GENETIC FACTORS ASSOCIATED WITH PERFORMANCE AND EXERCISE-ASSOCIATED WEIGHT LOSS IN IRONMAN TRIATHLETES

BY

LIESL DE MILANDER

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South Africa

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UCT/MRC Research Unit for Exercise Sciences and Sports Medicine
Sports Medicine Institute of South Africa
Boundary Road, Newlands, 7700
Cape Town
South Africa

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PRESENTATIONS AT INTERNATIONAL CONGRESSES

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2. Robson-Ansley, P., **de Milander, L.**, Collins, M. and Noakes, T.D. Acute interleukin-6 administration does not alter plasma soluble interleukin-6 receptor concentration following an acute bout of exercise. **11th Annual Congress of the European College of Sport Science**, Lausanne, Switzerland, 2006.

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1. **De Milander, L.**, Noakes, T.D. and Collins, M. The *IL-6* and *5-HTT* genes and performance during the South African Ironman Triathlons. **33rd Meeting of the Physiology Society of South Africa conference**, University of Cape Town, 2005 (Oral presentation).

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LIST OF ABBREVIATIONS

AAAD	Aromatic amino acid decarboxylase
ACE	The gene encoding for the angiotensin converting enzyme
ACSM	American College of Sports Medicine
ACTN3	The gene encoding for the alpha actinin isoform 3 of the skeletal muscle
ACTH	Adrenocorticotropic hormone
ADH	Antidiuretic hormone
ANG	Angiotensin
ANP	Atrial naturiuretic peptide
AP	Area postreamia
AQP1	The gene encoding for aquaporin 1
AQP2	The gene encoding for aquaporin 2
AT1	Angiotensin II receptor type 1
AT2	Angiotensin II receptor type 2
AVP	Arginine vasopressin
AVPR1a	Arginine vasopressin receptor type 1a
AVPR1b	Arginine vasopressin receptor type 1b
AVPR2	The gene encoding for the arginine vasopressin receptor type 2
AV3V	Anteroventral third ventricle
BCAA	Branched-chain amino acids
BDKRB2	The gene encoding for the bradykinin β2 receptor
BMI	Body mass index
bp	base pair
CNS	Central nervous system
COL5A1	The gene encoding for the α1 chain of the type V collagen
COL6A1	The gene encoding for the α1 chain of the type VI collagen
CSF	Cerebrospinal fluid
DA	Dopamine
EAH	Exercise-associated-hyponatremia
EAHE	Exercise-associated hyponatremic encephalopathy
ECF	Extracellular fluid

ET	Endothelin
FTO	The gene encoding for the fat mass and obesity associated protein
gp	Glycoprotein
GWAS	Genome-wide association studies
GH1	The gene encoding for growth hormone 1
HPA axis	Hypothalamic-pituitary-adrenal axis
ICF	Intracellular fluid
IL-6	Interleukin-6
IL-6R alpha	Interleukin-6 receptor alpha chain
in vivo	inside a living organism
ISF	Interstitial fluid
K+	Potassium
KKS	Kallikrein-kinin system
L allele	Long allele
L_G allele	A to G nucleotide substitution in the L allele
LPBN	Lateral parabrachial nucleus
MAO	Monoamine oxidase
MAO A	Monoamine oxidase type A
MAO B	Monoamine oxidase type B
MePO	Median preoptic nucleus
mRNA	Messenger RNA
Na+	Sodium
[Na+]	Sodium concentration
NaCl	Sodium chloride
[NaCl]	Sodium chloride concentration
NDI	Nephrogenic diabetes inspidus
NE	Norepinephrine
NOS3	The gene encoding for nitic oxide synthase 3
NTS	Nucleus of the tractus solitarius
OVLT	Organum vasculosum of the lamina terminalis
OXY	Oxytocin
PEA	Phenylethylamine

PVN	Paraventricular nuclei
RAS	Renin-angiotensin-system
PPARA	The gene encoding for the peroxisome proliferator-activated receptor alpha
PPARD	The gene encoding for the peroxisome proliferator-activated receptor delta
PPARGC1A	The gene encoding for the peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PPARGC1B	The gene encoding for the peroxisome proliferator-activated receptor gamma coactivator 1-beta
RFLP	Restriction fragment length polymorphism
rhIL-6	Recombinant human IL-6
S allele	Short allele
SFO	Subfornical organ
SNP	Single nucleotide polymorphism
SON	Supraoptic nuclei
TBW	Total body water
TFAM	The gene encoding for the mitochondrial transcription factor A
TPH	Tryptophan hydroxylase
TRHR	The gene encoding for the thyrotropin-releasing hormone receptor
TRP	Tryptophan
UCP2	The gene encoding for the mitochondrial uncoupling protein 2
UCP3	The gene encoding for the mitochondrial uncoupling protein 3
US	United States
VNTR	Variable number of tandem repeats
VO2 max	Maximum oxygen consumption or maximal oxygen uptake
WT	Water turnover
5-HIAA	Hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine or serotonin
5-HT1A	5-HT receptor subtype 1A
5-HTP	5-hydroxytryptophan
5-HTT	5-HT transporter or serotonin transporter

5-HTTLPR 5-HTT-gene-linked polymorphic region or serotonin transporter gene-linked polymorphic region

ABSTRACT

BACKGROUND

There is a large inter-individual variation in the physiological responses and performance of athletes during participation in endurance events, as well as power and sprint events. These multifactorial phenotypes are determined by a poorly understood complex interaction of genetic and environmental factors. Although investigators have identified individual factors, including specific genetic sequence variants that contribute to the endurance phenotype, the possible neurogenetic contribution to endurance athletic ability and an athletes' physiological responses to participation in endurance events remain unknown.

Changes in the levels of cytokines and neurotransmitters that play key signaling roles in the central nervous system (CNS) have been implicated in fatigue models and by implication endurance athletic ability. Exercise-induced increases in IL-6, central 5-HT and altered MAO-A can result in a deterioration of performance. The association of functional variants within the candidate genes *IL-6, 5-HTT* and *MAO-A*, that encode for proteins involved in physiological and biochemical pathways that have been implicated in overlapping peripheral (musculoskeletal) and central (brain/neural) fatigue models, with endurance performance were investigated in this thesis. In addition, the association of variants within candidate genes that regulate the homeostatic control of water balance during participation in endurance events were also investigated in this thesis. To this end, the *5-HTT* and *AVPR2* gene

were selected since they encode for key proteins that function in parallel with CNS pathways, regulating the homeostatic control of water balance.

AIMS AND OBJECTIVES OF THE THESIS

The aim of this thesis was to investigate candidate genes, mainly within the CNS, that may contribute to the variation in physiological responses (body weight changes) and athletic ability between athletes during participation in a 226 km Ironman triathlon. A genetic association approach was used in case-control studies to identify specific sequence variants within selected candidate genes. These candidate genes (*IL-6, 5-HTT* and *MAO-A*) were selected based on the biological function of their encoded proteins, which have been implicated in peripheral and central fatigue models. Candidate genes (*5-HTT* and *AVPR2*) that encode for proteins that play key roles in the neuro-endocrine control of total body water homeostasis were also investigated in this thesis. The objectives of the specific gene association studies, which addressed the primary aim of this thesis, were as follows:

- To investigate whether functional polymorphisms within candidate genes that alter production of cytokines (-174 *IL-6* G/C SNP) or components of the serotonergic system (44 bp insertion/deletion, also referred to as the long or L/short or S, polymorphism of the *5-HTT* gene and the 30 bp VNTR polymorphism within the *MAO-A* genes) are associated with ultra-endurance performance in triathletes who completed either the 2000 and/or 2001 South African Ironman Triathlons. **(Study 1)**

- To determine whether the 44bp *5-HTTLPR* (L/S) polymorphism, a functional genetic component of the serotonergic system, is associated with body weight changes during the 2000 and/or 2001 South African Ironman Triathlons. We propose that the S allele, associated with a lower 5-HTT expression and thereby a reduced 5-HT turnover, would hinder efficient neurotransmission resulting in reduced stimulation of brain thirst centres. The inhibition of drinking behaviour is likely to result in a larger decrease in body weight changes (a proxy of hydration status) during these events. **(Study 2)**
- To determine whether there were any associations between three single nucleotide polymorphisms (SNP's) (rs3761528, rs3761527 and rs4898457) within the *AVPR2* gene, and inter-individual variations in serum sodium and/or body weight changes in athletes competing in the 2000, 2001 and 2006 South African Ironman Triathlons. **(Study 3)**
- To investigate the genetic association of a polygenic profile that include results from a previously published study [1], and results presented in this thesis (Study 2 and Study 3) with body weight changes of triathletes. We propose that triathletes with a higher total genotype score (TGS) are more likely to experience greater relative body weight losses during the 2000 or 2001 South African Ironman Triathlons. **(Study 4)**

METHODS

Four hundred and sixty eight (66.8% of the entire field) self-reported Caucasian male triathletes who completed either the 2000 (n=119) and/or 2001 (n=330) South African Ironman Triathlons, and 200 apparently healthy Caucasian male

control participants (Con) who had not participated in an ultra-endurance event, such as the Ironman Triathlon, were recruited for Studies 1 and 2 of this thesis. As a result of incomplete sets of data only 449 triathletes, 119 from the 2000 and 330 from the 2001 events, were analysed in Study 2. The 2001 data was used for triathletes with complete sets of data for both events.

In addition, 658 self-reported Caucasian male triathletes that completed either the 2000 (n=171), 2001 (n=276) and/or 2006 (n=211) South African Ironman Triathlons were included in Study 3. Only the 2001, or subsequent 2000, data were used for triathletes with complete sets of data for more than one event. A total of 570 individual triathletes were therefore analysed in this study.

The participants included in Studies 1 and 2 were genotyped for the functional -174 G/C *IL-6* SNP, *MAO-A* 30 bp VNTR and/or 44 bp *5-HTTLPR* (L/S) polymorphisms, while the participants in Study 3 were genotyped for the rs3761528, rs3761527 and rs4898457 SNPs in the *AVPR2* gene.

To answer the final objective of this thesis the data in Studies 2 and 3, as well as the previously published paper, were combined for analysis in Study 4. Only 296 triathletes who had been genotyped for all three variants were analysed in this study. In order to combine analysis from the previously published paper only triathletes that completed either the 2000 and/or 2001 South African Ironman Triathlons were included in the analysis. As previously described in Studies 1 and 2, only the 2001 data was used for triathletes (n=78) with complete sets of data for both events.

RESULTS AND DISCUSSION

Genetic variants associated with ultra-endurance performance

No significant association between either the -174 *IL-6* G/C, 44 bp *5-HTTLPR* (L/S) and *MAO-A* 30 bp VNTR polymorphisms and endurance performance in the 2000/2001 South African Ironman Triathlons were found.

Genetic variants associated with weight changes during ultra-endurance events.

The novel findings of this thesis were that the functional 44bp *5-HTTLPR* (Study 2) and minor haplotypes constructed from SNPs rs3761528, rs3761527 and rs4898457 within the *AVPR2* gene (Study 3) were associated with body weight changes during the 2000, 2001 and 2006 South African Ironman triathlons. The SS genotype of the *5-HTTLPR* polymorphism was significantly (p=0.042) associated with a relative greater body weight loss in triathletes. There was a significant (X^2= 5.1, p=0.024) linear trend for the SS genotype amongst the three body weight loss groups, with the >5% body weight loss group having the highest percentage of SS genotype triathletes.

There were no statistically significant associations between the three individual *AVPR2* SNPs and serum sodium and body weight changes during the Ironman events. There was however a significant association (p=0.041, odds ratio 9.7, 95% confidence interval 0.7 to 165.6) between the combined minor haplotype

constructs (GCT, GTC and GCC) and body weight loss of triathletes. The minor haplotype constructs were associated with a >3% relative body weight loss during the race. The function, if any, of the three individual *AVPR2* SNPs rs3761528, rs3761527 and rs4898457 is however currently unknown. A possible explanation for the observed association with minor haplotype constructs is that the individual SNPs might be tightly linked to unidentified functional SNPs within regulatory or protein coding regions of the *AVPR2* gene or perhaps are in linkage equilibrium with another gene within close proximity on the X chromosome.

Polygenic profile associated with body weight loss during ultra-endurance events.

Another novel association of this thesis was that triathletes with a higher total genotype score (TGS) are more likely to experience greater relative body weight losses during the 2000 or 2001 South African Ironman Triathlons. Each triathlete was scored according to the number of 'maximum body weight loss' genotypes (*5-HTT* SS, *BDKRB2* +9/+9 and *AVPR2* minor haplotypes) that he possessed (Table 5.1). The TGS was calculated from the accumulative score at all three loci and expressed as a percentile.

The distribution frequency of the 'maximum body weight loss' genotypes *5-HTT* SS, *BDKRB2* +9/+9 and *AVPR2* minor haplotypes was associated with greater relative body weight losses during the Ironman events (X^2= 8.5, p=0.015). Furthermore, the possession of two 'maximum' genotypes was associated with

greater relative body weight losses in triathletes when compared to those possessing one or none 'maximum' genotypes. These results provide further support to our hypothesis that was previously addressed in the secondary objective of this thesis.

CONCLUSION

The findings of this thesis provide insight into the complexity of the mutifactorial endurance performance phenotype. The findings of this thesis demonstrated there were no direct associations between the *IL-6* –174 G/C, *5-HTT* 44 bp insertion–deletion, and *MAO-A* 30 bp VNTR gene polymorphisms and endurance performance in the South African Ironman Triathlons. The neurogenetic basis of the central governor hypothesis during performance requires further investigation. In addition, the novel findings of this thesis provide an invaluable contribution to understanding the genetic basis of inter-individual weight changes and total body weight (TBW) loss in athletes during ultra-endurance events such as the Ironman Triathlon. The biological and perhaps functional role of the genetic variants within the *AVPR2* gene still requires further investigation. Also, the search for additional variants that can possibly contribute to the "weight loss" profile of athletes during ultra-endurance events is warranted.

CHAPTER 1

INTRODUCTION AND SCOPE OF THESIS

There is a large inter-individual variation in performance and physiological responses of athletes competing in sporting events [2]. Some athletes have the ability to excel at a much higher or elite level, than their competitors, in spite of similar motivation, training and opportunities. Similarly, physiological responses of athletes with similar endurance abilities, such as weight changes that probably resemble fluid balance, vary significantly [3]. The inter-individual variation in athletic performance and the physiological responses to training and competition can be explained by the multifactorial nature of these phenotypes. These phenotypes (athletic performance and physiological responses to training and competition) are determined by complex and poorly understood interactions of both environmental and genetic factors [4]. It has been well documented that a correlation between body weight loss and endurance performance exists, with faster athletes experiencing greater body weight loss during events [5]. It is therefore possible that common intrinsic factors, including genetic factors, are associated with both performance and body weight loss during participation in an athletic event. In support of this, investigators have reported that a single nucleotide polymorphism within the *AQP1* gene is associated with body fluid loss in 10 km runners [6] and predict marathon performance in runners [7]. Theoretically, the biological function of the protein encoded for by the genes investigated in this study can be involved

in both performance and body weight loss and hence, the association of these genes with both phenotypes was investigated in this thesis.

At the biological level athletic performance and the physiological responses to exercise is determined by the integration of multiple physiological systems and biochemical processes. Physiological systems include, amongst others, skeletal muscle and central nervous (CNS) systems. Although biological systems and processes are adaptable or trainable and can be alerted to maximise performance for a particular sporting discipline, their adaptability is not infinite or the same for every individual [2]. The fixed component is set by an individual's genetic make-up that is inherited from their parents. To add to the complexity of the matter, athletic ability and physiological responses are also polygenic in nature and, therefore, it is the combination of small contributions from many different genes opposed to one single gene that contributes to the variation in the observed performance and physiological responses [5].

The search for candidate genes that could possibly contribute to athletic ability have originally focused on indentifying components within the musculoskeletal system [6-17]. The products of these genes were believed to alter physiological, biochemical and biomechanical processes locally within the skeletal muscle favouring athletic ability. Although there was initially great emphasis on the musculoskeletal system in identifying a polygenic profile for athletic performance phenotypes, there is a large body of literature implicating

the involvement of other physiological systems such as the CNS in sporting performance [18].

The central nervous system and changes in various neurotransmitters and cytokines have been suggested to play a role in mediating exercise capacity. In particular, the serotonergic pathway has been implicated in central fatigue, which is defined as a negative central influence that overrides an individual's conscious voluntary muscular effort resulting in a deterioration of performance. Although scientists have identified possible mechanisms of CNS fatigue during exercise, in particular endurance exercise [19-22], there has been much less focus on the possible neurogenetic contribution to the athletic ability phenotype.

The primary objective of this thesis was therefore to identify genetic components, which influence CNS function that could possibly contribute to the variation in physiological responses and athletic ability favouring the endurance phenotype. Candidate genes (*IL-6, 5-HTT* and *MAO-A*) that encode for proteins involved in physiological and biochemical pathways that have been implicated in overlapping peripheral (musculoskeletal) and central (brain/neural) fatigue models were selected. Additional candidate genes (*5-HTT* and *AVPR2*) of key components that function in parallel with central pathways, regulating the homeostatic control of water balance during endurance exercise, were also selected. Variants, of which most were functional, were identified within candidate genes and association studies (case-control design) were performed to ascertain any favourable effect on the endurance phenotype.

For the purpose of experimental investigation and to gain an insight into the polygenic phenotype of athletic ability and the variation in physiological responses during endurance events, chapter 1 reviews the current literature on the central neural regulation of effort and fatigue in endurance exercise (Section 1.2). Focus is particular to the role of the cytokine, interleukin-6 (Section 1.3), and the serotonergic system (Section 1.4). Further we review the complex integration of endocrine (Section 1.6.3) and central serotonergic (Section 1.6.4.1) systems in regulating fluid balance by assessing body weight changes as a surrogate measure of fluid balance during endurance events. Subsequent experimental chapters will use a candidate gene approach to achieve the aims of this study (Chapters 2 to 5). The association of genetic variants within the *IL-6, 5-HTT* and *MAO-A* genes with ultra-endurance performance during the 2000 and/or 2001 South African Ironman Triathlons will be reported in chapter 2. The association of variants within the *5-HTT* and *AVPR2* genes with body weight changes of triathletes during the 2000, 2001 and/or 2006 South African Ironman Triathlon events will be examined in chapters 3 and 4. The final chapter of this thesis will investigate the association of a polygenic profile with body weight loss during these South African Ironman Triathlons (Chapter 5).

LITERATURE REVIEW

INTER-INDIVIDUAL VARIATION IN PHYSIOLOGICAL RESPONSES OF ATHLETES COMPETING IN ENDURANCE EVENTS

1.1 ENDURANCE ATHLETIC ABILITY: A MULTIFACTORIAL, POLYGENIC PHENOTYPE

Athletic ability, which can be can be classified into either endurance or muscle-strength (sprint and power) phenotypes, is a multifactorial, polygenic phenotype [14, 23-25]. Endurance events are typically performed at low to medium intensities for a prolonged period of time. Strength-power events on the other hand are characterised by high intensity, short duration or intermitted bouts of exercise, such as sprinting. There are therefore distinct biological differences between the sprint/power and endurance phenotypes. Biological factors that have been shown to associate with endurance athletic ability include anthropometrical measures such as lower body mass and body mass index (BMI) [26], reduced body fat % [26], increased distribution of oxidative type I muscle fibres [27], increased mitochondrial expression and oxidative enzyme activity [28, 29], increased VO2 max [26] and improved running economy [30]. Strength trained athletes, on the other hand, have an increased distribution of type IIA and IIX muscle fibers [31], show increased mitochondrial glycolytic and neural adaptations to sustain higher muscle contraction velocities needed for greater force generation [32] and have increased muscular hypertrophy of

trained muscle groups that is phenotypically characteristic for this discipline [33].

As previously mentioned, athletic ability is determined by the complex and poorly understood interactions of both environmental and genetic factors [14, 24, 34]. Some of the environmental or non-biological factors predicting sporting success include (i) coaching behaviour and technique [35, 33], (ii) training and competition environments [37-39] (iii) the age at which the young athlete begins practicing deliberately for the sport [40, 41], (iv) the number of hours or distance of accumulated practice [42], (v) psychological and motivational factors [43-46], (vi) the implementation of the correct training volumes and methods at different stages of development [39, 47] (vii) to avoid training and competition-associated injury and burnout [48-50], (viii) training techniques [51, 52], (ix) recovery [53, 54], (x) talent identification [55, 56], (xi) competition strategies [57, 58], (xii) socio-cultural issues [59, 60] and (xiii) nutrition [61-63]. These are non-biological factors that can, when optimally implemented or altered, induce favourable adaptations to musculoskeletal and other physiological components to maximise sporting performance.

In spite of the fact that training and other factors can cause vast improvements in athletic ability, it has long been recognized that the inter-individual variation in athletic ability also has a genetic component. Advances in molecular genetics prompted the identification of the first genetic variants shown to associate with athlete performance over a decade ago [64]. Unfortunately these early studies resulted in the ‘single gene as magic bullet’ philosophy in some circles,

whereby single gene variants are proposed to predict either physical power or endurance ability. These include the alpha-actinin-3 (*ACTN3*) [17, 65] or angiotensin converting enzyme (*ACE*) genes [66] respectively. An article entitled “Gene test for child's sporting chance” written by Jemma Chapman was published in the Times online on the 20th December 2004. The following was included in the article “WANT to know if your child is naturally geared to become the next Kelly Holmes or Matthew Pinsent? A biotechnology company based in Australia has developed a DNA test that claims it can identify whether a child has the genetic make-up to excel in either sprint and power sports or endurance events. The test, available online in kit form for about $110 (£43) uses a DNA sample taken from a mouth swab …….” The company Genetic Technologies Limited markets the “ACTN3 sports gene test” for $200 and originally made the following claim on their webpage: “We are the only company in the world able to offer a genetic test to determine whether you are naturally geared towards sprint/power events, or towards endurance sporting ability.” (http://www.gtg.com.au/). It is now evident that endurance, sprint and power athletic ability is multifactorial and the result of interaction between many genes and environmental constrains [2, 67, 68].

Up until 2010, over 239 genetic polymorphic associations with athletic performance and health-related fitness phenotypes had been published [69, 70]. It is believed that these polymorphisms either directly or indirectly due to linkage with the functional variants alter the protein products or production, which in turn impact on key physiological and biochemical processes during exercise, favouring athletic performance phenotypes. Initial investigations

during the early 90's primarily focussed on underlying genetic components encoding for proteins involved in skeletal muscle biology and their association with exercise intolerance [71-74], or responsiveness and trainability in either endurance or muscle-strength phenotypes [9]. As such, we and others have investigated and continue to investigate various polymorphisms within the musculoskeletal system or metabolism encoding for genes that are believed to contribute to endurance capacity in triathletes (Table 1.1). Polymorphisms that have been associated with Ironman Triathlon performance include the angiotensin converting enzyme (*ACE*) I [75] and bradykinin β2 receptor (*BDKRB2*) -9 alleles [76], whereas the nitric oxide synthase 3 (*NOS3*) GG genotype was significantly underrepresented in faster triathletes [76]. Furthermore, the collagen, type V, alpha 1 (*COL5A1*) BstUI restriction fragment length polymorphism (RFLP), a component of the tendon apparatus and other connective tissues that has been associated with musculoskeletal stiffness, has been identified as a marker for endurance running performance during the 2006 and 2007 South African Ironman Triathlons. The *COL5A1* BstUI RFLP, BMI, age and running training during the last 15 weeks accounted for 30% of the variance in time to complete the run [77]. Also, the *COL6A1* rs35796750 SNP, specifically the TT genotype, has recently been shown to associate with endurance cycling performance, a cross-sectional analysis of the 226km cycle stage during four South African Ironman Triathlons [78].

In addition, various polymorphic associations with endurance performance have been reported in Russian Olympic triathletes. The XX genotype of the R577X polymorphism within the *ACTN3* gene was significantly underrepresented in the

Russian Olympic triathletes when compared to controls [79]. Other genes that include the *PPARA* rs4253778 G, *PPARD* rs2016520 C, *PPARGC1A* Gly482, *PPARGC1B* 203Pro, *TFAM* 12Thr, *UCP2* 55Val and *UCP3* rs1800849 T polymorphic variants were significantly overrepresented in the same cohort of Russian Olympic triathletes when compared to controls [23, 80, 81]. Nonetheless, there was no association of the *ACTN3* R577X [82] and *UCP3* rs1800849 C/T (-55 C/T) [11] polymorphisms with ultra-endurance performance during the South African Ironman Triathlons. The *GH1* T/A variant also showed no association with endurance performance in the same cohort of South African Ironman triathletes [83].

However, we cannot exclude the possible genetic contribution of understudied physiological systems such as psychological aptitude that been shown to contribute, at least in part, to athletic phenotypes [84, 85]. In contrast to the musculoskeletal system the biological mechanism or contribution of the central nervous system and psychological traits on sporting performance is relatively unknown.

Table 1.1: A summary of genetic association studies relating to endurance athletes.

Gene	Chromosome Location	Polymorphism	Endurance allele or genotype association	Reference
Alpha-actinin 3 (ACTN3)	11q13.1	R577X (rs1815739 C/T)	None XX underrepresentation	[82] [79]
Angiotensin converting enzyme (ACE)	17q23	insertion (I) / deletion (D)	I	[76]
Bradykinin β_2 receptor (BDKRB2)	14q32.1-q32.2	-9/+9	-9/-9	[76]
Collagen, type V, alpha 1 (COL5A1)	9q34.2-q34.3	BstUI RFLP T/C	TT	[77]
Collagen, type VI, alpha 1 (COL6A1)	21q22.3	rs35796750 T/C	TT	[78]
Growth hormone 1 (GH1)	17q23–q24	T/A	None	[83]
Nitric oxide synthase 3 (NOS3)	7q35-36	rs1799983 G/T	GG underrepresentation	[76]
Peroxisome proliferator-activated receptor alpha (α) (PPARA)	22q13.1	rs4253778 G/C	G	[86]
Peroxisome proliferator-activated receptor delta (δ) (PPARD)	6p21.2-21.1	rs2016520 T/C	C	[87]
Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PPARGC1A)	4p15.1	Gly482Ser	Gly482	[80]
Peroxisome proliferator-activated receptor gamma coactivator 1-beta (PPARGC1B)	5q33.1	Ala203Pro	203Pro	[80]
Transcription factor A, mitochondrial (TFAM)	10q21	Ser12Thr (rs1937 G/C)	12Thr	[80]
Uncoupling protein 2 (UCP2)	11q13	Ala55Val (rs660339 C/T)	55Val	[80]
Uncoupling protein 3 (UCP3)	11q13	rs1800849 C/T	None T	[11] [80]

1.2 THE CENTRAL NEURAL REGULATION OF EFFORT AND FATIGUE DURING ENDURANCE ACTIVITY

1.2.1 Peripheral versus central fatigue

In an attempt to explain superior endurance athletic ability many studies have focussed on possible markers or phenotypes of performance that would delay the effects, or premature onset, of fatigue [88, 89]. Exercise related fatigue is defined as the inability of an athlete to maintain a desired workload, with an increased sensation of 'tiredness' that ultimately results in an acute impairment of performance [90]. Traditionally, fatigue during exercise has been attributed to dysfunction in the mechanical contractile processes within the skeletal muscle during excessive exercise [18, 91, 92]. Physiological and biochemical changes such as metabolite (lactate) accumulation [93], substrate (glycogen) depletion [94, 95] and pH changes [96] have been identified within fatigued muscle and have therefore been suggested to be the cause of fatigue. This has been referred to as the "peripheral" model of fatigue [97-99].

More recently, the central nervous system (CNS) has also been suggested to play an important regulatory role in the onset of fatigue [99]. In the "central" model, exercise is regulated by neural command signals to maintain a submaximal level of skeletal muscle activation as a protective mechanism for homeostasis. This model requires afferent signals, also known as sensory feedback, from active contracting skeletal muscle and other physiological

systems to the brain. It is proposed that the brain makes calculations based on the physiological and metabolic status of the athlete and when necessary, adjusts the efferent signals from the brain, resulting in reduced recruitment of skeletal muscle in order to maintain a homeostatic environment [99-105]. There is evidence for a decrease in motor neuron drive during self-paced exercise [106, 107]. It is, therefore, not the skeletal muscle's inability to exert the required force, but more so an increase in perceived effort as athletes have to generate a more substantial motor command to achieve this [105, 107, 108]. It has also been suggested that "psychological" factors such as motivation and perception can influence CNS drive to the active contracting muscle resulting in premature or delayed onset of fatigue [105, 109]. Furthermore, the involvement of the CNS in the aetiology of central fatigue is supported by the observation of an unexplained debilitating fatigue that accompanies severe infections, chronic fatigue syndrome, certain diseases and various psychological disorders that undoubtedly is unrelated to skeletal muscle [18].

It has been proposed that exercise induced changes in specific neurotransmitters and neuromodulators may play an important role in the onset of exercise related fatigue and will therefore be reviewed in detail in the following sections [18, 110].

1.2.2 Cytokines and neurotransmitters

Exercise induced changes in various brain neurotransmitters (serotonin, dopamine and acetylcholine) and neuromodulators (ammonia and various

cytokines) have been suggested to be possible mediators in the onset of exercise related fatigue [18]. Whilst not excluding the involvement of other neurotransmitters, the central fatigue hypothesis implicates serotonin (5-HT), that when elevated impairs CNS signaling resulting in deterioration of performance [111]. The potential role of the serotonergic system in this hypothesis will be explored in detail in section 1.4 of this review.

Furthermore, elevated cytokine concentrations associated with Chronic Fatigue Syndrome, which manifests as debilitating symptoms of fatigue and exercise intolerance, have been implicated at the CNS level [18]. Interleukin-6 (IL-6) production during exercise precedes that of any other cytokine and has, therefore, been proposed to act as a central messenger to the brain contributing to the "central fatigue" hypothesis [112]. For the purpose of this literature review, focus was specific to the role of IL-6 (section 1.3) and serotonergic system (section 1.4) signaling during endurance exercise.

1.3 IL-6: A CIRCULATING FATIGUOGEN

1.3.1 The biological role of IL-6

The polypeptide, IL-6, belongs to a family of cytokines that makes use of a common signaling membrane-bound receptor subunit, glycoprotein (gp) 130, as well as the IL-6 specific soluble receptor subunit gp80 (also known as IL-6Ralpha) [113]. This signaling molecule exerts a pleiotropic endocrine, autocrine and paracrine effect on many different cell types, thereby facilitating

cell-to-cell communication in an integrative neuroendocrine and immune system network [114-116]. IL-6 is produced by most organs within the human body (immune and non-immune tissues) and plays an important regulatory role in mediating inflammation, the acute phase response and hematopoiesis. This cytokine elicits pro- and anti-inflammatory effects, depending on the in vivo environmental circumstances, and regulate growth, differentiation and the survival of many cell types (Figure 1.1) [113, 116, 117].

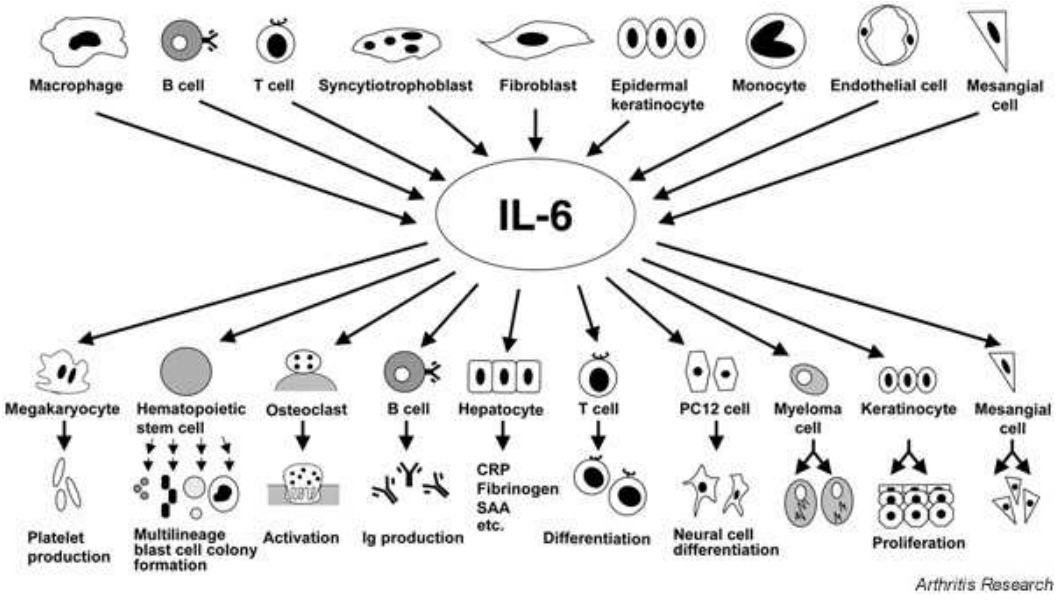

Figure 1.1. A schematic presentation of IL-6 producing cells (top) that exert a variety of different biological activities on various target cells (bottom). Image adopted from Naka et al. [118].

Contrary to its regulatory function in health, imbalances in IL-6 signaling are involved in the pathogenesis of autoimmune-diseases (reviewed by Mihara et al. [119]). These disease states clinically manifest with an up-regulation in pro-inflammatory cytokines [120]. Consequently, increased circulating IL-6 levels induce a chronic systemic inflammation with altered immune responses and debilitating “sickness” behaviour (lethargy, anorexia, depression, sleepiness and cognitive disturbances) [121]. IL-6 also plays an integral signaling role during exercise [122].

1.3.2 IL-6 response to exercise

Large increases in IL-6 levels (up to 100-fold), which precede that of any other cytokine, have been observed in response to prolonged exercise [123, 124]. The extent of the increase in circulating IL-6 levels is related to the mode, intensity and duration of exercise [125]. Higher concentrations have been reported for running versus cycling, indicating an association with the degree of muscle contraction per se and disruptions within the muscle cytoskeletal matrices [126, 127]. In addition, the kinetics of the IL-6 response depends on the type of muscle contraction. During concentric exercise, plasma IL-6 concentrations peak at cessation of exercise and decline during the post-exercise period [128]. Conversely, during eccentric contraction (more correctly referred to lengthening under tension) IL-6 release is associated with muscle damage and reaches peak levels well after cessation of exercise [127, 129, 130]. Nonetheless, the IL-6 contribution as a result of muscle damage to that of total circulating plasma levels is marginal [125, 131]. The greatest increase in

IL-6 concentrations have been observed during prolonged (endurance) exercise; implicating duration as the main determinant for IL-6 amplitude [124, 132, 133].

Steensberg and co-workers (2000) as well as others [125, 130] have identified the active contracting skeletal muscle as the primary source for this exponential increase in circulating IL-6 levels. Other contributing sources include the peritendinous tissue [134] and brain [135].

1.3.3 Metabolic effects of the "myokine" IL-6

Muscle-derived IL-6 not only mediates exercise-associated immune changes, but also mediates exercise-associated metabolic changes in a paracrine or endocrine manner and has, therefore, been newly defined as a "myokine" [136]. Myokines include all cytokines and peptides that are produced, expressed and released by the muscle fibres with resultant paracrine or endocrine effects [136]. Muscle-derived IL-6 has been suggested to maintain or deteriorate the ability to exercise by regulating energy metabolism in the liver and adipose tissue, as well as to, activate the hypothalamus.

1.3.3.1 Energy metabolism

There is clear evidence for the stimulatory action of IL-6 on hepatic glucose release [137, 138], adipocyte lipolysis during exercise-induced muscle glycogen depletion [137, 139, 140] and activation of the hypothalamic-pituitary-adrenal (HPA) axis [141]. Wallenius et al. [142] demonstrated the lipolytic effect of IL-6 administration, in decreasing body weight, within IL-6 deficient mice that developed mature-onset obesity, compared to wild-type control mice. Adipose IL-6 production is suppressed during exercise, but elevated post-exercise acting in a paracrine lipolytic manner [143].

An increased IL-6 mRNA expression, transcriptional rate and protein release have been observed during exercise when muscle glycogen stores were in a depleted state [127, 128, 144, 145]. This increase in IL-6 release, however, was attenuated following carbohydrate supplementation [126, 129]. Furthermore, IL-6 administration has been demonstrated to act directly on hepatocytes [138], resulting in an increase in circulating blood glucose and a decrease in hepatic glycogen content [146, 147]. IL-6 release from the working skeletal muscle and uptake by the liver, therefore, facilitates hepatic glucose release to help maintain blood glucose homeostasis as glucose uptake by muscles increases during prolonged exercise. It is therefore hypothesized that this mechanism accounts for the attenuated IL-6 response during glucose and/or carbohydrate supplementation [125].

1.3.3.2 Central activation

Maintaining blood glucose homeostasis is also crucial for metabolic function of the CNS. Within the CNS, IL-6 is produced by activated astrocytes [148] and hypothalamic nuclei [149], which can be enhanced following prolonged stress, such as endurance exercise. Under normal conditions IL-6 concentrations are low, with elevations during brain injury, inflammation, hypoxia and certain diseases. IL-6 produced centrally acts in a paracrine manner on central structures [135]. Also, systemic circulating IL-6 concentrations, predominantly from skeletal muscular origin, have been proposed to act as a feedback mechanism on central structures by crossing over the blood brain barrier and thereby, contributing to the “central fatigue” hypothesis [112].

