Genetic Modifiers of Caffeine and Endurance Performance in Athletes

by

Nanci Susan Guest

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Nutritional Sciences
Faculty of Medicine
University of Toronto

© Copyright by Nanci S. Guest 2019
Abstract

**Background:** Caffeine is used as an ergogenic aid by athletes to improve performance. However, the effects of caffeine differ between athletes. This may be due to genetic differences affecting caffeine metabolism and caffeine response during exercise. **Objective:** The aim was to determine whether variation in the *CYP1A2* gene, which affects caffeine metabolism, and the *HTR2A* gene, which encodes serotonin receptors and may affect caffeine response, modify the ergogenic effects of caffeine during a 10-km cycling time trial. **Methods:** Competitive male athletes (n=101; age: 25 ± 4 years) completed the time trial under three conditions: 0, 2 or 4 mg of caffeine per kg body mass, using a split-plot randomized, double-blinded, placebo-controlled design. DNA was isolated from saliva and genotyped for polymorphisms in *CYP1A2* (rs762551) and *HTR2A* (rs6313). **Results:** Overall, 4 mg/kg caffeine decreased cycling time by 3% versus placebo. A significant (p <0.0001) caffeine-gene interaction was observed with *CYP1A2*. In
those with the AA genotype, cycling time improved by 4.8% and by 6.8% compared to placebo in 2 mg and 4 mg/kg caffeine groups (mean ± SEM; 17.0 ± 0.3 min and 16.6 ± 0.3 min vs. 17.8 ± 0.4 min, p = 0.0005, p < .0001), respectively. No effects were observed among those with the AC genotype, and those with the CC genotype performed worse at 4 mg/kg by 13.7%. Among those with the HTR2A CC genotype, cycling time improved by 5.8% and 7.9% compared to placebo in 2 mg and 4 mg/kg caffeine groups. No effects were observed in those with the CT or TT genotypes. In CYP1A2 AAs (n=49; fast metabolizers) a caffeine-HTR2A interaction (p=0.001) resulted in HTR2A CCs improving cycling performance by 7.9% and 12.6% compared to placebo in 2 mg and 4 mg/kg caffeine groups, respectively. No effects were observed among those with CTs or TTs. In CYP1A2 AC/CC slow metabolizers HTR2A genotype had no effect.

Conclusion: Caffeine improves endurance performance and the effects are greater in those with the CYP1A2 AA or HTR2A CC genotypes. Both genotypes should be considered when deciding whether an athlete should use caffeine for enhancing endurance performance.
Acknowledgments

I would first like to express my sincere gratitude to my supervisor Professor Ahmed El-Sohemy for his continuous support of my doctoral research and related projects, and for his patience, inspiration and commitment to ensuring that I made the most of this journey.

Ahmed possesses the perfect balance of toughness and sensitivity. He is ultra-tough to ensure you meet and exceed the highest standards, because that is what the upper echelons of the world will expect from you. But this was never at the exclusion of compassion, sensitivity and encouragement, when needed. Whether it be the writing of manuscripts or the preparation of conference talks, I was pushed to perfection and for that I am forever grateful. When not pushing me to work hard and be my best, Ahmed allowed his relaxed nature and witty humor to entertain and buffer much of the seriousness that is required when striving for the pinnacle of your capabilities.

In our lab, our actions were expected to always be carried out with mindfulness and integrity, and never with short cuts. And we maintain our composure. He modeled this behavior, which earned my respect and trust in his leadership. This journey has forever changed me, and I like who I am. I am confident. Thank you for this guidance.

As an older student I arrived with experience, knowledge and wisdom of my own. But I left with much more than I could have ever imagined. I have had the highest level of training and mentorship that is expected from a world-class university. But I received more. I had the privilege of receiving the guidance of an insightful and exceptional individual. Doors have opened and opportunities have arisen. I see the world differently. I am inspired. For this and everything else I am truly grateful to Ahmed, my supervisor.

I would also like to thank my thesis committee, which enabled me to learn far beyond what my PhD project entailed. Prof. Richard Bazinet who is known for thinking outside of the box and asking the most difficult questions, from this I learned greatly. I had no choice but to think critically, and to keep pushing my own boundaries. Not only would Richard take the time to answer my specific questions, but he would also extend the conversation to include how this
knowledge fits into the real world, whether it be academia, the media or the farm. I always left the conversation feeling enlightened.

Dr. Jason Vescovi was likely the most understanding of who I am and the multiple hats I wear. He shared his experience and expertise on how we can be highly successful as both scientists and practitioners in the world of sport. Jason constantly kept me thinking about which hat I was wearing and how this impacted the dissemination of my knowledge and my message. We will work together again I’m sure!

Dr. Paul Corey is not only a wealth of knowledge as a biostatistician, but a veteran of patience and understanding. There was never a feeling of being rushed, and I always walked away feeling wiser and better equipped. I feel more confident and informed in such a specialized area that was very foreign to me in my initial years. Paul also has a dry and sharp sense of humor that was cherished and appreciated.

And I can’t forget my lab mates who were always there in any way needed: Dr. Bibiana Garcia-Bailo, Dr. Daiva Nielsen, Dr. Sara Mahdavi, Dr. Ohood Alharbi, Dennis Wang, Neshat Deljooanesh and a special thanks to Alicia Jarosz and Dr. Joseph Jamnik who were instrumental in ensuring that the 800+ hours of athlete training sessions in my RCT ran smoothly!

I would also like to thank my love Dr. Brendan Frey who came into my life in the last 15 months of this journey. He was not only by my side emotionally and spiritually, but he was also able to occasionally step in as a professor and give me some tangible guidance! He filled me with confidence, enthusiasm and love during my most stressful final months. We as scientists see the world differently than others and having this unique connection to my partner has turned out to be magic. I feel fortunate.

Last but not the least, and most importantly I would like to thank my mom for her unconditional love and endless support in every way. My mom was a single mom to an only child. There is no human being on this planet that I am closer to. She is my role model for success and female empowerment. She is a pillar of strength, determination and intelligence. I can’t help but think it was in fact those characteristics passed down from her genes that helped me accomplish this
goal! As a child she instilled confidence in me, loved me, encouraged me and provided me every opportunity and experience imaginable. She celebrated every accomplishment with enthusiasm no matter how small. She still does. She ensured that my path was a rainbow-colored brick road – filled with love, happiness, joy and magic. I have made her very proud and that is a gift to both of us. She always calls me her star, but she will always be the brightest light in my Universe.
# Table of Contents

Acknowledgments........................................................................................................ iv
Table of Contents........................................................................................................ vii
List of Tables ................................................................................................................ ix
List of Figures ................................................................................................................ x

## Chapter 1

1 Introduction .................................................................................................................. 1

## Chapter 2

2 Background: Caffeine in Exercise and Sport ............................................................. 5
   2.1 Introduction ........................................................................................................... 5
   2.2 Caffeine Legality in Sport ..................................................................................... 7
   2.3 Caffeine Pharmacokinetics ................................................................................... 9
   2.4 Mechanism of Action ......................................................................................... 12
   2.5 Caffeine in Low Doses .................................................................................... 17
   2.6 Interindividual Variation in Response to Caffeine ........................................... 18
   2.7 Alternative Caffeine Sources ........................................................................... 31
   2.8 Caffeine and Endurance ..................................................................................... 35

## Chapter 3

3 Rationale, Hypotheses and Objectives .................................................................. 36
   3.1 Rationale .............................................................................................................. 37
   3.2 Overall Hypothesis ............................................................................................ 38
   3.3 Objectives ........................................................................................................... 38

## Chapter 4

4 Caffeine, CYP1A2 genotype and endurance performance in athletes ................ 41
   4.1 Abstract .............................................................................................................. 41
   4.2 Introduction ......................................................................................................... 42
   4.3 Methods .............................................................................................................. 44
      4.3.1 Subjects and Recruitment. .......................................................................... 44
      4.3.2 Experimental Design ................................................................................. 44
      4.3.3 Parameters of Assessment .......................................................................... 45
      4.3.4 Genotyping ................................................................................................. 47
   4.4 Results ................................................................................................................. 50
      4.4.1 Subject Characteristics ............................................................................... 50
      4.4.2 Time Trial Performance ............................................................................. 51
   4.5 Discussion ............................................................................................................ 61

## Chapter 5

5 Caffeine, HTR2A and CYP1A2 genotypes, and endurance performance in athletes 67
   5.1 Abstract .............................................................................................................. 68
   5.2 Introduction ......................................................................................................... 69
   5.3 Methods .............................................................................................................. 71
      5.3.1 Statistical Analyses (specific to Chapter 5) ................................................... 72
   5.4 Results ................................................................................................................. 72
      5.4.1 Subject Characteristics ............................................................................... 72
      5.4.2 Performance by HTR2A genotype (n=100). ................................................. 75
5.4.3 Performance by HTR2A genotype in CYP1A2 fast metabolizers (n=49) ..........75
5.4.4 Performance by HTR2A genotype in CYP1A2 slow metabolizers (n=51) .......76
5.4.5 Cycling Time Scatterplots for HTR2A Genotypes ........................................76
5.4.6 Effect Size (ES) .......................................................................................78
5.5 Discussion .................................................................................................82
Chapter 6 ..................................................................................................87
Discussion, Limitations and Future Directions ..................................................87
6 General Discussion ......................................................................................88
   6.1 Summary of Results .............................................................................92
   6.2 Limitations ..........................................................................................95
     6.2.1 Study Design and Population .........................................................95
   6.3 Future Research ...................................................................................96
   6.4 Thesis Implications and Conclusion ....................................................97
References ....................................................................................................99
Appendix 1 ..................................................................................................120
Appendix 2 ..................................................................................................130
List of Tables

TABLE 4.1. Descriptive characteristics of participants by CYP1A2 (rs762551) genotype ........61
TABLE 4.2. TT time and caffeine dose by CYP1A2 (rs762551) genotype with and without visit.................................................................................................................................62
TABLE 5.1. Descriptive characteristics of participants by HTR2A (rs6313) genotype ...............80
List of Figures

Figure 4.1 10-km cycling times for all subjects (n = 101) under each caffeine treatment ........63
Figure 4.2 10-km cycling time by caffeine dose and CYP1A2 genotype. .........................64
Figure 4.3 Change in 10-km cycling time to completion between genotypes .....................65
Figure 4.4 Scatterplot: 10 km cycling times for AA, AC, CC genotypes..........................66
Figure 5.1 Average 10-km cycling time by caffeine dose and HTR2A genotype .................84
Figure 5.2 Average 10-km cycling time by caffeine dose and HTR2A genotype in CYP1A2
genotypes ................................................................................................................................85
Figure 5.3 10 km cycling times for HTR2A CC, CT, TT genotypes in CYP1A2 fast
metabolizers ................................................................................................................................86
Figure 6.1 Consort diagram: Randomized Controlled Trial..............................................103
List of Appendices

Appendix 1: GHPALQ General Health Physical Activity Lifestyle Questionnaire ..................123
Appendix 2: Study Information and Consent Forms ..........................................................133
Chapter 1

Introduction
1 Introduction

Ergogenic aid supplements are a focus of intense study in the field of sports nutrition [1]. Many supplements, including caffeine, have been shown to provide direct benefits to performance and indirect benefits such as optimization of intense training sessions. The appropriate use of supplements can offer benefits to athletes but may also sometimes be harmful to an athlete’s health or performance [2]. Caffeine has become ubiquitously used by individuals pursuing fitness or sport, whether they be gym-goers or elite athletes, and there is keen interest in better understanding the impact of caffeine on various types of exercise performance [3-5].

Athletes frequently use caffeine in training and competition because of its reported performance-enhancing effects [4-18]. Numerous studies have investigated the effect of supplemental caffeine on exercise performance, but there is considerable inter-individual variability in the magnitude of these effects [3, 5, 8], as well as a lack of an effect [19, 20], when compared to placebo. Notably, a recent meta-analysis reporting on 56 endurance time trials in athletes (79% cycling), showed that 52 had positive results while 4 had negative results (placebo group performed better than caffeine group). The percent difference between the caffeine and placebo group ranged from −3.0% to 15.9% [21]. These inter-individual differences might be partly due to variation in genes that are associated with caffeine metabolism and caffeine response [22]. Studies that report on the effects of caffeine on performance have been inconsistent despite having similar study designs, subjects and dose of caffeine.

Over 95% of caffeine is metabolized by the CYP1A2 enzyme, which is encoded by the CYP1A2 gene, and is involved in the demethylation of caffeine into the primary metabolites paraxanthine, theophylline and theobromine [23]. The -163A>C (rs762551) single nucleotide polymorphism
(SNP) has been shown to alter CYP1A2 enzyme inducibility and activity [24, 25], and has been used to categorize individuals as ‘fast’ or ‘slow’ metabolizers of caffeine. Individuals with the AC or CC genotype (slow metabolizers) have an elevated risk of myocardial infarction [26], hypertension [27], and pre-diabetes [28] with increasing caffeinated coffee consumption, whereas those with the AA genotype show no such risk. In addition, a few studies have shown that the rate of caffeine metabolism could also have implications for sports performance, but the findings remain equivocal [15, 29].

_HTR2A_ rs6313 also known as T102C or C102T, is a SNP that encodes for the serotonin receptor 2A (5-HT2A), a G protein-coupled receptor that serves as a primary target for serotonin signaling [30]. Caffeine has been shown to increase serotonin release, but it is unclear if this is associated with the expression of 5-HT2A receptors. 5-HT2A receptors have been associated with blood pressure and vasoconstriction [31-35], dopamine release [36] and various aspects of behaviour such as motivation, mood, movement and pain perception [37-39].

The aim in this work was to determine whether variation in the _CYP1A2_ gene, which affects caffeine metabolism, the _HTR2A_ gene, which encodes serotonin receptors and may affect caffeine response, or combinations of those two genes, modify the ergogenic effects of caffeine during a 10-km cycling time trial in competitive male athletes.
Chapter 2

Review of Literature
2 Background: Caffeine in Exercise and Sport

2.1 Introduction

Caffeine is the world’s most widely consumed psychoactive substance, and naturally occurs in
dozens of plant species, including coffee, tea and cocoa. Caffeine is ingested most frequently in
the form of a beverage such as coffee, soft drinks and tea, although the consumption of many
functional beverages, such as energy drinks, has been on a steady rise in the past two decades
[40]. In western countries, approximately 90% of adults consume caffeine on a regular basis,
with dietary caffeine consumption of U.S. adult men and women estimated at approximately 200
mg/day in a 2009–2010 survey [41-43]. It was also reported that approximately 35% of dietary
caffeine was consumed away from home, with 43% consumed outside of any defined meal, e.g.
in coffee breaks [43]. In young adults and exercising individuals, there has also been a rise in the
consumption of other caffeine-containing products, such as energy drinks [40, 42], ‘pre-workout
supplements’, chewing gum, energy gels and chews, aerosols and many other novel caffeinated
food products [44]. Caffeine-containing products have a range of doses per serving, from 1 mg in
milk chocolate up to >300 mg in some dietary supplements [45].

Caffeine and its effects on health have been a longstanding topic of interest, and caffeine
continues to be a dietary compound of concern in public health, as indicated by extensive
investigations [46-49]. At the same time, caffeine has become ubiquitous in the sporting world,
where there is keen interest in better understanding the impact of caffeine on various types of
exercise performance. Accordingly, caffeine has dominated the ergogenic aids and sport
supplements research domain over the past several decades [3-5].
Caffeine in Sport: A Brief History

In the early days (1900s) of modern sport, concoctions of plant-based stimulants, including caffeine and other compounds such as cocaine, strychnine, ether, heroin and nitroglycerin, were developed secretly by trainers, athletes and coaches, in what appears to be evidence for early day ergogenic aids designed to provide a competitive advantage [50]. The use of various pharmaceutical cocktails by endurance athletes continued until heroin and cocaine became restricted to prescriptions in the 1920s, and further when the International Olympic Committee (IOC) introduced antidoping programs in the late 1960s [51].

Some of the earliest published studies on caffeine came from two psychologists and colleagues William Rivers and Harald Webber, at Cambridge University, who both had an interest in disentangling the psychological and physiological effects of substances like caffeine and alcohol. Rivers and Webber, using themselves as subjects, investigated the effects of caffeine on muscle fatigue. The remarkably well-designed studies carried out around 1906-1907 used double-blinded placebo-controlled trials and standardization for diet (i.e. caffeine, alcohol), and were described in a 1907 paper in the Journal of Physiology [52]. Significant research on caffeine and exercise performance with more subjects, different sports and variables such as trained and untrained, began and continued through the 1940s [50, 53]. However, it was a series of studies investigating the benefits of caffeine in endurance sports in the Human Performance Laboratory at Ball State University in the 1970s, many led by David Costill [54, 55], that sparked a generation of research on the effects of caffeine in exercise metabolism and sports performance.
Along with naturally occurring sources, such as coffee, tea and cocoa, caffeine is also added to many foods, beverages and novelty products, such as perky jerky, peanut butter and candy, in both synthetic (e.g. powder) and natural (e.g. guarana, kola nut) forms. Synthetic caffeine is also an ingredient in several over-the-counter and prescription medications, as it is often used in combination with analgesic and diuretic drugs to amplify their pharmacological potency [56].

Approximately ninety-six percent of caffeine consumption from beverages comes from coffee, soft drinks and tea [57, 58]. Additionally, there are varying levels of caffeine in the beans, leaves and fruit in dozens of plants, resulting in great interest in herbal and other plant-based supplements [59-62]. Further, caffeine containing energy drink consumption [63-67] and co-ingestion of caffeine with (e.g. “pre-workouts”), or in addition to, other supplements (e.g. caffeine + creatine) is popular among exercising individuals [68-75]. To date, the preponderance of caffeine and exercise performance literature has utilized anhydrous caffeine (capsule) [8, 76-81] for ease in standardizing doses and creating the placebo form of the supplement. There is also a growing body of literature studying the effects of using alternate forms of caffeine during exercise [44] such as coffee [6, 54, 82-90], energy drinks, herbal formulas [91] and ‘pre-workout’ formulas, among others.

2.2 Caffeine Legality in Sport

Antidoping rules apply to most sports especially in those where athletes are competing at national and international levels. The International Olympics Committee (IOC) continues to recognize that caffeine is frequently used by athletes because of its reported performance-enhancing or ergogenic effects [1]. Caffeine was added to the list of banned substances by the IOC in 1984 and the World Anti-Doping Agency (WADA) in 2000. A doping offense was
defined as being urinary caffeine concentrations exceeding a cut-off of 15 μg/ml. In 1985, the threshold was reduced to 12 μg/ml [92]. The cut-off value was chosen to exclude typical amounts ingested as part of common dietary or social coffee drinking patterns, and to differentiate it from what was considered to be an aberrant use of caffeine for the purpose of sports performance enhancement [93].

The IOC and WADA removed the classification of caffeine as a “controlled” substance in 2004, leading to a renewed interest in the use of caffeine by athletes. However, caffeine is still monitored by WADA, and athletes are encouraged to maintain a urine caffeine concentration below the limit 12 μg/ml urine which corresponds to 10 mg/kg body mass orally ingested, which is more than triple the intake reported to enhance performance [94, 95]. Interestingly, caffeine is also categorized as a banned substance by the National Collegiate Athletic Association (NCAA), if a urinary caffeine concentration exceeds 15 μg/ml, which is greater than the “monitored substance” level set for WADA [96], and also well above amounts that are deemed ergogenic.

A comparison made between results obtained in 2004 and results obtained before the removal of caffeine from the WADA doping list indicates that average caffeine concentrations decreased after the withdrawal of caffeine from the list of prohibited substances [92]. The overall percentage of positive samples between the two periods remained the same although the percentage of positive samples noticed in cycling increased after the removal of caffeine from the doping list [92]. Reports on over 20,000 urine samples collected and analyzed after official national and international competitions between 2004 and 2008, found overall prevalence of caffeine use across various sports to be about 3 out of 4 athletes, with the highest use among
endurance athletes [97]. Caffeine use in endurance sports continues to be widespread, and endurance activities also appear to be the focus for the bulk of caffeine-exercise research.

