Running head: PREDICTABILITY OF ACUTE MOUNTAIN SICKNESS

Determining the Predictability of Acute Mountain Sickness using a Regression Analysis

Ceilidh M. Boyd

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Supervisor: William J. Montelpare, PhD

Faculty of Kinesiology

Lakehead University

Thunder Bay, Ontario

Abstract

AMS (Acute Mountain Sickness) is an altitude-related illness that occurs as a direct result of hypoxia. To date there is no consistent diagnostic measurement tool, leaving AMS as a subjectively-measured illness. An exploratory retrospective analysis was completed to determine the predictability of several physiologic and genetic parameters on AMS. It was hypothesized that there would be a genetic influence on physiologic variables, suggesting the potential of a genetic biomarker for AMS. The results indicate that none of the variables determine an individual's susceptibility to AMS. The major finding of the present study is that in the AMS present group, saturation of peripheral oxygen (SpO_2) may be predicted by the genes that encode the angiotensin converting enzyme (ACE), and the endothelial nitric oxide synthase (eNOS) ($p=0.03$), suggesting the potential of ACE and/or NO as genetic biomarkers of AMS.

Key words: acute mountain sickness, hypoxia, secondary regression analysis, physiologic susceptibility, endothelial nitric oxide synthase, angiotensin converting enzyme

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List of Abbreviations

ACE	Angiotensin Converting Enzyme
AMS	Acute Mountain Sickness
BMI	Body Mass Index
eNOS	Endothelial Nitric Oxide Synthase
HACE	High-Altitude Cerebral Edema
HAPE	High-Altitude Pulmonary Edema
HPVR	Hypoxic Pulmonary Vasoconstriction Response
HVR	Hypoxic Ventilatory Response
NO	Nitric Oxide
$PA0_2$	Pressure of Alveolar Blood Oxygen
PaO_2	Pressure of Arterial Blood Oxygen
PCO_2	Partial Pressure of Carbon Dioxide
PIO_2	Pressure of Inspired Oxygen
PO_2	Partial Pressure of Oxygen
SaO_2	Arterial Oxygen Saturation
SpO_2	Saturation of Peripheral Oxygen
$\dot{V}_A/\dot{Q}$	Ventilatory Perfusion

Chapter 1: Introduction

1.1 Introduction

Each year millions of people ascend to high altitude for a variety of purposes such as recreational, religious, and military activities. The most common acute altitude illnesses can be divided into three categories; Acute Mountain Sickness (AMS), High-Altitude Cerebral Edema (HACE) and High-Altitude Pulmonary Edema (HAPE) (Ward et al., 2000). This study focuses on AMS, the least life-threatening and most common of the three illnesses. Predicting which individuals will experience AMS as a result of the decreased oxygen availability that occurs at high altitude remains an elusive practice, and further diagnostic information will help to reveal an individual's susceptibility to AMS.

The main determinants of AMS are: the altitude attained, the rate of ascent, the degree of physical exertion, and an individual's physiologic susceptibility (Burtscher et al., 2004; Hackett and Roach, 2001; Basnyat and Murdoch, 2003; Chen et al., 2008; Gallagher and Hackett, 2004). For many high-altitude ascendants, there may not be sufficient time or logistical support to utilize staged ascent profiles (Beidleman et al., 2004). An understanding of individual susceptibility would allow for personally prescribed ascent plans, and would forewarn highly susceptible people that they are at a greater risk of developing symptoms.

One group that is particularly vulnerable to AMS is religious pilgrims who ascend to high altitude in order to worship at shrines and holy lakes. An example of one such religious pilgrimage is the Janai Purnima festival that is held annually in Nepal. During this festival, many Nepali Hindus and Buddhists partake in a religious pilgrimage involving a trek from their lowland residences to Lake Gosainkund at 4154 m. Previous research suggests that AMS occurs

with unusually high incidence during this trek (Basnyat et al., 2000). The trek involves a very fast rate of ascent with a high degree of physical exertion, two of the main determinants of AMS. An additional contributor to high incidence rates may include fasting, which many of the religious extremists partake in during the trek (particularly women). A better understanding of individual susceptibility to AMS might determine which pilgrims should take additional time to reach the lake, or should perhaps consider taking preventative medication.

1.2 Study Overview

This study examines the relationship between non-invasive, easily measured variables in an attempt to determine the ability of these variables to predict the occurrence of AMS within a Nepali population participating in the Janai Purnima Festival. These variables include; sex, age, body mass index (BMI), heart rate, systolic blood pressure, diastolic blood pressure, arterial oxygen saturation (SaO_2) as measured by saturation of peripheral oxygen (SpO_2), angiotensin converting enzyme (ACE), and endothelial or endogenous nitric oxide synthase (eNOS) level. Several statistical analyses are performed to assess the relationship between these retrospectively measured variables and to determine if there is a correlation between these variables and susceptibility to AMS.

1.3 Purpose

The purpose of the present study is to determine if easily measured variables are correlated with the incidence of AMS amongst individuals participating in the Janai Purnima Festival.

1.4 Significance

Objective analysis of AMS remains nonexistent. Having the ability to objectively measure easily obtained variables would allow for more reliable measurement of this illness, and could help reduce future incidence rates by suggesting that susceptible individuals allow for more time to ascend. Slower ascent rates allow the body time to acclimatize, significantly reducing AMS incidence (Hackett and Roach, 2001). Highly susceptible individuals may also want to consider taking preventative medication such as acetazolamide (Hackett and Roach, 2001), or may want to avoid altitude exposure entirely.

1.5 Hypothesis

The hypothesis for the present study is that exploratory analyses using retrospective data will provide insight into an individual's susceptibility to AMS. This analysis will contribute to previous research by providing further information to the pre-existing wealth of knowledge that currently remains under debate. It is further hypothesized that while SpO_2 remains an insubstantial predictor of AMS, genetic influences on SpO_2 levels will be present, and that these influences may predict AMS susceptibility.

Chapter 2: Review of Literature

2.1 Altitude/Barometric Pressure

In the nineteenth century, Bert (1878) recognized that the detrimental effects of high-altitude were due to the diminished partial pressure of oxygen at reduced barometric pressure (Åstrand and Rodahl, 1986). Barometric pressure decreases upon ascent to higher altitude as a result of less atmospheric weight compressing gas molecules (Ward et al., 2000). This is in accordance to Boyle's law, which states that, at a constant temperature, the pressure of a given mass of a gas is inversely proportional to its volume. This means that the percentage of oxygen at sea level is the same as the percentage of oxygen on the summit of Mount Everest, but the availability of oxygen is decreased because of the increased volume resulting in decreased pressure.

Factors other than altitude also affect barometric pressure. The relationship of barometric pressure to altitude is dependent on both latitude and season (Zafren and Honigman, 1997; Ward et al., 2000; Gallagher and Hackett, 2004). As the latitude moves progressively closer to the equator, atmospheric mass increases due to increased convection and radiation (Gallagher and Hackett, 2004, Harris et al., 1998). Thus, in the northern hemisphere, the partial pressure of oxygen decreases as one moves further northward (Harris et al., 1998; Zafren and Honigman, 1997, Gallagher and Hackett, 2004). Seasonal variations result in winter pressures that are significantly lower than in the summer (Zafren and Honigman, 1997). This trend is important for extreme altitudes with most ascent expeditions being planned for the higher pressure summer months when the partial pressure of oxygen is higher.

While most of the undesirable effects of altitude on the body are due to the decreased oxygen availability, other environmental stressors contribute to the extreme conditions found at high altitude. The decreased temperature results in hypothermia and the lack of humidity can cause dehydration (Ward et al., 2000; Gallaher, 2004). These environmental stressors have a huge impact on the body's physiologic regulatory systems at high altitude, and while they are not the focus of this review of literature, they do deserve mention.