These findings suggest the role of IL-6 within a regulatory loop consisting of feedback mechanisms regulating energy metabolism (liver, adipose tissue) and central activation (hypothalamus) to either maintain or deteriorate the ability to exercise.

1.3.4 The role of IL-6 in the perception of fatigue

1.3.4.1 At Rest

The administration of recombinant human IL-6 (rhIL-6) to healthy individuals at rest has been associated with an increased sensation of fatigue, altered ability

to concentrate and disrupted sleep patterns [150]. Conversely, the administration of an IL-6 antagonist (humanized anti-IL-6 receptor antibody) to patients diagnosed with IL-6 related immune-inflammatory diseases (Castleman's disease and rheumatoid arthritis) resulted in an immediate attenuation of the symptom of fatigue [151]. Changes associated with elevated levels of plasma IL-6 include malaise, fatigue, elevated levels of "stress hormones" such as adrenocorticotrophic hormone and cortisol, and increased heart rate and core temperature [114, 152-156].

1.3.4.2 Exercise

During prolonged exercise the contracting skeletal muscle contributes largely to the total plasma IL-6 produced, suggesting that skeletal muscle IL-6 production during exercise might be involved in the perception of fatigue [157]. In support, findings from a performance related study within our laboratory showed that intravenous rhIL-6 administration, prior to a 10km time trial run, increased the sensation of physical and psychological fatigue ultimately resulting in a decrement in performance. Furthermore, we observed a concurrent increase in plasma IL-6 and prolactin (by implication, serotonin production within the brain) concentrations during exercise. This has led to the conclusion that IL-6 may possibly act as a circulating 'fatiguogen', resulting in an increased serotonergic system activation, which may play a role in the development or perception of fatigue, during exercise [22].

1.4 CENTRAL ACTIVATION: THE SEROTONERGIC SYSTEM

1.4.1 The biological role of serotonin

Serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter synthesised from the amino acid l-tryptophan that involves a two-step metabolic process mediated by the enzymes tryptophan hydroxylase (TPH) and the aromatic amino acid decarboxylase (AAAD) [158]. Eighty to ninety percent of the body's total serotonin production occurs peripherally within various cell types. However, the major source of production is the enterochromaffin cells of the gastrointestinal tract, where it acts locally in a paracrine manner to regulate intestinal movements [159]. This neurotransmitter also regulates many other physiological processes within multiple organ systems through the activation of myenteric interneurons and afferents that activate regulatory processes, reflexes and transmit information to the CNS [159, 160]. These processes include the regulation of energy balance and food intake, endocrine function, and cardiovascular and pulmonary physiology [160].

The remainder of the body's total serotonin production occurs centrally and primarily within the median raphe nuclei, where most serotonergic neurons are localised [161]. Serotonin is probably best known for its neurotransmitter function within the brain, modulating all human behavioural and neuropsychological processes. These include mood, perception, reward, anger, aggression, appetite, memory, sexuality and attention [160].

Serotonergic dysregulation has been implicated in the pathogenesis of psychiatric and neurological disorders [162].

1.4.2 Serotonergic neurotransmission within the brain

The biosynthesis of serotonin occurs within the neuron's axon terminal followed by uptake into presynaptic storage vesicles (Figure 1.2). These vesicles transform to secretory vesicles that attach to the presynaptic membrane, where after serotonin is released into the synaptic cleft [163]. This process is regulated by presynaptic autoreceptors that ensure appropriate serotonin release from the presynaptic neuron needed for physiological activation of postsynaptic receptors. Consequently, postsynaptic receptors activate corresponding secondary messenger systems or stimulate target organs. In addition, the amount of serotonin released is proportional to the serotonin content within serotonergic neurons following uptake into storage vesicles. Therefore, removal of serotonin from the synaptic cleft, back into presynaptic neurons, by 5-HT transporters (5-HTT) is essential for effective neurotransmission [158].

Furthermore, extra- and intra-neuronal 5-HT is degraded by the enzyme monoamine oxidase (MAO), which is localised to the outer mitochondrial membrane. Two isoforms of the enzyme exist: type A (MAO A) that predominantly metabolises 5-HT and norepinephrine (NE), and type B (MAO B) that metabolises phenylethylamine (PEA) [164]. Both isoenzyme forms have

an equal preference for dopamine (DA), although dopaminergic neurons exclusively contain the MAO A type [165, 166]. The two isoenzymes are differentially expressed in various peripheral tissues and centrally in neurons. MAO A is present primarily in the gastrointestinal tract, portal system, and peripheral and central adrenergic neurons [167-169], whereas MAO B is more abundant in blood platelets and the human basal ganglia [170]. The distribution of MAO A and B in the human brain differs in various regions [171]. MAO B is the primary isoenzyme in the brain that accounts for 80% of total MAO activity [172] and has been shown to increase with age [173]. Although the precise function of MAO B in the brain is still unknown, MAO A plays a vital role in maintaining concentrations of DA, 5-HT and NE within extra- and intra-neuronal compartments [172, 174].

Effective neurotransmission is, therefore, modulated by an appropriate equilibration of presynaptic 5-HT storage, release and removal from the synaptic cleft by 5-HT transporters, and the degradation by MAO A [158].

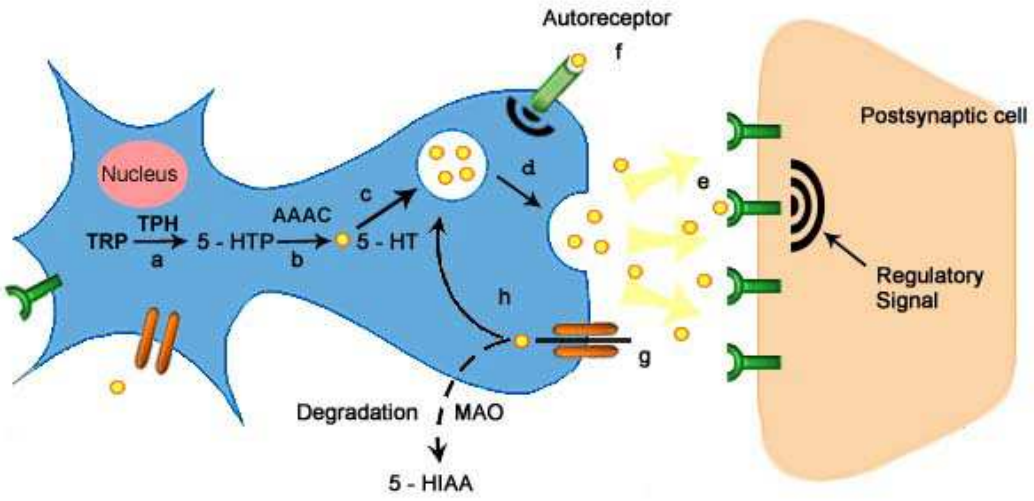

Figure 1.2. A schematic illustration of serotonin synthesis, neurotransmission and turnover within the brain. **(A)** The precursor tryptophan (TRP) is converted to 5-hydroxytryptophan (5-HTP) by the enzyme tryptophan hydroxylase (TPH). **(B)** 5-HTP is converted to 5-hydroxytryptamine (5-HT, serotonin) by aromatic amino acid decarboxylase (AAAC). **(C)** 5-HT is taken up into storage vesicles. **(D)** 5-HT is released from the storage vesicles into the synaptic space. **(E)** 5-HT can activate different subtype 5-HT receptor families (1, 2, 3, 4, 5, 6 and 7) that signal respective pathways inside the postsynaptic neuron. **(F)** 5-HT activates the presynaptic 5-HT1A autoreceptor that signal the inhibition of serotonin production and release into the synaptic space. **(G)** Serotonin transporters (5-HTT) facilitate the re-uptake of 5-HT into the presynaptic 5-HT terminals. **(H)** Within the presynaptic 5-HT terminals, 5-HT would either be taken up by the storage vesicles or degraded by monoamine oxidase (MAO). Figure adapted from Wong et al. [175] with slight modifications.

1.4.3 Impaired serotonergic neurotransmission: a proposed mechanism in centrally mediated fatigue during endurance exercise

Early reports by Newsholme [110] suggested that changes in amino acid concentrations during exercise could increase the synthesis, concentration and release of 5-HT within the brain resulting in central fatigue. The amino acid tryptophan (precursor for 5-HT synthesis) competes with other amino acids, such as branched-chain amino acids (BCAA) for transport into the brain. During prolonged exercise the plasma ratio of free tryptophan/BCAA increases, therefore favouring the transport of tryptophan into the brain and also the synthesis and release of 5-HT which may result in central fatigue [111, 176]. Several studies have confirmed an increase in cerebral 5-HT in response to exercise [177-179].

Cerebral 5-HT assist in regulating mood, arousal and appetite and elevated concentrations during sustained exercise is believed to increase the mental effort necessary to sustain athletic activity [111]. In support, Blomstrand and co-workers observed that BCAA ingestion during prolonged exercise was associated with a decrease in perceived exertion and improved physical and mental performance [180-182]. Other studies failed to show an association with BCAA administration and improved physical performance, however the observation of improved mental agility or cognitive performance was consistent [183, 184]. Possible confounding factors include the time period of BCAA ingestion and the duration of exercise. On the contrary, another prediction of

the central fatigue theory is that tryptophan ingestion would accelerate mental fatigue and possibly deteriorate physical performance [176]. Only one animal study thus far has reported a reduced performance following tryptophan ingestion [185].

In addition, performance related studies have demonstrated the significant effect of pharmacological drugs (acting as 5-HT receptor agonists and antagonists) in altering exercise capacity. The administration of a 5-HT agonist, which would simulate an increased 5-HT synthesis and release, impaired running performance in rats in a dose-response manner, whereas a 5-HT antagonist improved running performance [19-21]. Moreover, Dwyer and Browning [186] reported a desensitisation in 5-HT1A receptors in rats following 6 weeks of endurance training on a treadmill, which implicates an adaptive response to the fatiguing effects of increased cerebral serotonin in order to improve performance.

1.4.3.1 Serotonin transporter (5-HTT)

In support of previous findings, by using a pharmacological approach, the manipulation of 5-HTT has also been shown to play a role in physical performance and by implication central fatigue. Human laboratory trials have shown that the administration of a 5-HTT inhibitor, acting as a 5-HT agonist upon acute administration, can deteriorate physical performance [187]. In addition, Strachan and Maughan [188] reported a higher 5-HTT density within

the blood platelet plasma membranes of endurance-trained male athletes compared to sedentary males. The 5-HT system within blood platelets corresponds well with that of central serotonergic neurons and has, therefore, helped with investigations to elucidate effects of cerebral 5-HT [158]. Lastly, it is proposed that altered cerebral 5-HTT activity is associated with psychiatric disorders that include impulsive behaviour and negative mood states such as depression and anxiety [189-191]. Perception and motivation has been known to have an influence on physical performance [192]. However, the affect of a negative mood state or behaviour, such as aggression and impulsivity, on physical performance is still unclear.

1.4.3.2 Monoamine oxidase A (MAO-A)

Although platelets predominantly contain MAO B, platelet MAO concentration correlates with that of cerebrospinal fluid hydroxyindoleacetic acid (5-HIAA), a break-down product of serotonin and have, therefore, been proposed as a predictor of cerebral 5-HT activity [193]. A decreased platelet MAO activity, which has been proposed to represent a lower level of cerebral 5-HT turnover, has been associated with personality traits for e.g. impulsiveness, aggressive behaviour and sensation seeking; for review see [165]. Conversely, higher levels of MAO that represent a higher level of cerebral turnover and more efficient neurotransmission have been shown to correlate with persistence.

In summary, mechanisms involving the role of elevated cerebral 5-HT and the possible effect on CNS fatigue have received much interest during the past two decades. The serotonergic system plays a vital role in regulating mood and behaviour, which has been proposed to influence endurance performance in either a positive or negative manner. The pharmacological use of 5-HTT inhibitors and pre- and post-synaptic receptor agonists and antagonists has, therefore, helped in understanding their complex physiological function as well as dysfunction in mental disorders. Based on their observations, Davis and Bailey [18] hypothesised that an increase in brain serotonin may induce central fatigue through the inhibition of the dopaminergic system and/or by decreasing arousal and motivation to perform. Therefore, a high 5-HT/DA ratio would result in a decrease in motivation, lethargy, sleepiness and loss of motor coordination all of which favours deterioration in performance [18].

1.5 CANDIDATE GENES FOR ENDURANCE PHENOTYPES

As mentioned previously (Section 1.1), the search for genetic loci that influence physical performance traits have identified over 239 genetic polymorphic associations with these phenotypes [70, 194]. Continuous advances in genetic methodology and the completion of the Human Genome Project in 2003 have enabled scientists to perform whole genome scans aimed at examining genetic variation across the entire genome to identify possible genetic associations with a particular observable trait. These genome-wide association studies (GWAS) have proved informative in identifying a number of genetic regions showing associations with variation in human physical performance traits; for review see [70]. De Moore and colleagues [195] published the first GWAS on habitual physical activity level where they investigated 1.6 million SNPs in 2622 individuals obtained from two cohort studies. Another GWAS focused on skeletal muscle traits that identified SNPs in the thyrotropin-releasing hormone receptor (*TRHR*) gene that associated with lean body mass [196]. Lastly, multiple genetic loci, in particular the *FTO* (fat mass and obesity associated protein) locus, associated with obesity-related traits that confer to variation in BMI [197]. However, the GWAS method has also received much criticism for some claim it's not hypothesis-driven and there is an unprecedented possibility for false positive results due to the numerous statistical tests performed [198]. It also requires large sample sizes and is expensive. Most studies in the field of exercise science have therefore focused on genetic association studies. Although sample sizes are generally smaller, and in many cases under-

powered, this approach resides on investigating only a selected few variants within a “candidate” gene that has been selected on strong biological reasoning or understanding that could influence a particular phenotypic trait. This method can often yield associations that are difficult to replicate. However, it is much more cost effective than GWAS and therefore better suited for initial exploratory genetic studies. Moreover, due to the smaller sample size required for statistical significance, this method was used in case-control studies in this thesis. The endurance performance related candidate genes investigated in this thesis contained functional polymorphisms that alter the expression of the protein product produced. These proteins are proposed to play a key role in biological processes implicated in central fatigue and by implication the development of fatigue during endurance exercise.

1.5.1 The *-174* G/C (rs1800795) SNP within the *IL-6* gene

Fishman et al. [199], have identified a G to C single nucleotide polymorphism at base pair (bp) position -174 within the *IL-6* gene’s promoter region, which alters the transcriptional response of this gene to stimuli. Each individual only carries one of the three genotypes GG, GC or CC. Higher plasma IL-6 concentrations have been reported in individuals with a GG genotype, compared to the lower plasma IL-6 concentrations in individuals with a CC genotype [199].

The *IL-6* G allele has recently been shown to associate with sprint/power sports performance in elite Spanish athletes [200, 201]. A replication of this finding in

two Caucasian cohorts consisting of elite Israeli and Spanish/Israeli athletes, however, did not show any association [202]. Also, the -174 *IL-6* G/C polymorphism showed no association with maximal force output in Finish males [203]. Nonetheless, the notion that the *IL-6* G allele may favour sprint/power sports performance seems plausible as increased levels of IL-6 (G-allele) facilitate muscle repair and hypertrophy following exercise-induced damage [204].

As previously discussed in this chapter (Section 2.3.2), prolonged exercise results in large amounts of IL-6 being produced, which has been associated with both physical and mental fatigue [22]. We propose that the *-174* IL-6 GG polymorphism would result in increased circulating IL-6 levels in response to the stimuli of prolonged exercise. This would exacerbate the sensation of fatigue and thereby decrease endurance performance. The *IL-6* gene was, therefore, selected as a candidate gene that may play a role in affecting an athlete's athletic ability and contribute to the observed inter-individual variation in performance during ultra-endurance events. This hypothesis will be explored in Chapter 2 of this thesis.

1.5.2 The *5-HTT* gene-linked polymorphic region (*5-HTTLPR*) insertion/deletion polymorphism within the *SLC6A4* gene

The serotonin transporter gene-linked polymorphic region (*5-HTTLPR*) is an intensively studied locus with countless studies that have investigated

polymorphic associations with neuropsychiatric phenotypes [205]. Heils et al. [206] identified a 20-23 base pair repeat polymorphism within the 5'- regulatory promoter region of the serotonin transporter gene (*SLC6A4*), termed the *5-HTTLPR*. This polymorphism was considered functionally biallelic: one allele consisting of 14 repeats (the short (S) variant) and the other of 16 repeats (the long (L) variant). The S allele, opposed to L allele, is associated with a lower 5-HTT expression in membranes [206]. More recently an additional A to G SNP within the L allele that contributes to functional allelic variation was detected [205]. The L_G allele drives expression equivalently to that of the S allele. However, it should be noted that the observed L_GL_G genotype frequency within the Caucasian population is relatively low, ranging from 1-3% in Finnish and U.S whites [205] with a trivial contribution to the variation in genotype expression. The A to G SNP was therefore not considered in this thesis. In addition, the effect of the *5-HTTLPR* on central 5-HT levels may differ between sexes and race [207].

As previously discussed (Section 1.3.3.1), the affect of a negative mood state or behaviour, such as aggression and impulsivity, on physical performance is still unclear. Studies conducted on non-clinical cohorts reported significant associations between the *5-HTTLPR* S allele and behavioural changes that include decision making under risk [208], impulsivity [209-212] and aggression [209, 213]. Others, however, observed no association with cognitive function [214] or subsets (motor, attentional and non-planning) of impulse behaviour [215]. These aforementioned studies reported stronger associations and tendencies with males than females [208, 209, 212, 215]. Furthermore,

serotonin also plays a vital role in regulating appetite (Section 2.4.1) and the *5-HTTLPR* S allele has been implicated in eating disorders and suggested to present a risk factor for anorexia nervosa [216, 217]. In support, Bah et al. [218] reported a lower BMI for SS genotype carriers in a healthy population. Such a phenotypic trait can favour endurance athletic ability [26].

Lastly, the *5-HTTLPR* has been implicated in elite endurance status. Park et al. [219] reported a higher frequency of the S allele within a cohort of elite endurance athletes. This abstract was never published in full and the physiological explanation for this finding remains unclear. In fact, we expected the opposite (L allele association with endurance status) as a functionally lower 5-HTT expression (SS genotype) will maintain higher 5-HT levels within the synaptic cleft resulting in a lower 5-HT turnover activity and impaired neurotransmission. As previously discussed in this literature review (Section 1.3.3.2), such impairment can increase central fatigue which favours deterioration in endurance performance [18, 158]. Further investigation is warranted to elucidate the possible link/association with endurance athlete status. Hence, the *5-HTT* (*SLC6A4*) gene was selected as a candidate that may affect endurance athletic ability and contribute to the observed inter-individual variation in performance during ultra-endurance events. This hypothesis will be also explored in Chapter 2 of this thesis.

1.5.3 The 30 bp VNTR polymorphism within the *MAO-A* gene

The *MAO-A* and *MAO-B* genes are arranged tail-to-tail at position Xp11.23 - Xp11.4 on the X chromosome. Several polymorphisms in the *MAO-A* gene have been identified and investigated with a proposed influence on behavioural or physiological variability in humans [220-223]. However, these genetic variants are non-functional (no effect on transcriptional expression or activity). Sabol et al. [224] demonstrated a transcriptional variation of the *MAO-A* gene, as a result of a 30 bp variable number of tandem repeats (VNTR) polymorphism located approximately 1-1.2 Kb upstream of the transcription start site. This polymorphism can produce either one of the following 4 alleles: 3, 3.5, 4 or 5 copies of a 30bp repeat sequence. Alleles with the 3.5 or 4 copies have a 2-10 fold higher transcriptional efficiency than those with 3 or 5 copies of the repeat sequence [224].

Although the *MAO-A* VNTR polymorphism have been implicated in risk taking [225, 226] and aggressive behaviour in humans [227], results from various studies that have investigated different population groups have been controversial [228]. Nonetheless, higher transcriptional activity alleles (3.5 or 4 repeats) have been linked to risk taking attitudes [226], whereas the lower transcriptional activity alleles (3 or 5 repeats) associate with more optimal (non-impulsive) discussions under risk [229]. Newman [230] reported the first gene-environment association with the *MAO-A* VNTR and aggressive behaviour in rhesus monkeys, where allelic variation in MAOA activity were sensitive to social experiences during early development. Following discussions on the

selective advantage of this gene, at the 73rd Annual Meeting of the American Association of Physical Anthropologists, the media adopted the term “warrior” gene [231]. This embarked a further debate, known as the “warrior” gene hypothesis, where allelic *MAO-A* variations were implicated in warrior-like traits of Maori tribe males [232]. The use of the term “warrior” gene has received much criticism however, as the genetic association with aggression have been generalised across populations. Furthermore, there is no direct scientific evidence to support the claim that the *MAO-A* gene confers “warrior” traits in Māori tribe males [233]. The association of the *MAO-A* gene with endurance performance during the Ironman triathlon will also be explored in Chapter 2 of this thesis.

1.6 BODY WEIGHT LOSS DURING ULTRA ENDURANCE EVENTS

In the previous section, this review focused on physiological and biochemical pathways that have been implicated in overlapping peripheral (musculoskeletal) and central (brain/neural) fatigue models. In addition, we focused on identifying candidate genes (*IL-6, 5-HTT* and *MAO-A*), that influence CNS function, that could possibly contribute to the variation in physiological responses and athletic ability favouring the endurance phenotype. To further elucidate the underlying genetic contribution to the variation in physiological responses in endurance athletes, this section will discuss candidate genes (*5-HTT* and *AVPR2*) within key systems that function in parallel with central pathways, regulating the homeostatic control of water balance during endurance exercise.

1.6.1 Total body water and fluid compartments

Approximately 55-65 % of an individual's weight constitutes total body water (TBW), which can vary slightly with age, gender and amount of body fat [234]. The TBW content is divided into intracellular fluid (ICF) (2/3 of TBW) and extracellular fluid (ECF) (1/3 of TBW) compartments [235]. The ECF is further divided into the following 3 compartments, i) interstitial fluid (ISF) surrounding cells that do not circulate, ii) plasma circulating as the extracellular component of blood, and iii) a small volume of transcellular fluid that include digestive juices, cerebrospinal fluid (CSF) and mucus. The main solutes within the ICF are potassium (K^+) and organic anions, while sodium (Na^+) is the primary solute

within the ECF [234].

The maintenance of water balance and the homeostatic control of osmolality, which constitutes the concentration of solutes per given kilogram of fluid, is crucial in preserving cell size for optimum cellular function. Thus, the maintenance of a 2:1 intracellular to extracellular water ratio is crucial to ensure osmotic equilibrium and homeostasis. This equilibrium is primarily dictated by membrane pumps that extract Na^+ from the inside to the outside of cells with K^+ moving in the opposite direction. This maintains the trans-membrane Na^+ and K^+ gradients and hence the ECF and ICF volumes [234]. Fluid losses can occur from both the ICF and ECF compartments, but not necessarily in equal amounts. For example, the loss of NaCl, that occur with vomiting or diarrhoea, will result in a greater fluid loss from the ECF compartment as opposed to water loss alone, whereas a hypertonic solution added to the ECF will cause an osmotic gradient with a fluid shift from the ICF to the ECF compartment, until the osmolality of both compartments is again the same. Excretion of the added Na^+, will in time, restore the 2:1 (ICF:ECF) volume ratio.

Under normal physiological conditions daily water loss can occur through i) an insensible manner of which we are not consciously aware. This includes evaporation from the respiratory tract and diffusion through the skin (~700ml/day), ii) sweating that can substantially reduce water stores (up to 2L/hour) dependent on ambient conditions and exercise intensity [236], iii) faeces that accounts for a marginal water loss (~100ml/day) and lastly iv) urinary water excretion regulated via the kidneys [237].

1.6.2 The regulation of water homeostasis

Daily fluid losses from either the ECF or ICF compartments are compensated by reflex endocrine and neural responses, which are activated by osmo-, sodium and baroreceptors, in order to maintain homeostasis. Osmo- and/or sodium receptors are located in the splanchnic/hepatoportal vasculature and anterior hypothalamus of the brain [238]. Activation will occur in response to an increase in effective osmolality of the ECF as occurs with the ingestion of sodium containing (salty) substances, which will subsequently induce intracellular dehydration (fluid shift from the ICF compartment to the ECF to restore the osmotic equilibrium). Conversely, baroreceptors are activated with the onset of hypovolaemia (decreased ECF volume resultant from ECF losses with hemorrhage, vomiting, diarrhea and sweating) and stimulate sympathetic reflexes that increase heart rate and vasoconstriction to maintain arterial pressure [239]. In conjunction with the immediate effect of the sympathetic reflexes, the excretion of renal, adrenal and pituitary hormones exert their actions to decrease water and sodium losses.

1.6.3 Reflex endocrine mechanisms

Reflex endocrine activation serves to minimise the excretion of free water and/or renal solutes, in particular Na^+, in an attempt to counterbalance perturbations resulting from unregulated water losses or gains [234]. These responses include arginine vasopressin (AVP) secretion, renin-angiotensin-

aldosterone system activation, sympathetic activation and reduced renal solute and water excretion; see Table 1.2 for summary.

However, these compensatory responses can not replenish fluid and electrolyte (primarily Na^+) losses and thus behavioural responses that include thirst and salt appetite are also activated [239].

This thesis will primarily focus on water balance during endurance exercise and thus detail the two key mechanisms involved i.e. AVP secretion and the stimulation of thirst.

Table 1.2: The mechanisms involved in the homeostatic control of water balance. The various renal, adrenal and pituitary hormones that aid in reducing water and sodium losses through regulated excretion and activated behavioural mechanisms.

Water balance regulation	
Reflex Mechanisms	**Function**
Sympathetic	
• Vasoconstriction	Maintain arterial pressure
• ↑ heart rate	
Endocrine	
• Nor-epinephrine (NE)	Activate α_1-noradrenergic receptors that increase Na^+ reabsorption in kidneys
• Epinephrine	Vasoconstriction, reduced urinary Na^+ and K^+ excretion
• Renin	Vasoconstriction, stimulate secretion of AVP and aldosterone
• Arginine Vasopressin (AVP)	↑ water reabsoption by the kidneys ↑ urine osmolality ↑ arterial blood pressure
• Oxytocin (OXY)	Sodium excretion (natiuresis) by the kidneys
• Aldosterone	↑ water and Na^+ reabsorption and secretion of K^+ by the kidneys ↑ arterial blood pressure
• Adrenocorticotropic hormone (ACTH)	Stimulate secretion of aldosterone
• Glucocorticoids	↑ water reabsoption ↑ arterial blood pressure Negative feedback inhibition for ACTH secretion
• Atrial natriuretic peptide (ANP)	↑ water and Na^+ excretion Inhibits renin and reduces aldosterone secretion Vasodilation
• Endothelin (ET)	Regulate water and Na^+ excretion and vascular resistance depending on receptor isoform (ET_A and ET_B)
Behavioural Mechanisms	**Function**
Thirst	Replace fluids lost (see Figure 1.4 for detailed illustration)
Salt appetite	Replace ECF Na^+ deficit, however only consciously stimulated with pathological conditions (e.g. Addison's disease [240])

1.6.3.1 Arginine vasopressin (AVP) secretion

Antidiuretic hormone (ADH), which has now been named arginine vasopressin (AVP), is a peptide hormone best known for its potent stimulatory effect on water re-absorption within the kidneys, but can also induce moderate vasoconstriction. As its name therefore implies (e.g. a pressor) this hormone plays a vital role in regulating blood volume and pressure. AVP is synthesized within hypothalamic supraoptic (SON) and paraventricular (PVN) nuclei and stored within neurosecretory granules in the posterior pituitary ready for release into the bloodstream [241]. These pituitary stores are substantial to ensure the maximum antidiuretic effect of this hormone in conditions of constant dehydration for as long as one week [234]. AVP act at the kidneys and blood vessels in response to stimulation, such as dehydration or haemorrhage, sensed by osmo- and/or barroreceptors [242]. AVP can act on three receptor types e.g AVPR1a in kidneys, liver and vasculature, AVPR1b in the anterior pituitary and AVPR2 mainly in kidneys [243-245].

1.6.3.1.1 Osmotic regulation

Osmoreceptors located within the circumventricular organs (organs in the brain with an incomplete blood-brain barrier) and the splanchnic/hepatoportal vasculature detect changes in osmolarity, which send electrochemical signals to the anterior pituitary that stimulate the synthesis and release of AVP [239, 242]. Circulating AVP acts on V2 receptors (AVPR2) in the kidney that

increase water permeability of collecting tubules with subsequent water re-absorption resulting in a decreased urinary flow and an increased urine osmolality. The extreme sensitivity of AVP to small changes in osmolality (0-1% above basal levels) is sufficient to induce a significant increase in plasma AVP concentration with a proportional increase in urine concentration. However, at omolalities that are 5-10mOsm/kg H2O (2-4%) above basal levels, antidiuresis and by implication urinary osmolality has reached a maximum despite further increases in circulating AVP [234]. Therefore, a 0.5-2 pg/ml increase in AVP will induce an augmented response on renal antidiuresis and subsequent decreased urinary flow as opposed to a 2-5 pg/ml concentration [246]. More so, the hormonal effects of AVP are experienced within minutes and with its extreme sensitivity to small fluctuations in plasma osmolality, regulation of water excretion is corrected on a minute-to-minute basis [234]. AVP is also regulated by changes in blood volume.

1.6.3.1.2 Volaemic regulation

A significantly greater degree of plasma volume or blood pressure reduction ranging from 4-15% across different species is required to consistently stimulate drinking. It is postulated that this delayed stimulatory response serves as a protective adaptation to accommodate greater plasma volume and blood pressure fluctuations, resultant from orthostatic pooling of blood in the lower body from an erect posture. This will prevent overdrinking and improper antidiuresis in response to a decreased ECF volume that is in fact only

momentarily maldistributed [234]. Hence, volaemic changes are less effective in stimulating AVP secretion and subsequent thirst compared to slight changes in osmolality [234, 247]. Therefore, only severe states of hypovolaemia (e.g. blood volume reductions of >10%) are sufficient in stimulating AVP secretions and thirst. More so, an increased osmolality collectively with significant hypovolaemia will result in an augmented stimuli for AVP secretion compared to euvolaemic states [234].

Other non-osmotic stimuli for AVP secretion include the sensation of nausea [248], hypoglycaemia [249], nicotine [250], plasma volume contraction [251, 252], elevated body temperature [253], as well the circulating endocrine factors IL-6 [254], angiotensin II [255], corticosterone, oxytocin and brain natiuretic peptide [252, 256].

In summary, AVP secretion and resultant renal mechanisms are sufficient to maintain plasma osmolality within a narrow margin of basal levels and if these mechanisms prove inadequate, thirst is stimulated to restore homeostasis. However, under normal physiological conditions humans consume more water in an unregulated manner (e.g. intrinsic water in ingested foods, social or habitual drinking of caffeine and alcoholic beverages) than is necessarily required and, therefore, water balance is maintained through the regulation of excretion rather than of intake [234, 257].

1.6.3.1.3 Activation of the Kallikrein-kinin system (KKS)

Kinins are released from the precursor, kininogen, through the action of tissue and plasma enzymes (kininogenases), of which kallikrein is best known. Kinins act as local hormones on β1 and β2 receptors. The most known effects i.e. vasodilation, diuresis and natriuresis are mediated through β2 receptor activation. Since 1965, a relationship between the renal KKS and AVP was suggested whereby AVP infusion resulted in an increased release of renal kinins that attenuated the antidiuretic effect of AVP [258, 259]. The counteractive response by kinins on AVP action was mediated through β2 receptors [260] and the dipsogenic effect of endogenous bradykinin via β2 receptors has now been established [261, 262]. Whether the activity of bradykinin is exclusively peripheral is unknown, however the involvement of central angiotensin (ANG)-related pathways have been implicated [262].

1.6.4 Thirst

Thirst is a subjective perception that generates the urge to consume fluids and acts as the body's defence to prevent large body fluid deficits. Thirst is stimulated as a regulatory behavioural mechanism in response to fluid losses, hypertonicity of the ECF or increased concentrations of dipsogenic hormones i.e renin and ANG ll [234, 263]. Activation occurs as inhibitory and excitatory influences that arise as a result of osmotic changes in circulating hormones and neural input from baroreceptors during hypovolaemia and hypotension that

stimulate brain thirst centres [239]. Small increases of 1-2% in plasma osmolality above basal levels are sufficient to stimulate thirst in mammals [239]. However, as previously mentioned in section 1.6.3.1.2, significant greater changes ranging from 4-15% decreases in plasma volume or blood pressure are required to effectively stimulate drinking [234, 263-265].

1.6.4.1 Stimulation of brain thirst centres

Signals that activate brain thirst centres arise from both the ICF and ECF compartments. Osmoreceptors located within the circumventricular organs (e.g subfornical organ (SFO), organum vasculosum of the lamina terminalis (OVLT) and area postreamia (AP)) and the splanchnic/hepatoportal vasculature detect changes in osmolarity that activate afferent sensory feedback and the production of humoral factors (see Figure 1.3). These afferent nerves and circulating factors communicate to the CNS on current body fluid status and cardiovascular homeostasis (for review see [239, 263]).

The nucleus of the tractus solitarius (NTS) in the hindbrain serves as the main entry point to the brain for systemically derived input from the IXth and Xth cranial nerves. Afferents derive information from arterial baroreceptors, cardiopulmonary and other visceral receptors pertaining to the status of blood volume, by implication the volume of the ECF compartment. It is proposed that the ECF is monitored by osmo- and/or sodium receptors in splanchnic/hepatoportal vasculature in the periphery and osmosensitive regions

in the brain (circumventricular organs), most likely the SFO and AP that project to and/or through the anteroventral third ventricle (AV3V).

The volume of the ICF compartment is monitored by osmo- and/or sodium receptors located in the periphery and in the brain's AV3V, mainly the median preoptic nucleus (MePO) and organum vasculosum of the lamina terminalis (OVLT). Circulating factors such as the peptides ANG II and atrial naturiuretic peptide (ANP) cannot enter the blood-brain barrier and must act via the circumventricular organs. Stimulated 'thirst' centres (circumventricular organs) communicate via ascending adrenergic and descending angiotensinergic systems to forebrain structures that result in the acquisition of motor patterns to ingest water and sodium. Conversely, hindbrain serotonergic pathways provide inhibitory feedback to forebrain structures to prevent excessive intake [239].

