known that ANG II production also occurs through pathways other than the "classical" RAS pathway. These pathways include those involving the serine proteases tonin and chymase (Dostal *et al.* 1992, Richard *et al.* 2001). Purified tonin converts angiotensinogen to ANG I and ANG II (Dostal *et al.* 1992). Similarly, human heart and mammary artery chymase has been reported as a major serine protease implicated in ANG II production in the heart and arteries (Dostal *et al.* 1992, Richard *et al.* 2001). In the rat, chymase hydrolyzes ANG I to give an inactive product.

As ACE is present in tissues other than the vascular endothelium, part of the circulating ANG II may be derived from the conversion of ANG I produced in tissues outside the circulation (Danser *et al.* 1992).

Physiological Functions of ANG II

Angiotensin II is the most important biologically active product of the RAS in circulation. The functions of ANG II (octapeptide) include:

1) Indirect positive chronotropic and inotropic effect on the heart via sympathetic nerves and the adrenal medulla, where it stimulates noradrenaline synthesis and release by sympathetic nerves. ANG II facilitates adrenergic neurotransmission in the heart (Dostal *et al.* 1992). The indirect chronotropic effect is enhanced by the direct action of ANG II on myocardial tissue (possibly by stimulation of Ca^{2+} influx during the plateau of the cardiac action potential). It also decreases blood flow through the coronary artery (Dostal *et al.* 1992). It is not certain whether these effects are mediated through circulating or locally produced angiotensin II (Dostal *et al.* 1992). ANG II is thought to facilitate the activity of the sympathetic nervous system by binding to presynaptic sympathetic neurons and augmenting the release of norepinephrine (Townsend *et al.* 1993).

2) Angiotensin II has a strong pressor effect; calculated on a molar basis, ANG II is a 40 times stronger vasoconstrictor than noradrenaline (Meyer BJ 1988, Nakahara *et al.* 2000). It increases both systolic and diastolic blood pressure by working directly and indirectly (via sympathetic nerves) on bloodvessels. It also constricts renal vessels and decreases glomerular filtration. (Because ANG II stimulates the production of vasodilator prostaglandins (PG), this effect is more or less cancelled out.)

3) ANG II stimulates thirst when injected intravenously or when applied directly to the hypothalamus (dipsogenic effect of ANG II).

4) ANG II also stimulates the release of ADH; together these two hormones play an important role in the maintenance of fluid homeostasis in the body.

5) ANG II together with Ca^{2+} stimulates aldosterone synthesis and secretion. High $[Na^+]$ and/or low $[K^+]$ decreases the effect of ANG II on aldosterone – and *visa versa*.

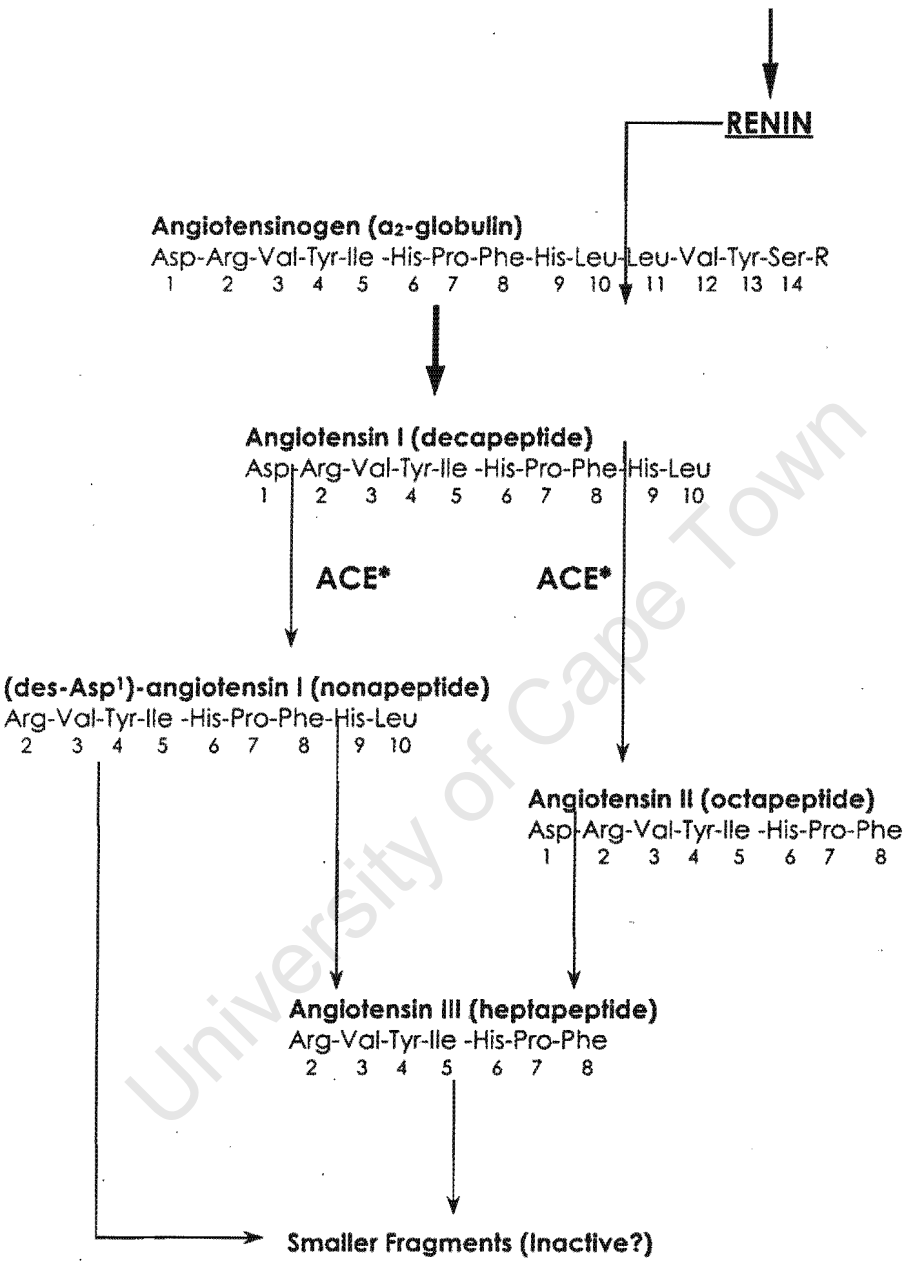
6) ANG II increases prostaglandin synthesis.

7) ANG II induces the expression of vascular endothelial growth factor (VEGF) by vascular smooth muscle cells (Fabre *et al.* 1999). VEGF is an endogenous angiogenic cytokine, which up-regulates the production of nitric oxide (NO), an important regulatory molecule for angiogenesis. Available data suggest that NO production may be critical for VEGF-induced angiogenesis (Fabre *et al.* 1999). VEGF is a secreted heparin-binding growth factor that is specific for vascular endothelial cells (Lueng *et al.* 1989).

8) ANG II activates myocyte apoptosis (Liu *et al.* 1998).

Figure 1.4: Main steps in the production and degradation of angiotensin.

*Angiotensin Converting Enzyme. Adapted from: Meyer BJ 1988.



Through the interplay of these functions, ANG II regulates intravascular volume, vascular resistance, cardiac function and overall cardiovascular homeostasis. Angiogenesis, vascularization and increased microcapillary density of skeletal muscle are important performance enhancing adaptations to endurance and other types of exercise.

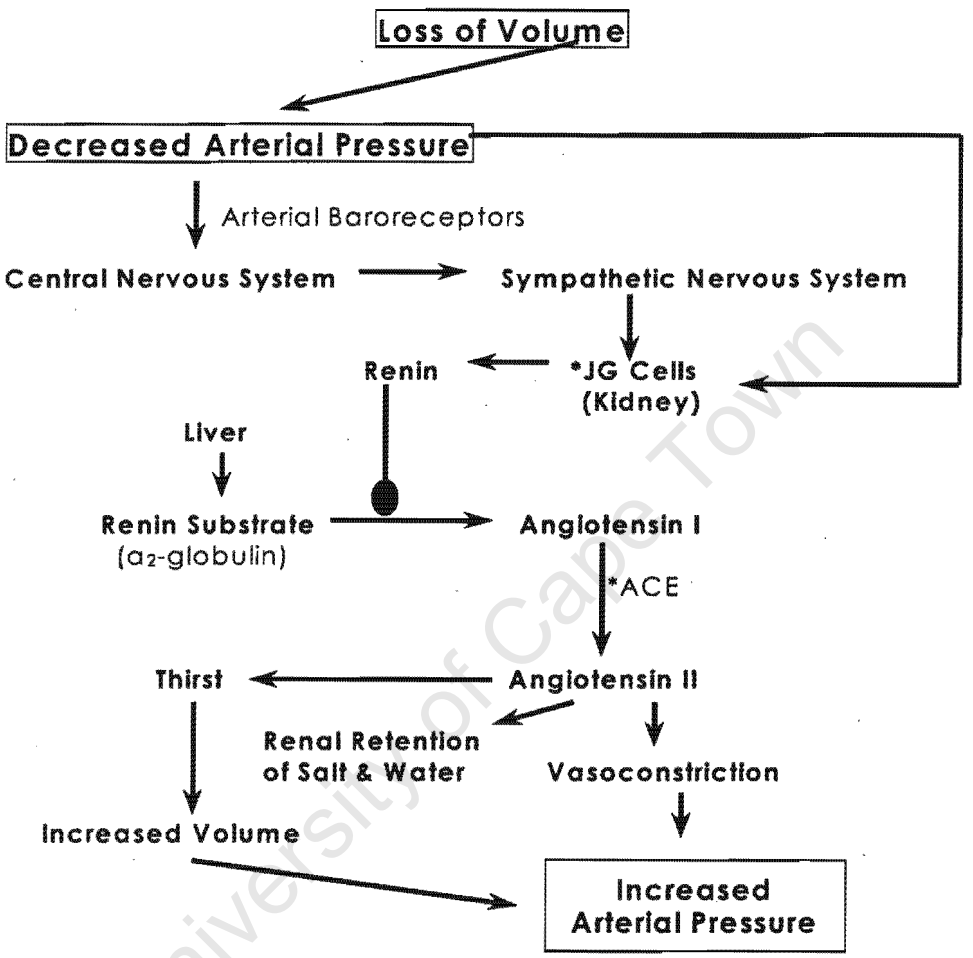
Additional Supportive Functions of ANG II

ANG II stimulates liver glycogenolysis through glycogen phosphorylase activation by a cyclic AMP independent mechanism (DeWitt and Putney 1983). DeWitt and Putney (1983) reported that the activation of glycogenolysis, as measured by [^{3}H] glucose release after addition of ANG II, in cultured guinea pig hepatocytes has Ca^{2+}-dependent and Ca^{2+}-independent components. Calcium release from an intracellular pool stimulates the initial response; this phase is independent from extracellular Ca^{2+}. The second phase occurs only in the presence of Ca^{2+} influx into the cell (DeWitt and Putney 1983).

Insulin mediated uptake of 6,6-d_2-glucose is increased by pressor doses of ANG II (Townsend *et al.* 1993). Townsend and DiPette (1993) investigated the effect of a 3-hour hyperinsulinemic euglyceamic glucose clamp in the presence or absence of pressor doses of ANG II (~15 ng/kg/min) on insulin-mediated glucose uptake in 14 normotensive men (Townsend *et al.* 1993). Blood pressure increased by 20/15 mmHg in the presence of ANG II. Glucose uptake increased by 15% and glucose oxidation by 25% when insulin alone was compared to insulin plus ANG II (Townsend *et al.* 1993). The authors conclude that the mechanism for the increase in uptake and oxidation of glucose may involve a redirection of blood flow to skeletal muscle with an increase in skeletal muscle glucose uptake during ANG II and insulin infusion or a direct biochemical action of ANG II (Townsend *et al.* 1993). Both pressor doses of ANG II and insulin infusions have been shown to increase skeletal muscle blood flow by up to 50%, which may have a positive effect in actively exercising skeletal muscle (Townsend *et al.*1993).

Other physiological effects include activation of voltage-sensitive Ca^{2+} channels which leads to increased free cytosolic Ca^{2+} and to an increased inotropic effect (or increased mechanical activity) and, an activation of phospholipase C resulting in increased levels of inositol triphosphate, diacylglycerol and protein kinase C (PKC) translocation (Dostal *et al.* 1992, Yamazaki *et al.* 1995). The latter may contribute to cardiac cell growth (Dostal *et al.* 1992). Cardiac hypertrophy is seen with regular endurance exercise.

Figure 1.5: The renin-angiotensin mechanism of arterial pressure regulation.
*Angiotensin converting enzyme



Angiotensin II also stimulates the induction of platelet-derived growth factor (PGDF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), endothelin-1 (ET-1) and other autocrine growth factors in vascular smooth muscle cells and vascular cell adhesion molecule-1 (VCAM-1) (Otani *et al.* 1998, Tummala *et al.* 1999). Mechanical loading on cultured myocytes induces phosphorylation cascades (PKC, MAP kinase) (Yamazaki *et al.* 1995).

ANG II Receptors

ANG II elicits its many functions through ANG II receptors of which at least two types have been identified, namely angiotensin II type 1 (AT_1) and angiotensin II type 2 (AT_2) (Danser *et al.* 1999). Neonatal myocytes have both AT_1 and AT_2 receptor subtypes. Mechanical stretch increases the expression of both subtypes three fold *in vitro* (Liu *et al.* 1998). Up-regulation of myocyte AT receptors (both AT_1 and AT_2) also occurs acutely following infarction (Liu *et al.* 1998). ANG II promotes myocyte growth (in both size and protein content) through activation of AT_1 receptors on myocyte membranes (Liu *et al.* 1998). Continuous increased hemodynamic overload as induced by exercise stress or hypertension causes the autocrine release of ANG II, which activates signal transduction and ultimately leads to myocardial hypertrophy (Nakahara *et al.* 2000, Yamazaki *et al.* 1995). Myocytes taken from the left-ventricle of post infarcted hearts are more sensitive to ANG II induced hypertrophy and achieve a greater magnitude of hypertrophy (40-45% higher hypertrophic response) than normal control cells (Liu *et al.* 1998). This effect is completely blocked by the AT_1 receptor blockers, including Losartan (AT_1 antagonist) (Liu *et al.* 1998). In cardiac tissue, ANG II binds to AT_1 receptors to up-regulate protein synthesis and cell growth with resultant cardiac hypertrophy (Liu *et al.* 1998, Dostal *et al.* 1992). Liu *et al.* (1998) suggests that AT_2 receptors appear not to be involved in the ANG II mediated up-regulation of myocyte growth. Cardiac cells with membrane AT receptors include sympathetic nerve terminals, cardiomyocytes and fibroblasts (Dostal *et al.* 1992). It has been demonstrated that a 1-2 week ANG II infusion causes significant left ventricular hypertrophy in rats and cultured human myocytes in the absence of cellular loading and contractile activity (Liu *et al.* 1998, Dostal *et al.* 1992).

Angiotensin III

The functions of angiotensin III (ANG III) and ANG II differ only slightly. ANG II is more effective on a molar basis than ANG III, except in the stimulus of aldosterone synthesis where ANG III is more potent. There are also indications that ANG III activates tryptophan hydroxylase in the brain, which increases serotonin synthesis and release by these cells.

Through its direct and indirect action on the cardiovascular system and through its effect on aldosterone synthesis, the RAS plays an important role in both the short- and long-term control of blood pressure. Although a strong pressor, angiotensin plays a more important role in the long-term control of blood pressure through aldosterone and ADH than in its acute action and direct control (Meyer BJ 1988).

ACE and Bradykinin

Endothelial ACE degrades BK to its inactive metabolites BK1-5 (Arg-Pro-Pro-Gly-Phe) and BK 1-7 (Linz *et al.* 1999, Fabre *et al.* 1999, Jaspard *et al.* 1993, Murphey *et al.* 2000). BK1-5 is a stable metabolite ($t_{1/2}$ = 80-90 minutes in humans) of BK, which reflects the sum of vascular, endothelial and serum ACE activity (Murphey *et al.* 2000). The ratio of BK1-5 to circulating BK (BK1-5:BK ratio) has been used as marker for ACE activity (Murphey *et al.* 2000). Bradykinin is a strong vasodilator with an anti-proliferative (anti-trophic) effect and thus contributes to the cardio-protective effect of ACE inhibitors in hypertensive patients (Murphey *et al.* 2000).

BK exerts its vasodilatory action through the direct stimulation of B_2 kinin receptors, causing the synthesis of vasodilator compounds such as endothelium derived hyperpolarizing factor (EDHF), prostacyclin and NO (Linz *et al.* 1999). NO is produced through the L-arginine-NO pathway. This pathway was shown to promote growth of endothelial cells via the activation of B_1 kinin receptors (Fabre *et al.* 1999). BK is produced in several types of inflammation where mononuclear cells are involved (Costerousse *et al.* 1993). Furthermore, BK inhibits thrombin-induced platelet activation through the activation of guanylyl cyclase by NO and subsequent synthesis of cyclic GMP (cGMP), and stimulates the release of tissue-type plasminogen activator (t-PA) from the vascular endothelium (Meyer BJ 1988, Murphey *et al.* 2000, Marieb EN 1989). BK (and substance P) is also implicated in aspects of the immune response and inflammation. These include lymphocyte proliferation, neutrophil chemotaxis, phagocytosis and the release of inflammatory mediators (Costerousse *et al.* 1993).

What is the ACE insertion/deletion polymorphism?

The ACE I/D polymorphism* comprises the presence (insertion, I) or absence (deletion, D) of a 287-base pair (bp) Alu repeat element in intron 16 of the human ACE gene (*DCP 1)* (Rieder *et al.* 1999). All Alu repeat sequences are part of the Alu family, named after the *Alu I* restriction enzyme, which generates characteristic fragment patterns upon digestion of human or mammalian DNA (Blackburn and Gait 1996). Alu repeats have sequences similar to that of signal recognition particles involved in protein synthesis. All Alu repeats fall into a class of DNA called short interspersed elements or SINEs. They comprise short stretches of DNA, usually a few hundred base pairs long. Almost all SINEs are pseudogenes, derived from small RNA's (Blackburn and Gait 1996).

The literature suggests that SINEs do not fulfill any major function, or significantly harm the host DNA (Blackburn and Gait 1996). SINEs have been termed " *selfish* " DNA's or parasitic because " *their only function is their propagation through the genomes they inhabit* " (Blackburn and Gait 1996). It is uncertain whether these sequences are truly parasitic and

perhaps some undiscovered property, which helps the host in some yet unknown way, is awaiting discovery (Blackburn and Gait 1996). About 5% of the human genome is made up of SINEs (around 750 000 copies) (Mueller and Young 1998).

* The official name for the gene encoding ACE is *DCP 1* and the two alleles are *DCP 1*D and DCP 1*I.*

How does the ACE I/D polymorphism influence the expression of ACE?

The ACE I/D polymorphism is a major determinant of circulating ACE levels (Huang *et al.* 1998, Samani *et al.* 1996). The deletion allele is associated with higher ACE activity, both in circulation and in body tissues. Mean plasma ACE in subjects homozygous for the D-allele is approximately twice that of subjects homozygous for the I-allele, with I heterozygotes having intermediate values (Tiret *et al.* 1992, Rigat *et al.* 1992). Danser *et al.* (1995) determined that ACE activity in cardiac tissue was highest in subjects homozygous for the D allele. McKenzie *et al.* (1995) could show no significant differences in either systolic and diastolic blood pressure or serum ACE levels when grouping 98 hypertensive subjects and 156 normotensive controls by ACE I/D genotype. This remained true even when data was adjusted for the confounding effects of age, gender and BMI. The I/D genotype accounted for ~9% of the total variance in serum ACE level which is less than estimated by Rigat *et al.* (1990).

Rigat *et al.* (1990) and Tiret *et al.* (1992) showed that strong linkage disequilibria exist between the ACE I/D polymorphism and a gene locus controlling plasma ACE levels. Serum ACE levels are highly influenced by multiple quantitative-trait loci's (QTLs) (McKenzie *et al.* 1995). As much as 44 to 52% of the total variability in ACE activity, in French nuclear families and Jamaican families of African Caribbean descent, is accounted for by a QTL unlinked to the ACE gene and in strong linkage disequilibrium with the ACE I/D polymorphism (Samani *et al.* 1996, Rigat *et al.* 1992, McKenzie *et al.* 1995). A second QTL is located within or close to the ACE locus and accounts for 27-28% of the total variability in ACE (Tiret *et al.* 1992, McKenzie *et al.* 1995). McKenzie *et al.* (1995) estimated that 79% of the total variability in serum ACE levels is influenced by these two QTLs (McKenzie *et al.* 1995). Quantitative-trait loci can be described as the inheritance or expression of a phenotype, which is determined by many genes at different loci (Mueller and Young 1998). Each of these genes exert a small additive effect with no one gene being dominant over another. Recently, researchers reported the existence of a point mutation in the stalk region of the ACE gene, Pro1199Leu (Kramers *et al.* 2001). This mutation causes a dramatic five-

fold increase in serum ACE activity by a much more efficient clipping of membrane-bound mutant ACE than in the wild-type enzyme.

Tiret and associates (1992) determined the plasma ACE activity and ACE I/D genotype of 98 nuclear families in an effort to elucidate whether the ACE I/D genotype contributed to the genetic regulation of circulating ACE levels. All participants were selected based on the absence of acute or chronic pathology and any treatment that would alter the phenotype. ACE levels were significantly higher in offspring than in parents ($P < 0.001$) and significantly higher in sons than in daughters ($P < 0.001$) (Tiret *et al.* 1992). Age accounted for 10% of the variance in offspring and the authors suggest hormonal regulation activated during puberty as a possible basis for the variability (Tiret *et al.* 1992). ACE activity was not associated with blood pressure in parents, but showed a significant correlation with systolic ($r = 0.19$; $P = 0.006$) and diastolic blood pressure ($r = 0.13$; $P = 0.05$) in offspring (Tiret *et al.* 1992). ACE levels showed highly significant familial similarity ($\chi^2 = 30.56$, $P < 0.001$). Linkage and segregation analysis revealed that the ACE I/D polymorphism is unlikely to be the gene locus directly affecting the variability of plasma ACE, but could be a marker in strong linkage disequilibrium with this locus (Tiret *et al.* 1992). Individual plasma ACE levels are highly stable when repeated measures are taken over time (Tiret *et al.* 1992). Interindividual ACE levels differ by as much as five fold (Tiret *et al.* 1992). Environmental and hormonal parameters are only weakly associated with ACE levels (Tiret *et al.* 1992).

The *DCP 1* gene is not gender linked and allele frequencies are therefore the same in men and women (Murphey *et al.* 2000).

Murphey *et al.* (2000) studied the effect of ACE genotype on the *in vivo* metabolism of intra-arterially administered BK. Plasma ACE activity was the highest in those with the DD genotype (36.8±6.2 U/ml), intermediate in those with the ID genotype (25±3.3 U/ml), and lowest in those with the II genotype (20.3±2.3 U/ml) ($P = 0.027$ for effect of number of D-alleles) (Murphey *et al.* 2000). Concentrations for BK were 762±242 (II), 469±50 (ID) and 545±104 (DD) fmol/ml and the venous BK1-5:BK ratio correlated with plasma ACE activity ($r^2 = 0.16$, $P = 0.039$) (Murphey *et al.* 2000). Degradation as measured by the BK1-5:BK ratio was greatest in D homozygotes and lowest in I homozygotes (Murphey *et al.* 2000). This study shows an association between the ACE D-allele and augmented degradation of BK in humans *in vivo*. The author cautions that although there seems to be diminished BK degradation by ACE in the I homozygotes, BK may be degraded by alternative pathways such as aminopeptidase P (Murphey *et al.* 2000). The author suggests furthermore, that the BK1-5:BK ratio may be used as a marker for vascular ACE activity in humans (Murphey *et al.*

2000). Nitric oxide regulates ACE expression and activity (Linz *et al.* 1999). A chronic inhibition of endothelial NO synthase (ecNOS) results in up regulation of both vascular and cardiac ACE activity.

ACE and disease

Abnormal ACE levels occur in the course of several granulomatous diseases, including sarcoidosis (Kem and Brown 1990, Tiret *et al.* 1992). High ACE levels are routinely used in the diagnosis of sarcoidosis (Tiret *et al.* 1992). A change in the synthesis of ACE, together with those of BK and nitric oxide, are associated with cardiovascular diseases such as hypertension, atherosclerosis and coronary heart disease (Linz *et al.* 1999, Tummala *et al.* 1999).

The deletion (D) allele is a risk factor for myocardial infarction (MI), cardiomyopathy, left ventricular hypertrophy and coronary artery disease in patients formerly considered at low risk of developing MI and has been linked to cardiovascular disease (Danser *et al.* 1995, Murphey *et al.* 2000, Cambien *et al.* 1992). Researchers attribute this association to an increased formation of ANG II in individuals who carry the D-allele and to an increased degradation of the cardio protective BK (Murphey *et al.* 2000). Tummala *et al.* (1999) indicated that ANG II contributes to atherogenesis by increasing expression of VCAM-1, which is a vascular inflammatory protein and through the production of $\bullet O_2^-$. VCAM-1 is present in the endothelium and underlying smooth muscle in early atherosclerotic lesions (Tummala *et al.* 1999).

Cambien and associates (1992) reported a significantly higher incidence of the ACE DD genotype in subjects with MI (n = 610) than in controls (n = 733) (P = 0.007). This was especially true for subjects with a low body-mass index (BMI) and with low circulating plasma levels of ApoB (P < 0.0001) (Cambien 1992). The presence of hypertension, as defined by a diastolic blood pressure above 100 mmHg (> 100 mmHg) or by treatment with anti-hypertensive medication, showed a similar distribution across genotypes: 22.0%, 17.7% and 20.3% in DD, ID and DD genotypes respectively had hypertension (Cambien *et al.* 1992). The authors propose that the ACE DD genotype is a new risk factor for MI in middle-aged men, particularly in those previously considered at low risk (low BMI, low ApoB) (Cambien *et al.* 1992).

Elevated ACE levels as determined by ACE genotype may, through increased conversion of ANG I to ANG II, lead to higher tissue and circulating levels of ANG II. This holds true for

cardiac tissue where the ACE DD genotype leads to higher ACE levels (Danser *et al.* 1995). It is known that ANG II plays a key role in the pathogenesis of hypertension, intimal hyperplasia and cardiac hypertrophy (Nakahara *et al.* 2000). Since ANG II has known proliferative and remodeling effects, the higher ACE levels associated with the ACE DD genotype may explain the association between the ACE deletion polymorphism and cardiac disease (Danser *et al.* 1995, Nakahara *et al.* 2000).

Samani *et al.* (1996) conducted a meta-analysis of studies published on the association of the ACE gene D-allele with myocardial infarction. The authors included 15 studies with a total of 3394 cases (MI) and 5479 controls (n = 8873). The meta-analysis supports the proposed linkage between the D-allele and increased risk of MI and coronary artery disease and between an increased familial risk of developing MI and the D-allele (Samani *et al.* 1996). The authors caution that the ACE I/D genotype's main value will probably be at the level of elucidating the mechanism of the association and not in the stratification of individual risk for MI (Samani *et al.* 1996).

In a prospective study over 9 years by Huang *et al.* (1998), it was concluded that the ACE I/D polymorphism is an important and independent risk factor for the development of coronary heart disease (CHD) in patients with non-insulin-dependent diabetes mellitus (NIDDM) (Table 1.3, p21). 83 patients, aged 40 to 65 years were evaluated over a period of 9.1 years. None had CHD at entry. At 9 years, 21 patients (37.5%) of the original group of 83 had developed CHD and/or MI (Huang *et al.* 1998). The D-allele was significantly associated with development of CHD ($P = 0.033$) (Huang *et al.* 1998). Genotype distributions were in Hardy-Weinberg equilibrium and allele frequencies in the healthy subgroup were similar to those of healthy Caucasian populations. "*The best model in the logistic regression analysis showed ACE genotype ($P = 0.0105$) and age ($P = 0.0407$) to be the significant risk factors for CHD. In this model, the efficiency, i.e., the percentage of subjects correctly classified as being with or without CHD, was 89%*" (Tiret *et al.* 1992). Researchers have also correlated levels of ACE, prorenin and ANG II to the severity of diabetic retinopathy and nephropathy (Tiret *et al.* 1992), and have demonstrated that diabetes mellitus was less frequent in patients with the II genotype (Nakahara *et al.* 2000).

Nakahara *et al.* (2000) examined the association between ACE genotype and heart weight. Heart weight in the DD genotype was significantly higher than that in the ID ($P < 0.01$) and II ($P < 0.05$) genotypes. Heart weight was also correlated to age ($P < 0.0001$) and coronary stenosis index ($P < 0.0019$) (Nakahara *et al.* 2000). Although it was shown that ACE

genotype is a predictor of heart weight, it was a less effective predictor than history of hypertension and age (Nakahara *et al.* 2000).

Table 1.3: ACE genotype and allele frequency in 83 NIDDM patients according to CHD status at 9.1 year follow-up examination. (Huang *et al.* 1998)

	GENOTYPE							
	II		ID		DD		ALLELE FREQUENCY	
	n	%	n	%	n	%	I	D
CHD^-	8	18.6	24	55.8	11	25.6	0.47	0.53
CHD^+	3	7.5	17	42.5	20	50	0.29	0.71*

CHD^-, without CHD; CHD^+, with CHD.

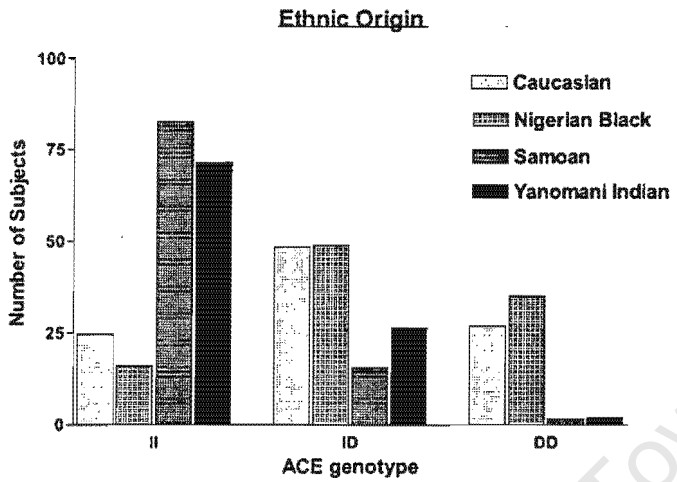
*$P = 0.033$ (χ^2) for allele frequency

Association of the ACE I/D Polymorphism with Ethnic Origin

There is a recognised association between cardiovascular disease, hypertension and different ethnic groups (Barley *et al.* 1991). Barley and associates (1994) studied the effect of ethnicity on the distribution of the ACE I/D polymorphism. The ACE genotype was determined in four different ethnic groups. The study group was made up of 186 Caucasian subjects of European origin from a London hospital; 80 Nigerian Blacks sampled from a working population in Ibadan, Nigeria; 58 Samoans; 49 native Yanomani Indians living in Brazil and Venezuela (Barley *et al.* 1994). Figure 6.1, p22 shows the genotype distribution among these groups.