Altitude and air temperature are inversely related, with temperature decreasing about 1°C for each 150 m of increase in elevation (Åstrand and Rodahl, 1986; Hainsworth et al., 2007). Humans exhibit peripheral vasoconstriction upon exposure to cold in an attempt to slow down heat loss and to maintain core temperature (Mazzeo and Fulco, 2006). This peripheral vasoconstriction increases the susceptibility of the extremities to injuries that may result from cold exposure (e.g. frostbite). Acute cold exposure also causes an increase in metabolism through shivering, which helps to maintain body temperature. This increase in metabolism increases the demand for oxygen, placing additional stress on system adaptation (Harris et al., 1998).

As altitude increases, air becomes increasingly dry. The low humidity enhances dehydration as the water loss via the respiratory tract is greater at high altitude than at sea level (Åstrand and Rodahl, 1986). This dehydration has been found to exaggerate the sympathetic activation occurring upon ascent to high altitude (Bärtsch and Gibbs, 2007).

2.2 Hypoxia

Hypoxia is defined as "an oxygen deficiency caused by a reduction in the partial pressure of oxygen in ambient air due to either the ambient barometric pressure or the oxygen concentration of the inspired gas" (Mazzeo and Fulco, 2006, p. 564). Ascent to high altitude

causes a reduction in the ambient barometric pressure requiring physiologic adjustments to minimize the effects of hypoxia and maintain cellular function. Oxygen is critical to normal cellular function because it is an essential part of adenosine triphosphate (ATP) production via the electron transport chain (ETC) which occurs in mitochondria (West, 2004). Immediate physiologic adjustments preserve cerebral oxygen delivery despite a reduction in the partial pressure of oxygen (Bärtsch and Gibbs, 2007). The physiologic adjustments that occur at high altitude are known as acclimatization (Zafren and Honigman, 1997; Gallagher and Hackett, 2004; Favret and Richalet, 2007). Immediate responses such as hyperventilation and tachycardia can partially ensure oxygen delivery to tissues at high altitudes (Wilson et al., 2009). The variability of adaptation to high altitude appears to have a genetic basis and likely depends on individual variations in anatomy and physiology (Rupert and Koehle et al., 2006). Given adequate time, most individuals have the capacity to acclimatize up to 5500 meters above sea level (Gallagher and Hackett, 2004). Above this elevation, additional deterioration of systems exceeds the body's ability to compensate for decreased oxygen availability (Gallagher and Hackett, 2004). All forms of high-altitude illnesses originate from hypoxia, and the resulting hypoxemia, or low blood oxygen (Gallagher and Hackett, 2004).

The barometric pressure decrease that occurs with progressive ascent to higher altitudes results in a decrease in the pressure of the inspired oxygen (PIO_2), alveolar oxygen (PAO_2) and arterial blood oxygen (PaO_2) respectively (Gallagher and Hackett, 2004). These pressure decreases ultimately result in a reduction of arterial oxygen saturation (SaO_2) or hypoxemia (Gallagher and Hackett, 2004; Bärtsch and Saltin, 2008). The body undergoes several physiologic changes in an attempt to maintain oxygen transport throughout the body. Some of the factors found to be responsible for initiating the physiologic changes seen at high altitude are

increases in hypoxia-inducible factor (HIF-1α), vascular endothelial growth factor (VEGF), and erythropoietin (EPO) (Mazzeo and Fulco, 2006). Respiratory, cardiovascular and endocrine systems all experience acute physiologic changes via the autonomic nervous system (ANS) working together to adapt to the decreased oxygen availability that exists at high altitude (Mazzeo and Fulco, 2006).

The immediate response to decreased oxygen availability occurs within the respiratory system. Ventilatory and acid-base balance adjustments are involved in maintaining the homeostasis of the oxygen transport system (Winslow, 1984). Gas exchange is driven by the partial pressure difference between the gas in the alveolar space and the plasma of the capillaries; this gradient is reversed for carbon dioxide (Hughes, 2007). A decrease in the partial pressure of oxygen in ambient air will result in a lower driving pressure for oxygen diffusion from air into the blood (Weil et al., 2007). Ventilation increases proportionally to the degree of hypoxemia detected at the peripheral chemoreceptors (Gallagher and Hackett, 2004).

Hypoxemia causes a disturbance in the carbon dioxide-oxygen gradient ($\uparrow CO_2$: $\downarrow O_2$). The ratio of inspiratory flow to pulmonary capillary blood flow determines alveolar oxygen and carbon dioxide concentrations (Mazzeo and Fulco, 2006). Reduced PAO_2 results in a discrepancy in the ventilation-perfusion ratio ($\dot{V}_A/\dot{Q}$). $\dot{V}_A/\dot{Q}$ is defined as the ratio of alveolar ventilation to alveolar capillary blood flow in the lung (Sherwood, 2008). A mismatch of this ratio can lead to hypoxemia and hypercapnia, or high blood carbon dioxide (Hughes, 2007; Wagner et al., 1986). This difference in the $\dot{V}_A/\dot{Q}$ is demonstrated as high carbon dioxide content and is relayed via changes in the concentration of hydrogen ions in the blood, and is detected by the peripheral chemoreceptors, located in the arch of the aorta and the carotid body (Hughes, 2007; Duffin, 2007; Bernardi, 2007). Ventilation depends on the drive of both

peripheral and central chemoreflexes (Duffin, 2007). When peripheral chemoreceptors are stimulated in response to hypoxemia, it is in addition to the central chemoreflex drive that is already present (Duffin, 2007). Of the peripheral chemoreceptors, the carotid chemoreceptors elicit a greater response to hypoxia than do the aortic chemoreceptors (Levine, 2000). Carotid chemoreceptors are also directly sensitive directly to decreased PaO_2, in addition to $\dot{V}_A/\dot{Q}$ (Levine, 2000). Peripheral chemoreceptor stimulation results in afferent signals being sent to the cardiovascular centre in the medulla oblongata, leading to an increase in both sympathetic and parasympathetic activity (Levine, 2000). In addition to the peripheral receptor response, the central chemoreceptors located in the medulla oblongata are sensitive to an increase in PCO_2 (Levine, 2000; Collier et al., 2008). In an effort to reduce the relatively high concentration of carbon dioxide in the blood, the body blows off excess CO_2 through hyperventilation. Hyperventilation causes an increase in the volume of both inspired and expired air that exceeds metabolic requirements (Plowman, 2008). This hyperventilation stimulated by a decrease in PO_2 is called the Hypoxic Ventilatory Response (HVR). The HVR causes an increase in the amount of carbon dioxide that is exhaled, decreasing the amount of carbon dioxide in the blood while simultaneously increasing the volume of inspired oxygen (Mazzeo and Fulco, 2006). The HVR results in reduced levels of CO_2 and consequently a rise in arterial blood pH that is called respiratory alkalosis (occurs at $PCO_2 < 38$ mmHg), which is sensed by the peripheral and central chemoreceptors (Plowman, 2008). Alkalosis results in conflicting inhibitory messages being sent to the medulla oblongata, the result of which is a limitation in hyperventilation (Plowman, 2008). Respiratory alkalosis is usually not life threatening, however prolonged alkalosis is not compatible with normal body functions (Samaja et al., 1997). When alkalosis reaches extreme levels over several hours, it may impair the functions of the central nervous system and can

diminish the hyperventilatory response necessary to maintain an adequate arterial partial pressure at altitude by modifying the action of the peripheral chemoreceptors (discussed later) (Samaja et al., 1997; Cumbo et al., 2005). Respiratory alkalosis is usually compensated for within 24-48 hours by bicarbonate excretion from the kidneys (Harris et al., 1998; Hainsworth, 2009). The degree of hyperventilation that occurs at altitude is proportional to the extent that hypoxemia and hypocapnia are detected by the peripheral chemoreceptors (Gallagher and Hackett, 2004).