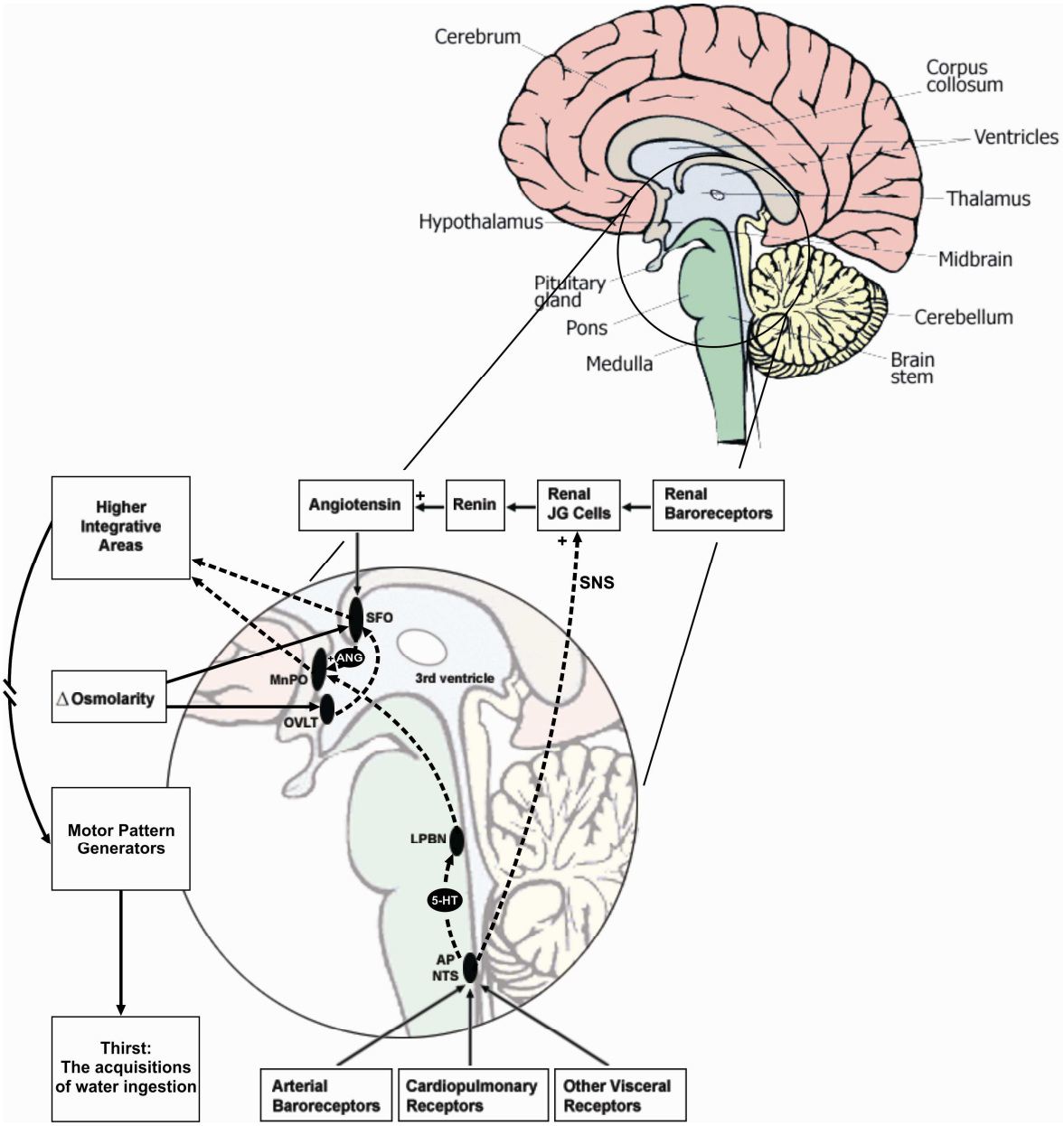

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Figure 1.3. Diagram illustrating the neural and hormonal signaling of thirst in the brain and the central neural pathways that mediate sensory integration of signals for the control of drinking. Peripheral voleamic, baro- and other visceral receptors send inhibitory and excitatory inputs via afferent nerves that project mainly to the nucleus of the tractus solitarius (NTS). Angiotensin (ANG) acts in the form of ANG II that activates angiotensin type I (AT1) receptors in the subfornical organ (SFO). Hormonal input to the SFO is carried in descending, possibly angiotensinergic pathways. Changes in osmolarity signal structures along the organum vasculosum of the lamina terminalis (OVLT). Information from descending pathways is carried to forebrain structures in the anteroventral third ventricle (AV3V). Ascending information derived from arterial, cardiovascular and other visceral receptors is carried from hindbrain to forebrain structures via noradrenergic cell signaling. A serotonergic (5-HT) inhibitory pathway that projects from the area postreamia (AP) and NTS to the lateral parabrachial nucleus (LPBN) protects against excessive water intake.

1.6.4.2 Renin- angiotensin-system

The role of the renin-angiotensin-system (RAS) in thirst and sodium appetite has been reviewed extensively by Fitzsimons [266]. The RAS is present in various organs (periphery) and the brain (central), involving a cascade of chemical interactions that cause the formation of angiotensin peptides. The potent dipsogenic peptide ANG II is synthesised in a two step chemical process. Renin cleaves angiotensinogen to form ANG I, which is converted to ANG II via the action of angiotensin-converting enzyme (ACE). The juxtaglomerular apparatus in the kidney is the major source of renin production, which is secreted into the blood, lymph or peripheral tissues. ANG II can act via two receptor subtypes type 1 (AT1) and type 2 (AT2) [267]. However, most known functions are associated with AT1 receptor activity. Circulating ANG II

activate various autonomic, endocrine and behavioural responses. These include (i) the contraction and hypertrophy of vascular smooth muscle, (ii) activation of the sympathetic nervous system and the release of adrenomedullary hormones, (iii) secretion of aldosterone, (iv) release of pituitary hormones, and (v) regulation of fluid balance via tubular reabsorption of sodium and water in the kidneys. Angiotensin II is also produced centrally and plays an important role in cardiovascular and fluid homeostasis via descending angiotensinergic pathways that stimulate drinking and sodium ingestion (Figure 1.3).

The powerful dipsogenic stimulus of renin and its effector peptide ANG II have been shown in several mammalian studies whereby systemic administration or injections directly into the brain result in water intake [263, 266].

In summary, Johnson and Thunhorst [239] propose that fluid losses from either the ICF or ECF compartments will result in the activation of different sensory signals (e.g. osmotic, hormonal, neural) that communicate information via different afferent pathways (e.g. blood, nerves) to central structures on the current status of total body water and cardiovascular homeostasis. This will activate a complex neural circuitry that activate compensatory autonomic (e.g. sympathetic) and endocrine reflex responses, resulting in circulatory hormones acting as efferents and in turn stimulate brain centres to induce the conscious sensations of thirst and salt appetite. Serotonergic pathways provide inhibitory feedback to forebrain structures to prevent excessive fluid intake [239].

1.6.5 Sweating and fluid balance during prolonged exercise

During prolonged exercise fluid and electrolyte loss occurs as a result of sweat evaporation from the skin, which assists with cooling to facilitate thermoregulation. Sweating can account for an approximate 92% of all water [268] and 87% of all sodium [269] loses during exercise. Humans typically consume less fluid than they lose as sweat during exercise and so do not fully restore the water deficit lost due to sweating [270, 271]. Furthermore, athletes competing in events such as running, cycling and triathlon can easily lose up to 6% body weight during exercise in the heat [272, 273]. The amount of water lost is related to exercise intensity and ambient conditions which include temperature, humidity and wind speed [274]. Sweating removes water from both the ECF and ICF compartments but not necessarily in equal amounts [269]. Under-replaced hypotonic sweat generally causes hypertonicity as would “pure” water losses (such as those found in highly trained individuals whereas sweat sodium concentrations in sweat are very low). It is the resulting ECF hypertonicity from hypotonic sweat losses that draw water out of the ICF compartment, which then leads to cellular shrinkage and dysfunction. Sweating can therefore challenge the preservation of an osmotic equilibrium, which is vital for the maintenance of circulatory volume to ensure cardiovascular function [275, 276]. Also, the sweat [NaCl] is reduced in response to higher sweat rates and heat acclimatisation; an adaptation to preserve Na^+ [277, 278]. However, with large sweat losses the ECF compartment will ultimately become hypertonic (increased concentration of electrolytes in body fluid) [279].

During the initial stage of exercise, plasma volume will typically decrease due to hydrostatic forces [280-282]. An increase in blood pressure at the start of exercise will force fluid from the vascular compartment into the interstitial space. The acute decrease in blood plasma volume will increase the hematocrit and thereby the oxygen carrying capacity per unit of blood to the exercising skeletal muscle [283]. Over time fluid shifts from other compartments are favoured to preserve the plasma volume needed to maintain optimum filling conditions for the heart [269, 284]. At cessation of exercise the plasma volume deficit is restored via a fluid-flux into the vascular space. Following prolonged exercise this auto-restoration response can be superfluous, resulting in the expansion of plasma volume well above baseline levels [285-289]. Apart from the auto-restoration response the influx of plasma proteins into the vascular space, through an increased permeability of the capillaries with exercise, also causes plasma volume expansion above baseline levels during prolonged endurance exercise.

Lower sweat rates have been observed following fluid restrictions during prolonged exercise, which have been attributed to ECF hypertonicity. An increased serum osmolality detected by osmosensitive neurons in the hypothalamus or a resultant high interstitial osmotic pressure could possibly restrict fluid availability to the sweat glands [290]. Field observations indicate that athletes drink sparingly during events and typically do not replace the entire fluid deficit they generate as a result of sweating. This has been termed “voluntary dehydration” [272, 291], although it is in fact involuntary as it is neither consciously perceived nor is it a conscious behaviour choice. Athletes

can comfortably tolerate a 2-3 kg weight loss during competition, which was believed to resemble total fluid loss and subsequent dehydration. It was therefore suggested that thirst is an imperfect index in restoring fluid deficit [276] and that the fluid deficit can only be restored following the consumption of electrolyte containing food or fluid [292, 293]. When athletes have attempted to increase their fluid consumption to match sweat rates, some have reported gastrointestinal discomfort, possibly due to different gastric emptying rates and tolerance between individuals [273, 294-296].

1.7 FLUID REPLACEMENT DURING PROLONGED EXERCISE

1.7.1 Traditional studies and recommendations

To observe the physiological changes that occur in athletes during prolonged exercise, laboratory studies have attempted to simulate racing and environmental conditions to induce dehydration. It was proposed incorrectly that dehydration was associated with lower sweat rates that would compromise the dissipation of heat resulting in an elevated core temperature and increased the risk for the development of heat illness and deterioration in performance [276, 297]. In response to these findings the American College of Sports Medicine (ACSM), in conjunction with other athletic associations, established guidelines encouraging athletes to replenish all fluid lost during exercise to prevent becoming dehydrated. It was proposed that this would reduce the risk for heat associated medical complications and thereby enhance performance [276, 298, 299].

1.7.2 Challenging excessive fluid replacement

In the past decade this body of research has been challenged by Noakes and colleagues [300, 301] as it has become apparent that these recommendations are superfluous to ultra endurance events held in a moderate climate, such as the South African Ironman triathlon, and seem more detrimental to performance

rather than to aid performance. These recommendations are based on laboratory findings conducted in climate chambers with inappropriate convective cooling. Saunders et al. [302] demonstrated that when exercising indoors and exposed to higher wind velocities, reflective of an outdoors environment, subjects' rectal temperatures were significantly lower and performance increased. However, contrary to what was proposed as a beneficial aid by former laboratory studies [276, 297, 303], an increased fluid consumption had no effect on lowering rectal temperature or enhancing performance [302].

In support, Sharwood and co-workers [304, 305] documented that within 871 athletes competing in the South African Ironman triathlon the faster athletes experienced greater fluid losses (hypohydration) in comparison to slower participants. They concluded that hypohydration does not necessarily pose a threat to performance and that minor dehydration during the Ironman Triathlon in a moderate climate is not associated with increased rectal temperatures in athletes. Similarly, it was reported that during the 2004 Western Australia Ironman well trained athletes were able to effectively thermoregulate despite a significant weight loss (3%) [306]. It is now apparent that weight loss, originally implied as dehydration from subsequent water loss, does not cause an important increase in core temperature during prolonged exercise [304, 305].

Athletes exercising at a moderate intensity for a prolonged period should be aware of the risk of dehydration, which can become exacerbated in hot humid conditions. However they should be mindful not to overdrink. Considering the

complication of fluid overload during ultra-distance events, Noakes have recommended a fluid intake of 500ml/h for the majority of non-competitive ultra-distance runners, which closely resembles ad libitum fluid intakes observed during ultra-distance events [273, 307]. In fact, Noakes [300] advocated ad libitum drinking.

1.7.3 Fluid homeostasis and internal water stores

As early as 1933 it was postulated that the maintenance of osmolality, rather than body mass, is crucial during exercise [308]. Therefore, the maintenance of body mass is not necessarily needed to maintain fluid homeostasis for endurance exercise [282]. In fact, blood [Na^+] cannot be regulated appropriately during exercise unless some body mass, by implication fluid loss through sweating, is lost during exercise. It is important to bear in mind that 1 kg weight lost during prolonged exercise doesn't necessarily constitute 1 kg volume fluid deficit from the extracellular fluid compartment. In fact, Nolte and Noakes [309] recently highlighted methodological flaws that biased the conclusion in an investigation where the change in body mass during exercise reflected the change in TBW alone. It has been shown that an athlete having lost up to 3 % of his body weight during an endurance event can have a euhydrated TBW status as a result from intracellular water release from the liver and skeletal muscle following glycogen oxidation [289, 310]. More so, the oxidisation of glycogen and triglycerides can account for at least a 2 kg of body weight loss during ultra-endurance multisport events [311, 312]. Also, Fusch et

al. [313] suggested that well trained athletes are able to store greater amounts of glycogen-bound water compared to untrained individuals. It is therefore plausible to consider that “carbo-loading” provides internal water storage aiding in a training adaptation to facilitate endurance capacity.

The rate of total body water turnover (WT) has been identified as an index for water homeostasis and subsequent health [314]. Researchers have shown that exercising and endurance trained athletes have an increased WT as a result of increased metabolic water production and insensible water loss and are therefore able to tolerate a greater body water loss [313, 315, 316].

1.7.4 Fluid overload and exercise-associated-hyponatraemia (EAH)

It is evident that endurance trained athletes can maintain an euhydrated TBW state during an endurance event and serum sodium concentrations (pre- to post-race) in normal physiological range despite a 3kg body weight loss [317]. Hence, fluid overload as a result from superfluous drinking habits can occur and consequently result in exercise-associated-hyponatraemia (EAH) or its fatal complication, exercise-associated hyponatraemic encephalopathy (EAHE) [3, 318-326]. Hyponatraemia is clinically defined as an electrolyte disturbance in which the serum sodium concentration falls below 135mmol/l and presents with symptoms of weakness, vomiting, headache, confusion and in extreme cases grand mal seizures, pulmonary edema, respiratory arrest and coma [327-329]. During the 1996 New Zealand Ironman Triathlon fluid intakes of 6.2 to 16 L

were recorded in triathletes that developed EAH [330]. Amended recommendations of conservative drinking reduced the occurrence of EAH in the New Zealand Ironman Triathlon from 22 % in 1997 to 3 % in 1998 [289].

Contrary, sodium supplementation had no protective effect in restoring sodium balance in runners that drank to excess, but in fact, worsened fluid overload as a result of fluid retention [331]. Also, female ultra-distance athletes have lower sweat rates, smaller fluid compartments and have been observed to consume greater amounts of fluids relative to their body weight compared to males during competition [332]. Ayus [333] proposed that hormonal levels within females may also impact on the susceptibility to develop EAH. This generates a concern for a possible increased risk for female runners developing EAH [324, 330, 332, 334]. However, EAH can also develop in the presence of modest fluid intakes. In such cases it is proposed that renal and hormonal dysfunction, causing fluid retention, can induce fluid overload [317, 334]. In a clinical review, Noakes and co-workers [3] proposed three independent biological mechanisms that can cause EAH and EAHE: (i) over-drinking during exercise as the primary cause, (ii) inadequate suppression of antidiuretic hormone (ADH) secretion resulting in inappropriate fluid retention and (iii) either osmotic inactivation of circulating sodium or the failure to mobilize osmotically inactive sodium from internal stores. Inappropriate AVP (previously named ADH) secretion can result from non-osmotic stimuli during endurance exercise (previously discussed in section 1.6.3.1.2) and exacerbate the development of EAH [3, 252, 335]. Other factors that can cause inappropriate fluid retention such as renal dysfunction will be discussed below.

1.7.5 Genetic mutations that alter renal function and thereby water and sodium homeostasis.

The condition nephrogenic diabetes inspidus (NDI) is characterised by the inability of the kidney to conserve water in response to AVP secretion. The hereditary form of NDI is the result of an X-linked genetic defect in either the *AVPR2* (90% of all cases) or aquaporin 2 (*AQP2*, 10% of all cases) genes that result in a loss of function or dysregulation of these receptors [336, 337]. To date more than 211 mutations within the *AVPR2* gene that causes NDI have been identified [338]. Of the 15 different types of mutations causing NDI, missense mutations (55.83%) are the most prominent [338].

More recently, a disease associated gain-of-function mutation within codon 137 of the arginine vasopressin receptor 2 (*AVPR2*) gene that caused inappropriate water reabsorption in infants [339-341] and a familial case [342] have been identified. Under normal physiological circumstances the *AVPR2* receptor is activated through the ligand binding of AVP. However, this gain-of-function receptor is AVP-independent, resulting in inappropriate water reabsorption by the kidneys and a subsequent increase in total body water content that causes the serum sodium concentrations to fall resulting in severe hyponatraemia [339, 342, 343]. Based on these findings, the *AVPR2* is an ideal candidate gene to consider when investigating inter-individual variation in total body water lost

during ultra-endurance events. This hypothesis will be also explored in Chapter 4 of this thesis.

1.8 SUMMARY AND CONCLUSIONS OF LITERATURE REVIEW

There is no doubt that heritage plays a key role in the inter-individual variation in the performance and the physiological responses to exercise observed between athletes. Although a plethora of studies have investigated the contribution of single genetic components to the endurance phenotype it has become more apparent that endurance athletic ability is polygenic in nature. Although recent studies have adopted a polygenic approach in establishing an endurance profile, the focus is nonetheless still centered on peripheral systems. In this literature review we identified mechanisms that not only act peripherally, but also centrally, that undoubtedly play a role in central signaling during exercise and when manipulated, can impact on performance. However, the genetic contribution to central mechanisms and how it impacts on psychological aptitude and physiological responses during endurance exercise is still unclear and warrants further investigation.

Also, these central pathways play a vital role in the regulation of thirst and by implication the homeostatic control of water balance during endurance exercise. Although significant weight changes have been documented in Ironman triathletes an underlying genetic contribution is yet to be discovered. In support, recent studies have identified genetic mutations in peripheral systems, such as the *AVPR2* gene, that results in kidney dysfunction and inappropriate water reabsoption in infants.

In conclusion, the reviewed literature support the significant role of overlapping peripheral (musculoskeletal) and central (brain/neural) components in regulating physiological responses, such as body water losses and athletic performance during endurance events. Candidate genes that may contribute to the inter-individual variation in these physiological responses between athletes and by implication the endurance phenotype have been identified for investigation in the following chapters of this thesis.

1.9 AIMS AND OBJECTIVES OF THE THESIS

The primary aim of this thesis was to investigate candidate genes, that influence CNS function, that may contribute to the variation in physiological responses and athletic ability between athletes, thereby favouring the endurance phenotype. A genetic association approach was used in case-control studies to identify specific sequence variants (single nucleotide polymorphisms, SNP's) within selected candidate genes. These candidate genes (*IL-6, 5-HTT* and *MAO-A*) were selected based on the biological function of their encoded proteins which have been implicated in overlapping peripheral (musculoskeletal) and central (brain/neural) fatigue models. The objectives of the specific gene association studies which addressed the primary aim of this thesis were as follows:

- To investigate whether functional polymorphisms within candidate genes that alter production of cytokines (-174 *IL-6* G/C SNP) or components of the serotonetgic system (44 bp insertion/deletion, also referred to as the long or L/short or S, polymorphism of the *5-HTT* gene and the 30 bp VNTR polymorphism within the *MAO-A* genes) are associated with ultra-endurance performance in triathletes who completed either the 2000 and/or 2001 South African Ironman Triathlons. **(Study 1)**

The secondary aim of this thesis was to investigate candidate genes that may be associated with body weight changes in athletes during ultra-endurance

events. A genetic association approach following case-control studies was used to identify candidate genes (*5-HTT*, which have been investigated in Study 1 and *AVPR2*). These candidate genes encode for proteins that play key roles in the neuro-endocrine control of total body water homeostasis. Although the *5-HTT* gene has not been investigated in this regard, there is evidence for disease-causing mutations within the *AVPR2* gene that cause excess water reabsorption by the kidneys and clinically manifest with hyponatraemia. Therefore, the objectives of the specific gene association studies which addressed the secondary aim of this thesis were as follows:

- To determine whether the 44bp *5-HTTLPR* (L/S) polymorphism, a functional genetic component of the serotonergic system, is associated with body weight changes during the 2000 and/or 2001 South African Ironman Triathlons. We propose that the S allele, associated with a lower 5-HTT expression and thereby a reduced 5-HT turnover, would hinder efficient neurotransmission resulting in reduced stimulation of brain thirst centres. The inhibition of drinking behaviour is likely to result in a larger decrease in body weight changes (a proxy of hydration status) during these events. **(Study 2)**
- To determine whether there were any associations between three single nucleotide polymorphisms (SNP's) (rs3761528, rs3761527 and rs4898457) within the *AVPR2* gene, and inter-individual variations in serum sodium and/or body weight changes in athletes competing in the 2000, 2001 and 2006 South African Ironman Triathlons. **(Study 3)**

The final aim of this thesis was to determine whether a polygenic profile for body weigh changes in athletes during participation in an ultra-endurance event exists. The objective of the study that addressed the third aim of this thesis was:

- To investigate the genetic association of a polygenic profile that include results from a previously published study, and results presented in this thesis (Study 2 and Study 3) with body weight changes of triathletes. We propose that triathletes with a higher total genotype score (TGS) are more likely to experience greater relative body weight losses during the 2000 or 2001 South African Ironman Triathlons. **(Study 4)**

CHAPTER 2

THE INTERLEUKIN-6, SEROTONIN TRANSPORTER AND MONOAMINE OXIDASE A GENES AND ENDURANCE PERFORMANCE DURING THE 2000 AND 2001 SOUTH AFRICAN IRONMAN TRIATHLON

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2.1 INTRODUCTION

As discussed in the literature review (Section 1.1), there is a growing body of evidence that have identified genetic components that partly contribute to athletic ability. To date both, the angiotensin converting enzyme (*ACE*) I allele and bradykinin β2 receptor (*BDKRB2*) -9 allele have been associated with ultra-endurance performance in the Ironman triathlon [75, 76]. In addition, Saunders et al. [76] also reported that the overall finishing times of the triathletes during the 2000 or 2001 event with a *NOS3* GG genotype together with a *BDKRB2* +9 allele were significantly slower than those triathletes with other genotype combinations. The combined *NOS3 BDKRB2* genotypes, BMI and age accounted for 15% of the variance in the actual overall race time of the

triathletes during these events [75]. Recently, the *COL5A1* BstUI RFLP and *COL6A1* rs35796750 polymorphisms, a component of the tendon apparatus that has been associated with musculoskeletal stiffness, have been identified as markers for endurance running and cycling performance respectively, during the South African Ironman Triathlons. The *COL5A1* BstUI RFLP, BMI, age and running training during the last 15 weeks accounted for 30% of the variance in time to complete the run [77], whereas the *COL6A1* TT genotype associated with endurance cycling performance [78]. Polymorphisms within the *GH1* [83], *UCP3* [11] and *ACTN3* [344] genes have on the other hand not been shown to be associated with performance in the Ironman Triathlon. The *UCP3* T allele, however, was significantly overrepresented in a cohort of Russian Olympic triathletes when compared to controls [80]. It has been proposed that the products of these genetic variants alter physiological and metabolic processes locally within skeletal muscle which favour endurance athletic ability.

In addition to the musculoskeletal system, the central nervous system (CNS) has also been proposed to play a role in endurance performance [99]. Manipulation of the cytokine IL-6 and the neurotransmitter serotonin have been shown to alter physical performance [19-22]. It is proposed that increased levels of circulating cytokines and impaired serotonergic neurotransmission contribute to the onset of central fatigue resulting in decreased physical performance, as previously discussed in the literature review (Section 1.2-1.4).

As previously discussed in chapter 1, evidence from our laboratory has shown a concurrent increase in plasma IL-6 and prolactin (by implication, serotonin

production) concentrations during exercise, following rhIL-6 administration [22]. Furthermore, serotonergic neurotransmission is regulated by receptors, re-uptake transporters and the degradation enzyme monoamine oxidase A (MAO-A). Investigators have demonstrated deterioration and improvement in running performance of rodents following the administration of 5-HT receptor agonists and antagonists respectively [21]. Also, a higher serotonin transporter (5-HTT) density has been reported within the blood platelet plasma membranes of endurance-trained male athletes compared to their sedentary counterparts [188]. Lastly, a lower MAO-A enzyme activity has been observed within the hippocampus of Spontaneously-Running-Tokushima-Shikoku (SPORTS) rats, which display a voluntary increase in wheel running activity [345]. Based on these findings, researchers have suggested that serotonergic pathways play a key role in centrally mediated fatigue and by implication, endurance performance [18, 176].

Apart from a single conference preceding publication [219], to our knowledge no studies have investigated the association of these variants with endurance performance and athletic ability. The aim of this first study in this thesis was to investigate whether functional polymorphisms within candidate genes that alter production of cytokines (-174 *IL-6* G/C SNP) or components of the serotonetgic system (44 bp insertion/deletion, also referred to as the long or L/short or S, polymorphism of the *5-HTT* gene and the 30 bp VNTR polymorphism within the *MAO-A* genes) are associated with ultra-endurance performance in triathletes who completed either the 2000 and/or 2001 South African Ironman Triathlons.

2.2 METHODS

2.2.1 Participants

The Ironman Triathlon is a multi-sport ultra-endurance event consisting of a 3.8 km swim, 180 km cycle and a 42.2 km run in immediate succession which has to be completed within 17 hours (www.ironman.com). Prior to the 2000 and 2001 South African Ironman events, each entrant was sent a complete explanation of the study and invited to participate [305]. Seven-hundred and one self-reported Caucasian male triathletes completed either the 2000 (n=272) and/or 2001 (n=544) races, of which 115 completed both events [75]. For the triathletes that completed both the 2000 and 2001 events only their 2001 data was included in this study. During race registration, the 468 (66.8% of the entire field) triathletes included in this study completed an informed consent (Appendix 2) and a personal particulars questionnaire (Appendix 3). Only 26 and 57 female triathletes completed the 2000 and 2001 events respectively. Due to the sample size and differences in performance between male and female triathletes, the female triathletes were not included in this thesis. Similarly only 7 and 11 triathletes from other population groups (self-reported) completed the 2000 and 2001 events respectively. Due to the possible effects of population stratification only the self-reported Caucasian male triathletes were therefore included in this thesis. Age, height, body weight, country of birth, sex and ethnic background were determined from the completed

questionnaires. Body mass index (BMI) was calculated as self-reported weight (kg) divided by self-reported height in meters squared (m^2). The triathletes were divided into the fastest (Fast Triath, n = 156), middle (Mid Triath, n = 156) and slowest (Slow Triath, n = 156) finishing male Caucasian triathlete tertile groups respectively. Overall race and split times were obtained from the race organisers after the event.

Two hundred apparently healthy Caucasian male control participants (Con) who had not participated in an ultra-endurance event, such as the Ironman Triathlon, were recruited from the greater Cape Town Metropolitan area. Approval for this study was obtained from the race organisers and the Human Research Ethics Committee of the Faculty of Health Sciences, University of Cape Town, South Africa (references: 005/2000 and 099/2001; Appendix 4).

2.2.2 DNA extraction

During registration, approximately 4.5 ml of venous blood was collected into an EDTA vacutainer tube from each subject via venipuncture of the forearm vein. Blood samples were stored at 4 ^{o}C until total DNA extraction, as described by Lahiri and Nurnberger [346], with previously described modifications [76].

Briefly, the blood samples were transferred to sterile 15 ml polypropylene tubes, to which 10 ml of TKM1 buffer (10 mM Tris-HCl pH 7.6, 10 mM KCl, 10 mM MgCl2 and 2 mM EDTA) containing 2.5% Nonidet P-40 was added to lyse the

red blood cells. After incubating at room temperature for 10 minutes, the white blood cells (WBC) were pelleted by centrifugation at 1200 x g for 10 minutes at room temperature and washed at least once with 5 ml of TKM1 buffer. The washed WBC pellets were resuspended in 800 µl of TKM2 buffer (10 mM Tris-HCl pH 7.6, 10 mM KCl, 10 mM MgCl2, 0.4 M NaCl2 and 2 mM EDTA) containing 50 µl of 10% SDS and then incubated for at least 60 minutes at 55 ºC to lyse the WBC. One hundred and fifty µl of 5 M NaClO4 and 500 µl of chloroform was added to each sample, which was then thoroughly mixed by vortexing for 15-20 seconds. The samples were transferred to 1.5 ml microfuge tubes and the protein precipitated by centrifugation at 15 000 x g (13 000 rpm) for 5 minutes at room temperature. Five hundred µl of the top aqueous phase was transferred to a new microfuge tube containing 1 ml of absolute ethanol. The sample was then mixed and the DNA pelleted by centrifugation at 13 000 rpm for 2 minutes at room temperature. The precipitated DNA was air dried for at least 30 minutes and then resuspended in 200 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Each sample containing tube was incubated at 65 µC for 15 minutes before being stored at 4 ^{o}C until PCR analysis.

2.2.3 Genotyping

2.2.3.1 IL-6 gene

A 228 base pair (bp) fragment corresponding to the -348 to -121 region of the *IL-6* gene promoter, which contained the functional -174 G/C single nucleotide

polymorphism (SNP), was PCR amplified using the forward primer 5'-TTT TCT CTT TGT AAA ACT TCG TGC ATC ACT T-3' (the modified nucleotide is underlined), in which SfaN1 and NlaIII restriction nuclease sites within the wild type promoter sequence are destroyed, and reverse primer 5'-TGG GGC TGA TTG GAA ACC TTA TTA AG-3' as previously described with slight modifications [199] (Figure 2.1). Briefly, the PCR reactions were carried out in a final volume of 50 µl containing at least 100 ng of DNA, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.5 mM MgCl2, 2.5 mM of each nucleotide (dATP, dTTP, dCTP and dGTP), 20 pmol of each primer and 0.5 units of Taq DNA polymerase (Southern Cross Biotechnology (Pty) Ltd, S.A.). The samples were amplified with a PCR Express Thermal Cycler (Hybaid Limited, Middlesex, UK) using the following conditions: an initial denaturing step for 5 minutes at 94 ^{o}C; followed by 35 cycles of denaturing for 60 seconds at 94 ^{o}C, annealing for 105 seconds at 60 ^{o}C and extension for 60 seconds at 72 ^{o}C; and a final extension step at 72 ^{o}C for 3 minutes.

The PCR products were digested with the restriction endonuclease SfaN1 (5'-GCATNNNNN/N-3') to produce fragments of 196 bp and 32 bp for the C allele and 137 bp, 59 bp and 32 bp for the G allele. Genotypes were confirmed by also digesting the PCR products with NlaIII (5'-CATG/N-3') to produce 123 bp, 56 bp and 49 bp fragments for the C allele and 172 bp and 56 bp fragments for the G allele. The digestion fragments were resolved on 8 % polyacrylamide gels and visualised under UV light after ethidium bromide staining (Figure 2.2). Images were captured using an UVItec Gel Documentation System (UVItec Limited, UK).

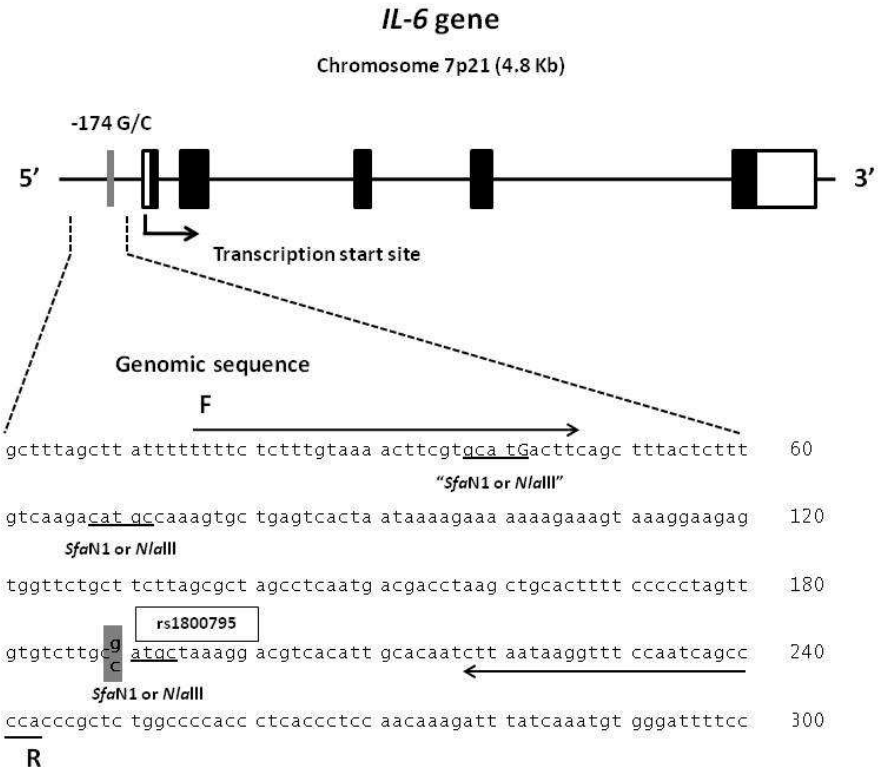

Figure 2.1: A schematic representation of the exon (boxes) and intron (horizontal lines) boundaries of the *IL-6* gene. Untranslated and translated regions of the exons are indicated by clear and solid black boxes, respectively. The -174 G/C single nucleotide polymorphism (SNP) (rs1800795) is represented as a grey box within the proximal promoter. The diagram was constructed from information Entrez Gene database (www.ncbi.nlm.nih.gov/gene) hosted by the National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health (Bethesda, MD, USA). A 300 bp genomic sequence within the proximal promoter region containing the 228 bp PCR fragment is also indicated in the figure. The binding positions of the forward (F) and reverse (R) primers (solid arrows), designed to amplify the PCR fragment containing the SfaN1 and NlaIII restriction fragment length polymorphisms (RFLPs; rectangular grey box in genomic sequence) are indicated in the 300 bp promoter sequence. The recognition (underlined) sequence of the SfaN1 (5'-GCATCNNNNN/N-'3) and NlaIII (5'-CATG/N-'3) restriction enzymes are indicated on the genomic sequence. The mutated nucleotide (G to C) with the forward primer is upper case. This mutation destroys the restriction sites shown in parenthesis.

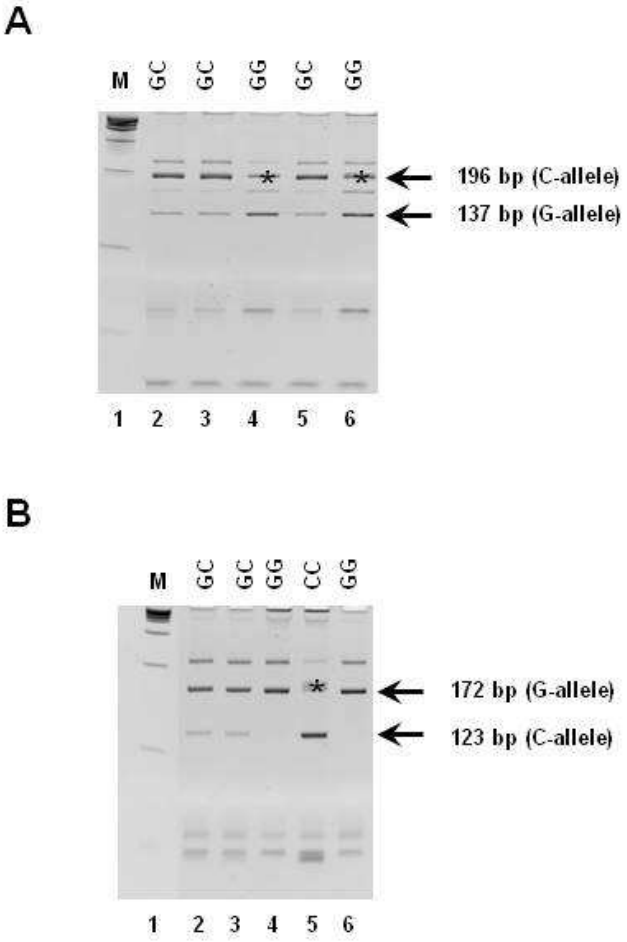

Figure 2.2: Typical 8% polyacrylamide gels showing the genotype analysis of the -174 G/C *IL-6* polymorphism following digestion of the 228 bp PCR products with the restriction endonuclease **(A)** SfaN1 and **(B)** NlaIII. As indicated, digestion by SfaN1 produced fragments of 196 bp and 32 bp for the C allele and 137 bp, 59 bp and 32 bp for the G allele. Because of the presence of a non-specific band that co-migrated with the 196 bp C allele band (indicated with an asterisk), the genotypes were confirmed by also digesting the amplicons with NlaIII to produce 123 bp, 56 bp and 49 bp fragments for the C allele and 172 bp and 56 bp fragments for the G allele. The non-specific band that migrates around the 172 bp G allele band after NlaII digestion is indicated with an asterisk. Lane numbers are indicated at the bottom of each gel. M, represents the 100bp DNA molecular weight ladder and the assigned genotype of each sample is indicated at the top of each lane.

2.2.3.2 5-HTT gene

The functional 44bp 5-HTT linked polymorphic region (*5-HTTLPR*) located 1 Kb upstream from the transcription start site of the *5-HTT* (*SLC6A4*) gene was PCR amplified using the following forward, 5'-GGC GTT GCC GCT CTG AAT TGC-3' and reverse 5'-GAG GGA CTG AGC TGC ACA ACC CAC-3' primers to produce 484 bp short (S) and/or 528 bp long (L) fragments [206] (Figure 2.3). The PCR reactions were carried out as described in section 2.2.3.1, except that the PCR reaction also contained 0.5 µl of 5 % dimethyl sulfoxide solution. The PCR conditions were as follows: an initial denaturing step for 3 minutes at 95^{o}C; followed by 35 cycles of denaturing for 30 seconds at 95 ^{o}C, annealing for 30 seconds at 61 ^{o}C and extension for 30 seconds at 72 ^{o}C; and a final extension step at 72 ^{o}C for 7 minutes. Amplified fragments were resolved on 4 % polyacrylamide gels and visualised under UV light after ethidium bromide staining (Figure 2.4). Images were captured using the UVItec Gel Documentation System (UVItec Limited, UK).