ACE genotype distributions were 24.7 (II), 48.4 (ID) and 26.9% (DD) for Caucasians; 16.2, 48.8 and 35% for Nigerian Blacks ($\chi^2 = 3.1$, $P = 0.08$); 82.8, 15.5 and 1.7% for Samoans ($\chi^2 = 63.2$, $P < 0.001$) and 71.4, 26.5 and 2.0% for Yanomani Indians ($\chi^2 = 40.4$, $P = 0.001$). Both the Samoan and Yanomani groups were significantly different from the Caucasian European group. Frequency distribution for the I-allele in the Samoan and Yanomani groups were significantly higher than those for the Caucasian and Black groups. Frequencies for the D-allele in the Samoan and Yanomani groups were significantly lower than those for the Caucasian and Black groups. Frequency distributions were not different between Yanomani and Samoans.

Figure 1.6: Genotype distribution of the ACE I/D polymorphism in Caucasian, Nigerian Black, Samoan and Yanomani Indians. Barley *et al.* (1994)



Researchers have suggested that the ACE gene polymorphism could enhance endurance performance at high altitude (Montgomery *et al.* 1999). This association has led Rupert and associates (1999) to genotype Quechua speaking natives of the Andean Altiplano in South America. The people of this tribe live at altitudes greater than 3000m. The I-allele was found to be significantly higher than in Caucasians of European descent, but was not different from frequencies found in lowland Native American populations.

1.2 The ACE I/D Polymorphism and Exercise Performance.

1.2.1 Studies Reporting Significant Association

The ACE I-allele is the first genetic marker to be associated with elite athletic performance and excellence. In their paper in Nature entitled '*Human gene for physical performance*', Montgomery *et al.* (1998) stated that " *the insertion allele is **strongly associated** with elite endurance performance* ".

Cardiovascular endurance depends largely on the performance of the cardiovascular system, particularly as regards to arterial compliance, cardiac hypertrophy and vascularization of skeletal muscle (Gayagay *et al.* 1998). The RAS plays an important role in the regulation of cardiac and vascular physiology. Very important in the RAS is the hormone angiotensin II.

Angiotensin II has a trophic effect on heart muscle cells and there is evidence for a local skeletal muscle RAS, which may influence muscle growth (Montgomery *et al.* 1998, Gayagay *et al.* 1998). Angiotensin II is a strong arterial constrictor and plays an important role in the regulation of blood pressure. Angiotensin II has known growth-promoting (growth factor) activity on vascular smooth muscle and there is strong evidence that it may play a role in the regulation of autocrine/paracrine mechanisms including the adrenergic system (Evans *et al.* 1994). It has further also been suggested that local renin-angiotensin levels may affect the function of the gut, adipose tissue and skeletal muscle and work in on other metabolically active tissues to influence the body's energy balance (Montgomery *et al.* 1999).

ACE is responsible for the conversion of inactive ANG I to vaso-active ANG II by enzymatic cleavage of two amino acid residues.

The insertion/deletion (*I/D*) polymorphism in intron 16 of the human angiotensin-converting enzyme gene is a major determinant of circulating ACE levels (Huang *et al.* 1998). The deletion (D) allele is associated with higher ACE levels, both in circulation and in body tissues (Tiret *et al.* 1992, Rigat *et al.* 1992). It is the insertion (I) allele however, that is associated with aerobic endurance performance (Montgomery *et al.* 1998). Table 1.5 (p28-9), summarizes studies on the association between the ACE I/D polymorphism and sporting performance.

Montgomery *et al.* (1997) reported a strong relationship between the ACE I/D polymorphism and exercise-induced growth (hypertrophy) of the left ventricle (LV). The study group consisted of 460 male recruits of the British Army of which 150 subjects failed to complete the study (n = 310). Measurements were taken before and after a 10-week basic training program. LV thickness and LV mass index was calculated from ECG data recorded before and after the 10 weeks of training. All physical characteristics were the same across genotypes. The genotype distribution was 24.3% II, 55% ID and 20.7% DD. End-diastolic LV volume differed between genotypes with II>ID>DD; P = 0.009. Training induced LV growth in all subjects (P < 0.0001) and the magnitude of growth was strongly genotype associated. LV mass changed with +2.0, +38.5 and +42.3g for II, ID and DD respectively (P < 0.0001). It was concluded that exercise-induced LV hypertrophy, which is important in endurance exercise is strongly influenced by the presence of the D-allele.

Montgomery *et al.* (1998) reported an anabolic response to exercise in 123 British Army recruits. Of the 78 recruits completing the study, those who were homozygous (II) or heterozygous (ID) for the I-allele showed the greatest increase in the duration for which they

could perform repetitive elbow flexion with a 15 kg weight. The improvement was eleven-fold greater for those with the II genotype compared to those with the DD genotype. This result could however have been a result of adaptation to training and the muscle being stronger and was not tested at a higher absolute load.

Montgomery *et al.* (1998) also found the I-allele to be associated with endurance performance among 25 high-altitude mountaineers ascending mountains above 7000m without oxygen. Of the 15 climbers who ascended mountains above 8000m, six were homozygous (II) and nine heterozygous (ID) for the I-allele (I-allele frequency 0.65). Montgomery suggests that the association of the I-allele with elite endurance performance might be derived from increases in substrate delivery due to increased capillary density and cardiac output.

Montgomery and co-workers (1999) further strengthened the evidence for an association between the ACE I/D genotype and physical performance. 123 consecutive Caucasian male army recruits from a British Army regiment were chosen. 42 subjects did not complete the study (n = 81). Subjects underwent an identical 10-week basic training program. Genotype distribution was 24% II, 57% ID and 19% DD and was in Hardy-Weinberg equilibrium. There were no correlations between ACE genotype and physical characteristics (no genotype-phenotype correlation). The D-allele strongly influenced the change in body composition following 10 weeks of training (P = 0.05). These measurements were taken by bioelectric impedance, magnetic resonance imaging (MRI) and direct measurement of skinfolds. Chest and waist circumferences were significantly smaller in those with the D-allele after training, while neck circumference increased in those with the II genotype. Montgomery concluded that the I-allele is associated with an anabolic response to training (Montgomery *et al.* 1999).

Gayagay and associates (1998) conducted a study on a group consisting of 64 Australian national rowers, 41 of whom represented their country in the 1996 Atlanta Summer Olympic Games, and 118 controls. Control samples were taken from healthy volunteers or blood donors from the NSW Blood Bank (Gayagay *et al.* 1998). All subjects were Caucasian. Allele frequencies were 0.57 (I) and 0.43 (D) for rowers and, 0.43 (I) and 0.57 (D) for controls, with the I-allele being significantly higher in rowers ($P < 0.02$, $\chi^2 = 5.93$). Genotype distributions followed the same trend with the rowers having excess of the II genotype (P = 0.03, $\chi^2 = 6.91$). All frequencies were in Hardy-Weinberg equilibrium. Gayagay *et al.* (1998) proposed that a gene or genetic factor associated with the ACE gene may provide some advantage in endurance-dominated sports performance. These results are however difficult to interpret as rowing performance is dependent on both aerobic endurance and power.

endurance and power. Trent *et al.* (1998) and Gayagay *et al.* (1998) concluded that the ACE gene I-allele is the first genetic marker to be associated with athletic excellence in endurance sports.

It is of interest that Trent *et al.* (1998) also genotyped the study group for polymorphisms of the AT_1 and AT_2 receptors. There were however, no observed differences in the polymorphisms associated with the AT_1 and AT_2 receptors.

Myerson *et al.* (1999) studied the ACE I/D polymorphism's effect on elite performance in runners (n = 91) selected by the British Olympic Association as potential candidates to represent Great Britain at the 2000 Summer Olympic Games. Statistical analysis revealed a trend towards an increasing I-allele frequency with distance run as seen in Table 1.4. The authors concluded that the I-allele might be associated with endurance performance. Their data suggests that the I-allele frequency is higher in distance runners than healthy controls and increases with distance run (Myerson *et al.* 1999).

Myerson and colleagues (1999) showed a significant difference in I-allele frequency between 64 Olympic level swimmers and 1906 healthy controls. The I-allele frequencies were 0.40 and 0.49 for swimmers and controls respectively (P = 0.034). A significant excess of the D-allele was found in events taking less than 2 minutes. The shorter events have a great power component and as ANG II is a known cellular growth factor, the higher ACE levels associated with the D-allele may account for its possible effect through alterations in myocyte fiber size (Myerson *et al.* 1999, Montgomery *et al.* 1997).

Table 1.4: The ACE I/D polymorphism in elite Olympic level runners over various distances. (Myerson *et al.* 1999)

		ACE GENOTYPE			
DISTANCE RUN	**N**	**DD**	**ID**	**II**	**I-ALLELE FREQUENCY**
		ALL RUNNERS			
≤200 m	20	0.45	0.40	0.15	0.35†
400-3 000 m	37	0.19	0.57	0.24	0.53†
≥ 5 000 m	34	0.18	0.41	0.41	0.62†
All distances	91	0.24	0.47	0.29	0.52

† P = 0.009

Williams *et al.* (2000) examined training-induced changes in mechanical working efficiency of skeletal muscle. 58 Caucasian army recruits were genotyped and grouped into 35 II and 23 DD genotype. Change in work efficiency (delta efficiency) was calculated as the "percentage ratio of the change in work performed per minute to the change in energy expended per minute". An increase of 8.62% relative to base values for II and a decrease of –0.39% for DD genotype was observed. The authors concluded that the ACE I-allele confers an enhanced mechanical efficiency to trained muscle (Williams *et al.* 2000).

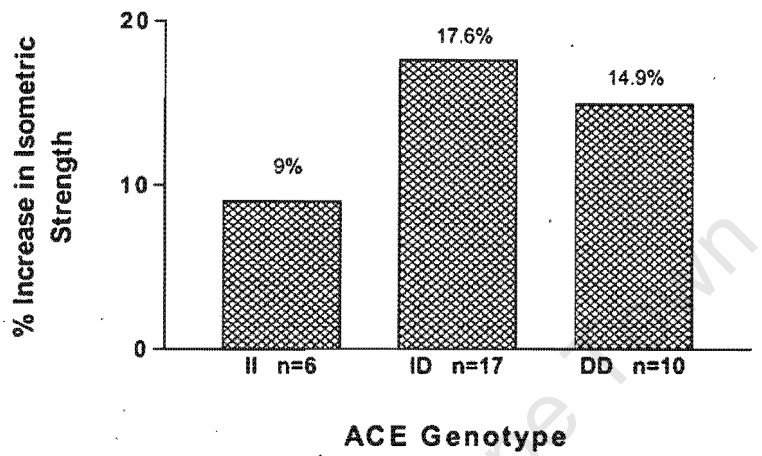
In their study on British Olympic candidates, Myerson *et al.* (1999) showed an association between the I-allele and sporting performance in elite swimmers (P = 0.034) (Myerson *et al.* 1999). Woods *et al.* (2001) hypothesized that the ratio of the I- and D-alleles in swimmers competing over different distances would differ in a manner similar to that of Olympic runners in the study of Myerson *et al.* (1999) where a linear trend was found between the I-allele and distance run. Samples were collected from 56 elite Caucasian swimmers competing at the European and Commonwealth championships and from 47 non-elite swimmers from an American college team. ACE genotypes and D-allele frequencies were compared to those of a large (n = 1248) age-matched (19.7±2.5 years) Caucasian control group and with other groups quoted in the literature. Significant differences were shown for the D-allele only in the elite swimmers and only when compared to the largest (n = 615-1906) control groups. An excess in the D-allele was shown in swimmers competing over distances shorter than 400m ("*power oriented distances*") (Woods *et al.* 2001). No differences were shown between controls and swimmers competing over distances longer than 400m. When comparing the elite swimmers to the smaller control groups (n = 114 and 189), no significant differences were shown. The fact that significant results were found when a small select group was compared to a large control group suggest that 'genotype enrichment' could have taken place to some extent.

Research has shown the ACE I-allele to be associated with a high VO_{2max} in postmenopausal women (Hagberg *et al.* 1998). Hagberg *et al.* (1998) investigated the relationship between ACE genotype, VO_{2max} and maximal exercise hemodynamics in women with different activity levels. ACE genotype distribution were 0.21 II, 0.57 ID and 0.22 DD; similar to that of the general population (0.23 II, 0.49 ID and 0.28 DD). Female athletes with the II genotype had substantially faster 5- and 10 km race times compared to matched athletes with the DD genotype. ACE II genotype carriers had a significantly higher VO_{2max} (6.3 ml/kg/min higher, $P < 0.05$) than carriers of the DD genotype. Hagberg suggest that the observed difference was the "result of genotype-dependent differences in a-v$D0_2$ and not of stroke volume or maximal cardiac output" (Hagberg *et al.* 1998).

Fatini *et al.* (2000) genotyped 28 elite Italian soccer players and 155 sedentary male controls. Genotype distribution and D-allele frequencies were the same for both athletes and controls. Allele frequencies were 36% and 37% for the I-allele and 64% and 63% for the D-allele in athletes and controls respectively (χ^2= 0.02, P = 0.88). ACE genotypes were 14% and 13% for II, 43% and 48% for ID and 43% and 39% for the DD genotype in athletes and controls respectively (χ^2 = 0.23, P = 0.89). The authors concluded that exercise-induced LV mass changes following seven months of training were associated with the presence of the ACE D-allele (Fatini *et al.* 2000).

"*Individual responses to strength training vary considerably and could be influenced by genetic variables*" (Folland 2000). This statement led Folland *et al.* (2000) to investigate the effect of the ACE I/D polymorphism on the response to isometric and dynamic strength training in 33 male volunteers with no experience of strength training. Subjects followed a nine-week strength-training program consisting of three training sessions per week. One leg of each subject performed only isometric training while the other leg performed only dynamic training; legs were randomly assigned to a specific form of exercise. There were no differences in pre-training strength for the three ACE genotypes. A significant response to isometric training was found to be strongly genotype dependant as can be seen in Figure 1.7. Strength gains were associated with the presence of the D-allele over all strength measurements taken. Dynamic training was a weaker stimulus to strength gains and was not genotype dependant (Folland *et al.* 2000). This is the first study to report a genetic locus that affects individual response to strength training.

Figure 1.7: ACE genotype and the response to functional overload. The graph shows the increase in isometric strength following 9 weeks of isometric training in 33 untrained male subjects (II, n=6; ID, n=17; DD, n=10). (Adapted from: Folland *et al.* 2000)



STUDY	SUBJECTS	*N*	CONTROL	VARIABLE	SIGNIFICANCE	ETHNIC GROUP
Montgomery *et al.* 1997 (52)	British Army recruits	140	Within group analysis	Exercise-induced left ventricular growth following a 10-week basic training period	$P < 0.0001$	Caucasian
Montgomery *et al.* 1998 (54)	1. Army recruits	78	Within group analysis	Repetitive elbow flexion with a 15kg barbell following a 10-week basic training period	$P < 0.05$	Caucasian
	2. High-altitude mountaineers	25	1906[1]	Ascending mountains above 7000m without supplementary oxygen	$P = 0.02$	Not indicated
Gayagay *et al.* 1998 (31)	National level rowers	64	114	Genotype distribution	$P = 0.03$	Caucasian
Hagberg *et al.* 1998 (35)	Post-menopausal women; 19 sedentary, 19 physically active, 20 athletes	58	Within group analysis	VO_{2max} and maximal exercise hemodynamics	$P < 0.05$	Not indicated
Montgomery *et al.* 1999 (51)	British Army recruits	81	Within group analysis	Change in body composition after 10-week basic training period	$P < 0.05$	Caucasian
Myerson *et al.* 1999 (57)	1. Olympic level runners; grouped n = 20 ≤ 200m, n = 37 400-3000m, *n* = 34 ≥5000m, all distances; 79 Caucasian *	91	1906[1], Within group analysis	Genotype distribution with distance run	$P = 0.009$ for linear trend, skew toward I allele in ≥5000m and toward D allele in ≤ 200m	Caucasian, black
	2. Olympic level swimmers	64	1906[1]	Genotype distribution	$P = 0.034$	Not indicated
Taylor *et al.* 1999 (84)	Elite athletes; 26 hockey, 25 cyclists, 21 skiers, 15 track and field, 13 swimmers, 7 rowers, 5 gymnasts, 8 other **	120	685[2]	Genotype distribution	No association	Caucasian
Williams *et al.* 2000 (89)	Army recruits; 35 II, 23 DD genotype	58	Within group analysis	Training induced changes mechanical working efficiency of skeletal muscle	↑8.62% II ↓0.39% DD	Caucasian
Fatini *et al.* 2000 (26)	Elite soccer players	28	155	Left ventricular mass in response to 7 months training	$P = 0.89$	Caucasian

Rankinen *et al.* 2000d (71)	Male elite endurance athletes; 59 cross-country skiing, 40 biathlon, 2 nordic combined, 20 long-distance running, 19 middle distance running, 48 road cycling	192	189	Genotype distribution	$P = 0.264$	Caucasian
Rankinen *et al.* 2000b (69)	Sedentary volunteers; 476 Caucasian from 99 families, 248 black from 104 families	724	Within group analysis	Response of VO_{2max} to 20-week endurance-training program	No association	Caucasian, black
Folland *et al.* 2000 (28)	Recreationally active male volunteers	33	Within group analysis	Response of quadriceps muscle to strength training	$P < 0.05$	Not indicated
Woods *et al.* 2001 (91)	Swimmers; 56 Commonwealth & European Championship, 47 non-elite American College level	56	1248[3]	Genotype distribution	$P < 0.05$†	Caucasian
Alvarez *et al.* 2000 (1)	Elite athletes; 25 cyclists, 20 distance runners, 15 handball players	60	400	Genotype distribution	$P < 0.05$	Caucasian
Sonna *et al.* (2001) (82)	US Army recruits; 62 male, 85 female	147	Within group analysis	Genotype distribution and response to basic training measured by standardized US Army fitness tests	No association	Caucasian, African-American, Hispanics, Asians, Native American

n = actual number of elite/experimental group that completed training period
[1] British males free from clinical cardiovascular disease
[2] electoral roll community control group
[3] compared with 4 control groups *n* = 615-1906
† only for truly elite Commonwealth & European Championship swimmers *n* = 56 competing in distances of less than 400m

Table 1.5: Summary of ACE I/D polymorphism and exercise performance studies

1.2.2 Studies Reporting No Association

Taylor *et al.* (1999) reported no differences in ACE genotype frequency between a group of elite athletes and sedentary individuals. The study group consisted of 81 male and 39 female elite athletes (n = 120) from a variety of sporting disciplines and 347 male and 338 female controls (n = 695). All athletes had obtained Australian national colours in their sport. All sports were classified as "highly aerobic", and included hockey, cycling, skiing, track and field, swimming, rowing and gymnastics. Genotypes were 30 and 29% for DD, 47.5 and 49% for ID, 22.5 and 22% for II in athletes and controls respectively (Taylor *et al.* 1999). Allele frequencies were 54 and 53% for the D-, 46 and 47% for the I-allele, for athletes and controls respectively. The classification of these sports as aerobic may not be true and the authors did not indicate whether for example, cyclists were road (more aerobic) or track (power dependant) and the type of track and field events athletes took part in, which makes interpretation of results problematic.

Rankinen *et al.* (2000d) studied the ACE I/D polymorphism in 192 male endurance athletes (mean VO_{2max} of 78.6 ± 3.2) and 189 sedentary male controls (mean VO_{2max} of 36.4 ± 7.4). Athletes were from Canada, Germany, Finland and the United States and competed in cross-country skiing, biathlon, Nordic combined, long-distance running, middle-distance running and road cycling. All athletes had competed at national and international level. The control group was from the same geographic areas as the athletes and of approximately equal number to that of the athletes. All were healthy Caucasians. ACE I/D genotype frequencies did not differ among sports. Values were 0.248, 0.495 and 0.257 in skiers, 0.256, 0.436, 0.308 in runners, and 0.292, 0.438, 0.271 in cyclists for the II, ID and DD genotypes respectively (χ^2 = 7.66, P = 0.264). Genotype frequencies were similar for countries of origin (χ^2 = 7.66, P = 0.264). No differences were observed for either allele or genotype frequencies between athletes and controls. Genotype frequencies were 0.265 and 0.196 (II), 0.464 and 0.476 (ID), and 0.271 and 0.328 (DD), for athletes and controls respectively (χ^2 = 3.09, P = 0.214). Allele frequencies were 0.497 and 0.434 for I, and 0.503 and 0.566 for the D-allele (χ^2 = 2.84, P = 0.096). The authors concluded that the study does not support the hypothesis that the ACE I/D polymorphism has an influence on performance in endurance sports (Rankinen *et al.* 2000d).

As part of the HERITAGE Family Study, Rankinen *et al.* (2000b) examined the relationship between ACE I/D genotype and physical performance related phenotype. The study group consisted of 476 sedentary Caucasian subjects from 99 families and 248 sedentary black subjects from 104 families. The Caucasian group consisted of 229 male and 247 female

subjects and the black group of 88 men and 160 women. Subjects completed a 20-week endurance-training program. Pre- and post measurements were taken for a maximal, a submaximal and a submaximal/maximal cycle ergometer test. The training program increased VO_{2max} by 16.7 ± 9.4, 17.0 ± 8.9, 22.6 ± 11.2 and 17.4 ± 8.9% in Caucasian parents, Caucasian offspring, black parents and black offspring, respectively. Maximal workrates were increased by 28.6 ± 14.7, 28.5 ± 13.4, 36.1 ± 25.3 and 30.9 ± 15.0% in Caucasian parents, Caucasian offspring, black parents and black offspring, respectively. Allele frequencies were 0.468 (I) and 0.532 (D) for Caucasians, and 0.416 (I) and 0.584 (D) for black subjects. Genotype frequencies were in Hardy-Weinberg equilibrium for both races. In Caucasians, none of the major endurance phenotypes tested were associated with ACE I/D genotypes. Baseline HR at 60 and 80% of VO_{2max} showed association with ACE genotype due to higher values for the ID genotype in black parents. Baseline lactate concentrations at 60 and 80% of VO2max workload were weakly associated with the I/D polymorphism. DD homozygotes showed significantly lower lactate concentrations at 60 and 80% of VO_{2max} workloads. All responses to the 20-week endurance training program were similar for all ACE genotypes in both races for Black- and Caucasian parents and for black offspring. Caucasian offspring of the DD genotype showed the greatest increases in VO_{2max}, VO_2 at 80% of max, maximal power output, work rate at 60 and 80% of VO_{2max} and HR decrease at 50W (P = from 0.042 to 0.0001 when adjusted for age, gender, body mass, phenotype). Ventilation also showed some association with the ACE I/D polymorphism. Cardiac output, stroke volume and lactate training responses were not associated with the ACE I/D polymorphism. The authors agree that the insertion/ deletion polymorphism in intron 16 of the ACE gene does not contribute to endurance related phenotypes in sedentary individuals or to physiological responses after following a 20-week progressive endurance-training program (Rankinen *et al.* 2000b).

From the literature review, it is clear that studies report conflicting results regarding the association of the I-allele with enhanced endurance performance, and the association of the D-allele with training induced enhanced muscle power. The studies with positive outcomes (ie. association between the ACE I/D polymorphism and exercise performance) were mostly from one research group (H. Montgomery, J. Folland, S. Myerson, D. Woods; references: Myerson *et al.* 1999, Montgomery *et al.* 1997, 1998, 1999, Woods *et al.* 2001 and Folland *et al.* 2000). It seems that throughout their work, Montgomery *et al.* used the same control group consisting of 1906 sedentary individuals. There is a risk of decreasing power/resolution if a cohort is too homogenous. If a particular set of alleles are 'enriched' in a population and maintained there, 'patients' and 'controls' will harbor them. This implies that

when a particular allele is analyzed in a population where enrichment has taken place, a false positive result will be obtained.

1.3 The ecNOS G894T Polymorphism.

Nitric Oxide Synthase: Basic Physiology and Occurrence.

The mammalian nitric oxide synthase (NOS) enzyme exists in three known isoforms (Fösterman *et al.* 1998). NOS I (NOS1, ncNOS, cNOS) is a constitutively expressed, low-output enzyme present in neurons, myocytes, lung, kidney, testis, skin and adrenal gland among others (Fösterman *et al.* 1998, Fernández-Alfonso and González 1999). NOS II (NOS2, iNOS) is an inducible, high-output enzyme, expressed by activated macrophages (Fösterman *et al.* 1998). NOS III (NOS3, ecNOS, ecNOS), also a low-output constitutively expressed enzyme, is found in endothelial-, smooth muscle- and neuronal cells, bone marrow, platelets and cardiac myocytes among others (Fösterman *et al.* 1998)

Nitric oxide synthase is the enzyme responsible for the synthesis of the vasodilator and anti-proliferative agent nitric oxide (NO) from the amino acid L-arginine. Nitric oxide is produced by the reaction of molecular oxygen with L-arginine to give L-citrulline and NO. NADPH is oxidized to $NADP^+$ in the reaction:

$$\mathbf{O_2 + L\text{-}Arginine} \xrightarrow{NADPH \rightarrow NADP^+} \mathbf{L\text{-}Citrulline + NO}$$

NO is an important regulating factor of the renin-angiotensin system (RAS) at various levels. Evidence exists that angiotensin II (ANG II) may also have a regulatory function on the generation of NO (Fernández-Alfonso and González 1999). New data suggest that ANG II and NO could be viewed as, and integrated into, a homeostatic system directed at the regulation of vascular structure and function (Fernández-Alfonso and González 1999).

Nitric Oxide and the RAS

As discussed in section 1.2, p6, the angiotensin-I converting enzyme (ACE) cleaves ANG I to form the main active peptide of the RAS, namely ANG II. All the physiological functions of ANG II are transmitted through receptors specific for this peptide named AT_1 and AT_2.

NO regulates ACE expression and activity (Fernández-Alfonso and González 1999). A chronic inhibition of endothelial NO synthase (ecNOS) results in up-regulation of both vascular and cardiac ACE activity. Research into the effect of NO on the activity of ACE

suggests that NO might be a direct modulator of ACE activity (Fernández-Alfonso and González 1999). ANG I has no direct vasoconstrictor action, so vasoconstriction elicited by addition of nanomolar amounts (physiological range) to blood vessels reflect the conversion of ANG I to ANG II, and indirectly ACE activity (Fernández-Alfonso and González 1999). Endothelial derived NO reduces the contractile effect of ANG I under these conditions without affecting any contractions elicited by ANG II at the same concentrations. This suggest that NO has a direct effect on ACE activity and might be necessary for the modulation of the conversion rate of ANG I to ANG II (Fernández-Alfonso and González 1999). L-arginine, which serves as substrate for NOS, also reduces serum ACE activity and plasma ANG II levels (Fernández-Alfonso and González 1999). It is currently unclear whether the effect on ACE is mediated by NO or its major metabolites namely NO^{2-}, and/or peroxynitrite ($ONOO^-$). The mechanism of the inhibition remains to be elucidated, but may involve the interaction of NO or its metabolites with the active center of NOS and/or nitrosilation of the cysteine- or tyrosine-residues (Fernández-Alfonso and González 1999).

NO regulates ANG II receptors *in vitro* by decreasing AT_1 receptors through down-regulation of AT_1 gene expression (Fernández-Alfonso and González 1999). Down-regulation occurs as result of a decrease in DNA binding protein acting on the promoter region of the AT_1 receptor gene.

The binding of ANG II to AT_1 receptors causes the release of NO and an increase in the production of cGMP. In the vascular wall, which has AT_1 receptors in both the endothelium and vascular smooth muscle, contraction (vasoconstriction) is the result of direct and simultaneous stimulation of AT_1 receptors in both of these tissues. Stimulation of endothelial AT_1 receptors and subsequent release of vasodilatory NO impairs the direct vasoconstriction caused by ANG II (Fernández-Alfonso and González 1999). Although speculative, it has been suggested that the activation of AT_2 receptors by ANG II may result in bradykinin-dependent increase in NO production, leading to an increase in cGMP production (Fernández-Alfonso and González 1999).

Exercise and shear stress produced by increased blood flow up-regulate the expression of ecNOS (Fösterman *et al.* 1998). ecNOS expression is down-regulated in pulmonary endothelial cells under hypoxic conditions (Fösterman *et al.* 1998). Hypoxia induced down-regulation may contribute to the enhanced endurance performance noted in some individuals training at high altitude. Down-regulated and thus decreased ecNOS leads to decreased production of NO, which is anti-proliferative and would inhibit angiogenesis. The ventilatory response associated with high altitude causes alkalosis, which causes diuresis and a

concomitant decrease in blood pressure. As a result of the lower NO and blood pressure, the proliferative effect of ANG II (which is also decreased at altitude $\xrightarrow{altitude}$ ↓ ACE ⟶ ↓ ANG II) would be more pronounced on angiogenesis and up-regulate angiogenesis in skeletal muscle and other tissue.

Human NOS III mRNA is encoded by 26 exons consisting of 21-22kbp genomic DNA (Fösterman *et al.* 1998). It is a single copy gene, which has been mapped to region q35-36 on chromosome 7. The mRNA encodes a 133 kDa protein. Minisatellites are formed from 9-24bp repeats with the total length varying between 0.5kb and 30kb. A 27 bp repeat has been identified in intron 4 and dinucleotide repeats $(CA)_n$ (intron 13; intron 18) have also been identified within the NOS III gene (Fösterman *et al.* 1998). A variant of the human endothelial NO synthase gene was identified within exon 7. This polymorphism consists of a G→T transversion at nucleotide 894 in the ecNOS complementary DNA (cDNA) (G894T, ecNOS cDNA) resulting in Glu298Asp substitution at protein level (Philip *et al.* 1999).

Some studies have attempted to link polymorphisms of the NOS III gene to a risk of developing cardiovascular disease (Philip *et al.* 1999, Yoshimura *et al.* 2001). Recently, a study by Philip *et al.* (1999) showed coronary bypass patients with the 894T-allele to have an enhanced responsiveness to α-adrenergic (phenylephrine) stimulation. The authors suggest a lesser production of NO in these patients may be the causative factor. The allelic variant may be involved in a conformational change in the ecNOS protein, which may induce an alteration in the NO pathway.

It is uncertain and remains to be determined whether the G894T polymorphism of the ecNOS gene gives rise to direct functional alterations in the NO pathway.

1.4 The ecNOS G894T Polymorphism and Exercise Performance.

Rankinen *et al.* (2000c) examined the association between the ecNOS G894T polymorphism and endurance training induced changes in blood pressure in the HERITAGE Family Study. A cohort of 471 normotensive Caucasians from 99 families undertook a 20-week endurance training program consisting of three cycle ergometer exercise sessions per week. Blood pressure was measured pre-, during and post-exercise. Endurance exercise induced significant changes in resting heart rate, systolic- (SBP50) and diastolic blood pressure (DBP50), cardiac output (Q) and rate pressure product (RPP50, an index of the myocardial workload) during submaximal exercise performed at 50W on a cycle ergometer. The response of DBP50 to endurance training showed a significant association (P = 0.0005) with

the ecNOS G894T genotype. Results were similar for men and women and Glu298 allele homozygotes showed a 3.1mmHg greater reduction in DBP50 than Asp298 homozygotes. The ecNOS G894T genotype accounted for 2.3% of the DBP50 variance. Glu298 homozygotes showed a greater reduction in SBP50 than did Asp298 homozygotes. The Glu298 homo- and heterozygotes also had a greater reduction in RPP50 than did Asp298 homozygotes following 20 weeks of endurance training (Rankinen *et al.* 2000c). The authors concluded that the ecNOS G894T polymorphism was associated with decreases in submaximal exercise diastolic pressure and rate-pressure product induced by endurance training. No association studies between the ecNOS G894T polymorphism and elite level exercise performance have been published.