2.3 Caffeine Pharmacokinetics

Caffeine (1,3,7-trimethylxanthine) is an odorless white powder that is soluble in both water and lipids and has a bitter taste. It is rapidly absorbed from the gastrointestinal tract, mainly from the small intestine but also in the stomach [98]. In saliva, caffeine concentration reaches 65%–85% of plasma levels, and is often used to non-invasively monitor compliance for ingestion or abstinence of caffeine [99]. Caffeine is effectively distributed throughout the body by virtue of being sufficiently hydrophobic to allow easy passage through all biological membranes, including the blood-brain barrier [100]. When caffeine is consumed it appears in the blood rapidly and within several minutes, with peak caffeine plasma concentrations after oral administration reported to occur at times (T_{max}) ranging from of 30–120 min [79, 101-103]. Absolute bioavailability reaches near 100% as seen in studies reporting areas under the plasma concentration-time curves (AUC) [101]. Once caffeine is absorbed, there appears to be no hepatic first-pass effect (i.e., the liver does not appear to remove caffeine as it passes from the gut to the general circulation), as evidenced by similar plasma concentration curves when administered by either an oral or intravenous route [104]. Caffeine absorption from food and beverages does not seem to be dependent on age, gender, genetics or disease, or the consumption of drugs, alcohol or nicotine. However, the rates of caffeine metabolism and breakdown appear to differ between individuals through both environmental and genetic influences [42, 105, 106].
Over 95% of caffeine is metabolized in the liver by the Cytochrome P450 1A2 (CYP1A2) enzyme, a member of the cytochrome P450 mixed-function oxidase system, which metabolizes and detoxifies xenobiotics in the body [107]. CYP1A2 catalyzes the demethylation of caffeine into the primary metabolites paraxanthine (1,7-dimethylxanthine), theobromine (3,7-dimethylxanthine) and theophylline (1,3-dimethylxanthine), which account for approximately 80%, 11%, and 5%, respectively, of total caffeine elimination [23, 108]. These three caffeine metabolites undergo further demethylations and oxidation to urates in the liver with about 3-5% remaining in caffeine form when excreted in the urine [109, 110]. While the average half-life (t1/2) of caffeine is generally reported to be between 4 and 6 h, it may range from 1.5 to 10 h in adults [101]. The wide range of variability in caffeine metabolism is due to several factors. The rate of caffeine metabolism may be inhibited or decreased with pregnancy or use of hormonal contraceptives [106], increased or induced by cigarette smoking [24] or modified in either direction by certain dietary factors [111] and/or variation in the CYP1A2 gene, which will be discussed later [24, 25, 106, 111].

Several studies have also shown that the form of caffeine or its vehicle for entry into the body can modify the pharmacokinetics [100, 103, 112, 113]. One small trial evaluated T_{max} for a variety of beverages that included different volumes but all contained 160 mg of caffeine, and reported 0.5, 0.5 and 2 hr for coffee, tea and cola, respectively [114]. In another study, involving seven subjects, caffeine plasma concentrations peaked rapidly at 30 min for capsule form, whereas cola and chocolate absorption was delayed and produced lower plasma concentrations which peaked at roughly 90-120 min after treatment. That study also did not control for volume of administered solution (capsules and chocolate ingested with 360 ml water and 800 ml cola) [103]. Liguoria et al. evaluated a 400 mg dose of caffeine in 13 subjects and reported salivary
caffeine $T_{\text{max}}$ values of 42, 39 and 67 min, respectively, for coffee, sugar-free cola and capsules, although volume was again not standardized (coffee – 12 oz, sugar-free cola – 24 oz, capsules – volume of administered fluid not reported) [12]. The impact of temperature or rate of ingestion of caffeine has also been investigated, amidst concerns that cold energy drinks might pose a danger when chugged quickly, compared to sipping hot coffee. In a study [102] that compared five conditions, that included: slow ingestion (20 min) of hot coffee, and 2- or 20-min ingestion of cold coffee and energy drinks. Similar to other caffeine pharmacokinetic studies [103, 114], White et al reported that although rate of consumption, temperature and source (coffee vs energy drink) may be associated with slight differences in pharmacokinetic activity, these differences are small [102].

Chewing gum formulations appear to alter pharmacokinetics, as much of the caffeine released from the gum through mastication can be absorbed via the buccal cavity, which is considered faster due to its extensive vascularization, especially for low molecular weight hydrophobic agents [115]. Kamimori et al. [113] compared the rate of absorption and relative caffeine bioavailability from chewing gum compared to capsule form of caffeine. Although caffeine administered in the chewing gum formulation was absorbed at a significantly faster rate, the bioavailability was near comparable to that of the capsule formulation for the 100 and 200 mg caffeine dose groups. These pharmacokinetic findings are useful for military and sport purposes, where there is a requirement for rapid and maintained stimulation over specific periods of time. Chewing gum may also be advantageous due to reduced digestive requirements, where absorption of caffeine in other forms (capsule, coffee etc.) may be hindered by diminished splanchnic blood flow during moderate to intense exercise. Finally, the growing prevalence of caffeinated nasal and mouth aerosols administered directly in the mouth, under the tongue or
inspired may affect the brain more quickly through several mechanisms [44], but there are few studies to date to support this claim. The administration of caffeine inspired via aerosol into the oral cavity appears to produce a caffeine pharmacokinetic profile comparable to administration of a caffeinated beverage [112]. Nasal and mouth aerosols will be discussed further in another section.

2.4 Mechanism of Action
The effects of caffeine in reducing fatigue and increasing alertness and wakefulness through its stimulating properties is well recognized by those involved in activities or occupations where sustained energy expenditure or mental focus and vigilance is required [116, 117]. Most individuals involved in shift-work, long-haul trucking, the military or competitive athletics are very familiar with the properties of caffeine that aid in improving performance during long periods of sleep deprivation and physical exhaustion [117, 118].

Several mechanisms have been proposed to explain the ergogenic effects of caffeine, including increased myofibrillar calcium availability [119, 120], optimized exercise metabolism and substrate availability [80], and stimulation of the central nervous system (CNS) [121-123]. One of the earlier leading mechanisms associated with the ergogenic effects of caffeine stemmed from the observed adrenaline (epinephrine)-induced enhanced free-fatty acid (FFA) oxidation after caffeine ingestion and consequent glycogen sparing, resulting in improved endurance performance [54, 80, 124]. However, this substrate-availability hypothesis was challenged and eventually dismissed, where after several performance studies it became clear that the increased levels of FFAs appeared to be higher earlier in exercise when increased demand for fuel via fat oxidation would be not be expected [122, 125, 126]. This observation did not explain the consistent performance improvements in exercise of much longer duration (>60 min), where
glycogen becomes the limiting factor to continued exercise and greater reliance on FFAs as a fuel source would be necessary [127]. Importantly, several studies employing a variety of exercise modalities and intensities failed to show a decrease in respiratory exchange ratio (RER) and/or changes in serum FFAs which would be indicative of enhanced fat metabolism during exercise when only water was ingested [125, 128-131]. Ingestion of lower doses of caffeine (1–3 mg·kg\(^{-1}\) of body mass), which do not result in significant physiological responses (i.e. RER, changes in blood lactate, glucose), also appear to deliver measurable ergogenic effects, offering strong support for the central nervous system (CNS) as the origin of reported improvements [16, 79, 122, 132]. As such, focus has shifted to the action of caffeine within the central and peripheral nervous systems, which could alter the rate of perceived exertion (RPE) [133-138], and muscle pain [135, 139-141], and possibly the ability of skeletal muscle to generate force [135].

Caffeine does appear to have some direct effects on muscle which may contribute to its ergogenicity. The most likely pathway that caffeine may benefit muscle contraction is through calcium (Ca\(^{2+}\)) mobilization, which facilitates force production by each motor unit [119, 120, 132, 142]. Fatigue caused by the gradual reduction of Ca\(^{2+}\) release may be attenuated after caffeine ingestion [120, 143]. Similarly, caffeine may work, in part, in the periphery through increased Na\(^{+}/K^{+}\) pump activity to potentially enhance excitation contraction coupling necessary for muscle contraction [144].

Caffeine appears to employ its effects at various locations in the body, but the strongest evidence suggests that the main target is the CNS, which is now widely accepted as the main mechanism by which caffeine alters mental and physical performance [122]. Caffeine is believed to exert its
effects on the CNS via the antagonism of adenosine receptors, leading to increases in neurotransmitter release, motor unit firing rates, and pain suppression [135, 139-141, 145]. There are four distinct adenosine receptors, adenosine A₁, A₂A, A₂B and A₃, that have been cloned and characterized in several species [146]. Of these subtypes, A₁ and A₂A, which are highly concentrated in the brain, appear to be the major targets of caffeine [147]. Adenosine is involved in numerous processes and pathways, and plays a crucial role as a homeostatic regulator and neuromodulator in the nervous system [148]. The major known effects of adenosine are to decrease the concentration of many CNS neurotransmitters, including serotonin, dopamine, acetylcholine, norepinephrine and glutamate [147-149]. Caffeine, in turn, via adenosine receptor blockade, increases the concentration of these neurotransmitters [147, 149]. This results in positive effects on mood, vigilance, focus, and alertness in most, but not all, individuals [150, 151]. Researchers have also characterized aspects of adenosine A₂A receptor function related to cognitive processes [152] and motivation [153, 154]. In particular, several studies have focused on the functional significance of adenosine A₂A receptors and the interactions between adenosine and dopamine receptors, in relation to aspects of behavioral activation and effort-related processes [152-155].

The last decade has seen a growth in better understanding of CNS fatigue even though, historically, it is well-documented that “psychological factors” can affect exercise performance and that a dysfunction at any step in the continuum from the brain to the peripheral contractile machinery will result in muscular fatigue [156, 157]. Dopamine and serotonin have also been implicated in causing fatigue in the CNS, so it is not clear how, if caffeine increases all neurotransmitter concentrations, it could alleviate fatigue. One suggestion is that the caffeine-induced increases in excitatory neurotransmitters might dominate
those causing fatigue, with the stimulating characteristics overriding the latter, and leading to increased alertness and arousal to alleviate fatigue. Another possible explanation involves the existence of several types of adenosine, dopamine and serotonin receptors which may possess opposing actions [121, 158]. Increases in brain serotonin can have important effects on arousal, sleepiness, and mood that could be linked to altered perceptions of effort and muscular fatigue [159]. Animal studies have provided strong evidence that brain dopaminergic activity is necessary during physical activity and is likely to affect endurance performance [160]. Indeed, brain dopamine has been linked with increased arousal, motivation, muscular coordination, and increased endurance performance [160-162]. There is also some evidence of an association between increases in dopamine receptors and alertness, which suggests that caffeine might enhance arousal, in part, by upregulating dopamine receptors [36, 163].

The serotonin receptor 2A (5-HT2A) has been shown to modulate dopamine release, through mechanisms involving regulation of either dopamine synthesis or dopaminergic neuron firing rate [36, 164]. If caffeine acts by upregulating dopamine receptors and 5-HTR2A receptors have been shown to modulate dopamine release, then one possible mechanism is that alterations in 5-HTR2A receptors may therefore affect dopamine release and upregulation of dopamine receptors [163, 165]. A possible mechanism may involve 5-HTR2A receptors coupled with dopamine release and upregulation of dopamine receptors, which affect dopamine release and consequently impacts alertness, pain and motivation and effort [122].

Muscle pain has been shown to negatively affect motor unit recruitment and skeletal muscle force generation proportional to the subjective scores for pain intensity [166, 167]. In one study, progressively increased muscle pain intensity caused a gradual decrease of motor firing rates. However, this decrease is not associated with a change in motor unit membrane properties, and
therefore demonstrates a central inhibitory motor control mechanism with an efficacy correlated to the nociceptive activity [166]. Other studies also indicate that muscle force inhibition by muscle pain is centrally mediated [168]. Accordingly, caffeine-mediated CNS mechanisms, such as dopamine release [169], are likely imputable for pain mitigation during high intensity exercise [133, 139-141, 168, 170-172]. Although there appears to be strong evidence supporting the analgesic effects of caffeine during intense exercise, others have found no effect [133, 173].

The attenuation of pain during exercise as a result of caffeine supplementation may also result in a decrease in the rating of perceived exertion (RPE) during exercise. Acute caffeine ingestion has been shown to alter RPE, where effort may be greater under caffeine conditions, yet it is not perceived as such [4, 136-138]. A meta-analysis [4] identified 21 studies using mostly healthy male subjects (74%) between the ages of 20 and 35 years and showed a 5.6% reduction in RPE during exercise following caffeine ingestion. An average improvement in performance of 11% was reported across all exercise modalities [4]. Others have not found changes in RPE with caffeine use [173]. A more recent study by Green et al. [174] also showed that when subjects were instructed to cycle at specific RPE (effort) levels under caffeine conditions, the higher perceived intensity did not necessarily result in greater work and improved performance in all subjects equally. The authors noted that individual responses to the caffeine may explain their unexpected findings.

5-HTR2A receptors are encoded by the HTR2A gene, that serves as a primary target for serotonin signaling [30], and variations in the gene have been shown to affect 5-HTR2A receptor activity [175, 176]. This may therefore modulate dopamine activity, which may help to elucidate some of
the relationships among neurotransmitters, genetic variation and caffeine response, and the subsequent modifications to exercise performance.

The role of the CNS and its ‘motor drive’ effect was nicely shown by Davis et al. [177] who examined the effects of injections of caffeine directly into the brains of rats on their ability to run to exhaustion on a treadmill. In this controlled study, rats were injected with either vehicle (placebo), caffeine, 5’-N-Ethylcarboxamido adenosine (NECA), an adenosine receptor agonist, or caffeine NECA together. Rats ran 80 min in the placebo trial, 120 min in the caffeine trial and only 25 min with NECA. When caffeine and NECA were given together, those effects appeared to cancel each other out, and run time was similar to placebo. When the study was repeated with peripheral intraperitoneal (body cavity) injections instead of brain injections, there was no effect on run performance. The authors concluded that caffeine increased running time by delaying fatigue through CNS effects, in part by blocking adenosine receptors [177].

Caffeine also appears to enhance cognitive performance more in fatigued than well-rested subjects [178-180]. This phenomenon is also apparent in exercise performance [21] both in the field [181] and in the lab [16, 182].

2.5 Caffeine in Low Doses

Along with choosing to use alternate forms of caffeine (discussed in section 2.8) for convenience, personal preference or speed of delivery, the use of the low doses of caffeine is a fervent area of interest for both researchers and consumers.

Lower caffeine doses (2-4 mg/kg) have not only been shown to be ergogenic [12, 16], but may be more desirable in order to avoid the potential adverse side effects of higher doses (6-9 mg/kg), such as anxiety and agitation [183], and sleep disturbances [184], especially for athletes training
or competing at night. Caffeine at low doses (1-3 mg/kg) has been found to be just as effective at increasing endurance performance as moderate to high doses (6-9 mg/kg) in several ([16, 17, 94, 185]), but not all [186] studies. This finding, notwithstanding, low doses not exhibiting the same physiological responses found with moderate and high doses, such as increased epinephrine, norepinephrine, FFA and glycerol levels [79].

In one study [79], runners (n = 8) performed a running trial after consuming one of three different doses of caffeine (3, 6 or 9 mg/kg) or a placebo 1h before exercise. The highest dose of caffeine produced significantly higher levels of plasma FFA, but the moderate and low dose did not produce any significant changes in plasma FFA. Despite this, a significant increase in endurance performance was found following caffeine ingestion at low and moderate doses. This not only illustrates the effectiveness of lower doses but also supports the notion that the ergogenic effects of caffeine are unlikely to be as a result of substrate metabolism or glycogen sparing, but a function of its effects on the CNS. However, given that there were only 8 subjects in each of the 3 dose groups, inter-individual variability may be a cause for concern regarding the interpretation of the results.

2.6 Interindividual Variation in Response to Caffeine

Within the myriad of studies examining caffeine in endurance performance, the differences in benefits of caffeine between individuals do not appear to be influenced by sex, age, VO2 max, type of sport, or the dose of caffeine [5, 7, 21]. Nevertheless, while most exercise studies reliably show endurance performance enhancement with caffeine use [4-18], there is substantial inter-individual variability as to the magnitude of these effects [19, 20, 187-189].
For example, in a study of caffeine effects in runners, Graham et al. [8] reported that endurance benefits were associated with caffeine supplementation overall, but the magnitude of the improvements ranged from 5% to 87% and 10% to 156% in running and cycling time-to-exhaustion trials, respectively. Similarly, Doherty et al. [9] found that during treadmill running time-to-exhaustion, 9 out of 14 subjects improved, while 5 subjects did not, during the caffeine versus placebo trials. Wiles et al. [10] also found that the mean improvement during a 1-kilometer (km) cycling time trial was 3% overall under caffeine conditions, but individual results ranged from one subject performing worse to another improving their performance by 6%.

In contrast to most caffeine-performance studies, some investigations such as that by Roelands et al. [20] report no ergogenic effect of caffeine in a study involving trained male cyclists. The authors concluded that inter-individual differences in response to caffeine might be responsible for the lack of overall performance improvement, as 50% of subjects improved while 50% worsened, in the caffeine compared to the placebo trial. Similarly, Skinner et al. [187] found no effect of caffeine at 2, 4, or 6 mg/kg versus placebo in a rowing time trial, which may have been due, in part, to the large variation in individual response to caffeine. The authors noted that this consideration is often overlooked in caffeine performance studies, and due to infrequent reporting of individual data it is difficult to determine the extent to which variation in responses may be occurring. The performance of some individuals is often in stark contrast to the average findings reported, which may conclude beneficial, detrimental or no effect of caffeine on performance.
Genetics

Genetic variants affect the way we absorb, metabolize, utilize and excrete nutrients, and gene-diet interactions that affect metabolic pathways relevant to health and performance are now widely recognized [190]. Twin studies have also revealed a strong heritability (30-77%) for caffeine consumption behaviours [191], and several studies have associated genetic variation to habitual caffeine intakes [192-194]. In the field of nutrigenomics, caffeine is the most widely researched compound with several randomized controlled trials investigating the modifying effects of genetic variation on exercise performance (see Chapter 4 and 5; [15, 195-197]).

Numerous studies have investigated the effect of supplemental caffeine on exercise performance, but there is considerable inter-individual variability in the magnitude of these effects [3, 5, 8] or in the lack of an effect [19, 20] when compared to placebo. These inter-individual differences appear to be partly due to variations in genes such as \textit{CYP1A2} and possibly \textit{ADORA2} and \textit{HTR2A} which are directly or indirectly associated with caffeine metabolism or general caffeine response [22].

Up to 95% of ingested caffeine is metabolized by the \textit{CYP1A2} enzyme, which is encoded by the \textit{CYP1A2} gene and is involved in the demethylation of caffeine into the primary metabolites paraxanthine, theophylline and theobromine [23]. The -163A>C (rs762551) single nucleotide polymorphism (SNP) has been shown to alter \textit{CYP1A2} enzyme inducibility and activity [24, 25], and has been used to categorize individuals as ‘fast’ or ‘slow’ metabolizers of caffeine. Individuals with the AC or CC genotype (slow metabolizers) have an elevated risk of myocardial infarction [26], hypertension and elevated blood pressure [27, 198], and pre-diabetes [28], with increasing caffeinated coffee consumption, whereas those with the AA genotype show
no such risk. Additionally, regular physical activity appears to attenuate the increase in blood pressure induced by caffeine ingestion, but only in individuals with the AA genotype [198].

The first caffeine, exercise and genetics study [15] examined the effects of caffeine and CYP1A2 genotype in trained male cyclists. Subjects cycled through two 40-km time trials, following consumption of either 6 mg/kg of caffeine or placebo 60 min beforehand. There was a significant caffeine-CYP1A2 interaction where AA genotypes (fast metabolizers) improved by 4.9%, which was a significantly greater performance improvement than the 1.8% reported in C-allele carriers (slow metabolizers). In those with AA genotypes, caffeine improved cycling performance by at least 1 min for 15 out of 16 subjects, while in C allele carriers only 10 of 19 subjects improved by ≥1 min. The 4.9% is greater than the 3% consistent improvements seen in previous studies using similar doses [5, 12].