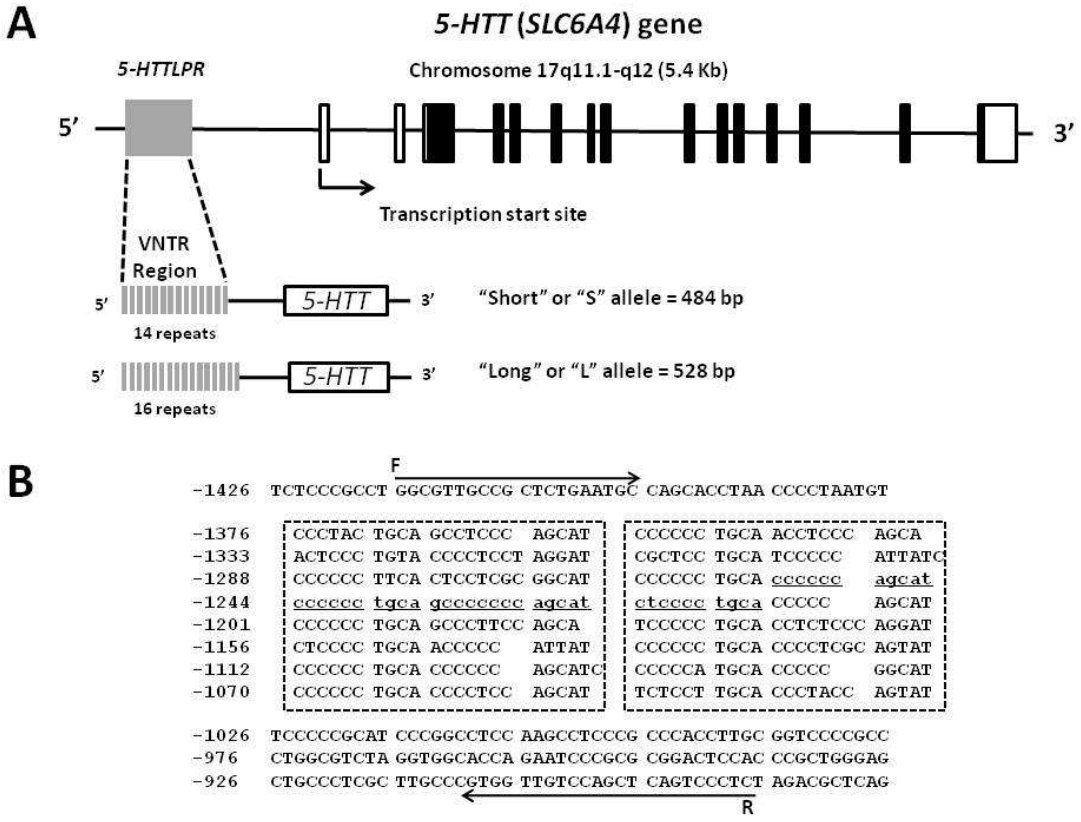

Figure 2.3: (A) A schematic representation of the exon (boxes) and intron (horizontal lines) boundaries of the *5-HTT* gene. Untranslated and translated regions of the exons are indicated by clear and solid boxes, respectively. The functional 44bp 5-HTT linked polymorphic region (*5-HTTLPR*) located 1 Kb upstream from the transcription start site is located within the grey box. The *5-HTTLPR* contains either 16 (long or L allele) or 14 (short or S allele) variable number of tandem repeats (VNTR) polymorphism. Each repeat (grey box) is approximately 22 bp in length. The diagram was constructed from information Entrez Gene database (www.ncbi.nlm.nih.gov/gene) hosted by the National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health (Bethesda, MD, USA). **(B)** The genomic sequence containing the VNTR polymorphism (dashed boxes). The additional 2 repeats in the L allele are in lower case and underlined. The binding positions of the forward (F) and reverse (R) primers (solid arrows), designed to amplify the PCR fragment containing the L (528 bp) and S (484 bp) alleles are indicated.

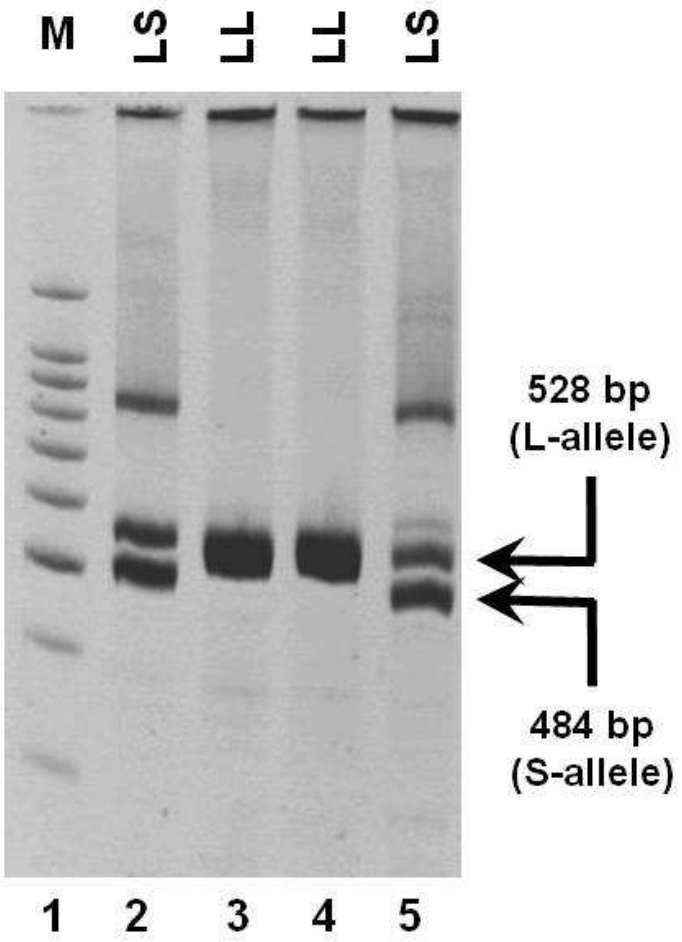

Figure 2.4: A typical 4% polyacrylamide gels showing the genotype analysis of the functional 44bp *5-HTTLPR* polymorphic region located 1 kb upstream of the *5-HTT* gene. Lane numbers are indicated at the bottom of the gel. M, represents the 100bp DNA molecular weight ladder and the assigned genotype of each sample is indicated at the top of each lane.

2.2.3.3 MAO-A gene

The functional 30 bp VNTR polymorphism of the *MAO-A* gene, was PCR amplified using the forward 5'-ACA GCC TGA CCG TGG AGA AG-3' and reverse 5'-GAA CGG ACG CTC CAT TCG GA-3' primers [347]. This produced either 306, 321, 336 or 366 bp fragments which corresponded to the 3-, 3.5-, 4- or 5-repeats of the 30 bp VNTR sequence respectively (Figure 2.5). The PCR reactions were carried out in a final volume of 50 µl containing at least 100 ng of DNA, 10 mM KCl, 10 mM (NH4)2SO4, 20 mM Tris-HCl, 2 mM MgSO4, 0.1% Triton X-100 (pH 8.8) MgCl2, 2.5 mM of each nucleotide (dATP, dTTP, dCTP and dGTP), 20 pmol of each primer and 0.5 units of Taq DNA polymerase (Southern Cross Biotechnology (Pty) Ltd, S.A.). The PCR samples were amplified as follows: an initial denaturing step for 5 minutes at 94 ^{o}C; followed by 35 cycles of denaturing for 1 minute at 94 ^{o}C, annealing for 1 minute at 62^{o}C and extension for 1 minute at 72 ^{o}C; and a final extension step at 72 ^{o}C for 5 minutes. Amplified fragments were resolved on 4 % polyacrylamide gels and visualised under UV light after ethidium bromide staining (Figure 2.6). Images were captured using the UVItec Gel Documentation System (UVItec Limited, UK).

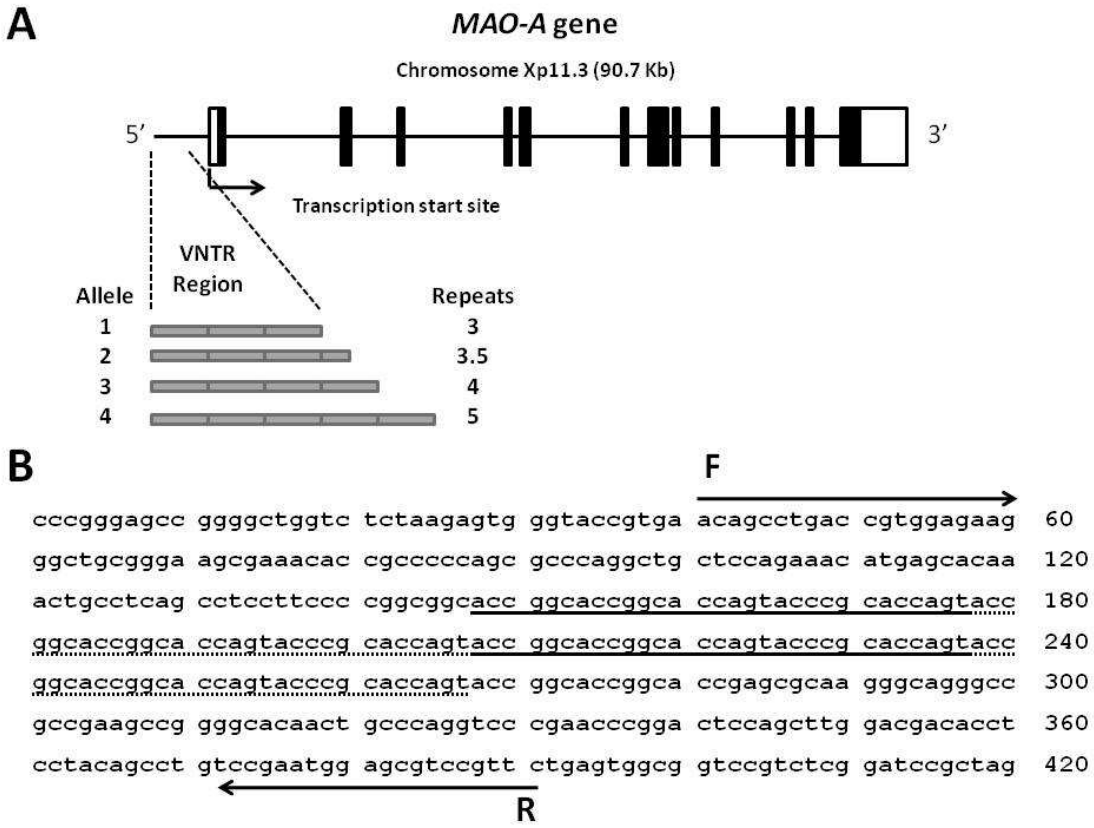

Figure 2.5: (A) A schematic representation of the exon (boxes) and intron (horizontal lines) boundaries of the *MAO-A* gene. Untranslated and translated regions of the exons are indicated by clear and solid boxes, respectively. The four functional variable number of tandem (30 bp) repeats (VNTR) and the repeat sizes are indicated within the 5'-flanking regulatory region of the gene. The diagram was constructed from information Entrez Gene database (www.ncbi.nlm.nih.gov/gene) hosted by the National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health (Bethesda, MD, USA). **(B)** The genomic sequence containing the VNTR polymorphism. The 30 bp are underlined with a solid or dashed line. The binding positions of the forward **(F)** and reverse **(R)** primers (solid arrows), designed to amplify the PCR fragment containing the VNTR alleles are indicated.

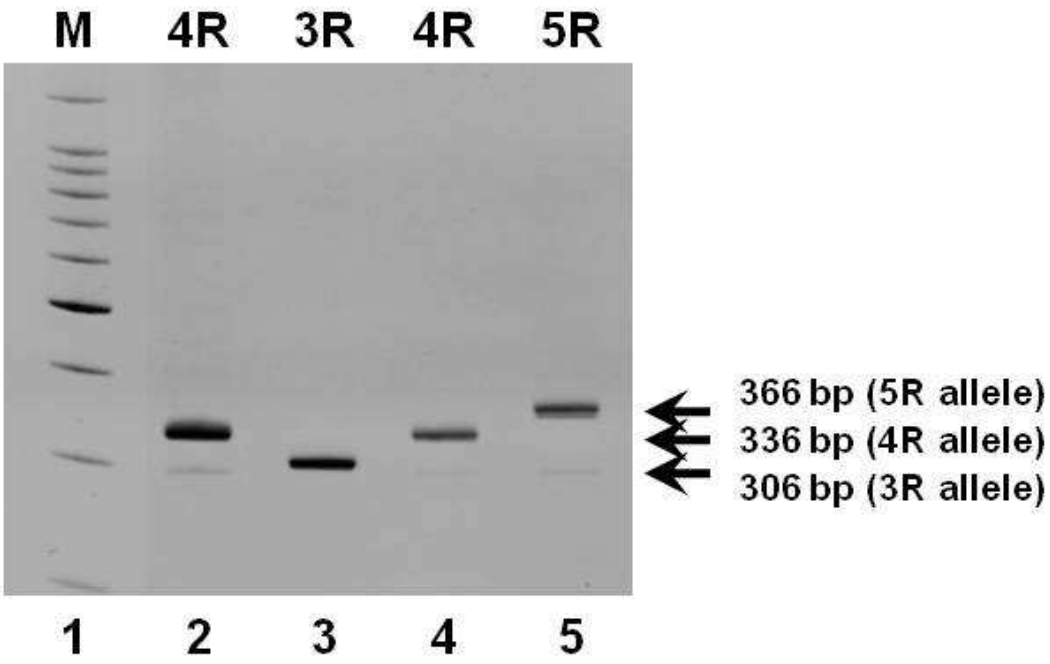

Figure 2.6: A typical 4% polyacrylamide gel showing the genotype analysis of the functional 30 bp VNTR polymorphism of the *MAO-A* gene. Lane numbers are indicated at the bottom of the gel. M, represents the 100bp DNA molecular weight ladder and the assigned genotype (number of repeats) of each sample is indicated at the top of each lane. The *MAO-A* gene is located on the X chromosome and only male participants were analysed. Therefore only on copy of the gene is present.

2.2.4 Environmental conditions

Details of the environmental conditions in Gordons Bay (55 km outside Cape Town) on the two race days were provided by the South African Weather Service. During the 2000 and 2001 events, the dry bulb temperature at midday was 21.7 ^{o}C (ranging from 17.0 to 23.9 ^{o}C) and 20.0 ^{o}C (ranging from 15.6 to 20.9 ^{o}C), respectively. The average humidity during the 2000 event was 68% (ranging from 46 to 87%) and 63% (ranging from 48 to 79%) during the 2001 event. The average wind speed was 4.6 m/s during 2000 and 6.4 m/s during 2001. The sea temperature was 16 ^{o}C and 15 ^{o}C during the 2000 and 2001 events respectively.

2.2.5 Statistical analysis

Data were analysed using STATISTICA version 8.0 (Stat-soft Inc., Tulsa, OK, USA) and GraphPad InStat version 2.05a (GraphPad Software, San Diego, CA, USA) statistical programmes. Where applicable, data are presented as means ± SD with the number of participants with non-missing data for each variable in parentheses. Pearson's Chi-squared analysis was used to detect differences in the genotype and allele frequencies, as well as in the percentage of South African-born individuals, between the triathlete and control groups. Hardy-Weinberg equilibrium was established using the Genepop web version 3.1c program (http://genepop.curtin.edu.au/). A one-way analysis of variance (ANOVA) was used to determine any significant differences between the

physiological characteristics of the triathlete and control groups, statistical significance was accepted when $p<0.05$. Where the overall F value was significant, a Tukey's honest significant difference post hoc test was used to identify where the differences were. The required sample sizes, to achieve 80% power with an α of 0.05, of the various groups were determined as previously described [75].

2.2.6 Participants characteristics

Athletes within the Fast (n=156), Mid (n=156) and Slow (n=156) Triath groups finished the event on average 10.9 (range 8.7 to 11.8), 12.5 (range 11.8 to 13.3) and 14.3 (range 13.3 to 16.1) hours respectively (Table 2.1). The 468 triathletes included in this study were representative of the entire field of 701 triathletes who completed either the 2000 or 2001 events [75]. In addition, there was a strong correlation ($r=0.833$, $p<0.001$) between the 2000 and 2001 race finishing times (n=119) for triathletes who completed both Ironman events (Appendix 1, Additional material, Figure A1).

The three triathlete and control groups were similarly matched for height ($p=0.806$) (Table 2.1). The Con group was, however, significantly younger than all the triathlete groups ($p<0.001$), while both the Fast ($p=0.004$) and Mid ($p=0.020$) Triath groups were significantly younger than the Slow Triath group. The Fast Triath group weighed significantly less ($p\leq0.004$) and had a lower BMI ($p<0.001$) than either the Mid Triath, Slow Triath or Con groups. The Con group was also significantly heavier ($p<0.001$) with a higher BMI ($p<0.001$) than Mid Triath group. There were significantly less South African-born triathletes within the Fast Triath group ($p<0.013$), while there were significantly more South Africa-born participants in the control group ($p<0.007$), when compared to the other groups. In addition, there were significantly less South African-born

triathletes in the Slow Triath group compared to the Mid Triath group (P=0.048, Table 2.1).

Apart from the *5-HTT* heterozygous individuals (LS) who were significantly younger (31.6 ± 8.6 yrs, n=325) than the homozygous individuals (LL, 33.8 ± 9.6 yrs, n=211, p=0.016), there were no observed genotype effects on any of the other physiological or performance characteristics (Table 2.2).

Table 2.1: General physiological and performance characteristics of the fastest (Fast Triath), middle (Mid Triath) and slowest (Slow Triath) finishers of the 2000 and/or 2001 South African Ironman Triathlons and the control (Con) groups.

	Fast Triath (n=156)	**Mid Triath** (n=156)	**Slow Triath** (n=156)	**Con** (n=200)	**p-value**
Age (years)	33.2 ± 6.0 (156) [c,f]	33.7 ± 7.4 (156) [d,g]	36.4 ± 9.6 (147) [e,f,g]	27.8 ± 9.8 (191) [c,d,e]	<0.001
Height (cm) [a]	180.5 ± 6.4 (143)	180.5 ± 6.2 (135)	180.9 ± 7.5 (146)	181.2 ± 7.6 (193)	0.806
Body Weight (kg) [a]	74.8 ± 7.4 (154) [c,f,h]	78.5 ± 8.1 (155) [d,h]	80.2 ± 10.4 (155) [f]	82.9 ± 11.6 (196) [c,d]	<0.001
BMI ($kg.m^{-2}$) [b]	22.9 ± 1.5 (142) [c,f,h]	24.1 ± 1.9 (134) [d,h]	24.6 ± 2.5 (145) [f]	25.2 ± 3.2 (191) [c,d]	<0.001
SA Born (%)	48.7 (74) [c,f,h]	74.5 (114) [d,g,h]	63.5 (99) [f,g,e]	86.5 (167) [c,d,e]	<0.001
Overall Time (min)	654 ± 41 (156) [f,h]	750 ± 26 (156) [g,h]	861 ± 46 (156) [f,g]	N/A	<0.001

With the exception of South African (SA) born, which is expressed as a frequency, values are expressed as mean ± SD. The number of participants with non-missing data for each variable is in parentheses. The total number of participants in each group (n) is also in parentheses.
N/A = non-applicable.
[a] Height and body weight are self-reported normal values; [b] Body mass index (BMI) was calculated as weight in kilograms (kg) divided by height in metres squared.
[c] $p<0.001$ Fast Triath vs Con; [d] $p\leq0.007$ Mid Triath vs Con; [e] $p<0.001$ Slow Triath vs Con;
[f] $p<0.013$ Fast Triath vs Slow Triath; [g] $p\leq0.048$ Mid Triath vs Slow Triath; [h] $p\leq0.004$ Fast Triath vs Mid Triath.
cm, centimetres; min, minutes.

Table 2.2: The *IL-6, 5-HTT* and *MAO-A* genotype effects on the physiological and performance characteristics of triathletes that completed the 2000 or 2001 South African Ironman Triathlons.

	IL-6			P-value	5-HTT			P-value	MAO-A		P-value
	GG	GC	CC		LL	LS	SS		"low-activity" [c]	"high-activity" [d]	
Age (years)	33.3 ± 9.4 (188)	31.9 ± 8.9 (329)	31.8 ± 8.7 (109)	0.215	33.8 ± 9.6 (211) [e]	31.6 ± 8.6 (325) [e]	33.1 ± 8.7 (109)	0.017 0.015[e]	32.6 ± 9.1 (221)	32.6 ± 9.0 (381)	0.932
Height (cm) [a]	180.3 ± 8.0 (178)	180.8 ± 6.5 (303)	181.1 ± 6.7 (106)	0.622	180.5 ± 7.2 (195)	181.0 ± 6.8 (307)	180.9 ± 7.2 (100)	0.644	181.0 ± 6.9 (205)	180.7 ± 7.2 (357)	0.560
Body Weight (kg) [a]	80.1 ± 11.0 (192)	79.0 ± 9.9 (328)	79.0 ± 9.8 (107)	0.468	79.2 ± 10.3 (210)	79.2 ± 9.6 (326)	79.6 ± 10.4 (110)	0.940	79.2 ± 10.0 (221)	79.0 ± 10.0 (379)	0.772
BMI ($kg.m^{-2}$) [b]	24.7 ± 2.9 (177)	24.2 ± 2.5 (301)	24.1 ± 2.6 (104)	0.089	24.4 ± 2.5 (193)	24.2 ± 2.5 (305)	24.4 ± 3.0 (100)	0.806	24.2 ± 2.7 (204)	24.1 ± 2.5 (353)	0.864
Swim Time (min)	69 ± 13 (126)	71 ± 14 (220)	68 ± 12 (74)	0.286	70 ± 13 (144)	70 ± 13 (230)	70 ± 14 (70)	0.884	70 ± 14 (153)	70 ± 13 (259)	0.727
Cycle Time (min)	390 ± 45 (123)	391 ± 42 (211)	382 ± 39 (73)	0.285	389 ± 45 (141)	340 ± 39 (222)	387 ± 41 (66)	0.863	387 ± 44 (147)	389 ± 42 (251)	0.580
Run Time (min)	284 ± 50 (127)	285 ± 48 (219)	282 ± 47 (72)	0.914	283 ± 50 (148)	285 ± 46 (226)	284 ± 47 (70)	0.899	284 ± 49 (155)	282 ± 48 (256)	0.675
Overall Time (min)	757 ± 102 (130)	757 ± 92 (230)	744 ± 90 (75)	0.562	752 ± 99 (152)	757 ± 88 (237)	751 ± 90 (73)	0.799	753 ± 96 (160)	752 ± 93 (268)	0.849

Values are expressed as mean ± SD. The total number of participants (n) is in parentheses, with the number of participants with non-missing data for each variable in parentheses.

[a] Height and body weight are self-reported normal values; [b] Body mass index (BMI) was calculated as weight in kilograms (kg) divided by height in metres squared; [c] "low-activity" represent the 3- and 5- combined and [d] "high-activity" the 3.5- and 4- combined repeats of the MAO-A VNTR polymorphism.

cm, centimetres; min, minutes.

2.2.7 *IL-6, 5-HTT* and *MAO-A* genotype and allele frequencies

There were no significant differences in the relative *IL-6, 5-HTT* and *MOA-A* genotype or allele distributions when the Fast Triath, Mid Triath, Slow Triath and Con groups were compared (Figure 2.7). In addition there were no significant differences in the relative distributions when the *MAO-A* alleles were combined to represent either high (3.5- or 4-repeats) or low (3- or 5-repeats) enzyme activity alleles [348] between the three triathlete and control groups (X^2=2.4, p=0.497) (Figure 2.8). The effects of population stratification cannot be excluded, however, similar *IL-6, 5-HTT* and *MAO-A* genotype and allele frequency distributions were observed when only the South African-born individuals were analysed (Appendix 1, Additional material, Figure A2). Furthermore, the observed genotype and allele frequencies reported for Caucasian males in this chapter are similar to what have been previously documented in the literature (Appendix 1, Additional material, Table A1) [205, 224, 349]. Both the *IL-6* (Fast Triath p=0.671, with the exception of Mid Triath p=0.022, Slow Triath p=0.619, Con p=0.564) and *5-HTT* (Fast Triath p=0.869, Mid Triath p=0.137, Slow Triath p=0.745, Con p=0.8825) genotype distributions for the triathlete and control groups were in Hardy-Weinberg equilibrium.

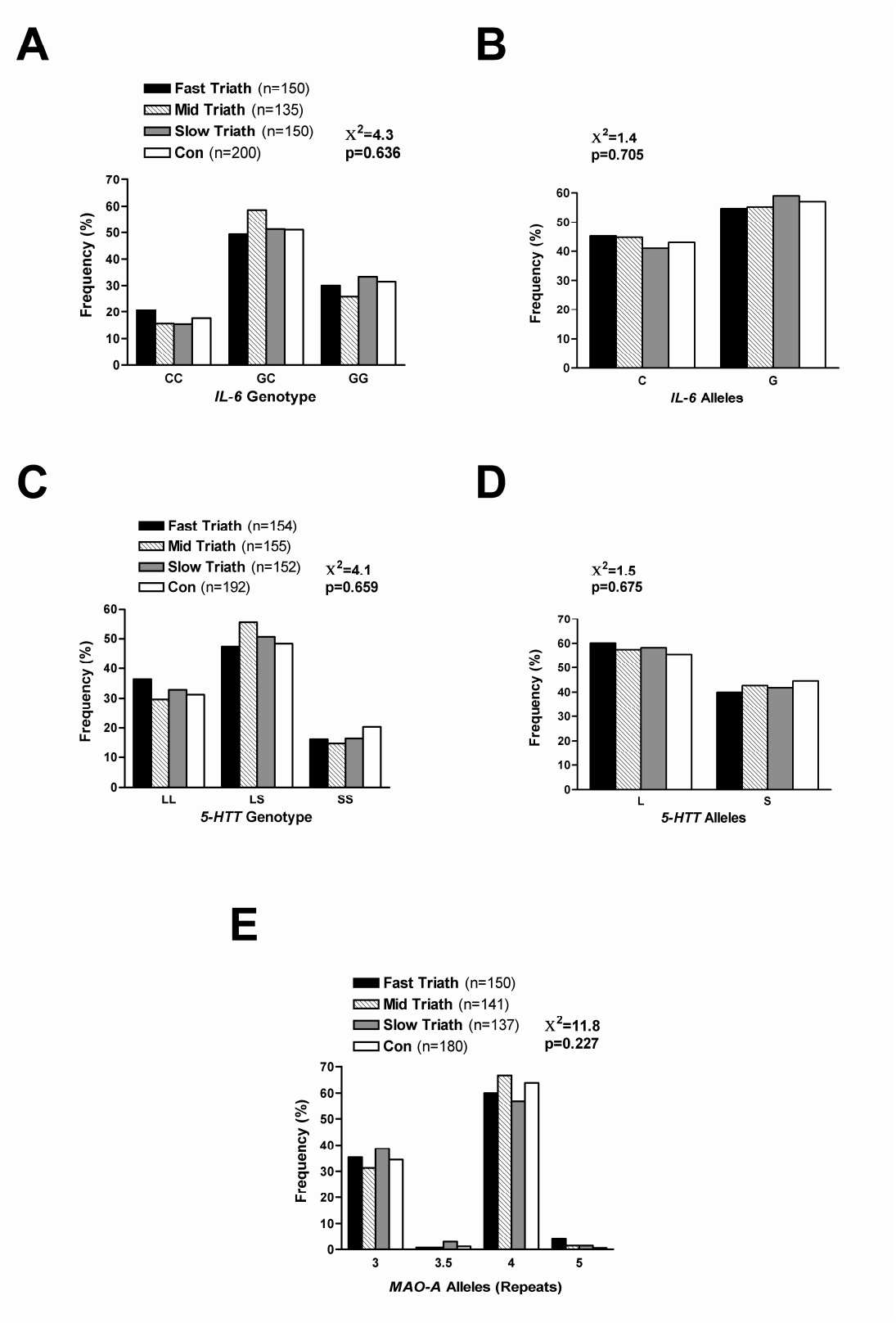

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Figure 2.7: The relative **(A)** genotype and **(B)** allele frequencies of the -174 G/C polymorphism within the *IL-6* gene of the fastest (Fast Triath), middle (Mid Triath), and slowest (Slow Triath) finishing male Caucasian triathletes, as well as, the control (Con) groups. The relative **(C)** genotype and **(D)** allele frequencies of the *5-HTTLPR* insertion (L)/deletion (S) polymorphism within the *SLC6A4* gene of the Fast Triath, Mid Triath, Slow Triath and Con groups. **(E)** The relative individual allele frequencies of the 30 bp VNTR polymorphism within the *MAO-A* gene of the Fast Triath, Mid Triath, Slow Triath and Con groups. The triathletes completed either the 2000 and/or 2001 South African Ironman Triathlon. For technical reasons not all the samples within each group were genotyped for all three polymorphisms. The actual number of genotyped samples within each group for the individual polymorphisms are indicated in the figure.

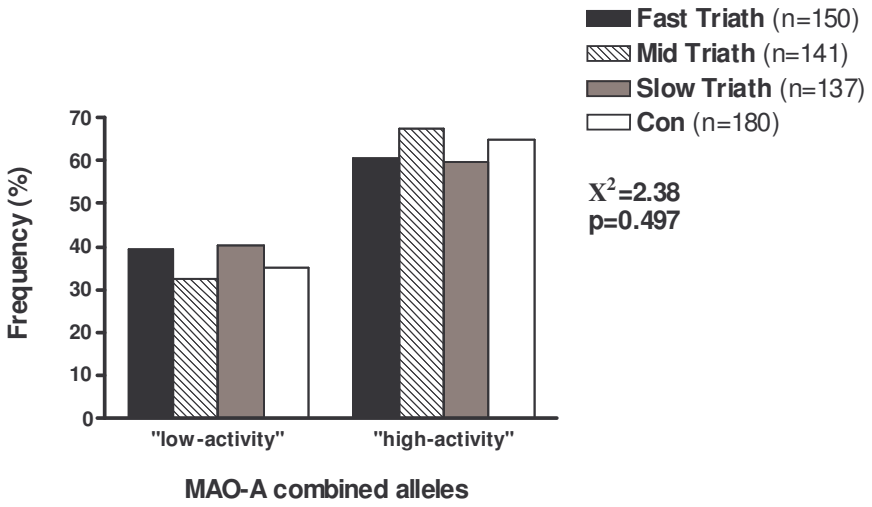

Figure 2.8: The relative genotype frequency of "high-" and "low-activity" combined alleles of the *MAO-A* VNTR polymorphism of the fastest (Fast Triath), middle (Mid Triath), and slowest (Slow Triath) finishing male Caucasian triathletes, as well as, the control (Con) groups. The triathletes completed either the 2000 and/or 2001 South African Ironman Triathlon. For technical reasons not all the samples within each group were genotyped for all three polymorphisms. The actual number of genotyped samples within each group is indicated in the figure.

Table 2.3 summarises the individual and combined results for the proposed advantageous *IL-6* CC and *5-HTTLPR* LL genotypes, as well as, the *MAO-A* "low-activity" allele. No significant differences were detected in the individual or combined genotype and allele distributions of the triathlete and control groups. In support of this, there were no differences between the overall and split times of the triathletes *IL-6, 5-HTT* and *MOA-A* genotype groups (refer to table 2.2).

Table 2.3: The frequencies of the proposed advantageous individual and combination *IL-6*, *5-HTT* and *MAO-A* genotypes of the fastest (Fast Triath), middle (Mid Triath) and slowest (Slow Triath) finishers of the 2000 and/or 2001 South African Ironman Triathlons and the control (Con) groups.

	All Triath	Fast Triath	Mid Triath	Slow Triath	Con	P-value[a]	P-value[b]
***IL-6* CC genotype**	17.2 (75/435)	20.7 (31/150)	15.6 (21/135)	15.3 (23/150)	17.5 (35/200)	0.936	0.597
5-HTT LL genotype	33.0 (152/461)	36.4 (56/154)	29.7 (46/155)	32.9 (50/152)	31.2 (60/192)	0.754	0.624
MAO-A "low activity" genotypes	37.4 (160/428)	39.3 (59/150)	32.6 (46/141)	40.1 (55/137)	35.0 (63/180)	0.642	0.497
***IL-6* CC and/or *5-HTT* LL genotypes**	43.9 (188/428)	48.0 (71/148)	40.3 (54/134)	43.2 (63/146)	43.2 (83/192)	0.941	0.623
***IL-6* CC and/or *MOA-A* "low activity" genotypes**	47.9 (193/403)	51.4 (76/148)	44.4 (55/124)	47.3 (62/131)	51.1 (92/180)	0.879	0.601
***5-HTT* LL and/or *MOA-A* "low activity" genotypes**	60.3 (254/421)	61.5 (91/148)	58.6 (82/140)	60.9 (81/133)	53.7 (94/175)	0.161	0.474
***IL-6* CC, *5-HTT* LL and/or *MOA-A* "low activity" genotypes**	66.7 (264/396)	68.5 (100/146)	64.2 (79/123)	66.9 (85/127)	66.3 (116/175)	0.929	0.906

Values are expressed as a percentage. The actual and total number of participants is in parentheses.
"low activity" represents the combined 3- and 5-repeats of the 30 bp VNTR polymorphism.
[a] All Triath vs. Con;
[b] Fast Triath vs. Mid Triath vs. Slow Triath vs. Con

2.3 DISCUSSION

The first novel finding of this study within the thesis was that there was no significant association between either the -174 *IL-6* G/C, 44 bp *5-HTTLPR* (L/S) and *MAO-A* 30 bp VNTR polymorphisms and endurance performance in the 2000/2001 South African Ironman Triathlons. The second finding of this study was to be expected with observed differences in age, body weight and BMI between the triathlete groups, with the faster triathletes being younger and weighing less than their slower counterparts.

Contrary to the findings within this study, Park et al. [219] reported a significant over-representation of the *5-HTTLPR* deletion or short (S) allele in elite long distance runners compared to elite short distance runners. These results should, however, be considered preliminary as the sample size used in the report was very small and insufficient for genetic association studies [350, 351], and no biological explanation for the result was given. Further, there are no studies that have examined the role of variants within the *IL-6* or *MAO-A* genes in endurance athletic ability, although numerous studies have demonstrated the effects of their protein products, including *5-HTT*, and manipulations within their biological systems in altering endurance exercise capacity [22, 187, 345].

Although we did not observe an association of the functional 44 bp insertion/deletion (L/S) *5-HTT* and *MAO-A* 30 bp *VNTR* polymorphisms with performance in this study, there is, evidence to support the involvement of 5-HT in endurance exercise performance. As previously discussed within the

literature review (Chapter 1, Section 1.4.3.1), the Central Fatigue Hypothesis support the notion that a decreased 5-HT intraneuronal concentration, as a likely result of increased 5-HTT protein synthesis (associated with the insertion or L allele), would delay the onset of fatigue and favour endurance exercise capacity. An additional A/G single nucleotide polymorphism within the *5-HTTLPR* has recently been identified and is now functionally characterised as triallelic (L_G , L_A and S allele) [205]. The dominance of this mutation is very low and would probably have no significant impact on our results [352]. However, further investigation of this triallelic polymorphism within these Ironman Triathletes is needed to conclude a full analysis of the *5-HTTLPR* and its role in endurance performance.

In addition, the adequate degradation of intraneuronal 5-HT by the enzyme MAO-A is vital for effective neurotransmission and the administration of inhibitors, by implication a decreased MAO-A activity, have been associated with increased exercise behaviour in rats [345]. Furthermore, it has also been proposed that MAO-A synthesis can be affected by other components [345], possibly hormones [353], that exclude genetic transcriptional factors. This could possibly explain the lack of an association between the *MAO-A* 30 bp VNTR polymorphism and performance within this population of Ironman triathletes.

The G-allele of the functional -174 *IL-6* G/C polymorphism, also investigated in this study, is associated with a higher plasma IL-6 concentration than the C-allele [199]. We demonstrated the fatiguing effect of elevated plasma IL-6

concentrations, by rhIL-6 administration, in treadmill running in healthy males [22]. It was, therefore, hypothesized that the lower IL-6 transcription C-allele would favour endurance performance. However, we did not observe any association of the -174 *IL-6* G/C polymorphism with performance within the 2000 and/or 2001 South African Ironman Triathletes.

Queries have arisen regarding the appropriateness of recruiting athletes from multi-disciplined sports (e.g. triathletes) as opposed to single disciplined sports (e.g. runners) in genetic studies as this increases the inherent phenotypic heterogeneity of the study population [354]. The Ironman triathlon, however, consists of three endurance disciplines (swim, cycle and run) combined over a total distance of 226 km and athletes completing this event within the allowed time are considered to display the endurance phenotype regardless of their strength in any of the three disciplines. In fact, the *ACE* "endurance" (insertion) allele has been shown to be associated with endurance in swimming, cycling and running [12, 355-357]. It is therefore unlikely that the lack of association between the *IL-6, 5-HTT* and *MAO-A* genes and endurance performance is due to methodological flaws in this study design. Variants within the angiotensin converting enzyme (*ACE*), bradykinin β2 receptor (*BDKRB2*) and nitric oxide synthase 3 (*NOS3*) genes have previously been shown to be associated with the performance of these triathletes [75]. In addition, as discussed in the literature review (Section 1.1), variants within other genes have been shown to be associated with Russian Olympic triathletes [23, 80, 81].