1.5 Aims and Objectives

The aim of the present research project was to determine whether the ACE I/D and ecNOS G894T polymorphisms contribute to the variation in performance seen between elite and control rugby players and between elite and control roadrunners. Several studies have indicated that the ACE I/D polymorphism may be a useful marker for athletic performance in both endurance and power-oriented sports. At the inception of this project it was reasoned that the I-allele of the ACE I/D polymorphism may be found in higher frequencies in distance runners and that the D-allele may be found in higher frequencies in rugby players (mixed power and endurance sport). A positive outcome would mean that the ACE I/D polymorphism might be a useful marker for athletic performance. This project also aimed to introduce a variety of new molecular techniques into a basic biochemistry laboratory.

The ecNOS G894T genotyping was done by Dr Marios Cariolou, Human Population Research Unit, The Cyprus Institute of Neurology and Genetics, Nicosia, Cyprus. This work was done as part of an ongoing collaboration between the researchers at the UCT/MRC Research Unit for Exercise Science and Sports Medicine and Dr Cariolou. Sample collection, DNA extractions and data analysis on the ecNOS G894T polymorphism was done as part of the present research project.

1.6 Plan of the Thesis

This thesis was structured to include five chapters, appendices and a list of references cited.

- Chapter 1 consists of a discussion on the development of the field of exercise genetics up to the present, a section on the physiology of the renin-angiotensin system and an overview of exercise studies on the ACE I/D polymorphism. A brief overview of the ecNOS G894T polymorphism, the physiology of the nitric oxide synthase system and the aims of this project conclude this chapter.
- Chapter 2: Materials and methods. This chapter describes all sample collection procedures, athlete selection and consent forms and questionnaires used in the present project. It includes a detailed description of DNA extraction methods, ACE genotyping using PCR and statistical methods.
- Chapter 3: Results. Tables and graphs describing both the SA rugby and SA roadrunner cohorts.
- Chapter 4: A critical discussion of the results in Chapter 3.

- Chapter 5: Concluding remarks, tentative conclusions and future prospects in the field of marker identification for athletic performance.
- Appendices include South Africa's best ever marathon-running times, all Consent Forms and Questionnaires, ethics approval, Buffers and Solutions, Reagents and Supplier Contact Details and the database described in Chapter 2.

CHAPTER 2

MATERIALS AND METHODS

2.1 SAMPLE COLLECTION

Blood samples

After signed informed consent (2.3, p43) was obtained, a 5ml venous blood sample was collected from the anterior or medial cubital vein into a purple top vacutainer tube containing ethylenediaminetetraacetic acid (EDTA) and mixed gently but thoroughly by inversion to prevent clotting. Medical doctors or nursing sisters took all the blood samples. The tubes were labelled immediately with the person's name and surname or a code that corresponded to a code on the consent form and questionnaire. At large marathons like the Two Oceans and the South African (SA) Marathon Championships the runners' race number were used as a code to number the tubes. This was done to save time when people were recruited as they finished the race and samples had to be taken quickly. The numbers were later cross-referenced with official race results, which included race times and the participant's full name. All runners were approached in the winner's circle after the race and consent forms were signed before samples were taken.

All freshly collected samples were immediately put on ice after they were drawn and before being transported. At marathon road races, the whole sampling process would usually take around two to three hours from the time the race leaders finished until the last runners crossed the line. Samples were kept on ice to prevent possible heat associated cell lysis and subsequent degradation of genomic DNA by deoxyribonuclease (DNase) present in the cell lysate. Being kept on ice did not freeze the samples.

Samples were kept at 4° C if they could be processed within five to seven days or frozen at –20 ° C if this was not the case. The latter blood samples from runners or other athletes that could not be processed within reasonable time were centrifuged (Sanyo MSE MISTRAL 200R, SANYO Gallenkamp PLC) at 2000 rpm for 10 minutes and the buffy coat (white cell layer) was removed by pipette (Gilson P1000, LASEC, Cape Town). One milli-liter (ml) Freeze Mix (Appendix 4, Buffers and Solutions, p124) at room temperature was then added to the buffy layer in 1.8ml CryoTubes™ (AEC-Amersham) in a 1:1 volume/volume (v/v) ratio and carefully mixed by inversion after which it was stored at –80° C, until it could be

processed. The ratio of Freeze Mix to buffy layer is only an approximation and the Freeze Mix works well if the ratio is close to 1:1 v/v.

Buccal samples

Buccal mouthwash samples were collected in numbered sterile 50ml polypropylene Corning centrifuge tubes with screw-on caps (Adcock Ingram CR Care (PTY) Ltd, Division: Sterilab Services) containing 15ml sterile 0.9% sodium chloride (NaCl). The tubes were handed out with the consent form and questionnaires and the sampling procedure was explained to the subject. To collect buccal cells, subjects had to pour the 0.9% NaCl solution into their mouth and vigorously rinse with the solution without gargling. Subjects were instructed to lightly abrade the insides of the cheeks with their teeth to loosen more buccal cells. The subjects handed back their mouthwash sample together with the completed forms and the sample number was noted on each individual form and on the tubes containing the buccal sample. Samples were kept on ice and processed immediately, where possible, or stored at 4° C and processed within three days of sampling.

Geographical sample collection areas

Runners' samples were collected in the Western Cape and Mpumalanga. The Western Cape samples, which made up the majority of samples, came from road races held in the greater Cape Town and Stellenbosch. Races included The Cape Town Marathon and SA Marathon Championships (1999 and 2000), the Two Oceans marathon (1999 and 2000), The First People's Marathon 1999, the Winelands Marathon and half marathon (1999), the 20 Miler Classic (1999) and Ironman South Africa (2000 and 2001). Samples were also taken from most of the SA athletes who competed in the 2000 Sydney Olympics and from individual elite athletes that were tested at the Sport Science Institute of South Africa during 1999-2001. Samples were taken in Malelane, Mpumalanga at the Selati 32 km and 10 km (2001) because of a need to increase samples of black control runners.

Rugby samples were collected in the Western Cape, Gauteng and Mpumalanga. Most of the elite samples were collected from various national rugby teams tested in Cape Town at the Sport Science Institute of South Africa's High Performance Laboratory. Samples were also taken from the Fedsure Stormers who regularly train at the Institute. Elite samples were also taken in Witbank, Mpumalanga, SA from the Pumas and in Pretoria, Gauteng, SA from the Northern Bulls. Controls samples were taken from university residence and club rugby teams at the University of Stellenbosch.

Permission

All samples taken from individuals who chose to participate in this study were taken after obtaining permission from the relevant sports governing bodies and team doctors and/or race organisers. For rugby samples permission were granted by Dr Ismael Jakut (Chief Medical Officer, South African Rugby Football Union (SARFU)) to take samples of players contracted by, and from teams governed by SARFU. All race organisers, and in the case of the Two Oceans marathon the medical co-ordinator, were approached for permission to take samples at all the races mentioned in 2.1, p39.

2.2 SELECTION OF ELITE ATHLETES AND CONTROLS

Roadrunners

Running and in particular, road running is a world sport. Competitive runners compete over distances ranging from under 5 km to ultra distance events of 100 miles (160 km) or more. Locally, because our weather permits, runners compete year round and there are racing opportunities every weekend of the year in most regions of South Africa.

Most elite level runners would have started running at school level. Here they would compete at school track and field in summer or cross-country meets in winter organised between schools. The better runners would compete at provincial track and field or cross-country championships and if selected would compete at the South African track and field or cross-country championships. In high school (age 14-18), runners usually start to specialize at a specific running distance. Specialization directs a runner's career towards either sprinting (100m, 200m and 400m and hurdling events) or middle/long distance (distances upward of 800m) running. Truly elite runners usually start to emerge at this age (post-pubescent) and continue their competitive careers up into their mid-thirties and race on the lucrative world road running circuit, which includes the great city marathons such as the Boston, New York, London, Berlin and Tokyo marathons.

For the purposes of this project an ELITE runner (EIR) was defined as a runner being able to run a 5 km road race in under 15 minutes and/or 10 km in under 30 minutes and/or 21.1 km (half marathon) in under 70 minutes (≤ 1 hour 10 minutes) and/or 42.2 km (marathon) in less than 145 minutes (≤ 2 hours 25 minutes). Runners were included in the elite group if they were able to run any one of the distances (5 km, 10km, 21.1km 42.2km) in times equal to or faster than the cut-off times. These cut-off times were decided on after consultation with Professor Mike Lambert and Dr Andrew Bosch of the UCT/MRC Research Unit for Exercise

Science and Sports Medicine, Sports Science Institute of South Africa (SSISA). Elite marathon runners were identified using statistical running data on SA best ever marathon-running times provided by Riël Hauman (Statistical Editor Runner's World) (Appendix 1, p116).

A CONTROL runner (ConR) was defined as a runner being able to run a 5 km road race in upward of 25 minutes and/or 10 km in upward of 50 minutes and/or 21.1 km (half marathon) in more than 120 minutes (≥ 2 hours) and/or 42.2 km (marathon) in more than 180 minutes (≥ 3 hours). Runners were included in the control group if they were able to run any one of the distances (5 km, 10km, 21.1km 42.2km) in times equal to or slower than the cut-off times. Because of the *Gaussian* (normal) population distribution of the performance of runners (Figure 4.2, p107) a large proportion of the samples collected were excluded from this study.

Rugby players

Rugby is a major sport in South Africa and various other Northern and Southern Hemisphere countries. It is played in the winter months and is well organized from school to national and international level.

Most senior level players would have started playing rugby at age ten or 11 and continued throughout their school career (approximately 8 years). After school the player joins a local rugby club, where he progresses to senior level teams while playing in local league and other matches. At the larger South African universities like Stellenbosch, Pretoria, Free State and the Randse Afrikaanse Universiteit (RAU), where most of the students reside in campus residences, the university rugby clubs might have several thousand members playing in internally organised so-called 'residence league' matches. Players participating at this level do so for the enjoyment of the game of rugby and most do not strive to reach national or international (Springbok and Super 12) level of play. These players practice once or twice per week for 1.5 to 2 hours and play 90-minute (45 minutes a side) mid-week or weekend matches.

If a player shows exceptional skill and ability, he would be invited to play for the province his club resides in. To make the provincial team a player would first be required to play selection games. The player is then contracted to play for the provincial team or even the international Super 12 or one of the national Springbok teams. During the rugby season, which lasts for approximately ten months, these players are tested on a weekly basis on the playing field and usually practice five or more days per week for upwards of two hours per day. At this level rugby is a physically demanding high intensity intermittent power sport requiring

sprinting ability as well as endurance, physical strength and a high level of hand eye co-ordination.

The rugby study group consisted of 187 male subjects, aged 26 ± 7 (mean, *N* ± standard deviation, sd) years. All elite (ER) subjects (*N* = 78) were volunteers from various SA National rugby teams, which were tested by the High Performance Laboratory, Sports Science Institute of South Africa (Newlands, Cape Town, South Africa) from 1999 to 2001 and provincial teams competing in the international Super 12 Series. 78 samples from SA elite rugby players were collected but 11 samples were excluded from the study cohort on grounds of ethnicity (black *N* = 5, coloured *N* = 6). An elite Caucasian-only cohort and a Caucasian-only control group to avoid possible racial gene skew were used (Barley *et al.* 1991, 1994). The number of samples in the elite group represents about four-and-a-half rugby teams.

Control (CR) samples for the study were collected from Stellenbosch university residence rugby club teams (*N* = 120). The number of samples in the control group represents eight rugby teams. All subjects completed a lifestyle questionnaire and gave their signed informed consent to participate in a genetic study.

2.3 CONSENT FORMS AND QUESTIONNAIRES

Informed consent

All participants in this project and subsequently in the larger project entitled "*Genetic Markers for Athletic Ability, Exercise Performance and Susceptibility to Exercise Induced Injury*" of which this project formed a part, had to sign an informed consent form.

By signing the consent form, the participants indicated that they were fully informed about the study on the " *Genetic Basis of Human Athletic Ability* ". They also agreed to donate five milli-litres (ml) of venous blood and complete the lifestyle questionnaire. The consent form assured subjects that all information collected during the " *Genetic Basis of Human Athletic Ability* " study would be treated with the strictest confidentiality and would only be used for scientific research on the " *Genetic Basis of Human Athletic Ability* ". DNA samples would be used for the purposes of this study as well as the " *Genetic Markers for Athletic Ability, Exercise Performance and Susceptibility to Exercise Induced Injury* " project of which it forms a part, and not for any other purpose. In addition, they were assured that names and personal particulars would not be released under any circumstances and they were also informed that they were free to withdraw from the study at any time, if they wished to do so.

In retrospect, it was realised that the Informed Consent form should also have included an indication of what would happen to the DNA samples once the study was completed. The remaining DNA samples would either be used for further genetic studies using markers for athletic ability and athletic performance or would be destroyed. The choice of what would happen to the samples should have been given to the athletes.
A copy of the consent form is included in Appendix 2, p118).

Lifestyle questionnaire

For the purpose of this research project and related projects that would use the DNA samples that were collected for this study, a lifestyle questionnaire was constructed. With the "*GENETIC BASIS OF ATHLETIC ABILITY QUESTIONNAIRE*" the aim was to collect information on the subject's general phenotypic characteristics and athletic ability. All the DNA samples are still in storage at the MRC/UCT Research Unit for Exercise Science and Sports Medicine, Sport Science Institute of South Africa (SSISA).

Basic contact information was included in case there was a need to resample. Contact information included Surname and First name, Postal and E-mail addresses, Phone and Cell numbers. The contact information would also be used at a later stage to inform the subject on the study outcome, if necessary or required. The DNA extraction and ACE I/D and ecNOS G894T genotyping of samples collected at Ironman 2000 and 2001 was part of a larger multi-discipline study conducted by researchers at SSISA. Triathletes who volunteered for this study were informed *via* email on all aspects that were researched including the outcome of the ACE I/D and ecNOS G894T genotyping.

The questionnaire included general phenotypic data. This data would be used to study possible genotype-phenotype interaction. Phenotypic data included Height (cm), Weight (kg), Gender, Blood and Rh Group, and Ethnic Group. Ethnic groups include: Black/African, White, Indian, Coloured (Mixed Ancestry), Asian and Other. The " *mixed ancestry population in the Cape Province of SA*" is an " *anthropologically distinct population group* ", historically made up of three ethnic groups, which originated in Southern Africa (San, Khoi-Khoi, West African Negro, Madagascan), Asia (Javanese) and Western Europe (Greenberg *èt al.* 1991, 1990).

> " *The Southern African component of this mixed ancestry population includes the San (Bushman), Khoi-Khoi (Hottentot) and Bantu-speaking Negroes. Additional genetic endowment was provided by Negro slaves imported from Madagascar and the West African coast, Asian immigrants from Ceylon, India, Java and Sumatra and Caucasians from Western Europe* " (Greenberg 1990).

Date of birth was also included to determine the subject's age at the time of sampling.
During the course of the study, it was realised that most of the black runners had different ancestral backgrounds. Ethnic origin (ancestry) was subsequently included in the questionnaire but because of the late stage of its inclusion and because of the subsequent incompleteness of the data for the samples used in the present study it was decided not to include ancestry in the analysis. The ancestral backgrounds of both the mother and father were requested.

This study targeted sportsmen from the entire Southern African region and therefore Nationality and Place of Birth was included in the questionnaire. In addition, the study collaborator, Dr Marios Cariolou of the Human Population Genetics Research Unit, Cyprus Institute of Neurology and Genetics, Nicosia, Cyprus, researched the Handedness of the participants and whether the subjects smoked or not and subsequently, this was also included in the questionnaire.
A copy of the lifestyle questionnaire is included in the appendix (Appendix 2, p118).

Sporting details and achievements

In the sporting details and achievements section subjects were asked about the type(s) of sport they competed in, time of involvement and whether they were amateurs or professional athletes (status). Subjects were also asked about the highest level they had competed at for their specific sport (provincial, national or international). Rugby players, were asked about the highest level of play they had achieved in their careers. Unfortunately this was not asked from the outset of sample collection and resulted in incomplete data on level of play and was subsequently not included for statistical analysis. Lastly, subjects were asked about their achievements and to disclose the highest sporting honours that they have achieved.

Training details (road running only)

A separate section was added for the roadrunners to collect data on their running ability. This was deemed important and was used to determine whether samples would be included and grouped into elite or control groups. The "Road Running Questionnaire" included questions on the Running distance (marathon or half/marathon) of the race where the samples were collected. It also included Years Involved in distance running and a section to enter their Personal Best times for 5 km, 10 km, 21.1 km and 42.2 km. Subjects were also asked about the average training per week they had done over the past year and in what year they had done their first marathon or road running event.

2.4 DNA EXTRACTION METHODS

The MRC/UCT Research Unit for Exercise Science and Sports Medicine biochemistry laboratory at the Sport Science Institute of South Africa, Newlands, Cape Town is equipped for general analysis of blood and muscle biopsy samples taken from test subjects at the various cycle ergometer, treadmill, environmental and metabolic laboratories used by the students and researchers of the MRC/UCT Research Unit for Exercise Science and Sports Medicine. Until 1999 blood samples were mainly analysed for lactate, glucose, fatty acids, creatine kinase, adenylate kinase and other enzymes and compounds important in the study of exercise metabolism. Muscle samples were routinely analysed for fatty acid and glycogen content. The present genetic study was the first such a study undertaken by a researcher at this laboratory and subsequently led to the introduction of various new DNA extraction techniques, as well as the use of the polymerase chain reaction (PCR) in the laboratory.

All biological samples were considered to be biologically hazardous. All samples were handled while wearing gloves and discarded into suitable waste containers for incineration. This included all blood collection tubes, pipette tips, needles, gloves and centrifugation tubes.

2.6.1 PARZER'S RAPID METHOD FOR EXTRACTING GENOMIC DNA FROM BLOOD (Parzer and Mannhalter 1991)

Parzer's Rapid Method was the first DNA extraction method to be introduced. It was found to be too laborious and did not suit this project where many hundreds of samples were to be processed within short periods of time. It was also found that Parzer's Rapid Method did not give a high enough DNA yield for the purposes of the major long-term project. In addition the SSISA laboratory did not have the refrigerated centrifuge that was needed for this method at the time and the centrifuge step had to be done in another laboratory, which was laborious.

MATERIALS:

1. Cell Lysis Buffer (Appendix 4, p124), Wash Buffer (Appendix 4), Sarkosyl solution (Appendix 4), Ammonium acetate solution (Appendix 4), Proteinase K (Appendix 4) (keep on ice), Guanidine HCl solution (Appendix 4), cold absolute ethanol (EtOH), 1x TE Buffer (Appendix 4).
2. 250ml polypropylene centrifugation tubes with screw caps (SORVALL®, Separation Scientific, Cape Town), pipettes (Gilson P1000, P200 or Finnpipette®), 200-1000µl and 40-200µl, and a 1-5ml Finnpipette® (AEC-Amersham), Jencons Powerpette Plus

rechargeable pipette controller (Scientific Group) sterile plastic 10ml pipette (LP ITALIANA SPA, #161010) and polypropylene 5ml and micropipette tips (200-1000µl Labtip Blue, #94300220, 5-200µl Labtip Yellow, #94300120, Labtips), water bath at 60 °C, ice, SORVALL® RC5C PLUS centrifuge (Separation Scientific), SLA 1500 Super-Lite (Separation Scientific), vortex, biological waste container, heating block at 37 °C.

METHOD:

Method for 5ml whole blood (or buffy coat from 5ml blood).

1. Mix blood or buffy layer thoroughly by inversion with 50ml Cell Lysis Buffer in 250ml polypropylene centrifugation tubes and incubate on ice for 15 minutes.
2. Centrifuge at 6800 ×G for 10 minutes at 4°C in a SORVALL® RC5C PLUS centrifuge using a SLA 1500 Super-Lite rotor. Decant the supernatant into the biological waste container.
3. Resuspend the remaining pellet thoroughly in 10ml Wash Buffer by gently pipetting up and down using a Gilson P1000 pipette. Top up to 40ml with Wash Buffer using a Jencons Powerpette Plus rechargeable pipette controller and 10ml sterile plastic pipette. Centrifuge the resulting white cell suspension at 6300 ×G for 10 minutes at 4°C in a SORVALL® RC5C PLUS centrifuge using a SLA 1500 Super-Lite rotor.
4. Carefully decant the supernatant into the biological waste container.
5. Vortex the pellet until it is resuspended in the remaining supernatant.
6. To the vortexed white cell suspension, add the following in the order stipulated:

6.1 350µl 20% Sarkosyl solution using a Gilson P1000 pipette.

6.2 250µl 7.5M Ammonium acetate solution using a Gilson P1000 pipette (room temperature).

6.3 3,5ml 6M Guanidine solution using a 1-5ml Finnpipette®.

6.4 125µl cold Proteinase K solution using a Gilson P200 pipette.

7. Incubate the covered tubes in a water bath at 60°C for 10-15 minutes until all the white cells have lysed and the solution is clear.
8. Cool to 0 °C on ice for 10-15 minutes. Precipitate the DNA by adding 10ml ice-cold 100% EtOH and mixing gently by inverting the tube. The DNA is spooled off with a sterile hooked glass rod or an autoclaved yellow micropipette tip.
9. Air dry the DNA by inverting the open Eppendorf tubes on clean tissue paper and leave to dry for 30 to 60 minutes. Resuspend the DNA in 500µl 1×TE Buffer. Store at 4°C in a labelled Eppendorf tube.

2.6.2 WIZARD® GENOMIC DNA PURIFICATION KIT

The WIZARD® Genomic DNA purification kit (Promega, Whitehead Scientific) was used for the preparation of high molecular weight (HMW) DNA according to the manufacturers instructions from fresh and frozen blood samples of uncoagulated blood. The WIZARD® kit was also used for the processing of frozen whole blood samples that had accumulated for three to four months after it was realized that the PARZER'S RAPID (Parzer and Mannhalter 1991) method was not suitable to fulfil our needs. The buffy coats of the accumulated samples were stored at –80 °C after being mixed with Freeze Mix (Appendix 4, p124) in a ratio of approximately 1:1 v/v. The high cost per sample processed using the WIZARD® Genomic DNA purification kit was found to be too high for the numbers of DNA isolations required and this led to the introduction and subsequent modification of the MODIFIED LAHIRI (Lahiri *et al.* 1991) method. However, due to availability of funds for that part of the project, the WIZARD® Genomic DNA purification kit was used for the processing of the Ironman South Africa 2000 samples. In an attempt to save on the cost and improve the final yields of DNA, the Protein Precipitation Solution (Promega, Whitehead Scientific) used in the WIZARD® kit was temporarily used in the MODIFIED LAHIRI method for the precipitation of protein.

2.6.3 MODIFIED LAHIRI (Lahiri *et al.* 1991)

Most standard DNA extraction methods make use of hazardous organic solvents including phenol, chloroform and isoamyl alcohol and also prolonged digestion of protein with Proteinase K. The MODIFIED LAHIRI method overcomes the need for the use of any hazardous organic solvents by the salting out of proteins with saturated sodium chloride (NaCl) or sodium perchlorate ($NaClO_4$). It is an economical, rapid and safe method for the processing of large numbers of blood samples. In the MODIFIED LAHIRI method the salting out step using NaCl was replaced with the more effective precipitation of protein using the Promega Protein Precipitation Solution. It was found that by using this modification, final DNA concentrations would average around 500ng/µl from 5ml of venous blood. These values may seem high but exercise causes white cell migration into the circulation and thus increases circulating white cell counts (Nieman 1997) and all blood samples were taken post exercise. To further reduce cost, the Promega Protein Precipitation Solution step was later replaced with a salting out step using sodium perchlorate, which is a chaotropic salt and thus effective at increasing protein conformational stability and in decreasing its solubility.

MATERIALS:

1. Low Salt Buffer (TKM1, Appendix 4, p124), High Salt Buffer (TKM2, Appendix 4), Protein Precipitation Solution (PPS), 10% SDS (Appendix 4), Isopropanol, 70% Ethanol, 1x TE Buffer (Appendix 4).
2. 2x Falcon® 15 ml High Clarity Polypropylene tubes with screw caps (BD Biosciences), Gilson P1000 and P200 pipettes (AEC-Amersham), Jencons Powerpette Plus rechargeable pipette controller (Scientific Group), sterile plastic 10ml pipettes and 1000µl and 200µl polypropylene micropipette tips (Labtips), water bath at 53-54 °C, marked 1.5ml Eppendorf tubes (Merck Laboratory Supplies Pty. Ltd), bench top centrifuge (Sanyo MSE MISTRAL 200R, SANYO Gallenkamp PLC), Heraeus Biofuge Fresco bench top microcentrifuge (Separation Scientific), vortex, biological waste container.

METHOD:

Method for 5ml whole blood (or buffy coat from 5ml blood).

1. Transfer 5ml whole blood or buffy layer (with EDTA, which is used as the anti-coagulant) into a 15ml polypropylene screw cap centrifugation tube and add 5ml Low Salt Buffer (TKM1).
2. Add 125µL Nonidet P-40[1] (now called Igepal CA-630) to lyse the cells and mix well by inversion.
3. Centrifuge at 2200 rpm in a standard bench top centrifuge for 10 minutes at room temperature to pellet the white blood cells. Carefully pour off the supernatant into the biological waste container and save the pellet. Steps 1 to 3 are repeated twice to produce an almost white pellet.
4. Resuspend the pellet in 0.8ml High Salt Buffer (TKM2) to which 50µL of 10% SDS is added. Pipette gently up and down to prevent frothing of the SDS using a Gilson P1000 pipette to mix and incubate in a waterbath for 10 minutes at 53-54 °C.
5. Add 1.7ml Protein Precipitation Solution and vortex for 20 seconds to precipitate the protein.
6. Centrifuge at 2000×G in a standard bench top centrifuge for 5 minutes to pellet the precipitated protein.
7. Pour the supernatant into a tube containing two volumes isopropanol and gently invert the tube several times until the DNA precipitates.
8. Centrifuge at 2000×G for 30 seconds in a standard bench top centrifuge to pellet the DNA. Discard the supernatant.
9. Wash the DNA pellet using 70% ethanol and centrifuge for 30 seconds in a bench top centrifuge to pellet the DNA.

10. Air dry the DNA by inverting the open Eppendorf tubes on clean tissue paper and leave to dry for 30 to 60 minutes. Resuspend the DNA in 500µl 1×TE Buffer. Store at 4°C in an Eppendorf tube.

[1] Low Salt Buffer (TKM1) can be made up and autoclaved without NP-40. NP-40 is then added as in step 2 in the MODIFIED LAHIRI method. NP-40 can also be added to TKM1 at 25 ml/L before it is autoclaved. The same volume of TKM1 is then used in step one and step two is omitted.

Table 2.1: MODIFIED LAHIRI DNA extraction method. Laboratory quick reference table for different blood sample volumes. TKM1, Low Salt Buffer; TKM2, High Salt Buffer; PPS, Protein Precipitation Solution (Promega, Whitehead Scientific)

Blood volume (ml)	TKM1 (ml)	TKM2 (µl)	PPS (ml)
2	4	340	0.7
2.5	5	425	0.8
3	6	510	1.0
3.5	7	595	1.2
4	8	680	1.3
4.5	9	765	1.5
5	10	850	1.7
5.5	11	935	1.8
6	12	1020	2.0

2.6.4 BUCCAL MOUTHWASH DNA EXTRACTION

Some athletes and especially the elite rugby players refused to give venous blood samples for DNA extraction purposes. It was assumed that they were needle shy or had a real problem with our taking samples because they feared being tested for illegal performance enhancing substances (doping). Even after explaining the aims of the study, they refused to give samples. Meyerson *et al.* (1999) mentioned using a buccal mouthwash in their study on British athletes with Olympic potential. The method was obtained from H. Montgomery, UCL Department for Cardiovascular Genetics, Rayne Institute, London *via* email correspondence (h.montgomery@ucl.ac.uk). This method has a low DNA yield but DNA concentrations were sufficient for the polymerase chain reaction.

Caution: Chloroform ($CHCl_3$) is used in this method. Chloroform is a halogenated hydrocarbon which is nephrotoxic and hepatotoxic and is metabolically activated by the microsomal enzyme system (Cytochrome P-450). Use with caution.

MATERIALS:

1. Lysis Buffer (Appendix 4, p124), 5M $NaClO_4$, Chloroform, 100% Ethanol, 70% ethanol, 1x TE Buffer (Appendix 4), sterile 0.9% NaCl solution (Appendix 4), 5M $NaClO_4$ (Appendix 4).
2. Autoclaved marked 1.5ml Eppendorf tubes (Merck Laboratory Supplies Pty. Ltd), sterile 50ml polypropylene Corning centrifuge tubes with screw-on caps (Adcock Ingram CR Care (PTY) Ltd), Gilson P1000 and P200 pipettes (AEC-Amersham), autoclaved 1000µl and 200µl micropipette tips (Labtips), bench top centrifuge (Sanyo MSE MISTRAL 200R, SANYO Gallenkamp PLC), Heraeus Biofuge Fresco micro centrifuge (Separation Scientific), vortex, biological waste container.

METHOD:

Method for Buccal cells collected in 15ml 0.9% NaCl solution.

1. Label two sets of 1.5ml Eppendorf tubes.
2. Spin down the sputum sample, which was obtained in a 50ml polypropylene tube with a screwcap at 3000 rpm for 2 minutes in a standard bench top centrifuge.
3. Pour off the saliva and saline into a biological waste container leaving the buccal cells at the bottom of the tube.
4. Add 500µl Lysis Buffer and mix well by pipetting up and down using a Gilson P1000 pipette.
5. Transfer the cell suspension into one of the labeled Eppendorf tubes. Add 150µl 5M $NaClO_4$ and mix well by pipetting using a Gilson P1000 pipette.
6. Add 500µl chloroform and mix vigorously by pipetting using a Gilson P1000 pipette while being careful not to spill the content of the Eppendorf tube as it is very full at this stage.
7. Centrifuge in a standard bench top microcentrifuge for 3 minutes at 14 000 rpm or maximum speed.
8. Remove all of the supernatant containing the DNA using a Gilson P1000 pipette and transfer into the second labelled Eppendorf tube.
9. Add 1000µl ice cold 100% ethanol and agitate by inverting the tube to precipitate the DNA. Spin the DNA down for 0.5 to 1 min at 14000 rpm in a microcentrifuge.
10. Pour off the supernatant leaving the DNA pellet.
11. Wash the pellet in 70% ethanol to remove DNA destabilising salts. Centrifuge at 14000 rpm for 30 seconds in a microcentrifuge to pellet the DNA.
12. Pour off the ethanol and air dry the DNA pellet.
13. Air dry the DNA by inverting the open Eppendorf tubes on clean tissue paper and leave to dry for 30 to 60 minutes. Resuspend the dried DNA pellet in 50 to 100µl 1×TE-buffer and leave at room temperature for 24 hours. Store at 4°C.