To maintain tissue oxygenation despite the reduction in oxygen availability, the cardiovascular system responds to hypoxia via the ANS (Favret and Richalet, 2007). One of the most powerful responses to hypoxic stress is driven by the two branches of the ANS: the sympathetic and parasympathetic responses (Favret and Richalet, 2007). Within the medulla oblongata of the brain stem, there are three neural cardiovascular centres which determine the hypoxic response of the cardiovascular system (Levine, 2000). The first is the cardioacceleratory centre which sends messages via sympathetic neurons causing an increase in heart rate and force of ventricular contraction (Levine, 2000). The heart's ventricles are solely innervated by sympathetic nerve fibres, and in hypoxic conditions, these fibres send signals to increase activity (Sherwood, 2008). The second is the cardioinhibitory centre which sends parasympathetic signals via the vagus nerve, which decrease in a hypoxic situation. Reduction of these inhibitory responses combined with an increase in excitatory responses cause an increase in heart rate. Finally, the vasomotor centre located in the medulla oblongata uses the sympathetic nervous system to innervate the smooth muscles of the arterioles (Plowman, 2007). The vasomotor sympathetic response to hypoxia is dependent on the arterioles that are recruited; the skeletal muscle arterioles vasodilate, while the visceral arterioles vasoconstrict (Levine, 2000; Bärtsch and Saltin, 2008).

The immediate consequences of exposure to acute hypoxia are a decrease in parasympathetic activity to the sinoatrial node, increased sympathetic activity, and sympathoadrenal activation (discussed later) (Levine, 2000). These responses result in an increased heart rate and left ventricular stroke volume causing an increase in cardiac output (Bärtsch and Gibbs, 2007; Bernardi, 2007). An increase in cardiac output results in a faster distribution of blood throughout the body, which results in an increased rate of oxygen delivery to the peripheral tissues (Mazzeo, 2008). A decrease in parasympathetic activity to the sinoatrial node reduces the inhibitory influence, causing an increased heart rate (Sherwood, 2008; Levine, 2000; Bernardi, 2007).

After a few days at high altitude, cardiac output begins to return to normal values despite continued elevation of heart rate. This may be due to a loss of plasma volume, which is believed to occur in order to increase hematocrit (Hainsworth et al., 2007). Vogel et al. (1974) suggested that the rise in peripheral resistance and blood pressure may lead to a decreased venous return, filling pressure, and stroke volume and thus decreases cardiac output (Hanna, 1999).

The response of blood pressure to hypoxia results from the complex interactions between several factors, including the effects of the chemoreceptors, hyperventilation, and central and peripheral contrasting effects on hypoxia and hypoxemia (Lanfranchi et al., 2005). During the first few hours of exposure, hypoxic vasodilation results in a slightly decreased systemic blood pressure (Bärtsch and Gibbs, 2007). Despite this initial fall in systolic blood pressure, mean arterial blood pressure is maintained (Mytton et al., 2008). After a few hours at high altitude, blood pressure increases due to catecholamine release via the sympathoadrenal response (Hanna, 1999). Blood pressure depends on the balance between hypoxic vasodilation and the vasoconstriction effect of sympathetic activation. Studies involving blood pressure have been

controversial in the past with some findings showing that it changes little overall at high altitude (Bernard et al., 1998, Ward et al., 2000). Recent studies, however, suggest that during a prolonged stay at altitude, a gradual increase in systolic and diastolic blood pressure, and therefore in the mean arterial pressure, occur. The reason for increased blood pressure is believed to be due to an increased autonomic response caused by fluid loss (Handler, 2009). Fluid loss occurs largely as a result of respiratory loses from hyperventilation (due to HVR). A consequence of fluid loss is that total blood volume is decreased, resulting in increased blood viscosity and therefore increased blood pressure (Handler, 2009; Kanstrup et al., 1999). Blood pressure is also related to catecholamine release (discussed later) (Handler, 2009).

After acute exposure to high altitude, the baroreflexes, located in the aortic arch and carotid bodies, remain functional. Initially, hypoxia causes hypotension in the systemic system. This is detected by the baroreceptors, leading to sympathetic activation and parasympathetic withdrawal (Somers et al., 1991). After blood pressure increases due to the sympathoadrenal response, baroreflexes are reset to higher pressures and higher values of heart rate and sympathetic nerve activity without experiencing changes in sensitivity (Passino et al., 2007; Halliwill et al., 2003; Hainsworth et al., 2007).

Another physiologic response that occurs in response to hypoxia occurs in the lungs. The pulmonary vascular response to hypoxia is to increase pulmonary arteriolar resistance (Maggiorini, 2003). The exact mechanism responsible for the initiation of the hypoxic pulmonary vasoconstriction response (HPVR) remains unknown (Fishman, 2004). The HPVR occurs rapidly, resulting in an increase in pulmonary vascular resistance and pulmonary arterial pressure (Hainsworth et al., 2007; Brown et al., 2006). This increase in pulmonary resistance and pressure causes blood to be redirected to regions of the lungs that are not usually perfused

(Gallagher and Hackett, 2004; Fishman, 2004; Brown et al., 2006). This response may provide an increased area for gas exchange to occur, helping to restore $\dot{V}_A/\dot{Q}$ (Ward et al., 2000). Dubowitz and Peacock (2007) hypothesized that HPVR may exacerbate hypoxemia, increasing incidence of AMS, however an association between HPVR and AMS has yet to be proven.

Originally, studies involving dogs, rats and rabbits showed that hypoxia stimulated the response of the sympathoadrenal system in a hypoxic environment. It is now well documented that humans share this response (Hainsworth et al., 2007). Increased sympathetic activity occurs in response to a hypoxic stimulus (Lanfranchi et al., 2005). Evidence of sympathetic activation has been shown in studies assessing levels of catecholamines in plasma and urine (Bernardi, 2007).

Hypoxemia is detected by the peripheral chemoreceptors causing sympathetic activation of the adrenal medulla, stimulating the release of the catecholamines epinephrine and norepinephrine (Mazzeo, 2005; Plowman, 2008). Catecholamines act both as neurotransmitters and as hormones and they have regulatory properties that improve oxygen availability (Mazzeo and Fulco, 2006). Within four hours of exposure to hypoxia, epinephrine is released, causing stimulation of β-adrenergic receptors, which increases oxygen delivery to tissues by increasing heart rate and contractility, leading to an increased cardiac output (Gallagher and Hackett, 2004; Mazzeo and Fulco, 2006; Mazzeo, 2008). Epinephrine also causes vasodilation within the skeletal muscle, which increases oxygen delivery to the periphery (Mazzeo and Fulco, 2006). Increased resting levels of norepinephrine in acute hypoxic conditions in individuals while at rest is currently still under debate (Mazzeo and Fulco, 2006; Appenzeller and Martignoni, 1996; Cunningham et al., 1965). It has consistently been found, however, that the additional stress of exercise in hypoxic conditions stimulates norepinephrine release, resulting in stimulation of the

β-adrenergic receptors, causing an increase in cardiac output, heart rate, peripheral vascular tone, blood pressure, and pulmonary perfusion by stimulation of the response (Gallagher and Hackett, 2004; Veligo, 1999; Wolfel et al., 1994; Kanstrup et al., 1999).