The second finding of this study was to be expected. Age, body weight and BMI differed between the triathlete groups, with the faster triathletes being younger and weighing less than their slower counterparts. It has been documented that age and BW correlate with performance [358-301], but the age difference of three years observed between the three triathletes groups within this study is probably inconsequential. In addition, the percentage South African born individuals were the least within the Fast Triath group. This can be accounted for by foreign professional triathletes who are more likely to travel to compete at international level compared to slower recreational triathletes. The highest percentage of South African born individuals was observed within the control group, which was as a resulted from local recruitment.

A limitation of this study is the fact that a possible genotype effect on training could not be excluded. Physical activity and cardiorespiratory fitness can modulate the genotype effect observed on physiological phenotypes. The endothelin 1 locus Lys198Asn [361], angiotensinogen (*AGT*) A-20C and angiotensin II receptor type 1 (*AGTR1*) A1166C polymorphisms [362] have been associated with blood pressure phenotypes in response to training. In this study the self-reported training data collected from participants were partial and incomplete and therefore not suitable for analysis. Further research is warranted to determine whether the *IL-6* G/C, *5-HTTLPR* L/S and *MAO-A* 30 bp VNTR genotypes play a role in training adaptation.

In conclusion, these results presented in this chapter of the thesis do not support the direct association of the -174 *IL-6* G/C, *5-HTTLPR* (L/S) and *MAO-*

A 30 bp VNTR polymorphisms investigated in this study with endurance athletic performance in the 2000 and/or 2001 South African Ironman Triathletes. Besides performance, there is also inter-individual variation in other physiological variables, such as body weight change and serum [Na^+] concentrations [304, 330], during participation in ultra-endurance events. These variables can, if not normally regulated, result in medical complications [3]. The focus of the remaining chapters of this thesis therefore is to investigate the possible genetic contribution to these observed changes in physiological variables during the South African Ironman Triathlon.

CHAPTER 3

DIPSOGENIC EFFECT OF THE 5-HTT GENE IN WEIGHT CHANGES DURING THE 2000 AND 2001 SOUTH AFRICAN IRONMAN TRIATHLONS

The data presented in this chapter has been published in the following article: Saunders, C.J., **de Milander, L.**, Hew-Butler, T., Xenophontos, S.L., Cariolou, M.A., Anastassiades, L.C., Noakes, T.D. and Collins, M. Dipsogenic genes associated with weight changes during Ironman Triathlons. **Human Molecular Genetics**, 2004; 15(20):2980-7.

3.1 INTRODUCTION

In the previous study (Chapter 2) we investigated the role of the candidate genes *IL-6, 5-HTT* and *MAO-A* with actual performance times during the 2000 and/or 2001 South African Ironman Triathlons. Although we did not observe an association with any of these three genes and actual overall performances during the triathlon, the serotonergic system plays a vital role in regulating thirst, as previously discussed in the literature review Section 1.6.4.1. This behavioral mechanism is initiated via circulatory peptides that signal the activation of brain centers that induce the sensation of thirst and the desire to drink, whereas inhibitory serotonergic (5-HT) pathways prevent against excessive fluid intake [266].

Ultra-endurance events such as the Ironman Triathlon challenge the homeostatic control of total body water (TBW) due to large amounts of water losses through sweating. Total body weight (BW) losses of up to 12% have been measured in competing Ironman triathletes [305, 330]. There is however a large variation amongst athletes in total body weight losses during participation in ultra-endurance events [3]. Although this variation is partly due to the drinking behaviour of athletes, an inter-individual variation in the homeostatic control of TBW during prolonged endurance exercise exists [3].

The primary aim of this second study was therefore to determine whether the 44bp *5-HTTLPR* (L/S) polymorphism, a functional genetic component of the serotonergic system, is associated with body weight changes during the 2000 and/or 2001 South African Ironman Triathlons. We propose that the S allele, associated with a lower 5-HTT expression and thereby increased synaptic serotonin levels, would activate inhibitory pathways reducing the stimulation of brain thirst centres. The inhibition of drinking behaviour is likely to result in a larger decrease in body weight changes (a proxy of hydration status) during these events.

3.2 METHODS

3.2.1 Participants

As previously described in Section 2.2.1., 468 (66.8% of the entire field) self-reported Caucasian male triathletes who completed either the 2000 and/or 2001 South African Ironman Triathlons were recruited to participate in this study. These events consisted of a consecutive 3.8 km swim, 180 km cycle and a 42.2 km run. Prior to participation in this study, each participant completed an informed consent (Appendix 2) and personal particulars questionnaire (Appendix 3), as detailed in Section 2.2.1. Four hundred and forty-nine triathletes, 119 from the 2000 and 330 from the 2001 events, were included in this study. The 2001 data was used for triathletes (n=78) with complete sets of data for both events.

Approval for this study was obtained from the Research and Ethics Committee of the Faculty of Health Sciences, University of Cape Town, South Africa (reference numbers: 005/2000 and 099/2001; Appendix 4)

3.2.2 DNA extraction

Blood sampling and DNA extractions were performed as previously described in Section 2.2.2.

3.2.3 Genotyping the *5-HTT* gene

DNA samples were genotyped for the 44bp *5-HTTLPR* insertion/deletion polymorphism, located 1kbp upstream from the transcription start site of the *5-HTT* (*SLC6A4*) gene, using the polymerase chain reaction (PCR) technique as previously described Section 2.2.3.2. (Figure 2.3.). The amplified fragments were resolved on 4% polyacrylamide gels and visualised under UV light after ethidium bromide staining (Section 2.2.3.2., Figure 2.4.). Images were captured using the UVItec Gel Documentation System (UVItec Limited, UK) and the sizes of the DNA fragments determined. As detailed within Section 2.2.3.2., PCR products consisted of 484 bp short (S) and/or 528 bp long (L) fragments (Figure 2.4.).

3.2.4 Biochemical analysis and weight determinations

For the determination of pre- and post-race serum [Na^+], ~4.5 ml of venous blood from each subject was collected in a lithium heparin vacutainer tube at race registration (1 to 3 days prior to the event) and again within 10 min after completing the event. The samples were immediately centrifuged at 3000 x g for 10 min at 4^oC and stored at -20^oC until analysis. Concentrations were determined by using an Easylyte PLUS Na/K/Cl analyzer (Medica Corporation, Bedford, MA, USA) as previously described [304]. The changes in serum [Na^+] and [K^+] were calculated as the difference between the pre- and post-race

concentrations divided by the pre-race concentration and expressed as a percentage.

Each triathlete was weighed on the morning of the race wearing swimming attire and immediately after completing the race in their running outfit without shoes. The weights were corrected for standard clothing worn at the time of measurement [304]. The percentage change in body weight lost or gained during the race was calculated as the difference between the pre- and post-race body weights divided by the pre-race body weight and expressed as a percentage. Also, fuel utilization can account for a small percentage in body weight loss [311, 312], however this calculation was utilised as an indirect measure for triathletes' hydration status. Only triathletes that completed the race and were considered to be euhydrated (0 to 3% body weight loss) or dehydrated (>3% body weight loss) were analysed in this study [3]. Triathletes that were classified as dehydrated were further divided into 3-5% and >5% body weight loss groups. The four triathletes that were over-hydrated were not included in this study.

3.2.5 Statistical analysis

Data were analysed using STATISTICA version 8.0 (Stat-soft Inc., Tulsa, OK, USA) and GraphPad InStat version 2.05a (GraphPad Software, San Diego, CA, USA) statistical programmes. Where applicable, data are presented as means ± SD with the number of participants with non-missing data for each variable in

parentheses. Pearson's Chi-squared analysis was used to detect differences in the genotype and allele frequencies, as well as in the percentage of South African-born individuals, between the different weight loss groups. Hardy-Weinberg equilibrium was established using the Genepop web version 3.1c program (http://genepop.curtin.edu.au/). A one-way analysis of variance (ANOVA) was used to determine any significant differences between the physiological characteristics of the triathlete and control groups, statistical significance was accepted when $p<0.05$. Where the overall F value was significant, a Tukey's honest significant difference post hoc test was used to identify where the differences were. The required sample sizes of the various groups were determined as previously described [75].

3.3 RESULTS

3.3.1 Participants characteristics

As previously described in Section 2.2.4., the environmental conditions for the 2000 and 2001 South African Ironman Triathlons were relatively mild. In addition, the 468 triathletes that were recruited for this study were representative of the entire field of 701 triathletes who completed either the 2000 or 2001 events, as previously described in Section 2.2.1. Only 449 (64.1%) self-reported Caucasian male triathletes, 119 from the 2000 and 330 from the 2001 event from whom pre- and post-race weights, and complete genotype data were obtained, were included in this study. The 2001 data were used for triathletes (n=78) with complete sets of data for both events and thus there were no duplicates within the above mentioned sample size for this particular study.

Triathletes' age ($r=0.162$, $n=449$, $p=0.001$), pre-race ($r=0.217$, $n=425$, $p<0.001$) and post-race ($r=0.267$, $n=439$, $p<0.001$) body weights, absolute ($r=0.190$, $n=417$, $p<0.001$) and relative ($r=0.256$, $n=417$, $p<0.001$) body weight changes, BMI ($r=0.330$, $n=406$, $p<0.001$) and pre-race serum [Na^+] ($r=0.150$, $n=395$, $p=0.003$) were all positively correlated with their overall finishing time (Figure 3.1.). There were significant negative linear relationships between post-race serum [Na^+] versus their overall finishing time ($r=-0.145$, $n=344$, $p=0.007$, data

not shown) and relative changes in body weight ($r=-0.379$, $n=312$, $p<0.001$, Figure 3.2.) during the South African Ironman triathlons.

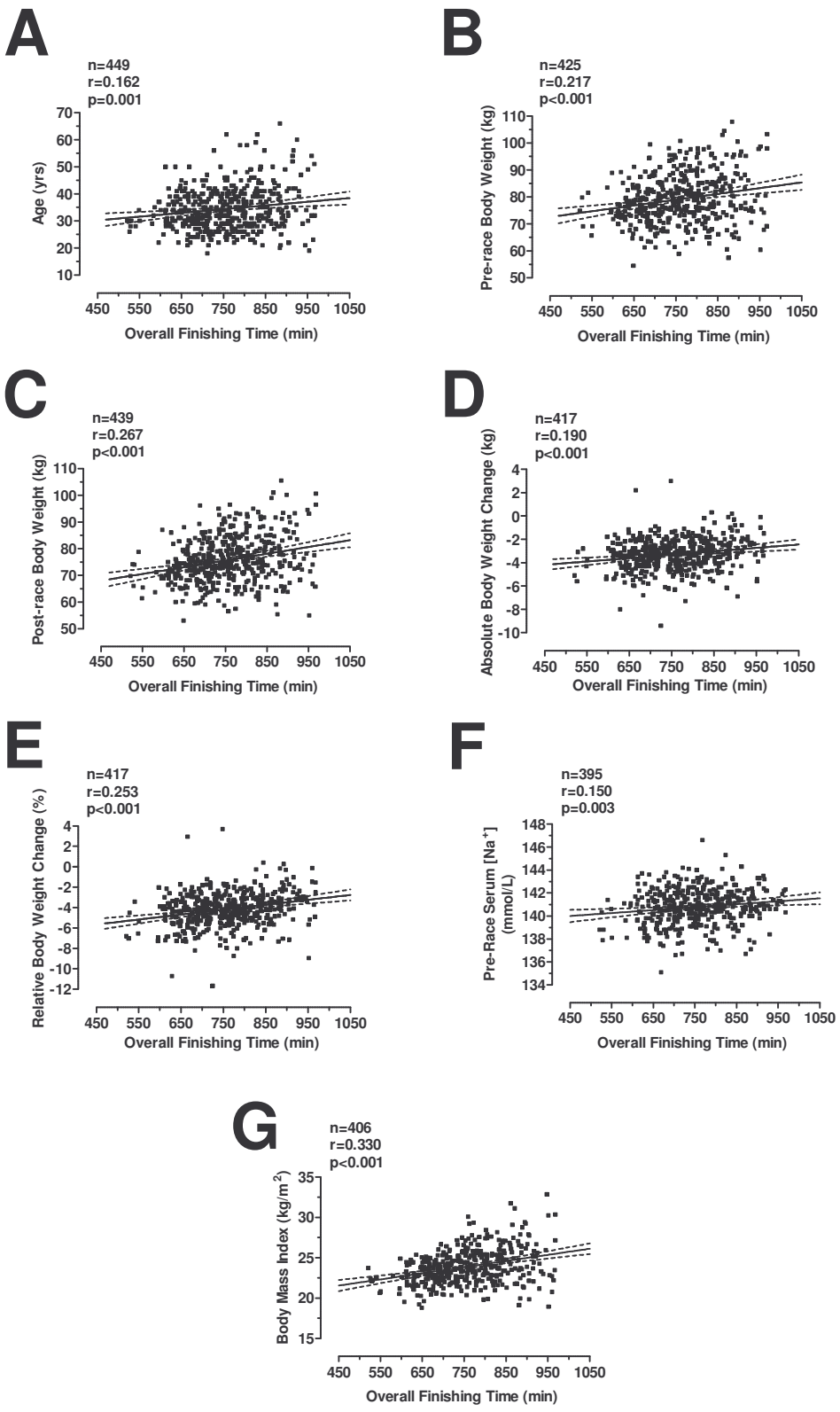

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Figure 3.1: Positive linear regressions of male Caucasian triathletes' **(A)** age, **(B)** pre-race and **(C)** post-race body weights, **(D)** absolute and **(E)** relative body weight changes (%), **(F)** pre-race serum [Na^+] and **(G)** body mass index with overall finishing time during the 2000 and 2001 South African Ironman Triathlons. The solid line is the line of best fit while the dashed lines are the 95% confidence intervals.

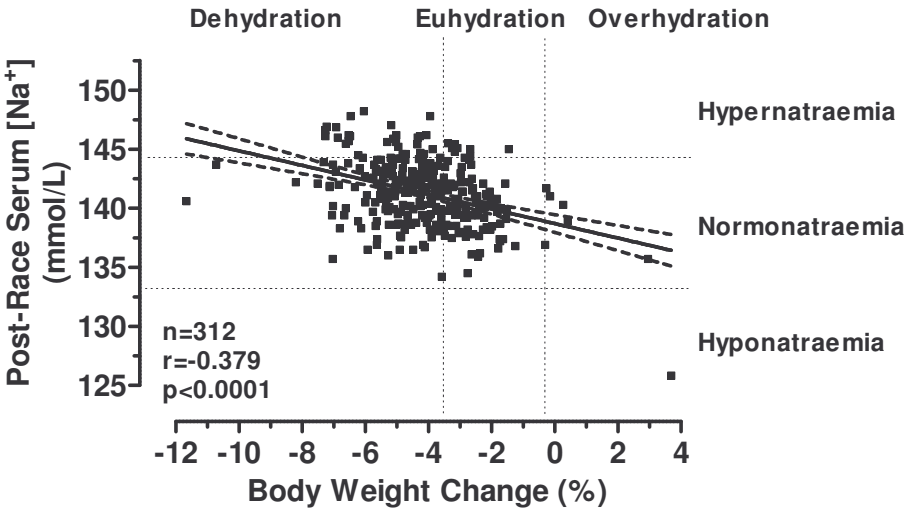

Figure 3.2: Relationship between post-race serum [Na^+] and relative body weight (BW) changes during the 2000 and 2001 Ironman Triathlon events. Dehydration, euhydration and overhydration regions, as well as hypernatraemia, normonatraemia and hyponatraemia regions are indicated. The solid line is the line of best fit while the dashed lines are the 95% confidence intervals.

3.3.2 Body weight and serum [Na+] changes

Three hundred and ninety-three (99%) triathletes started the Ironman events with a serum [Na^+] within the normal physiological range (135 to 145 mmol/l), whilst the remaining two triathletes had higher pre-race serum [Na^+] (145.3 and 146.8 mmol/l). As summarized in Table 3.1, triathletes completed the Ironman events with various combinations of percentage body weight change, which was used as an indirect measure of hydration status (dehydration, euhydration and overhydration) and serum sodium status (hypernatremia, normonatraemia and hyponatraemia). As previously described in this study (Section 3.2.4.), triathletes that were dehydrated (>3% body weight loss) were divided into two groups (>3-5% and >5%) to investigate possible linear trends for genotype effects on body weight loss during the triathlons. It should be noted that there was a significant positive linear relationship between the 2000 and 2001 relative changes in body weights ($r=0.506$, $n=95$, $p<0.001$), but not the 2000 and 2001 post-race serum [Na^+] ($r=0.270$, $n=52$, $p=0.053$), for triathletes that completed both Ironman events (Figure 3.3.).

When triathletes were divided into three groups (0-3, >3-5 and >5%) according to their relative body weight loss during the Ironman events, the triathletes within each group were similarly matched for age, height, pre- and post-race body weights, BMI and pre-race serum [Na^+] (Table 3.2). As expected, there were significant ($p>0.001$) differences in the absolute and relative body weight changes between the 0-3, >3-5 and >5% body weight loss groups, with relative

body weight changes of -2.2 ± 0.7 % (range: -0.13 to -2.98%), -4.0 ± 0.6% (range: -3.03 to -4.99%) and -6.1 ± 1.1% (range: -5.01 to -11.66%) respectively. Furthermore, there were significant differences in post-race serum [Na^+], overall finishing and split (swim, cycle and run) times between the three body weight loss groups. On average, the triathletes within the >5% body weight loss group, which were considered to be the most dehydrated, finished the Ironman events with the highest post-race serum [Na^+] and fastest overall race times. In addition, there was a significant linear trend (X^2= 3.9, p=0.049) for the percentage of South African born triathletes to be under-represented in the group that lost the most weight. (>5%) This was expected since they were also the fastest finishers and had the most international entrants (Table 3.2).

Table 3.1: Summary of all the triathletes finishing the 2000 (n=119) or 2001 (n=330) South African Ironman Triathlons with various combinations of body weight changes and post-race serum sodium concentrations following the event

	Body weight gain	Body weight loss (0-3%)	Body weight loss (>3-5%)	Body weight Loss (>5%)	Subtotal	Body weight Change (no data)	Total
Hypernatraemia (>145 mmol/l)	0	0	10	16	26	2	28
Normonatraemia (135–145 mmol/l)	3	67	137	76	283	30	313
Hyponatraemia (<135 mmol/l)	1	1	1	0	3	0	3
Subtotal	4	68	148	92	312	32	344
Post-race serum sodium [Na^+] not determined	0	23	54	28	105	0	105
Total	4	91	202	120	417	32	449

Triathletes who gained body weight during the event were considered to be overhydrated, those with a body weight loss between 0% and 3% were considered to be euhydrated, and those with a body weight loss >3% were considered to be dehydrated [3].

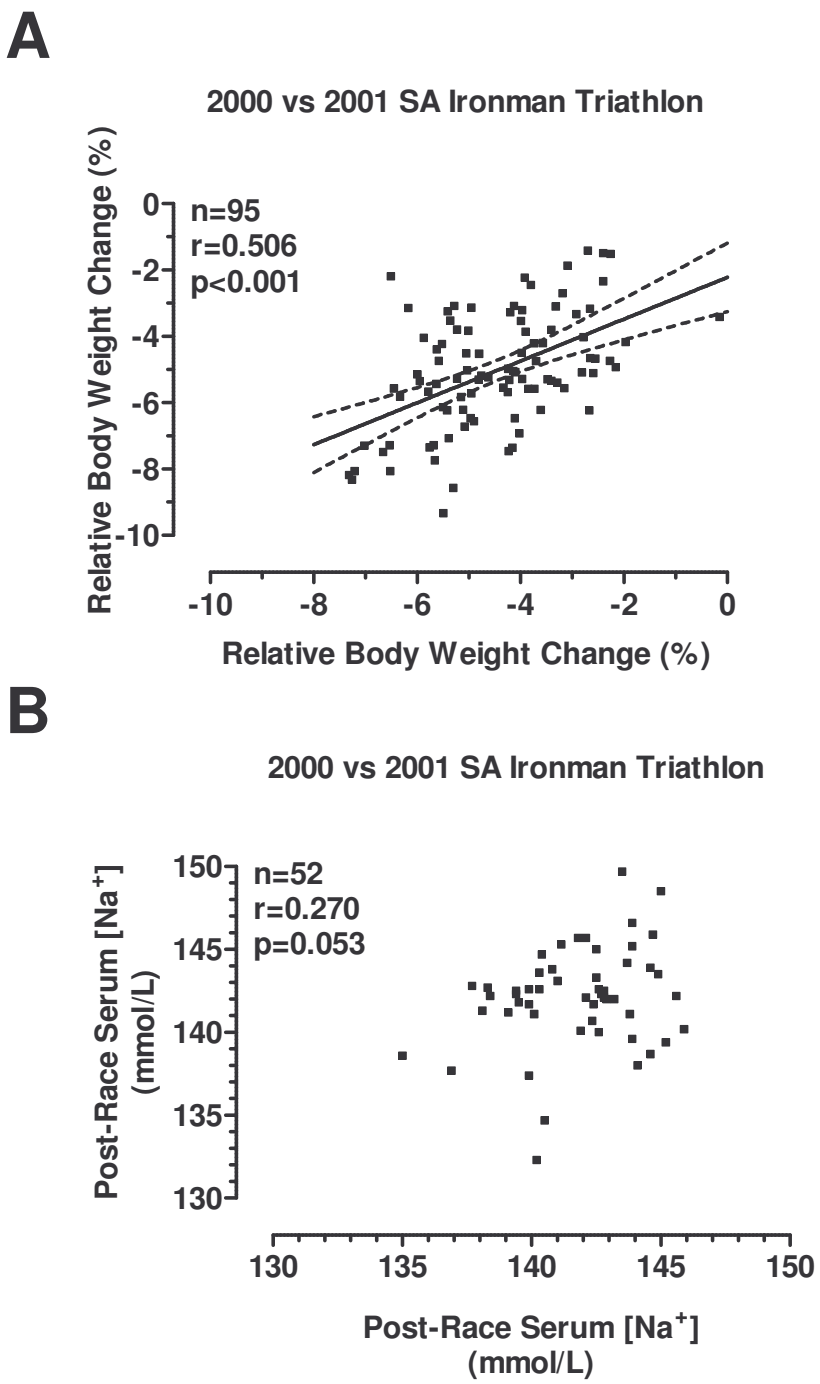

Figure 3.3: Positive linear regressions of male Caucasian triathletes' **(A)** relative body weight change (%) and **(B)** post-race serum [Na^+] between the 2000 and 2001 South African Ironman Triathlons for triathletes that completed both events. The solid line is the line of best fit while the dashed lines are the 95% confidence intervals.

Table 3.2: The general physiological and performance characteristics of triathletes that completed either the 2000 or 2001 South African Ironman Triathlon events, categorized according to their change in body weight within the three different body weight loss (0-3, >3-5 and >5%) groups.

	Body weight loss (0-3%) (N=91)	**Body weight loss (>3-5%)** (N=202)	**Body weight loss (>5%)** (N=120)	**P-value**
Age (years)	34.5 ± 9.2 (91)	34.1 ± 7.9 (202)	34.8 ± 7.0 (120)	0.910
Height (cm)	181.3 ± 6.5 (83)	180.3 ± 7.3 (185)	180.8 ± 5.9 (109)	0.708
BMI ($kg.m^{-2}$)	23.7 ± 2.2 (82)	24.1 ± 2.3 (184)	23.8 ± 2.0 (109)	0.482
South African Born (%)	67.0 (91)	66.5.8 (200)	54.4 (114)	0.070
Pre-race Body weight (kg)	78.4 ± 9.4 (91)	79.5 ± 9.3 (202)	79.3 ± 8.0 (120)	0.691
Post-race Body weight (kg)	76.6 ± 9.6 (91)	76.3 ± 8.9 (202)	74.5 ± 7.7 (120)	0.215
Absolute Body weight change (kg)	-1.7 ± 0.6 (91)[a,b]	-3.2 ± 0.6 (202)[a,c]	-4.8 ± 0.9 (120)[b,c]	<0.001[a] <0.001[b] <0.001[c]
Relative Body weight change (%)	-2.2 ± 0.7 (91)[a,b]	-4.0 ± 0.6 (202)[a,c]	-6.1 ± 1.1 (120)[b,c]	<0.001[a] <0.001[b] <0.001[c]
Pre-race [Na^+] (mmol/l)	140.8 ± 1.6 (82)	140.9 ± 1.6 (179)	140.7 ± 1.4 (99)	0.512
Post- race [Na^+] (mmol/l)	140.1 ± 2.3 (68)[a,b]	141.4 ± 2.4 (148)[a,c]	142.2 ± 2.7 (92)[b,c]	0.004[a] <0.001[b] 0.049[c]
Swim (min)	73 ± 14 (90)[b]	71 ± 13 (192)[c]	66 ± 11 (115)[b,c]	<0.001[b] 0.004[c]
Cycle (min)	402 ± 41 (85)[b]	393 ± 40 (185)[c]	378 ± 41 (113)[b,c]	<0.001[b] 0.013[c]
Run (min)	302 ± 52 (90)[a,b]	283 ± 43 (187)[a]	275 ± 46 (116)[b]	0.008[a] <0.001[b]
Overall Time (min)	790 ± 95 (91)[a,b]	760 ± 84 (202)[a,c]	729 ± 90 (120)[b,c]	0.036[a] <0.001[b] 0.016[c]

With the exception of South African (SA) born, which is expressed as a frequency, values are expressed as mean ± SD. The number of participants with non-missing data for each variable is in parentheses. The total number of participants in each group (n) is also in parentheses.

3.3.3 *5-HTT* genotype effects on weight loss

The *5-HTTLPR* genotype distributions of triathletes included in this study were in Hardy-Weinberg equilibrium (p=0.134). There was no significant difference (X^2= 7.2, p=0.127) in the *5-HTTLPR* genotype distribution between the three body weight loss groups (Table 3.3.). However, when combining the dominant L-allele genotypes (LS and LL), there was a significant (X^2= 5.1, p=0.024) linear trend for the SS genotype amongst the three body weight loss groups, with the >5% group (20.5% SS) having the highest percentage, followed by the >3-5% group (16.3% SS) and the 0-3% group (8.8% SS) having the least (Table 3.3. and Figure 3.4.).

Table 3.3: Serotonin transporter linked polymorphic region (*5-HTTLPR*) genotype distributions within the three different body weight loss (0-3, >3-5 and >5%) groups of triathletes during the 2000 or 2001 South African Ironman Triathlons.

Genotype	Body weight loss (0-3%)	Body weight loss (>3-5%)	Body weight loss (>5%)
5-HTT	n=91	n=202	n=117
SS	8.8 (8)	16.3 (33)	20.5 (24)
LS	55.0 (50)	54.0 (109)	44.4 (52)
LL	36.3 (33)	29.7 (60)	35.0 (41)

Values are expressed as a percentage, with the number of participants in parentheses. 5-HTT genotype distribution, X^2 =7.2, p=0.127 and SS genotype and L allele (LS and LL genotypes) distribution, X^2 =5.3, p=0.069 and linear trend X^2 =5.1, p=0.024.

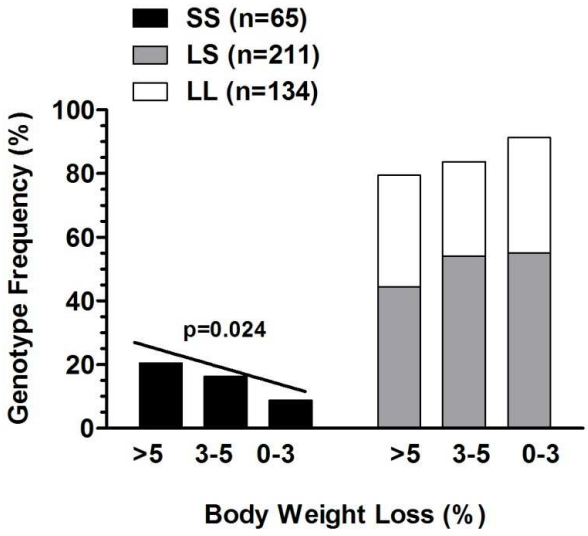

Figure 3.4: The *5-HTTLPR* genotype distributions for triathletes with the SS or combined L-allele (LS and LL) genotypes across the three body weight loss groups.

3.3.4 *5-HTT* genotype effects on physiological and performance variables

To investigate possible genotype and allele effects on physiological characteristics, the triathletes containing LL or LS genotypes were grouped for the dominant L-allele (Table 3.4). There were no significant differences in age, height, body weights and absolute body weight change, serum [Na^+], percentage South African born or overall and split times between the L-allele and SS genotype groups (Table 3.4). Triathletes with the SS genotype did however have a significant ($p=0.048$) lower BMI than triathletes with the L-allele. Also, there was a significant difference in the relative body weight changes between the two groups. The triathletes with an L-allele lost on average -4.1 ± 1.7% (n=348) of their pre-race body weight, compared to SS genotype carriers who lost on average -4.5 ± 1.7 (n=66) of their pre-race body weight. Lastly, as reported in the previous chapter, there was no *5-HTTLPR* genotype or allele effects with performance of the triathletes included in this study.

Table 3.4: The serotonin transporter linked polymorphic region (*5-HTTLPR*) genotype effects on the physiological and performance characteristics of triathletes that completed the 2000 or 2001 South African Ironman Triathlons.

	L allele[a] (n=375)	SS genotype (n=70)	P-value
Age (years)	34.4 ± 8.0 (375)	34.1 ± 7.3 (70)	0.733
Height (cm)	180.6 ± 6.7 (345)	180.7 ± 6.8 (60)	0.904
BMI ($kg.m^{-2}$)	24.0 ± 2.2 (342)	23.4 ± 1.7 (60)	0.048
South African Born (%)	62.7 (370)	58.8 (68)	0.638
Pre-race Body weight (kg)	79.4 ± 9.3 (355)	78.2 ± 7.2 (67)	0.325
Post-race Body weight (kg)	76.1 ± 9.0 (367)	74.4 ± 6.8 (68)	0.162
Absolute Body weight change (kg)	-3.2 ± 1.4 (348)	-3.6 ± 1.4 (66)	0.069
Relative Body weight change (%)	-4.1 ± 1.7 (348)	-4.5 ± 1.7 (66)	0.042
Pre-race [Na^+] (mmol/l)	140.8 ± 1.6 (330)	140.6 ± 1.7 (61)	0.393
Post- race [Na^+] (mmol/l)	141.4 ± 2.8 (290)	141.0 ± 2.6 (50)	0.417
Swim (min)	70 ± 13 (362)	70 ± 14 (67)	0.914
Cycle (min)	390 ± 42 (351)	387 ± 41 (64)	0.606
Run (min)	284 ± 48 (359)	283 ± 46 (66)	0.925
Overall Time (min)	756 ± 93 (375)	751 ± 88 (70)	0.658

With the exception of South African (SA) born, which is expressed as a frequency, values are expressed as mean ± SD. The number of participants with non-missing data for each variable is in parentheses. The total number of participants in each group (n) is also in parentheses.
[a] LL and LS genotypes

3.4 DISCUSSION

The first novel finding of this study was that the SS genotype of the functional 44bp *5-HTTLPR* polymorphism within the *5-HTT* (*SLC6A4*) gene was significantly (p=0.042) associated with relative greater body weight changes of triathletes during the 2000 or 2001 South African Ironman Triathlons. The second finding of this study was that triathletes with the SS genotype had a significantly (p=0.048) lower BMI compared to L allele triathletes. The third finding was that triathletes who experienced the greatest relative body weight loss (>5% group), finished the Ironman events with a significant higher serum [Na+], faster overall finishing and split (run, cycle and swim) times compared to smaller body weight loss groups.

To our knowledge, this is the first study that has reported a novel association with the SS genotype of the functional 44bp *5-HTTLPR*, within the *5-HTT* gene, with greater body weight losses within Ironman triathletes. Functionally, the S (short) or deletion allele is associated with a decreased 5-HTT protein synthesis [206]. We propose that biologically this will result in a reduced re-uptake of serotonin from the synaptic cleft and, thereby, increased levels of extracellular cerebral serotonin, which can impair effective neurotransmission [189]. We hypothesized that individuals with the SS genotype would experience a lesser drive for thirst as a result of cerebral serotonin's inhibition of the perception of thirst. Our finding, therefore, substantiates our hypothesis that the SS genotype would effectively reduce the stimulation of brain thirst centers, thereby allowing

for a greater reduction in total body water (TBW), observed as body weight lost, and by implication dehydration. We observed a significant (X^2= 5.1, p=0.024) linear trend for the SS genotype amongst the three body weight loss groups, with the >5% group having the highest percentage of SS genotype triathletes.

The second finding of this study was an observed significant lower BMI in SS genotype triathletes compared to their LL genotype counterparts. Serotonergic pathways have well been known to regulate the behavioural mechanism of appetite and food intake [363], whereby an increased availability of synaptic serotonin would exert an inhibitory influence on food intake [218]. Our results are, therefore, not surprising and support recent findings by Bah and co-workers who observed a significant overrepresentation of the *5-HTTLPR* SS genotype in underweight (BMI<20) males [218].

An additional third finding was that triathletes that lost the most body weight during the event and were considered the most dehydrated, finished the race with significant higher serum [Na^+]. This, however, is no novel finding to the existing literature. Reports from as early as 1968 documented an inverse relationship between serum [Na^+] and evaporative weight loss in unacclimatised males during exercise and acute heat stress [364]. Furthermore, a battery of studies that investigated serum [Na^+] and weight changes in athletes during endurance running [3, 322, 365-368] and Ironman Triathlon [3, 304, 330, 369] events reported the same inverse relationship. As previously discussed in section 1.6.1 the maintenance of tonicity or an osmotic equilibrium is vital to help defend cell volume (intracellular fluid compartment) during exercise. Thus,

fluid loss through sweating needs to exceed hypotonic fluid intake to maintain a 2:1 intracellular to extracellular water ratio and thereby plasma osmolality within the normal physiological range [370]. An overall weight loss is thus physiologically expected during prolonged exercise. Serum Na^+ is the main solute within the extracellular fluid (ECF) and following prolonged and/or excessive sweating a significant reduction in ECF volume would, subsequently, increase the tonicity within this fluid compartment [364, 371, 372].

Furthermore, triathletes that lost the most relative body weight (>5%) were faster, within each event and overall, compared to smaller body weight loss groups. Again our finding support previous studies that observed greater dehydration levels in faster runners during races [5]. These triathletes (>5% body weight loss) were generally assumed to be “lighter” and subsequently faster [373]. Also, well trained and heat acclimatised athletes are adapted to sustain higher sweat rates [374-376], thereby allowing for greater body weight losses and dehydration [3]. As such, not only would these athletes be adapted to further dehydrate and become “lighter”, they are perhaps likely to also be the better trained and faster athletes. In addition, faster runners spend shorter periods of time on the event course and are, therefore, exposed to a shorter time frame in which to rehydrate. Also, the possibility of experiencing gastric discomfort, by drinking larger volumes of fluid whilst running at a fast pace, might restrict drinking frequency and volumes [273, 294-296]. These physiological factors could perhaps justify the observation that triathletes who experienced greater body weight losses, had higher post-race serum [Na^+] concentrations and finished the event faster. It is, however, important to bear in

mind that although the SS genotype was associated with relative greater body weight losses and smaller BMI's within triathletes there is no association between the 44bp *5-HTTLPR* and performance within these Ironman triathletes, as previously investigated in Chapter 2. It is, therefore, highly unlikely that the observed association of the SS genotype with athletes' relative body weight was confounded by their performance. Furthermore, it should be noted that the possible use of anti-depressant drugs, of which serotonin re-uptake inhibitors are most commonly prescribed, amongst triathletes was unknown.

Although we acknowledge that confounding factors such as muscle fuel utilisation, urination, defecation and respiration also contributed to overall body weight loss, it was assumed that all triathletes were exposed to the same environmental conditions and experienced a relative similar energy fuel demand. Therefore, pre- and post-race body weights were used as an indirect measure to determine changes in body fluid volumes, as previously described in this study (Section 3.2.4.). Our observed results, however, need to be confirmed under strictly controlled laboratory conditions to exclude these possible confounding factors.

In conclusion, the study presented in this chapter found that the SS genotype of the 44bp *5-HTTLPR* within the *SLC6A4* gene is associated with relative greater body weight losses, and by implication water loss, during the 2000 and 2001 South African Ironman triathlons. These findings support the involvement of serotonergic pathways in the regulation of thirst and drinking behaviour.