2.6.5 DNA FROM CLOTTED BLOOD (Salazar *et al.* 1998)

On occasion, clotted blood samples were received that had not been taken in purple top vacutainers with EDTA added as an anticoagulant. This method was used to extract the DNA from clotted blood.

MATERIALS:

1. 0.9% saline (Appendix 4, p124), Buffer 1 (Appendix 4), Buffer 2 (Appendix 4), 5M NaCl (Appendix 4), isopropanol, 70% Ethanol, 1x TE Buffer (Appendix 4).

2. Dounce Homogeniser, 2x Falcon® 15 ml High Clarity Polypropylene tubes with screw caps (BD Biosciences), Gilson P1000 and P200 pipettes (AEC-Amersham), 1-5ml Finnpipette® (AEC-Amersham), autoclaved 5ml, 1000µl and 200µl micropipette tips (Labtips), water bath at 56 °C, standard bench top centrifuge (Sanyo MSE MISTRAL 200R, SANYO Gallenkamp PLC), vortex, marked 1.5ml Eppendorf tubes (Merck Laboratory Supplies Pty. Ltd), biological waste container.

METHOD:

Method for 5ml clotted blood.

1. Homogenize clotted blood in 5ml 0.9% saline using a hand held sterile dounce homogenizer[2], transfer to a marked 15ml polypropylene tube and centrifuge in a bench top centrifuge at 3000 rpm for 10 minutes.
2. Discard the supernatant into a biological waste container.
3. Homogenize the pellet in a dounce homogeniser in 5ml 0.9% saline and add another 3ml 0.9% saline using a 1-5ml Finnpipette® to wash the homogenate.
4. Transfer the homogenate to a 15ml polypropylene tube and centrifuge at 1200 ×G for 5 minutes in a bench top centrifuge.
5. Discard the supernatant into a biological waste container.
6. Add 5ml Buffer 1 to the pellet and pipette up and down using a Gilson P1000 to mix. Centrifuge at 1200 ×G for 5 minutes in a bench top centrifuge.
7. Repeat step 6 twice, discarding the supernatant into a biological waste container each time.
8. Resuspend the white pellet in 1.1ml Buffer 2 and mix well by pipetteting up and down using a 1-5ml Finnpipette®. Incubate in a waterbath at 56 °C for 15 minutes.
9. Add 0.5ml 5M NaCl using a Gilson P1000 and vortex for 30 seconds followed by centrifugation at 1200 ×G for 5 minutes in a bench top centrifuge.
10. Transfer the supernatant to a new marked 15ml polypropylene tube and add 1.5ml isopropanol using a 1-5ml Finnpipette®. Gently invert the tube to precipitate the DNA.
11. Spin the DNA down for 1 minute at 1200 ×G in a bench top centrifuge.
12. Pour off the supernatant and wash the DNA pellet in 5ml 70% EtOH. Centrifuge at 1200 rpm for 1 minute and pour off the 70% EtOH.
11. Air dry the DNA by inverting the open 15ml polypropylene tubes on clean tissue paper and leave to dry for 30 to 60 minutes. Resuspend the dried DNA pellet in 300µL 1x TE-buffer and transfer to marked Eppendorf tubes. Store at 4° C.

[2] Clean the dounce homogeniser after each use by rinsing three times with 100% ethanol followed by autoclaved dH_2O.

2.5 DNA CONCENTRATION AND PURITY

If the resuspended DNA is reasonably pure, it is possible to do a spectrophotometric determination of the DNA concentration. This method is based on the fact that DNA and other nucleic acids absorb ultraviolet (UV) light between 254 and 260 nm with a maximal absorbance at a wavelength of 260 nm. Because the concentration of base pairs available to absorb the UV light is a direct function of the concentration of the DNA itself, the A_{260} (absorbance at 260 nm) can be used to measure the overall concentration of DNA in a solution.

For DNA, the conversion between an A_{260} (also called OD or optical density) reading and the DNA concentration is as follows:
A_{260}/OD of 1.0 = a concentration of 50 µg/ml of double-stranded DNA

Typically, an A_{280} reading is also taken of the DNA sample. This is because proteins absorb UV light maximally at this wavelength. The A_{280} reading is thus a measure of the degree of protein contamination of the DNA sample. If the DNA sample is completely pure, then the A_{260}:A_{280} ratio (calculated by dividing the A_{260} by the A_{280}) will be 1.8. If the ratio is significantly less than this, then the sample is too contaminated with protein for the spectrophotometric method to be used accurately.

All DNA concentration and purity determinations were performed using a BECKMAN DU®-62 (Beckman Coulter, Inc.) spectrophotometer. All tubes used were labelled with corresponding sample codes.

Ten micro-litres genomic DNA was diluted in 490µl distilled water. Diluted DNA was pipetted into a Starna standard quartz cuvette (Merck Laboratory Supplies Pty. Ltd) (10mm light pathway, 1ml volume) and the absorbency measured at 260 nm (1 OD unit = 50 µg/ml DNA) and at 280 nm. Concentrations were calculated for linear double-stranded DNA (dsDNA) using a dilution factor of 1:50. For example, sample JB-16 had an OD A_{260} = 0.068 and OD A_{280} = 0.040; DNA concentration and purity was calculated as follows:

[DNA] (µg/ml) = A_{260} × dilution factor × 50 = 0.068 × 50 × 50 = 170 µg/ml
A_{260}:A_{280} = 0.068/0.040 = 1.7

After each sample was read, the cuvette was rinsed twice with dH_2O and once with 96% EtOH to clean the chamber and wiped with a soft tissue paper to clean the outer surfaces.

2.6 DNA STORAGE

All samples were stored in 1×TE buffer (Appendix 4, p124) in 1.5ml Eppendorf tubes at 4 °C during the course of this project.

2.7 BASIC PROTOCOL FOR PCR

The polymerase chain reaction (PCR), invented in 1983 by Kary Mullis, is widely used in molecular biology and is a revolutionary technique with many applications (Mullis KB 1990). PCR produces very specific exact copies of short lengths of the target DNA in virtually unlimited numbers. PCR-based diagnostic tests are often used in the identification of viral- and bacterial infection and in the detection of known DNA mutations associated with genetic diseases (Post and Ehrlich 2000).

Reaction mixture

ACE I/D genotyping was undertaken using the following PCR conditions:
100 ng DNA, 20 pmoles forward (ACE 1) and reverse (ACE 3) primers, 15 pmole insertion specific primer (ACE 2), 200 μM dNTPs (Promega, Whitehead Scientific), 1x *Taq* Polymerase Buffer, 1.5 mM $MgCl_2$, 1.25U *Taq* Polymerase (*Taq* polymerase obtained from Dr A Katz, Department of Medical Biochemistry, UCT, katz@curie.uct.ac.za) in a 40 μl total reaction volume.

Primers

ACE I/D polymorphism specific primers (Chiang *et al.* 1998) were obtained from Integrated DNA Technologies, Inc. (IDT, Whitehead Scientific). Forward, reverse and insertion specific primer positions were confirmed on DNASIS® Max Version 1.00.000.057 (Hitachi Software Engineering Co., Ltd.). The ACE gene intron 16 sequence was obtained from PUBMED NUCLEOTIDE (X62855, http://www.ncbi.nlm.nih.gov/entrez /query.fcgi?CMD=Pager&DB=nucleotide). See Figure 2.1, p56 for detail.

Table 2.2: Primers used to amplify the ACE I/D polymorphism in intron 16 of the ACE gene.

Primer direction	Name	Primer Sequences
Forward (5'→3')	ACE 1	CAT CCT TTC TCC CAT TTC TC
Reverse (5'→3')	ACE 3	ATT TCA GAG CTG GAA TAA AAT T
Insertion (5'→3')	ACE 2	TGG GAT TAC AGG CGT GAT ACA G

$MgCl_2$ concentration

Magnesium chloride ($MgCl_2$) concentration is also very important for effective and efficient PCR amplification. Low efficiency of PCR amplification and/or non-specific products may result if insufficient or excess $MgCl_2$ is used. The optimal concentration can be determined by using an $MgCl_2$ concentration range from 1.0 to 4.5 mM.

Annealing temperatures of primers.

Guanine≡cytosine (G≡C) base pairs have one more hydrogen bond than adenine=thymine (A=T) base pairs. This makes G≡C base pairs more heat stable than A=T base pairs. A DNA sequence with a high percentage G≡C base pairing will need a higher temperature to denature than a sequence with less G≡C pairs. When an optimal annealing temperature for a set of primers must be determined, a starting point to work from is often calculated from the GC content of the primer set. The value obtained is called the melting temperature (T_m) and represents the theoretical temperature where 50% of DNA strands will denature. Various formulae exist for the calculation of the T_m-value. A common formula used to calculate the T_m-value of short oligonucleotides (up to 20 bp) is:

$$T_m = 4(G+C)+2(A+T)$$

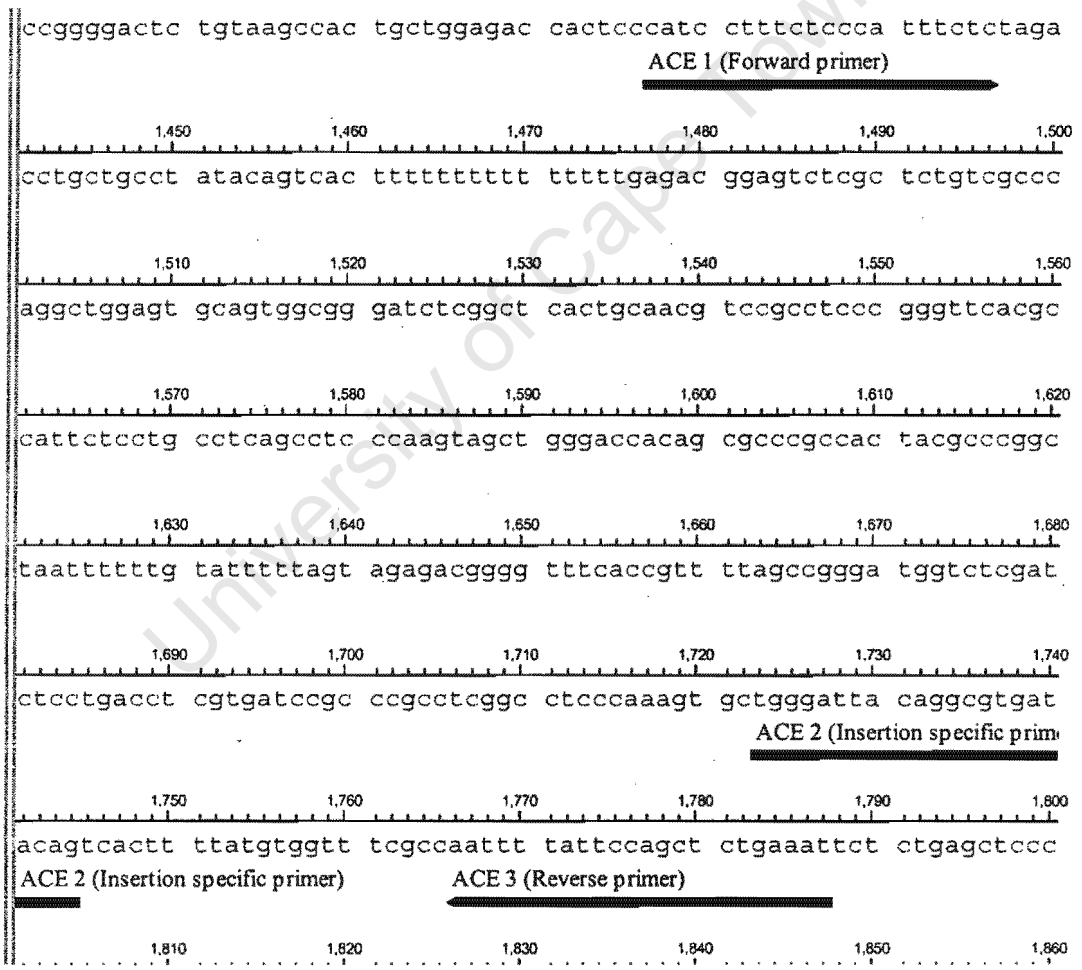
Where T_m = calculated melting time; G = guanine; C = cytosine; A = adenine; T = thymine.

The temperature at which annealing will take place is dependent on the primer with the lowest T_m. Under ideal conditions both primers should anneal at the same temperature. When optimizing annealing conditions the PCR reaction should be performed at various temperatures starting at 5 °C below the calculated T_m.

The following temperature cycling scheme was used in a HYBAID PCR Sprint thermal cycler (Scientific Group, Johannesburg) to amplify the ACE I/D polymorphism:

Five minutes initial denaturation at 94 °C.
Followed by 30 cycles of: 60 seconds denaturation at 94 °C, 60 seconds annealing at 55 °C and 90 seconds extension at 72 °C.
Followed by 7 minutes extension at 72 °C.

Figure 2.1. Position of the ACE I/D polymorphism directed primers on intron 16 of the ACE gene.



Caution: **Ultraviolet (UV) radiation is dangerous, particularly to the eyes. Make sure that the UV light source is shielded and always wear protective eyewear and/or a facemask that blocks UV light.**

2.8 POLYACRYLAMIDE DNA SEPARATION GELS (Ausubel 1987)

Ethidium Bromide (EtBr) is carcinogenic, mutagenic and moderately toxic. Inhalation, swallowing, or absorption through the skin in very small amounts can cause considerable damage to health, and may be lethal. EtBr is an intercalating florescent dye and is used to detect DNA in agarose and polyacrylamide gels.

Precaution: All contact with the human body must be avoided. Use gloves at all times when handling gels and solutions containing EtBr. If you feel unwell, seek medical advice immediately! Use with extreme caution and check for spills periodically with a portable UV light.

Acrylamide and its polymers are extremely toxic, mutagenic and carcinogenic. Unpolymerized polyacrylamide acts as a cumulative neurotoxin. It can do serious neurological damage and the damage is compounded by each subsequent exposure. In its powder form it is easily inhaled and must therefore be handled in a fume hood.

Precaution: All contact with the human body must be avoided. Masks, gloves and safety glasses must always be worn when working with polyacrylamide powder. Prepare gels using pre-prepare liquid stock solutions.

ACE specific PCR products were separated by polyacrylamide gel electrophoresis (PAGE) on vertical 2mm × 150mm × 150mm, 7% polyacrylamide gels. The PAGE system used was constructed by UCT technical personnel and was made of Perspex. A PAGE system such as the BIO-RAD PROTEAN® II or Mini-PROTEAN® II can also be used. The gels were buffered in 1 × Tris-Borate electrophoresis buffer (TBE, Appendix 4, p124) and run at 140 Volt for 60 minutes.

Polyacrylamide gels have the advantage that they form gels with a much more controlled pore size than agarose. This makes polyacrylamide gels useful for the separation of DNA fragments of 100 bp or less. These small DNA fragments tend to run through agarose gels. The polyacrylamide gel matrix is also much stronger than agarose and it is possible to load larger quantities of material onto acrylamide gels because of well size. Pore formation is a chemical process and results from the combined action of polymerization and cross-linking. The resulting gel is very regular and of very uniform pore size.

Polyacrylamide gels are composed of two chemicals, namely monoacrylamide and N,N' methylene bis acrylamide (bis). Ammonium persulphate (AMPS) and N,N,N'N'-Tetramethyl ethylenediamine (TEMED) generates free radicals that cause the acrylamide monomers to polymerise and the bis molecules to form cross-links between the polyacrylamide chains. The size of the pores is controlled by the ratio of bis to acrylamide, which determines the extent of the cross-linking. Pore size is also determined by the overall percentage of acrylamide. Cross-linking is maximal at an acrylamide to bis ratio of 19:1 (or 38:2). Overall acrylamide concentration varies between three percent (w/v) and 20%. For detail on preparing polyacrylamide gels to separate various ranges of nucleotides refer to Table 2.3, p59.

Materials:

Select the acrylamide percentage appropriate for the size of the oligonucleotides to be separated (Table 2.3, p59). Glass gel plates, gel combs and 2mm spacers, power pack, 96% EtOH for cleaning plates, petroleum jelly, Gilson P200 (AEC-Amersham), 200 µl tips (Labtips), 1-5ml Finnpipette® (AEC-Amersham) and 5ml tips (Labtips), large paper clamps, TEMED, 10% AMPS solution (Appendix 4, p124), 100 ml glass beaker, plastic Pasteur pipette, 6x DNA loading buffer (Appendix 4), ethidium bromide staining solution (Appendix 4), 1x TBE buffer (Appendix 4).

Method:

1. Prepare gel plates by cleaning very well by washing in water with a detergent and wiping them thoroughly with 96% EtOH.
2. Assemble the glass gel plates and 2mm spacers down the left and right sides and across the bottom to form a watertight seal to prevent unpolymerized gel solution from leaking out. Clamp the glass plates on the left, right and bottom with large paper clamps to prevent movement of the spacers during handling. Applying small amounts of petroleum jelly where spacers join and on spacer surfaces will help keep spacers in place and help

form a watertight seal. In all cases follow the manufacturers instruction to assemble the electrophoresis apparatus.

3. Follow the guidelines for the preparation of 50ml polyacrylamide gels in Table 2.3, p59 to select the proper gel concentration according to the sizes of the DNA fragments to be separated. Pipet all reagents into a 100 ml glass beaker using the FinnPipette® 5ml and 200-1000 µl pipettes and appropriate tips. Stir the solution to mix using a 5 ml pipette tip. Caution: Wear gloves when working with polyacrylamide.

Table 2.3: Compositions of poly-acrylamide gels of different percentages and resolution ranges. TBE, Tris-Borate electrophoresis buffer; bp, base pair; AMPS, ammonium persulfate.

	Milli-litres (ml) of reagents to cast gels of various concentrations				
% Gel	**10x TBE**	**40% Acrylamide**	**10% AMPS**	**dH_2O**	**Resolution Range**
3.5	5	4.37	0.30	Up to 50ml	1000-2000 bp
5	5	6.25	0.30	Up to 50ml	80-500 bp
8	5	10	0.30	Up to 50ml	60-400 bp
12	5	15	0.30	Up to 50ml	40-200 bp
15	5	18.75	0.30	Up to 50ml	25-150 bp
20	5	25	0.30	Up to 50ml	6-100 bp

4. As an optional step, the acrylamide solution can be deaerated by applying a vacuum. This reduces the chance that air bubbles will form when thick gels are poured.
5. Add 25µl TEMED to the acrylamide solution and stir using a 5 ml pipette tip.
6. Add 350µl 10% AMPS to the acrylamide solution and stir using a 5 ml pipette tip.

The acrylamide solution can be kept at 4 °C to slow down the polymerisation reaction.

7. Pour the acrylamide solution into the space between the two gel plates. With very thin gels it may be necessary to use a syringe to introduce the solution into the space between the plates. Avoid trapping air bubbles and lightly tap the sides to remove if present.
8. Insert the appropriate comb between the two gel plates. Take care to prevent air bubbles from becoming trapped under the comb.
9. Allow the acrylamide to polymerise in a horizontal position at room temperature for 20-60 minutes.

Polymerised gels may be stored for one to two days at 4 °C before they are used. If stored, PAGE gels must be sealed in plastic wrap and a small amount of 1x TBE (Appendix 4, p124) must be applied to the top of the gel to prevent the gel from drying. After polymerisation the comb and bottom spacer can be removed and the gel can be attached to the electrophoresis tank in a vertical position with the notched side facing toward the upper buffer tank.

10. Fill both the upper and lower reservoirs with 1x TBE buffer using a plastic pasteur pipette and buffer to remove excess unpolymerized acrylamide and air bubbles from the wells and also from the bottom of the gel.
11. Mix the PCR samples with 2µl 6x loading buffer and load the samples (generally 5-20µl depending on well size) into the wells using a Gilson P20 or FinnPipette® 5-40µl pipette and appropriate tips. Loading buffers increase the density and add colour to the sample thereby simplifying the loading process. They run through gels at predictable rates. Bromophenol blue runs at approximately the same rate as a DNA fragment of 300 bp. Xylene cyanol FF runs at the same rate as a 4 kb DNA fragment.
12. Connect the power pack with the positive electrode connected to the bottom reservoir and run at 140 Volts for 60 minutes or until the dyes in the loading buffer has run the desired distance. Turn the power off and disconnect the power pack.
13. The gel can now be carefully removed from both of the glass plates and stained in a staining solution with 0.5 µg/ml ethidium bromide in 1xTBE at room temperature for 15 minutes. The DNA can now be visualized by under UV light.

All gels were photographically reproduced and filed.

2.9 STATISTICAL ANALYSIS

All statistical analysis was done using StatSoft, Inc. (2003). STATISTICA (data analysis software system), version 6. www.statsoft.com.

A Chi-square test (χ^2) was used to compare ACE I/D and ecNOS G894T allele and genotype frequencies between elite subjects and controls. The Chi-square (X^2) statistical test was used to " *test the null hypothesis that the proportions are equal or, equivalently, that factors or characteristics are independent or not associated.* " (Dawson-Saunders and Trapp 1994).

Hardy-Weinberg equilibrium implies that gene and genotype frequencies are constant from generation to generation. The validity of the Hardy-Weinberg equilibrium depends on various

assumptions, some may cause large and others small deviations from equilibrium. The goodness of fit between observed and expected frequencies was statistically tested using a chi-square test.

Differences between body weight, height, age, BMI and personal best running times, ACE I/D genotype and ethnic origin were tested with a one-way analysis of variance (ANOVA). The One-Way ANOVA procedure is used to test the hypothesis that there are differences in the means of two or more groups (Dawson-Saunders and Trapp 1994). The Tukey honestly significant difference (HSD) test for unequal number of samples (*N*) was used as Post-hoc test. "*This post hoc test (or multiple comparison test) can be used to determine the significant differences between group means in an analysis of variance setting. The Tukey HSD test is generally more conservative than the Fisher LSD test but less conservative than Scheffe's test.* " (Dawson-Saunders and Trapp 1994). Statistical significance was defined as $p < 0.05$.

The power of a study is defined as " *the probability of rejecting the null hypothesis when it is false or of concluding the alternative hypothesis when it is true. Some people think of power as the capability of a study to detect a true difference.* " (Dawson-Saunders and Trapp 1994). It is very difficult to determine the power of a study in a case such as the present study where a phenotype (distance running or rugby playing ability) which is determined by many genes (*one-to-many relation of phenotypes to genes*) is used to classify or select groups of subjects. A generally accepted rule is to use 30 subjects per genotype to detect true differences with significant power; the ACE I/D polymorphism has three possible genotypes namely II, ID and DD and 90 subjects would thus be required.

Because of the importance of interaction between loci in multifactorial traits such as aerobic endurance, a basic attempt was made to give an indication whether there is any form of interaction between the two loci examined in this project. Cluster analysis was used to give an indication of linkage distance between genotypes and phenotypes.

CHAPTER 3

RESULTS

3.1 Rugby: ACE I/D Polymorphism

General phenotypic characteristics:

The overall study group collected consisted of 78 elite male rugby players (ER) and 120 male controls subjects (CR). 11 coloured (mixed ancestry) and black subjects were excluded from this analysis to prevent a possible racial skew in the results. Thus, the study group presented here consisted of 67 elite Caucasian subjects and 120 Caucasian controls. All subjects were born in South Africa (SA).

Table 3.1a summarizes the general phenotypic characteristics of the elite and control groups. Table 3.1b, p63 shows the highest level of play of SA elite rugby players and control subjects. 70 control subjects did not supply data on level of play.

Table 3.1a: General characteristics of SA elite rugby players and control subjects.

	Elite	Control
N	67	120
Age (years)	26.8 ± 4.8	26.4± 8.9
Height (cm)	186.1 ± 7.9*	182.3 ± 6.2
Body mass (kg)	100.8 ± 14.1*	85.6 ± 10.5
Body mass index (kg/m^2)	28.9 ± 3.4*	25.7 ± 2.8

Values are mean ± standard deviation (sd)

* $p \leq 0.05$

Table 3.1b: Highest level of play of SA elite rugby players and control subjects.

Elite	*N*	Control	*N*
Springboks – Senior	23	**University residence/club**	31
Springboks – U21	10	**High school**	11
Super 12	9	**No rugby played**	8
Other national teams including SA development, SA-A and Upcoming Springboks	25	**Unknown**	70
Total	67	**Total**	120

General phenotypic characteristics of SA elite rugby players and control subjects compared to ACE I/D genotype:

Table 3.2 summarises the data for all general phenotypic characteristic compared to the ACE I/D genotype and arranged in ER and CR groups.

Table 3.2: ACE I/D genotypes in SA elite and control rugby players.

	ACE I/D genotype	Elite (N = 67)	Control (N = 120)
Weight (kg)			
	DD	98.8±13.9*	88.1±11.1
	ID	103.3±15.5*	85.6±10.6
	II	99.5±10.9*	81.3±8.0
Height (cm)			
	DD	185.6±8.4	183.4±5.6
	ID	186.8±7.2	182.1±7.0
	II	185.1±9.4	181.1±7.4
BMI (kg/m^2)			
	DD	28.4±3.1	26.2±2.9
	ID	29.5±3.9	24.8±2.5
	II	28.6±2.6	26.9±3.4

All values are mean ± sd; $p \leq 0.05$

* Significant association between all ACE I/D genotypes, weight and elite status. ACE genotype distribution in SA elite rugby players and control subjects:

ACE I/D genotypes were determined for elite rugby players and control subjects as indicated in Table 3.3. The ACE I/D genotype distribution was in Hardy Weinberg equilibrium.

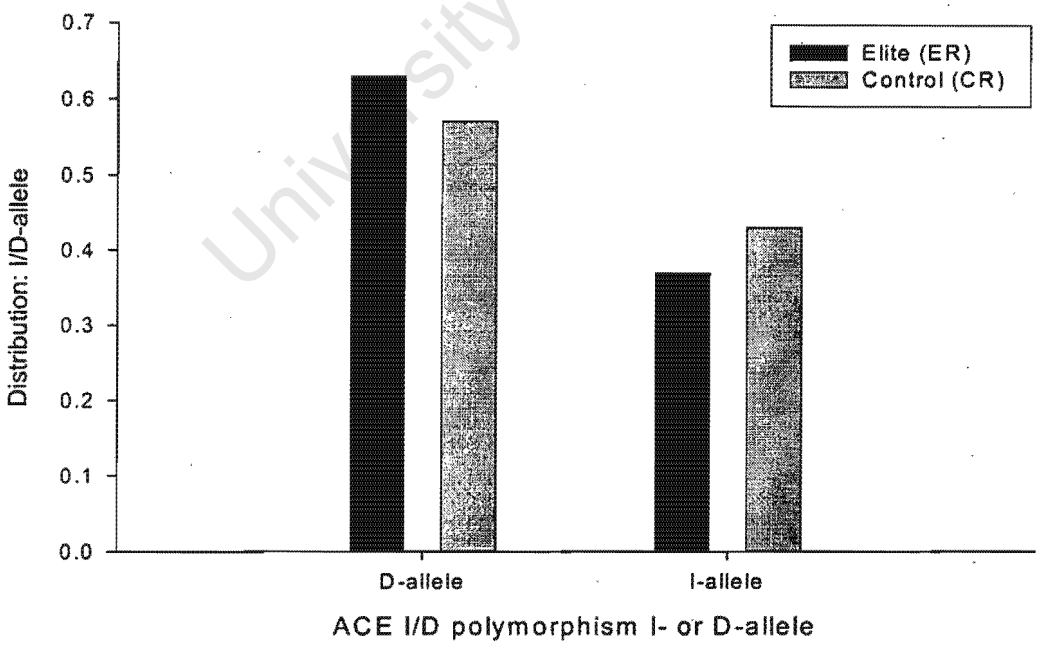
Table 3.3: ACE genotype distribution in SA elite rugby players and control subjects.

ACE I/D genotype	Elite (ER) (*N*=67)	Control (CR) (*N*=120)
DD	41.8(28)	31.7(38)
ID	41.8(28)	50.8(61)
II	16.4(11)	17.5(21)

df, degrees of freedom, Pearson Chi-Square: 2.02, df = 2, $p = 0.36$

ACE I- and D- allele frequency in SA elite rugby players and control subjects:

Figure 3.1: ACE I- and D-allele frequency in SA elite rugby players and control subjects.



Key: *The frequency of the ACE I- and D-alleles in the ER and CR groups was 0.62 and 0.58 for the D-allele, and 0.37 and 0.43 for the I-allele, respectively. (Pearson Chi-square: 1.09, df = 1, p = 0.30).*

3.2 Rugby: ecNOS G894T Polymorphism

General phenotypic characteristics compared to ecNOS G894T genotype:

The ecNOS G894T genotyping was done by Dr Marios Cariolou, Human Population Research Unit, The Cyprus Institute of Neurology and Genetics, Nicosia, Cyprus. This work was done as part of an ongoing collaboration between the UCT/MRC Research Unit for Exercise Science and Sports Medicine and Dr Cariolou. Table 3.4 summarises the data for all general phenotypic characteristics arranged by ecNOS G894T genotype and into ER and CR groups. The sample size of the elite group was small and this could have influenced results.

Table 3.4: ecNOS G894T genotypes in SA elite and control rugby players.

	ecNOS G894T Genotype	Elite (*N* = 35)	Control (*N* = 93)
Weight (kg)			
	GG	98.4 ± 14.6*	83.2 ± 9.7
	TG	104.6 ± 17.6*	85.3 ± 10.3
	TT	103.0 ± 7.3*	86.4 ± 8.9
Height (cm)			
	GG	186.1 ± 7.9	180.5 ± 7.2
	TG	184.1 ± 7.8	182.3 ± 6.1
	TT	188.4 ± 7.1	183.1 ± 5.8
BMI (kg/m^2)			
	GG	27.9 ± 3.3**	25.3 ± 2.3
	TG	30.9 ± 5.1	25.6 ± 2.9
	TT	29.0 ± 2.3	26.6 ± 2.5

All values are mean ± sd; $p \leq 0.05$

* Significant association between all ecNOS G894T genotypes and weight and elite status.

** Significant association between ecNOS GG genotype, BMI and elite status.

ecNOS G894T genotype in SA elite and control rugby players:

The frequency distribution of the ecNOS G894T genotype in the ER and CR groups was determined in SA rugby players. The ecNOS G894T genotype distribution is shown in Table 3.5.