Consistencies of high altitude illness incidence rates within family members, and high recurrence rates within individuals upon multiple ascents to high altitude suggest that genetic differences may explain individual variability in acclimatization (Montgomery et al., 1998; Wang et al., 2008; Rupert and Koehle et al., 2006). Patel (2003) found that the angiotensin converting enzyme (ACE) may be a potential locus of importance in regards to performance at altitude, identifying that the insertion (I) allele, representing the presence of an intronic *Alu* segment, may offer beneficial qualities over the deletion (D) allele, representing an absence of this segment (Montgomery et al., 1998; Koehle et al., 2006; Woods et al., 2000). The endocrine renin-angiotensin system is important in controlling the circulatory system through its effects on blood pressure and water balance (Bigham et al., 2008; Koehle et al., 2006). Circulating ACE aids in the maintenance of circulatory homeostasis by converting angiotensin I, a vasodilator, into angiotensin II, a vasoconstrictor (Montgomery et al., 1998; Patel et al., 2003). Furthermore, in 2006, Koehle et al. found that the renin-angiotensin system, which the ACE gene is a component of, contributes to regulation of plasma volume. This may explain why fluid retention has been associated with AMS, however confirmation of this remains incomplete at this time (Loeppky et al., 2005). ACE levels in plasma have been found to differ greatly between unrelated individuals, but are consistent within families, similarly to the incidence of AMS (Woods et al., 2000).

According to Woods et al. (2000),

> Large interindividual differences in plasma ACE levels exist but are similar within families, suggesting a strong genetic influence. The human *ACE* gene* is found on Chromosome 17 and contains a restriction fragment length polymorphism consisting of the presence (insertion, *I*) or absence (deletion, *D*) of a 287 base pair Alu repeat sequence in Intron 16. (p. 416)

Patel et al. (2003) found that elite performance during exercise at high altitude was associated with lower levels of ACE, which was found to be related to the presence of the I-allele (Woods et al., 2000; Patel et al., 2003; Montgomery et al., 1998). Montgomery et al. (1998) suggest the I-allele may cause differences in substrate utilization, altered mitochondrial density, or raised myoglobin content. This would allow an increase in cardiac output and muscle capillary density causing an increase in oxygen delivery, thus permitting increased performance at high altitude.

Previous studies indicate that there is a significant genetic influence on SpO_2 in high-altitude residents, however it is not known if this is true for lowlanders (Woods et al., 2002). An excess of the I-allele has also been reported in elite high-altitude mountaineers (Montgomery et al., 1998; Woods et al., 2002). An enhanced HVR is found in those with the ACEII genotype, which may contribute to higher SpO_2 and tissue oxygenation levels (Patel et al., 2003). HVR alone has not proven to be an accurate predictor of AMS susceptibility. Montgomery et al. (1998) suggested that HVR in conjunction with other characteristics may provide those with the ACEII genotype with a genetic advantage.

Nitric oxide (NO) has the potential to affect both cardiovascular and cerebral hemodynamics. NO is a lipophilic, inorganic, gaseous molecule generated largely within endothelial cells, readily diffusing from its producer to target cells (Busse and Fleming, 1995; Toda et al., 2009; Searles, 2006). Certain factors influence the production and the availability of NO, including the availability of substrates and cofactors (Chatterjee and Catravas, 2008). In order for NO synthesis to occur, certain substrates are required: there must be oxygen, calmodulin (a calcium binding protein), and L-arginine (an amino acid), which is then converted to citrulline. Four cofactors must also be present, including: nicotinamide adenine dinucleotide phosphate (NADPH), tetrahydrobiopterin (BH_4), flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) (Chatterjee and Catravas, 2008).

In order to maintain regular bodily functions, appropriate amounts of NO must exist. NO is released as it is synthesized, and is not stored in the body (Casas, 2006). Reduced availability of NO is considered an important factor associated with vascular disease (Chatterjee and Catravas, 2008). Excessive production of NO is both neurotoxic and cytotoxic, and may result in inhibition of DNA synthesis and of mitochondrial respiration (Paakkari and Lindsberg, 1995).

The family of isoform enzymes responsible for NO synthesis in tissues are known as NO synthases (Radomski, 1995). According to Radomoski (1995), there are three isoforms of the protein: neuronal NO synthase (nNOS) which is found in neuronal tissue; endothelial or endogenous NO synthase (eNOS) which is found in vascular endothelium; and inducible or immunological NO synthase (iNOS) which is an isoform originally described in macrophages. The endothelium is the major source of NO found in the vasculature (Chatterjee and Catravas, 2008).

The eNOS isoform is a relaxant, causing vasodilation and therefore a decrease in vascular resistance and blood pressure, and an increase in blood flow (Janigro et al., 1994; MacNaul and Hutchinson, 1993; Zhang et al., 2006). Vascular endothelium helps to regulate vascular tone and homeostasis (Toda et al., 2009). According to Török (2008), the vascular endothelium produces large amounts of vasodilators to oppose the vasoconstriction that occurs as a result of the activity of the sympathetic nervous system at altitude.

Furthermore, eNOS contributes to cerebral arterial dilatation causing an increase in cerebral blood flow and a decrease in cerebral vascular resistance (Toda et al., 2009; Sessa, 2004). Cerebral blood flow is one of the most important factors in the regulation of brain function (Toda et al., 2009). Autoregulation of cerebral blood flow is based on the relationship between oxygen supply and metabolic demand (Van Mil et al., 2002). A decrease in oxygen supply or an increase in metabolic demand results in a decrease in vasomotor tone, allowing an increase in cerebral blood flow to meet the oxygen demands of the brain (Van Mil et al., 2002). As mentioned, oxygen is one of the substrates necessary for the formation of NO. Therefore, in a hypoxic condition, one of the substrates is reduced, thus altering the production of NO. A decrease in exhaled NO has been found to occur, and Brown et al. (2006) suggest that this reduction in expired NO is likely related to HPVR as previously discussed (Brown et al., 2006).

Exchange of materials between the blood and the brain interstitial fluid is strictly limited by the blood brain barrier (BBB), which is comprised of endothelial cells, and protects the brain from harmful chemicals crossing from the blood into the brain (Janigro, 1994; Sherwood, 2008). The BBB consists of both anatomical and physiologic protective features. Anatomically, the cells of the capillaries in the brain are joined by tight junctions that prevent substances from entering into the brain (Sherwood, 2008). Physiologically, substances must be exchanged through the

cells, allowing lipid soluble substances such as oxygen, carbon dioxide, alcohol and steroid hormones to dissolve easily through the lipid plasma membrane, while all other substances, including glucose amino acids, and ions such as sodium and potassium, are transported by highly-selective membrane-bound carriers (Sherwood, 2008). Nitric oxide (NO) has been associated with the permeability of the BBB (Janigro, 1994; Toda and Okamura, 2003). Toda et al. (2003) recognize NO as an important intercellular messenger in cerebral and peripheral hemodynamics, and as a signal molecule in the central nervous system, the peripheral nervous system, and the cardiovascular system (Janigro, 1994; Busse and Fleming, 1995; Toda et al., 2009; Lefebvre, 1995). eNOS seems to be necessary for proper functioning of the BBB. Hypoxia-induced cerebral vasodilatation is thought to activate the trigeminovascular system, sensitizing small fibres that are thought to accumulate close to the nerve, perhaps causing the headache which has been found to be associated with AMS (Hackett and Roach, 2001).

The gene associated with eNOS (*NOS3*) is located on chromosome 7q35-36 (Wang, Koehle, & Rupert, 2009). There are several single nucleotide polymorphisms of this gene. One variation is the Glu298Asp variant, where a guanine(G)-thymine(T) substitution at exon 7 leads to a glutamate–aspartate substitution (Casas, 2006; Wang, Koehle, & Rupert, 2009). The missense Glu298Asp variant has been associated with cardiovascular disease, and is detectable by an increased concentration of expired NO (Park et al., 2004; Casas et al., 2008; Wang, Koehle, & Rupert, 2009). According to Dosenko et al., 2006, the decrease in NO production may be due to a functional or quantitative deficiency of eNOS, or to a degradation of the enzyme.