CHAPTER 4

THE ARGININE VASOPRESSIN RECEPTOR 2 (AVPR2) GENE, BODY WEIGHT AND SODIUM CHANGES DURING THE SOUTH AFRICAN IRONMAN TRIATHLONS

The data presented in this chapter has been published in the following article:
De Milander, L., Ah Kun, M., September, A.V., Schwellnus, M.P., Noakes, T.D. and Collins, M. AVPR2 Gene and Weight Changes During Triathlons. **International Journal of Sports Medicine**, 2012; 33(1):67-75.

4.1 INTRODUCTION

In the previous chapter (Chapter 3) of this thesis we reported an association with the functional *5-HTTPLR* in the *5-HTT* (*SLC6A4*) gene with body weight changes in triathletes during the 2000 and 2001 South African Ironman Triathlons. Specifically, triathletes with a SS genotype lost on average significantly more body weight during the event when compared to those with a L allele. This finding indirectly supports a role of the serotonergic system in regulating thirst and provides evidence for the underlying genetic contribution to the observed variation in total body water (TBW) lost during participation in ultra-endurance events. However, it is likely that there are also other contributing genetic variants within other systems than can account for body weight loss, by implication TBW losses, during an ultra-endurance event.

As discussed in Section 1.6, the maintenance of homeostasis and by implication an osmotic equilibrium is vital for cellular function. Compensatory mechanisms such as thirst and hormonal reflexes aim to replenish and minimize insensible water losses, respectively. Nevertheless, observations have shown that there is a wide range in body weight and serum [Na^+] changes, as a result from sweating, in athletes competing in endurance events [3]. As such inappropriate water replacement can lead to dysregulation of sodium homeostasis resulting in exercise-associated hyponatraemia (EAH) (Serum [Na^+] <135 mmol/L) and its fatal complication, exercise-associated hyponatraemic encephalopahty (EAHE). Over the past two decades the incidence of EAH and EAHE has increased substantially in ultra-endurance events [317, 328, 377, 378]. There is substantial proof that EAHE is due to abnormal fluid retention in those who drink to excess during prolonged exercise usually lasting more than 4 hours [319, 327, 379].

Thus Noakes and co-workers [3] have proposed that EAH and EAHE are caused by three independent biological mechanisms: (i) over-drinking during exercise as the primary cause, (ii) inadequate suppression of antidiuretic hormone (ADH) secretion resulting in inappropriate fluid retention and (iii) either osmotic inactivation of circulating sodium or the failure to mobilize osmotically inactive sodium from internal stores. The large inter-individual variation in the regulation of serum sodium and water balance that becomes apparent during prolonged endurance exercise [3], could also partly be attributed to genetic factors has not yet been documented.

Recently, a disease-causing gain-of-function mutation within codon 137 of the arginine vasopressin receptor 2 (*AVPR2*) gene that clinically manifests with

hyponatraemia have been identified [339-341]. This mutated receptor is constitutively active (functions independently from the hormonal regulation of arginine vasopressin (AVP)), resulting in inappropriate water reabsorption by the kidneys and a subsequent increase in TBW content that causes the serum sodium concentrations to fall [339, 341].

Since disease-causing mutations within the *AVPR2* gene can cause hyponatraemia in patients as a result of excess water reabsorption by the kidneys [339, 380], the *AVPR2* is an additional ideal candidate gene contributing to inter-individual variation in changes in serum [Na^+] and/or BW in endurance athletes.

Accordingly the aim of this study was to determine whether there were any associations between three single nucleotide polymorphisms (SNP's) (rs3761528, rs3761527 and rs4898457) within the *AVPR2* gene, and inter-individual variations in serum sodium and/or body weight changes in athletes competing in the 2000, 2001 and 2006 South African Ironman Triathlons. We hypothesize that common sequence variants within the *AVPR2* gene partly determine the inter-individual variation in electrolyte and fluid changes in athletes participating in prolonged exercise.

4.2 METHODS

4.2.1 Participants

A total of 658 self-reported Caucasian male triathletes that completed either the 2000 (n=171), 2001 (n=276) and/or 2006 (n=211) South African Ironman Triathlons consented to participate in this study. These triathletes represented 64% and 26% of the entire field of male triathletes who completed the 2000/2001 combined and 2006 events respectively. Eighty-one triathletes completed more than one event, of which 51 completed the 2000 and 2001 events, 15 the 2001 and 2006 events, and 8 triathletes finished the 2000 and 2006 events. Seven triathletes completed all three events. Data were therefore collected during more than one event for 88 triathletes (51, 15, 8, 2x7). Only the 2001, or subsequent 2000 event, data were used for triathletes with complete sets of data for more than one event. There was a strong correlation ($r=0.861$, $p<0.001$) between the finishing times for triathletes that completed more than one event. A total of 570 individual triathletes were therefore analysed in this study.

As described in section 2.2.1., this annual international event which débuted in South Africa in 2000 comprises of a 3.8 km swim, 180 km cycle and a 42.2 km run (www.ironman.com). Prior to the 2000, 2001 and 2006 events each entrant was invited to participate in the study. Triathletes were recruited at race registration and completed an informed consent (Appendix 2) and a personal particulars questionnaire (Appendix 3), as previously described in section 2.2.1. Age, height,

normal body weight, body mass index (BMI), country of birth, gender and ethnic background were determined from the completed questionnaires. This study was conducted in accordance with the ethical standards and laws as described by Harris and Atkinson [381] and approved by the Research Ethics Committee of the Faculty of Health Sciences, University of Cape Town (reference numbers: 005/2000, 099/2001 and 425/2005; Appendix 4).

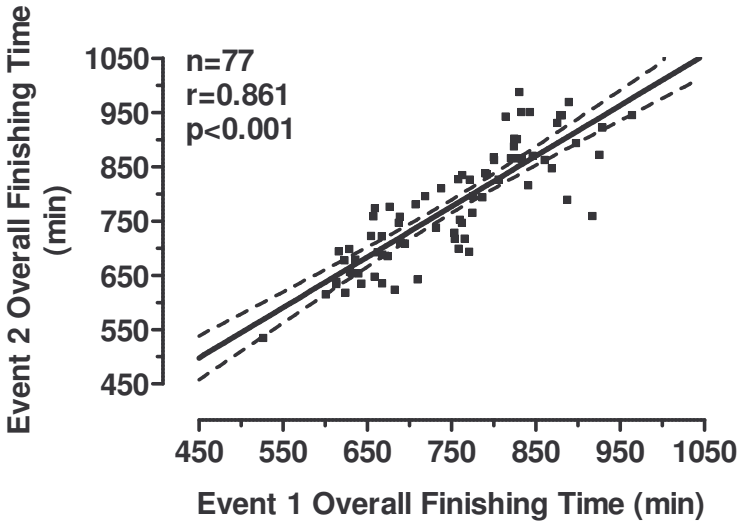

Figure 4.1: Positive linear regression of male Caucasian triathletes' overall finishing times between their first (event 1) and. second (event 2) South African Ironman Triathlon events. The solid line represents the line of best fit and the dashed lines are the 95% confidence intervals.

4.2.2 DNA extraction

Blood sampling and DNA extractions were performed as previously described in section 2.2.2.

4.2.3 SNP selection

Single nucleotide polymorphisms (SNPs) were selected for the *AVPR2* gene, which has been mapped to the long arm of chromosome X (Xq28) (http://www.ncbi.nlm.nih.gov), and flanking regions using the databases hosted by the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov), the Seattle SNP database (http://pga.mbt.washington.edu) and International HapMap Project (http://www.hapmap.org/index.html.en) (Table 4.1). Five exonic SNPs were initially identified for *AVPR2* of which 3 were nonsynonomous (change the amino acid sequence in the gene product,) and 2 were synonomous (do not alter the amino acid sequence in the gene product) (Figure 4.2.). From the databases, the rare allele of all these 5 exonic SNPs have, however, not been identified in Caucasian populations. These SNPs were therefore not informative for this study population and were consequently not selected for genetic analysis in this study. Subsequently seven additional SNPs with a high heterozygosity (minor allele frequency was >20%) was identified within a 10.3 Kb region which contained the *AVPR2* gene and its 5'-region (Table 4.2). Two of these informative SNPs (rs3761528 and rs3761527) were identified within the first intron of the *AVPR2* gene. SNPs rs3761528 and rs3761527

each contained a C/T transversion at positions 152,689,296 and 152,689,471 within intron 1 respectively. In addition, a third SNP, rs4898457, was selected within the promoter region. This SNP contained an A/G transversion at position 152,679,203 (Table 4.1 and Figure 4.2.). As illustrated in figure 4.2 (Bottom panel), the same two major *AVPR2* haplotypes (GTT and ACC) that are produced by the seven SNPs within the 10.3 Kb region could also be identified with these three SNPs. Thus, SNPs rs4898457, rs5945169, rs7052686, rs5945369, rs4898458, rs3761528 and rs3761527 produce the same haplotype block as the three identified SNPs rs3761528, rs3761527 and rs4898457. A haplotype represents a set of closely linked alleles which are inherited together and represents a segment on a chromosome [382]. Participants were therefore genotyped for the SNPs rs3761528, rs3761527 and rs4898457 within the *AVPR2* gene.

Table 4.1: SNPs within a 20 Kb fragment of chromosome X (nucleotides 152,678,000 to 152,698,000), which contain the *AVPR2* gene as well as its 5'- and 3'-flanking intergenic regions. The fragment also contains the 3'-end of the downstream neighbouring Rho GTPase activating protein 4 (*ARHGAP4*) gene (nucleotide 152,693,677 to 152,698,000).

Number	SNP	Nucleotide Position	Ob Het	Pred Het	HW P value	Minor Allele Frequency	Minor Allele
1	rs7065317	152,678,569	0.023	0.023	1.000	0.011	A
2	rs12007705	152,678,822	0.000	0.000	1.000	0.000	C
3 [a,b]	rs4898457	152,679,203	0.378	0.401	0.463	0.278	A
4	rs5945366	152,681,903	0.000	0.000	1.000	0.000	G
5 [b]	rs5945169	152,682,884	0.370	0.380	0.797	0.256	C
6 [b]	rs7052686	152,683,309	0.378	0.401	0.463	0.278	T
7	rs5987180	152,685,163	0.000	0.000	1.000	0.000	C
8 [b]	rs5945369	152,686,651	0.370	0.380	0.797	0.256	T
9	rs12011997	152,687,128	0.000	0.000	1.000	0.000	C
10 [b]	rs4898458	152,688,537	0.378	0.401	0.463	0.278	G
11	rs12559136	152,689,001	0.024	0.024	1.000	0.012	A
12 [a,b]	rs3761528	152,689,296	0.391	0.401	0.463	0.278	G
13 [a,b]	rs3761527	152,689,471	0.413	0.429	0.638	0.311	G
14	rs4898372	152,691,202	0.065	0.043	1.000	0.022	C
15 [c]	rs5196	152,691,465	0.000	0.000	1.000	0.000	A
16 [c]	rs5199	152,691,987	0.000	0.000	1.000	0.000	T
17 [c]	rs5200	152,692,247	0.000	0.000	1.000	0.000	C
18 [c]	rs5201	152,692,840	0.000	0.000	1.000	0.000	G
19 [c]	rs5203	152,693,026	0.000	0.000	1.000	0.000	G
20 [b,d]	rs2070097	152,697,101	0.435	0.464	0.117	0.367	C
21 [d]	rs3213441	152,697,421	0.000	0.000	1.000	0.000	C
22 [d]	rs5945371	152,697,690	0.000	0.000	1.000	0.000	T

Data downloaded from the International HapMap Project (http://www.hapmap.org/index.html.en).
SNP, single nucleotide polymorphism; Ob Het, Observed heterozygosity; Pred Het, Predicted heterozygosity; HW = Hardy- Weinberg.
[a] SNPs selected in this study for genotyping.
[b] SNPs with a minor allele frequency >20%.
[c] SNPs within the coding region of the *AVPR2* gene.
[d] SNPs within the 3'-end of the downstream neighbouring (*ARHGAP4*) gene (reverse orientation).

Table 4.2: Primers sequences for the tetra-primer amplification refractory mutation system–polymerase chain reaction (T-ARMS–PCR) of the single nucleotide polymorphisms (SNPs) within the 5'-flanking region of the *AVPR2* gene.

SNP	Name[1]	Primer sequences	Amplicon size (bp)[2]
rs4898457	AVPR A (Fo)	5'- CCACACTCCCACCCCTGAACTGCTATTA - 3'	*K 384* G 260 A 178
	AVPR B (Ro)	5'- CCATACTGCCTATCACACAGCTTGAACC - 3'	
	AVPR C (Fi)	5'- CATTCTCCTGCTTTGTGACAAATAC**A**C**G** - 3'	
	AVPR D (Ri)	5'- GCCAAAAGACATGTGCAGAAAAAGC**A**A**T** - 3'	
rs3761528	AVPR E (Ro)	5'- GTCCCCAATAACCGCATGCTGTCACTAA - 3'	*K 353* T 278 C 130
	AVPR F (Fo)	5'- GTTAGCATGGATTCCTCGCCCTAGCCGG - 3'	
	AVPR G (Ri)	5'- TGTGCAATGGGACTTGATGATTGAG**T**G**G** - 3'	
	AVPR H (Fi)	5'- ACCCCTGCCCATCTTACCACCGTAG**G**C**T** - 3'	
rs3761527	AVPR I (Ro)	5'- ACATCACACCCCGCATCTTTGCCATGTT - 3'	*K 355* T 225 C 185
	AVPR J (Fo)	5'- CCATTGCACAGAAGGGGAAGCTGAGGTG - 3'	
	AVPR K (Ri)	5'- CTTCCTAATGCCACCCAGATCAGTG**T**A**G** - 3'	
	AVPR L (Fi)	5'- CTGCCAGTTGAGGGAAGCCATCTCC**G**A**T** - 3'	

[1]F: forward, R: reverse, o: outer (common) and i: inner (allele-specific) primers are indicated in parentheses. Deliberate mismatches (shown in bold) have been introduced to increase the specificity of the allele-specific primers. [2]K size of the control amplicon (shown in italics).

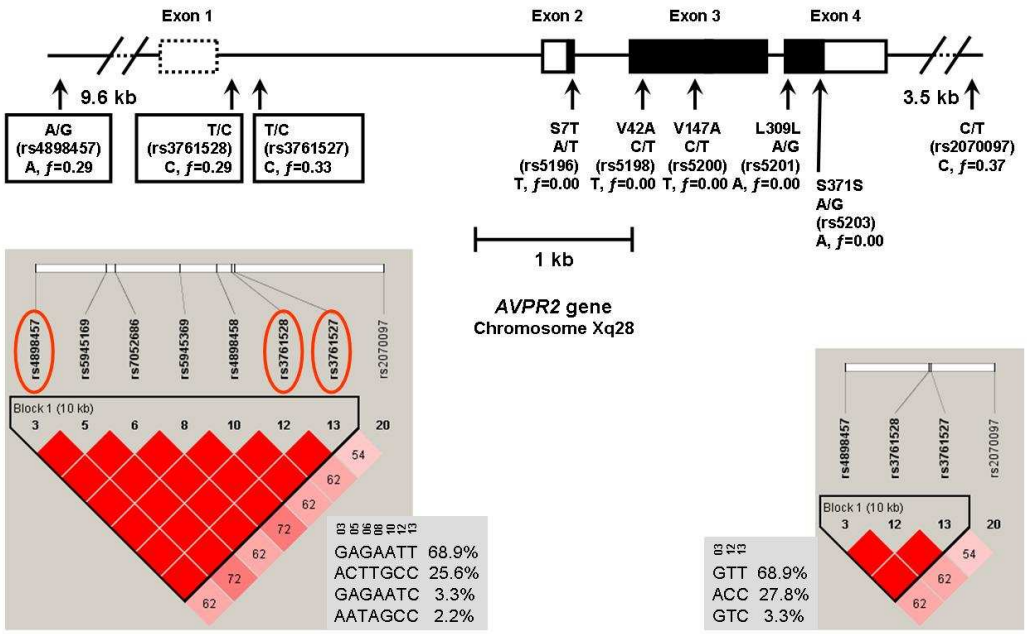

Figure 4.2: (Top panel) A schematic representation of the exon (rectangles) and intron (horizontal lines) boundaries of the *AVPR2* gene. Exon numbers are indicated. Untranslated and translated regions of the exons are indicated by clear and solid rectangles, respectively. Exon 1 is represented by a dashed rectangle because it is present in ensembl database but not the NCBI database. There are also differences in the size of exon 3 and the flanking introns between the databases. All the exonic SNPs identified from the NCBI database and only four of the flanking SNPs have been annotated. The accession numbers as well as the minor alleles and their frequencies are also annotated. The three SNPs used in this study are boxed. **(Bottom panels)** Linkage disequilibrium maps of the haplotype block of all the identified informative SNPs around the *AVPR2* gene (left), as well as only the 3 SNPs used in this study (right). The dark shading indicates pairwise linkage values of 100 (high probability that the pair of SNPs will be inherited together). The linkage values of the lighter shaded blocks are indicated. The triangle indicates an inherited haplotype block within the Caucasian population. The specific haplotype sequences and frequencies are also indicated. All the information used to construct this figure was obtained from databases hosted by the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov), ensemble project (http://www.ensembl.org), the Seattle SNP database (http://pga.mbt.washington.edu), and International HapMap Project (http://www.hapmap.org).

4.2.4 PCR conditions

DNA fragments were PCR amplified by constructing a four primer pair for each SNP based on the tetra-primer amplificatory refraction mutation system (T-ARMS) method [383]. The primer sequences and amplified product sizes for each SNP are indicated in table 4.2. These primer sequences were constructed in accordance with the specific sequence files for each of the *AVPR2* SNPs published on the NCBI database (http://www.ncbi.nlm.nih.gov).

The PCR reactions were carried out in a final volume of 40 µl containing at least 100 ng of DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.0 mM MgCl2, 0.2 mM of each nucleotide (dATP, dTTP, dCTP and dGTP), 20 pmol of each primer and 0.5 units of Taq DNA polymerase (New England Biolabs, Ipswich, Massachusetts, USA). The samples were amplified with a PCR Express Thermal Cycler (Hybaid Limited, Middlesex, UK) using the following conditions: an initial denaturing step for 15 minutes at 95^{o}C; followed by 5 cycles of denaturing for 25 seconds at 95^{o}C, annealing for 45 seconds at 70^{o}C and extension for 30 seconds at 72^{o}C; a further 27 cycles of denaturation for 25 seconds at 95^{o}C, annealing for 45 seconds at 50^{o}C and extension for 30 seconds at 72^{o}C and a final extension step at 72^{o}C for 10 minutes. Amplified fragments were resolved on 2% agarose gels and visualised under UV light following SYBER®Gold nucleic acid gel stain (Figure 4.3). Images were captured using the UVItec Gel Documentation System (UVItec Limited, UK). All the triathletes were genotyped for at least one of the three SNPs. Five hundred and thirty-nine (94.6%), 539 (94.6%) and 553 (97.0%) of the 570 triathletes included in this study were genotyped for rs3761528, rs3761527 and rs4898457, respectively.

A small subset of the samples could not be genotyped for all three SNPs for technical reasons.

Since only male triathletes were included in this study and the *AVPR2* gene is X lined, each triathlete's haplotype was constructed for the *AVPR2* gene using SNPs rs4898457, rs3761528 and rs3761527.

4.2.5 Biochemical analysis and weight measurement and calculations

As previously described section 3.2.4., pre- and post-race serum [Na^+] were determined from ~4.5 ml of venous blood, collected from each subject at race registration (n=514, 90.2%) (1 to 3 days prior to the event) and again within 10 min after completing the event (n=423, 74.2%). The samples were immediately centrifuged and stored at -20^oC until analysis as previously described [304]. The changes in serum [Na^+] were calculated as the difference between the pre- and post-race concentrations divided by the pre-race concentration and expressed as a percentage.

Also, triathletes' pre- (n=556, 97.5%) and post-race (n=552, 96.8%) weights were measured and corrected for standard clothing worn at the time [304] from which the percentage change in body weight lost or gained during the race was calculated, as previously detailed in section 3.2.4. This calculation was utilised as an indirect measure of the triathletes' hydration status. As described in the previous chapter (Chapter 3), the triathletes who gained body weight during the event were

considered to be overhydrated, those with a body weight loss between 0% and 3% were considered to be euhydrated, and those with a body weight loss >3% were considered to be dehydrated [3].

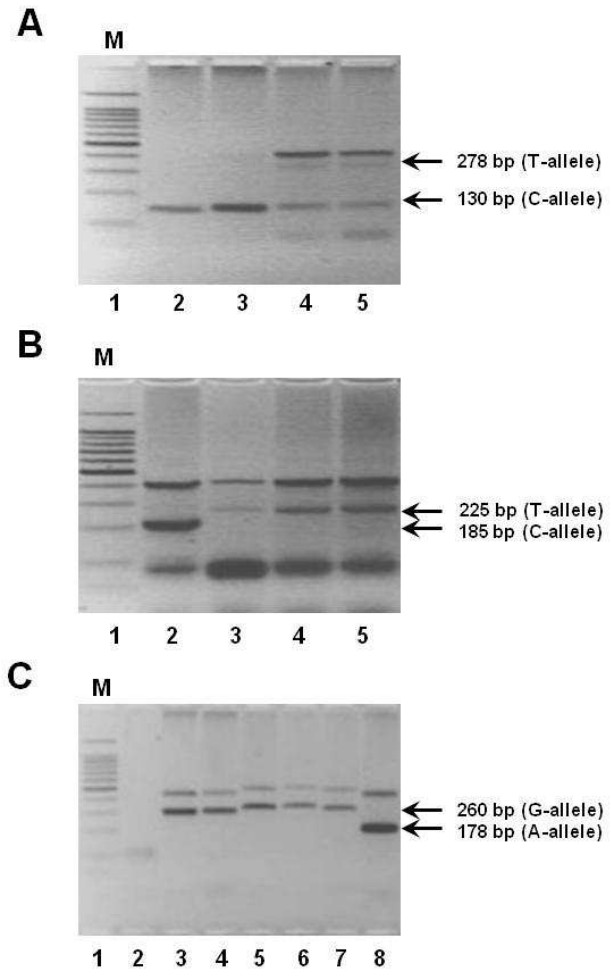

Figure 4.3: Typical 2% agarose gels showing the genotype analysis of the *AVPR2* **(A)** rs3761528, **(B)** rs3761527 and **(C)** rs4898457 SNPs with separated PCR products. Lane numbers are indicated at the bottom of each gel. M, represents the 100bp DNA molecular weight ladder.

4.2.6 Statistical analysis

Data were analysed using STATISTICA version 8.0 (Stat-soft Inc., Tulsa, OK, USA) and GraphPad InStat version 2.05a (GraphPad Software, San Diego, CA, USA) statistical programs. Where applicable, data are presented as means ± SD with the number of participants with non-missing data for each variable in parentheses. Pearson's Chi-squared analysis (X^2) was used to detect differences in the genotype and allele frequencies, as well as in the percentage of South African-born individuals, between the different weight loss groups. Hardy-Weinberg equilibrium was established using the Genepop web version 3.1c program (http://genepop.curtin.edu.au/). A one-way analysis of variance (ANOVA) was used to determine any significant differences between the physiological characteristics of the three different weight loss groups, statistical significance was accepted when $p<0.05$. Where the overall F value was significant, a Tukey's honest significant difference post hoc test was used to identify where the differences were.

4.3 RESULTS

4.3.1 Participants characteristics

The triathlete groups, representing each Ironman Triathlon event, were similarly matched for height, weight, BMI and percentage South African born (Table 4.3). As the 2006 triathlete group was significantly ($p<0.001$) older than the other groups, the overall finishing and split times for each individual event (swim, cycle and run) were corrected for age. Although the swim times of the 2006 triathlete group were significantly slower ($p<0.001$), there were no significant differences in the cycle, run and overall finishing times between the three triathlete groups.

As previously reported in the previous chapter, the triathletes' age ($r=0.190$, $n=561$, $p<0.001$), BMI ($r=0.356$, $n=510$, $p<0.001$), relative ($r=0.237$, $n=539$, $p<0.001$) body weight change and pre-race serum [Na^+] ($r=0.089$, $n=507$, $p=0.045$) were all positively correlated with their overall finishing time (Figure 4.4). Conversely, there were negative linear relationships between post-race serum [Na^+] ($r=-0.116$, $n=422$, $p=0.017$), and relative serum [Na^+] changes ($r=-0.149$, $n=410$, $p=0.002$) with overall finishing time (Figure 4.4).

Table 4.3: General physiological and performance characteristics of the triathletes that participated in the 2000, 2001 and/or 2006 South African Ironman Triathlons, as well as the combined (All) individual triathletes.

	2000 (N=171)	**2001** (N=276)	**2006** (N=211)	**P-value**	**All** (N=570)
Age (years)	34.6 ± 8.5 (118)[a]	34.3 ± 7.9 (276)[b]	38.1 ± 8.4 (207)[a,b]	< 0.001[a] < 0.001[b]	35.4 ± 8.3 (568)
Height (cm)	180.7 ± 6.8 (104)	180.8 ± 6.7 (257)	180.1 ± 6.7 (191)	0.596	180.5 ± 6.7 (520)
Body Weight (kg)	76.9 ± 9.5 (118)	78.5 ± 8.6 (274)	78.1 ± 9.4 (201)	0.270	77.9 ± 9.1 (561)
BMI ($kg.m^{-2}$)	23.6 ± 2.2 (104)	24.1 ± 2.1 (255)	24.0 ± 2.3 (190)	0.221	23.9 ± 2.2 (517)
South African Born (%)	59.5 (69)	63.2 (172)	73.2 (145)	0.122	62.6 (357)
Swim (min)	71 ± 12 (148)[a]	70 ± 14 (273)[b]	88 ± 16 (205)[a,b]	< 0.001 [a] * < 0.001 [b]	76 ± 17 (548)
Cycle (min)	397 ± 42 (134)	390 ± 43 (270)	402 ± 39 (201)	0.100 *	394 ± 41 (531)
Run (min)	291 ± 52 (168)	284 ± 46 (258)	293 ± 53 (200)	0.406 *	288 ± 49 (541)
Overall Time (min)	767 ± 95 (170)	759 ± 93 (276)	783 ± 95 (202)	0.172 *	766 ± 93 (563)

Except for South African born, which is expressed as a percentage, the rest of the values are expressed as mean ± SD. The total number of participants (n) with non-missing data is in parentheses. The maximum number (N) of participants in each category is also indicated. The group All consists all the combined triathletes, and for those who have complete sets of data for more than one event only their 2001 or 2000 data were included. *Values co-varied for age. [a] P-value for 2000 vs 2006; [b] P-value for 2001 vs 2006.

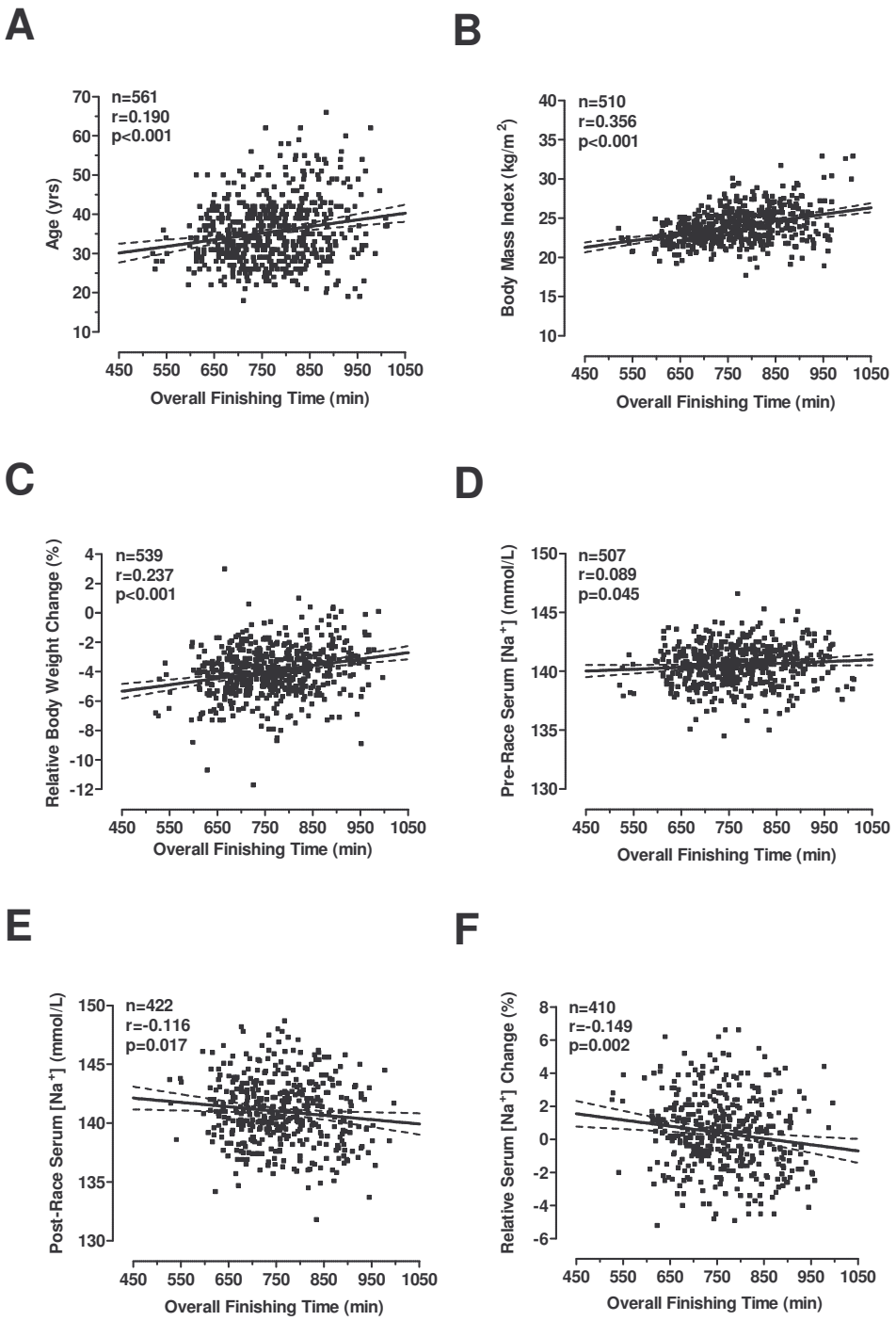

Figure 4.4: Positive linear regressions of male Caucasian triathletes' **(A)** age, **(B)** body mass index, **(C)** relative body weight change (%), **(D)** pre-race serum [Na^+] and negative linear regressions of **(E)** post-race serum [Na^+] and **(F)** relative serum [Na^+] change with overall finishing time during the 2000, 2001 and/or 2006 South African Ironman Triathlons. The solid line is the line of best fit while the dashed lines are the 95% confidence intervals.

4.3.2 Body weight and serum [Na^+] changes

Pre- and post-race body weights were recorded in 540 (94%) of the 570 triathletes and were matched between the three event groups (Table 4.3.). However, the 2000 Ironman event group experienced a greater absolute (-3.9 ± 1.6kg, $p<0.001$) and relative (-4.9 ± 2.0%, $p<0.001$) body weight loss during the race compared to the other groups, while the 2001 group lost more body weight (-3.2 ± 1.4kg, -4.0 ± 1.6%, $p=0.004$) than the 2006 (-2.7 ± 1.5kg, -3.4 ± 1.8%) group. In addition, significantly higher pre- and post-race serum [Na^+] were recorded in the 2000 Ironman group when compared to the other groups, whilst pre- and post-race serum [Na^+] were higher in the 2001 than the 2006 Ironman group (Table 4.4). All mean pre- and post-race serum [Na^+] were in the normal clinical range. There were however no differences in the absolute and relative serum [Na^+] changes between groups following the event.

Table 4.4: Changes in body weight and serum electrolyte concentrations of the triathletes that participated in the 2000, 2001 and/or 2006 South African Ironman Triathlons, as well as the combined (All) individual triathletes.

	2000 (N=171)	**2001** (N=276)	**2006** (N=211)	**P-value**	**All** (N=570)
Pre-race Body weight (kg)	78.9 ± 9.1 (169)	79.8 ± 8.7 (276)	79.8 ± 10.1 (195)	0.526	79.3 ± 9.3 (556)
Post-race Body weight (kg)	74.9 ± 8.6 (169)	76.6 ± 8.5 (276)	76.6 ± 9.3 (189)	0.085	76.1 ± 8.9 (552)
Absolute Body weight change (kg)	-3.9 ± 1.6 (167)[a,c]	-3.2 ± 1.4 (276)[b,c]	-2.7 ± 1.5 (176)[a,b]	< 0.001[a] 0.004[b] < 0.001[c]	-3.1 ± 1.4 (540)
Relative Body weight change (%)	-4.9 ± 2.0 (167)[a,c]	-4.0 ± 1.6 (276)[b,c]	-3.4 ± 1.8 (176)[a,b]	< 0.001[a] 0.004[b] < 0.001[c]	-4.0 ± 1.7 (540)
Pre-race [Na^+] (mmol/L)	141.2 ± 1.6 (123)[a,c]	140.7 ± 1.5 (268)[b,c]	140.0 ± 1.7 (208)[a,b]	< 0.001[a] < 0.001[b] 0.015[c]	140.5 ± 1.7 (514)
Post-race [Na^+] (mmol/L)	141.7 ± 2.9 (112)[a]	141.2 ± 2.6 (226)[b]	140.4 ± 3.1 (157)[a,b]	< 0.001[a] 0.011[b]	141.0 ± 2.8 (423)
Absolute [Na^+] change (mmol/L)	0.4 ± 3.3 (104)	0.6 ± 3.0 (222)	0.6 ± 3.3 (157)	0.850	0.5 ± 3.1 (411)
Relative [Na^+] change (%)	0.3 ± 2.3 (104)	0.4 ± 2.1 (222)	0.5 ± 2.3 (157)	0.851	0.4 ± 2.2 (411)

Values are expressed as mean ± SD. The total number of participants (n) with non-missing data is in parentheses. The maximum number (N) of participants in each category is also indicated. The group All consists all the combined triathletes, and for those who participated in more than one event only their 2001 or 2000 data were included. [a] P-value for 2000 vs 2006; [b] P-value for 2001 vs 2006; [c] P-value for 2000 vs 2001.

Five hundred and ten (99%) triathletes started the race with a serum [Na^+] within the normal physiological range (135 to 145 mmol/L), whilst three triathletes had higher (>145 mmol/L) pre-race serum [Na^+]. Post-race serum [Na^+] was recorded in 423 (74%) triathletes who completed the event, of which, 382 (90.3%) were within the normal distribution (135 to 145 mmol/L). Six (1.4%) and 35 (8.3%) triathletes completed the event with either hypo- or hypernatremia, respectively (Table 4.5). Overall there was a wide range of changes in relative serum [Na^+] (-5 to 6%) and body weight (-11 to 3%) within triathletes during the race (Figure 4.5). Eight (1.5%) triathletes gained body weight during the race and were considered to be over-hydrated, whereas 145 (26.8%) triathletes completed the race euhydrated (relative body weight loss 0-3%) and 387 (71.7%) triathletes lost >3% of their body weight and were considered to be “dehydrated” (Table 4.5). There was a negative linear relationship between post-race serum [Na^+] ($r=-0.353$, $n=408$, $p<0.0001$, Figure 4.6) and relative body weight change during the event.

Table 4.5: Summary of all the triathletes finishing either the 2000 (n=111), 2001 (n=276) or 2006 (n=183) South African Ironman Triathlons with various combinations of body weight changes and post-race serum sodium concentrations following the event.

	Body weight gain	Body weight loss (0-3%)	Body weight loss (>3%)	Subtotal	No weight change data	Total
Hypernatraemia (>145 mmol/L)	0	3	30	33	2	35
Normonatraemia (135–145 mmol/L)	8	104	257	369	13	382
Hyponatremia (<135 mmol/L)	0	1	5	6	0	6
Subtotal	8	108	292	408	15	423
Post-race serum sodium [Na^+] not determined	0	37	95	132	15	147
Total	8	145	387	540	30	570

Triathletes who gained weight during the event were considered to be overhydrated, those with a body weight loss between 0% and 3% were considered to be euhydrated, and those with a body weight loss >3% were considered to be dehydrated [3].