Some later endurance-type studies either did not observe any impact of the CYP1A2 gene on caffeine-exercise studies [29, 199], or reported benefits only in slow metabolizers [195]. There are several reasons that may explain discrepancies in study outcomes including smaller sample sizes (<20 subjects) that cause very low numbers and/or no subjects with the CC genotype [195, 199, 200], and shorter distance or different type (power versus endurance) of performance test [195], compared to those that reported improved endurance after caffeine ingestion in those with the AA genotype of CYP1A2 [15]. The effects of genotype on performance appear to be most prominent during exercise of longer duration or an accumulation of fatigue (aerobic or muscular endurance) [7, 21]. Fast metabolizers may quickly metabolize caffeine and achieve the benefits of caffeine metabolites as exercise progresses, or override the short duration of negative impacts (the initial stages of exercise), whereas the adverse effects of restricted blood flow and/or other
impacts of adenosine blockage in slow metabolizers are likely to remain for a longer duration [201, 202]. Indeed, in a study of basketball performance in elite players, caffeine improved repeated jumps (muscular endurance), but only in those with the AA genotype, however, there was no genotype effect in the other two performance components of the basketball simulation [203]. Similarly, a cross-over design of 30 resistance-trained men found that caffeine ingestion resulted in a higher number of repetitions in repeated sets of three different exercises, and for total repetitions in all resistance exercises combined, which resulted in a greater volume of work compared to placebo conditions, but only in those with the CYP1A2 AA genotype [197]. Taken together, the weight of the evidence does provide growing support for the role of CYP1A2 in modifying the effects of caffeine ingestion on aerobic or muscular endurance-type exercise, although additional studies are warranted.

The ADORA2A gene is another potential genetic modifier of the effects of caffeine on performance. The adenosine A2A receptor, encoded by the ADORA2A gene, has been shown to regulate myocardial oxygen demand and increase coronary circulation by vasodilation [201, 202]. The A2A receptor is also expressed in the brain, where it has significant roles in the regulation of glutamate and dopamine release, with associated effects on insomnia and pain [204, 205]. The antagonism of adenosine receptors after caffeine ingestion is modified by the ADORA2A gene, which may allow greater improvements in dopamine transmission in some genotypes versus others [152]. Dopamine has been associated with motivation and effort in exercising individuals, and this may be the mechanism by which differences in response to caffeine are manifested [122, 152, 153]. As previously mentioned, dopamine and serotonin have also been implicated in causing fatigue in the CNS, therefore polymorphisms associated with dopamine and serotonin receptors are also of interest. The serotonin receptor 2A (5-HT2A),
encoded by the *HTR2A* gene, has been shown to modulate dopamine release, through mechanisms involving regulation of either dopamine synthesis or dopaminergic neuron firing rate [36, 164]. 5-HT2A receptors in smooth muscle cells have also been linked to vasoconstriction [37] and may also be implicated in the variability in response to caffeine during exercise.

If caffeine acts by upregulating dopamine receptors, and 5-HTR2A receptors have been shown to modulate dopamine release, then one possible mechanism is that alterations in 5-HTR2A receptors may therefore affect dopamine release and upregulation of dopamine receptors [163, 165]. Therefore 5-HTR2A receptors coupled with dopamine release and upregulation of dopamine receptors (which also affect dopamine release), may consequently impacts alertness, pain and motivation and effort [122]. Although, to date, no studies have investigated 5-HT2A receptors and caffeine ingestion during exercise, 5-HT2A receptors have been associated with blood pressure and vasoconstriction [31-35], dopamine release [36] and various aspects of behaviour such as motivation, mood, movement and pain perception [37-39]. In addition, a common polymorphism in the *HTR2A* gene (rs6313) has been associated with behavior and mood disorders [49, 50] and cardiovascular disease [51].

Currently, only one small pilot study has examined the effect of the *ADOR2A* gene (rs5751876) on the ergogenic effects of caffeine under exercise conditions [206]. Twelve female subjects underwent a double-blinded, crossover trial comprising two 10-min cycling time trials following caffeine ingestion or placebo. Caffeine benefitted all six subjects with the TT genotype but only one of the six C allele carriers. Further studies are needed to confirm these preliminary findings and include a larger sample to distinguish any effects between the different C allele carriers (ie. CT vs CC genotypes).
The ADORA2A rs5751876 genotype has also been implicated, by both objective and subjective measures, in various parameters of sleep quality, after caffeine ingestion, in several studies [207-210]. Adenosine promotes sleep by binding to its receptors in the brain, mainly A1 and A2A receptors, and caffeine exerts an antagonist effect, blocking this receptor and reversing the effects of adenosine and promoting wakefulness [207]. This action, as well as the potency of caffeine to restore performance (cognitive or physical) in ecological situations, such as highway-driving during the night [211], support the notion that the adenosine neuromodulator/receptor system is significantly involved in sleep–wake regulation. This action of caffeine may also serve athletes well under conditions of jetlag, and irregular or early training or competition schedules. Psychomotor speed relies on the ability to respond, rapidly and reliably, to randomly occurring stimuli which is a critical component of and characteristic of most sports [212]. Genetic variation in ADORA2A has been shown to be a relevant determinant of psychomotor vigilance in the rested and sleep-deprived state and modulates individual responses to caffeine after sleep deprivation [209]. In support of this notion, individuals who had the TT genotype for ADORA2A rs5751876 consistently had faster response times (in seconds) than C allele carriers after ingesting 400 mg caffeine during a sustained vigilant attention task after sleep loss [209].

The ADORA2A genotype has also been implicated in sleep quality. Consistent with the “adenosine hypothesis” of sleep, where the accumulation of adenosine in the brain increases sleep propensity, caffeine prolongs time to fall asleep, decreases the deep stages of non-rapid-eye movement (nonREM) sleep, reduces sleep efficiency, and alters the waking and sleep electroencephalogram (EEG) in frequencies, which reliably reflect the need for sleep [213-215]. Increased beta activity in nonREM sleep may characterize individuals with insomnia when compared with healthy good sleepers [216]. A functional relationship between the ADORA2A
genotype and the effect of caffeine on EEG beta activity in nonREM sleep have previously been reported [208], where the highest rise was in individuals with the CC genotype, approximately half in the CT genotype, whereas no change was present in the TT genotype. Consistent with this observation, in the same study, individuals with the CC and TC genotypes appeared to confer greater sensitivity towards caffeine-induced sleep disturbance compared to the TT genotype [208]. Taken together, it appears that individuals with the TT genotype for the rs5751876 SNP in the \textit{ADORA2A} gene may have better performance outcomes, faster response times and less sleep disturbance following caffeine ingestion.

\textit{Habitual Caffeine Intake}

The quantification of habitual caffeine intake is difficult, which is problematic for studies aiming to compare performance outcomes in habitual versus nonhabitual caffeine users. This concern is highlighted by reports showing large variability in the caffeine content of commonly consumed beverages, e.g. ~8- to 9-fold differences in caffeine content have been reported in coffee beverages purchased from similar retail shops [217] and in pre-workout supplements (PWS) [218]. Self-reported intakes may therefore be unreliable. Newly discovered biomarkers of coffee consumption may be more useful for quantifying intakes in the future, but currently, these are not widely available [219]. Different protocols for the length of the caffeine abstinence period preceding data collection is also a relevant factor in determining variability in performance outcomes. For example, in shorter caffeine abstinence periods e.g., 12-48 hrs, reversal of caffeine withdrawal effects may have positive effects on performance, i.e. alleviating the negative symptoms of withdrawal, which in itself may improve performance [220]. These
effects may be more pronounced in those genetically predisposed to severe withdrawal effects [221]. However, in one study 3 mg/kg caffeine significantly improved exercise performance in trained cyclists (n = 12) irrespective of whether a 4-day withdrawal period is imposed on habitual caffeine users [222]. Another study also reported increased endurance in habitual caffeine users (n = 6) regardless of a 0, 2- or 4-day abstinence period. The authors concluded that improved performance under caffeine conditions at 6 mg/kg is not related to prior caffeine habituation in recreational athletes [130].

Genes have been associated with habitual caffeine intake using GWAS research [193, 223], but it is important to highlight that these associations are not directly applicable to determining differences in performance outcomes in response to acute caffeine doses for regular or habitual caffeine users versus non-habitual users. Furthermore, associations between genes and habitual caffeine intake do not elucidate potential mechanisms by which caffeine intake behaviours may influence subsequent performance under caffeinated conditions [192, 194]. In animal studies, regular consumption of caffeine has been associated with an upregulation of the number of adenosine receptors in the vascular and neural tissues of the brain [224]. Although this did not appear to modify the effects of caffeine in one study [225], in another, chronic caffeine ingestion by mice caused a marked reduction in locomotor exploratory activity [226]. Changes in adenosine receptor number or activity has not been studied in humans.

There does not appear to be a consistent difference in the performance effects of acute caffeine ingestion between habitual and non-habitual caffeine users, and study findings remain equivocal. In one study, habitual stimulation from caffeine resulted in a general dampening of the epinephrine response to both caffeine and exercise, however, there was no evidence that this
impacted exercise performance [227]. Another study [228] examined the effect of 4 weeks of caffeine supplementation on endurance performance in eighteen low-habitual caffeine consumers who were randomly assigned to ingest caffeine or placebo for 28 days. Four weeks of caffeine ingestion resulted in an increased tolerance to acute caffeine supplementation in previously low habitual caffeine consumers, with the ergogenic effect of acute caffeine supplementation no longer apparent [228]. More recently, a double-blind, crossover, counterbalanced study was performed [229], where forty male endurance-trained cyclists were allocated into tertiles according to their daily caffeine intake: low (58 ± 29 mg), moderate (143 ± 25 mg), and high consumers (351 ± 139 mg). Participants completed three trials in which they performed simulated cycling time-trials under three conditions: caffeine (CAF: 6 mg/kg), placebo (PLA), and no supplement (CON). No correlation was observed between habitual caffeine intake and absolute changes (CAF – CON) in a ~30 min cycling time-trial performance with caffeine [229]. However, a major limitation of this study is the short 24-h caffeine withdrawal period in all groups which may have resulted in performance improvements due to the reversal of caffeine withdrawal effects, rather than impact of acute-on-chronic caffeine administration and the effects of habituation to caffeine on exercise performance [220, 230]. In addition, dependence on subject recall and the use of foreign food frequency questionnaires, where products differ greatly between countries, raise concerns. As mentioned earlier, there is wide variability in caffeine content of commonly consumed items, and as such, an objective measure (e.g., caffeine or metabolite levels) should be used to reported caffeine intakes [219, 230].

Based on these observations, the assumption that habitual and nonhabitual caffeine consumers will or will not respond differently to caffeine supplementation during exercise, requires further study.
Caffeine Timing

Caffeine appears to be most beneficial during times or in sports where there is an accumulation of fatigue. Therefore, ingestion of caffeine during exercise (mid/later stages) may be more beneficial than ingestion beforehand for some individuals.

A recent review [21] reported that the effect size of caffeine increases along with the increasing duration of the time trial event. This supports the notion that endurance athletes may benefit most from caffeine for performance enhancement. This also supports findings in other investigations that show ingesting caffeine at various time points including late in exercise, may be most beneficial [181].

For example, an early study [181] aimed to understand whether or not there were benefits to a common practice among endurance athletes, such as those participating in marathons and triathlons, which is to drink flat cola toward the end of an event. When researchers investigated the ingestion of a low dose of caffeine toward the end of a race (e.g., in the form of flat coke) it was found to have comparable effects as ingesting higher doses, such as ~5 or 6 mg/kg, ingested ~60 min before the race. The study also demonstrated that the effect was due to the caffeine and not the carbohydrate, which may also aid performance as fuel stores become depleted [181].

More recently, caffeine enhanced cycling performance when it was administered immediately prior to exercise, but not when administered 1 or 2 hours beforehand. This may have been due to
the continued increase in plasma caffeine concentrations during the cycling time trial, when athletes may become fatigued (i.e. 30 + minutes into exercise), as the trials also included a 15 min steady state cycling bout prior to the time trial [182]. Similarly, in a lab setting, a study of athletes (n = 15) completing 120 min of steady state cycling followed by a time trial under conditions of placebo and caffeine, found that the ingestion of both low and moderate doses of caffeine later in exercise was beneficial [16]. In contrast, another study [231] concluded that caffeine administered to athletes (n = 9) in the morning improved cycle time to exhaustion in the morning and evening, and subsequent dosing throughout the day was unnecessary to improve cycling performance in the evening.

Several of these studies showed significant interindividual variability, highlighting the need for more research to investigate individual responses. In the meantime, athletes are required to experiment with their own strategies as far as dosing and timing is concerned.

*Training Status*

Training status may mediate the magnitude of caffeine’s ergogenic effect, but studies have reported mixed results. Although a 2010 meta-analysis [142] did not find differences (p=0.08) in caffeine’s ability to enhance muscle endurance in untrained subjects more in than trained subjects, results were not derived from direct comparisons between trained and untrained subjects. Currently, only a few investigations [232-237] have included both trained and untrained subjects in their study design.
In a study of elite and occasional swimmers [236], it was reported that 250 mg of supplemental caffeine was ergogenic only for competitive swimmers and not recreational swimmers during repeated 100 m races. Although it is unknown what trait of trained subjects elicits these effects. Some have postulated that this is because athletes perform more reliably on a given task than nonathletes, which improves ability to detect small differences between treatments [238]. This may also be possibly due to the technical nature of swimming as untrained swimmers may have not been unable to take full advantage of potential improvements in whole muscle function via caffeine supplementation. Study authors also suggested that the intra- or extra-cellular adaptations to buffer H+, from specific training, are necessary to benefit from caffeine during swimming sprint performance [236].

In contrast to the above evidence regarding the importance of training status, other research has shown no effect. One study [237] showed similar performance improvements (1.0% and 1.1%) in 15 well-trained and 15 recreational runners performing an outdoor 5 km time trial after 5 mg / kg caffeine intake compared to the placebo trial. Similarly, Astorino et al. [234] found that overall, acute caffeine intake improved 10 km time-trial performance in both endurance-trained athletes and active men, with no differences seen between groups. Likewise, an investigation concluded that there was no ergogenic effect of caffeine at a dose of 5 mg/ kg on time to exhaustion in either endurance trained or untrained men [232].

More recently, Boyett et al. [235] investigated the interactions of 6 mg / kg caffeine on training status to time of day in twenty male subjects. Subjects completed four experimental trials consisting of a 3-km cycling time trial that were performed in randomized order for each combination of time of day (morning and evening) and treatment. They reported that both
untrained and trained subjects improved performance with caffeine supplementation in the morning; however only the untrained subjects improved when tested in the evening. These observations indicate that trained athletes are more likely to experience ergogenic effects from caffeine in the morning, while untrained individuals appear to receive larger gains from caffeine in the evening than their trained counterparts. This may further complicate the training status data with a possible temporal effect [235]. The concentration of adenosine receptors (the primary target of caffeine) do appear to be higher in trained compared to untrained individuals), but this has only been reported in animal studies [239]. Boyett et al. [235] speculated that the higher concentration of adenosine receptors may increase tissue sensitivity to any given concentration of adenosine.

Although some studies comparing training status of subjects support the notion [236] that training influences response to caffeine during exercise, most do not [232, 234, 237] and this was also the finding in a subsequent meta-analysis [142]. More research in this area is warranted.

2.7 Alternative Caffeine Sources

Sources other than commonly consumed coffee and caffeine tablets have garnered interest, including caffeinated chewing gum, mouth rinses, aerosols, inspired powders, energy bars, energy gels and chews, among others. While the pharmacokinetics [54, 240-243] and effects of caffeine on performance when consumed in a traditional manner, such as coffee [6, 83, 89, 137, 244-246] or as a caffeine capsule with fluid [89, 247-249] are well understood, curiosity in alternate forms of delivery (as outlined in pharmacokinetics section) have emerged due to interest in speed of delivery [44, 112].
**Caffeinated Chewing Gum**

Several investigations have suggested that delivering caffeine in chewing gum form may speed the rate of caffeine delivery to the blood via absorption through the extremely vascular buccal cavity [113, 250]. Therefore, caffeine via chewing gum may be absorbed via two passageways: the buccal mucosa in the oral cavity and/or gut absorption due to the swallowing of caffeine-containing saliva [113, 250, 251]. Kamimori and colleagues [113] compared the rate of absorption and relative caffeine bioavailability from caffeinated chewing gum and caffeine in capsule form. The results suggest that the rate of drug absorption from the gum formulation was significantly faster, and both gum and capsule formulations provided near comparable plasma caffeine concentrations to the systemic circulation. These findings suggest that there may be an earlier onset of pharmacological effects from caffeine delivered through the gum formulation. Increasing absorption via the buccal cavity may be preferential over oral delivery if consumed closer to or during exercise, as splanchnic blood flow is often reduced [252], potentially slowing the rate of caffeine absorption.

Several studies [14, 182, 253-255] have examined the potential ergogenic impact of caffeinated chewing gum on aerobic performance, commonly administered in multiple sticks. To note, all studies have been conducted using cycling interventions, with the majority conducted in well-trained cyclists. Results from these investigations suggest that caffeinated chewing gum delivered in total dosages ranging 200 – 300 mg, closer to initiation of exercise or during a prolonged endurance event may be most beneficial, specifically for individuals with a higher training status. However, more research is needed, especially in physically active and recreationally training individuals.
In anaerobic-type exercise, Paton et al. [256] administered 3-mg/kg caffeinated gum to male cyclists during repeat sprint cycling, resulting in greater attenuation of fatigue, compared to a placebo. The reduced fatigue in the caffeine trials equated to a 5.4% performance enhancement in power during sprints, in favor of caffeinated gum. Lastly, caffeinated gum positively influenced performance in two out of three soccer-specific (Yo-Yo Intermittent Recovery Test and CMJ) tests used in the assessment of performance in soccer players [257]. Additionally, to date, only Bellar et al. [258] has examined chewing gum with caffeine on cognitive function, specifically reporting improved alertness as assessed by a psychomotor vigilance test.

**Caffeine Mouth Rinsing**

Caffeine mouth rinsing (CMR; 5 – 20 seconds in duration) may have the potential to enhance exercise performance due to the activation of sensorimotor brain cortices [259]. Specifically, the mouth contains bitter taste sensory receptors that are sensitive to caffeine [260]. It has been proposed that activation of these bitter taste receptors may activate neural pathways associated with information processing and reward within the brain [260-262]. Further, physiologically, caffeinated mouth rinsing may reduce gastrointestinal distress potential [263, 264].

Several investigations on aerobic [195, 265-268] performance, as well as cognitive function [269, 270] and performance [271], following CMR have been conducted to date. One study [267] demonstrated ergogenic benefits of CMR on aerobic performance, reporting significant increases in distance covered during a 30 minute arm crank time trial performance. Likewise, in a separate study [266], a 5 second CMR (containing 32 mg of caffeine dissolved in 125ml water) improved 30 minute cycling performance, without concurrent increases in ratings of perceived
exertion or heart rate. CMR appears to be ergogenic in cycling to include both longer, lower-intensity and shorter high-intensity, protocols.

**Caffeinated Nasal Sprays and Inspired Powders**

The use of caffeinated nasal sprays and inspired powders are of interest. Three mechanisms of action have been hypothesized for caffeinated nasal sprays. Firstly, the nasal mucosa is permeable, making the nasal cavity a potential route for local and systemic substance delivery; particularly for caffeine, a small molecular compound (11, 12, 30, 31). Secondly, and similar to CMR, bitter taste receptors are located in the nasal cavity. Use of a nasal spray may allow for the upregulation of brain activity associated with reward and information processing [272]. Thirdly, but often questioned due to it’s unknown time-course of action, caffeine could potentially be transported directly from the nasal cavity to the central nervous system, specifically the cerebrospinal fluid and brain by intracellular axonal transport through two specific neural pathways, the olfactory and trigeminal [273, 274].

In two separate trials [259, 275], the effects of caffeinated nasal sprays containing 15 mg of caffeine per mL were examined, and no improvements were reported in either anaerobic or aerobic performance outcome measures [259]. Laizure et al. [112] compared the bioavailability and plasma concentrations of 100mg caffeine delivered via an inspired powder (AeroShot) and an oral solution. Both were found to have similar bioavailability and comparable plasma concentrations with no differences in heart rate or blood pressure.
2.8 Caffeine and Endurance Performance

Less than a 1% change in average speed is enough to affect medal rankings in intense Olympic endurance events lasting ~45 s to 8 min [247]. In other events, such as the men’s individual road race, the difference between the top three medalists was <0.01% [276]. At the highest level of sports, competitors will all be near their genetical potential, will have trained intensively, followed prudent recovery protocols, and will have exploited all strategies to improve their performance. The use of an ergogenic aid, when legal, safe and effective, is an alluring opportunity.

Caffeine has consistently been shown to improve endurance by 2-4% across dozens of studies using doses of 3-6 mg/kg body mass [5, 12, 18, 21, 277]. Accordingly, caffeine is one of the most prominent ergogenic aids and is used by athletes and active individuals in a wide variety of sports and activities. Most research focuses on endurance-type exercise, as this is the area in which caffeine supplementation appears to be most widely used and most likely beneficial in most, but not all, athletes [3-5]. For example, the caffeine concentration in over twenty thousand urine samples obtained for doping control from 2004 to 2008 was measured after official national and international competitions [92, 97]. The investigations concluded that roughly 74% of elite athletes used caffeine as an ergogenic aid prior to or during a sporting event, where endurance sports are the disciplines showing the highest urine caffeine excretion (and therefore prevalence) after competition [92, 97].