2.3 Acute Mountain Sickness

The World Health Organization (WHO) estimates that approximately 40 million people worldwide climb to high and extremely high altitudes (WHO, 2008). High-altitude illness is a general term for a group of potentially fatal conditions associated with ascent to high altitude (Rupert and Koehle et al., 2006; Basnyat and Murdoch, 2003; Ward et al., 2000). High-altitude illnesses can be grouped into two general classifications; acute and chronic (Rupert and Koehle et al., 2006; Bärtsch and Gibbs, 2007; Shoene et al., 2004). The acute illnesses commonly affect otherwise healthy people who ascend too rapidly to high altitude, and can be classified further into three subcategories: Acute Mountain Sickness (AMS) which is often benign, and two others which are potentially fatal, High-altitude Cerebral Edema (HACE) and High-altitude Pulmonary Edema (HAPE) (Ward et al., 2000). The pathophysiology behind these illnesses remains poorly understood. It is known, however, that the key factor leading to AMS is the increasingly hypoxic environment resulting from lower atmospheric pressure at high altitude (Fischer et al., 2004).

AMS usually develops between six and twelve hours upon arrival to high altitude (Ward et al., 2000; Hackett and Roach, 2001; Basnyat and Murdoch, 2003; Kapoor et al., 2004), but can occur as soon as one hour after arrival (WHO, 2008; Kapoor et al., 2004). Usually AMS resolves spontaneously within 1-3 days if no further ascent occurs and physical exertion is avoided (Hackett and Roach, 2001). Grant et al. (2002) and Honigman et al. (1995) have found that limited information can be gained about a person's response to altitude based on alteration of physiologic variables at sea-level. Thus far, there have been no consistent diagnostic findings which may accurately predict a person's susceptibility to AMS (Gallagher and Hackett, 2004).

Due to confounding factors such as study design inconsistencies and limitations, barometric pressure differences, and a variety of subjective measurement tools, the exact

incidence of AMS has been difficult to determine (Gallagher and Hackett, 2004; Maggiorini et al., 1993; Schneider et al., 2002). Globally, the reported incidence of AMS has varied from 10% to over 90% (Gallagher and Hackett, 2004). In a large study conducted by Honigman et al. in 1993 (n=3158), 25% of visitors traveling to moderate heights experienced AMS. A study in the Swiss Alps conducted by Maggiorini et al. in 1990 (n= 466) at four altitudes found that 9% of people were affected at 2850 m, 13% at 3050, 34% at 3650 m, and 52% at 4559 m. In a study at Lake Gosainkund at 4300 m in the Nepal Himalayas, 68% (n=228) of trekkers were found to suffer from AMS (Basnyat et al., 2000).

Assessment of the presence and severity of AMS is based on the quantification of subjective reporting of symptoms (Dellasanta et al., 2007). The questionnaires that exist produce inconsistent results as to whether or not someone is suffering from AMS. Roach and Kayser (2007) showed the limitations of comparing various results using differing scoring systems. In an attempt to increase reliability of assessment, the 1993 Lake Louise Consensus Committee created three criteria that needed to be met for an individual to be diagnosed as suffering from AMS. These criteria include: a recent gain in altitude, at least several hours spent at the new altitude, and the presence of a headache (Roach et al., 1993). In conjunction with these three criteria, at least one of the following symptoms must also be present; gastrointenstinal upset (anorexia, nausea or vomiting), fatigue or weakness, dizziness or lightheadedness, or difficulty sleeping. AMS can be classified as mild, moderate or severe on the basis of the number and severity of symptoms present (Birnbaum and Plourde, 2007).

The Lake Louise AMS Score was created in Canada and is a simplified and standardized scoring system that provides the most widely accepted measurement tool in the assessment of AMS (Roach et al., 1993; Ward et al., 2000). The major drawback of the Lake Louise Scoring

System is that it provides only a subjective symptom rating, making it less reliable for use in intersubject comparisons (Roach and Kayser, 2007). Measurable physiologic parameters determined from a comparison of large-scale epidemiological surveys with non-invasive physiologic measures would provide insight into objective measurements that allow for more reliable quantification of AMS (Roach et al., 2002).

The main risk factors for AMS are the altitude attained, the rate of ascent, the degree of physical exertion, and individual physiologic susceptibility (Burtscher et al., 2004; Hackett, 2001; Gallagher and Hackett, 2004; Basnyat and Murdoch, 2003; Chen et al., 2008). Oftentimes, it is inconvenient, impractical or too expensive to ascend at a rate that would ensure an absence of AMS for everyone. An understanding of individual susceptibility would provide information on who is likely going to suffer from AMS, and allow better planning of ascent profiles and limitations of physical activites. Several predictive tests have been proposed to determine individual susceptibility to AMS, however, none of these tests have proven reliable (Burtcher et al., 2008).

Certain factors that have been found to be linked to AMS susceptibility including: permanent residence at sea-level, previous history of high-altitude illness, age, sex, and fitness capacity at sea level (Honigman et al., 1993; Hackett and Roach, 2001; Gallagher and Hackett, 2004; Wiseman et al., 2006). Several studies have found that a previous history of high-altitude illness tends to increase the likelihood of that individual suffering AMS on subsequent ascents (Wiseman et al., 2006; Honigman et al., 1993; Bärtsch et al., 2001; Rupert and Koehle et al., 2006). These findings provide the basis for the theory of an innate predisposition to AMS (Rupert and Koehle et al., 2006), although genetic evidence remains inconclusive at present. It is currently hypothesized that more than one gene may be involved (Koehle et al., 2006).

Study findings from studies have varied drastically, making it impossible to conclude which populations are more or less at risk of experiencing AMS. For example, Ziaee et al. (2003), Schneider et al. (2002), O'Conner et al. (2004) and Chen et al. (2008) found no significant differences in AMS susceptibility between men and women. Honigman et al. (1993) and Vann et al. (2005) found that women had an increased likelihood of experiencing AMS symptoms. Contrary to both of these outcomes, Wagner et al. (2008) found that women had a reduced likelihood of presenting symptoms. Studies comparing the performance of men and women at altitude have even looked at the effects of the menstrual cycle which was found not to affect physical performance at high altitude (Beidleman et al., 1999; Takase et al., 2002).

Gallagher and Hackett (2004) found that AMS is reported equally in both sexes, but agrees with the findings of Roach et al. (2002) that it is less commonly seen in adults over the age of fifty than in younger adults or in children. A common belief is that younger people may be more prone to risk-taking behaviour, and may be more likely to disregard recommended ascent rates than an older population, placing younger people at higher risk of developing AMS (Vardy et al., 2005). Another hypothesis related to the BBB theory of AMS is that older populations might have a decreased risk of AMS because there is a decrease in brain size with advancing age, allowing for greater brain swelling before the symptoms onset (Wagner et al., 2008).On the contrary, Ziaee et al. (2003), Chen et al. (2008) and Schneider et al. (2002) found that age was not a determinant in those likely to be affected.

Increased fitness level is generally not thought to be protective against AMS (Hackett and Roach, 2001; Jafarian et al., 2008; Roach et al., 2000; Honigman et al., 1995; Birnbaum and Plourde, 2007; Bircher et al., 1994; Bärtsch et al., 2001). A study involving four subjects completed by Seedhouse and Blaber (2005) comparing ultraendurance athletes to moderately

trained athletes found that the more aerobically fit individuals may actually be more susceptible to a delayed onset of AMS. Obesity, on the other hand, categorized on the basis of Body Mass Index (BMI) and found it to be a risk factor for AMS (Wu et al., 2007; Ri-Li et al., 2003). The results of a study by Ri-Li et al. (2003) found that there were higher AMS scores, and lower SaO_2 values in obese individuals than there were in individuals of an ideal weight.