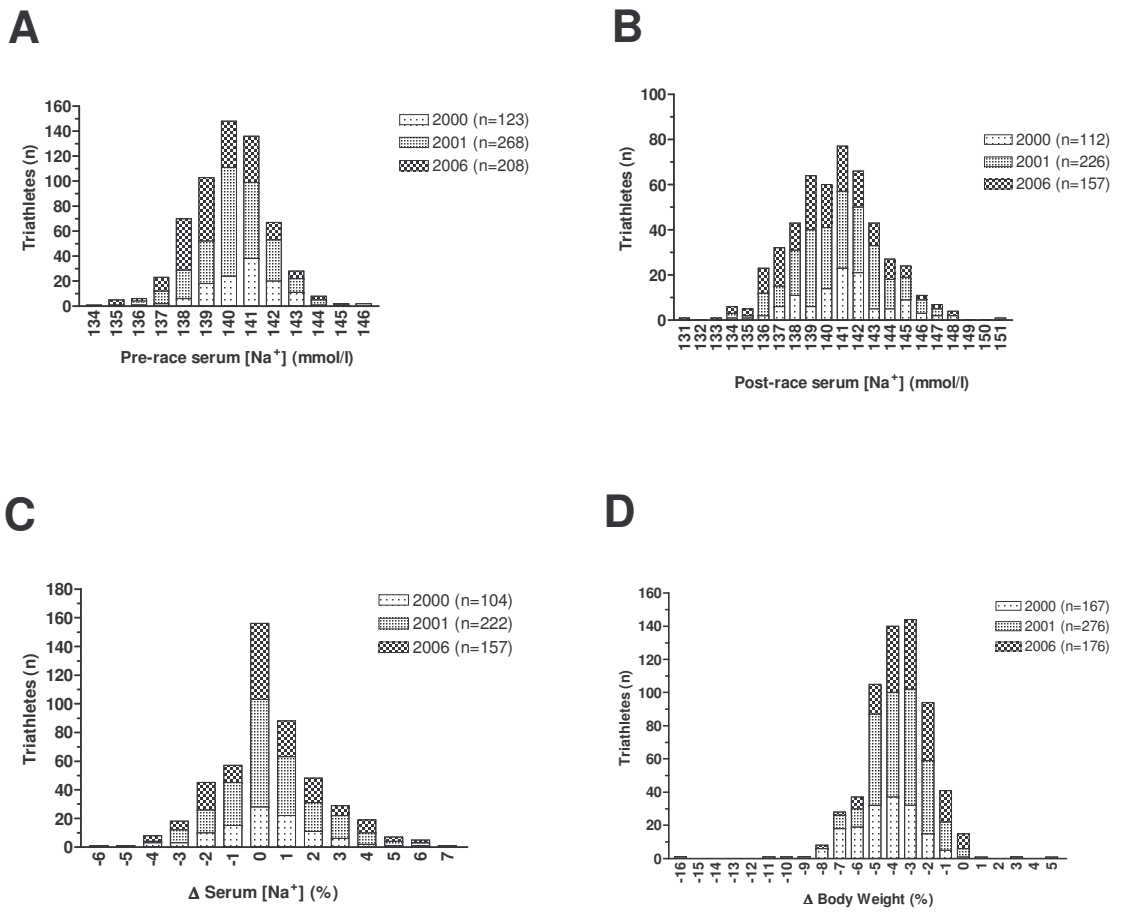

Figure 4.5: (A) Pre- and **(B)** post-race serum [Na^+] and **(C)** relative serum [Na^+] and **(D)** body weight changes within triathletes during the 2000, 2001 and/or 2006 Ironman Triathlon events.

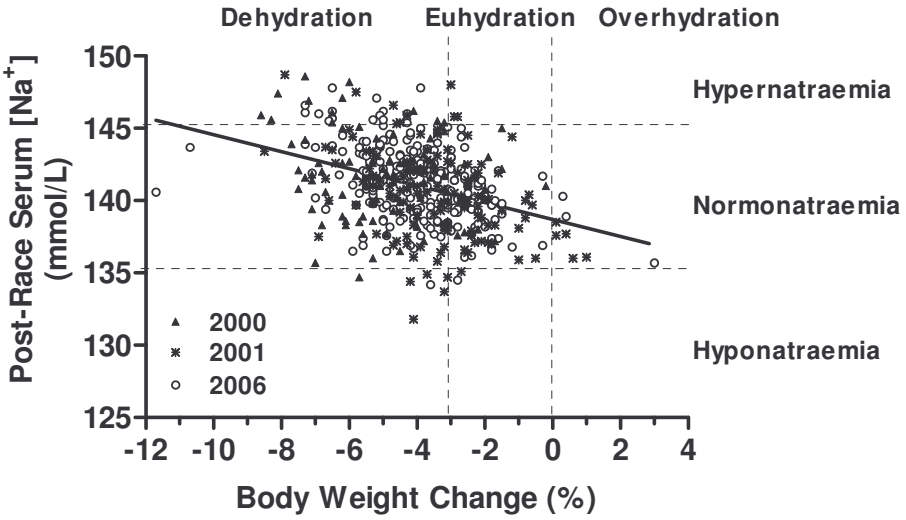

Figure 4.6: Relationship between post-race serum [Na^+] and relative body weight changes during the 2000, 2001 and/or 2006 Ironman Triathlon events. Dehydration, euhydration and overhydration regions, as well as hypernatraemia, normonatraemia and hyponatraemia regions are indicated. The solid line is the line of best fit.

4.3.3 Genotype effects on physiological and performance variables

Triathletes were genotyped for the rs3761528 (n=539), rs3761527 (n=539) and rs4898457 (n=553) SNPs within the *AVPR2* gene on chromosome X (Table 4.6). The participants included in this study were all male and therefore only carry one copy of this gene. There were no significant differences in the SNP's allele distributions between the three Ironman event groups.

Table 4.6: The *AVPR2* allele distributions frequencies for each single nucleotide polymorphism (SNP) within the 2000, 2001 and/or 2006 Ironman events and combined (All) groups.

AVPR2	**2000** (N=171)	**2001** (N=276)	**2006** (N=211)	**P-value**	**All** (N=570)
rs3761528	n=162	n=248	n=210		n=539
T	62.3 (101)	71.0 (176)	66.7 (140)	0.187	66.8 (360)
C	37.7 (61)	29.0 (72)	33.3 (70)		33.2 (179)
rs3761527	n=154	n=259	n=211		n=539
T	66.2 (120)	70.3 (182)	65.4 (138)	0.487	67.3 (363)
C	33.8 (52)	29.7 (77)	34.6 (73)		32.7 (176)
rs4898457	n=169	n=262	n=210		n=553
G	70.4 (119)	71.4 (187)	68.1 (143)	0.736	69.8 (386)
A	29.6 (50)	28.6 (75)	31.9 (67)		30.2 (167)

Values are expressed as a percentage, with the total number of participants (N) with a specific genotype in parentheses. The total number of participants (n) genotyped for each SNP is also indicated. The group All resembles all the combined triathletes and for those who have complete sets of data for more than one event only their 2001 or 2000 data were included.

Overall there were no significant allele differences between the rs3761527 and rs4898457 SNPs and age, height, weight and BMI (Table 4.6). The triathletes carrying a rs3761528 T allele were, however, significantly (p=0.03) older than those with a C allele, subsequently, performance variables were corrected for age. The overall allele distributions for each SNP within South African born triathletes were matched. Also, there were no significant allele interactions with the performance variables swim, cycle, run and overall finishing time. Lastly, the allele distributions for each SNP were matched when compared with relative BW loss (%) and post-race serum [Na^+].

4.3.4 *AVPR2* haplotypes

Two major: GTT (n=324, 64.8%), ACC (n=152, 30.4%) and four minor haplotypes: GCT (n=10, 2%), GTC (n=9, 1.8%), GCC (n=4, 0.8%) and ATT (n=1, 0.2%) were identified.

There were no significant difference in haplotype distributions (when the minor haplotypes were combined with either the GTT or ACC haplotypes) between the three body weight loss groups (p>0.1) or the normonatraemic and hypernatraemic groups (p>0.2). However, it is interesting to note that the >5% body weight loss group had the lowest percentage of triathletes with the GTT haplotype (61.2%, n=52), followed by the >3-5% group (63.5%, n=108), whereas the 0-3% group (74.4%, n=70) had the highest percentage of triathletes with the GTT haplotype (X^2 for linear trend = 3.6, p=0.057) (Figure 4.7). In addition, the distribution of all the

minor haplotypes (GCT, GTC and GCC) were clustered towards the >5% and >3-5% body weight loss groups (p=0.041, odds ratio 9.7, 95% confidence interval 0.7 to 165.6) (Figure 4.7). There was also a significant linear trend (p=0.010, X^2=6.7) for the distribution of minor haplotypes across the >5% (4.7%, n=6 minor haplotypes and 79 major haplotypes), >3-5% (3.6%, n=6 minor haplotypes and 164 major haplotypes) and 0-3% body weight loss groups (0%, n=94 major haplotypes).

Table 4.7: The physiological characteristics of the combined (2000, 2001 and 2006 events) triathletes for each single nucleotide polymorphism (SNP) allele within the *AVPR2* gene.

	rs3761528			rs3761527			rs4898457		
	T (N=360)	**C** (N=179)	**P-value**	**T** (N=363)	**C** (N=176)	**P-value**	**G** (N=386)	**A** (N=167)	**P-value**
Age (years)	36.0 ± 8.5 (359)	34.3 ± 7.5 (178)	0.03	35.6 ± 8.3 (362)	34.9 ± 8.1 (175)	0.33	35.8 ± 8.5 (385)	34.6 ± 7.9 (166)	0.15
Height (cm)	180.6 ± 6.5 (320)	180.7 ± 7.0 (169)	0.82	180.5 ± 6.5 (326)	180.6 ± 7.0 (166)	0.91	180.6 ± 6.5 (345)	180.4 ± 7.1 (159)	0.76
Body Weight (kg)	77.6 ± 8.9 (354)	78.6 ± 9.5 (176)	0.22	78.0 ± 8.9 (358)	78.3 ± 9.6 (173)	0.73	77.9 ± 8.9 (380)	78.2 ± 9.6 (164)	0.76
BMI ($kg.m^{-2}$)	23.8 ± 2.2 (318)	24.1 ± 2.3 (168)	0.22	23.9 ±2.2 (325)	24.1 ± 2.3 (165)	0.58	23.9 ± 2.2 (343)	24.0 ± 2.3 (158)	0.60
South African Born (%)	66.8 (349)	62.9 (170)	0.45	66.3 (353)	62.4 (165)	0.45	67.6 (373)	62.5 (160)	0.30
Swim (min)	76 ± 16 (347)	77 ± 18 (170)	0.11 *	76 ± 16 (351)	77 ± 18 (168)	0.49	76 ± 16 (372)	78 ± 18 (159)	0.22
Cycle (min)	395 ± 41 (336)	393 ± 42 (165)	0.88 *	396 ± 40 (338)	392 ± 41 (164)	0.30	395 ± 41 (359)	395 ± 42 (156)	0.91
Run (min)	288 ± 49 (340)	289 ± 50 (171)	0.61 *	290 ± 48 (342)	286 ± 49 (168)	0.36	287 ± 48 (365)	289 ± 51 (159)	0.65
Overall Time (min)	767 ± 92 (357)	765 ± 95 (175)	0.86 *	770 ± 92 (360)	760 ± 93 (172)	0.24	766 ± 92 (383)	768 ± 96 (163)	0.85
Relative BW change (%)	-3.9 ± 1.7 (343)	-4.0 ± 1.6 (167)	0.46	-3.9 ± 1.7 (347)	-4.2 ± 1.6 (162)	0.08	-4.0 ± 1.7 (369)	-3.9 ± 1.7 (155)	0.89
Post-race [Na^+] (mmol/L)	141.1 ± 2.8 (271)	140.7 ± 2.7 (131)	0.20	141.2 ± 2.9 (268)	140.6 ± 2.7 (131)	0.08	141.1 ± 2.9 (284)	140.6 ± 2.7 (123)	0.10

Values are expressed as mean ± SD, except for the South African-born triathletes which is expressed as a percentage. The total number of participants (n) with non-missing data is in parentheses. The total number of participants (N) genotyped for each SNP is also indicated.

* Co-varied for age

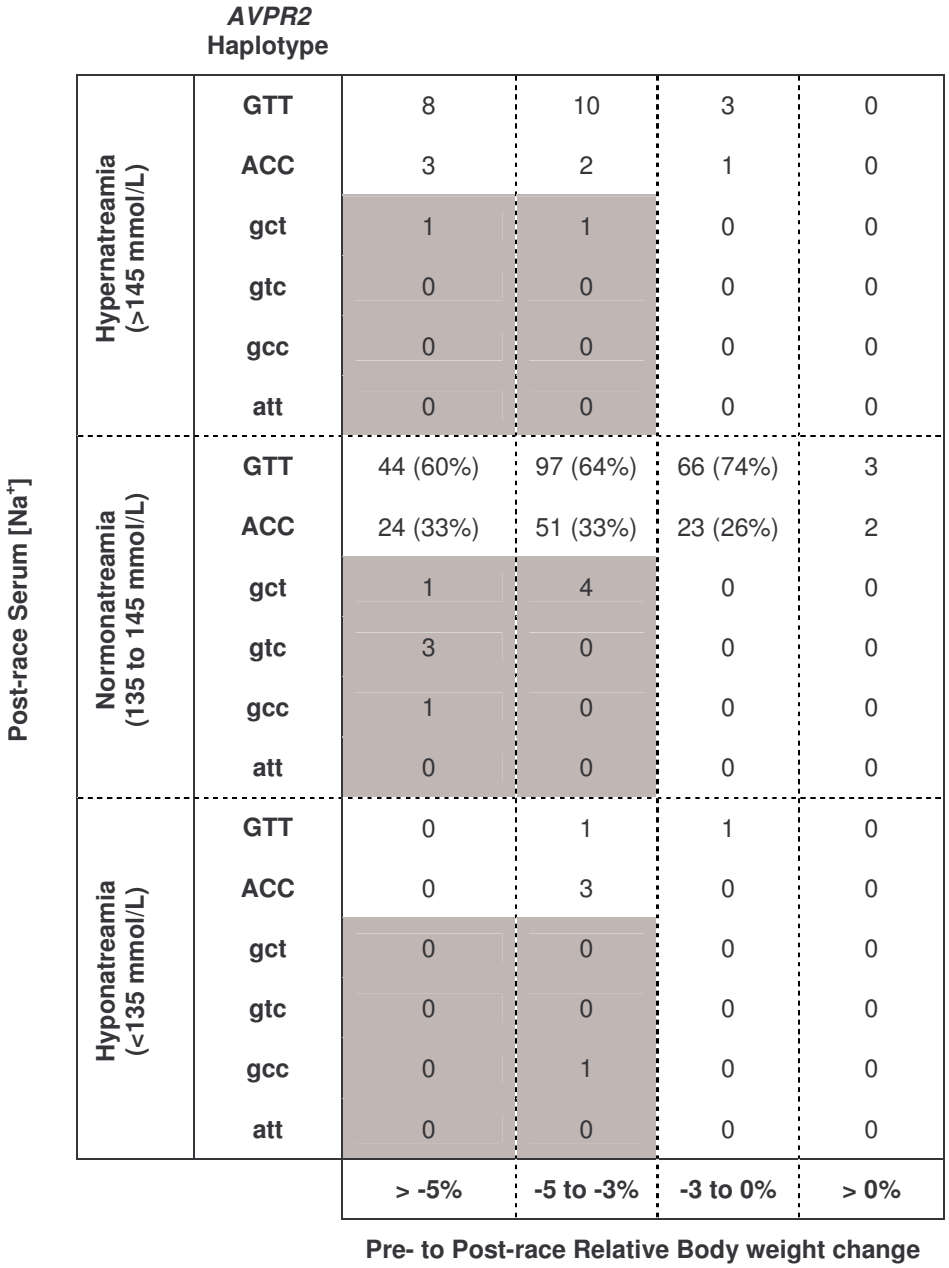

Figure 4.7: The categorical distribution of the six *AVPR2* haplotypes according to the triathletes' relative body weight changes and post-race serum [Na^+] during either the 2000, 2001 or 2006 Ironman Triathlon events. The dashed vertical lines differentiate the dehydrated (>-5% and -5 to -3% body weight change), euhydrated (-3 to 0% body weight change) and over-hydrated (>0% body weight change) categories. The dashed horizontal lines differentiate the hypo-, normo- and hypernatraemia groups. The two major haplotypes are in capital letters, while the four minor haplotypes are in lower case letters. The total number of participants (N) with a specific haplotype in each category is indicated. When the sample size of each category was large enough the relative contribution of each haplotype in that category is documented in parenthesis. The shaded area represents the clustering of the minor haplotypes within the >5% and 3-5% body weight loss groups.

4.4 DISCUSSION

The first novel finding of this study was the significant association (p=0.041, odds ratio 9.7, 95% confidence interval 0.7 to 165.6) between the combined minor haplotypes constructed from SNPs rs3761528, rs3761527 and rs4898457 (GCT, GTC and GCC) within the *AVPR2* gene and body weight loss during the 2000, 2001 and 2006 South African Ironman triathlons. There were, however, no significant associations between the three individual *AVPR2* SNPs and serum sodium and body weight changes during the Ironman events. The second finding of this study, as expected, was the previously reported (refer to chapter 3) association between body weight losses and higher serum [Na^+] following the race. The third finding was that the 2006 Ironman event swim leg was significantly slower than the 2000 and 2001 events.

Sweating is the primary source of water and electrolyte losses during exercise and can, following a prolonged period, induce hypertonicity (increased concentration of electrolytes in body fluid) [269, 279]. Consequently, compensatory endocrine reflex responses are activated to minimise water loss through the antidiuretic effect of arginine vasopressin (AVP). As previously detailed in section 1.6.3.1, this peptide hormone is released from the posterior pituitary and acts on V2 receptors within the kidney to increase water re-absorption resulting in decreased urinary flow and an increased urine osmolality [247, 384]. Case reports have documented an association with *AVPR2* gene mutations and chronic hyponatraemia in infants [385]. This mutation results in a constantly active AVPR2, thereby reducing the excretion of

free water. To date, more than 211 mutations that result in a constantly active AVPR2 have been identified [338].

In this study, the minor haplotype constructs GCT, GTC and GCC were associated with a >3% relative body weight loss during the race, while there were no associations with the three individual *AVPR2* SNPs The contrast in identifying common genetic variants oppose to rare variants as disease causal factors was discussed by Bodmer and Bonilla [386]. Rare variants are relatively population specific, have low frequencies ranging more or less from 0.1-1% and most have odds ratios (ORs) considerably greater than 2. These low frequency 'minor' alleles individually have small contributions to the overall inherited susceptibility of a disease. However, the summation of the effects of a series of dominantly and independently acting 'minor' alleles can have a high penetrance on relative risk for chronic diseases [386]. Colorectal cancer (CRC) is a classic example where 5% of cases are associated with inherited, dominant, familial mendelian susceptibility of highly penetrant mutations in the *APC* gene and 20-30% of cases are associated with lower penetrance variants with no familial pattern of inheritance [387, 388]. The function, if any, of the three common SNPs investigated within this study is however currently unknown. It is more likely that these SNPs are tightly linked to unidentified functional SNPs within regulatory or protein coding regions of the *AVPR2* gene. In addition, we cannot exclude the possibility that these SNPs are in linkage equilibrium with another gene that could be in close proximity to the *AVPR2* gene on the X chromosome. The inherited susceptibility is therefore referred to as 'multfactorial'. The importance of the association of minor SNPs with common phenotypes has only recently been appreciated [386, 389], therefore the association of minor haplotypes

with a common phenotype is not totally unexpected. Future work is required to identify these causal functional variants within the *AVPR2* or neighbouring gene(s).

Although, to our knowledge, AVPR2 or its gene expression has not been directly described in the sweat glands, Hew-Butler and colleagues [335] have reported a positive correlation between post-exercise plasma AVP concentrations and sweat sodium concentrations during 60 minute of steady state exercise at 60% of peak treadmill running speed. They proposed that AVP may affect sweat rate and sweat gland water reabsorption via AVPR2 [335]. Future work is however required to test this hypothesis.

As previously reported in chapter 3 and along with others [3, 305, 390], increased body weight losses were associated with higher serum [Na^+] following the race. This corroborates the opposite premise that body weight gain, resembling water overload, is associated with lower serum [Na^+] and the development of EAH and EAHE [3]. However, within this study 6 triathletes developed EAH without weight gain and were considered either euhydrated or dehydrated at the race finish. Importantly all these hyponatraemic triathletes were asymptomatic. In light of this, it is plausible to consider other factors that exclude the neural and endocrine control of free water ingestion and insensible losses, which can hinder sodium regulation. Such factors could include either failure to mobilise [Na^+] from osmotically inactive, exchangeable [Na^+] stores or, conversely, osmotic inactivation of [Na^+] during exercise [3].

More than 90% of the entire field of triathletes finished the race with normal serum sodium concentrations albeit having significant relative body weight losses of up to

11 %. Our results, therefore, substantiate the principle that the serum [Na^+] is generally well regulated during endurance exercise despite a wide range of body weight changes. Furthermore, although sweat is hypotonic, heat acclimatisation and a low sodium diet further reduce the sweat [Na^+] in trained athletes [391] thereby allowing for substantial weight losses whilst minimising sodium losses. Our results, therefore, emphasise the inter-individual variation and altered adaptation in sodium regulation of these endurance trained triathletes, which seem independent of AVPR2 regulation.

In addition, a possible limitation to this study was that individual variability in the endocrine regulation of AVP exist, which are very difficult to controlled for. These factors that can influence the release of AVP from the posterior pituitary could include a variation in osmotic set points between individuals as well as non-osmotic stimuli [247]. Non-osmotic stimuli include i) vomiting or the sensation of nausea [248], ii) plasma volume contraction [247], iii) increased core temperature [293], iv) hypoglycaemia [234] and v) circulating endocrine factors [252, 254, 256] that can override the osmotic regulation of AVP secretion. These biological factors could possibly explain why we failed to show an association with genetic variants of the *AVPR2* gene.

Although genetic association studies do not provide direct proof of the mechanisms involved in biological processes, the identification of genetic markers does provide insight into the possible mechanisms involved and which need to be tested. We acknowledge that a number of confounding factors such as muscle fuel utilisation, urination, defecation and respiration also contributed to overall body weight loss.

Inter-individual differences in fuel utilization and heat dissipation could not be excluded. These were not measured and this is a limitation of this study. However, these factors would be equally applicable to all the body weight loss subgroups.

An additional finding of this study was that the swim time for the 2006 Ironman event was significantly slower than the 2000 and 2001 events. The 2000 and 2001 events were held in Gordons Bay, 55 km outside Cape Town [305], and the 2006 event was held in Port Elizabeth. The change in geographical location, as well as the rough sea conditions observed on the 2006 event day, can account for this observation. Further, it should be noted that the distance markers for the swim can not be precisely measured for each Ironman event, as would be the case for markers on land.

In conclusion, we have demonstrated that there is a large inter-individual variation in serum [Na^+] and body weight changes within triathletes that completed the 2000, 2001 or 2006 South African Ironman Triathlons. The novel finding was an association with minor or 'rare' haplotype variants of the *AVPR2* gene and body weight loss during these endurance events. In addition, we have previously in this thesis (Study 2) shown a genetic dipsogenic effect of the *5-HTTLPR* in the *SLC6A4* gene in the 2000 and 2001 South African Ironman triathletes included in this study. These results, therefore, substantiate the multifactorial polygenic nature of body weight loss during an endurance event.

These preliminary findings highlight the need for further investigation to elucidate the biological and perhaps functional role of these genetic variants within the *AVPR2* gene.

CHAPTER 5

POLYGENIC PROFILE ASSOCIATED BODY WEIGHT CHANGES DURING THE SOUTH AFRICAN IRONMAN TRIATHLONS

5.1 INTRODUCTION

In the previous two chapters of this thesis we investigated genetic variants shown to be associated with the observed inter-individual variation in athlete's weight changes, a proxy of total body water (TWB) regulation, during participation in an Ironman Triathlon. We reported novel associations with variants within the *5-HTT* (Chapter 3) and *AVPR2* (Chapter 4) candidate genes with body weight changes during the South African Ironman Triathlons. As reviewed in chapter 1 of this thesis, both genes encode for proteins that play a key role in signaling pathways that regulate thirst and water balance. In addition, findings from our laboratory have also shown an association between the +9/+9 genotype of the bradykinin β2 receptor (*BDKRB2*) gene and body weight changes in the same cohort of Ironman triathletes [1]. As previously described in (Chapter 1, Section 1.6.3.1.3.), the bradykinin β2 receptor is a component of the kalikrein kinin system (KKS) and through the binding of bradykinin exerts a potent dipsogenic effect (i.e stimulation of thirst). Taken together these findings suggested that several genetic variants are associated with

TBW regulation during participation in an ultra-endurance event. We have therefore selected these three genetic variants to identify a polygenic 'body weight loss' profile as a result of participation in the Ironman triathlon using the method described by Williams and Folland [68].

Although not excluding other possible unidentified polymorphisms that might play a more important role, the aim of this last study of this thesis was to investigate the genetic association of a polygenic profile with body weight changes of triathletes. We propose that triathletes with a higher total genotype score (TGS) are more likely to experience greater relative body weight losses during the 2000 or 2001 South African Ironman Triathlons.

5.2 METHODS

5.2.1 Participants

Only the 296 triathletes who had been genotyped for all three variants were analysed in this study. These self-reported Caucasian male triathletes represented 63% of the total number of triathletes included in chapter 2. As previously described in section 2.2.1, these triathletes completed either the 2000 and/or 2001 South African Ironman Triathlons. The 2001 data was used for triathletes (n=78) with complete sets of data for both events.

As detailed in chapter 3 (Section 3.2.4.), triathletes were classified into three (0 to 3%, >3% to 5% and >5%) weight loss groups according to their respective relative body weight losses during the events. The calculated relative change in body weight loss was used as an indirect measure for triathletes' hydration status. Body weight loss groups 0 to 3% were considered to be euhydrated, whilst the >3% group were considered dehydrated [3].

5.2.2 Genotyping

DNA samples were previously genotyped for the *5-HTTLPR* (L/S) polymorphism in the *SLC6A4* gene (as described in Chapter 2, Section 2.2.3.2.)[206] and SNPs rs3761528, rs3761527 and rs4898457 in the *AVPR2* gene as detailed in chapter 4

(Section 4.2.4.). In addition, the same cohort of DNA samples was previously genotyped for the +9/-9bp sequence in exon 1 of the *BKBR2* gene [1].

5.2.3 Determination of the 'total genotype score' (TGS)

Each triathlete was scored according to the number of 'maximum body weight loss' genotypes (*5-HTT* SS, *BDKRB2* +9/+9 and *AVPR2* minor haplotypes) that he possessed (Table 5.1). Based on the data presented in chapters 3 (Table 3.3) and Saunders et al. [1] a recessive model was chosen as the appropriate scoring system for the *5-HTT* and *BDKRB2* genes, where the 'minimum body weight loss' homozygous genotypes as well as the heterozygous genotypes were scored zero. Each 'maximum body weight loss' (homozygeous) genotype received a score of two. Since the *AVPR2* gene is X-linked (refer to section 4.2.3.), each triathlete only carries a single copy. Taken together, the triathlete could therefore receive a minimum accumulated score of 0 (carrying none of the 'maximum body weight loss genotypes') or a maximum accumulated score of 6 (carrying all three of the 'maximum body weight loss genotypes' i.e. the *5-HTT* SS genotype, *BDKRB2* +9/+9 genotype and *AVPR2* minor haplotypes). With this scoring method the assumption is made that each SNP is acting with similar impact.

For each triathlete, a total genotype score (TGS) was calculated from the accumulative score at all three loci, as described by Williams and Folland [68]. TGSs were calculated by dividing the sum of the individual genotype scores by the maximum score obtainable (in this case 6) and multiplying by 100. A TGS of 100

represents the complete 'maximum weight loss' polygenic profile for triathletes during participation in the Ironman Triathlon and a TGS of 0 represents the perfect 'minimum weight loss' polygenic profile.

Table 5.1: A summary of the 'maximum and minimum body weight loss' genotypes for the *5-HTT, BDKRB2* and *AVPR2* genes.

	Body Weight Loss Genotypes	
	Maximum	**Minimum**
5-HTT	SS	LS LL
BDKRB2	+9/+9	+9/-9 -9/-9
AVPR2	Minor haplotypes [a]	Major haplotypes [b]

[a] GCT, GTC, GCC and ATT
[b] GTT and ACC

5.2.4 Statistical analysis

Data were analysed using STATISTICA version 8.0 (Stat-soft Inc., Tulsa, OK, USA) and GraphPad InStat version 2.05a (GraphPad Software, San Diego, CA, USA) statistical programmes. The data is presented as means ± standard deviations or a frequency with the number of participants with non-missing data for each variable in parentheses. TGSs were graphically represented as means ± 95% confidence intervals (CI). Chi-squared tests were used to detect differences in the distribution of categorical data. A one-way analysis of variance (ANOVA) was used to determine any significant differences between continuous data, which included the TGSs. Where the overall F value was significant, a Tukey's honest significant difference post hoc test was used to identify where the differences were. Statistical significance was accepted when $p<0.05$.

5.3 RESULTS

5.3.1 Participants characteristics

There were no significant differences in general and performance characteristics between the 296 triathletes included in this study that were recruited during the 2000 (n=83) and 2001 (n=213) Ironman Triathlons (Table 5.2).

Table 5.2: General physiological and performance characteristics of the triathletes that participated in the 2000 and 2001 South African Ironman Triathlons that was included in this study, as well as the entire group.

	2000 (N=83)	**2001** (N=213)	**P-value** [a]	**All** (N=296)
Age (years)	34.6 ± 8.4 (83)	33.9 ± 7.4 (213)	0.510	34.1 ± 7.6 (296)
Height (cm)	1801 ± 7 (74)	181 ± 7 (196)	0.933	181 ± 7 (270)
Body Weight (kg)	77.2 ± 9.9 (83)	79.1 ± 8.7 (212)	0.105	78.6 ± 9.1 (295)
BMI ($kg.m^{-2}$)	23.7 ± 2.4 (74)	24.2 ± 2.1 (195)	0.079	24.1 ± 2.2 (269)
South African Born (%)	57.5 (46)	67.1 (141)	0.163	64.5 (187)
Swim (min)	70 ± 11 (74)	69 ± 13 (210)	0.589	70 ± 13 (284)
Cycle (min)	395 ± 40 (66)	389 ± 41 (209)	0.260	390 ± 41 (275)
Run (min)	291 ± 52 (81)	284 ± 46 (198)	0.284	286 ± 48 (279)
Overall Time (min)	762 ± 92 (83)	756 ± 92 (213)	0.172	758 ± 92 (296)

Except for South African born, which is expressed as a percentage, the rest of the values are expressed as mean ± SD. The total number of participants (n) with non-missing data is in parentheses. The All group represents all the combined triathletes, and for those who have complete sets of data for both events only their 2001 data were included.
[a] 2000 vs. 2001.

5.3.2 Body weight changes

As described in chapter 3 (Section 3.2.4.), triathletes were divided into three groups (0-3, >3-5 and >5%) according to their relative body weight loss during the Ironman events (chapter 3, Table 3.1.). The general physiological characteristics of the 296 triathletes within the three different body weight loss groups are summarized in table 5.3 and were similar to the previously described characteristics in chapter 3 (Section 3.3.1., Table 3.2.).

5.3.3 Genotype effects on weight loss

Significant linear trends for the *5-HTT* SS genotype and *AVPR2* minor haplotypes amongst the three body weight loss (0-3, >3-5 and >5%) groups have previously been documented in this thesis (Table 3.3. and Table 4.6. respectively). In addition, Saunders et al. [1] reported a similar significant linear trend for the distribution of the *BDKRB2* +9/+9 genotype amongst the three body weight loss (0-3, >3-5 and >5%) groups.

Table 5.3: The general physiological and performance characteristics of triathletes that completed either the 2000 or 2001 South African Ironman Triathlon events, categorized according to their change in body weight within the three different body weight loss (0-3, >3-5 and >5%) groups.

	Body weight loss (0-3%) (N=63)	Body weight loss (>3-5%) (N=145)	Body weight loss (>5%) (N=88)	P-value
Age (years)	33.4 ± 8.2 (63)	34.0 ± 7.7 (145)	34.7 ± 7.3 (88)	0.577
Height (cm)	181.4 ± 6.5 (56)	180.6 ± 7.2 (133)	181.4 ± 5.7 (81)	0.598
BMI ($kg.m^{-2}$)	23.8 ± 2.0 (56)	24.3 ± 2.4 (132)	24.0 ± 2.1 (81)	0.303
South African Born (%)	74.6 (47)[a,b]	67.1 (96)[a,c]	52.4 (44)[b,c]	0.364[a] 0.010[b] 0.039[c]
Pre-race Body weight (kg)	78.9 ± 9.8 (63)	80.2 ± 9.5 (145)	80.2 ± 8.1 (88)	0.582
Post-race Body weight (kg)	77.2 ± 9.6 (63)	77.0 ± 9.17 (145)	75.5 ± 8.1 (88)	0.366
Absolute Body weight change (kg)	-1.7 ± 0.6 (63)[a,b]	-3.2 ± 0.6 (145)[a,c]	-4.8 ± 0.7 (88)[b,c]	<0.001[a] <0.001[b] <0.001[c]
Relative Body weight change (%)	-2.2 ± 0.7 (63)[a,b]	-4.0 ± 0.6 (145)[a,c]	-6.0 ± 0.9 (88)[b,c]	<0.001[a] <0.001[b] <0.001[c]
Swim (min)	72 ± 12 (62)[b]	71 ± 14 (137)[c]	66 ± 85 (11)[b,c]	0.011[b] 0.029[c]
Cycle (min)	402 ± 40 (59)[b]	392 ± 39.5 (133)[c]	379 ± 42 (83)[b,c]	0.002[b] 0.045[c]
Run (min)	304 ± 55 (62)[a,b]	283 ± 42 (132)[a]	278 ± 47 (85)[b]	0.009[a] 0.002[b]
Overall Time (min)	791 ± 96 (63)[a,b]	758 ± 86 (145)[a]	733 ± 93 (88)[b]	0.042[a] <0.001[b]

With the exception of South African (SA) born, which is expressed as a frequency, values are expressed as mean ± SD. The number of participants with non-missing data for each variable is in parentheses. The total number of participants in each group (n) is also in parentheses.

5.3.4 Polygenic effects on weight loss

Sixty-four % (n=190) of the triathletes did not have any of the 'maximum body weight loss' genotypes, (TGS=0) while 27% (n=80) and 9% (n=26) of the triathletes had at least one or two 'maximum' genotypes respectively (Table 5.4.). Interestingly, no triathlete possessed all three 'maximum body weight loss' genotypes (i.e. TGS=100).

There was a significant difference in the genotype distribution frequencies between weight loss groups when the triathletes with no 'maximum weight loss' genotype were compared with those with one or two 'maximum' genotypes (X^2= 8.5, p=0.015)(Table 5.4 and Figure 5.1). In addition, there was a significant linear trend for the distribution of the 'maximum' genotypes (one and two combined) between the three body weight loss groups, with the highest percentage of one and two 'maximum' genotypes in the >5% group (45.3%) followed by the >3-5% group (36.1%) and the 0-3% group (22.2%) having the least (X^2= 8.3, p=0.004)(Table 5.4 and Figure 5.1.). Similarly there was also a significant linear trend (p=0.009) in the genotype distribution frequencies when those with at least two 'maximum' genotypes were compared to the rest of the triathletes. Fifteen % of the triathletes in the group that lost the most weight (>5%) had at least two 'maximum body weight loss' genotypes, while only 3.1% of triathletes in the 0 to 3% weight loss group had two 'maximum' genotypes (Figure 5.1).

Table 5.4: The relative and absolute number of 'maximum body weight loss' genotypes within the three different weight loss (0-3, >3-5 and >5%) groups of triathletes participating in the 2000 or 2001 South African Ironman Triathlons, as well as the relative and absolute number for all the triathletes (Total).

Number of 'Maximum' Genotypes	Body Weight Loss Groups			Total
	0 to 3% (n=63)	**>3 to 5%** (n=147)	**>5%** (n=86)	**Total** (n=296)
0	**77.8** (49)	**63.9** (94)	**54.7** (47)	**64.2** (190)
1	**19.1** (12) *5-HTT* (4) *BKBR2* (7) *AVPR2* (1)	**28.6** (42) *5-HTT* (15) *BKBR2* (24) *AVPR2* (3)	**30.2** (26) *5-HTT* (7) *BKBR2* (17) *AVPR2* (2)	**27.0** (80)
2	**3.1** (2) *5-HTT + BKBR2* (2)	**7.5** (11) *5-HTT + BKBR2* (8) *5-HTT + AVPR2* (1) *AVPR2 + BKBR2* (2)	**15.1** (13) *5-HTT + BKBR2* (8) *5-HTT + AVPR2* (1) *AVPR2 + BKBR2* (4)	**8.8** (26)
3	**0**	**0**	**0**	

The 'maximum body weight loss' genotypes data is presented as a percentage (in bold) with the number of 'maximum' genotypes in parenthesis. The individual genotype breakdown for each cell is also indicated with the number in parenthesis.
0 vs. 1 or 2 'maximum' genotypes: P=0.015 and P for linear trend = 0.004.
0 or 1 vs. 2 'maximum' genotypes: P for linear trend = 0.009.