More recently, a meta-analysis reporting on 56 endurance time trials in athletes (79% cycling), found the percent difference between the caffeine and placebo group ranged from −3.0% to 15.9% [21]. This wide range in performance outcomes highlights the substantial inter-individual
variability in the magnitude of caffeine’s effects as reported. These inter-individual differences might be partly due to variation in genes that are associated with caffeine metabolism and caffeine response [22]. Studies that report on the effects of caffeine on performance have been inconsistent, therefore identifying potential difference due to genetic variation warrants investigation.

Chapter 3

Rationale and Objectives
3 Rationale, Hypotheses and Objectives

3.1 Rationale

Sport and exercise performance are significantly influenced by nutrition, yet individuals respond differently to the same foods, nutrients and supplements. Although there is a growing foundation of research linking gene-diet interactions on biomarkers of nutritional status, which impact athletic performance, there have been few randomized controlled trials examining the effects of genetic variation on performance in response to an ergogenic aid. Building this foundation will form the basis from which the field of sport nutrigenomics and personalized nutrition continues to develop.

Caffeine has widespread use in athletics in the form of coffee, tablets, energy drinks and “pre-workouts”, based on the belief that athletes can train harder or compete more successfully after ingesting caffeine. However, it appears that caffeine does not benefit all athletes and may in fact impair performance in some. Studies on caffeine and performance generally do not explore the basis for the interindividual variation in response, which has been well-documented in several studies. These differing performance outcomes might be due, in part, to interindividual differences in caffeine metabolism or caffeine response, as determined by known, unknown and plausible genetic modifiers of caffeine. Polymorphisms in the CYP1A2 and HTR2A genes may modifier an athlete’s response to caffeine and could explain inter-individual variability in performance under caffeine conditions. The CYP1A2 rs762551 polymorphism has been shown to modify endurance performance in cyclists, where only individuals with the AA genotype (fast metabolizers) showed significant improvements under caffeine conditions compared to placebo. The HTR2A rs6313
polymorphism has not yet been investigated, but it is a plausible modifier of caffeine response due to its effects on cardiovascular system, particularly blood flow, as well as potential mediating effects of dopamine action.

Providing athletes with individually tailored dietary and other performance-related information based on their DNA could yield a competitive edge and is of keen interest to sport dietitians and the sports community as a whole.

3.2 Overall Hypothesis

Genetic variation in caffeine metabolism or caffeine mediated neuro-signaling pathways modifies the ergogenic effects of caffeine in athletes. Individuals who are either CYP1A2 AA fast metabolizers of caffeine or HTR2A CC genotype will performance better under caffeine conditions.

3.3 Objectives

The overall objective of this study was to replicate previous reports showing ergogenic benefits of caffeine use in endurance performance, and to determine if known and plausible CYP1A2 and HTR2A genetic modifiers of caffeine would explain inter-individual variability in performance under caffeine conditions.

The specific objectives were to determine:

1. The effects of low (2 mg/kg) or moderate (4 mg/kg) doses of caffeine supplementation on endurance performance.
2. Whether variation in the \textit{CYP1A2} gene, which affects caffeine metabolism, modifies the ergogenic effects of caffeine in a 10-km cycling time trial.

3. Whether variation in the \textit{HTR2A} gene, which affects serotonin receptors, modifies the ergogenic effects of caffeine in a 10-km cycling time trial.

4. Whether variation in the \textit{HTR2A} gene within \textit{CYP1A2} genotypes, i.e. fast and slow metabolizers, modifies the ergogenic effects of caffeine in a 10-km cycling time trial.
Chapter 4

CAFFEINE, CYP1A2 GENOTYPE AND ENDURANCE PERFORMANCE IN ATHLETES

Adapted from:

4 Caffeine, CYP1A2 genotype and endurance performance in athletes

4.1 Abstract

**Purpose:** Many studies have examined the effect of caffeine on exercise performance, but findings have not always been consistent. The objective of this study was to determine whether variation in the CYP1A2 gene, which affects caffeine metabolism, modifies the ergogenic effects of caffeine in a 10-km cycling time trial. **Methods:** Competitive male athletes (n=101; age: 25 ± 4 years) completed the time trial under three conditions: 0, 2 or 4 mg of caffeine per kg body mass, using a split-plot randomized, double-blinded, placebo-controlled design. DNA was isolated from saliva and genotyped for the -163A>C polymorphism in the CYP1A2 gene (rs762551). **Results:** Overall, 4 mg/kg caffeine decreased cycling time by 3% (mean ± SEM) versus placebo (17.6 ± 0.1 vs. 18.1 ± 0.1 min, \( p = 0.01 \)). However, a significant \( p <0.0001 \) caffeine-gene interaction was observed. Among those with the AA genotype, cycling time decreased by 4.8% at 2 mg/kg (17.0 ± 0.3 vs. 17.8 ± 0.4 min, \( p = 0.0005 \)) and by 6.8% at 4 mg/kg (16.6 ± 0.3 vs. 17.8 ± 0.4 min, \( p < .0001 \)). In those with the CC genotype, 4 mg/kg increased cycling time by 13.7% versus placebo (20.8 ± 0.8 vs. 18.3 ± 0.5 min, \( p = 0.04 \)). No effects were observed among those with the AC genotype. **Conclusion:** Our findings show that both 2 and 4 mg/kg caffeine improve 10-km cycling time, but only in those with the AA genotype. Caffeine had no effect in those with the AC genotype and diminished performance at 4 mg/kg in those with the CC genotype. CYP1A2 genotype should be considered when deciding whether an athlete should use caffeine for enhancing endurance performance.
4.2 Introduction

Caffeine is frequently used by athletes because of its reported performance-enhancing or ergogenic effects [4-18]. Numerous studies have investigated the effect of supplemental caffeine on aerobic endurance performance, and while most reliably show performance enhancement with caffeine use [4-18], there is considerable inter-individual variability as to the magnitude of these effects [19, 20, 187-189]. For example, in a study of caffeine effects in runners, Graham et al. [8] reported that endurance benefits were associated with caffeine supplementation overall, but the magnitude of the improvements ranged from 5% to 87% and 10% to 156% in running and cycling time-to-exhaustion trials, respectively. Similarly, Doherty et al. [9] found that during treadmill running time-to-exhaustion, 9 out of 14 subjects improved, while 5 subjects did not, during the caffeine versus placebo trials. Wiles et al. [10] also found that the mean improvement during a 1-kilometer (km) cycling time trial was 3% overall under caffeine conditions, but individual results ranged from one subject performing worse to another improving their performance by 6%.

In contrast to most caffeine-performance studies, no ergogenic effect of caffeine was reported by Roelands et al. [20] in a study involving trained male cyclists. The authors concluded that inter-individual differences in response to caffeine might be responsible for the lack of overall performance improvement, as 50% of subjects improved while 50% worsened, in the caffeine compared to the placebo trial. Similarly, Skinner et al. [187] found no effect of caffeine at 2, 4, or 6 mg/kg versus placebo in a rowing time trial, which may have been due, in part, to the large variation in individual response to caffeine. The authors noted that this consideration is often overlooked in caffeine performance studies, and due to infrequent reporting of individual data it is difficult to determine the extent to which variation in responses may be occurring. The
performance of some individuals is often in stark contrast to the average findings reported, which may conclude beneficial, detrimental or no effect of caffeine on performance.

Studies that report on the effects of caffeine on performance have been inconsistent despite having similar study designs, subjects and dose of caffeine. These inconsistencies might be due, in part, to inter-individual differences in caffeine metabolism or caffeine response. Over 95% of caffeine is metabolized by the CYP1A2 enzyme, which is encoded by the CYP1A2 gene, and is involved in the demethylation of caffeine into the primary metabolites paraxanthine, theophylline and theobromine [23]. The -163A>C (rs762551) single nucleotide polymorphism (SNP) has been shown to alter CYP1A2 enzyme inducibility and activity [24, 25], and has been used to categorize individuals as ‘fast’ or ‘slow’ metabolizers of caffeine. Individuals with the AC or CC genotype (slow metabolizers) have an elevated risk of myocardial infarction [26], hypertension [27], and pre-diabetes [28] with increasing caffeinated coffee consumption, whereas those with the AA genotype show no such risk. In addition, a few studies have shown that the rate of caffeine metabolism could also have implications for sports performance, but the findings remain equivocal [15, 29, 195, 199].

The objective of this study was to determine the effects of low (2 mg/kg) or moderate (4 mg/kg) doses of caffeine supplementation on endurance performance, and whether variation in the CYP1A2 gene modifies these effects among competitive male athletes recruited from a variety of sports.
4.3 Methods

4.3.1 Subjects and Recruitment.

Recruitment was carried out at the University of Toronto, Ryerson and York University campuses, the Canadian Sport Institute of Ontario, local running/triathlon clubs and training gyms using posted flyers. A standardized email with study details and contact information was also sent to head coaches, program directors of sports teams or clubs, and some professional sport organizations with eligible athletes. Ethics approval was obtained from the University of Toronto Institutional Review Board, and the study was registered with clinicaltrials.gov (NCT 02109783). All subjects provided written informed consent and were informed that they could terminate their participation in the study at any time.

A total of 113 competitive male athletes from a variety of sports participated in the present study. Subjects were recruited from a wide range of sports that could be classified into three categories: endurance (e.g. marathon, triathlon, cycling, cross-country skiing), power (e.g. boxing, volleyball, dragon-boat, powerlifting) or mixed (e.g. soccer, rugby, basketball, swimming). All participants were training and/or competing for ≥ 8 hr per week, 9 out of 12 months per year, and for at least 3 years in their given sport. Eight athletes dropped out of the study due to a sport-related injury (n=3), school or work demands (n=2), unwillingness to abstain from caffeine (n=2), or relocation (n=1). Four subjects were excluded because of incomplete data. The remaining 101 athletes had a mean ± SD age of 25 ± 4 years and body mass of 81.3 ± 12.4 kg.

4.3.2 Experimental Design.

A split-plot randomized, double-blinded, placebo-controlled study design was used. Subjects completed 4 visits (~90-120 min each) that were approximately 1 week apart, in the exercise
laboratory at the Goldring Centre for High Performance Sport at the University of Toronto.

During the first laboratory visit, each subject had descriptive and anthropometric data collected, completed a maximal aerobic capacity test (VO$_2$-peak) and completed a questionnaire on general health, caffeine intake habits, and sport history. Subjects also provided a saliva sample for DNA analysis. Testing took place on weekdays and weekends, and the treatment visits were scheduled at the same time of day, every 7 days, for each athlete. Participants were instructed to maintain their regular diet and sleeping habits, avoid strenuous activity 48 hours before each visit, and abstain from caffeine one week prior to the first visit and for the duration of the data collection (4 weeks total). To ensure dietary consistency prior to testing across all visits, participants were advised on their first visit to consume meal(s) that could be easily replicated for all subsequent treatment visits. Participants were also reminded of their required meal composition by email or text message one day prior to each visit. On treatment visits 2-4, subjects were randomly assigned to ingest capsules containing either anhydrous caffeine (American Chemicals Ltd, Montreal, Quebec) at 2 or 4 mg/kg body mass or placebo (PLAC). The PLAC (dextrose) capsule was tasteless and had the same volume and color as the caffeine. After ingestion, the subjects sat quietly (completing questionnaires or using e-devices) in the laboratory for 25 minutes before commencing their warm-up and four exercise tests. Blood pressure and heart rate were measured after capsule ingestion and 3 minutes of sitting quietly, and again 20 min later, just prior to warm up. This protocol was repeated three times; one for each treatment (0, 2 or 4 mg/kg caffeine).

4.3.3 Parameters of Assessment.

Before testing, athletes were led through a brief standardized warm-up that consisted of light cycling and stretching for approximately 7 minutes. Physical tests were conducted in a standard
order to minimize fatigue: 1) Vertical Jump 2) Handgrip 3) Wingate 4) 10-km Cycling Time Trial. Only the results of the 10-km cycling time trial are reported here.

**Anthropometry.** Height was measured with a Harpenden stadiometer (Holtain, Crymych, UK) and body mass was measured by an electronic floor scale (AND FW-150K; Tokyo, Japan). Total body fat % was measured by BC-558 IronMan Segmental Body Composition Monitor (Tanita, Arlington Heights, IL, USA).

**Maximal Exercise Test (VO₂peak).** Subjects began the test at a work rate of 50 Watts (W) on a mechanically weighted and braked ergometer (Monark Ergomedic 839E), with load increases of 50 W each minute for the first two minutes, then 25 W each minute thereafter until volitional exhaustion. Gas exchange was measured by a portable metabolic system (Cortex Metamax 3B®), and maximal oxygen uptake (VO₂peak) was defined as the highest 1-minute oxygen value obtained during the test. VO₂peak power (W) was calculated by measuring the power output (W) at VO₂peak, and end power W_power was calculated as the power output (W) at volitional fatigue. Heart rate was monitored using a Polar Heart Rate Monitor (Lake Success, NY).

**Time Trial.** Subjects commenced the 10-km cycling time trial (last exercise) when blood lactate levels reached <2.5 mmol/L from the prior Wingate test. The time trial was conducted by setting the Ergomedic 839 E stationary bike to a constant resistance or power output, and each subject cycled 10-km at the specified resistance (Watts). Resistance was set at 65% W_power for all subjects as calculated from the VO₂peak test, which was the equivalent of 65-69% VO₂peak (varying between subjects but identical % used within each subject for all three treatment visits). The on-board computer automatically controls the degree of resistance by applying varying amounts of braking force on the belt. The computer of the stationary bike calculates the speed of
travel based on the cadence of pedalling (RPM), where a faster cadence would result in a faster speed. The 10-km time trial requires 1,667 rotations (6 m per rotation) to be completed; therefore, the power output did not affect the speed of the bike. Speed was altered only by how fast the subject pedalled (cadence). Therefore, different cadences (RPM) would result in different completion times for the 10-km time trial. Subjects were blinded to time, speed and heart rate, but were able to see distance traveled. Water was available ad libitum throughout the time trial. Heart rate was monitored throughout the test using a Polar Heart Rate Monitor (Lake Success, NY). Subjects estimated their Ratings of Perceived Exertion (RPE) on the basis of Borg’s rating scale (score ratings from 6-20, where 6 is no exertion, and 20 is extremely difficult) at 5-km and 9-km.

4.3.4 Genotyping.
Saliva samples were collected on visit 1 using the Oragene ON-500 kit for DNA isolation using standard procedures, as previously described [194]. Genotyping of the rs762551 SNP in the CYP1A2 gene was conducted using the Sequenom MassArray platform, as we have described previously [194]. Since there is evidence of a difference in enzyme activity between the three CYP1A2 genotypes [24, 25], we grouped individuals into AA (fast), AC (heterozygous slow) and CC (homozygous slow) for all analyses.

4.3.5 Statistical Analyses.
Data were analyzed using the SAS statistical package (SAS 9.4, SAS Institute Inc., USA), and are presented as mean ± SEM unless stated otherwise. Descriptive data (height, body mass, age, body fat, VO2peak [L•min⁻¹], VO2peak [ml•kg⁻¹•min⁻¹] dietary caffeine or caffeine used for sport, sport type distribution) were compared between genotypes using analysis of variance.
(ANOVA) or for sport type, using Chi-Square. Body mass was log-transformed before analysis, as it was not normally distributed. Using a classical split-plot design, the between subject variance was used to compare mean cycling times across the three genotypes while the within subject variance was used to compare placebo and the two caffeine doses. The order of the three visits was randomized across the subjects and visit was included as a co-variate in all analyses. Randomization was done using balanced permutations blocked by time of entry (randomization.com). The outcome variable was 10-km time trial time, and the initial analysis included the three predictor variables caffeine, gene, visit, along with the three 2-factor interactions and the one 3-factor interaction. After identifying a significant caffeine-gene (p<0.0001) interaction, each genotype was analyzed separately. This model was also used to assess RPE and HR between genotypes and within subjects between visits and caffeine treatments. The main effect of caffeine was assessed across all genotypes combined, which left two predictor variables: caffeine and visit, and the caffeine-visit interaction, with time trial time to completion as the outcome variable. Post-hoc Tukey adjustments for multiple comparisons were performed for all analyses. All p-values are two-tailed and p < 0.05 was used as the threshold for significance. Effect Sizes (ES) are presented as standardized differences between caffeine treatments (all subjects combined or for individual genotypes) using Cohen's $d = (M_2 - M_1) / SD_{pooled}$, where $SD_{pooled} = \sqrt{((SD_1^2 + SD_2^2) / 2)}$ [278]. Cohen [278] suggested that 0.2 be considered a 'small', 0.5 represents a 'medium' and 0.8 a 'large' effect size. For significant genotype and treatment p-values, the analysis of the effect of caffeine dose on the mean 10-km time trial time was completed with and without an adjustment for the visit variable, to establish whether visit was a confounder. This was completed for the main effect of caffeine for all subjects, as well as the effect of caffeine within each of the three genotypes. Sample size was
determined by power analysis calculations using a power of 0.8, and a medium effect size of 0.5. A power calculation based on two caffeine doses and three genotypes revealed that a sample size of 110 athletes will provide sufficient power for our analysis, based on a potential subject dropout rate of 10% [278].

4.3.6 Randomization

Randomization was completed by using balanced permutations blocked by time of entry (randomization.com). A study assistant (lab-mate) created a password-protected log for the randomization of treatments (0, 2 or 4 mg caffeine) for 120 subjects before the commencement of the study. The primary investigator and all exercise trial assistants were blinded to this information. The same assistant carried out the task of measuring and weighing the caffeine powder or placebo (dextrose) and making all capsules as he was notified of newly-recruited subject’s body mass (kg), prior to subject’s treatment visits. Three capsules were placed in one of three different envelopes labelled ‘Visit 1, 2 or 3’. The three capsules combined, equated to either 0 mg (dextrose; placebo), 2 mg or 4 mg per kg of caffeine and pertained to the body mass of the subject in kilograms. The completed envelopes where provided to the investigator as they were completed (~weekly). The subjects ingested all three capsules at each treatment visit, in order of Visit (1, 2 or 3) as labelled on the envelope, but the contents of the envelope where in random order and blinded to all assistants as well as the primary investigator. The capsule-making assistant did not attend or assist with any of the exercise trials, nor did he have any
contact with any of the subjects at any time. Allocation concealment was therefore successfully carried out.

4.4 Results

4.4.1 Subject Characteristics.
Of the 101 participants, 49% (n = 49) were homozygous for the A allele (AA), 43% (n = 44) were heterozygous (AC), and 8% (n = 8) were homozygous for the C allele (CC). These distributions are in Hardy-Weinberg equilibrium, and similar to frequencies reported previously in some other populations [24, 26]. The rs762551 polymorphism in the CYP1A2 gene was initially used to identify fast and slow metabolizers of caffeine. We discovered that another SNP in CYP1A2, rs2472300, is in 100% linkage disequilibrium with rs762551. As such, either polymorphism can be used to identify fast or slow metabolizers of caffeine. For rs2472300, GG corresponds to fast metabolizers whereas GA and AA are considered slow metabolizers. For rs762551, AA corresponds to fast metabolizers whereas AC and CC are considered slow metabolizers. In the present study, we genotyped subjects for both the rs2472300 and rs762551 SNPs and found 100% concordance, but we report the results for rs762551 because it is the one more commonly reported [15, 24-28, 195].

Descriptive characteristics of the three genotypes are shown in Table 4.1. There were no significant differences between the three genotypes for age, height, body fat, \( \text{VO}_2 \text{peak} \) \( (\text{L} \cdot \text{min}^{-1}) \), \( \text{VO}_2 \text{peak} \) \( (\text{ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}) \), dietary caffeine, caffeine used for sport or percent distribution of sport type (endurance, power or mixed; \( \chi^2 [4, N = 101] = 3.31 \)). The breakdown for sport type for all participants was as follows: endurance 42% (e.g. running, cycling, rowing); power 42% (e.g. baseball, powerlifting, boxing); and mixed 16% (e.g. basketball, rugby, hockey).
4.4.2 Time Trial Performance.