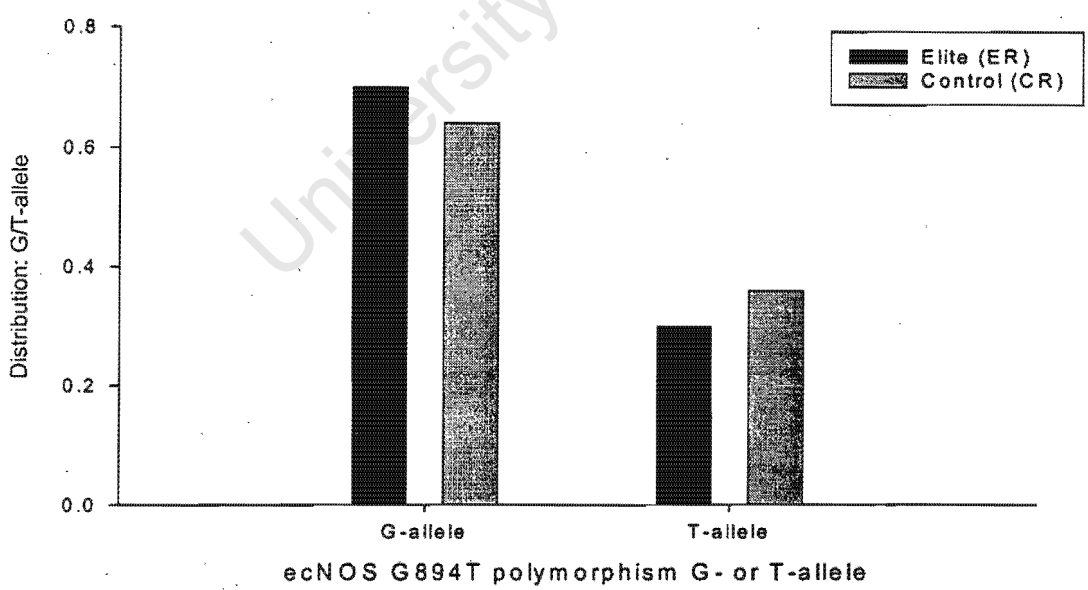
Table 3.5: ecNOS G894T genotypes in SA elite rugby players and control subjects.

ecNOS G894T genotype	Elite (ER) (*N*=35)	Control (CR) (*N*=93)
GG	51.4(18)	43.0(40)
TG	37.1(13)	41.9(39)
TT	11.4(4)	15.1(14)

Pearson Chi-Square: 0.78, df = 2, $p = 0.68$

ecNOS G894T G- and T-allele frequency in SA elite and control rugby players:

Figure 3.2: ecNOS G- and T-allele frequency in SA elite rugby players and control subjects.



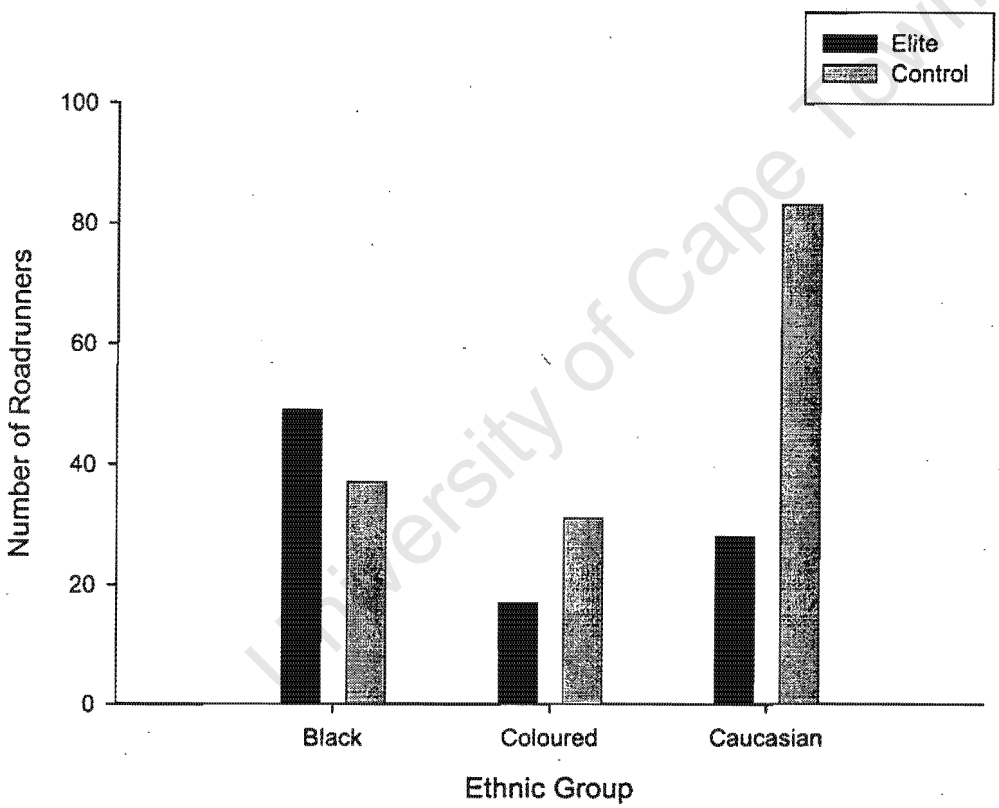
Key: ecNOS G/T-alleles in the ER and CR groups were 0.7 and 0.64 for the G-allele, and 0.3 and 0.36 for the T-allele respectively. G- and T-allele frequency was not significantly different (Pearson Chi-square: 0.82, df = 1, p = 0.37).

3.3 Distance runners: ACE I/D Polymorphism

General phenotypic characteristics:

The overall study group consisted of 94 elite South African roadrunners (EIR) and 133 controls (ConR). Since the ACE I/D polymorphism is not gender linked both male and female roadrunners were used. All subjects were from the Southern African region, which included South Africa, Namibia, Zimbabwe, Botswana and Swaziland.

Figure 3.3: Ethnic composition of the SA roadrunner ACE I/D genotype study group.



Key: *The study group consisted of 49 elite black, 17 elite coloured and 28 elite Caucasian roadrunners. The control group consisted of 37 black, 13 coloured and 81 Caucasian roadrunners.*

Table 3.6 summarises the general phenotypic characteristics of the elite and control SA roadrunner groups.

Table 3.6: General characteristics of elite and control SA roadrunners.

	Elite	Control
N	94	133
Age	29.1 ± 5.7	30.5 ± 9.3
Height, cm	174.2 ± 8.1	172.2 ± 9.4
Body mass, kg	62.7 ± 8.9*	67.0 ± 12.8
Body mass index, kg/m^2	20.8 ± 1.8*	22.7 ± 2.7
5km best times	15.7 ± 2.6*	19.7 ± 2.3
10km best times	31.6 ± 2.3*	41.5 ± 5.3
21.1km best times	70.9 ± 4.9*	92.1 ± 16.3
42.2km best times	146.6 ± 24.6*	198.7 ± 28.8

Values are mean ± standard deviation (sd); * $p \leq 0.05$;

General phenotypic characteristics compared to ethnic origin:

Table 3.7 summarises the data for all general phenotypic characteristics arranged by ethnic origin and into EIR and ConR groups.

Table 3.7: General characteristics of elite and control roadrunners.

	Ethnic origin	Elite	Control
Weight (kg)			
	Black	58.6 ± 6.2*	63.7 ± 9.9
	Coloured	62.7 ± 9.3*	72.4 ±12.4
	Caucasian	70.5 ± 7.8	65.8 ± 12.9
Height (cm)			
	Black	170.7 ± 7.9	168.8 ± 5.5
	Coloured	173.3 ± 4.2	172.4 ± 9.1
	Caucasian	179.6 ± 7.3*	172.1 ± 9.8
BMI (kg/m^2)			
	Black	20.2 ± 1.2	22.0 ± 2.8
	Coloured	20.3 ± 1.8*	24.1 ± 3.5
	Caucasian	21.8 ± 2.0	22.3 ± 2.9

All values are mean ± standard deviation (sd); height, cm; weight, kg; age, years; BMI, kg/m^2; * $p \leq 0.05$

ACE I/D genotype in elite and control South African roadrunners:

ACE I/D genotypes were determined for SA elite and control roadrunners as shown in Table 3.8. The ACE I/D genotype distribution was in Hardy Weinberg equilibrium.

To determine whether ethnic origin had any influence on the distribution of the ACE I/D genotype, the EIR and ConR cohorts were arranged into the three ethnic groups represented in this SA study namely coloured, Caucasian and black.

Table 3.8: ACE genotype distribution in SA elite and control roadrunners.

	ACE I/D genotype	Elite (EIR) (*N*=94)	Control (ConR) (*N*=133)
Distribution	**DD**	27.7(26)	26.7(35)
Distribution	**ID**	53.2(50)	51.1(67)
Distribution	**II**	19.1(18)	22.2(29)

Pearson Chi-Square: 1.55, df = 3, p = 0.67

Table 3.9: ACE genotypes in SA elite and control black, coloured and Caucasian roadrunners.

BLACK SUBJECTS	ACE I/D Genotype	Elite (EIR) (*N*=49)	Control (ConR) (*N*=35)
Distribution	**DD**	30.6(15)	40.0(14)
Distribution	**ID**	51.0(25)	31.4(11)
Distribution	**II**	18.4(9)	28.6(10)

Pearson Chi-square: 3.29, df = 2, $p = 0.19$

CAUCASIAN SUBJECTS	ACE I/D Genotype	Elite (EIR) (*N*=28)	Control (ConR) (*N*=80)
Distribution	**DD**	10.7(3)	22.5(18)
Distribution	**ID**	71.4(20)	60.0(48)
Distribution	**II**	17.9(5)	17.5(14)

Pearson Chi-square: 1.91, df = 2, $p = 0.38$

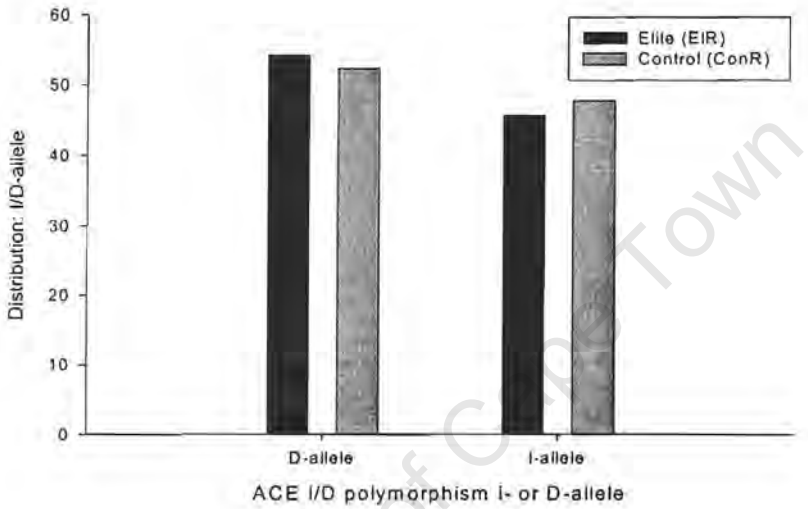
COLOURED SUBJECTS	ACE I/D Genotype	Elite (EIR) (*N*=17)	Control (ConR) (*N*=13)
Distribution	**DD**	47.1(8)	23.1(3)
Distribution	**ID**	29.4(5)	53.8(7)
Distribution	**II**	23.5(4)	23.1(3)

Pearson Chi-square: 2.25, df = 2, $p = 0.32$

The ACE I- and D-allele frequency in SA elite roadrunners and control subjects from different ethnic groups:

The ACE I/D allele frequency was determined for SA elite roadrunners and control subjects and is graphically represented in Figure 3.4.

Figure 3.4: ACE I- and D-allele frequency distribution in elite and control roadrunners.



Key: *The frequency of the ACE I- and D-alleles in the EIR and ConR groups was 0.54 and 0.53 for the D-allele, and 0.46 and 0.47 for the I-allele respectively. The ACE I/D-allele frequency distribution was not significantly different (Pearson Chi-square: 0.04, df = 1, p = 0.84).*

The ACE I/D allele frequency was determined in elite SA roadrunners and control subjects and grouped into the three ethnic groups represented in the present study. Data is shown in Table 3.10.

Table 3.10: The ACE I- and D-allele frequency in SA elite and control roadrunners.

BLACK SUBJECTS	ACE I/D allele	Elite (EIR) (*N*=98)	Control (ConR) (*N*=70)
Distribution	**D**	56.1(55)	55.7(39)
Distribution	**I**	43.9(43)	44.3(31)

Pearson Chi-square: 0.00, df = 1, p = 0.96

CAUCASIAN SUBJECTS	ACE I/D allele	Elite (EIR) (*N*=56)	Control (ConR) (*N*=160)
Distribution	**D**	46.4(26)	52.5(84)
Distribution	**I**	53.6(30)	47.5(76)

Pearson Chi-square: 0.61, df = 1, p = 0.43

COLOURED SUBJECTS	ACE I/D allele	Elite (EIR) (*N*=34)	Control (ConR) (*N*=26)
Distribution	**D**	61.8(21)	50.0(13)
Distribution	**I**	38.2(13)	50.0(13)

Pearson Chi-square: 0.83, df = 1, p = 0.36

To determine whether ethnic origin had any influence on the distribution of the ACE I/D genotype or ACE I/D alleles and personal best running times for the 5km, 10km, 21.1km and 42.2km road races, the EIR and ConR cohorts were arranged into the three ethnic groups represented in this SA study namely coloured, Caucasian and black (Table 3.11 and 3.12, p77).

Table 3.11: ACE genotypes arranged by race, compared to personal best time for different racing distances.

Ethnic origin	ACE I/D genotype	5km best times	10km best times	21.1k best times	42.2km best times
		Elite roadrunners			
Coloured	**DD**	15.3 ± 1.1	31.2 ± 2.3	68.7 ± 2.6	149.7 ± 11.5*
Coloured	**ID**	15.2	31.6 ± 0.5*	72.0 ± 3.7	153.5 ± 9.1
Coloured	**II**	19.1 ± 8.5	35.6 ± 8.11	76.5 ± 14.8	145.0
Caucasian	**DD**	15.2 ± 0.5	31.7 ± 0.9	70.3 ± 4.1	154.0 ± 22.6
Caucasian	**ID**	15.6 ± 1.0	31.7 ± 1.7*	72.7 ± 3.6*	140.1 ± 44.5*
Caucasian	**II**	15.9 ± 0.2	30.8 ± 2.1*	72.7 ± 2.4	153.5 ± 4.9
Black	**DD**	15.3 ± 1.6	31.4 ± 1.7	70.6 ± 5.4	151.7 ± 30.4
Black	**ID**	15.8 ± 2.8	31.7 ± 3.2*	70.8 ± 5.2	146.2 ±11.1
Black	**II**	14.4 ± 0.8	31.0 ± 1.9	67.1 ± 4.0	141.0 ± 9.6
		Control samples			
Coloured	**DD**	19.5 ± 3.5	40.5 ± 9.1	98.0 ± 18.1	243.0 ± 46.0
Coloured	**ID**	20.7 ± 3.3	44.4 ± 7.3	98.6 ± 16.5	215.7 ± 30.3
Coloured	**II**	18.0	36.0 ± 1.0	78.0 ± 3.4	167.3 ± 12.4
Caucasian	**DD**	19.7 ± 3.3	41.6 ± 6.9	91.3 ± 12.6	200.6 ± 26.7
Caucasian	**ID**	19.5 ± 2.0	41.1 ± 2.3	91.1 ± 19.3	192.4 ± 21.2
Caucasian	**II**	20.3 ± 2.3	42.3 ± 3.2	93.4 ± 8.8	200.9 ± 36.1
Black	**DD**	-	40.0	85.0	204.0
Black	**ID**	17.2	43.1 ± 10.2	94.6 ± 17.7	190.9 ± 28.3
Black	**II**	-	-	-	-

* $p \leq 0.05$; for running times compared to corresponding group (ethnic origin and ACE I/D genotype) in control group. All values are mean ± sd. – no running time available.

Table 3.12: ACE I- and D-allele frequency arranged by race, compared to personal best time for different racing distances.

Ethnic Origin	ACE I/D allele	5km best time	10km best time	21.1k best time	42.2km best time
		Elite roadrunners			
Coloured	**D**	15.3 ± 1.1	31.2 ± 2.3	68.7 ± 2.6	149.7 ± 11.5*
Coloured	**I**	18.1 ± 7.2	33.6 ± 5.5	73.5 ± 7.6	150.6 ± 8.1
Caucasian	**D**	15.2 ± 0.5	31.7 ± 1.0	70.3 ± 4.1	153.9 ± 22.6
Caucasian	**I**	15.7 ± 0.9*	31.4 ± 1.8*	72.7 ± 3.4*	142.0 ± 41.2*
Black	**D**	15.3 ± 1.6	31.4 ± 1.8	70.6 ± 5.4	151.7 ± 30.4
Black	**I**	15.5 ± 2.5	31.5 ± 3.0*	70.0 ± 5.7	144.8 ± 10.8
		Control roadrunners			
Coloured	**D**	19.5 ± 3.5	40.5 ± 9.1	98.0 ± 18.1	243.0 ± 46.0
Coloured	**I**	19.8 ± 2.9	41.2 ± 7.0	92.4 ± 16.8	199.6 ± 34.6
Caucasian	**D**	19.7 ± 3.2	41.6 ± 9.9	91.3 ± 12.6	200.6 ± 26.7
Caucasian	**I**	19.7 ± 2.0	41.4 ± 4.1	91.6 ± 17.5	194.5 ± 25.5
Black	**D**	-	40.0	85.0	204.0
Black	**I**	17.25	43.1 ± 10.2	94.6 ± 17.7	190.9 ± 28.4

* $p \leq 0.05$; for running times compared to corresponding group (ethnic origin and ACE I/D genotype) in control group. All values are mean ± sd. – no running time available.

3.4 Distance runners: ecNOS G894T Polymorphism

The ecNOS G894T genotyping was done by Dr Marios Cariolou, Human Population Research Unit, The Cyprus Institute of Neurology and Genetics, Nicosia, Cyprus. This work was done as part of an ongoing collaboration between the UCT/MRC Research Unit for Exercise Science and Sports Medicine and Dr Cariolou. Table 3.13 summarises the data for all general phenotypic characteristics arranged by ecNOS G894T genotype and into EIR (*N* = 75) and ConR (*N* = 99) groups.

Table 3.13: General characteristics of SA elite and control roadrunners arranged by ecNOS G894T genotype.

	ecNOS genotype	Elite	Control
Weight (kg)			
	TG	66.1 ± 11.0	68.2 ± 12.7
	GG	60.9 ± 7.0	66.8 ± 13.1
	TT	72.1 ± 4.9	67.6 ± 13.0
Height (cm)			
	TG	175.8 ± 8.7	174.2 ± 9.9
	GG	173.9 ± 7.1	171.1 ± 8.8
	TT	181.7 ± 10.7	171.6 ± 10.2
BMI (kg/m^2)			
	TG	21.4 ± 2.6	22.7 ± 3.1
	GG	20.5 ± 1.5*	22.9 ± 3.1
	TT	22.2 ± 1.5	22.0 ± 2.3

All values are mean ± sd; *N*, number of samples; * p ≤ 0.05

ecNOS G894T genotype in elite and control SA roadrunners.

The frequency distribution of the ecNOS G894T genotype in the EIR and ConR groups was determined in SA roadrunners. The ecNOS G894T distribution for the total roadrunner cohort is shown in Table 3.14. To determine if the ethnic origin of the athletes contributed to the significant difference shown for the ecNOS G894T genotype distribution between the EIR and ConR groups, roadrunners were grouped into the three ethnic groups represented in this study namely coloured, Caucasian and black (Table 3.15, p80).

Table 3.14: ecNOS G894T genotypes in SA elite and control roadrunners.

	ecNOS G894T genotype	Elite (EIR) (*N*=75)	Control (ConR) (*N*=99)
Distribution	**GG**	68.0(51)	48.5(48)
Distribution	**TG**	24.0(18)	40.4(40)
Distribution	**TT**	8.0(6)	11.1(11)

Pearson Chi-square: 6.72, df = 2, p = 0.03

Table 3.15: ecNOS G894T genotypes in black, Caucasian and coloured SA elite roadrunners and control subjects.

BLACK SUBJECTS	ecNOS G894T genotype	Elite (EIR) (*N*=42)	Control (ConR) (*N*=7)
Distribution	**GG**	81.0(34)	85.7(6)
Distribution	**TG**	14.3(6)	14.3(1)
Distribution	**TT**	4.7(2)	-

Pearson Chi-square: 0.35, df = 2, p = 0.83

CAUCASIAN SUBJECTS	ecNOS G894T genotype	Elite (EIR) (*N*=20)	Control (ConR) (*N*=78)
Distribution	**GG**	50.0(10)	42.3(33)
Distribution	**TG**	35.0(7)	46.2(36)
Distribution	**TT**	15.0(3)	11.5(9)

Pearson Chi-square: 0.82, df = 2, p = 0.66

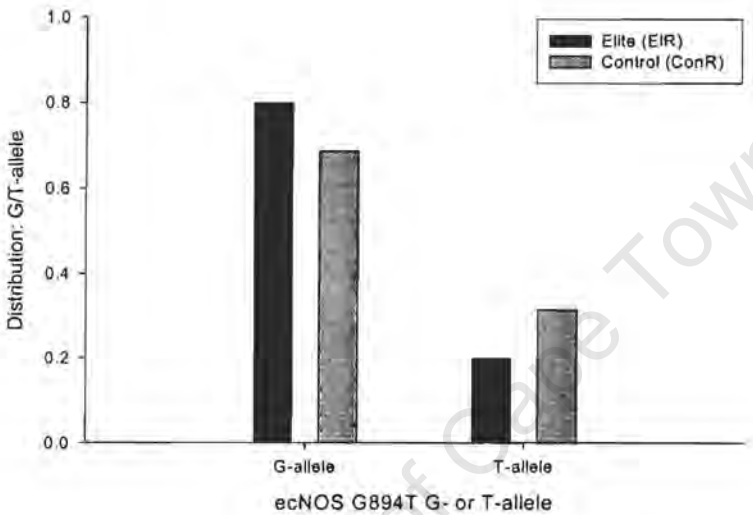
COLOURED SUBJECTS	ecNOS G894T genotype	Elite (EIR) (*N*=13)	Control (ConR) (*N*=13)
Distribution	**TG**	38.5(5)	23.1(3)
Distribution	**GG**	7.7(1)	7.7(1)
Distribution	**TT**	53.8(7)	69.2(9)

Pearson Chi-square: 0.75, df = 2, p = 0.68

ecNOS G894T G- and T-allele frequency in SA elite and control roadrunners.

The ecNOS G894T G/T-allele was determined for SA elite roadrunners and control subjects and is represented in Figure 3.5.

Figure 3.5: ecNOS G- and T-allele frequency distribution in elite SA roadrunners and control subjects.



Key: *The frequency distribution of the ecNOS G/T-alleles in the EIR and ConR groups were 0.8 and 0.69 for the G-allele, and 0.2 and 0.31 for the T-allele respectively. The frequency distribution was significantly different (Pearson Chi-square: 5.29, df = 1, p = 0.02) between the EIR and ConR groups.*

To determine whether one of the ethnic groups represented in this SA study contributed more to the significant difference between the EIR and ConR groups, samples were grouped by ethnic origin into coloured, Caucasian and black in Table 3.16.

Table 3.16: ecNOS G/T allele frequency in black, Caucasian and coloured SA elite roadrunners and control subjects.

BLACK SUBJECTS	ecNOS G894T allele	Elite (EIR) (*N*=82)	Control (ConR) (*N*=16)
Distribution	**G**	87.8(72)	93.7(15)
Distribution	**T**	12.2(10)	6.3(1)

Pearson Chi-square: 0.47, df = 1, p = 0.49

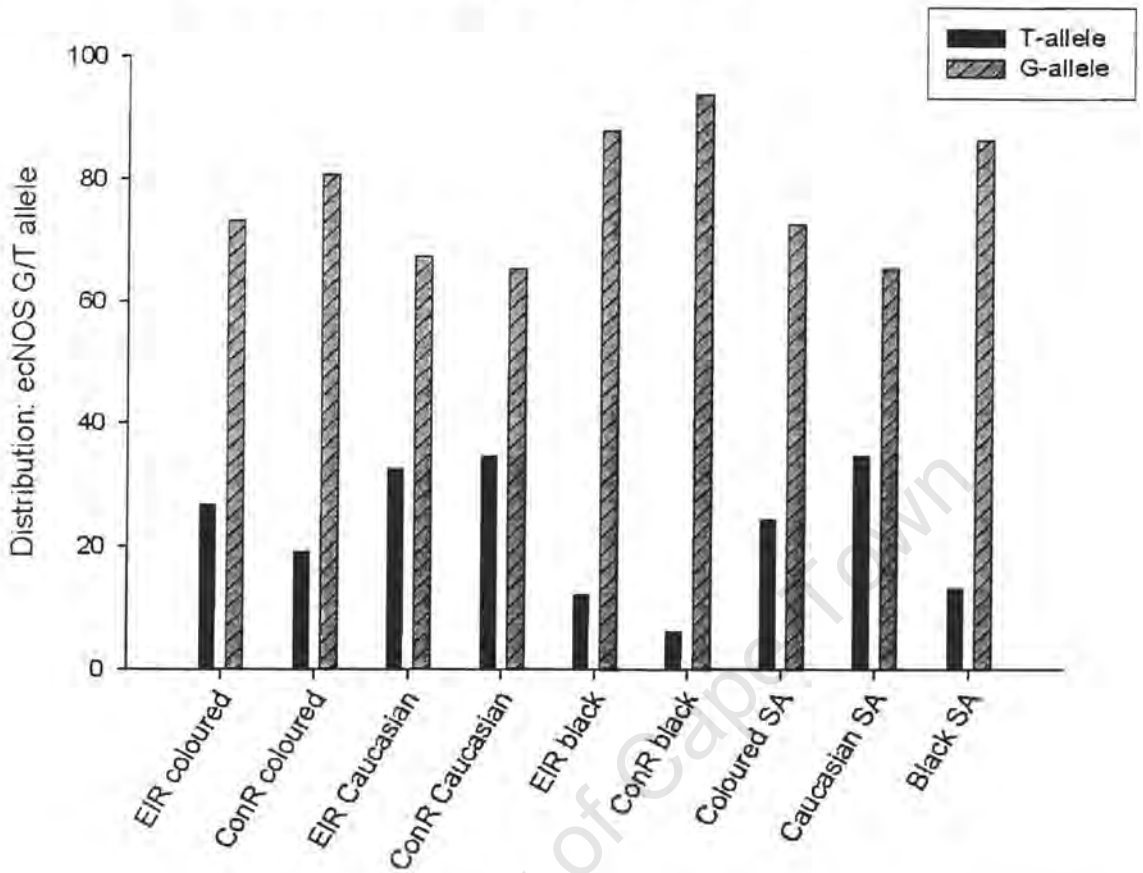
CAUCASIAN SUBJECTS	ecNOS G894T allele	Elite (EIR) (*N*=40)	Control (ConR) (*N*=156)
Distribution	**G**	67.5(27)	65.4(102)
Distribution	**T**	32.5(13)	34.6(54)

Pearson Chi-square: 0.06, df = 1, p = 0.80

COLOURED SUBJECTS	ecNOS G894T allele	Elite (EIR) (*N*=26)	Control (ConR) (*N*=26)
Distribution	**G**	73.1(19)	80.8(21)
Distribution	**T**	26.9(7)	19.2(5)

Pearson Chi-square: 0.43, df = 1, p = 0.51

Figure 3.6: The ecNOS G/T-allele frequency in SA elite roadrunners and control subjects and the general SA sporting population.



Key: *The distribution of the G-and T-allele in SA elite and control coloured, Caucasian and black roadrunners and in the general SA sporting population for the same ethnic groups.*

To determine whether ethnic origin had any influence on the distribution of the ecNOS G894T genotype and G- and T-allele frequencies and personal best running times for the 5km, 10km, 21.1km and 42.2km road races, the EIR and ConR cohorts were arranged into the three ethnic groups represented in this SA study namely coloured, Caucasian and black (Table 3.17, Table 3.18, p85).

Table 3.17: ecNOS G894T genotypes arranged by ethnic origin, compared to personal best time for different racing.

Ethnic origin	ecNOS G894T genotype	5km best time	10km best time	21.1km best time	42.2km best time
			Elite roadrunners		
Coloured	**TG**	14.0	32.7 ± 1.9*	73.3 ± 2.5*	159.0 ± 19.7
Coloured	**GG**	14.9 ± 1.0	31.0 ± 1.6	68.8 ± 2.5	149.1 ± 7.6*
Coloured	**TT**	29.0	45.0	87.0	-
Caucasian	**TG**	15.4 ± 1.1	31.6 ± 1.6*	71.6 ± 4.3	126.6 ± 16.9*
Caucasian	**GG**	16.0 ± 0.6	32.6 ± 1.3*	73.3 ± 2.8	155.8 ± 4.6
Caucasian	**TT**	14.8	31.7	71.9	-
Black	**TG**	15.2 ± 1.2	31.4 ± 1.9	68.3 ± 4.4	167.0 ± 5.3
Black	**GG**	15.4 ± 2.4	31.6 ± 2.9*	70.2 ± 5.2	144.9 ± 9.3
Black	**TT**	16.0	31.1 ± 1.2	69.0	148.0
			Control subjects		
Coloured	**TG**	23.5 ± 2.1	51.0 ± 5.6	114.0 ± 16.3	246.0 ± 7.0
Coloured	**GG**	17.9 ± 1.1	38.4 ± 5.1	87.4 ± 11.8	204.2 ± 43.1
Coloured	**TT**	21.0	40.0	90.0	195.0
Caucasian	**TG**	19.5 ± 2.5	41.2 ± 4.8	92.8 ± 11.8	197.0 ± 24.0
Caucasian	**GG**	19.1 ± 1.1	41.3 ± 4.5	88.7 ± 21.0	192.4 ± 27.2
Caucasian	**TT**	20.9 ± 3.1	41.0 ± 4.7	92.4 ± 12.0	202.6 ± 30.6
Black	**TG**	-	40.0	85.0	204.0
Black	**GG**	17.2	45.6 ± 10.8	94.6 ± 17.7	190.9 ± 28.3
Black	**TT**	-	-	-	-

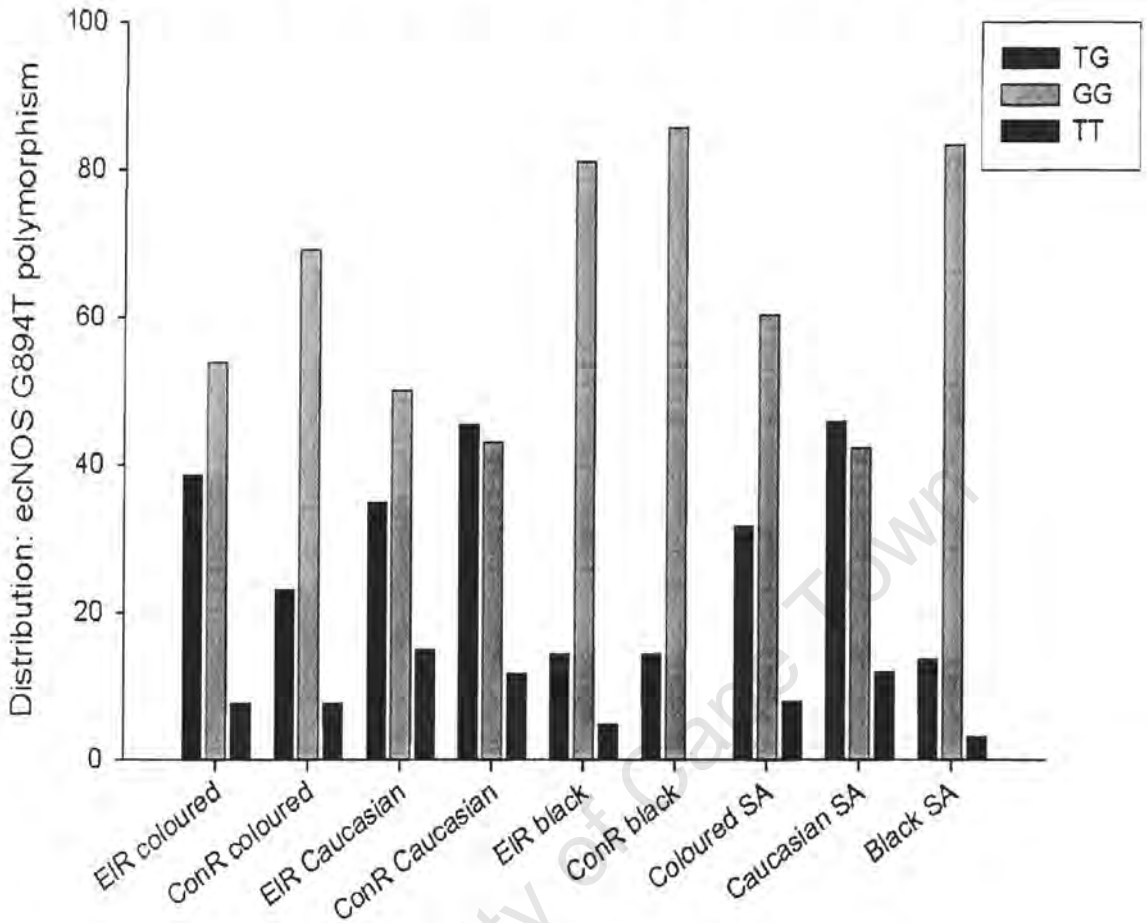
* $p \leq 0.05$; for running times compared to corresponding group (ethnic origin and ecNOS genotype) in control group. All values are mean ± sd. – no running time available.