Pulse oximetry is a commonly used, non-invasive approach to assessing SpO_2, thereby providing an estimate of SaO_2. All studies measuring SpO_2 show a drastic reduction as a result to hypoxia exposure (Roach et al., 2000). O'Conner et al. (2004), Roach et al. (2000), Roeggla et al. (1996), and Dyer et al. (2008) all found that this reduction was not associated with the presence or severity of AMS. Three studies (Lanfranchi et al., 2005; Burtscher et al., 2004; Tannheimer et al., 2001) found that participants with lower SpO_2 values tended to have higher AMS scores. Basnyat and Murdoch (2003) found that an SpO_2 of less than 85% was the strongest predictor of AMS. In 2000, Roach et al. found that SpO_2 values were associated with future symptoms of AMS, however today he believes that the association is weak largely due to interference that occurred in the oximeters and because testing was conducted on cold extremities resulting in decreased blood flow (Roach, personal communication, December 05, 2008).

Exercise at altitude results in additional stress further complicating the body's already complex physiologic response to hypoxia (Mazzeo, 2005). Altitude studies are often completed as convenience studies involving participants who have ascended for recreational or military purposes, often involving exercise. The body is then being exposed not only to the stress of decreased oxygen availability, but also to exercise, exacerbating the physiologic and metabolic responses (Roach et al., 2000; Beidleman et al., 2004; Mazzeo, 2005). A study by Roach et al

(2000) found that physical exertion contributes to the development of AMS. They found that symptoms were more pronounced in individuals exposed to an exercise protocol than those who were sedentary. These results are consistent with previous studies which have found that passive ascent yields lower AMS symptom reporting (Nishihara et al., 1998). The correlation between increased exercise and increased AMS incidence is likely due exercise amplifying the physiologic effects of hypoxia (Roach et al., 2000; Mazzeo, 2005). Exercise decreases SpO_2 levels, increases dehydration (via sweating and increased expiration), and increases ventilation, heart rate, blood pressure and cardiac output (Irmay et al., 2005; Shah et al., 2006). Dehydration may interfere with the acclimatization process by interfering with bicarbonate diuresis (Shah et al., 2006). Dehydration is believed by some to be protective against AMS, reducing cerebral edema by reducing the hydrostatic forces that may contribute to capillary leak (Shah et al., 2006). The major determinants of cerebral blood flow are PaO_2, arterial pressure of carbon dioxide ($PaCO_2$), and blood pressure, all of which are altered in the presence of hypoxia and exercise (Irmay et al., 2005; Shah et al., 2006).

Basnyat (2002) discusses the potential of neck irradiation or surgery as a potential risk factor increasing susceptibility to AMS. He discusses how both procedures may result in dysfunctional carotid bodies, resulting in a deficient HVR. Research on this topic is currently limited due to the low number of available participants, but further research would be helpful in determining if patients who have undergone either radiation or neck surgery are more susceptible to all of the high-altitude illnesses.

It is widely accepted that self-limiting AMS will likely result in HACE upon continued ascent (Roach and Hackett, 2001), suggesting that the two are on the same continuum (Basnyat and Murdoch, 2003; Gallagher and Hackett, 2004). The prevailing hypothesis for both AMS and

HACE points to a process within the central nervous system (Basnyat and Murdoch, 2003). HACE has been identified as a vasogenic edema, suggesting a role of the BBB (Hackett and Roach, 2001). Hackett (1999) defines brain edema as an abnormal accumulation of fluid within the brain parenchyma. Hackett and Roach (2001) have proposed a model to explain the pathophysiology of AMS and HACE. In this model, changes in PaO_2 and arterial pressure of carbon dioxide ($PaCO_2$) result in hypoxemia (Imray et al., 2005), causing various neurohumoral and hemodynamic responses. The result is increased cerebral vasodilation resulting in increased cerebral blood volume, altered permeability of the BBB, and cerebral edema (Hackett, 1999; Hackett and Roach, 2001; Roach et al., 2002; Van Osta et al., 2005; Weil et al., 2007). These changes may result in overperfusion in the brain as a result of microvascular and biochemical changes, and may lead to brain swelling resulting in increased intracranial pressure (Gallagher and Hackett, 2004; Clarke, 2006; Kinsey and Roach, 2003; Palma et al, 2006; Weil et al., 2007). Two studies examining cerebral blood flow in the brain showed that there were no differences in AMS-susceptible versus AMS-resistant people (Dyer et al., 2008; Fischer et al., 2004). Murdoch and Basnyat (2003) summarize the 2001 Hackett and Roach model:

> AMS occurs in people who have inadequate cerebrospinal capacity to buffer the brain swelling; those with a greater ratio of cranial cerebrospinal fluid to brain volume are better able to compensate for swelling through displacement of cerebrospinal fluid, and are less likely to develop AMS than people with a lower ratio.

This is generally referred to as the "tight fit" hypothesis (Hackett, 1999). This hypothesis is considered by some as an oversimplification, which might explain why AMS continues to be difficult to predict (Wilson et al., 2009). The conclusions of a recent study by Bailey et al. (2009) suggest that increased NO may predispose individuals to AMS in conjunction with hypoxia-

mediated cerebral vasodilatation and/or following intracellular accumulation of hypoxic-inducible factor-1α (HIF-1α), and vascular endothelial growth factor (VEGF), all of which may contribute to alteration of the BBB.

One study examining the brain found that right temporal cerebral dysfunction occurred prior to the onset of clinical symptoms of AMS (Fedderson et al., 2007). They conclude that Electroencephalogram (EEG)-detected regional right temporal dysfunction may serve as a predictor for AMS (Fedderson et al., 2007). Further inquiry into this hypothesis is required.

While it is generally accepted that the brain swells at high altitude, some studies neglect to show an increase in cerebral blood flow, which further complicates AMS etiology. Mehta et al. (2008) found there was no increase in cerebral pressure, and believe the headache associated with AMS is likely due to a hypoxia mediated release of VEGF, bradykinin and NO (Mehta et al., 2008). These substances may sensitize the pain nerve endings in the brain resulting in headache (Mehta et al., 2008; Roach and Hackett, 2001). NO is thought to sensitize small fibres conveying pain, and is thought to accumulate close to trigeminovascular nerve fibres causing high-altitude headache (Roach and Hackett, 2001). There has been no confirmation of this hypothesis at this point in time.

Loeppky et al. (2005) discuss the potential of early fluid retention affecting AMS. In this study, it was found that the AMS-susceptible group has increased water retention, compared with the AMS-resistant group. This retention is believed to occur because of increased circulating arginine vasopressin levels, which cause anti-diuresis and increased extracellular water levels (Roach and Hackett, 2001). This may prevent bicarbonate excretion (Pichler et al., 2008) which is known to increase hyperventilation. In other words, AMS-susceptible individuals have a blunted HVR as a result of fluid retention (Harris et al., 1998; Hainsworth et al., 2008). Fluid

intake was found to be similar between the two groups, despite the potential influence of nausea, a known symptom of AMS (Nerin et al., 2006). Further research is required in this area to discover if fluid retention actually causes a blunted HVR.

A physiologically-based hypothesis regarding susceptibility to AMS is related to an individual's ability to alter the efficiency of oxygen use at the level of the mitochondria to maintain SpO_2 at altitude (Wilson et al., 2009). This theory is relatively new, and is under review.