**All Subjects.** The average 10-km cycling times (n = 101) under the three treatments (0, 2 or 4 mg/kg caffeine) are shown in Figure 1.1. There was a significant (p = 0.04) main effect for treatment (2 or 4 mg/kg caffeine vs placebo) for all subjects, where 4 mg/kg caffeine decreased 10-km cycling time by 3% (-0.5 min [-0.2, -1.0]) compared to placebo (17.6 ± 0.3 vs. 18.1 ± 0.1 min, p = 0.01). There was no significant difference between 2 mg/kg and either 4 mg/kg caffeine or placebo.

**By Genotype.**

When subjects were stratified by caffeine dose (0, 2, 4 mg/kg) and genotype (Figure 4.2) there was a significant overall difference between genotypes (p = 0.002), as well as a caffeine-gene (p<0.0001) interaction. Thus, the three genotypes were analyzed individually to determine the effects of caffeine within each genotype.

**AA genotype (fast metabolizers).** Among those with the AA genotype, the caffeine effect remained significant, where 2 mg/kg caffeine decreased cycling time by 4.8% (-0.8 min [-0.3, -1.3]) compared to placebo (17.0 ± 0.3 vs. 17.8 ± 0.4 min, p = 0.0005), and by 6.8% (-1.2 min [-0.8, -1.7]) in 4 mg/kg compared to placebo (16.6 ± 0.3 vs. 17.8 ± 0.4 min, p <0.0001), but no difference was observed between 2 and 4 mg/kg caffeine.

**AC genotype (slow metabolizers).** In those with the AC genotype, there was no caffeine effect on cycling performance for any of the treatments (18.6 ± 0.4, 18.4 ± 0.5, 18.0 ± 0.5, for 0, 2 and 4 mg/kg, respectively; p = 0.43).
**CC genotype (slow metabolizers).** Among those with the CC genotype, 4 mg/kg caffeine increased cycling time by 13.7% (2.5 min [0.6, 4.5]) compared to placebo (20.8 ± 0.8 vs. 18.3 ± 0.5 min, p = 0.04), but no difference was observed between 2 mg/kg and either 4 mg/kg caffeine or placebo.

**Change in cycling time: placebo vs 2 mg or 4mg/kg caffeine.** Figure 4.3 shows the average change in cycling time (mean ± SEM) to completion between the (A) 2 mg/kg and (B) 4 mg/kg caffeine dose, compared to placebo. In Figure 4A (2 mg/kg vs placebo), there were no differences between any of the genotypes. Figure 4B shows a significant (p = 0.001) overall difference between genotypes, such that those with the CC genotype had the greatest change in 10-km time (although a worsening of performance with caffeine) compared to changes in time in the opposite direction in those with the AA (2.5 ± 1.0 min vs -1.2 ± 0.3 p<0.0001) and AC (2.5 ± 1.0 min vs -0.6 ± 0.4 min, p = 0.0015) genotypes, respectively.

**Time Trial Performance Scatterplot by Genotype.** Figure 4.4 shows individual data points representing 10-km time to completion for placebo (x-axis) and either (A) 2 mg/kg or (B) 4 mg/kg, (y-axis), for all subjects by genotype (AA, AC, CC). Data points below the line indicate faster times with caffeine. For those with the AA genotype, 35 (71%) and 40 (82%) out of 49 subjects performed better during 2 or 4 mg/kg, respectively, compared to placebo. In those with the AC genotype, 26 (59%) and 28 (64%) out of 44 subjects performed better during 2 or 4 mg/kg caffeine, respectively, compared to placebo. In those with the CC genotype, 2 (25%) and 1 (12%) out of 8 subjects performed better during 2 or 4 mg/kg respectively, compared to placebo.
**RPE and HR.** At 5-km, those with the AA genotype reported a 3% (0.5 min) lower RPE in the 4 mg/kg time trial compared to placebo (14.3 ± 0.3 vs 14.8 ± 0.2, p = 0.03), but there was no difference between 2 mg/kg (14.5 ± 0.3) and either 4 mg/kg caffeine or placebo. There were no differences in those with the AC genotype (15.1 ± 0.3, 15.5 ± 0.3, 15.0 ± 0.3) or CC genotype (14.1 ± 0.6, 14.3 ± 0.6, 15.5 ± 0.3) between any of the time trials at 0, 2 or 4 mg/kg caffeine, respectively. At 9-km there were no differences in RPE between any of the treatments within any of the genotypes. Heart Rate (HR) analysis was determined in those with the AA (n=46), AC (n=42) and CC (n=6) genotypes. In those with the AA genotype, there were no significant differences in HR (mean ± SEM) between any of the doses (167 ± 1, 169 ± 1, 168 ± 1 bpm for 0, 2 or 4 mg/kg caffeine, respectively). In those with the AC genotype, there was a 2.5% (4 bpm) increase in HR in 4 mg/kg compared to 2 mg/kg caffeine and placebo, respectively (171 ± 2 vs 167 ± 2 bpm, p = 0.007 and 167 ± 2 bpm, p = 0.005). In those with the CC genotype, there was a 2% (3 bpm) decrease in HR in those taking 4 mg/kg caffeine compared to both placebo and 2 mg/kg (160 ± 5 vs 157 ± 5 bpm, p = 0.03; 160 ± 5 vs 157 ± 5 bpm, p= 0.05), respectively.

**Effect Size (ES).** The main effect for caffeine (n = 101) in the 10-km time trial at the 4 mg/kg dose, resulted in a 3% (0.5 min) improvement and small ES, d =0.27, compared to placebo. However, in those with the AA genotype (n = 49) the 4.8% improvement with 2 mg/kg and the 6.8% (1.2 min) improvement with 4 mg/kg, both correspond to a medium ES: d = 0.4 and d = 0.63, respectively. In those with the CC genotype, the 13.7% impairment in performance in 4 mg/kg vs placebo resulted in a very-large ES, d =1.3.

**Treatment Blinding.** We collected responses from 86 subjects post-time trial, who were asked whether or not they thought they had consumed caffeine. Out of 172 caffeine trials, 31% (54) were correctly identified as caffeine-containing. Among the other 118 caffeine trials, 81% (96)
reported ‘no caffeine’ and 19% (22) reported ‘maybe caffeine’. Only 3% (3) of subjects correctly identified all three trials (i.e. 2 caffeine, 1 placebo).

**Familiarization.** A learning or visit effect due to familiarization with cycling on the Monark bike for the three treatment visits (plus cycling VO\(_2\)peak test) was expected in this group of athletes, where less than 6% were experienced cyclists. Although we observed well-balanced allocation of the three doses of caffeine across all three visits where \(X^2 (4, N = 101) = 2.01, p = 0.73\), we assessed the effect of visit within each genotype. In those with the AA genotype, cycling time decreased across visits, likely as a learning or familiarization effect. However, cycling time also decreased within each visit as caffeine dose increased from 0 mg to 2 mg/kg to 4 mg/kg, where ~33% of subjects would have ingested one of the three caffeine doses at each particular visit. Therefore, at each visit, each group consisting of one third of the 101 total subjects improved their performance in a dose dependent manner after caffeine ingestion from 0 mg to 2 mg/kg to 4 mg/kg. Table 4.2 shows the cycling time and caffeine dose by genotype with and without adjusting for visit. When analyzing the effect of caffeine and visit in each genotype individually, the caffeine effect remained significant in both the AA (p <0.0001) and CC (p = 0.04) genotypes. The predictive power (R\(^2\)) dropped from 0.85 in the model with all subjects (not shown) to 0.78 in the model with AA genotypes and 0.80 in the model with CC genotypes. When visit was not included in the model (Table 4.2, model 1), the caffeine effect remained significant in the AA genotype (p <0.0001) but decreased the predictive power of the model (R\(^2\) = 0.70). However, in those with the CC genotype, the caffeine effect was no longer significant, and R\(^2\) decreased to 0.56.
TABLE 4.1. Descriptive characteristics of participants by CYP1A2 (rs762551) genotype

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>AA (n = 49)</th>
<th>AC (n = 44)</th>
<th>CC (n = 8)</th>
<th>p¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height² (cm)</td>
<td>179 ± 7</td>
<td>177 ± 6</td>
<td>181 ± 10</td>
<td>0.15</td>
</tr>
<tr>
<td>Body mass (kg)</td>
<td>80.3 ± 12.2</td>
<td>79.7 ± 9.5</td>
<td>92.9 ± 24.9</td>
<td>0.07</td>
</tr>
<tr>
<td>Age (y)</td>
<td>24 ± 4</td>
<td>25 ± 5</td>
<td>25 ± 5</td>
<td>0.48</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>14.2 ± 4.4</td>
<td>13.8 ± 4.4</td>
<td>15.9 ± 6</td>
<td>0.49</td>
</tr>
<tr>
<td>VO₂peak (L·min⁻¹)</td>
<td>3.9 ± 0.8</td>
<td>3.8 ± 0.7</td>
<td>3.9 ± 0.6</td>
<td>0.74</td>
</tr>
<tr>
<td>VO₂peak (ml·kg⁻¹·min⁻¹)</td>
<td>49 ± 8</td>
<td>47 ± 12</td>
<td>44 ± 12</td>
<td>0.34</td>
</tr>
<tr>
<td>Caffeine Dietary³ (mg per day)</td>
<td>87 ± 18</td>
<td>80 ± 20</td>
<td>38 ± 24</td>
<td>0.61</td>
</tr>
<tr>
<td>Caffeine Sport⁴ (mg per day)</td>
<td>61 ± 13</td>
<td>89 ± 17</td>
<td>80 ± 74</td>
<td>0.49</td>
</tr>
<tr>
<td>Sport Type (%)</td>
<td></td>
<td></td>
<td></td>
<td>0.51</td>
</tr>
<tr>
<td>Endurance</td>
<td>46</td>
<td>49</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Power</td>
<td>45</td>
<td>45</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Mixed</td>
<td>62</td>
<td>25</td>
<td>13</td>
<td></td>
</tr>
</tbody>
</table>

¹p values were derived by using ANOVA, body mass variable was log transformed before analysis as it was not normally distributed, or for sport type by using Chi-Square.

²Mean ± SD (all values)

³Average dietary caffeine intake (excludes caffeine intake for sport)

⁴Average caffeine intake specifically for sport performance, i.e. training and competition (coffee, energy drinks, pre-workouts, gels, tablets etc.)
TABLE 4.2. Time Trial time and caffeine dose by CYPIA2 (rs762551) genotype with and without the visit variable

<table>
<thead>
<tr>
<th>rs762551</th>
<th>Adj</th>
<th>n²</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>R²</th>
<th>p³</th>
<th>p⁴</th>
<th>p⁵</th>
<th>p⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>Yes</td>
<td>147</td>
<td>17.8</td>
<td>16.9</td>
<td>16.7</td>
<td>0.76</td>
<td>&lt;0.0001</td>
<td>0.001</td>
<td>&lt;0.0001</td>
<td>0.50</td>
</tr>
<tr>
<td>AA</td>
<td>No</td>
<td>147</td>
<td>17.8</td>
<td>17.0</td>
<td>16.6</td>
<td>0.70</td>
<td>&lt;0.0001</td>
<td>0.007</td>
<td>&lt;0.0001</td>
<td>0.50</td>
</tr>
<tr>
<td>AC</td>
<td>Yes</td>
<td>132</td>
<td>18.4</td>
<td>18.5</td>
<td>18.1</td>
<td>0.86</td>
<td>0.47</td>
<td>0.87</td>
<td>0.75</td>
<td>0.44</td>
</tr>
<tr>
<td>AC</td>
<td>No</td>
<td>132</td>
<td>18.6</td>
<td>18.4</td>
<td>18.0</td>
<td>0.74</td>
<td>0.37</td>
<td>0.95</td>
<td>0.37</td>
<td>0.55</td>
</tr>
<tr>
<td>CC</td>
<td>Yes</td>
<td>24</td>
<td>18.5</td>
<td>19.4</td>
<td>21.0</td>
<td>0.68</td>
<td>0.05</td>
<td>0.60</td>
<td>0.04</td>
<td>0.23</td>
</tr>
<tr>
<td>CC</td>
<td>No</td>
<td>24</td>
<td>18.3</td>
<td>19.6</td>
<td>20.8</td>
<td>0.56</td>
<td>0.06</td>
<td>0.37</td>
<td>0.05</td>
<td>0.45</td>
</tr>
</tbody>
</table>

¹adjusted for visit
²number of visits = 3 x number of subjects in genotype
³overall p-value for comparison of three caffeine doses
⁴,⁵,⁶p-values for comparing time to completion between caffeine doses 0 and 2⁴, 0 and 4⁵, and 2 and 4 mg/kg⁶
10-km Cycling Time Trial by Caffeine Dose

FIGURE 4.1—Mean (SEM) 10-km cycling times for all subjects (n = 101) under each caffeine treatment: 0, 2 and 4 mg/kg body mass.

*There was a significant decrease (p = 0.01) in 10-km cycling time during the 4 mg/kg caffeine trial compared to placebo.

p-values were generated from a model adjusted for visit
FIGURE 4.2—Average (mean ± SEM) 10-km cycling time by caffeine dose and CYP1A2 genotype.

*2 mg/kg and **4 mg/kg caffeine trials significantly different from placebo (p < 0.0001; p = 0.0005, respectively).

*4 mg/kg caffeine trial significantly different (p = 0.02) from placebo.

p-values were generated from models of individual genotypes and adjusted for visit
FIGURE 4.3—Change in 10-km cycling time to completion between genotypes (A) For group mean (SEM) between 2 mg/kg caffeine and placebo and (B) 4 mg/kg caffeine and placebo.

*CC genotype significantly different from AC (p = 0.002) and AA (p < 0.0001).

CYP1A2 genotype (rs762551)
FIGURE 4.4 — 10 km cycling times for AA, AC, CC genotypes (A) 2 mg/kg versus 0 mg/kg caffeine. Data points below the identity line indicate faster cycling times during 2 mg/kg versus 0 mg/kg caffeine dose. (B) 4 mg/kg versus 0 mg/kg caffeine. Data points below the identity line indicate faster cycling times during 4 mg/kg versus 0 mg/kg caffeine dose.
4.5 Discussion

The current study examined the effects of caffeine and a genetic modifier of caffeine metabolism, CYP1A2 genotype, on 10-km cycling time trial performance in competitive male athletes after ingestion of caffeine at 0, 2 and 4 mg/kg body mass. Our results indicate that in the total population caffeine is ergogenic to endurance cycling performance, with a 3% improvement in cycling time at 4 mg/kg, but not at 2 mg/kg, which is consistent with previous studies using similar doses [5, 12]. However, we observed a significant caffeine-gene interaction where the improvements in performance were seen with both 2 and 4 mg/kg caffeine, but only in those with the AA genotype who are ‘fast metabolizers’ of caffeine. In that group, the 6.8% improvement in cycling time at 4 mg/kg, is greater than the 2-4% mean improvement seen in approximately 30 cycling time trial studies using similar doses [5, 11, 12, 17, 18, 188, 279]. The improvement in performance that we observed in the entire population at 4 mg/kg corresponds to a small effect size, d = 0.27, but in those with the AA genotype the effect corresponds to a medium effect size d = 0.63. Contrary to the beneficial effects we observed among those with the AA genotype, we found that 4 mg/kg caffeine impaired performance by 13.7% in those with the CC genotype who are ‘slow metabolizers’ of caffeine, and this corresponds to a very large effect size, d = 1.3. We found no effect of either dose in those who have the AC genotype.

Most studies on caffeine and performance do not explore the basis for the inter-individual variation in response, which has been well-documented in several studies [8-10, 17, 20]. For example, Jenkins et al. [17] examined the effects of caffeine on exercise performance in thirteen cyclists, and the inter-individual range for performance change with caffeine at 1, 2 or 3 mg/kg compared with placebo was –7.9% to 17.8%. Although 11 of 13 cyclists benefited from the 3 mg/kg dose, the authors noted that “the mean performance outcome did not reach statistical
significance due to two “non-responders” strongly influencing the mean and SEM, in addition to 8 of the 13 subjects performing worse on at least one caffeine condition versus placebo. Similarly, Paton et al. [14] found that caffeinated (~3-4 mg/kg) chewing gum improved overall performance in 20 male and female cyclists, but only 13 (65%) of the cyclists were considered ‘positive responders’ while 5 (20%) experienced ‘negative’ responses and the remaining 2 (15%) experienced no observable effect on cycling performance. The authors speculated that this variation in response may be related to differences in the rate of caffeine metabolism or absorption between individuals [14].

Acute caffeine ingestion has been shown to alter RPE, where effort may be greater under caffeine conditions, yet it is not perceived as such [4, 136]. Consistent with other studies [4, 136], our results showed a 3% decrease in RPE for the AA genotype at 5km after taking 4 mg/kg caffeine, which coincides with the group that had the fastest 10-km cycling time. Those with the CC genotype taking 4 mg/kg had a non-significant increase in RPE, which is consistent with the impaired performance in that group. Our findings suggest that caffeine does not lower RPE in all individuals. Similarly, a recent study by Green et al. [174] showed that when subjects were instructed to cycle at specific RPE (effort) levels under caffeine conditions, the higher perceived intensity did not necessarily result in greater work and improved performance in all subjects equally. The authors noted that individual responses to the caffeine may explain their unexpected findings.

In the present study, only those with the AA genotype who are fast metabolizers of caffeine benefited from caffeine during the 10-km time trial. There is some evidence that extended periods of blocked adenosine receptors may be detrimental to performance [36], and this may
explain the lack of benefit or diminished performance in slow metabolizers. Slower clearance of caffeine, and longer caffeine build-up in slow metabolizers has been associated with increased blood pressure [27], and this vasoconstriction may also have effects on both blood flow to the heart and muscles [201]. Resting myocardial blood flow does not appear to be affected by caffeine ingestion, but exercise-induced myocardial blood flow has been shown to decrease after caffeine ingestion [201]. Under exercise conditions the expected adenosine-mediated coronary vasodilation and subsequent increase in myocardial blood flow to match augmentation in cardiac work, is likely impaired by caffeine and could explain the impaired performance among slow metabolizers [201, 202]. It has also been postulated that caffeine metabolites, such as paraxanthine, may have ergogenic properties and would be generated more quickly in fast metabolizers, thereby providing benefits sooner than in slow metabolizers [15]. The initial ergolytic effects of impaired adenosine-mediated vasodilation experienced by fast metabolizers may be outweighed by their ability to expedite the production of these metabolites, which may be the source of ergogenicity.

Our findings are consistent with a previous study by Womack et al. [15] who observed a caffeine-gene interaction and improved time trial cycling performance with caffeine only in those with the AA genotype. In contrast, previous studies either did not observe any impact of the CYP1A2 gene on caffeine ergogenicity [29, 199], or reported benefits only in slow metabolizers [195]. Pataky et al. [195] reported an improvement in those with the AC genotype compared to the AA genotype after caffeine ingestion, however, the 3-km time trial performed in that study was a much shorter duration than in the present study or in the previous study [15]. Furthermore, that study [195] did not include any subjects with the CC genotype, which is the group that we found to have impaired performance after 4 mg/kg caffeine. Algrain et al. [29]
found no effect of 255 mg caffeine on a 15-minute cycling performance trial, and no modifying effect of CYP1A2 genotype. However, this dose of caffeine was likely too low to observe any effect, since most previous studies report an effect only at higher doses. That study also had a small sample size of 20 subjects (AA genotype, n=10; C-allele carriers, n = 9), did not differentiate between AC and CC genotypes among C-allele carriers, and included both males and females, which may have been a confounder due to potential gender differences in caffeine response by genotype [280]. Similarly, no effects of CYP1A2 on caffeine ergogenicity were observed in the study by Salinero et al. [199], but the very small sample size of only 10 subjects with the AA genotype, compared to 49 subjects with this genotype in the present study, make it unlikely that any significant findings would be detected. Importantly, that study used a 30-sec Wingate test, which is a measure of power or anaerobic capacity, and is not a valid measure of endurance [199]. Consistent with this notion, a meta-analysis of caffeine and exercise performance [7] showed that larger effect sizes with caffeine supplementation were more often reported in trials of longer duration. Therefore, the unmasking of the effects of genotype on performance may occur during exercise of longer duration and during an accumulation of fatigue, where caffeine often provides its greatest benefits, and where the adverse effects to slow metabolizers are more likely to manifest. And as previously mentioned, this may improve performance by allowing for a greater accumulation of the potentially ergogenic caffeine metabolites, in fast metabolizers.