Table 3.18: ecNOS G- and T-allele frequency arranged by ethnic origin, compared to personal best time for different racing distances.

Ethnic origin	ecNOS T/G allele	5km best time	10km best time	21.1km best time	42.2km best time
			Elite roadrunners		
Coloured	**T**	21.5 ± 10.6	35.8 ± 6.3*	76.7 ± 7.1*	159.0 ± 19.7
Coloured	**G**	14.9 ± 1.0	31.0 ± 1.6	68.8 ± 2.5	149.1 ± 7.6*
Caucasian	**T**	15.3 ± 1.0*	31.7 ± 1.5*	71.6 ± 4.0*	126.6 ± 6.2*
Caucasian	**G**	16.0 ± 0.6	32.6 ± 1.3*	73.3 ± 2.8	155.8 ± 4.6
Black	**T**	15.4 ± 1.0	31.3 ± 1.6	68.4 ± 4.0	163.2 ± 14.7
Black	**G**	15.4 ± 2.4	31.6 ± 2.5*	70.2 ± 5.2	144.9 ± 9.3
			Control subjects		
Coloured	**T**	22.6 ± 2.0	47.3 ± 7.5	108.0 ± 18.0	229.0 ± 29.8
Coloured	**G**	17.9 ± 1.1	38.4 ± 5.1	87.4 ± 11.8	204.2 ± 43.1
Caucasian	**T**	19.8 ± 2.6	41.1 ± 4.7	92.7 ± 11.7	198.3 ± 25.3
Caucasian	**G**	19.1 ± 1.1	41.3 ± 4.5	88.7 ± 21.0	192.4 ± 27.2
Black	**T**	-	40.0	85.0	204.0
Black	**G**	17.2	45.6 ± 10.8	94.6 ± 17.7	190.9 ± 28.3

* $p \leq 0.05$; for running times compared to corresponding group (ethnic origin and ecNOS genotype) in control group. All values are mean ± sd. – no running time available.

Figure 3.7: ecNOS G894T genotypes in SA elite and control roadrunners and the general sporting population arranged by ethnic origin.



Key: *The ecNOS G894T genotype distribution in the three ethnic groups and the overall coloured, Caucasian and black cohorts represented in this SA study.*

4.5 The ecNOS G894T genotype distribution in a population sample of South African athletes.

The ecNOS G894T genotype and allele frequency was determined (Table 3.19a, Figure 3.7, p86 and 3.19b, Figure 3.6, p83, respectively) in a South African population sample of athletes from all sporting disciplines (including running, rugby, triathlon and all Olympic disciplines, section 2.1, p39) for the three ethnic groups (coloured, black, Caucasian) included in the present study.

Table 3.19a: ecNOS G894T genotypes in SA Coloured, Black and Caucasian athletes.

	ecNOS G894T genotype	Coloured (*N*=63)	Caucasian (*N*=504)	Black (*N*=66)
Distribution	**GG**	60.4(38)	42.2(213)	83.3(55)
Distribution	**TG**	31.7(20)	45.8(231)	13.7(9)
Distribution	**TT**	7.9(5)	12.0(60)	3.0(2)

Pearson Chi-square: 43.45, df = 4, $p \leq 0.05$

Table 3.19b: ecNOS G/T allele frequencies in SA Coloured, Black and Caucasian athletes.

	ecNOS G/T allele	Coloured (*N*=119)	Caucasian (*N*=1005)	Black (*N*=124)
Distribution	**T**	24.4(29)	34.6(348)	13.7(17)
Distribution	**G**	75.6(90)	65.4(657)	86.3(107)

Pearson Chi-square: 25.51, df = 2, $p\ p \leq 0.05$

4.6 The ACE I/D genotype genotype distribution in a population sample of South African athletes.

The whole South African population sample of athletes from all sporting disciplines (including running, rugby, triathlon and all Olympic disciplines) was grouped together and the data was analysed to determine the distribution of the ACE I/D genotype in the three ethnic groups (coloured, black, Caucasian) included in the present study. The ACE I/D genotype and I/D-allele frequencies is shown in Table 3.20a and 3.20b.

Table 3.20a: ACE I/D genotypes in SA Coloured, Black and Caucasian athletes.

	ACE I/D genotype	Caucasian (*N*=517)	Coloured (*N*=86)	Black (*N*=147)
Distribution	**DD**	29.6(153)	34.9(30)	32.6(48)
Distribution	**ID**	51.3(265)	40.7(35)	49.0(72)
Distribution	**II**	19.1(99)	24.4(21)	18.4(27)

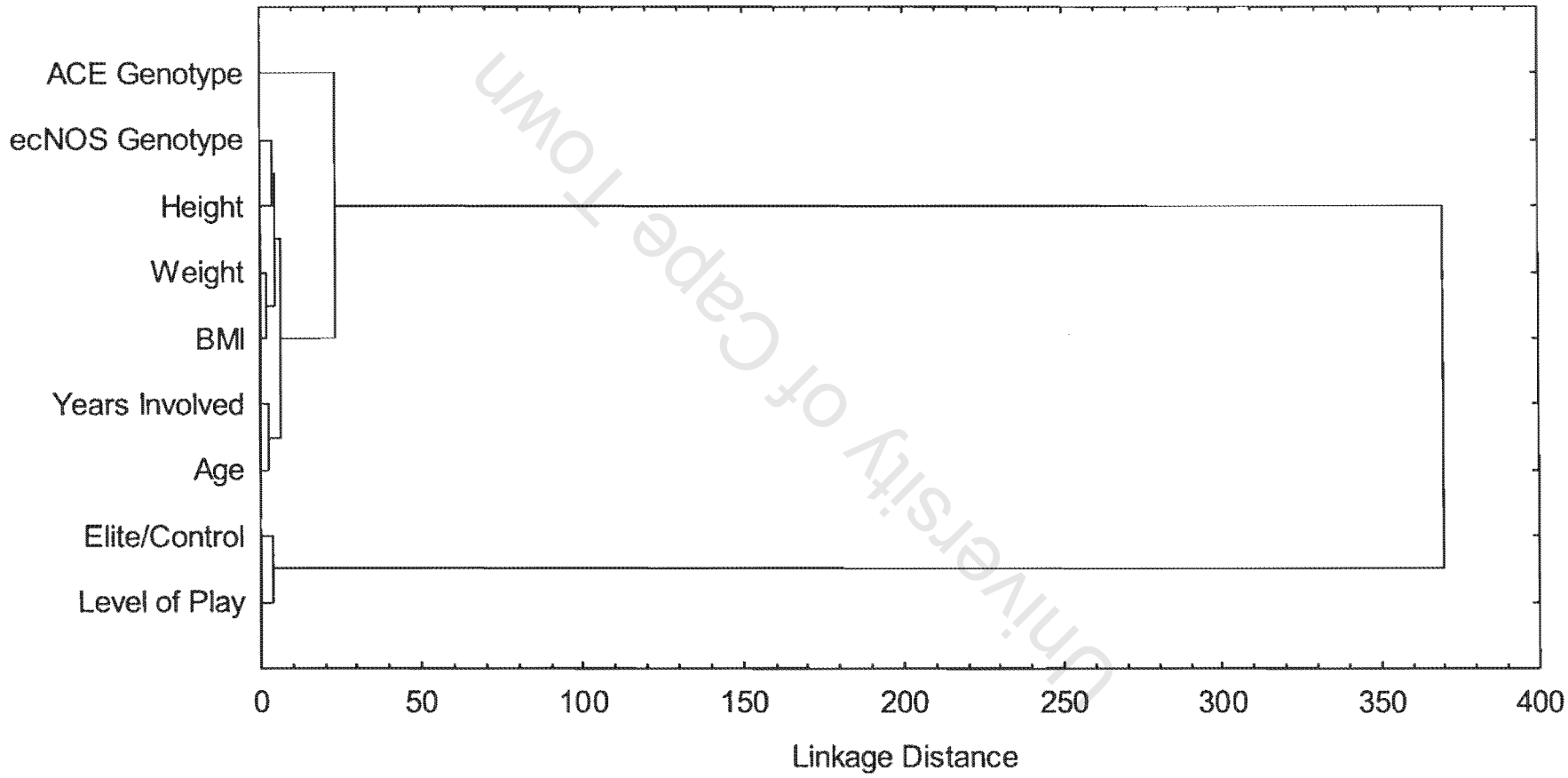
Pearson Chi-square: 3.73, df = 4, $p = 0.44$

Table 3.20b: ACE I/D allele frequencies in SA Coloured, Black and Caucasian athletes.

	ACE I/D allele	Caucasian (*N*=1034)	Coloured (*N*=172)	Black (*N*=294)
Count	**D**	51.8(536)	61.6(106)	65.3(192)
Distribution				
Count	**I**	48.2(498)	38.4(66)	34.7(102)
Distribution				

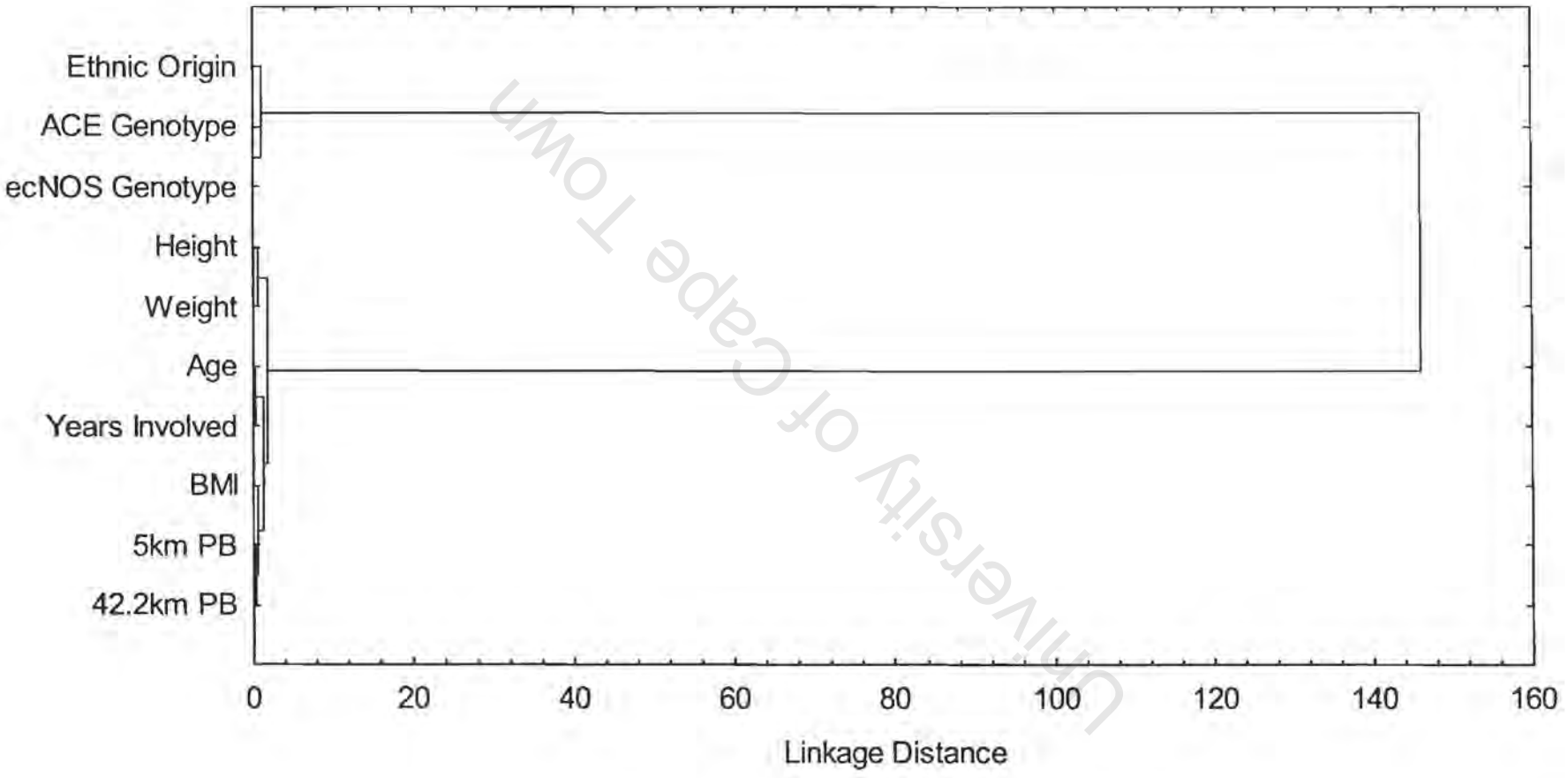
Pearson Chi-square: 19.68, df = 2, $p \leq 0.0001$

Figure 3.8: Horizontal hierarchal tree plot showing linkage distance between phenotypic characteristics, age, years involved and level of play in SA rugby players and ACE I/D and ecNOS G894T genotype.



Cluster analysis of the data for SA rugby players indicate that the ACE I/D and ecNOS G894T genotypes are more closely linked to phenotypic characteristics than to the level of play and elite/control status.

Figure 3.9: Horizontal hierarchal tree plot showing linkage distance between phenotypic characteristics, age and years involved running in SA roadrunners and ACE I/D and ecNOS G894T genotype.



Cluster analysis of the data for SA roadrunners indicate that the ACE I/D and ecNOS G894T genotypes are more closely linked to ethnic origin than any phenotypic characteristic. Personal best (PB) running times over five and 42.2km is more closely linked to the runner's phenotype than to the gene loci investigated in this project.

Interaction between the ACE I/D and ecNOS G894T loci.

Table 3.21: Interaction between the ACE I/D and ecNOS G894T loci in SA roadrunners.

Elite	DD	ID	II
GG	20.0(15)	34.7(26)	13.4(10)
TG	6.7(5)	13.4(10)	4.0(3)
TT	1.3(1)	4.0(3)	2.7(2)

Control	DD	ID	II
GG	8.3(8)	33.3(32)	7.5(7)
TG	10.4(10)	21.8(21)	8.3(8)
TT	2.1(2)	6.2(6)	2.1(2)

Table 3.21 shows the number of individuals in the elite and control roadrunner groups and the interaction between the two loci. There are a low percentage of individuals in both groups that are carriers of four mutant alleles. 20 percent of elite runners are wild type homozygotes for both loci compared to only eight percent of control runners. Although no formal statistical analysis was done, the distribution seems to be similar between the two groups.

Table 3.22: Interaction between the ACE I/D and ecNOS G894T loci in SA rugby players.

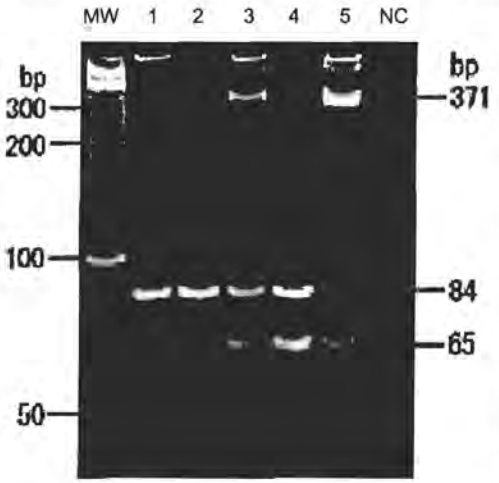
Elite	DD	ID	II
GG	13.3(4)	30.0(9)	-
TG	23.3(7)	13.3(4)	6.7(2)
TT	-	6.7(2)	6.7(2)

Control	DD	ID	II
GG	11.8(10)	27.1(23)	2.3(2)
TG	12.9(11)	18.9(16)	11.8(10)
TT	5.9(5)	8.2(7)	1.1(1)

Table 3.22 shows the frequency and number of individuals in the elite and control roadrunner groups and the interaction between the two loci. A low percentage of individuals in both groups are carriers of the four mutant alleles. The low number of elite rugby players that were genotyped for the ecNOS genotype has a negative influence on the elite table and may cause misinterpretation of the results.

Experimental results:

Figure 3.10: 7% PAGE gel of fragments produced by PCR genotyping of the ACE I/D variant.

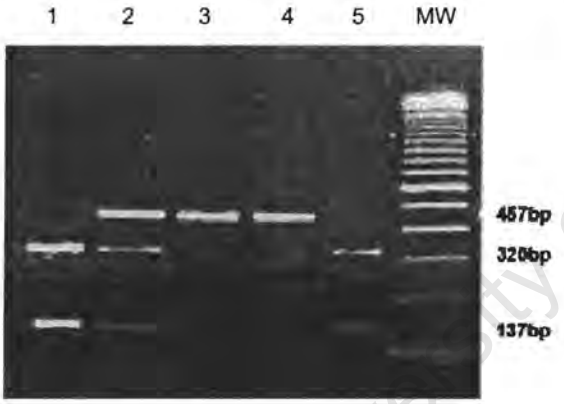


Key:

MW	Molecular weight marker
Lane 1, 2	II
Lane 3	ID
Lane 4	ID, in some cases, the 371bp fragment did not amplify clearly.
Lane 5	DD
NC	Negative control

The ACE variant is amplified using two flanking primers (ACE 1 and 3) and a insertion specific primer (ACE 2) which in combination produce the following fragments (Figure 2.1, page 56): **ACE II**: 371bp (ACE1/ACE3), 65bp (ACE1/ACE2); **ACE ID**: 371 + 85bp (ACE1/ACE3), 65bp (ACE1/ACE2); **ACE DD**: 85bp (ACE1/ACE3)

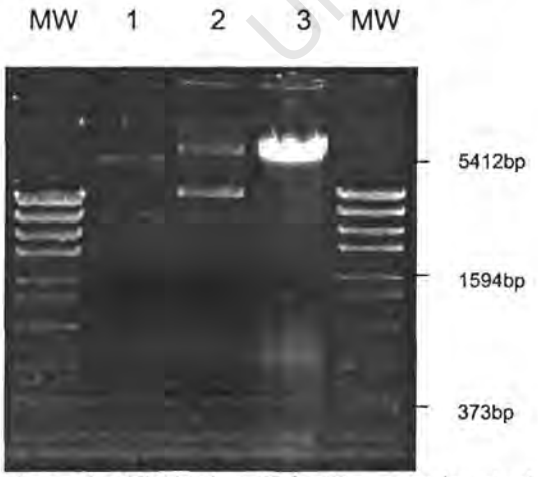
Figure 3.11: 3% agarose gel of fragments produced by PCR amplification and digestion with *BanII* of the ecNOS G894T variant.



Key:

Lane 1	GG
Lane 2	GT
Lane 3,4	TT
Lane 5	GG
MW	Molecular weight marker

Figure 3.12: Yield of DNA extracted by three different extraction methods.



Key:

MW	Molecular weight marker
Lane 1	DNA extracted by BUCCAL MOUTHWASH method
Lane 2	DNA extracted from CLOTTED BLOOD
Lane 3	DNA extracted by MODIFIED LAHIRI
MW	Molecular weight marker

One µl of DNA in TE-buffer was loaded on a 0.8% agarose gel to give an idea of yield and quality of DNA extracted by the different methods used in this project.

CHAPTER 4

DISCUSSION

The aim of this project was to test the hypothesis of a possible association between elite athletic status in rugby and distance (road) running with the ACE I/D and ecNOS G894T polymorphisms. The study did not detect a significant association between either of the polymorphisms and elite status in SA rugby players. A significant association was however shown between ethnic origin and running status, personal best running time and the ACE I/D genotype. A significant association was also shown between the ecNOS G894T genotype and the ecNOS G-allele frequency and elite status in road running, ethnic origin and personal best running time. This study also indicated that black SA athletes posses an significant excess of the ecNOS G-allele, coloured athletes have intermediate frequencies and SA Caucasians have the lowest frequencies.

4.1 The ACE I/D and ecNOS G894T polymorphisms in elite South African rugby players.

For the purposes of this study, an elite rugby player was defined as a player who has played rugby for a South African (SA) national team; this includes the Springbok, SA under-23, SA under-19, Upcoming Springboks and Seven aside teams. Players were also included in the elite group if they had played in the Super 12 international series played between South Africa, New Zealand and Australia. Control subjects were defined as rugby players who had not played rugby at an international level or for any of the SA national and provincial teams. Most of the control subjects were still involved in club rugby and played for the University of Stellenbosch rugby club or for male residence rugby teams (see Table 3.1b, p63).

Montgomery *et al.* (1998) found a significant association between the ACE I-allele and the ability of British Army recruits to perform elbow flexion with a 15 kg barbell. Recruits who were homozygous for the I-allele could perform elbow flexion for 11-fold longer duration after 10 weeks of basic training when compared to D homozygotes. Results were however not given relative to body mass. The authors concluded that the I-allele was associated with an improved and more efficient contraction of skeletal muscle and local muscle endurance. Montgomery *et al.* (1999) reported a significant excess of the I-allele among British Army recruits who showed the greatest anabolic response and the greatest changes in body morphology in response to 10 weeks of physical training. In subsequent studies, results

generated by Montgomery *et al.* (1999), Williams *et al.* (2000) and Woods *et al.* (2001) indicated a positive association between the I-allele and exercise associated changes in body morphology, anabolic training-induced responses in skeletal muscle, and improved muscle working capacity. In contrast to this, the I-allele was also shown to be associated with " *a lower training-induced cardiac hypertrophy* " (Montgomery *et al.* 1997). Table 4.1, p95 summarizes studies showing a positive correlation between the ACE I/D polymorphism and exercise induced improvements in muscle performance.

At the inception of this project, most studies showed that the I-allele was associated with endurance performance. This led the researchers in the present study to hypothesize that the ACE I/D genotype might contribute to elite status in SA rugby players. The present study however showed no correlation (p = 0.37, Table 3.3, p65) between the ACE I/D genotype and elite rugby player status in SA rugby players. Furthermore, no correlation was shown between ACE I- and D-allele frequency and elite status (Figure 3.1, p65). The distribution of the ACE I/D genotype was in Hardy-Weinberg equilibrium, which implies that the ACE I/D genotype frequency will usually be constant from generation to generation.

Rugby can be classified as a high-intensity intermittent power sport requiring superior physical strength, sprinting ability and endurance from rugby players at the elite level of play with matches lasting 90 minutes. From the phenotypic characteristic data (Table 3.1a, page 62) generated by this study, it is clear that elite SA rugby players are physically significantly larger in stature (body mass, height and BMI) than control players of the same age. This significant difference remained true for body mass and BMI when the elite and control SA rugby players were stratified by ACE I/D genotype (Table 3.2, p64). Although it has been indicated that a positive association does exist between the I-allele and changes in body morphology and anabolic responses in skeletal muscle to training in British Army recruits, an association could not be shown between the ACE I/D genotype and the physique of elite SA rugby players (Montgomery *et al.* 1999, Williams *et al.* 2000).

Table 4.1. Studies that have demonstrated a positive correlation between the ACE I/D polymorphism and muscle hypertrophy associated changes in skeletal muscle tissue.

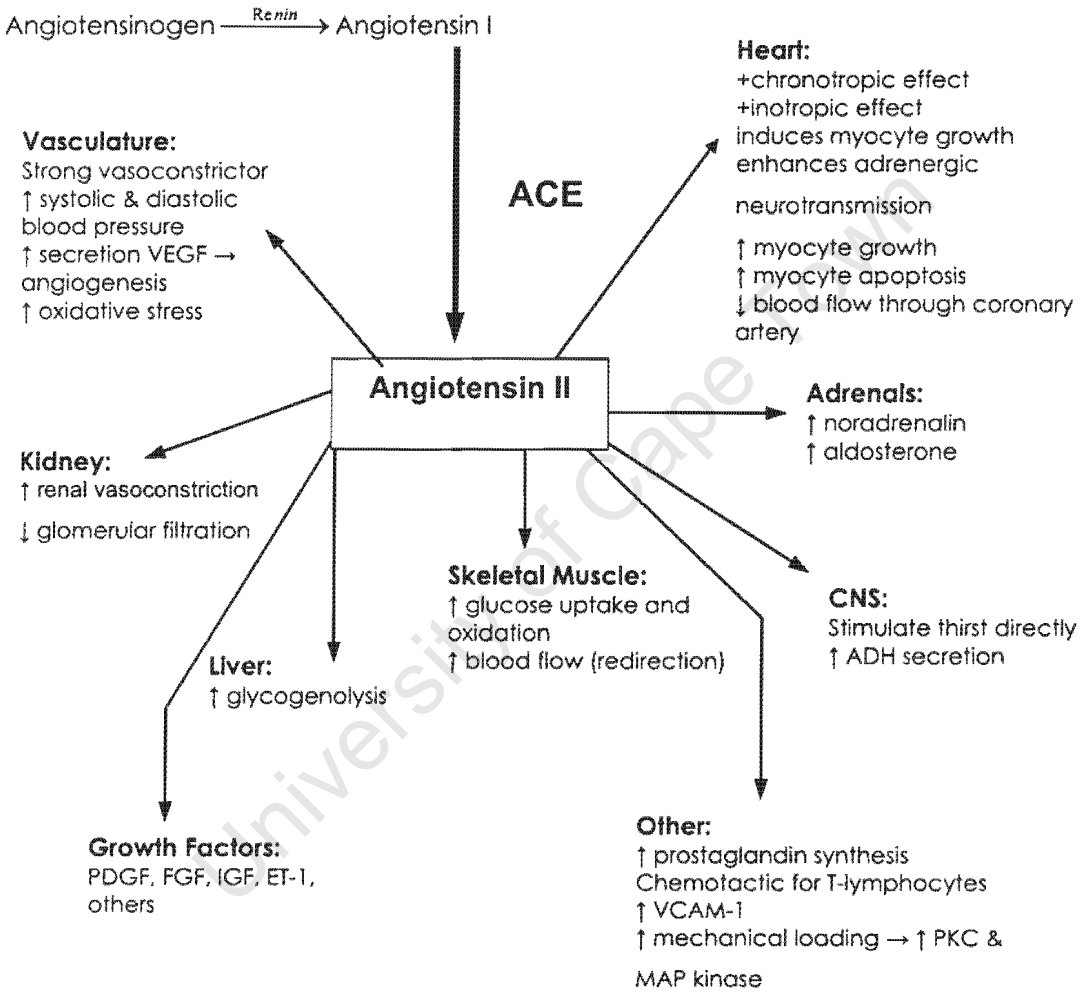
Study	Subjects	Variable	Significance	Allele associated with performance measure
Montgomery *et al.* 1997	British Army recruits	Left ventricular growth.	$P < 0.0001$	D-allele
Montgomery *et al.* 1998	British Army recruits	Repetitive elbow flexion with a 15kg barbell	$P < 0.05$	I-allele
Montgomery *et al.* 1999	British Army recruits	Change in body composition.	$P < 0.05$	I-allele
Myerson *et al.* 1999	Olympic level runners	Genotype distribution with distance run	$P = 0.009$ for linear trend, skew toward I allele in ≥5000m and toward D allele in ≤ 200m	D-allele associated with sprint distances; I-allele associated with longer distances
Myerson *et al.* 1999	Olympic level swimmers	Genotype distribution	$P = 0.034$	D-allele
Williams *et al.* 2000	British Army recruits	Mechanical working efficiency of skeletal muscle	↑8.62% II ↓0.39% DD	I-allele
Fatini *et al.* 2000	Elite soccer players	Left ventricular mass.	$P = 0.89$	D-allele
Folland *et al.* 2000	Recreationally active male volunteers	Response of quadriceps muscle to strength training	$P < 0.05$	D-allele
Woods *et al.* 2001	Swimmers; 56 Commonwealth & European Championship, 47 non-elite American College level	Genotype distribution	$P < 0.05$	D-allele
Alvarez *et al.* 2000	Elite athletes; 25 cyclists, 20 distance runners, 15 handball players	Genotype distribution	$P < 0.05$	I-allele
This study.	**Elite SA rugby players (*N*=67) and roadrunners (*N*=93).**	**Genotype distribution**	**No association**	**Low numbers could have been confounding factor. Should be regarded as pilot study.**

The sprinting and power generating ability of rugby players was also thought to be similar to the high power generating forces required by elite short (sprint) distance swimmers as investigated by Woods *et al.* (2001) and Myerson *et al.* (1999). In these studies, the D-allele was shown to be associated with power-oriented performance. Woods *et al.* (2001) showed that there was a " *significant excess of the D-allele* " in a group of 56 elite Caucasian swimmers who competed at the Commonwealth Games and/or European championships. This was true for only the 35 swimmers who competed in the sprint distances shorter than 400 meters (Woods *et al.* 2001).

The ACE D-allele is associated with higher serum and tissue ACE activity (Rigat *et al.* 1992, Tiret *et al.* 1992, Danser *et al.* 1995, Samani *et al.* 1996, Huang *et al.* 1998). Higher ACE activity would lead to an increase in angiotensin II production and an associated up regulation of various growth factors including insulin-like growth factor (IGF) and vascular endothelial growth factor (VEGF) (for more detail see Figure 4.1, p98) leading to muscle hypertrophy and associated strength gains (Jones and Woods 2003). To illustrate this, Folland *et al.* (2000) showed that the D-allele was associated with strength gains in the quadriceps muscle following a strength-training program. Montgomery *et al.* (1997) indicated that a correlation existed between the ACE D-allele and the magnitude of cardiac hypertrophy induced by a 10-week training program. In addition, Myerson and colleagues (2001) showed that the increase in left ventricular growth following a 10-week physical training program was greatest in men with the ACE DD genotype, intermediate in men with the ID genotype and lowest in men with the II genotype. These studies seem to indicate that the D-allele is associated with muscle hypertrophy and power-oriented performance in sports such as rugby.