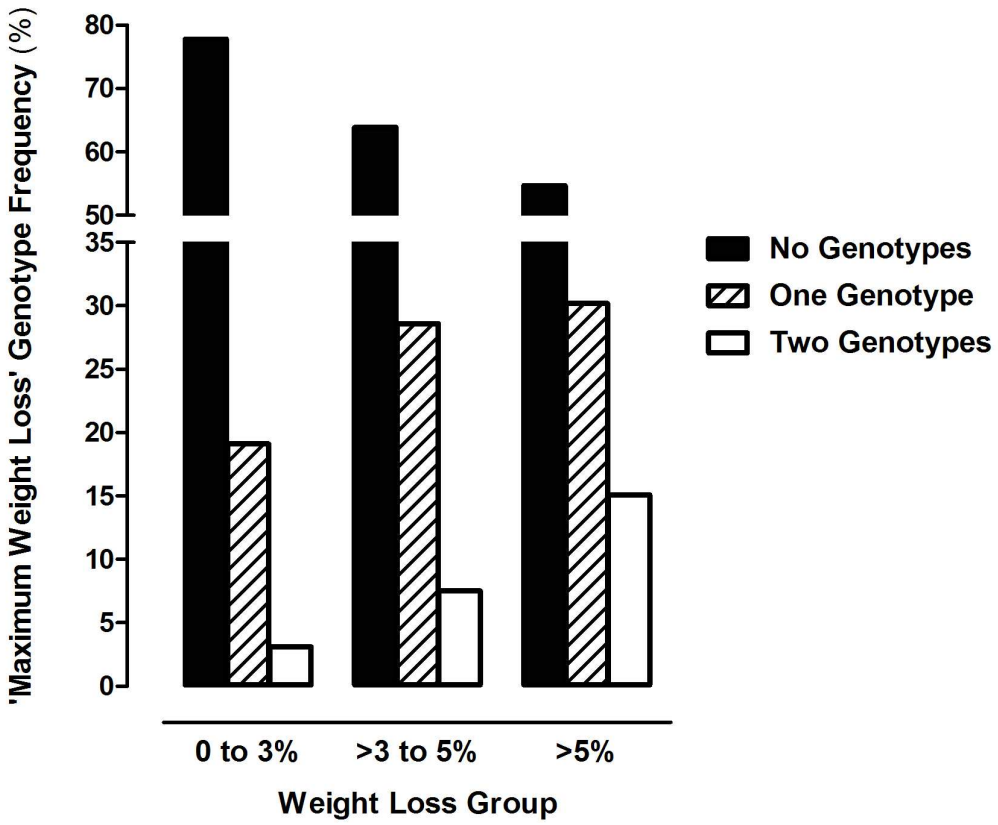

Figure 5.1: The frequency of the 'maximum weight loss' *5-HTT* (SS), *BKBR2* (+9/+9) and/or *AVPR2* (minor haplotypes) genotype distributions across the three body weight loss groups of the triathletes who competed in either the 2000 or 2001 South African Ironman Triathlons.

In addition, the TGS for each triathlete was calculated and the mean TGSs were compared between the three weight loss groups. There was significant difference (P=0.005) between the average TGSs of the 0 to 3% (9 ± 17%, n=63), >3 to 5% (15 ± 21%, n=147) and >5% (20 ± 25%, n=86) weight loss groups (Figure 5.2). Post-hoc analysis demonstrated that the average TGS of the >5% weight loss group was significantly ($p<0.05$) higher than the average TGS of the triathletes in the 0 to 3% weight loss group.

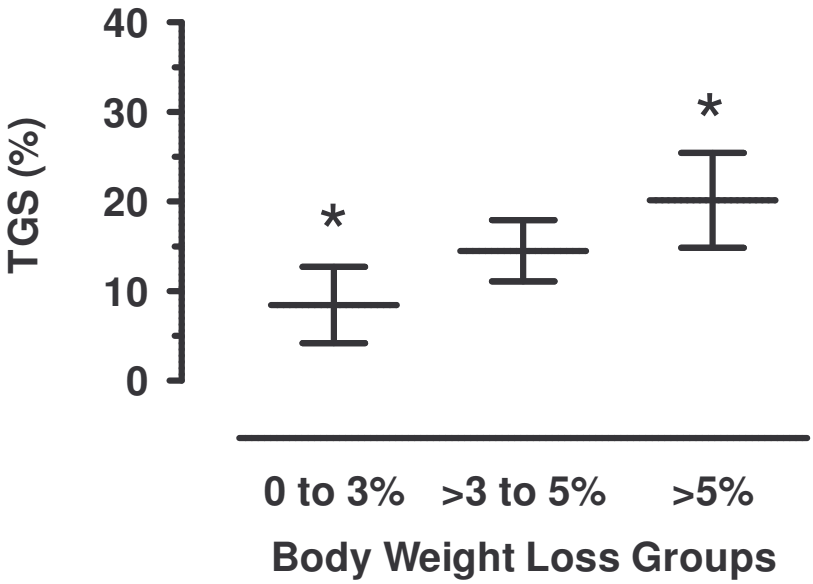

Figure 5.2: The mean total genomic score (TGS) (± 95% confidence intervals) for the triathletes within the three weight loss groups. The asterisk represents significant differences ($P<0.05$) between the body weight loss groups.

5.4 DISCUSSION

The main finding of this study was that triathletes with a higher total genotype score (TGS) are more likely to experience greater relative body weight losses during the 2000 or 2001 South African Ironman Triathlons. To our knowledge this is the first study that has investigated the three candidate genes *5-HTT, BDKRB2* and *AVPR2* as part of a genetic profile for body weight loss, and by implication TBW loss, in athletes during an ultra-endurance event.

The distribution frequency of the 'maximum body weight loss' genotypes *5-HTT* SS, *BDKRB2* +9/+9 and *AVPR2* minor haplotypes was associated with greater relative body weight losses during the Ironman events (X^2= 8.5, p=0.015). Also, the possession of two 'maximum' genotypes was associated with greater relative body weight losses in triathletes when compared to those possessing one or none 'maximum' genotypes. As previously discussed in this thesis (Chapter 3, Section 3.4), this finding is supported by our hypothesis that the *5-HTT* SS genotype would result in cerebral serotonin's inhibition of the perception of thirst, thus resulting in greater relative body weight changes. Furthermore, the *BDKRB2* -9 allele has been associated with increased gene transcription resulting in the blockade of AVP-induced water re-absorption that ultimately facilitates TBW loss. It would therefore seem contradictory for the *BDKRB2* +9/+9 genotype to be associated with body weight loss. Saunders et al. [1] provides the biological explanation that the diuretic effect of bradykinin is masked during cardiovascular exercise as a result of decreased vascular flow to the kidneys that will reduce glomerular filtration and urine

production. Therefore, the observed genetic association between greater relative body weight losses and the *BDKRB2* +9/+9 genotype provides evidence for the dipsogenic action of bradykinin via the β2 receptors. Thus a decreased β2 receptor activity would result in a reduced thirst drive and greater body weight loss. As discussed in chapter 4 of this thesis, the functional role of the *AVPR2* minor haplotypes is unknown. However, we cannot exclude possible other genetic factors that may have a larger impact on the polygenic nature of body weight loss during prolonged exercise

In conclusion, the study presented in this chapter provides evidence for the genetic contribution of genotypes *5-HTT* SS, *BDKRB2* +9/+9 and *AVPR2* minor haplotypes to a polygenic profile for relative greater body weight losses during the 2000 and 2001 South African Ironman Triathlon events. Furthermore, this study provides a unique yet informative approach to investigating the genetic contribution to multifactorial polygenic phenotypes.

CHAPTER 6

SUMMARY AND CONCLUSIONS

There is a large inter-individual variation in the fluid regulatory effector responses and performance during participation in endurance events. There is however no single physiological or non-physiological factor that determines how an athlete responds to participation in athletic events. Rather these phenotypes are determined by a poorly understood complex interaction of genetic and environmental factors. The multifactorial nature of these phenotypes adds to the complexity in elucidating the mechanisms involved. In spite of this investigators have successfully identified individual factors, including specific genetic variants that contribute to the endurance phenotype. The literature in this thesis provides a review (Chapter 1) of the various biological, environmental and genetic factors that have been shown to contribute to the endurance phenotype to date, with an emphasis on the triathlete. Although many genetic components that function within different physiological systems have been associated with athletic ability [392], none have been identified within the central nervous system (CNS). Cytokines, such as interleukin 6 (IL-6), and neurotransmitters, such as serotonin (5-HT), that play key signaling roles in the CNS have been implicated in fatigue models and thereby endurance athletic ability [18]. Genetic studies investigating the possible association of functional variants within genes encoding for proteins within the CNS with the endurance phenotype is lacking. Furthermore, the CNS also plays a vital integrative role together with endocrine and humeral factors in the regulation of total body water (TBW)

homeostasis during exercise and the control of drinking behaviour [234, 239, 263]. A large variation in body weight changes have been reported in athletes participating in ultra-endurance events. This variation is partly due to the drinking behaviour of athletes, however, an inter-individual variation in the homeostatic control of TBW during prolonged endurance exercise exists [3]. In support, recent findings from our laboratory have reported a genetic association between a component of the KKS system and weight loss in athletes during the Ironman Triathlon [1]. Furthermore, rare functional mutations of the AVPR2 gene have been identified which have led to abnormal water retention and fatal hyponatremia [339, 380]. The association of common polymorphisms within the *AVPR2* gene with TBW homeostasis during participation in ultra-endurance exercise warrants further investigation. The association of genetic variants in other biological pathways, such as the serotoninergic pathway, shown to regulate TBW homeostasis during exercise also warranted further investigation. In addition, the investigation of whether a possible polygenic profile exists, that include the combined effect of candidate genes in overlapping peripheral and central systems, would further our understanding into the nature of these multifactorial phenotypes. The findings from these studies will help in understanding and identifying the possible mechanisms involved.

Based on the findings discussed in the literature review (Chapter 1), functional polymorphisms within candidate genes *IL-6*, *5-HTT* and *MAO-A* were selected for possible association with endurance performance. Since the serotonergic system plays a key signaling role in brain thirst centers in generating thirst, the *5-HTT* gene was also investigated for possible association with weight changes during ultra-endurance events. In addition, rare functional mutations within the *AVPR2* gene that

result in inappropriate water retention by the kidneys have also been identified [339, 380]. These investigations served to launch future research delineating central versus peripheral mechanisms associated with endurance performance (once identified). In this concluding chapter of the thesis, the aims of the genetic association studies will be reiterated and followed by a summary of the results for each study addressing these aims. Limitations, future studies and clinical applications of this thesis work will also be discussed in this chapter.

- **Primary aim: To investigate whether the candidate genes *IL-6, 5-HTT* and *MAO-A* contribute to ultra-endurance performance.**

As previously mentioned, there is evidence for increased levels of plasma IL-6 and centrally produced serotonin to be associated with a decrement in endurance performance [19-22]. Also, Park et al. [219] reported a significant genetic association with the *5-HTTLPR* deletion or short (S) allele in elite long distance runners compared to elite short distance runners. On the contrary, we found no significant association between either the -174 *IL-6* G/C, 44 bp *5-HTTLPR* (L/S) and *MAO-A* 30 bp VNTR polymorphisms and endurance performance in the 2000/2001 South African Ironman Triathlons.

Although we did not show a direct association with the *IL-6, 5-HTTLPR* and *MAO-A* polymorphisms investigated in this study with endurance athletic performance, we cannot exclude the involvement of the serotonergic and other CNS signaling systems in endurance exercise. As previously discussed in Sections 1.4.3.1 and 1.4.3.2, changes in serotonergic system signaling can alter mood states and

personality traits. Some researchers have depicted personality traits such as socially extroverted, controlled, optimistic, emotionally stable and high psychic vigor in endurance athletes [393-395]. The *5-HTTLPR* and *MAO-A* 30 bp VNTR polymorphisms have shown associations with risk taking, sensation seeking, impulsiveness and aggressive behaviour, as reviewed in Chapter 1. In fact, Hugo [396] observed correlations between psychometric scores and performance variables in the 2007 South African Ironman Triathletes. Although the actual overall finishing times of triathletes did not significantly correlate with novelty seeking or reward dependence scores, there was tendency for a negative relationship between actual swim time and novelty seeking score, and a significant negative relationship between actual swim time and reward dependence score. Furthermore, the triathlete group had a higher average resilience score compared to that of a general population as depicted by Connor and Davidson [397]. It has therefore been suggested that such innate psychometric components combined with physical attributes distinguish between endurance participation or non-participation [398].

These findings therefore highlight the fundamental importance of the serotonergic or perhaps other CNS signaling systems in psychological health, mental toughness and certain personality traits, which undoubtedly play a role in sports participation and by implication the choice as well as the ability to compete and excel in ultra-endurance events.

A limitation to this particular study was the incomplete training data for the triathletes. Whether a possible gene x training interaction exists provides for future investigation.

Also, due to the fact that this study was performed in retrospect, psychological traits could not be investigated.

From a clinical point of view, the acute pharmacological manipulation of serotonergic system signaling and the response in altering endurance performance in animal models is known [21, 186, 399]. The long-term effects of serotonergic system or CNS manipulation on endurance performance and the interaction between psychometric traits and endurance exercise in athletes, however, require future investigation.

- **Secondary aim: To determine whether the candidate genes *5-HTT* and *AVPR2* predispose athletes to significant weight changes during ultra-endurance events.**

There is a large variation amongst athletes in total body weight losses during participation in ultra-endurance events. This can partly be explained by the drinking behaviour of athletes. However, an inter-individual variation in the homeostatic control of TBW during prolonged endurance exercise exists. We have previously discussed (Section 1.6.4.1) the physiological role of serotonin in the homeostatic control of TBW. Serotonin signals the activation of brain centers that inhibits the sensation of thirst and the desire to drink. In addition, gain-of-function mutations in the *AVPR2* gene can result in inappropriate water reabsoption thus hindering the homeostatic control of TBW. Such mutations can result in hyponatraemia, a clinical condition which have been documented to cause fatalities in endurance athletes. To our knowledge, the genetic contribution of the candidate genes *5-HTT* and *AVPR2*

with body weight changes, resembling TBW losses of Ironman triathletes, have not been investigated. In addition, findings from our laboratory have previously shown an association between the *BDKRB2* gene and body weight changes in the same cohort of Ironman triathletes [1].

The novel findings of this thesis were that the functional 44bp *5-HTTLPR* and minor haplotypes constructed from SNPs rs3761528, rs3761527 and rs4898457 within the *AVPR2* gene were associated with body weight changes during the 2000, 2001 and 2006 South African Ironman triathlons. The SS genotype of the *5-HTTLPR* polymorphism was significantly (p=0.042) associated with a relative greater body weight loss in triathletes. There was a significant (X^2= 5.1, p=0.024) linear trend for the SS genotype amongst the three body weight loss groups, with the >5% body weight loss group having the highest percentage of SS genotype triathletes.

Our finding, therefore, substantiates our hypothesis that the SS genotype would result in a decreased 5-HTT protein synthesis and increased levels of extracellular cerebral serotonin, which will reduce the stimulation of brain thirst centers, thereby allowing for a greater reduction in TBW.

We did not observe any significant associations between the three individual *AVPR2* SNPs and serum sodium and body weight changes during the Ironman events. There was however a significant association (p=0.041, odds ratio 9.7, 95% confidence interval 0.7 to 165.6) between the combined minor haplotype constructs (GCT, GTC and GCC) and body weight loss of triathletes. The minor haplotype constructs were associated with a >3% relative body weight loss during the race.

The function, if any, of the three individual *AVPR2* SNPs rs3761528, rs3761527 and rs4898457 is however currently unknown. A possible explanation for the observed association with minor haplotype constructs is that the individual SNPs might be tightly linked to unidentified functional SNPs within regulatory or protein coding regions of the *AVPR2* gene or perhaps are in linkage equilibrium with another gene within close proximity on the X chromosome.

It should be noted that the absence of quantitative evaluation of thirst and sodium palatability ratings, due to the retrospect nature of this investigation, in these athletes poses a limitation to this study.

- **Final aim: To investigate the association of a polygenic profile with body weight loss during ultra-endurance events.**

Considering the observed relationship between body weight loss and performance of triathletes in this thesis, we constructed a scoring method to assess the direct or indirect fit of this phenotype to that of the endurance athletic phenotype, which requires further investigation. Based on the novel associations with variants in the *5-HTT* (Chapter 3), *AVPR2* (Chapter 4) and the previously reported *BDKRB2* [1] genes, we selected these three candidate genes to identify whether a polygenic 'body weight loss' profile as a result of participation in the Ironman triathlon exists. Using a previously described method [68], each triathlete was scored according to the number of 'maximum body weight loss' genotypes (*5-HTT* SS, *BDKRB2* +9/+9 and *AVPR2* minor haplotypes) that he possessed (Table 5.1). The total genotype

score (TGS) was calculated from the accumulative score at all three loci and expressed as a percentile.

Another novel finding of this thesis was that triathletes with a higher TGS are more likely to experience greater relative body weight losses during the 2000 or 2001 South African Ironman Triathlons. The distribution frequency of the 'maximum body weight loss' genotypes *5-HTT* SS, *BDKRB2* +9/+9 and *AVPR2* minor haplotypes was associated with greater relative body weight losses during the Ironman events (X^2= 8.5, p=0.015). Furthermore, the possession of two 'maximum' genotypes was associated with greater relative body weight losses in triathletes when compared to those possessing one or none 'maximum' genotypes. These results provide further support to our hypothesis that was previously addressed in the secondary aim of this thesis.

It can however seem contradictory for the *BDKRB2* +9/+9 genotype to facilitate with body weight loss as the -9 allele has been associated with increased gene transcription resulting in the blockade of AVP-induced water re-absorption that ultimately facilitates TBW loss. A plausible biological explanation is the masking of the diuretic effect of bradykinin as a result of cardiovascular exercise that reduces vascular flow to the kidneys [1]. The *BDKRB2* +9/+9 genotype provides evidence for the dipsogenic action of bradykinin via the β2 receptors. Thus a decreased β2 receptor activity (*BDKRB2* +9/+9 genotype) would result in a reduced thirst drive and greater body weight loss.

The final aim of this thesis provides a unique yet informative approach to investigating the genetic contribution to multifactorial polygenic phenotypes.

*

In the process of answering the three aims of this thesis, additional observations that need mentioning were discovered. As expected the faster Ironman triathletes were younger, weighed less and had a lower BMI compared to their slower counterparts. Also, triathletes with the *5-HTT* SS genotype had a significantly ($p=0.048$) lower BMI compared to L allele triathletes. In support, the association between the *5-HTT* SS genotype and lower BMI scores (BMI<20) in males have previously been reported [218]. It is well known that serotonergic pathways regulate the behavioural mechanism of appetite and food intake [363] and it is therefore plausible to consider that an increased availability of synaptic serotonin would exert an inhibitory influence on food intake [218].

Another observation made in this thesis was the inverse relationship between serum $[Na^+]$ levels and dehydration status in triathletes. This, however, is no novel finding to the existing literature. An increase in the tonicity of the ECF is to be expected with an overall weight loss, a surrogate measure of total body water loss, consequently from prolonged and/or excessive sweating. Furthermore, triathletes that lost the most relative body weight (>5%) and were considered to be the most dehydrated were also faster the competitors compared to those that had smaller body weight losses. Again our findings corroborate observations from previous studies that reported greater dehydration levels in faster runners during races [5, 400]. Well trained and heat acclimatised athletes are adapted to sustain higher sweat rates

[374-376], thereby allowing for greater body weight losses and dehydration [3]. In addition, these athletes are assumed to be “lighter” and therefore faster [373]. Another possible explanation is that faster pace runners consume smaller volumes of fluid to minimise gastric discomfort [273, 294-296] and spend shorter periods of time on the event course and are, therefore, exposed to a shorter time frame in which to rehydrate. These physiological factors could perhaps justify the observation that triathletes who experienced greater body weight losses, had higher post-race serum [Na^+] concentrations and finished the event faster. However, even in the fastest of finishers, the serum sodium concentrations remained largely within the normal physiological range despite greater body weight losses.

The strengths and limitations of this particular study design presented in this thesis are important and need mentioning. The strength of this thesis was the robust study design for each of the association studies. The cases (performance and body weight loss) were well defined criteria that have previously been investigated in this regard and the sample sizes were sufficient for genetic investigation thereof. In addition, the hypotheses had strong biological reasoning and the investigation thereof warranted. It should however be noted that a possible confounding factor such as pharmacological drugs, which can alter serotonergic neurotransmission and AVP release, were not accounted for. Other restrictions that we had limited control of included weigh loss that was used as a proxy for dehydration status and environmental conditions during the Ironman events. We assumed that all triathletes experienced relative similar levels of fuel (energy) depletion and therefore had similar weight loss due to these factors.

It is important that the novel findings of this thesis, the associations of *5-HTTLPR* and minor haplotypes of the *AVPR2* with weight changes and by implication TBW loss, be confirmed with other independent groups participating in ultra-endurance events. As previously mentioned in Chapter 4, the unknown biological and perhaps functional role of the genetic variants within the *AVPR2* gene, needs further investigation. Chapter 5 presents a unique yet informative approach to investigating the genetic contribution to multifactorial polygenic phenotypes. This thesis, therefore, provides an invaluable contribution to understanding the genetic basis of inter-individual weight changes and TBW loss in athletes during ultra-endurance events such as the Ironman Triathlon. With the limited current literature in this particular field, this thesis provides a basis for future investigations.

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APPENDIX 1

ADDITIONAL MATERIAL

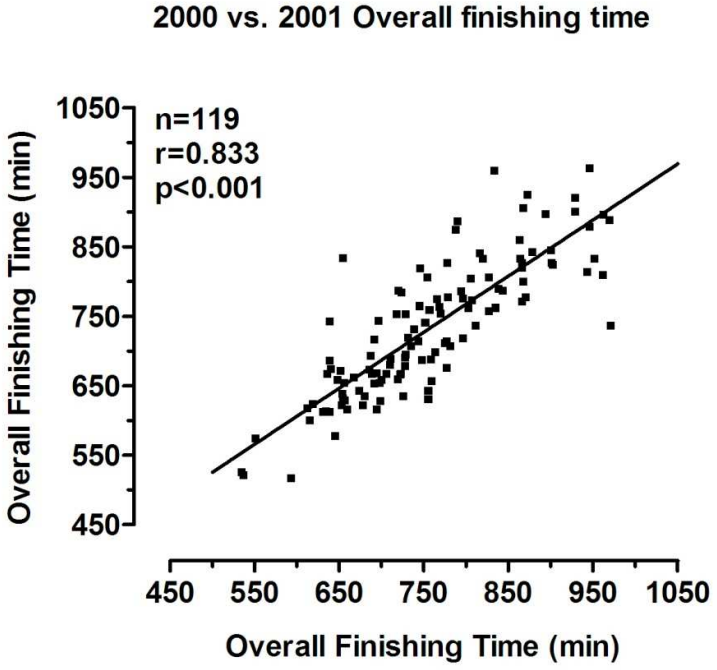
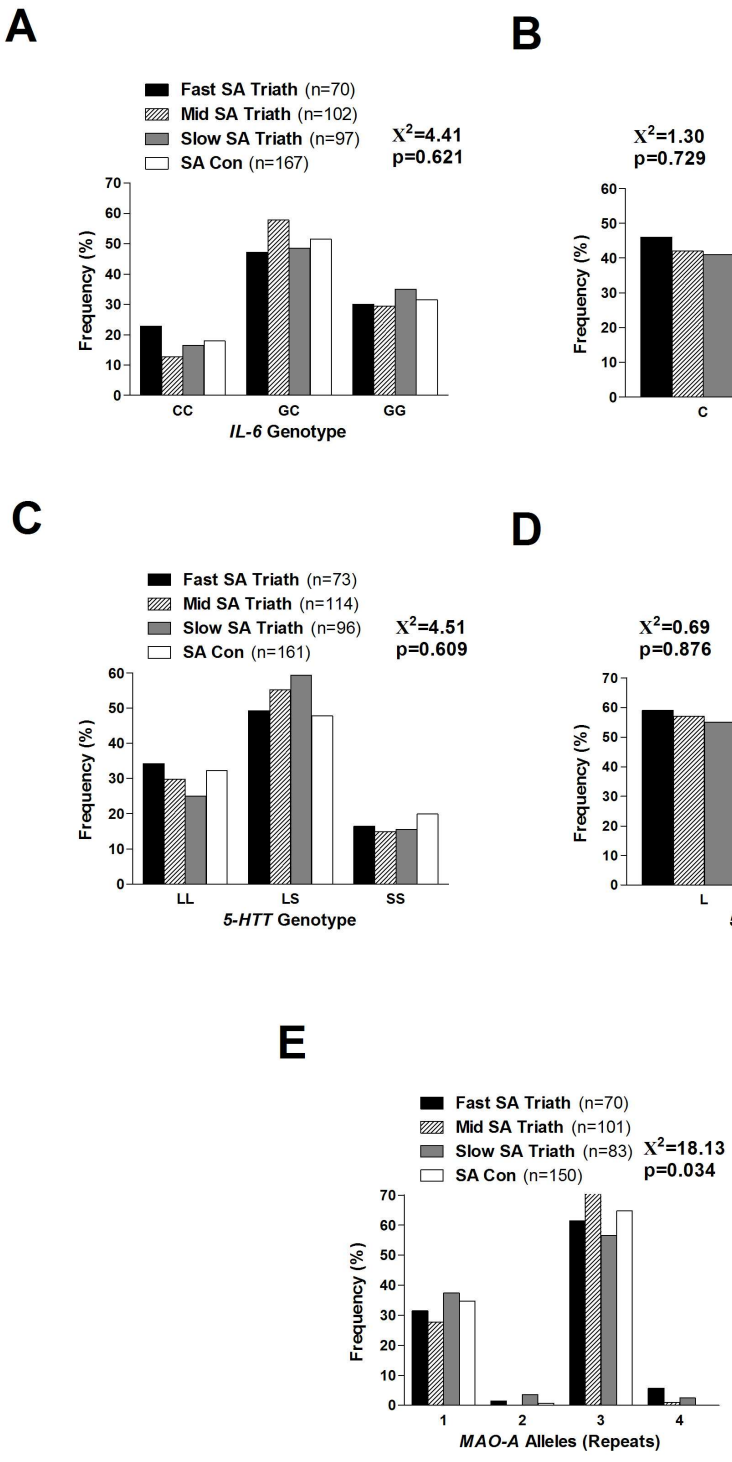


Figure A1: Positive linear regression of male Caucasian triathletes' overall finishing times between the 2000 and 2001 South African Ironman Triathlons for triathletes that completed both events.



Legend is on the following page.

Figure A2: The relative **(A)** genotype and **(B)** allele frequencies of the -174 G/C polymorphism within the *IL-6* gene of the fastest (Fast Triath), middle (Mid Triath), and slowest (Slow Triath) South African born finishing male Caucasian triathletes, as well as, the control (Con) groups. The relative **(C)** genotype and **(D)** allele frequencies of the *5-HTTLPR* insertion (L)/deletion (S) polymorphism within the *SLC6A4* gene of the Fast Triath, Mid Triath, Slow Triath and Con groups. **(E)** The relative individual allele frequencies of the 30 bp VNTR polymorphism within the *MAO-A* gene of the Fast Triath, Mid Triath, Slow Triath and Con groups. The triathletes completed either the 2000 and/or 2001 South African Ironman Triathlon. For technical reasons not all the samples within each group were genotyped for all three polymorphisms. The actual number of genotyped samples within each group for the individual polymorphisms are indicated in the figure.

Table 1A: The percentage genotype and allele frequencies of the -174 *IL-6* G/C, 44 bp *5-HTTLPR* (L/S) and *MAO-A* 30 bp VNTR polymorphisms in different Caucasian populations.

Population		Genotype Frequency					Allele Frequency		Reference
IL-6	**N**	**CC**	**GC**	**GG**			**C**	**G**	
Northern Ireland Caucasians	100	22.0	48.0	30.0			46.0	54.0	[347]
5-HTT		**SS**	**LS**	**LL**			**S**	**L**	
Finnish whites	771	15.0	52.0	33.0			40.0	60.0	[202]
MAO-A		**3r**	**3.5r**	**4r**	**5r**		**"Low-activity"**	**"High-activity"**	
White/Non-Hispanic	539	33.1	0.5	64.8	1.6		34.7	65.3	[221]

N represents the total number of subjects in each population group.

APPENDIX 2

Department of Human Biology

UCT/MRC RESEARCH UNIT FOR EXERCISE SCIENCE & SPORTS MEDICINE
Faculty of Health Sciences, University of Cape Town
Private Bag, Rondebosch 7700, South Africa
Tel: + 27 21 650 4561
Fax: + 27 21 686 7530

INFORMED CONSENT

Genetic variants associated with athletic ability within South African male ultra-endurance athletes study.

I, the undersigned, have been fully informed as to the nature of the genetic components associated with athletic ability within South African male ultra-endurance athletes study, to be performed by the UCT/MRC Research Unit for Exercise Science and Sports Medicine of the Department of Human Biology, University of Cape Town. I have agreed to donate five millilitres of venous blood that will be used for the extraction of genetic material and the identification of genetic markers that could influence athletic ability. I have also agreed to complete a personal particulars and sporting participation 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 within this study and have been informed that I am free to withdraw from this study at any given time if I wish to do so. I understand that my DNA samples will be destroyed on completion of the study on the genetic components associated with athletic ability within South African male ultra-endurance athletes, I also understand that I will be free to request that my DNA sample is destroyed before the completion of this study.

FULL NAME OF SUBJECT: _____
SUBJECT'S SIGNATURE: _____
INVESTIGATOR: _____
INVESTIGATOR'S SIGNATURE: _____
DATE: _____
WITNESSES 1. _____ 2. _____

APPENDIX 3

Department of Human Biology
UCT/MRC Research Unit for Exercise Science & Sports Medicine
Faculty of Health Sciences, University of Cape Town
Private Bag, Rondebosch 7700, South Africa
Tel: + 27 21 650 4561
Fax: + 27 21 686 7530

Genetic variants associated with athletic ability within South African endurance athletes questionnaires.

A. PERSONAL PARTICULARS			
Surname			
First Name			
Postal Address		Code	
E-mail address		Phone (day time)	( )
Date of birth	Y Y Y Y / M M / D D	Cell no.	
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, German, Italian)	Father	Unknown ☐	
	Mother	Unknown ☐	
Your Country of Birth			

B. SPORTING DETAILS Please record your sporting activities in order of importance			
	Main sport 1	**Other sport 2**	**Other sport 3**
Type of sport(s) you have participated in (please name)			
Current or past participation	Current ☐ Past ☐	Current ☐ Past ☐	Current ☐ Past ☐
Year started participation			
Number of years involved in the sport			
Hours of training per week			
Running distance (km) per week			

C. SPORTING INJURIES		
Have you suffered any sporting injuries that prevented you from running?	Yes ☐ No ☐	
If you answered YES above, please complete the following questions:		
1. When did your last injury occur? e.g. Year and month (1997/09)	Y Y Y Y / M M	
2. What type of injury?		
3. How long were you unable to run or train for?	1-2 weeks ☐ 2-3 weeks ☐ 1-2 months ☐ 2-3 months ☐ 3-4 months ☐ more than 5 months ☐	

D. RACING HISTORY		
1. Comrades marathon		
1.1 How many Comrades marathon(s) have you completed?		
1.2 First Comrades marathon completed?	Year	Finish Time (h/min)
	Y Y Y Y	...….h /…….min
1.2.1 Average distance run (km) per week prior to 1st Comrades race?	……. km/week	
1.2.2 Average training speed (min/km) prior to 1st Comrades race?	……. min/km	
1.3 Best Comrades performance?	Year	Finish Time (h/min)
	Y Y Y Y	...….h /…….min
1.3.1 Average distance run (km) per week prior to best Comrades race?	……. km/week	
1.3.2 Average training speed (min/km) prior to best Comrades race?	……. min/km	
1.4 Worst Comrades performance?	Year	Finish Time (h/min)
	Y Y Y Y	...….h /…….min
1.5 List in chronological order the years in which you ran the Comrades. (e.g. 1996, 1998, 1999, 2001, etc.)		
1.6 Highest training week (km) for Comrades? (Most kilometres run within a training week.)	…….km	

2. Two Oceans marathon (56km)		
2.1 How many Two Oceans marathon(s) have you completed?		
2.2 First Two Oceans marathon completed?	Year	Finish Time (h/min)
	Y Y Y Y	...……h /…….min
2.2.1 Average distance run (km) per week prior to 1st Two Oceans race?	…… Km/week	
2.2.2 Average training speed (min/km) prior to 1st Two Oceans race?	…… min/km	
2.3 Best Two Oceans performance?	Year	Finish Time (h/min)
	Y Y Y Y	...……h /…….min
2.3.1 Average distance run (km) per week prior to best Two Oceans race?	…… Km/week	
2.3.2 Average training speed (min/km) prior to best Two Oceans race?	…… min/km	
2.4 Worst Two Oceans performance?	Year	Finish Time (h/min)
	Y Y Y Y	...……h /…….min
2.5 List in chronological order the years in which you ran the two Oceans. (e.g. 1996, 1998, 1999, 2001, etc.)		
2.6 Highest training week (km) for Two Oceans? (Most kilometres run within a training week.)	……km	

3. Standard marathon (42km)		
3.1 How many standard marathon(s) (42km) have you completed?		
3.2 First marathon (42km) completed?	Year	Finish Time (h/min)
	Y Y Y Y	...….h /…….min
3.2.1 Average distance run (km) per week prior to 1st marathon (42km) race?	..... Km/week	
3.2.2 Average training speed (min/km) prior to 1st marathon (42km) race?	..... min/km	
3.3 Best marathon (42km) performance?	Year	Finish Time (h/min)
	Y Y Y Y	...….h /…….min
3.3.1 Name the marathon (42km) that was your best performance.		
3.3.2 Average distance run (km) per week prior to best marathon (42km) race?	..... Km/week	
3.3.3 Average training speed (min/km) prior to best marathon (42km) race?	..... min/km	
3.4 Worst marathon (42km) performance?	Year	Finish Time (h/min)
	Y Y Y Y	...….h /…….min
3.4.1 Name the marathon (42km) that was your worst performance.		
3.5 Highest training week (km) for a marathon (42km)? (Most kilometres run within a training week.)	……km	

4. Running time trials		
4.1 10km Personal best time trial performance	Year	Time (min/sec)
	Y Y Y Y	...….min /…….sec

APPENDIX 4

UNIVERSITY OF CAPE TOWN

Research Ethics Committee
Faculty of Medicine
Anzio Road, Observatory, 7925
Queries : Martha Jacobs
Tel : (021) 406-6492 Fax: (021) 406-6390
E-mail : Martha@medicine.uct.ac.za

01 March 2000

REC REF: 005/2000

Ms J Goedecke
BERU

Dear Ms Goedecke

THE CAPE IRONMAN TRIATHLON 2000: FLUID AND SODIUM BALANCE, PREDICTORS OF PERFORMANCE, AND MEDICAL CONSEQUENCES

Thank you for your application submitted to the Research Ethics Committee on 19 January 2000.

I have pleasure in informing you that the above study has been **formally approved** by the Research Ethics Committee on 24 February 2000.

Included is a list of Research Ethics Committee Members who have formally approved your protocol.

Please quote the above Reference number in all correspondence.

Yours sincerely,

PROFESSOR DM DENT
DEPUTY-CHAIRPERSON: RESEARCH ETHICS COMMITTEE
Queries: Martha Jacobs
Research Ethics Committee
Room 212 Werner and Beit
UCT Medical School
Anzio Road, Observatory, 7925
Tel: (021) 406-6492 Fax: (021) 406-6390
E-Mail: martha@medicine.uct.ac.za

UNIVERSITY OF CAPE TOWN

Research Ethics Committee
Faculty of Health Science
Anzio Road, Observatory, 7925
Queries : Xolile Fula
Tel : (021) 406-6492 Fax: 406-6390
E-mail : Xfula@curie.uct.ac.za

17 April 2001

REC REF: 099/2001

Dr. M. Collins
Sports Science Institute

Dear Dr. Collins

SOUTH AFRICAN IRONMAN 2001 RESEARCH PROJECTS

Thank you very much for submitting your application to the Research Ethics Committee for reviewal.

It is a pleasure to inform you that the committee has formally approved your study:

Please quote the above Reference number in all correspondence.

Yours sincerely

PROFESSOR CR SWANEPOEL
CHAIRPERSON

UNIVERSITY OF CAPE TOWN

Health Sciences Faculty
Research Ethics Committee
Room E53-24 Groote Schuur Hospital Old Main Building
Observatory 7925
Telephone [021] 406 6338 • Facsimile [021] 406 6411
e-mail: [illegible]

13 January 2006

REC REF: 425/2005

Assoc Prof MP Schwellnus
Department of Human Biology
UCT/MRC Research Unit for Exercise Science and Sports Medicine
Medical School

Dear Prof Schwellnus

THE PORT ELIZABETH IRONMAN TRIATHLON 2006: MEDICAL CONSEQUENCES FOLLOWING ENDURANCE SPORTS.

Thank you for your letter to the Research Ethics Committee dated 14 December 2005, addressing the issues raised by the committee. It is a pleasure to inform you that the Ethics Committee has formally approved the above mentioned study.

Please quote the REC. REF in all your correspondence.

Yours sincerely

PROF. T ZABOW
CHAIRPERSON