Two concerns often raised in caffeine-performance studies using cycling time trial protocols are 1) a learning effect and 2) the caffeine-placebo effect. To address the issue of familiarization, we included a visit variable in our statistical model, to control for potential confounding due to a learning effect. Although performance improved with each successive visit, the improved
performance with caffeine in those with the AA genotype occurred regardless of the order of treatment and the findings were the same with and without adjusting for visit. The caffeine-placebo effect can introduce a psychological factor [279] outside of the expected physiological effect, when a subject is not blinded to the treatment. Treatment blinding in the present study was successful with less than one third of caffeine trials identified correctly. Although a placebo benefit has been reported to occur in subjects who believe they have ingested caffeine [279, 281], this would not explain the benefits seen only in those with the AA genotype.

Our results also confirm the ergogenicity of lower caffeine doses (2-4 mg/kg) as previously reported [12, 16], but only within a specific genetic subset of individuals. Lower caffeine doses are more desirable in order to avoid the potential adverse side effects of higher doses (6-9 mg/kg), such as sleep disturbances [184], especially for athletes training or competing at night, as well as other adverse effects such as anxiety and agitation [183].

Although the results from the present study suggest a potential role of CYP1A2 genotype in influencing the ergogenic response of caffeine in competitive athletes from a variety of sports, care should be taken in extrapolating these findings to female, non-athletic or older populations. It is unknown if there is a similar genetic influence for other modes of exercise of high-intensity or short-duration, or whether other polymorphisms in CYP1A2 or other genes involved in the response to or metabolism of caffeine may modify the effects of caffeine during exercise.

In summary, we found that caffeine improves endurance performance at a dose of 2 and 4 mg/kg for fast metabolizers of caffeine who have the CYP1A2 AA genotype (rs762551). Among the slow metabolizers, there is either no effect (AC genotype) or impaired performance (CC genotype) under the caffeine conditions in this study. These results highlight the importance of
considering *CYP1A2* genotype when deciding whether athletes should use caffeine as an ergogenic aid to improve endurance performance.
Chapter 5

Caffeine, *HTR2A* and *CYP1A2* genotypes, and endurance performance in athletes
Caffeine, *HTR2A* and *CYP1A2* genotypes, and endurance performance in athletes

### 5.1 Abstract

**Background:** Caffeine is known to improve endurance performance, but the effects differ between athletes. This is due, in part, to genetic differences affecting caffeine metabolism. **Purpose:** The objective of this study was to determine whether variation in the *HTR2A* (serotonin receptor) gene, which may affect neurotransmitter signaling post-caffeine ingestion, modifies the ergogenic effects of caffeine during a 10-km cycling time trial, and how this gene may further modify performance between fast and slow caffeine metabolizers based on their *CYP1A2* genotype. **Methods:** Competitive male athletes (*n*=101; age: 25 ± 4 years) completed the cycling time trial under three conditions: 0, 2 or 4 mg of caffeine per kg body mass, using a split-plot randomized, double-blinded, placebo-controlled design. DNA was isolated from saliva and genotyped for a common polymorphism in the *HTR2A* gene (rs6313). **Results:** Overall, 4 mg/kg caffeine decreased cycling time by 3% versus placebo. When subjects were stratified by caffeine dose and *HTR2A* genotype, those with the CC genotype decreased cycling time by 5.8% and by 7.9% compared to placebo in 2 mg and 4 mg/kg caffeine groups (mean ± SEM of 17.8 ± 0.4 and 17.4 ± 0.4 vs. 18.9 ± 0.4 min, *p* = 0.0004, 0.001), respectively. No effects were observed in those with the CT or TT genotype in any of the trials. Among those with the AA genotype for *CYP1A2* (*n*=49; fast metabolizers), a significant caffeine-*HTR2A* interaction (*p*=0.001) was observed where the *HTR2A* CC genotype improved cycling performance by 7.9% (1.5 min) with 2 mg/kg caffeine and 12.6% (2.4 min) with 4 mg/kg caffeine compared to placebo (17.6 ± 0.4 and 16.7 ± 0.5 vs. 19.1 ± 0.6 min, *p* = 0.0002 and *p*<0.001), respectively. No effects were observed among the fast metabolizers who have the CT or TT genotype of *HTR2A*, nor were there any effects of caffeine by *HTR2A* genotype in *CYP1A2* AC/CC slow metabolizers. **Conclusion:** Our findings show that both 2 and 4 mg/kg caffeine improve 10-km cycling time in those with *HTR2A* CC genotype, with further benefits in those who also have the AA genotype for *CYP1A2*. Both *CYP1A2* and *HTR2A* genotypes should be considered when deciding whether an athlete should use caffeine for enhancing endurance performance.
5.2 Introduction

Many studies have examined the effect of supplemental caffeine on endurance performance, and most have shown that performance enhancements are associated with caffeine use [1-15]. However, there is considerable inter-individual variability as to the magnitude of these beneficial effects [16-20]. Studies that report on the effects of caffeine on performance have been inconsistent despite having similar study designs, subjects and dose of caffeine. These inconsistencies might be partly due to genetic differences affecting caffeine metabolism or caffeine response.

As discussed in Chapter 4, most studies reporting on caffeine and performance do not explore the basis for the inter-individual variation in response, which has been well-documented in several studies (see Chapter 4, [8-10, 187]). Although several trials have examined the impact of genetic variation on differences in performance as a response to caffeine, including the CYP1A2 genotype (rs762551) [15, 195-197, 199] and the ADORA2A genotype (rs5751876) [206], to date no studies have examined the effects of other polymorphisms associated with caffeine use and response. The CYP1A2 (rs762551) SNP has been shown to alter CYP1A2 enzyme inducibility and activity [21, 22], and has been used to categorize individuals as ‘fast’ or ‘slow’ metabolizers of caffeine. Several elevated health risks have been reported in individuals with the AC or CC genotype (slow metabolizers), including myocardial infarction [23], hypertension [24], and pre-diabetes [25], with increasing caffeinated coffee consumption, whereas those with the AA genotype show no such risk. CYP1A2 genotype may also have implications for sport.

Performance benefits among fast metabolizers have been reported in many (see Chapter 4 [10, 26-28]), but not all [29, 30], caffeine-exercise studies involving muscular or aerobic endurance-
type performance. The impact of CYP1A2 genotype in response to caffeine in other sport-types remains equivocal [29, 31, 32].

We have shown that 4 mg/kg caffeine improved 10-km cycling performance in 101 competitive athletes by 3% compared to placebo (see Chapter 4 [26]). Importantly, we observed a significant caffeine-CYP1A2 gene interaction where the improvements in performance were seen with both 2 and 4 mg/kg caffeine, but only in those with the AA genotype, who are ‘fast metabolizers’ of caffeine. In that group of fast metabolizers, there was a 4.8 and 6.8% improvement in cycling time at 2 and 4 mg/kg, respectively [26]. We also found that 4 mg/kg caffeine impaired performance by 13.7% in those with the CC genotype, who are ‘slow metabolizers’ of caffeine. We found no effect of either dose in those who have the AC genotype [26].

Here we examine the effects of serotonin or 5-hydroxytryptamine, a neurotransmitter and neuromodulator that is primarily found in the enteric nervous system located in the gastrointestinal (GI) tract but is also present in the central nervous system (CNS) [282]. The serotonin receptor 2A (5-HT2A), encoded by the HTR2A gene, is a G protein-coupled receptor that serves as a primary target for serotonin signaling [30]. Caffeine has been shown to increase serotonin release, but it is unclear if this is associated with the expression of 5-HT2A receptors. These receptors partly function as a developmental signal in the CNS and regulate a variety of physiological functions in the periphery, such as the GI tract [283], and in the cardiovascular system [34]. 5-HT2A receptors have been associated with blood pressure and vasoconstriction [31-35], dopamine release [36] and various aspects of behaviour such as motivation, mood, movement and pain perception [37-39].
The mechanism by which caffeine improves exercise performance includes processes originating in the CNS that appear to lower the perception of both effort and pain, which thereby influences motivation to maintain greater effort and work output during exercise [45, 46]. It has also been postulated that the adverse effects of caffeine during exercise, in some individuals such as slow caffeine metabolizers, may be due to adenosine receptor antagonism and vasoconstriction (see Chapter 4, [26, 47, 48]). 5-HT2A receptors in smooth muscle cells have also been linked to vasoconstriction [32, 33] and may also be implicated in the variability in response to caffeine during exercise. A common polymorphism in the HTR2A gene (rs6313) results in a C to T substitution, and has been associated with mood disorders [175, 284], and cardiovascular disease [176]. Therefore, we aimed to examine whether HTR2A genotype modifies the effect of caffeine on cycling performance. Our second objective was to determine whether any modifying effects of HTR2A genotype differ between CYP1A2 genotypes (fast and slow metabolizers).

5.3 Methods

Please refer to the Methods section in Chapter 4 for the following sections:

- Subjects and Recruitment
- Experimental Design
- Parameters of Assessment.
  - Anthropometry
  - Maximal Exercise Test (VO2peak).
  - Time Trial
- Genotyping
- Statistical Analyses (aside from the following)
5.3.1 Statistical Analyses (specific to Chapter 5)

Using a classical split-plot design, the between subject variance was used to compare mean cycling times across the three HTR2A genotypes while the within subject variance was used to compare placebo and the two caffeine doses. The order of the three visits was randomized across the subjects and visit was included as a co-variate in all analyses. The outcome variable was 10-km cycling time, and the initial analysis included the three predictor variables caffeine, HTR2A gene and visit, along with the three 2-factor and the one 3-factor interaction terms. The same model and procedures were used to analyze the effects of HTR2A genotype in fast metabolizers (CYP1A2 AA genotype) and slow metabolizers (CYP1A2 AC/CC genotype). Post-hoc Tukey adjustments for multiple comparisons were performed for all analyses. All p-values are two-tailed and p < 0.05 was used as the threshold for significance. Effect Sizes (ES) are presented as standardized differences between caffeine treatments using Cohen's d (see Chapter 4; [52]). For significant genotype and treatment p-values, the analysis of the effect of caffeine dose on the mean 10-km cycling time was completed with and without an adjustment for the visit variable, to establish whether visit was a confounder.

5.4 Results

5.4.1 Subject Characteristics

Of the 100 participants, 34% (n = 34) were homozygous for the C allele (CC), 48% (n = 48) were heterozygous (CT), and 18% (n = 18) were homozygous for the T allele (TT). These distributions are in Hardy-Weinberg equilibrium, and similar to frequencies reported previously in some other populations [53, 54].
Descriptive characteristics of the three genotypes are shown in Table 5.1. There were no significant differences between the three genotypes for age, height, body fat, VO$_2$peak (L•min$^{-1}$), VO$_2$peak (ml•kg$^{-1}$•min$^{-1}$), dietary caffeine, caffeine used for sport or percent distribution of sport type (endurance, power or mixed; $X^2$ [4, N = 100] = 0.91). The breakdown for sport type for all participants was as follows: endurance 42% (e.g. running, cycling, rowing); power 42% (e.g. baseball, powerlifting, boxing); and mixed 16% (e.g. basketball, rugby, hockey).

Please refer to the Results section in Chapter 4 for the following sections:

- Time trial performance in all subjects (note Chapter 4 included 101 subjects, however the values for the main effect of caffeine did not differ)
TABLE 5.1. Descriptive characteristics of participants by *HTR2A* (rs6313)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>CC (n = 34)</th>
<th>CT (n = 48)</th>
<th>TT (n = 18)</th>
<th>p¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height² (cm)</td>
<td>177 ± 7</td>
<td>178 ± 6</td>
<td>179 ± 7</td>
<td>0.25</td>
</tr>
<tr>
<td>Body mass (kg)</td>
<td>79.6 ± 12.1</td>
<td>80.5 ± 14.6</td>
<td>84.9 ± 9.1</td>
<td>0.34</td>
</tr>
<tr>
<td>Age (y)</td>
<td>24 ± 4</td>
<td>25 ± 5</td>
<td>25 ± 5</td>
<td>0.41</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>14.2 ± 4.4</td>
<td>13.8 ± 4.4</td>
<td>15.9 ± 6</td>
<td>0.13</td>
</tr>
<tr>
<td>VO₂peak (L•min⁻¹)</td>
<td>3.9 ± 0.8</td>
<td>3.8 ± 0.7</td>
<td>3.9 ± 0.6</td>
<td>0.65</td>
</tr>
<tr>
<td>VO₂peak (ml•kg⁻¹•min⁻¹)</td>
<td>49 ± 8</td>
<td>47 ± 12</td>
<td>44 ± 12</td>
<td>0.79</td>
</tr>
<tr>
<td>Caffeine Dietary³ (mg per day)</td>
<td>87 ± 18</td>
<td>80 ± 20</td>
<td>38 ± 24</td>
<td>0.20</td>
</tr>
<tr>
<td>Caffeine Sport⁴ (mg per day)</td>
<td>61 ± 13</td>
<td>89 ± 17</td>
<td>80 ± 74</td>
<td>0.55</td>
</tr>
<tr>
<td>Sport Type (%)</td>
<td></td>
<td></td>
<td></td>
<td>0.92</td>
</tr>
<tr>
<td>Endurance</td>
<td>34</td>
<td>49</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Power</td>
<td>37</td>
<td>44</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Mixed</td>
<td>25</td>
<td>56</td>
<td>19</td>
<td></td>
</tr>
</tbody>
</table>

¹p values were derived by using ANOVA, body mass variable was log transformed before analysis as it was not normally distributed, or for sport type by using Chi-Square.

²Mean ± SD (all values)

³Average dietary caffeine intake (excludes caffeine intake for sport)

⁴Average caffeine intake specifically for sport performance, i.e. training and competition (coffee, energy drinks, pre-workouts, gels, tablets etc.)
5.4.2 Performance by HTR2A genotype (n=100).

When examining the partial effect of HTR2A genotype, (i.e. irrespective of other variables in the model), there was no difference between genotypes (p = 0.35). This indicates that HTR2A genotype alone does not directly impact performance, and effects, if any, would require examining them in combination with the other variables. When examining the effect of caffeine dose (0, 2, 4 mg/kg) in combination with HTR2A genotype (Fig. 5.1), a significant interaction (p=0.035) was found. Thus, the three genotypes were analyzed individually to determine the effects of caffeine within each genotype.

Among those with the CC genotype, the caffeine effect was significant, where 2 mg/kg caffeine improved cycling time by 5.8% (-1.1 min [-0.62, -2.31]) compared to placebo (17.8 ± 0.4 vs. 18.9 ± 0.4 min, p = 0.0004), and by 7.9% (-1.5 min [-1.74, -3.09]) in 4 mg/kg compared to placebo (17.4 ± 0.5 vs. 18.9 ± 0.4 min, p =0.001), but no difference was observed between 2 and 4 mg/kg caffeine. In those with the CT or TT genotype, there was no effect of caffeine on time trial performance at any of the doses (Fig. 5.1).

5.4.3 Performance by HTR2A genotype in CYP1A2 fast metabolizers (n=49).

When examining the effect of caffeine dose (0, 2, 4 mg/kg) in combination with HTR2A genotype among the AA genotype for CYP1A2 (i.e. fast metabolizers; Fig. 5.2A), a significant interaction (p=0.001) was found. Among those with the CC genotype for HTR2A there was an improvement with caffeine (Fig. 5.2A). In that group, 2 mg/kg caffeine improved cycling time by 7.9% (-1.5 min [-0.62, -2.31]) compared to placebo (17.6 ± 0.4 vs. 19.1 ± 0.6 min, p = 0.0004), and by 12.6% (-2.4 min [-1.74, -3.09]) with the 4 mg/kg dose compared to placebo (16.7 ± 0.5 vs. 19.1 ± 0.6 min, p =0.001), but no difference was observed between 2 mg/kg and 4
mg/kg caffeine. In those with the CT or TT genotype for \textit{HTR2A}, there was no effect of caffeine on time trial performance at any of the doses.

\textbf{Comparing \textit{HTR2A} CC genotype to CT and TT genotype} (Fig. 5.2A) In fast metabolizers, there was a significant difference between the change in time in the 0 mg versus the 4 mg/kg caffeine trial in those with the CC (n=16) compared to CT genotype (n=26). Specifically, the improvement in cycling time (i.e. 0 mg to 4 mg/kg) differed by -1.20 min [-0.2, -2.4]; \( p = 0.04 \), where those with the CC genotype received greater improvements. There was no difference in the CC compared to the TT genotype, which is likely due to the small number of subjects with the TT genotype (n=7).

\textbf{5.4.4 Performance by \textit{HTR2A} genotype in \textit{CYP1A2} slow metabolizers (n=51).}

When examining the effect of caffeine dose (0, 2, 4 mg/kg) in combination with \textit{HTR2A} genotype among the CC genotype for \textit{CYP1A2} (i.e. slow metabolizers; Fig. 5.2B), no significant interaction (\( p=0.41 \)) was found, and there was no effect of caffeine on cycling performance at any of the doses and in any of the three \textit{HTR2A} genotypes.

\textbf{5.4.5 Cycling Time Scatterplots for \textit{HTR2A} Genotypes in \textit{CYP1A2} fast metabolizers (n=49)}

Fig. 5.3 shows individual data points representing 10-km time to completion for placebo (x-axis) against the 10-km time to completion (y-axis) for either (A) 2 mg/kg or (B) 4 mg/kg caffeine doses for fast metabolizers, with different \textit{HTR2A} genotypes (CC, CT, TT). The identity line corresponds to the case when the placebo cycling time is equal to either (A) the 2 mg/kg cycling time or (B) the 4 mg/kg cycling time. Data points below the line indicate faster cycling times with caffeine.
For those with the CC genotype in HTR2A, all 16 subjects improved cycling time with 4 mg/kg caffeine compared to placebo. The variability in the improvement across subjects is small relative to the magnitude of the improvement, including when data was analyzed with and without two outliers (far right; ~24.5 min for placebo). In contrast, for those with the CT genotype, cycling times are distributed both above and below the identity line, indicating both improvements and impairments in performance for caffeine at 4 mg/kg. For those with the TT genotype, 5 out of 7 subjects are below the identity line, but the variability in improvement across the subjects relative to the small magnitude in improvement as well as the small number of subjects (n=7) indicates that an improvement in TT genotype cannot be inferred (Fig. 5.3B). These results are consistent with the statistical analyses.

5.4.6 RPE and HR for HTR2A genotype in CYP1A2 fast metabolizers (n=49)

There were no differences in RPE (mean ± SEM) at either 5- or 9-km, between any of the time trials at 0, 2 or 4 mg/kg caffeine, respectively, within any of the HTR2A genotypes (CC, CT, TT).

HR analysis was determined in those with the CC (n=15), CT (n=25) and TT (n=6) genotypes. There were no differences in HR (mean ± SEM) between any of the time trials at 0, 2 or 4 mg/kg caffeine, respectively, within any of the HTR2A genotypes (CC, CT, TT).
5.4.7 Effect Size (ES).

For subjects with the *HTR2A* CC genotype (n=34), the effect of caffeine on 10-km cycling time at 2 mg and 4 mg/kg doses, compared to placebo, resulted in 5.8% (1.1 min) and 7.9% (1.5 min) improvements with medium ES’s of d=0.47 and d=0.60, respectively. However, among subjects that also had the *CYP1A2* AA genotype (n=16), the effect of caffeine on 10-km cycling time at 2 mg and 4 mg/kg doses, compared to placebo, resulted in 7.9% (1.5 min) and 12.6% (2.4 min) improvements with a medium ES of d=0.6 and a large ES of d=1.1, respectively.
FIGURE 5.1 Average (mean ± SEM) 10-km cycling time by caffeine dose and HTR2A genotype.

*2 mg/kg and **4 mg/kg CAF trials significantly different from placebo (p\(^1\) = 0.0004; p = 0.001, respectively).

\(^1\)p-values were generated from an adjusted model.
FIGURE 5.2 Average (mean ± SEM) 10-km cycling time by caffeine dose and \textit{HTR2A} genotype in:

A) \textit{CYP1A2} AA genotype (fast metabolizers)

*2 mg/kg and **4 mg/kg caffeine trials significantly different from placebo (p = 0.0002; p = <0.0001, respectively).

B) \textit{CYP1A2} AC/CC genotype (slow metabolizers). No significant differences in cycling time within or between genotypes.