Reasons for the present study on SA rugby players demonstrating no association may be related to the homogeneity of the study population and the study design. All the subjects were Caucasian males and were born in Southern Africa. The elite rugby players were selected if they had played for the Springbok team, the national U23 or U19 teams, the national Seven aside team or in the Super 12 series. An improvement on the composition of the elite cohort would be to select only current and past Springbok players. During the course of the present study, ex-Springbok players were contacted through letters with a very poor response. It was found that these players were hard to contact as SARFU could only supply a list of past Springbok players names and could not supply their contact details. Past and present Springbok players who reside in the Western Cape were contacted through Western Province Rugby.

Figure 4.1. The physiological functions of angiotensin II in the human body.
PDGF, platelet derived growth factor; FGF, fibroblast growth factor; IGF, insulin-like growth factor; ET-1, endothelin-1; VCAM-1, vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor; PKC, protein kinase C; MAP kinase, mitogen activated protein kinase; ACE, angiotensin-I converting enzyme; ADH, antidiuretic hormone.



A second improvement on the present study would be to divide the elite group (consisting of past and present Springbok players only) into forward (more power-oriented) and back players (mixed power and endurance). A problem with this approach, as with the previous suggestion, is that the numbers needed for significant power in such a study is around 100 individuals. The elite rugby players are exceptionally hard to locate as explained and would have tc be recruited on an individual basis which is very time consuming and costly.

4.2 The ACE I/D polymorphism in elite South African roadrunners.

For the purposes of this study, an elite roadrunner was defined as an athlete who had run a standard marathon in 2:25.00 (< 2 hours 25 minutes) or faster. Athletes were also included if they had run a half-marathon in 1:10.00 (< 1 hour 10 minutes) or faster, and/or had run a 10 km race in 0:30.00 minutes (< 30 minutes) or faster and/or had run a 5 km race in 0:15.00 (< 15 minutes) or faster. Control subjects were defined as roadrunners who had run a standard marathon in 3:00.00 (> 3 hours) or slower. Runners were also included in the control group if they had run a half-marathon in 2:00.00 or (> 2 hours) or slower, and/or had run a 10 km in 50 minutes (> 50 minutes) or slower and/or had run a 5km in 30 minutes (> 30 minutes) or slower. Table 3.6, p70 shows the mean ± sd of the personal best running times of both the elite and control groups. These times do not agree with the cut-off times mentioned above because of the selection strategy followed. Athletes were included in the elite or control groups if any one of their running times met the standards for inclusion. The result was that some runners could run all the times faster (elite) or slower (control) than the inclusion times and some could only make one of the times. The remaining race times were included in the analysis for completeness. Both male and female runners were included in the elite and control groups. This approach was also followed by various other authors (Myerson *et al.* 1999, Gayagay *et al.* 1998, Taylor *et al.* 1999). Because the ACE gene and I/D polymorphism is not gender linked the mixed gender cohorts did not influence the outcome of these studies. All elite female runners (*N*=2) were met the inclusion criteria for male runners. Female control runners (*N*=25) met all inclusion criteria for male control runners.

The results of the present study did not support the hypothesis of an association between the ACE I/D genotype and elite runner status in a mixed ethnic cohort of SA roadrunners (Table 3.8, p72). The distribution was the same in the general Caucasian population: 25% II, 50% ID and 25% DD (Jones and Woods 2003). There was also no association between the ACE I/D polymorphism and ethnic origin between elite runners and controls (Table 3.9, p73). Analysis also failed to demonstrate an association between the ACE I- and D-allele, elite roadrunner status (Figure 3.4, p74) and ethnic origin (Table 3.10, p85). It is however, realized that the results are confounded by a small sample size and a heterogeneous cohort in terms of ethnic origin. Elite runners had a significantly lower body mass (kg) and body mass index (BMI, kg/m^2) than control runners (Table 3.6, p70). Elite Caucasian roadrunners were significantly taller and elite black runners had a significantly lower body mass compared to matched control runner groups when they were arranged by ethnic origin (Table 3.7, p71). When arranged by ACE I/D genotype, elite Caucasian roadrunners with the ID genotype had significantly faster personal best times over 10km, 21.1km and 42.2km than control runners

of the same genotype (Table 3.11, p76). The same was true for elite Caucasian runners with the II genotype for 10km, black and coloured elite runners with the ID genotype over 10km and coloured runners over 42.2km with the DD genotype. Because of the small sample sizes involved caution must be exercised in the interpretation of these results. When the same analysis was performed for the ACE I- and D-alleles, elite Caucasian runners with the I-allele had significantly faster personal best times over all distances (5km to 42.2km).

Current research has shown that the I-allele is associated with endurance performance as indicated by the studies by Myerson *et al.* (1999) on Olympic runners, by Gayagay *et al.* (1998) on elite rowers and by Montgomery *et al.* (1998) on high altitude mountaineers and is also associated with lower serum and tissue ACE activity (Rigat *et al.* 1992, Tiret *et al.* 1992, Danser *et al.* 1995, Samani *et al.* 1996, Huang *et al.* 1998). ACE activity is lowest in I homozygotes and intermediate in I heterozygotes with the highest activity being found in D homozygotes. Low ACE activity increases the nett production of vasodilatory substances including angiotensin-(1-7) and bradykinin (BK) through decreased degradation (Jones and Woods, 2003). The physical endurance enhancement and increased muscle efficiency seen in subjects homozygous for the I-allele is thought to be a result of the increased vasodilatation and coupled increased substrate delivery and metabolite removal in skeletal muscle (Jones and Woods, 2003). But, in the words of Myerson *el al.* (1999) " *Any attemps to explain the association of the I-allele with endurance performance must remain speculative. It seems likely that the ACE I/D polymorphism is in linkage disequilibrium with factors influencing the expression of the neighboring growth hormone gene.* ". Table 4.2, p101 summarizes studies showing a positive correlation between the I-allele and muscle performance and endurance.

4.3 Ethnic background of the roadrunners.

The cohort of the study was made up of SA black, colored and Caucasian runners in both the elite and control groups. Previous studies have shown that the distribution of the ACE I/D genotype differs between groups from different (Caucasian, Nigerian black, Samoans, Yanomami Indians) ethnic backgrounds (Barley *et al.* 1994).

Because of the observed differences in the ACE I/D genotype distribution between different population groups (Table 3.20a, p88), it is reasonable to assume that this may have had some impact on the outcome of the present study. However, a study by Myerson *et al.*

different ethnic backgrounds (79 Caucasian, 12 black) the I-allele was associated with the distance run (Myerson *et al.* 1999). Myerson states that:

> " *The racial mix of the cohort may have influenced our results, as the ACE genotype distribution is influenced by race. The association of the I allele with endurance performance is, however, unlikely to have been due to the effects of race alone. First, only blacks and Caucasians were represented among the athletes studied, and it has been shown that ACE genotype distributions in healthy African and Caribbean blacks in the UK are no different from those of healthy Caucasians in a British population sample.* " (Myerson *et al.* 1999).

An improvement on the current study would be to increase the number of subjects in both the elite and control groups for all three of the ethnic groups represented in this study. The definition of what constitutes elite running ability also needs revision. This would increase the power of the study. The problem with being restricted to recruiting and sampling in the Western Cape is that SA athletes who do not compete in the major races held in this region, notably the Two Oceans marathon (the SA marathon championships is no longer held in Cape Town), cannot be reached. If researchers could travel to more of the major SA road races like the 90km Comrades marathon in Kwazulu-Natal (run between Durban and Petermaritzburg), more athletes could be reached and recruited. A second approach could be to select one or two ethnic groups for the study to focus on. This would exclude the problem of ethnic origin influencing the outcome of the study.

Table 4.2. Studies showing a positive correlation between the ACE I-allele, muscle performance and endurance.

Study	Subjects	Variable	Significance
Montgomery *et al.* 1997	British Army recruits	Exercise-induced left ventricular growth following a 10-week basic training period	$P < 0.0001$
Montgomery *et al.* 1998	High-altitude mountaineers	Ascending mountains above 7000m without supplementary oxygen	$P = 0.02$
Gayagay *et al.* 1998	National level rowers	Genotype distribution	$P = 0.03$
Myerson *et al.* 1999	Olympic level runners; grouped n = 20 ≤ 200m, n = 37 400-3000m, *n* = 34 ≥5000m, all distances; 79 Caucasian	Genotype distribution with distance run	$P = 0.009$ for linear trend, skew toward I allele in longer distances and toward D allele in sprint distances
Alvarez *et al.* 2000	Elite athletes; 25 cyclists, 20 distance runners, 15 handball players	Genotype distribution	$P < 0.05$
Sonna *et al.* (2001)	US Army recruits; 62 male, 85 female	Genotype distribution and response to basic training measured by standardized US Army fitness tests	No association
This study.	**Elite and control roadrunners.**	**Genotype distribution.**	**I-allele in SA elite Caucasian and black runners.**

4.4 Competitive level of runners.

As described in Materials and Methods (page 40-1), the times chosen as the upper limits for the elite runners and lower limits for the control runners were chosen after consultation with Dr. A Bosch (Running Division, High Performance Laboratory, Sports Science Institute of South Africa) and Prof M Lambert (Head of High Performance Laboratory, Sports Science Institute of South Africa). Both Dr Bosch and Prof Lambert are considered experts in the field of running and both are lecturers in exercise science at the MRC/UCT Research Unit for Exercise Science and Sports Medicine.

When examining the published literature, most studies assume that their cohorts consist of elite athletes. Myerson *et al.* (1999) studied "*Olympic-standard runners*" but made no mention of their actual running ability or phenotypical characteristics such as their maximal oxygen uptake (VO_{2max}) values, which would indicate their running status. In contrast, Rankinen *et al.* (2000d) studied the distribution of the ACE I/D genotype (0.271 DD, 0.464 ID, 0.265 II) in 192 elite Caucasian endurance athletes competing in cross-country skiing, biathlon, Nordic combined, long-distance and middle-distance running and cycling. All athletes had a " *VO_{2max} of at least 75 ml/kg/minute [mean 78,6 ± 3.2 (SD)]* ". This is the only published study that supplies such phenotypic data to indicate the elite status of the subjects. All the sports represented in the Rankinen cohort are endurance sports and all require similar endurance training (gene-environment interaction) techniques and put similar physiological and biochemical demands on the human body. Although the negative (no association) outcome of that study has been criticized by various authors because of the different sporting disciplines of the athletes in the study cohort, it is the opinion of this researcher that this is the only published study on the ACE I/D genotype and its distribution among elite athletes that uses a truly elite cohort (as indicated by the VO_{2max} values). The measure by which the elite athletes were selected (here simplistically defined, it is realized that it is more complex), namely the maximal oxygen uptake or VO_{2max} is a " *fundamental measure"* of performance in the exercise sciences because the attaining of a *" high VO_{2max} requires the integration of a high level of ventilatory, cardiovascular and neuromuscular functions* " (McArdle *et al.* 1996). Olympic level male cross-country skiers, middle-distance runners and cyclists have average maximal oxygen uptakes of 84 ml/kg/min, 79 ml/kg/min and 75 ml/kg/min, respectively (McArdle *et al.* 1996). The range for the athletes in the Rankinen cohort was 75.0 to 92.9 ml/kg/min (Rankinen *et al.* 2000d), which put the cohort well above the average for Olympic level athletes.

The present SA study's elite cohort included past Olympic and Commonwealth Games champions and medalists and national champions over various distances. All the elite runners had the ability to run one or all of the distances (5 km, 10 km, 21.1 km, 42.2 km) in faster than the specified cut-off times. Although VO_{2max} values were not measured on any of the athletes because samples were taken after races, it is known that for an endurance athlete to be able to compete at a high level in events lasting longer than 15 minutes a VO_{2max} of greater than 70 ml/kg/min is required (Hawley *et al.* 1997).

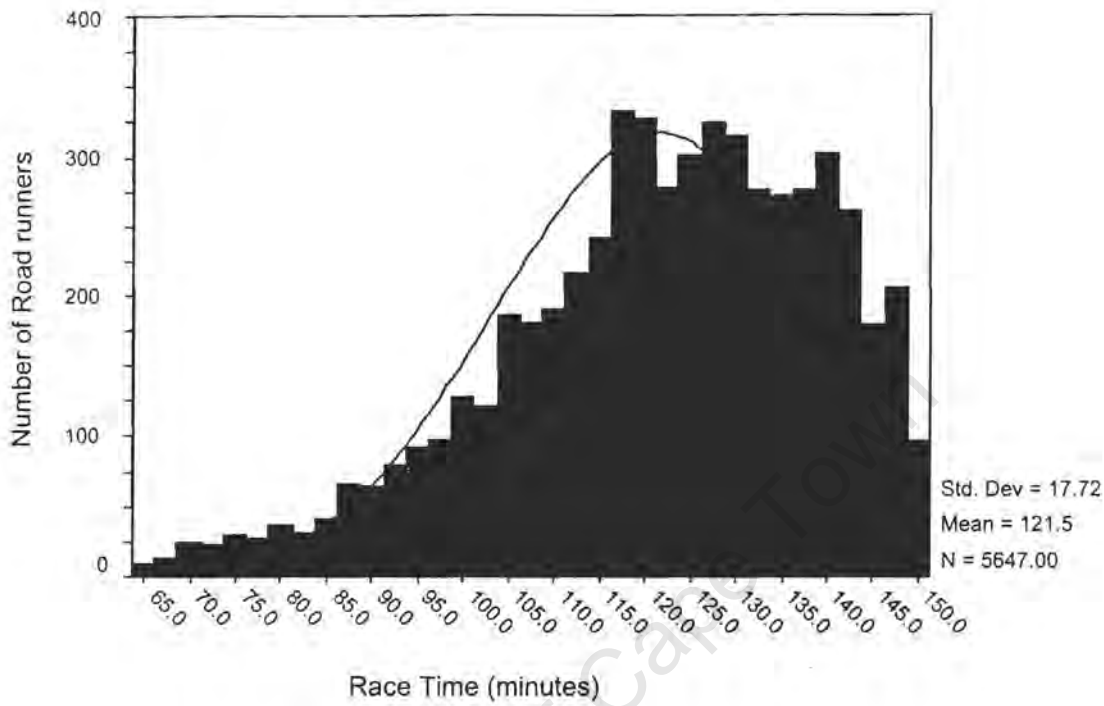
4.5 Sample Collection.

Although there are no published data to support the idea of a Gaussian (Normal) distribution in the performance (times) of roadrunners, during sample collection it was found that most runners finished races (10 km, 21.1 km, 42.2 km) in the time zone between the inclusion criteria for the present study. To illustrate this idea, the race times for the 5647 competitors in the 2003 Two Oceans half marathon was plotted in a histogram and a normal curve was fitted to show the distribution of runners in road races (Figure 4.2, p104).

The personal best times of the elite runners that were chosen (based on best times) for the present study lay in the tail of the normal curve. It was especially hard to find runners that were able to run times faster than those set as limits for inclusion into the elite group. Except for subject recruitment and sample collection at the SA Marathon Championships (1999/2000) and the Two Oceans Marathon (1999/2000) which are two major races on the SA running calendar, it was common to collect only between one and five samples for elite runners per race. If it were, for example, possible to collect samples at the 2003 Two Oceans half marathon, only the first 30 runners of 5647 (that is 0.53%) would have qualified for inclusion in the elite roadrunner cohort. Because it was not possible for this present study to recruit runners and take samples in geographical areas other than the Western Cape, many runners were sampled on more than one occasion (duplicate samples were destroyed). If samples could have been taken from other geographical areas of South Africa, more samples would have been collected in the elite group. The same holds true for the control group.

In retrospect, it is possible that the times set for inclusion into the elite group were somewhat unrealistic and slower times would have increased the number of subjects. However, this change in the selection criteria could have an effect opposite to what the aim was and the study group would become more heterogeneous.

Figure 4.2: The Gaussian running time distribution of runners (*N* = 5647) in the 2003 Two Oceans half marathon.



4.6 The two- *versus* three-primer method for PCR detection of the ACE I/D polymorphism.

Some of the controversy that exists around the use of the ACE I/D polymorphism as a marker for athletic ability centers on the differences in the methodological approaches used in the detection of the insertion and deletion (I/D) alleles. The different approaches seem to have different sensitivities for the detection of the D allele. The original PCR method reported by Rigat *et al.* (1992) infrequently led to mistyping of D homozygotes (DD) as D heterozygous (ID) with an estimated frequency of between five and ten percent (Chiang *et al.* 1998). The PCR method described by Rigat *et al.* (1992) made use of a two-primer system. The sense and anti-sense primers were designed to flank the area in intron 16 of the *DCP1* gene (17q23) where the ACE I/D polymorphism occurs. PCR products included a 190 bp fragment in the case of the D-allele and a 490 bp fragment where an I-allele occurs (Rigat *et al.* 1992). See Table 4.3, p105 for detail.

Table 4.3. Study outcomes and ACE I/D genotyping methods used.

Author	Method	Major Finding
Montgomery et al. (1997)	Three-primer†	D-allele associated with LV growth in mountaineers and British Army recruits following basic training.
Montgomery et al. (1998)	Three-primer†	I-allele associated with endurance performance in high altitude mountaineers and British Army recruits.
Hagberg et al. (1998)	Two-primer‡	ACE II-genotype associated with significantly higher VO_{2max} in post-menopausal women.
Gayagay et al. (1998)	Three-primer†	I-allele associated with endurance performance in elite rowers.
Myerson et al. (1999)	Three-primer†	Increasing I-allele frequency with distance run in Olympic runners.
Montgomery et al. (1999)	Three-primer†	I-allele associated with anabolic response in British Army recruits following basic training.
Rankinen et al. (2000)	Three-primer†	No association.
Fatini et al. (2000)	Two-primer‡	D-allele associated with LV growth.
Folland et al. (2000)	Three-primer†	D-allele associated with greater strength gains.
Woods et al. (2001)	Three-primer†	D-allele excess in elite sprint distance swimmers.

‡ Rigat *et al.* (1992)

† Evans *et al.* (1994)

Most studies, including this present study, have used a three-primer method to genotype the ACE I/D polymorphism. The three-primer system was used to ensure avoiding mistyping of the ID genotype as DD (Chiang *et al.* 1998). Preferential amplification of the D allele was found to be common with using the two-primer system suggested by Rigat *et al.* (1992). The three-primer technique was reported to be 100% repeatable in the typing of the ACE gene polymorphism (Chiang *et al.* 1998). Evans *et al.* (1994) proposed a method where a third 'nested' oligonucleotide was used. The third oligonucleotide was positioned inside the insertion allele (Alu sequence) and minimized the chances of mistyping the I-allele as a D-allele. This technique was reported to be 100% effective in typing of the ACE I/D genotype (Evans *et al.* 1994).

4.7 The ecNOS G894T polymorphism in elite South African rugby players and distance runners.

For the purposes of this study, an elite rugby player was defined as a player who has played rugby for a South African (SA) national team; this includes the Springbok, SA under-23, SA under-19 and Seven aside teams. Players were also included in the elite group if they had played in the Super 12 international series. Control subjects were defined as rugby players who had not played rugby at an international level or for any of the SA national teams. The study collaborator, Dr Marios Cariolou of the Human Population Genetics Research Unit, The Cyprus Institute of Neurology and Genetics, Nicosia, Cyprus, undertook this section of the genotyping.

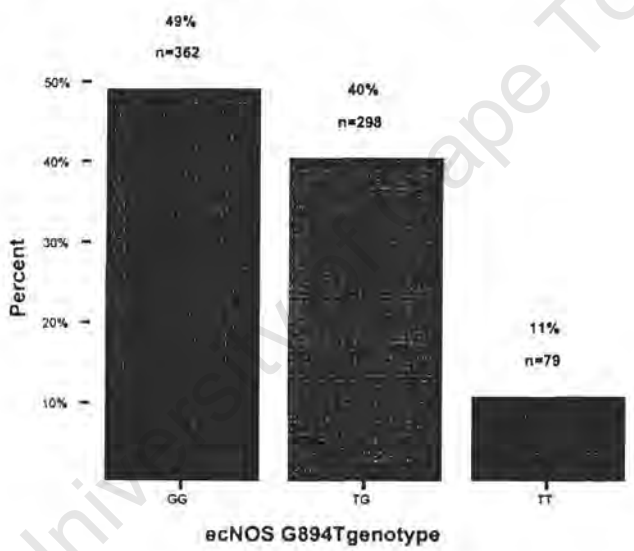
The present study showed no correlation between ecNOS G894T genotype and elite rugby player status in SA Caucasian rugby players. Furthermore, no correlation was demonstrated between ecNOS G- and T-allele frequency and elite status.

For the purposes of this study, an elite roadrunner was defined as an athlete who had run a standard marathon in 2:25.00 (< 2 hours 25 minutes) or faster. Athletes were also included if they had run a half-marathon in 1:10.00 (< 1 hour 10 minutes) or faster, and/or had run a 10 km race in 0:30.00 minutes (< 30 minutes) or faster and/or had run a 5 km race in 0:15.00 (< 15 minutes) or faster. Control subjects were defined as roadrunners who had run a standard marathon in 3:00.00 (> 3 hours) or slower. Runners were also included in the control group if they had run a half-marathon in 2:00.00 or (> 2 hours) or slower, and/or had run a 10 km in 50 minutes (> 50 minutes) or slower and/or had run a 5km in 30 minutes (> 30 minutes) or slower. Table 3.6, p70 shows the mean ± sd of the personal best running times of both the elite and control groups. These times do not agree with the cut-off times mentioned above because of the selection strategy followed. Athletes were included in the elite or control groups if any one of their running times met the standards for inclusion. The result was that some runners could run all the times faster (elite) or slower (control) than the inclusion times and some could only make one of the times. The remaining race times were included in the analysis for completeness.

The results presented in this study indicate an association between the ecNOS G894T genotype distribution and elite roadrunner status in SA runners (Table 3.14, p79). Elite runners carried an excess of the G-allele (Figure 3.5, p81). When data was arranged according to ethnic origin (Table 3.15, p80 and Table 3.16, p82), it emerged that SA elite

black runners had excess of the G-allele when compared to Caucasian and coloured runners had intermediate levels and this contributed to the observed difference. Black control runners also had an excess of the G-allele. The excess of the G-allele in the black elite group thus caused the significant result found when elite and control runners of all ethnicities were compared. When the total cohort of samples from SA sportsmen from different sporting backgrounds was analyzed to give an indication of the distribution of the ecNOS variant in an SA population sample, it was found that black athletes had a significant excess of the G-allele (Table 3.19a and 3.19b, p87). The values for Caucasians generated by this SA study compares well with published frequencies for Caucasian subjects (0.623) for the G-allele and (0.377) for the T-allele (Rankinen *et al.* 2000c).

Figure 4.3: The ecNOS G894T distribution in a mixed race South African population sample of athletes from a variety of sporting disciplines.



NO, produced by endothelial NO synthase is one of the most important mediators of vascular tone and blood pressure. Only one known study involving the ecNOS G894T polymorphism and sporting performance has been published. In this study Rankinen *et al.* (2000c) compared the blood pressure response before and after 20 weeks of endurance training in subjects participating in the HERITAGE Family Study. The authors showed a positive association between endurance training induced decreases in diastolic blood pressure and rate pressure product while performing submaximal exercise (Rankinen *et al.* 2000c). Rankinen states that: " *Our data suggest that the Glu298Asp polymorphism has a role in the long-term adaptation of hemodynamic phenotypes to endurance training rather than in short-term response to a single bout of exercise.* " (Rankinen *et al.* 2000c). At this time it is

uncertain whether the G894T polymorphism of the ecNOS gene gives rise to direct functional alterations in the NO pathway.

It is known, however, that exercise and shear stress produced by increased blood flow up-regulate the expression of ecNOS (Fösterman *et al.* 1998). It is also known that NO is an important regulating factor of the renin-angiotensin system (RAS) at various levels, and that angiotensin II (ANG II) may also have a regulatory function on the generation of NO (Fösterman *et al.* 1998). New data show that ANG II and NO form a homeostatic system directed at the regulation of vascular structure and function (Fösterman *et al.* 1998). At this stage, it is unfortunately not certain whether the ecNOS G894T polymorphism has any definite effect on the functional integrity and NO generating ability of NO synthase.

4.8 Gene-environment interaction.

> " *Gene-environment interaction (GEI) studies relate the magnitude of physiological response to a uniform environmental stimulus with variation in a candidate gene.* " (Montgomery and Dhamrait 2002).

In the present SA study's context GEI could be the nature or type of training that individual athletes are subjected to and the amount (hours per week) and duration of training sessions as well as the total number of months or years over which training takes place. The scale or magnitude of the response can be attenuated or lessened by various factors including nutrition and nutritional status, environmental conditions such as ambient temperature, humidity and altitude and also by rest from activity. Montgomery and Dhamrait (2002) state that:

> " *The ideal GEI study thus seeks 1) subject homogeneity (similar individuals of identical race and sex), 2) stimulus homogeneity (nature, magnitude, and duraution), and 3) selection of an appropriate phenotype to assess.* " (Montgomery and Dhamrait 2002).

In any sport related GEI study it is virtually impossible to meet all three of the above-mentioned criteria. " *Subject homogeneity* " and the " *selection of an appropriate phenotype to assess* " are the easier of the two criteria to meet. The second point " *stimulus homogeneity* " is the most difficult to meet. This can only be done in a controlled training

environment where everything the subject does is monitored and controlled by the researcher. For stimulus homogeneity, all subjects must do exactly the same type and amount of exercise at the same relative intensity. In studies where subjects are recruited at random, it is virtually impossible to control for " *stimulus homogeneity* ". For example, in the road running cohort of the present SA study, some of the elite subjects ran upwards of 160km per week while others ran less than 60km per week and both met the inclusion criteria of the elite group. Some of the control subjects also ran upward of 160km per week and others ran 30km and less per week. Some of the elite runners had a 20 year running history while others had only one year of experience or less. The magnitude of the body's physiological and biochemical response in these individuals can obviously not be realistically compared or be related back to just one variant in one candidate gene (in this the case ACE I/D and ecNOS G894T polymorphisms) because of the differences in the accumulated interaction between the gene(s) and the environment. It is also necessary to realize that a phenotype such as having a high VO_{2max} and having the ability to run at a high, sustained percentage of the VO_{2max} cannot be related back to one single candidate gene. It is known that " *there is a one-to-many relation of phenotypes to genes* " (one gene-many phenotypes)(Griffiths *et al.* 1999). This means that many different genes can affect a single phenotype. VO_{2max} could for example be influenced or affected by the many genes that code for proteins associated with and involved in the biosynthesis of red blood cells, capillaries and compounds important for the uptake and release of oxygen. A single gene, like the DCP 1 gene (which codes for angiotensin-I converting enzyme) for example, affects many different phenotypes like blood pressure and muscle hypertrophy through different physiological pathways. This " *one-to-many relation of gene to phenotypes* " is called *pleiotropy* and is " *inferred from the observation that mutations selected for their effect on one specific character are often found to affect other characters of the organism* " (Griffiths *et al.* 1999).

Another important point is that there is a risk of decreasing power/resolution if a cohort is too homogenous. If a particular set of alleles are 'enriched' in a population and maintained there, 'patients' and 'controls' will harbor them. This implies that when a particular allele is analyzed in a population where enrichment has taken place, a false positive result will be obtained.

CHAPTER 5

CONCLUDING REMARKS

The work described in this thesis makes three tentative conclusions regarding the ACE I/D and the ecNOS G894T polymorphisms' influence on exercise performance and status as markers for performance in elite athletes. Firstly, it was found that in contrast to other published work on the distribution of the ACE I/D genotypes in endurance athletes, there was no difference in the distribution between South African elite roadrunners and control subjects. The ACE I/D genotype distribution was the same as those published in the literature for the general Caucasian population. This supports the research by Rankinen *et al.* (2000). Secondly, the distribution of the ACE I/D polymorphism is not significantly different between the different ethnic groups represented in this study.

A significant association was demonstrated between the ecNOS G894T genotype distribution and the ecNOS G-allele frequency and elite status in road running, ethnic origin and personal best running time. This study also indicated that black SA athletes posses a significant excess of the ecNOS G-allele, coloured athletes have intermediate frequencies and SA Caucasians have the lowest frequencies. This is important for the interpretation of the results of the present study and future studies will have to include this aspect in their sample selection strategies and analysis. The sample sizes are small and need to be increased to draw a more definite conclusion.

The interpretation of these results and how they may ultimately contribute to elite status in endurance athletes and in roadrunners in particular is confounded by many factors. The genetic factors that contribute to a phenotype such as being an elite distance runner are difficult to determine. A study such as this one that includes one or two polymorphisms in two possible candidate genes is very difficult to interpret because of the effect of one gene on many phenotypes and also the effect of many genes on one phenotype. It is the opinion of this researcher that one single polymorphism in one gene cannot be used as a marker for athletic ability in a phenotype that is made up of so many factors such as aerobic endurance. Hawley *et al.* (1997) states that the requirements for an endurance athlete to compete at an elite level are: "*...(1) a high (>70 ml kg^{-1} min^{-1}) maximal aerobic power (VO_{2max}); (2) the ability to maintain a high percentage of VO_{2max} for sustained periods; (3) a high power output or speed at the 'lactate threshold'; (4) the ability to withstand high absolute power outputs or speeds and resist the onset of muscular fatigue; (5) an efficient/economic technique; and (6) the ability to utilize fat as a fuel during sustained exercise at high work rates.* ". An ideal

genetic marker would have to in some way influence most biochemical and physiological factors that contribute to aerobic endurance. This is highly unlikely as endurance performance (for example) is not a monogenic phenotype and is most likely polygenic.

There is more than one research approach that could be followed in the identification of markers for athletic ability. This study needs to be based on larger sample numbers for the identification of genetic markers (gene polymorphisms) that may influence the expression of proteins, which may be important in endurance or power sport. This one gene approach is very slow and results are difficult to interpret due to the influence of gene-environment interaction and population homogeneity, determining what constitutes a homogenous population sample and the problems associated with this approach. In studies on elite athletes a further problem arises in that individual athletes are difficult to contact and to encourage to provide samples for genetic studies.

An approach that would provide much more information on the possible location of genes that are important to a certain type of exercise modality would make use of DNA microarray technology. A simple experiment would involve taking (pre-) muscle biopsy samples from untrained individuals, then subjecting them to either endurance specific or power specific training programs. After a certain period of training (post-) muscle biopsy samples would again be taken. Here one can choose whether the object is to determine the acute or the chronic affect of training on gene expression in skeletal muscle. Messenger RNA (mRNA) is then extracted from the pre- and post muscle biopsy samples and individually hybridized to a skeletal muscle gene specific DNA microarray containing hundreds or even thousands of muscle specific probes. A comparison of results obtained from pre- and post-exercise DNA microarrays would indicate which genes are expressed at high, intermediate and low levels following acute or long-term exposure to exercise. The DNA sequences of highly expressed genes can be obtained from the National Center for Biotechnology Information (NCBI, U.S. National Library of Medicine, 8600 Rockville Pike, Bethesda, MD 20894, http://www.ncbi.nlm.nih.gov/) and can then be analyzed for the presence of various DNA polymorphisms which may be useful as markers of athletic ability. In the present researchers opinion this approach would yield the most accurate information on the possible location of putative genetic markers for athletic ability. This approach was not taken at the inception (1999) of this research project because of the cost factor and the University of Cape Town did not have a micro-array facility at the time.