Raised body temperature, a sign of an increased sympathetic response, and AMS have been found to be correlated (Maggiorini et al., 1997), suggesting that a rise in body temperature after rapid ascent to high altitude is a sign of AMS. Contrary to these findings, Loeppky et al. (2003) found that greater heat loss occurred during early AMS, suggesting increased hypoxic vasodilation in spite of enhanced sympathetic drive. More research is required to clarify these contradictory findings.

Treatment of AMS includes descent, supplemental oxygen, hyperbaric therapy, and acetazolamide ("Diamox") or dexamethasone. Acetazolamide is a carbonic anhydrase inhibitor, which acts as a respiratory stimulant by promoting bicarbonate excretion (Ward et al., 2000). A decrease in bicarbonate causes an increase in ventilation, improving PIO_2, and therefore SpO_2 (Ward et al., 2000; Golja et al., 2008). Dexamethasone is a potent anti-inflammatory steroid which can mask AMS. It is not recommended to continue ascent after taking dexamethasone, as it does not alleviate potential causes of AMS (i.e. lowered SpO_2). To avoid further development of symptoms, the person affected should avoid exertion and halt further ascent until symptoms subside (Hackett and Roach, 2001; Gallagher and Hackett, 2004). If AMS is moderate to severe, it is recommended that descent occur immediately, and that further ascent should not occur

(Hackett and Roach, 2001). A graded ascent (rest day every 600–1200 m) and a slow ascent rate (maximum 300-600 m/d) should allow adequate time for acclimatization and reduce the risk of all types of AMS (Hackett and Roach, 2001; Koehle, personal communication, April 04, 2009).

The present study limits some of the confounding variables such as; rate/type of ascent, barometric pressure/altitude attained and inconsistencies in testing procedures. Furthermore, by using a specific population, this study limits potential racial, socioeconomical, and other lifestyle differences that may exist in a more general population. Whether or not race affects AMS susceptibility currently remains unknown (Handler, 2009).

2.4 Janai Purnima Festival

The geography of Nepal is uncommonly diverse. Landlocked between China and India, Nepal is roughly trapezoidal in shape (Explore Nepal, n.d.). Nepal is 800 kilometres long and 200 kilometres wide, occupying a total land area of 147,181 square kilometres (National Geographic, n.d.). The northern portion of the country contains ten of the world's fourteen highest mountains (Mayhew, 2006). Nepal is divided into three physiographic areas: the Mountain, Siwalik ("Hill") region and Terai ("moist land") regions (Nepal: Geography, 1991). The majority of the population (over 50%) lives in the Terai, the lowest altitude region (Mayhew, 2006).

There are five climactic zones in Nepal. These zones may be defined by varying altitude as; the tropical and subtropical zones, which lie below 1,200 metres, the temperate zone (1,200 to 2,400 m), the cold zone (2,400 to 3,600 m), the subarctic zone (3,600 to 4,400 m), and the arctic zone, which lies above 4,400 m (Mayhew, 2006).

Over eighty percent of Nepal's population practices the main religion, Hinduism (The World Factbook, 2009). The second largest religion is Buddhism (10.7%) (The World Factbook, 2009). Both religions share common temples and worship common deities, and consider Shiva as the divine being. Nepal is home to the largest Shiva temple in the world, the Pashupatinath Temple.

The participants tested in this study were religious pilgrims participating in the Janai Purnima Festival. During the festival Hindu men perform their annual change of Janai ("sacred thread"), a yellow cotton string worn across the chest or tied around the wrist of the right hand (Shah, 2006). According to Hindu rules the Janai must be worn everyday of their lives, and the cord must be changed without fail by a Brahman Priest on the Purnima ("full moon") (Shah, 2006).

The festival takes place annually during the full moon in August. During the festival, several thousand Nepali people take part in a pilgrimage that involves a rapid ascent from the Terai (under 2000 m) to higher than 4000 m in less than three days (Basnyat, 2000). While the festival takes place throughout the country, a very grand religious gathering occurs at Lake Gosainkund, where the many of the pilgrims bathe in the lake to eradicate their sins prior to receiving their new Janai from Bhramin Priests (Janai Purnima, 2009).

The religious pilgrims who participated in this study ascend for religious rather than recreational purposes, and as such, generally did not have extensive mountaineering experience. While the majority of pilgrims that take part in the Janai Purnima Festival are Hindu, a large Buddhist population partake as well (Koehle, personal communication, March 19th, 2009).

As a consequence of the quick rate of ascent, one of the major risk factor described earlier, many pilgrims suffer from AMS. A study completed by Basnyat et al in 2000 determined

that the incidence of pilgrims experiencing AMS during the Janai Purnima Festival was as high as 68% (n=228), one of the highest recorded rates.

A study examining energy requirements at high altitude found that severe environmental conditions and intense physical exertion led to increased individual energy requirements (Barnholt, 2006). During the festival, a large percentage of the pilgrims fast, forcing them to adapt to the physiologic effect of hypoxia and the metabolic effects of energy imbalance concurrently, likely contributing to the extremely high incidence of AMS. Caloric restriction at high altitude decreases the available glucose, contributing to a shift in substrate use from carbohydrates to alternative fuel sources such as fat or ketones to support energy needs (Barnholt, 2006). Furthermore, ingestion of carbohydrates increases the production of carbon dioxide, which stimulates ventilation and improves SpO_2 in hypoxic conditions (Golja, 2008).

Chapter 3: Methodology

3.1 Background Information

Every August, approximately 12,000 Nepali people make the pilgrimage from their lowland homes to Lake Gosainkund at 4380 m in Langtang, Nepal to honour the full moon in the Janai Purnima Festival (Koehle et al. et al., 2005; Shah et al. et al., 2006). Most of these pilgrims ascend from approximately 2000 m over a span of one to three days (Shah et al., 2006). Typically, the pilgrims ascend the final 2200 metres to the lake in 1-2 days. This rapid rate of ascent results in an unusually high incidence of AMS (68%) (Basnyat et al, 2000; Koehle et al., 2005).

The data collected for use in this research were obtained by emergency medical teams set up to provide care for the ascending pilgrims. In 48 hours, the teams treated people with a variety of conditions, including altitude-related illnesses. After treatment, patients with AMS were invited to participate in the study. Treating physicians clinically identified subjects with AMS and then determined their Lake Louise score using the self-assessment questionnaire (Roach et al., 1993). Healthy non-AMS participants were other festival attendees, and were contacted by researchers outside the clinic. Once participants were recruited and consented, they were screened to exclude those with significant comorbidities that may have affected their susceptibility to AMS (e.g. respiratory or cardiac disease) and those taking preventative medication (i.e. acetazolamide). Once accepted into the study, a physical exam was conducted, and anthropometric, historical and physiologic data were obtained while the participants were at rest.

3.2 Research Design

The present study is a retrospective analysis using previously collected data from 2005 during the Janai Purnima Festival. The use of information involved in this study was approved by, and permission was granted by, the researchers who completed the primary study. Prior to data collection, approval was acheieved by the University of British Columbia Clinical Ethics Review Board, and informed consent was obtained from all participants. The Research Ethics Board at Lakehead University reviewed the study protocol and confirmed that the present study did not require additional Research Ethics Board approval, as it is a retrospective analysis.

The focus of the primary study was genetic predisposition to AMS susceptibility. In addition to the primary data from these studies, the researchers obtained additional, non-invasive, physiologic and anthropological measurements which provide the data for the present study.

The 2005 study consisted of 40 participants (n=40). The variables measured included: Lake Louise Score, sex, age, height, mass, heart rate, systolic blood pressure, diastolic blood pressure, SpO_2, ACE genotype, and eNOS genotype.