\textsuperscript{1}p-values were generated from an adjusted model.
FIGURE 5.3 - 10 km cycling times for HTR2A CC, CT, TT genotypes in CYP1A2 fast metabolizers (A) 2 mg/kg versus 0 mg/kg caffeine. Data points below the identity line indicate faster cycling times during 2 mg/kg versus 0 mg/kg caffeine dose. (B) 4 mg/kg versus 0 mg/kg caffeine. Data points below the identity line indicate faster cycling times during 4 mg/kg versus 0 mg/kg caffeine dose.
5.5 Discussion

The current study is the first to examine caffeine, HTR2A (rs6313) genotype, a genetic modifier of serotonin receptor activity, and exercise performance. Specifically, the effects of HTR2A genotype on 10-km cycling time trial performance in competitive male athletes after ingestion of caffeine at 0, 2 and 4 mg/kg body mass. The effects of HTR2A genotype were also examined in CYP1A2 subgroups: fast and slow caffeine metabolizers.

Our results indicate that in the total subject population (n = 100), regardless of genotype, caffeine is ergogenic to endurance cycling performance, with a 3% improvement in cycling time at 4 mg/kg caffeine (see Chapter 4, [196]), which is identical to those results observed in Chapter 4 with one less subject in the current analysis. When stratifying by HTR2A genotype we did not observe a caffeine-HTR2A gene interaction. However, upon further exploration, we found significant improvements in performance in both 2 and 4 mg/kg caffeine trials, but only in those with the CC genotype. In that group, the 5.9% and 7.9% improvements in cycling time at 2 and 4 mg/kg caffeine, respectively, are greater than the 2-4% mean improvement seen in numerous time trial cycling studies using similar doses [5, 12, 18, 21].

The results pertaining to the effects of the HTR2A CC genotype were additionally supported when analyses were carried out in CYP1A2 fast metabolizers only (n = 49). In fast metabolizers, those with the HTR2A CC genotype experienced 7.9% and 12.6% improvements in cycling times at 2 and 4 mg/kg caffeine, respectively, corresponding to impressive ES’s of d = 0.63 and d = 1.2, respectively. Notably, these improvements are nearing the upper range of mean improvements seen in a recent meta-analysis reporting on 56 endurance time trials in athletes (79% cycling), where the percent difference between the caffeine and placebo group ranged from
−3.0% to 15.9% [21]. Our results on the effects of genetic differences in both the \textit{CYP1A2} and the \textit{HTR2A} genes on the benefits of caffeine in endurance performance may be helpful in providing greater insights into the reasoning behind the wide discrepancies in caffeine-exercise study outcomes.

It is known that the \textit{HTR2A} gene modifies serotonin receptor activity, which suggests possible mechanisms that warrant further investigation. It is well-known that caffeine’s adenosine antagonism results in an increase in the concentration of many CNS neurotransmitters, including serotonin, dopamine, acetylcholine, norepinephrine and glutamate [147-149]. Although caffeine is known to increase serotonin, variability in the activity of 5-HTR2A receptors through \textit{HTR2A} genotype may modulate individual serotonin effects during exercise after caffeine ingestion.

Serotonin has been implicated in the pathogenesis of hypertension because of its ability to induce vasoconstriction via stimulation of 5-HTR2A receptors [34]. Accordingly, the rs6313 SNP in \textit{HTR2A} has been associated with cardiovascular disease [176]. The characteristics associated with variation in 5-HT2A receptor activity or availability relevant to caffeine response include: increased vasoconstriction [31, 34], thereby affecting blood flow to cardiac or skeletal muscles; dopamine release [36], thereby affecting alertness or focus; and receptor availability, thereby potentially affecting pain response [38] and pain processing [39]. These observations suggest potential mechanisms by which \textit{HTR2A} variants may impact endurance performance.

Post-mortem studies have confirmed the presence of 5-HTR2A receptors in several dopaminergic regions of the brain [285]. 5-HTR2A receptors have been shown to modulate dopamine release, through mechanisms involving regulation of either dopamine synthesis or dopaminergic neuron firing rate [36, 164]. There is also some evidence of an association between increases in D$_2$ and D$_3$ dopamine receptor availability in the ventral striatum (reward center of
the brain) and alertness, which suggests that caffeine might enhance arousal, in part, by upregulating D2 and D3 dopamine receptors [36, 163]. If caffeine acts by upregulating D2 and D3 dopamine receptors and 5-HTR2A receptors modulate dopamine release, then one possible mechanism is that alterations in 5-HTR2A receptors may therefore affect dopamine release and upregulation of dopamine receptors [163, 165]. High availability of dopamine D2 and D3 receptors and 5-HT2A receptors has also been associated with high pain intensity, although chronic musculoskeletal pain is associated with low baseline availability of striatal dopamine D2 and D3 receptors [37]. A possible mechanism may involve 5-HTR2A receptors coupled with dopamine release and upregulation of dopamine receptors, which affect dopamine release and consequently impacts alertness, pain and motivation and effort [122].

Given that the activity of 5-HTR2A receptors is modified by HTR2A rs6313, several observations may provide clues to our understanding of the associated mechanism, although it should be noted that there does not appear to be any studies that also include caffeine use. The HTR2A rs6313 TT genotype has been implicated in cardiovascular effects where it has been associated with acute myocardial infarction (AMI) in 255 non-fatal AMI cases compared to 255 controls [176]. However, the C-allele has been associated with hypertension where the CC and CT genotypes were more prevalent among female, but not male, elderly hypertensives, compared to age-matched normotensive controls [286]. Similarly, a study examining rs6311, which is in high linkage disequilibrium with rs6313, also found that elevated blood pressure (>130/85 mm Hg) was associated with what corresponds to the HTR2A C-allele [287]. In contrast, a study conducted on 198 hypertensive patients of Chinese descent and 164 healthy controls did not find a correlation between the rs6313 polymorphism and hypertension [288]. Associations between
HTR2A polymorphism and hypertension in two large Japanese populations (n = 2,968 total) were also not found [289]. However, an interaction between the HTR2A C-allele and a polymorphism of the endothelin-1 (ET-1) gene was investigated and was found to be associated with hypertension [289]. Finally, in a case-control study involving 99 stroke patients and a comparable number of controls, the prevalence of the TT genotype in cases for ischemic stroke was significantly higher than in controls [290].

Although the aforementioned studies did not provide information on receptor upregulation or availability, in a study of 152 depressed and 63 healthy subjects, the TT genotype was associated with higher 5-HTR2A receptor binding kinetics in platelets, with higher binding in the TT compared with TC genotype in the 63 healthy controls [291]. Higher binding activity in 5-HTR2A receptors may enable greater serotonin stimulation and signalling, which may thereby result in increases in vasoconstriction and other effects previously mentioned. Although it is difficult to speculate on how the CC genotype benefited from caffeine while the T-allele carriers did not, the above observations suggest that it may be a result of a combination of enabling vasodilation through adenosine antagonism. This, in addition to possible increases in dopamine and dopamine receptor stimulation, which may in turn provide higher tolerances to pain and lower perceived effort, or enhanced arousal. It should also be noted that none of these investigations included treatment with caffeine, which may modify these outcomes. Without further experimental research, it is not possible to accurately identify the mechanism by which variations in the HTR2A gene impact the benefits of caffeine in endurance performance.
In summary, the performance enhancing effects of caffeine are greatest in those with the CC genotype of \textit{HTR2A}. These effects were even greater among individuals who were also fast metabolizers of caffeine. Together, this body of evidence indicates that among fast metabolizers, individuals with the CC genotype for \textit{HTR2A} may benefit most from taking caffeine to reduce cycling time, compared to T-allele carriers and compared to individuals that are slow metabolizers, regardless of their \textit{HTR2A} genotype.
Chapter 6

Discussion, Limitations and Future Directions
6 General Discussion

Caffeine is one of the most prominent ergogenic aids and is used by athletes and active individuals in a wide variety of sports and activities. Most research focuses on endurance-type exercise, as this is the area in which caffeine supplementation appears to be most widely used and most likely beneficial in most, but not all, athletes [3-5]. The generic ‘one-size-fits-all’ approach to supplementation is no longer acceptable. Genetic differences are known to impact a number of metabolic pathways and affect how individuals respond to nutrients and supplements, such as caffeine, which may result in helpful, harmful or neutral effects on sports performance. Most studies on caffeine and performance do not explore the basis for the inter-individual variation in response, which has been well-documented in several studies [8-10, 17, 20].

In this study, we found that genetic differences that impact performance appear to be partly due to variation in genes such as CYP1A2 [22] and HTR2A, which are associated with caffeine metabolism and caffeine response during exercise. Caffeine was shown to improve endurance performance for fast metabolizers of caffeine who have the CYP1A2 AA genotype, as well as in those with the HTR2A CC genotype, compared to placebo.

When examining the HTR2A genotypes, significant improvements in performance in both 2 and 4 mg/kg caffeine trials were seen, but only in those with the CC genotype. In that group, there was a 5.9% and 7.9% improvement in cycling time at 2 and 4 mg/kg caffeine, respectively. This study is the first to examine the effects of HTR2A genotype, a genetic modifier of serotonin receptor activity, on 10-km cycling time trial performance in competitive male athletes after ingestion of caffeine. Interestingly, when we restricted the analysis to fast metabolizers (CYP1A2 AA), although the sample size dropped by 51% from 100 subjects to 49, the statistical
significance of the caffeine-\textit{HTR2A} interaction strengthened from $p = 0.07$ to $p = 0.001$. Among fast metabolizers, those with the \textit{HTR2A} CC genotype experienced a 7.9\% and 12.6\% improvement in cycling time at 2 and 4 mg/kg caffeine, respectively, corresponding to impressive ES’s of $d = 0.63$ and $d = 1.2$. Accordingly, we have shown that subjects are most likely to benefit from caffeine if they have both the \textit{HTR2A} CC genotype and the \textit{CYP1A2} AA genotype, as opposed to one or the other. For example, possessing the \textit{HTR2A} CT or TT genotype was not beneficial to performance even if the subject was a \textit{CYP1A2} AA fast metabolizer. The cycling improvement of up to 12.6\%, seen in both genotypes combined at either 2 or 4 mg/kg caffeine, are greater than the 2-4\% mean improvement seen in numerous time trial cycling studies using similar doses [5, 12, 18, 21]. Notably, these improvements are nearing the upper range of mean improvements seen in a recent meta-analysis reporting on 56 endurance time trials in athletes (79\% cycling), where the percent difference between the caffeine and placebo group ranged from $-3.0\%$ to $15.9\%$ [21].

As shown, caffeine is known to cause both positive and negative effects on performance. The positive effects are likely due to decreased feelings of exertion. And contrary to the beneficial effects observed among those with the \textit{CYP1A2} AA genotype, 4 mg/kg caffeine impaired performance by 13.7\% in those with the CC genotype who are ‘slow metabolizers’ of caffeine, which corresponds to a very large effect size, $d = 1.3$. There was no effect of either dose in those who have the AC genotype. There is some evidence that extended periods of blocked adenosine receptors may be detrimental to performance [36], and this may explain the lack of benefit or diminished performance in slow metabolizers. The negative effects may be due to vasoconstriction which has been implicated elsewhere in both the \textit{CYP1A2} and \textit{HTR2A} polymorphisms examined here. Intuitively, it would seem that slow metabolizers have more
opportunity to be exposed to caffeine and would therefore experience greater ergogenic effects. However, it appears that CYP1A2 fast metabolizers may experience the beneficial effects acutely, and then they clear the caffeine before it can cause the adverse effects. Conversely, the slow metabolizers perhaps cannot clear the caffeine quickly enough and experience the negative effects, which may override the short-lived beneficial effects.

Slower clearance of caffeine, and longer caffeine build-up in slow metabolizers has been associated with increased blood pressure [27], and this vasoconstriction may also have effects on both blood flow to the heart and muscles [201]. 5-HT2A receptors have also been associated with blood pressure and vasoconstriction [37-40], and variations in the HTR2A gene were also shown to impact the benefits of caffeine in endurance performance in this study. Among the slow metabolizers, there is either no effect (CYP1A2 AC genotype) or impaired performance (CYP1A2 CC genotype) under the caffeine conditions. In HTR2A CT and TT genotypes there was also no effect of caffeine on performance.

Another possible mechanism for benefit may include caffeine’s ability to upregulate D2 and D3 dopamine receptors, where 5-HTR2A receptors modulate dopamine release [163, 165]. This upregulation of dopamine receptors may modulate dopamine release, which may occur differently in some individuals due to HTR2A genotype, and consequently impact alertness, pain and motivation and effort [122]. It has also been postulated that caffeine metabolites, such as paraxanthine, may have ergogenic properties and would be generated more quickly in fast metabolizers, thereby providing benefits sooner than in slow metabolizers [15]. In contrast, previous studies either did not observe any impact of the CYP1A2 gene on caffeine ergogenicity [29, 199], or reported benefits only in slow metabolizers [195].
Modification of RPE during exercise, perhaps due to increases dopamine [122], is of particular interest since decreasing the perception of effort may allow athletes to generate more power and capacity for work. Similar to findings in the present study, caffeine ingestion has been shown to decrease RPE, where efforts appear to be greater under caffeine conditions [4, 136, 292]. In this study a 3% decrease in RPE for the AA genotype at 5km after taking 4 mg/kg caffeine, which coincides with the group that had the fastest 10-km cycling time. There was no change in RPE however across any of the HTR2A genotypes or between caffeine doses within each genotype (CC, CT, TT). These findings suggest that caffeine does not lower RPE in all individuals.

In summary, the performance enhancing effects of caffeine are greatest in those with either the CC genotype of HTR2A or the AA genotype of CYP1A2. These effects were even greater among individuals who were HTR2A CC genotype and also fast metabolizers of caffeine, i.e., possessing the CYP1A2 AA genotype. Together, this body of evidence indicates that among fast metabolizers, individuals with the CC genotype for HTR2A may benefit most from taking caffeine to reduce cycling time, compared to T-allele carriers and compared to individuals that are slow metabolizers, regardless of their HTR2A genotype.

These findings highlight the relevance of genetic differences when optimizing an athlete’s nutrition and supplementation regimen. It appears as though, in the future, there will be several genotypes involved in the determination of the likelihood of successful performance outcomes with caffeine ingestion.
6.1 Summary of Results

Objective 1: Determine if the effects of low (2 mg/kg) or moderate (4 mg/kg) doses of caffeine supplementation on endurance performance.

Results: There was a significant (p = 0.04) main effect for treatment (2 or 4 mg/kg caffeine vs placebo) for all subjects, where 4 mg/kg caffeine decreased 10-km cycling time by 3% (0.5 min) compared to placebo (17.6 ± 0.3 vs. 18.1 ± 0.1 min, p = 0.01). There was no significant difference between 2 mg/kg and either 4 mg/kg caffeine or placebo.

Objective 2: Determine whether variation in the CYP1A2 gene, which affects caffeine metabolism, modifies the ergogenic effects of caffeine in a 10-km cycling time trial.

Results: Among those with the AA genotype, cycling time improved by 4.8% at 2 mg/kg and by 6.8% at 4 mg/kg caffeine, compared to placebo. In those with the CC genotype, 4 mg/kg caffeine impaired cycling time by 13.7% versus placebo. No effects were observed among those with the AC genotype

Objective 3: Determine whether variation in the HTR2A gene, which affects serotonin receptors, modifies the ergogenic effects of caffeine in a 10-km cycling time trial.

Results: Among those with the CC genotype, cycling time improved by 5.8% at 2 mg/kg and by 7.9% at 4 mg/kg caffeine, compared to placebo, but no difference was observed between 2 and 4 mg/kg caffeine. In those with the CT or TT genotype, there was no effect of caffeine on time trial performance at any of the doses.
**Objective 4**: Determine whether variation in the *HTR2A* gene within *CYP1A2* genotypes, i.e. fast and slow metabolizers, modifies the ergogenic effects of caffeine in a 10-km cycling time trial.

**Results**: Among those with the AA genotype for *CYP1A2* (i.e. fast metabolizers), those with the CC genotype for *HTR2A* improved cycling time with caffeine at 2 mg/kg by 7.9% and by 12.6% with the 4 mg/kg dose compared to placebo, but no difference was observed between 2 mg/kg and 4 mg/kg caffeine. In those with the CT or TT genotype for *HTR2A*, there was no effect of caffeine on time trial performance at any of the doses.
FIGURE 6.1 CONSORT DIAGRAM: RANDOMIZED CONTROLLED TRIAL

Assessed for eligibility:
18 months ongoing (n = 113)

Excluded (n = 8) during first 11 months
- Sport-related injured (n = 3)
- Unwilling to abstain from caffeine (n = 2)
- School or work demands (n = 2)
- Relocation (n = 1)

Randomized Treatments (n = 105)

All subjects (n = 105)
allocated to all treatments in random order
Caffeine: 0, 2, 4 mg/kg body mass.

Lost to follow up: n/a

Study 1 (CYP1A2)
Analyzed (n = 105)
Excluded from analysis:
incomplete data (n = 4)
Final Analysis n=101

Study 2 (HTR2A)
Analyzed (n = 105)
Excluded from analysis:
incomplete data (n = 5)
Final Analysis n=100
6.2 Limitations

6.2.1 Study Design and Population

The results from the present study suggest a potential role of the \textit{CYP1A2} and \textit{HTR2A} genotypes in influencing the ergogenic response of caffeine. The results are limited to male athletes, as females were not included in this study. This is an important factor to address in future, as gender disparity in the current scientific literature has been reported [293]. For example, one study found that the average percentage of female participants per research article across three major sport science journals over a three-year period ranged from 35\% to 37\% [294]. Sex-specific factors should be further investigated. For example, the present study included examination of caffeine metabolism and the \textit{CYP1A2} gene, which has been shown to be inhibited by oral contraceptives [106]. Further, exercise performance has been shown to be impacted by different phases of the menstrual cycle [293, 295-297]. Because of this, if the present study were extended to include females, the schedule of weekly visits may result in unreliable data. Finally, recruitment of >50 female athletes aged 18 to 35 years who were not taking oral contraceptives would likely have proven difficult, especially in the university setting.

The findings were in competitive athletes and in a younger population, therefore care should be taken in extrapolating these findings to non-athletic or older populations. It is unknown if there is a similar genetic influence for other modes of exercise of high-intensity or short-duration, or whether other polymorphisms in \textit{CYP1A2}, \textit{HTR2A} or other genes may be involved in the response to or the metabolism of caffeine.
As far as the study design, each subject’s genotype was determined after data collection, which carries a risk of having small numbers of subjects in some genotypes. Recruiting to have similar genotype frequencies across our study population may have improved power to interpret our findings in the genotype groups that had small numbers, like 7 or 8 subjects.

Lastly, the study design was chosen to enable data collection in performance measurements that encompassed four essential parameters of athletic performance. These included power, strength, anaerobic capacity and aerobic capacity. Due to time constraints, the inclusion of an endurance test that was longer than 10 km or 20 min would have resulted in a session that was well over 2 hrs and not practical for the scope of the study. The current treatment sessions lasted 100 to 120 min. Therefore, the endurance measurement was shorter compared to some studies that include time trials lasting 40 km or close to one hour.

### 6.3 Future Research

The findings of this research indicate that two genetic modifiers of caffeine, $CYP1A2$ and $HTR2A$ genotypes, are associated with endurance performance in competitive athletes under caffeine conditions compared with placebo.

Future trials should aim to recruit subjects by genotype in order to have similar numbers in each genotype to confirm or reject current findings in smaller genotype groups (i.e. 7 or 8 subjects). In future studies, other genes with the potential to modify caffeine responses such as those that encode adenosine or dopamine receptors or serotonin transporters, with known polymorphisms, should be investigated.
Future research examining other doses of caffeine such as 3 mg per kg body mass, other types and distances or time of exercise (endurance >60 min), and performance responses in older subjects also warrant further investigation. Lastly it is important for future research to address gender disparity in the current scientific literature [294], by including females in their study design [293].

6.4 Thesis Implications and Conclusion

There are several practical implications of this study. The present findings show that CYP1A2 AA fast metabolizers benefit from caffeine use, and athletes possessing this genotype may want to experiment with different doses of caffeine to determine personal responses. Similarly, the HTR2A CC genotype is also beneficial, but is likely to offer improvements only if an individual is also a CYP1A2 AA fast metabolizer. Another key and novel finding is that it is the first study to show an ergolytic outcome for endurance performance in those with the CYP1A2 CC genotype after ingestion of caffeine. Athletes should use caution and potentially avoid caffeine use if they have the CYP1A2 CC genotype and may want to carefully monitor their performance under caffeine conditions if they carry the CYP1A2 AC genotype which was shown to have no effect on performance when compared to placebo.