Focus areas for future research: flux generating points and the key enzymes of the intermediate metabolism (including those associated with glycolysis, the Krebs cycle and

oxidative phosphorylation); genes associated with muscular development and hypertrophy, including the insulin-like growth factors (IGF) and human growth hormone, their receptors and factors associated with the up- and down-regulation of these factors; heme synthesis; signaling pathways and factors associated with the activity regulation of mitochondrial biogenesis.

In summary, it was concluded that although differences does exist in the distribution of both the ACE I/D and ecNOS G894T polymorphisms between SA roadrunners from the various ethnic groups represented in this study, it is difficult to make definite conclusions due to the effect of gene-environment interaction, ethnic origin, subject heterogeneity and sample size.

INDEX OF APPENDICES

S.A. MEN BEST TIMES EVER: 42.2 KM

02:06:33	GERT THYS		121171	TOKIO	140299
02:07:28	JOSIAH THUGWANE		150471	FUKUOKA	071297
02:07:45	GERT THYS		121171	CHICAGO	111098
02:07:52	GERT THYS		121171	BOSTON	200498
02:08:04	ZITHULELE SINQE		090663	PORT ELIZABETH	030586
02:08:06	JOSIAH THUGWANE		150471	LONDEN	130497
02:08:07	DAVID TSEBE		091166	BERLYN	270992
02:08:15	WILLIE MTOLO		050564	PORT ELIZABETH	030586
02:08:30	GERT THYS		121171	BEPPU	040296
02:08:58	MARK PLAATJES	(10)	010661	PORT ELIZABETH	040585
02:09:10	LAWRENCE PEU		130266	LONDEN	130497
02:09:29	WILLIE MTOLO		050564	NEW YORK	011192
02:09:31	GERT THYS		121171	FUKUOKA	051293
02:09:36	ANDRIES KHULU		210872	BERLYN	280997
02:09:41	ERNEST SELEKE		051259	PORT ELIZABETH	310384
02:09:50	DAVID TSEBE		091166	PORT ELIZABETH	240290
02:09:54	EZAEL TLHOBO		040564	PRAAG	240598
02:09:56	EZAEL TLHOBO		040564	REIMS	201096
02:10:17	WILLIE MTOLO		050564	ROTTERDAM	170494
02:10:18	WILLIE MTOLO	(20)	050564	KAAPSTAD	300488
02:10:18	MARTIN NDIVHENI		020265	BERLYN	290996
02:10:22	XOLILE YAWA		290962	LONDEN	020495
02:10:29	LAWRENCE PEU		130266	FUKUOKA	061292
02:10:29	MARK PLAATJES		010661	LOS ANGELES	030391
02:10:29	JOSEPH SKOSANA		210663	DURBAN	200791
02:10:32	WILLIE MTOLO		050564	PORT ELIZABETH	040585
02:10:32	DAVID TSEBE		091166	DURBAN	200791
02:10:39	THOMPSON MAGAWANA		230359	PORT ELIZABETH	040585
02:10:39	MAKHOSONKE FIKA		200172	PARYS	040499
02:10:41	MARK PLAATJES	(30)	010661	LOS ANGELES	060388
02:10:47	DAVID TSEBE		091166	DURBAN	100989
02:10:47	MICHAEL SCOUT		051262	DURBAN	200791
02:10:48	DAVID TSEBE		091166	PORT ELIZABETH	290789
02:10:51	ZITHULELE SINQE		090663	STELLENBOSCH	020587
02:10:53	PETER TSHIKILA		220662	PORT ELIZABETH	290789
02:10:55	THABISO MOQHALI		071267	LONDEN	120492
02:10:56	SIMON MPHULANYANE		100970	LONDEN	180499
02:10:57	XOLILE YAWA		290962	BERLYN	260993
02:11:00	ERNEST TJELA		161054	PORT ELIZABETH	030586
02:11:01	WILLIE MTOLO	(40)	050564	STELLENBOSCH	020587
02:11:07	ABEL MOKIBE		030765	KAAPSTAD	280392
02:11:13	GERT THYS		121171	REIMS	201096
02:11:18	LAWRENCE PEU		130266	PARYS	020495
02:11:19	JOHANNES MAREMANE		270965	REIMS	201096
02:11:24	JOHN SEBATA		070156	PORT ELIZABETH	240290
02:11:35	WILLIE MTOLO		050564	LONDEN	020495
02:11:37	FRANK POOE		021274	BOSTON	190499
02:11:39	ERNEST TJELA		161054	COLUMBUS	081187
02:11:39	JOHANNES MABITLE		150967	LONDEN	020495
02:11:40	GERT THYS	(50)	121171	OHTSU	140393
02:11:42	JOHAN DREYER		111158	KUILSRIVIER	020783

02:11:44	GABASHANE RAKABAELE		030948	PORT ELIZABETH	010582
02:11:46	JOHNNY HALBERSTADT		021049	CHICAGO	260982
02:11:46	JOSIAH THUGWANE		150471	KAAPSTAD	250296
02:11:47	ZITHULELE SINQE		090663	KAAPSTAD	280392
02:11:47	ERNEST TJELA		161054	KAAPSTAD	070387
02:11:49	NIXON NKODIMA		260469	KAAPSTAD	250296
02:11:51	DEREK MAY		020755	HOUSTON	180187
02:11:51	ZITHULELE SINQE		090663	DURBAN	300887
02:11:54	XOLILE YAWA	(60)	290962	KYONGJU	200394
02:11:55	GERT THYS		121171	GOLD COAST	130797
02:11:56	JAN TAU		181160	KAAPSTAD	280392
02:11:56	XOLILE YAWA		290962	BERLYN	240995
02:12:01	ZITHULELE SINQE		090663	KYONGJU	200394
02:12:03	EZAEL TLHOBO		040564	REIMS	121097
02:12:07	DAVID TSEBE		091166	BERLYN	260993
02:12:08	EWALD BONZET		251251	STELLENBOSCH	100983
02:12:10	GEOFF BACON		310842	PORT ELIZABETH	060980
02:12:10	BERNARD ROSE		020753	KAAPSTAD	050383
02:12:10	JOHANNES MAREMANE	(70)	270965	HIROSHIMA	060394
02:12:14	ERNEST TJELA		161054	PORT ELIZABETH	010887
02:12:14	DAVID TSEBE		091166	KAAPSTAD	300488
02:12:14	DAVID TSEBE		091166	PORT ELIZABETH	280790
02:12:14	THABISO MOQHALI		071267	DURBAN	200791
02:12:17	MARK PLAATJES		010661	COLUMBUS	131188
02:12:17	SIMON MPHULANYANE		100970	SAN SEBASTIAN	231197
02:12:19	JOHNNY HALBERSTADT		021049	DURBAN	090979
02:12:19	JAN TAU		181160	ENSCHEDE	130693
02:12:22	BERNARD ROSE		020753	PORT ELIZABETH	010582
02:12:27	GABASHANE RAKABAELE	(80)	030948	PORT ELIZABETH	201079
02:12:32	MARTIN NDIVHENI		020265	PORT ELIZABETH	110395
02:12:32	THABISO MOQHALI		071267	NEW YORK	121195
02:12:33	WILLIE MTOLO		050564	ROTTERDAM	180493
02:12:35	THOMPSON MAGAWANA		230359	STELLENBOSCH	020587
02:12:36	JOSIAH THUGWANE		150471	ATLANTA	040896
02:12:36	ANDRIES KHULU		210872	BERLYN	200998
02:12:37	THABISO MOQHALI		071267	PORT ELIZABETH	240290
02:12:40	FRANK POOE		021274	KAAPSTAD	280299
02:12:45	ABNER CHIPU		020172	BOSTON	190499
02:12:47	FERDIE LE GRANGE	(90)	240848	PORT ELIZABETH	230474
02:12:48	DANIEL RADEBE		120465	MELBOURNE	111098
02:12:50	THOMPSON MAGAWANA		230359	FAURE	111080
02:12:53	DEREK MAY		020755	SACRAMENTO	021284
02:12:54	LAWRENCE PEU		130266	PARYS	210496
02:12:55	ERNEST TJELA		161054	MUNCHEN	080588
02:12:56	THABISO MOQHALI		071267	BOSTON	170495
02:12:57	BERNARD ROSE		020753	DURBAN	020881
02:12:57	FRANS NTOALE		080850	PORT ELIZABETH	310384
02:13:00	JOHN MONYATSO		000000	KAAPSTAD	280299
02:13:01	BERNARD ROSE	(100)	020753	PORT ELIZABETH	090581
02:13:02	JOHNNY HALBERSTADT		021049	EUGENE	130981
02:13:02	JOHN SEBATA		070156	KAAPSTAD	300488
02:13:04	JOHANNES MABITLE		150967	KYONGJU	240396
02:13:09	JOHNNY HALBERSTADT		021049	HOUSTON	240182
02:13:09	FRANK POOE		021274	KAAPSTAD	220298
02:13:10	JOHNNY HALBERSTADT		021049	PORT ELIZABETH	201079
02:13:11	DAVID PHALATSE		151263	PORT ELIZABETH	240290
02:13:11	THABISO MOQHALI		071267	HONOLULU	141297
02:13:11	THABISO MOQHALI		071267	BELGRADO	250498

APPENDIX 2

CONSENT FORMS AND QUESTIONNAIRES

DNA is the blueprint of what makes us human and must be treated in a respectful manner by the scientists that study the information stored in it.

In retrospect, it was realised that the Informed Consent and the general information sheet handed out to the participants in this study should have included the following:

1. **A short but detailed description of the Genetic Markers for Human Athletic Ability research project and its aims.**
2. **A short detailed description of the ACE I/D genotype, the polymorphism under investigation in this study.**
3. **An indication should also have been included as to what would happen to the DNA samples after the completion of the study. The samples would have been either destroyed, or with the permission of the athlete, would be used for further studies on genetic markers for athletic ability.**
4. **A statement entitling the athlete to withdraw his/her DNA sample at any stage before the completion of the study.**
5. **A statement indicating that all data will be analysed anonymously.**

BERTY LIFE CHAIR OF EXERCISE AND SPORTS SCIENCE IN THE
BIOENERGETICS OF EXERCISE RESEARCH UNIT
OF THE MEDICAL RESEARCH COUNCIL (MRC)
AND THE UNIVERSITY OF CAPE TOWN

Sports Science Institute of South Africa Boundary Road Newlands 7700 South Africa
115 Newlands 7725
Telephone (021) 6867330 International 27-21 6867330 Fax (021) 6867530

18 February 1999

The Genetic Basis of Athletic Ability

Ve are interested in studying the genetic basis of athletic ability at the MRC/UCT ioenergetics of exercise research unit. To do this study we would like to draw blood amples from top athletes who excel or have excelled in a variety of sports. We ould therefore appreciate it if you would be prepared to participate in this study.

o take part in this study, you will be asked to donate five millilitres of venous blood fter signing a consent form. You will also be asked to complete a short lifestyle and porting history questionnaire which should only take a few minutes. All the nformation that is collected during the course of the investigation will be treated with ne strictest confidentiality and will only be used for scientific research purposes. our names and personal particulars will not be released under any circumstances.

you would like to participate in the study, please meet us at the medical tent after ne race and enjoy a coke while we draw your blood.

	Dr Malcolm Collins, PhD	Mr Jan Bekker, B.Sc. (Hons)
Phone:	(021) 686 7330 ext 292	(021) 686 7330 ext 297
E-mail:	mcollins@sports.uct.ac.za	jbekker@sports.uct.ac.za

LIBERTY LIFE CHAIR OF EXERCISE AND SPORTS SCIENCE IN THE
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AND THE UNIVERSITY OF CAPE TOWN

Sports Science Institute of South Africa Boundary Road Newlands 7700 South Africa
115 Newlands 7725
Telephone (021) 6867330 International 27-21 6867330 Fax (021) 6867530

18 Februarie 1999

Die Genetiese Basis van Sportprestasie

Ons doen 'n studie op die genetiese basis van prestasie in sport by die MRC/UCT Bioenergetics of Exercise Research Unit (Sport Science Institute of South Africa). Om die studie uit te voer benodig ons bloedmonsters van huidige top atlete, of van atlete wat in die verlede presteer het, in 'n verskeidenheid van sportsoorte. Ons sal dit waardeer as u bereid sal wees om aan hierdie studie deel te neem.

Vir deelname aan die bogenoemde studie sal daar van u gevra word om 5ml veneuse bloed te skenk na die invul van 'n vrywaringsvorm. 'n Kort lewensstyl en oefening geskiedenis vraelys moet ook ingevul word. Al die inligting versamel deur die loop van die studie word as streng konfidensieël beskou en sal slegs vir navorsings doeleindes gebruik word. Name en persoonlike besonderhede sal onder geen omstandighede bekend gemaak word nie.

As u bereid is om deel te neem aan die studie, ontmoet ons na die wedloop by die mediese tent waar u 'n Coke kan geniet terwyl ons u bloed trek.

	Dr Malcolm Collins, PhD	Jan Bekker B.Sc (Hons)
Telefoon:	(021) 686 7330 uitbr 292	(021) 686 7330 uitbr 297
E-mail:	mcollins@sports.uct.ac.za	jbekker@sports.uct.ac.za

LIBERTY LIFE CHAIR OF EXERCISE AND SPORTS SCIENCE

UNIVERSITY OF CAPE TOWN

LIBERTY LIFE CHAIR OF EXERCISE AND SPORTS SCIENCE IN THE
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PO Box 115 Newlands 7725
Telephone (021) 6867330 International 27-21 6867330 Fax (021) 6867530

INFORMED CONSENT

I, the undersigned, have been fully informed about the MRC/UCT Bioenergetics of Exercise Research Unit study on the genetic basis of athletic ability. I have agreed to donate about five millilitres of venous blood. I have also agreed to complete a lifestyle and sporting history questionnaire and understand that all the information that is collected during the study will be treated with the strictest confidentiality and will only be used for scientific research purposes. I understand that my name and personal particulars will not be released under any circumstances.

I agree to participate in the study and I have been informed that I will be free to withdraw from the study at any time if I so wish.

FULL NAME OF SUBJECT: _____

SUBJECT'S SIGNATURE: _____

INVESTIGATOR: _____

INVESTIGATOR'S SIGNATURE: _____

DATED at _____ this _____ day of _____ 2000.

AS WITNESSES:

1. _____ 2. _____

LIBERTY LIFE CHAIR OF EXERCISE AND SPORTS SCIENCE IN THE BIOENERGETICS OF EXERCISE RESEARCH UNIT

OF THE MEDICAL RESEARCH COUNCIL (MRC)
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Telephone (021) 6867330 International 27-21 6867330 Fax (021) 6867530

GENETIC BASIS OF ATHLETIC ABILITY QUESTIONNARE
GENETIESE BASIS VAN SPORTPRESTASIE VRAELYS

PERSONAL PARTICULARS PERSOONLIKE BESONDERHEDE					
Name Naam					
Postal Address Pos Adres					
E-mail		Phone Telefoon			
Date of Birth Geboorde Datum		Cell			
Height Lengte		Sex Geslag	Male Manlik ☐	Female Vroulik ☐	
Race Ras		Weight Gewig		Age Ouderdom	

TRAINING DETAILS OEFENING BESONDERHEDE				
Sporting activity Sport aktiwiteit				
Years involved in distance running Jare deelgeneem in lang afstand hardloop				
Personal best time Persoonlike beste tyd	5 km	10 km	21.1 km	42.2 km
Year of first marathon Jaar van eerste maraton				
Average training per week (km/week) over the last year Gemiddelde oefening per week (km/week) oor laaste jaar				

NO _____

LEFT OR RIGHT HANDED

SMOKER OR NON-SMOKER

Nationality: _____

Place of Birth: _____

Have you had Achilles Tendon problems? Yes No

ABO blood group? A B AB O Pos Neg

RUGBY QUESTIONNAIRRE/VRAELYS

Name/Naam	

This questionnairre will be used to determine your highest level of play. Please mark the appropriate box with an 'X'.
Hierdie vraelys sal gebruik word om jou hoogste vlak van spel te bepaal. Dui asseblief die hoogste vlak met 'n 'X' aan.

HIGH SCHOOL/HOëRSKOOL: **(Name/Name _____)**

1st Team/Span		2nd Team/Span		Other/Ander		
Provincial/ Provinsiaal (Craven week)		SA Schools/ SA Skole		SA U19 SA O19		Province/Provinsie (_____)

UNIVERSITY/UNIVERSITEIT: **(Name/Name _____)**

Residence league/ Koshuis liga		1st Team/ Span			2nd Team/ Span			Other/ Ander	
University Team/ Universiteit Span		U19/ O19	U21/ O21		Senior 1st		Senior 2nd		Other/ Ander

CLUB/KLUB: **(Name/Name _____)**

U19/ O19		U21/ O21		Senior 1st		Senior 2nd		Other/ Ander	

PROVINCIAL TEAM/ PROVINSIALE SPAN: **(Name/Name _____)**

U19/ O19		U21/ O21		Senior 1st		Senior 2nd		Other/ Ander	

NATIONAL TEAMS/NASIONALE SPANNE:

U19/ O19		U21/ O21		**Springbok (senior)**		**Sevens**		**Other/ Ander**	

ACHIEVEMENTS/PRESTASIES:
Please state any other significant rugby achievements. Enige ander belangrike rugby prestasie.

Achievement / Prestasie	

Other/ Ander Sport:
Please indicate sport and level of participation. Noem die sport en vlak van deelname.

Sport	

GENETIC BASIS OF ATHLETIC ABILITY QUESTIONNAIRE

A. PERSONAL PARTICULARS			
Surname			
First Name			
Postal Address		Code	
E-mail address		Phone	
Date of birth		Cell	
Height (cm)		Gender	male ☐ female ☐
Weight (kg)			
Ethnic group (Only Required and Used for Research Purposes)	Black/African ☐ White ☐ Indian ☐ Mixed Ancestry (Coloured) ☐ Asian ☐ Other ☐		
Ancestry: Tribal or national background (eg Xhosa, Dutch, Zulu)	Father	Unknown ☐	
	Mother	Unknown ☐	
Nationality		Smoke	Yes ☐ No ☐
Place of Birth		Handedness	Left ☐ Right ☐
Do you know your blood group?	Yes ☐ A ☐ B ☐ AB ☐ O ☐ No ☐ Pos ☐ Neg ☐		

SPORTING DETAILS AND ACHIEVEMENTS	
Type of Sport	
Sport Details (specify distance or height; position(s) played in team; etc)	
Years involved in this Sport	
Professional or Amateur	
Competed at the provincial, national or international level	
Achievements (competitions or tournaments in which athlete or team has won awards or world records)	

APPENDIX 3

ETHICS APPROVAL

This project is part of a larger project entitled "Genetic Markers for Athletic Ability, Exercise Performance and Susceptibility to Exercise Induced Injury" for which formal ethics approval was received from the Faculty of Health Sciences Research Ethics Committee in 1999, ref number #202/99.

Institutional ethics approval was reapplied for in 2003, and was received in May 2003. REC REF: 114/2003

Research Ethics Committee
Faculty of Health Science
E46-26 Old Main Building, Groote Schuur Hospital, Observatory, 7925
Queries : Xolile Fula
Tel : (021) 406-6492 Fax: 406-6411
E-mail : Xfula@curie.uct.ac.za

14 May 2003

REC REF: 114/2003

Dr M Collins
Human Biology

Dear Dr Collins

THE GENETIC BASIS OF HUMAN ATHLETIC PERFORMANCE

Thank you for submitting your study to the Research Ethics Committee for reviewal.

The extension of the study is approved but the investigator should note the reservations which have been expresses in the post hoc submission of the study and the need for consent process to be followed in research protocols.

Please quote the above Rec. reference number in all correspondence

Yours sincerely

PROF T ZABOW
CHAIRPERSON

APPENDIX 4

BUFFERS AND SOLUTIONS

40% Acrylamide stock solution (38:2)

38% Acrylamide

2% Bis-Acrylamide

Store in dark bottle at 4 °C

Acrylamide gel buffer (1× TBE)

100ml 10× TBE buffer

dH_2O up to 1000ml

10% Ammonium Persulfate (10% AMPS)

1g Ammonium persulphate

10ml dH_2O

The solution may be stored at 4 °C for several weeks.

Ammonium Acetate Solution (Parzer's Rapid)

7.5 M ammonium acetate

Store at 4 °C.

Buffer 1 (Clotted Blood)

10 mM TRIS pH 8

10 mM KCl

10 mM $MgCl_2$

2 mM EDTA

2.5% TRITON X-100

Autoclave

Store at room temperature

Buffer 2 (Clotted Blood)

10 mM TRIS pH 8

10 mM KCl

10 mM $MgCl_2$

2 mM EDTA

0.4 M NaCl

1% SDS
Autoclave
Store at room temperature

Cell Lysis Buffer (Parzer's Rapid)
109,5 g sucrose
200 mM MgCl (take 1ml of 5 M Stock Solution)
1 M stock Tris-HCl (take 10mls, make up to 850 ml with dH_20)
10 ml Triton X-100
Make up to 1000 ml with dH_2O
pH 8
Autoclave
Store at room temperature

EDTA Stock Solution
100 mM EDTA pH 8.0

For 500 ml, start with 450ml dH_2O, add NaOH pellets to pH 7.5. More EDTA will go into solution as the pH approaches 8.0, so add gradually and let pellets dissolve. Continue to pH 8.0 with 6.0 N NaOH solution. Autoclave.

Store at room temperature.

Ethidium bromide staining solution
10 mg/ml
Light Sensitive! Store in dark bottle in refrigerator.

Freeze Mix
17.8 g Trisodium citrate
2.4 g $NaH_2PO_4.2H_2O$
2.8 g $Na_2HPO_4.2H_2O$
1 g Na azide
400 ml Glycerol
Make up to 1000 ml with dH_2O
Filter sterilize using Falcon 7104 bottle top filter (BD Biosciences Rivonia) with 0.22 micron cellulose nitrate membrane.
Store at room temperature.

6x Gel-loading buffer

0.25% bromophenol blue

0.25% xylene cyanol FF

30% glycerol in dH_2O

Glycerol Loading Dye, 6X

0.26% bromophenol blue

30% glycerol in H20

Store at 4 °C.

Guanidine HCl (Parzer's Rapid)

6.0 M Guanidine HCl

Autoclave

Store at room temperature

High Salt Buffer (TKM2)

10 mM Tris HCl pH 7.6

0,4 M NaCl

10 mM KCl

10 mM $MgCl_2$

2 mM EDTA

Autoclave

Store at room temperature

Lysis Buffer (Mouthwash)

1 M Tris-HCl (pH 8.0)

0.5 M EDTA (pH 8.0)

6 M NaCl

10% SDS

Autoclave

Store at room temperature

Low Salt Buffer (TKM1)

10 mM Tris HCl pH 7.6

10 mM KCl

10 mM $MgCl_2$

2 mM EDTA

25 ml/L Igepal CA-630 (previously known as NP-40)

Autoclave

Store at room temperature

NaCl Stock Solution

5 M NaCl

Autoclave

Store at room temperature

0.9% NaCl solution

9g NaCl

1000ml dH_2O

Autoclave

Store at room temperature

Proteinase K solution (Parzer's Rapid)

10 mg/ml Proteinase K

Freeze in aliquots.

Sarkosyl Solution (Parzer's Rapid)

20% Na laurylsarcosinate

You may need to heat to get into solution.

pH to 7.0 - 7.2 with 1.0 M HCl.

Autoclave

Store at room temperature

Freeze in aliquots.

10× Taq Polymerase Buffer

100 mM Tris-HCl (pH 9.0)

500 mM KCl

1.0% Triton® X-100

15mM $MgCl_2$

1× TE Buffer

10 mM Tris-Cl (pH 8.0)

1 mM EDTA

Autoclave before use. Store at room temperature.

Tris Stock Solution

1 M Tris-HCl pH 8.0

Autoclave

Store at room temperature

10× Tris-borate (TBE) Electrophoresis Buffer (1L)

107.8g Tris

~ 55g boric acid

7.44g di-sodium EDTA•$2H_2O$

Add the components in the order listed above to ~ 800ml dH_2O. It is important to add less than the total amount of boric acid. Stir until completely dissolved and check the pH. Adjust the pH to 8.3 with the remaining boric acid and bring the volume to 1000ml with dH_2O.

Wash Buffer (Parzer's Rapid)

10 mM NaCl

10 mM EDTA (pH 8.0)

Autoclave

Store at room temperature

APPENDIX 5

REAGENTS AND SUPPLIER CONTACT DETAILS

REAGENT NAME	SOUTH AFRICAN SUPPLIER	CATALOGUE #
100% Ethanol (C_2H_5OH) ■	Saarchem (Merck)	983
Acetic Acid Glacial (CH_3COOH)	AnalaR	BB10001P
Acrylamide (C_3H_5NO) ■	Sigma-Aldrich (Pty) Ltd.	A-3553
Agarose	Promega, Whitehead Scientific	V3121
Ammonium acetate ($NH_4C_2H_3O_2$)	Saarchem (Merck)	112220 EM
Ammonium persulphate ($(NH_4)_2S_2O_8$)	Sigma-Aldrich (Pty) Ltd.	A-3678
Bis-acrylamide	Roche	1 685 830
Boric Acid (H_3BO_3)	Promega, Whitehead Scientific	H5001
Bromophenol blue ($C_{19}H_{10}Br_4O_5S$)	Saarchem (Merck)	143 75 00 CB
Cell lysis solution	Promega	A7933
Chloroform ($CHCl_3$) ■	Saarchem (Merck)	159 504
di-Sodium hydrogen phosphate dehydrate ($Na_2HPO_4.2H_2O$)	Saarchem (Merck)	5822870
dNTP set (PCR grade)	Roche	
EDTA ($C_{10}H_{16}N_2O_8$) ■	Saarchem (Merck)	223 602
EDTA di-sodium salt ($C_{10}H_{14}N_2O_8Na_2.2H_2O$)	Saarchem (Merck)	223 60 20 EM
Ethidium Bromide ($C_{21}H_{20}BrN_3$) ■ T+	Sigma-Aldrich (Pty) Ltd.	E-8751
Glycerol ($C_3H_5(OH)_3$)	Saarchem (Merck).	267 65 20 LC
Guanidine HCL	Sigma-Aldrich (Pty) Ltd.	G-9277
Hydrochloric acid (HCl)	Saarchem (Merck)	306 30 54 LCA
IGEPAL CA-630 (Nonidet P-40)	Sigma-Aldrich (Pty) Ltd.	I-3021
Isoamyl Alcohol	Sigma-Aldrich (Pty) Ltd.	I-9392
Isopropanol, Propan-2-ol (C_3H_8O) ■ ■	Saarchem (Merck)	818766

Magnesium Chloride ($MgCl_2.6H_2O$)	NT Laboratory Supplies (Pty)Ltd.	R1480
N,N',-Methylene-bis-Acrylamide	ICN Biomedicals	800173
Nuclei lysis solution	Promega	A7943
Phenol (C_6H_5OH)	Sigma	P-1037
Potassium Chloride (KCl)	Saarchem (Merck)	504 202
Protein Precipitation Solution	Promega, Whitehead Scientific	A7953
Proteinase K	Sigma-Aldrich (Pty) Ltd.	P-2308
SDS, Sodium dodecyl sulphate ($C_{12}H_{25}OSO_2ONa$)	Saarchem (Merck)	113760
Sodium Acetate ($CH_3COONa.3H_2O$)	LASECHEM	L538
Sodium azide (NaN_3)	Riedel-deHaën (Merck)	13412
Sodium Chloride (NaCl)	Saarchem (Merck)	582 232 EM
Sodium dihydrogen orthophosphate dihydrate ($NaH_2PO_4.2H_2O$)	Saarchem (Merck)	582 26 80 EM
Sodium hydroxide (NaOH)	Saarchem (Merck)	582 32 00 EM
Sodium laurylsarkosinate ($C_{15}H_{28}NO_3Na$)	Sigma-Aldrich (Pty) Ltd.	L-5125
Sodium Perchlorate ($NaClO_4$)	Sigma-Aldrich (Pty) Ltd.	S-3564
Sucrose ($C_{12}H_{22}O_{11}$)	Saarchem (Merck)	588 15 EM
TEMED ($C_6H_{16}N_2$) N,N,N'N'-Tetramethyl ethylenediamine	Sigma-Aldrich (Pty) Ltd. Riedel-de Haën (Sigma)	T-9281
TRIS	Roche	708 976
TRIS-HCl	ICN Biomedicals	152172
TRITON-X100	Boehringer Mannheim	789 704
Xylene cyanol ($C_{25}H_{27}N_2O_7S_2Na$)	Saarchem (Merck) (Serva)	38505

Flammable Toxic Irritant, harmful

SUPPLIERS:

Promega, Whitehead Scientific, 89 Buitenkant Street, Brackenfell, Cape Town 7560, Tel: (27) 21 981 1560, Fax: (27) 21 981 5789, E-mail Address: info@whitesci.co.za

Sigma-Aldrich (Pty) Ltd., PO Box 10434, Ashton Manor 1630, Tel. (011) 979 1188, Fax. (011) 979 1119
www.sigmaaldrich.com/Area_of_Interest/Europe_Home/South_Africa.html

Merck (Pty) Ltd., Merck Laboratory Supplies, Tel: 011 345 9000, www.merck.co.za

Merck (Pty) Ltd. Western Cape Regional Office, Tel: 021 551 2410

ICN Biomedicals, Laboratory Specialties, P.O. Box 1513, 197 Fabriek Street, Bromhof, Strydom Park, Randburg 2125, Phone: +27 11 792 6790, Fax: +27 11 793 1064, www.labspec.co.za

LABORATORY & SCIENTIFIC EQUIPMENT CO., P.O. Box 2110, Capetown 8000, Tel. 27 21 53 17 504, Fax 27 21 53 17 562, e-mail: sales@lasec.co.za

AEC-Amersham (PTY) Ltd, 15th & Virginia St. Marlboro, Sandton, P.O. Box 1596, Kelvin 2054, Tel. 27 1144 44330, Fax. 27 1144 45457, e-mail: info@aecam.co.za

Adcock Ingram CR Care (PTY) Ltd, Division: Sterilab Services, P.O. Box 6888, Johannesburg 2000, SA, Phone: 27-11-494-8000, Fax: 27-11-494-8305, Products: Plastics

Roche Products (Pty.) Ltd., P.O. Box 129, Isando 1600 Gauteng RSA, South Africa, Tel +27-11-928 8700, Fax +27-11-974 7405

APPENDIX 6

DATABASE DISK

The database that was used during this project and for related projects is supplied on the disk. The database file was originally managed on Microsoft® Access 2000 (9.0.4402 SR-1).

Password: cyprus

.pdf versions of the main chapters of the thesis is also included on the disk (open in Adobe® Acrobat or Acrobat Reader).

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