This study also potentially adds important information for use in genetic testing, where including the HTR2A rs6313 and CYP1A2 rs762551 in future genetic tests may add greater precision to caffeine supplementation recommendations.
In summary, this study examined the association between \textit{CYP1A2} and \textit{HTR2A} genotypes and endurance exercise performance in competitive male athletes that participate in a variety of sports. Caffeine improved endurance performance overall, but the effects were much greater in those with the \textit{CYP1A2} AA genotype, or the or \textit{HTR2A} CC genotype in combination with the \textit{CYP1A2} AA genotype. Both genotypes should be considered when deciding whether an athlete should use caffeine for enhancing endurance performance.

In conclusion the findings of this study support the hypothesis that genetic differences modify endurance performance in athletes under caffeine conditions, compared to placebo.
References


Appendix 1: General Health, Lifestyle and Physical Activity Questionnaire

Main Sport ___________________________ Years in Sport _____________________

1. Gender
   - Male
   - Female
   Today’s Date ____________
     - Day
     - Month
     - Year

2. Age ______

3. Date of Birth ________________
   - Day
   - Month
   - Year
   Country of Birth ________________

4. Highest level of education:
   - Elementary
   - High school
   - Some college or undergraduate training
   - College or Undergraduate degree received
   - Graduate degree received

5. Please describe your ethnicity (write as many as necessary).
   __________________________________________________________

6. Please indicate your blood type: and Rh factor:
   - A
   - B
   - AB
   - O
   - positive
   - negative
   - don’t know
   - don’t know

7. Do you currently, or have you ever, experienced headache attacks (including migraines, cluster headaches, etc.)?
   - Yes
   - No

At what age did these attacks start? _______yrs old

How long ago was your last headache attack? _____________________

How many headache attacks have you had in the last year? _________

How long do the attacks usually last? _____________________
8. Within the past **two weeks**, have you had

<table>
<thead>
<tr>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>a piercing, tattoo, acupuncture?</td>
<td></td>
</tr>
<tr>
<td>medical or dental procedure?</td>
<td></td>
</tr>
<tr>
<td>a vaccination or immunization?</td>
<td></td>
</tr>
<tr>
<td>a flu?</td>
<td></td>
</tr>
<tr>
<td>an infection?</td>
<td></td>
</tr>
<tr>
<td>a fever?</td>
<td></td>
</tr>
</tbody>
</table>

9. Have you ever had a sport-related injury (e.g., torn ACL, Achilles or rotator cuff, sprained ankle, concussion etc.)?  
   - [ ] Yes
   - [ ] No (Go to Question 10)

Please list all injuries:
__________________________________________________________________
__________________________________________________________________
__________________________________________________________________
__________________________________________________________________

Have you had any surgery related to any of these injuries? ______ If yes, please describe
__________________________________________________________________

Have you had the same injury more than once? ______ If yes, please describe
__________________________________________________________________

Do you have an injury right now? ______ If yes, please describe:__________________
__________________________________________________________________

10. Please indicate if you have ever been diagnosed with any of the following conditions:

<table>
<thead>
<tr>
<th>Condition</th>
<th>√ if YES</th>
<th>Age or Date of Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>High cholesterol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High blood pressure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Depression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anxiety disorder</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chest pain or shortness of breath</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney problems</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food allergies, specify</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other allergies, specify</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asthma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Health conditions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------------</td>
<td>----------</td>
<td></td>
</tr>
<tr>
<td>Cancer, specify</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crohns</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ulcerative Colitis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ulcer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thyroid conditions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Celiac disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diverticulitis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arthritis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteoporosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other condition,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>specify</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

11. Has any family member ever been diagnosed with any of the above conditions?

<table>
<thead>
<tr>
<th>Your….</th>
<th>Health conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mother</td>
<td></td>
</tr>
<tr>
<td>Father</td>
<td></td>
</tr>
<tr>
<td>Brothers</td>
<td></td>
</tr>
<tr>
<td>Sisters</td>
<td></td>
</tr>
</tbody>
</table>

12. What medications have you taken within the last month?

<table>
<thead>
<tr>
<th>Medication name</th>
<th>Reason</th>
<th>Amount</th>
<th>Frequency (e.g. daily, 3x/wk)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
13. What vitamins, minerals, herbal supplements or other nutritional / sport supplements have you taken within the last month (e.g., multivitamin, protein powder, creatine, gingko)?

<table>
<thead>
<tr>
<th>Name</th>
<th>Reason</th>
<th>Amount</th>
<th>Frequency (e.g. daily, 3x/wk)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

14. Please list any food restrictions (e.g., gluten, carbohydrate, fat, salt etc.) or special diets you are have been on in the last month (e.g., Paleo, South Beach, vegan) and the reason (e.g., health, religious or other reasons).

<table>
<thead>
<tr>
<th>Food Restrictions/ Special Diet</th>
<th>Reason</th>
<th>How long?</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

15. Do you currently smoke (at least 1 cigarette per day for 1 month or longer)?

❑ Yes ❑ No

If yes, how many years have you been smoking? ____________

How many cigarettes do you smoke per day? ____________

Are you a past smoker (have previously smoked at least 1 cigarette per day for 1 month or longer but have not smoked at least 1 cigarette per day in the last month)?

❑ Yes ❑ No

If yes, when did you quit (approximate date)? ____________

How many years did you smoke? ____________
How many cigarettes did you smoke per day? ____________

16. Do you consume CAFFEINE beverages, shots (e.g. 5-hour energy) or tablets SPECIFICALLY to improve your training and/or sport performance?

❑ Yes, as coffee or espresso, latte etc. What type? ____________ How much? ________ How often? ____________

❑ Yes, as an energy drink or shot. What type? ____________ How much? ________ How often? ____________

❑ Yes, in tablet form (e.g. wake-ups) or in a supplement
Provide dose if known ____________ milligrams. How often? ____________

❑ Yes, in another form - please specify ____________ How much? ________ How often? ____________

❑ No, I don’t use caffeine for training and/or sport performance

17. Do you currently, or have you ever, consumed caffeine-containing beverages (e.g., coffee, energy drinks or “shots”, tea, cola) regularly (NOT specifically for sport)

❑ Yes, I currently consume them regularly

❑ Yes, I used to consume them regularly but do not anymore

❑ No, I have never regularly consumed them (GO TO Q20)

18. If you now or in the past regularly consume(d) caffeine, please indicate next to each of the following withdrawal symptoms the degree to which you experience(d) them up to 48 hours after ceasing to consume caffeine-containing beverages, tablets or shots.

<table>
<thead>
<tr>
<th>SYMPTOM</th>
<th>Don’t know</th>
<th>None</th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Headache</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tiredness/ Fatigue</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decreased energy/activeness</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decreased alertness/attentiveness</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drowsiness/ Sleepiness</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decreased contentedness/well-being</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Depressed mood</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Difficulty concentrating</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Irritability</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Foggy/ Not clearheaded</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>“Flu-like” symptoms</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nausea/ Vomiting/ Upset stomach</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscle pain/ Stiffness</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anxiety/ Nervousness</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other, please specify ______________</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
19. Do you experience any of the following effects **up to 12 hours** after consuming one caffeine-containing beverage (e.g., coffee, tea, cola), tablet or shot?

<table>
<thead>
<tr>
<th>EFFECT</th>
<th>Don’t know</th>
<th>None</th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Headache</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Increased energy/activeness</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Increased alertness/attentiveness</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elevated mood</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Increased heart rate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anxiety/ Nervousness</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Panic attacks</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Restlessness</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agitation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tremors/ Jitters/ Shakiness</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dizziness</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insomnia/ Impaired sleep</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upset stomach</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laxative effect</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other, please specify________</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

20. Do you try to avoid or limit your consumption of caffeine?
   - [ ] No (GO TO Q21)
   - [ ] Yes
   
   If yes, please indicate the reason(s) below

<table>
<thead>
<tr>
<th>REASON</th>
<th>YES</th>
<th>NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fear of dependence/addiction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fear of experiencing withdrawal symptoms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Increased heart rate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Increased blood pressure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anxiety/ Nervousness</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Panic attacks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Restlessness</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agitation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tremors/ Jitters/ Shakiness</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dizziness</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insomnia/ Impaired sleep</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upset stomach</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laxative effect</td>
<td></td>
<td></td>
</tr>
<tr>
<td>General health</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dislike taste of caffeine-containing beverages</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other, please specify_______________</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
21. What other food(s), beverage(s) or ingredient(s) do you **avoid/dislike**?

<table>
<thead>
<tr>
<th>Food, beverage, ingredient</th>
<th>Reason you avoid/dislike</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**PHYSICAL ACTIVITY**

The following questions are related to your physical activity level at work, in sports and in your leisure/free time.

Please fill in the blanks with your response or CIRCLE your response when given a choice.

1. What is your main occupation? (state “student” if full-time student)

   ________________________________

2. At work/school I sit:

   1) never  2) seldom  3) sometimes  4) often  5) always

3. At work/school I stand:

   1) never  2) seldom  3) sometimes  4) often  5) always

4. At work/school I walk:

   1) never  2) seldom  3) sometimes  4) often  5) always

5. At work/school I lift heavy loads:

   1) never  2) seldom  3) sometimes  4) often  5) always

6. After work/school I am tired:

   1) never  2) seldom  3) sometimes  4) often  5) always

7. At work/school I sweat:

   1) never  2) seldom  3) sometimes  4) often  5) always

8. In comparison with others my own age I think my work/school is physically:
1) much heavier  2) heavier  3) as heavy  4) lighter  5) much lighter

9a. Do you participate in a sport?
   ____ no (If no, go to question 10 on the next page and continue)
   ____ yes: If yes, continue...
Which sport do you play most frequently?

9b. How many hours a week do you play/perform/train in this sport?
   ____ less than 1 hr/wk
   ____ 1 – 2 hr/wk
   ____ 3 – 4 hr/wk
   ____ more than 4 hr/wk

9c. How many months a year do you play/perform/train in this sport?
   ____ less than 1 month
   ____ 1-3 months
   ____ 4-6 months
   ____ 7-9 months
   ____ more than 9 months

9d. How many years have you competed in this sport? ___________

9e. Do you play/perform a second sport?
   ____ no (If no, go to question 10 below and continue)
   ____ yes: If yes, continue….
What is this second sport? ________________________________

9f. How many hours a week do you play/perform this sport?
   ____ less than 1 hr/wk
   ____ 1 – 2 hr/wk
   ____ 2 - 3 hr/wk
   ____ 3 – 4 hr/wk
   ____ more than 4 hr/wk

9g. How many months a year?
   ____ less than 1 month
   ____ 1-3 months
   ____ 4-6 months
   ____ 7-9 months
   ____ more than 9 months

9h. Do you have a record holder or championship title in this sport? If yes, please describe
   ____________________________________________

                                       127
10. In comparison with others my own age I think my physical activity during leisure time (NOT your main or 2nd sport) is:
   1) much more  2) more  3) the same  4) less  5) much less

11. During leisure time I sweat:
   1) very often  2) often  3) sometimes  4) seldom  5) never

12. During leisure time I play sports:
   1) very often  2) often  3) sometimes  4) seldom  5) never

13. During leisure time I watch television:
   1) very often  2) often  3) sometimes  4) seldom  5) never

14. During leisure time I walk:
   1) very often  2) often  3) sometimes  4) seldom  5) never

15. During leisure time I cycle:
   1) very often  2) often  3) sometimes  4) seldom  5) never

16. How many minutes do you walk and/or cycle per day to and from work, school, and shopping?
   ____ less than 5 min/d
   ____ 5 – 15 min/d
   ____ 15 – 30 min/d
   ____ 30 – 45 min/d
   ____ more than 45 min/d
17. On a usual weekday and weekend day in the **last month**, how much time did you spend on each of the following activities? *Total for each type of day should add up to 24 hours.*

<table>
<thead>
<tr>
<th>Activity</th>
<th>Effects</th>
<th>Usual weekday (hours/day)</th>
<th>Usual weekend day (hours/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sleeping</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sitting or reclining activity</strong> (studying, eating, reading, desk work, computer activity, watching TV, etc.)</td>
<td>• Minimal movement&lt;br&gt;• Minimal exertion</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Light activity</strong> (office work, driving a car, strolling, standing, etc.)</td>
<td>• Some movement&lt;br&gt;• Weak exertion</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Moderate activity</strong> (housework, light sports, regular walking, golf, yard work, lawn mowing, easy gym workout/yoga, coaching sports etc.)</td>
<td>• † heart rate&lt;br&gt;• Moderate exertion</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Vigorous activity</strong> (strenuous sports, jog/run, heavy weight-lifting, power yoga, aerobic dancing, swimming, rollerblading, hiking, cycling, etc.)</td>
<td>• ††† heart rate&lt;br&gt;• Strong exertion&lt;br&gt;• Get out of breath&lt;br&gt;• Sweating</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

= 24 hours = 24 hours

**Contact Information**

Full Name: ____________________________________________

Address ____________________________________________

__________________________________________

Phone number (______) ____________________________

e-mail (please print clearly) ___________________________

*Thank You!*
Appendix 2:

Research Consent Information

Title of Research Study: Caffeine, Genetic Variation and Athletic Performance

Principal Investigator:
Nanci S. Guest, MSc, RD, CSCS (Sport Dietitian; Strength and Conditioning Specialist)
Dept. of Nutritional Sciences, University of Toronto
Phone 416-873-3312

Faculty Supervisor:
Dr. Ahmed El-Soehemy
Dept. of Nutritional Sciences, University of Toronto
Phone: 416-946-5776

Conflict of Interest: None.

This form provides all of the information we think you need to decide if you want to take part in this study. If you have any questions after you read this form, please call Nanci at 416-873-3312. You should not sign this form until you are sure that you understand everything on it.

Purpose of the Research

Numerous studies have investigated the effect of caffeine on exercise, but many are not able to draw conclusions regarding caffeine and athletic performance, partially due to the phenomenon of “responders” and “non-responders”. Previous research in our lab has identified genetic variations among individuals that affect caffeine metabolism and its various physiological effects. We hypothesize that the inconsistencies in studies on caffeine use and performance may be due to genetic variations among athletes. Varsity athletes will take part in an intervention of two different doses of caffeine versus placebo, using a randomized double-blinded placebo-controlled design that will test various exercise protocols that are associated with performance across multiple sports. Saliva samples will be taken for genotyping and identification of genetic variations related to caffeine metabolism. Not only is the outcome of value to determine which genotypes (athletes) are likely to gain a performance advantage in various sports, but also to identify athletes who may possess a genotype linked to adverse health risks associated with high caffeine intakes.

Description of the Research

We will ask approximately 100 University of Toronto varsity and club athletes to take part in this study. The study office is in the Performance Lab in the Athletic Center, at the University of Toronto. To be eligible for this study, you must be an athlete in good health, who has been competing in your sport for at least 3 years at any level of competition. The sports we are assessing include volleyball, gymnastics, swimming, track and field, triathlon and marathon.
If you agree to participate, we will take your blood pressure, weigh, height, body fat measurements, finger prick (blood lactate) and a saliva sample for DNA analysis. We will ask you to complete three
questionnaires about your general health and lifestyle, eating habits, and sport history. We will ask you to complete the questionnaire while you wait the 60 min post-caffeine or placebo ingestion, before starting the exercise tests. But you may also take the questionnaires with you to complete at your convenience. During the 60 min waiting period you may do any activity that does not require physical exertion e.g. study, read, text, other electronic device use.

The Exercise Tests Include:

Day 1 – VO2 Max
Day 2, 3, 4 – Handgrip, Vertical Jump, Wingate, 7.5 km Time Trial (Cycling)

A more detailed description of these exercises can be explained to you in person or over the phone. Each Day (1-4) is separated by 5-8 days. We will aim for 7 days in between visits. The entire time allocated to this study is not expected to exceed 150 min per visit (including the 60 min wait for the caffeine to be adequately absorbed into your bloodstream). You will be asked to abstain from caffeine for 1 week prior to Day 1 of the research project, and for the duration of your next 3 visits. All visits are booked 5-8 days apart, for a total of 22-30 days study duration.

Right to refuse or withdraw

Your participation in this study is voluntary. You may withdraw from the study at any time. You may decline to answer any question or to complete any part of the procedures/tasks. You may also request to have your DNA sample destroyed at any time. If you wish to do this, please make this request in writing to Dr. Ahmed El-Sohemy, Dept. of Nutritional Sciences, 150 College Street, University of Toronto, Toronto, ON, M5S 3E2.

Risks and Benefits

There is a direct benefit to you as an athlete from this study. The knowledge gained will help to determine the effect of genes, the type of exercise and your potential benefits or risk of caffeine ingestion in your given sport with your given genetic (DNA) profile. The kind of genetic information from the DNA sample will tell you how fast or slow you metabolize caffeine and how this may affect your personal health and your athletic performance. Although highly unlikely, there is a risk that if people other than the researchers got your genetic information they would misuse them. We think the chance of this ever happening to you is very small. We will protect the confidentiality of your saliva sample by assigning it a specific code. We will not keep your name and address with the sample, only the code number. Only the principal researcher or an individual she authorizes will be able to tell which is your sample. Your sample will be stored after the completion of the study. The DNA will be stored anonymously so that as new genes are discovered it is possible for research in this area to continue.
You will also receive approximately $600 worth of information directly related to your sport performance:

1. Dietary Assessment and Consultation with Sport Dietitian
2. Genetic Variations in Caffeine Metabolism
3. Body Composition Assessment
4. VO2 Max Test
5. Wingate Test
6. Vertical Jump Test
7. Strength Test

Confidentiality and Privacy

The information that we collect will be used for research purposes only. Your name will not be attached to any of the information we collect during the exercise tests, or to the DNA sample. You will not be identified by name when the data are analyzed, or in any publication that arises from the study. All personal information that can be identified with your name for this study will be held securely at the study office at the University of Toronto in locked cabinets in locked rooms. Your name and address are linked to your study number, for future follow-up purposes, in a database that is protected by passwords and kept in locked offices with controlled access, only available to the study staff of this research team. All analyses and reports will use groups of data, so that no one individual can be identified.

Publication of Results

The results of this study may be presented at scientific conferences, seminars or other public forums and they may be published, but you will not be identified.

Future Follow-up

On one of the questionnaires we will ask the following question: “Would you be interested in being contacted for a follow-up study?” Any follow-up would be done within 5 years of the start of the study. We ask you this now so that if you are not interested in doing further questionnaires then we would not contact you. Any contact we would have with you would be to clarify any answers in your present set of questionnaires.

Compensation for Injury

In no way does signing this form waive your legal rights nor relieve the investigator, sponsors or involved institutions from their legal and professional responsibility.

Request for more information

If you have any questions or concerns regarding the research or your participation in it, now or at any time in the future, please feel free to contact the Study Coordinator, Nanci Guest, telephone 416-873-3312. She will answer any questions you have.

You may also talk to someone who is not involved in the study at all but who can advise you on your rights as a subject. You may call: University of Toronto Research Ethics Board 416-978-5585.
Caffeine, Genetic Variation and Athletic Performance - Consent Form

The research study described above has been explained to me and a copy of the Information Sheet / Consent Form has been provided for me to keep. Any questions that I have asked have been answered to my satisfaction. I have been informed of the alternatives to participation in this study, including the right not to participate and the right to withdraw at any time. This includes the destruction of the exercise test results and/or the DNA sample if I request it. The potential risks, harms and discomforts have been explained to me and I also understand the benefits of participating in the research study.

I understand that I have not waived my legal rights nor released the investigators, sponsors, or involved institutions from their legal and professional duties. I know that I may ask now, or in the future, any questions that I have about the study or the research procedures. I have been assured that records relating to me will be kept confidential and that no information will be released or printed that would disclose my identity without my permission unless required by law. I have been given sufficient time to read and understand the above information.

I further understand that:

1. I am being asked to complete three questionnaires, concerning my general health and lifestyle, my dietary intake and caffeine habits and my physical activity / sport history.
2. I am being asked to do one exercise test on Day 1 of my visit to the lab, and four exercise tests on three subsequent visits. Prior to these tests (visit 2, 3, 4) I will be asked to ingest a capsule containing a placebo material (non-caloric fiber) or caffeine at 3 mg or 6 mg per kilogram of my body weight.
3. I am being asked to have my height, weight, blood pressure and body fat level measured.
4. I am being asked for a finger prick blood sample after one of my exercise tests on visit 2, 3 and 4.
5. I am being asked to abstain from caffeine for 1 week prior to the study and for the study duration (24-30 days)

I have read the Information Sheet that describes the study, and I agree to participate.

Participant’s Name (please print) Participant’s Signature Date

____________________________________________________________________________

Witness’ Name (please print) Witness’ Signature Date

____________________________________________________________________________