3.3 Measurement Tools

3.3.1 Lake Louise Score

The Lake Louise AMS Score is a standardized questionnaire, created to measure AMS symptoms objectively. The test involves participants completing a five question survey conveying various symptoms regarding headache, gastrointestinal symptoms, fatigue/weakness or both, dizziness or lightheadedness, and difficulty sleeping (Roach et al. 1993). The score from

each question is then added to determine a numerical score ranging from zero to fifteen, with zero being low and fifteen being high. The mostly widely accepted cut off score for a positive AMS test is a score of greater than or equal to three; a score of two or less results in a negative score, and thus the absence of AMS.

The Lake Louise Acute Mountain Sickness Scoring System self-assessment questionnaire was used to determine the Lake Louise AMS Score. This score was then used to determine the presence or absence of AMS for the present study, with three or greater representing a positive score (Roach et al, 1993). Please refer to appendix A for a detailed description of the Lake Louise Acute Mountain Sickness Scoring System.

3.3.2 Age, Sex, and Previous History of AMS

Age, sex and previous history of AMS were assessed and recorded during an interview with participants via a translator.

3.3.3 Heart Rate

Heart rate was assessed with subjects resting supine on mats on the floor, separated from the rest of the clinic by a divider. Subjects wore a heart rate strap from a commercially available heart-rate monitor (Polar S810, Polar Electro, Kempele, Finland). After a 5-minute rest period, heart rate was collected on a beat-by-beat basis for ten minutes.

3.3.4 Pulse Oximetry

After a five minute rest period, pulse oximetry was measured while participants were lying in the supine position on the floor for a period of ten minutes using a Nonin 8500 portable

finger oximeter (Nonin Medical Inc., Plymouth MN, U.S.A.). The device was placed on the index finger of the dominant hand.

3.3.5 Blood Pressure

Blood pressure was measured by auscultation using a manual sphygmomanometer by an attending physician. Blood pressure was measured and recorded at the end of the examination while the participants were at rest.

3.3.6 Height

Height was measured in centimetres.

3.3.7 Weight

Weight was measured in kilograms using a digital bathroom scale.

3.3.8 ACE/NO

Buccal mucosal scrapings were taken from four locations in the mouth using an endocervical sampling cytobrush (CooperSurgical Inc., Trumbull, CT, USA) and stored dry in envelopes for transport (King et al., 2002). On return to Canada, genomic DNA was extracted from the sample brushes (Saftlas et al., 2004). For a detailed description of the DNA sampling and genotyping, please refer to appendices B and C.

3.4 Data Collection and Storage

Data were received via password protected email from the primary researcher in Microsoft Excel format. The data were then transferred to the present researcher's personal computer, and a second master database was created in and kept in an alternate location, matching the participants to their unique study number. All personal information regarding participants was kept confidential and were stored under password protection.

3.5 Statistical Analyses

The analyses were completed using Statistical Analysis Software (SAS). The following procedures were analyzed.

1) Body Mass Index (BMI) was calculated using the following formula:

$$\frac{\text{mass}}{\text{height X height}}$$

2) Mean Arterial Pressure (MAP) was calculated using the following formula:

$$\frac{(2 \text{ x diastolic blood pressure}) + \text{systolic blood pressure}}{3}$$

3) Frequency tables were created for all categorical variables (sex, ACE, NO)
4) The univariate function was used to obtain descriptive statistics for all continuous variables (Lake Louise Score, age, BMI, heart rate, systolic blood pressure, diastolic blood pressure, and SpO_2), including mean, standard deviation, minimum values, maximum values, standard error and confidence intervals (CI).

5) The correlation procedure was completed to assess the correlation between variables.
6) Generalized linear modelling was completed to assess significance of predictive ability of certain variables onto others.
7) Lake Louise Score was then divided into those determined to have a presence of AMS (AMS+), and those that did not display symptoms (AMS-). This was done in order to determine which characteristics were common amongst each group.
8) Frequency tables were created using the AMS+ and AMS- groups for all categorical variables (sex, ACE, NO).
9) Generalized linear modelling was completed for each new level of Lake Louise Score (AMS+ and AMS-), using SpO_2 as the dependent variable, and ACE and NO as the predictor variables. This procedure was completed to examine the predictive ability of ACE and NO on SpO_2, as previous research suggested that either or both may be accurate predictors (see review of literature).
10) Generalized linear modelling was completed for each new level of Lake Louise Score (AMS+ and AMS-), using each of the continuous variables (age, BMI, heart rate, systolic blood pressure, diastolic blood pressure, and SpO_2). This was completed to assess the predictive capabilities of each of the measured variables.
11) As the NO group TT (thymine-thymine) was so small, TT and TG (guanosine) were combined, creating two groups for NO, instead of three. A chi square analysis was completed to determine the significance of NO on Lake Louise Score.

Chapter 4 – Results

All raw data were received in Microsoft Excel (2003) spreadsheets from the primary researcher. The data were then imported into a SAS (version 9) data file and the following procedures were completed; means, frequency, correlation, generalized linear model, and regression analysis. Data were collapsed into New Lake Louise Score in order to analyze the data based on the presence (AMS+) or the absence (AMS-) of AMS, as described by Roach et al., 1993.

4.1 Descriptive Statistics

Table 4.1.1 presents the number of observations (N), means, standard deviations (SD), minimum values (min), maximum values (max), standard errors (SE), and 95% confidence intervals ($CI_{95\%}$) for anthropometric and physiologic measurements. There were 107 participants involved in this study; however only data for approximately 40 participants included all variables in the data set. The age range was from 17 to 76, and the average age was 33.38 ± 13.26. Average BMI was 22.2 ± 7.94; average heart rate was 88.00 ± 15.78; average systolic blood pressure was 124.13 ± 13.93; average diastolic blood pressure was 75.75 ± 12.30; and average SpO_2 was 81.12 ± 6.70.

Table 4.1.1

Anthropometry and Physiologic Measurements

Variable	N	$\bar{X}$	SD	Min	Max	SE	$CI_{95\%}$
Age	107	33.38	± 13.26	17	76	1.28	33.38 ± 2.51
BMI	107	22.20	± 3.41	15.20	32.73	0.33	22.20 ± 0.65
HR	40	88.00	± 15.78	61.04	118.34	2.49	88.00 ± 4.88
SBP	41	118.71	± 13.93	86.00	143.00	2.17	118.71 ± 4.25
DBP	41	81.93	± 12.30	56.00	115.00	1.92	81.93 ± 3.76
SpO_2	43	81.14	± 6.70	66.00	92.00	1.02	81.14 ± 2.00

Note: BMI = mass/height^2 HR = mean heart rate; SBP = systolic blood pressure; DBP = diastolic blood pressure; SpO_2 = arterial oxygen saturation

4.2 Frequency Information

Table 4.2.1 presents the frequency information for all the categorical variables. Of the 107 participants, 78.5% of the population was male, while 21.5% was female. One important aspect of this study was the identification of the gene for ACE. The most frequently occurring gene for ACE was the heterozygote ID (43), while the least frequent was the homozygote DD (22). For the NO gene, the guanine homozygote (GG) was the most prominent, and was found in 60.19 % of the population.

Table 4.2.1

Frequency Information for All Categorical Variables

Variable	Frequency	Percent
Sex		
Male	84	78.5
Female	23	21.5
ACE		
DD	22	21.36
ID	43	41.75
II	38	36.89
NO		
GG	62	60.19
TG	36	34.95
TT	5	4.85

Note: ACE = angiotensin converting enzyme (D = deletion, I = insertion); NO = nitric oxide (G = guanine, T = thymine)

4.3 Percent Distribution of Lake Louise Score

Figure 4.3.1 presents the percent distribution of the Lake Louise Score for the 107 participants. Fifty-one percent of people within the population had a Lake Louise Score of zero. The scores ranged from zero to twelve out of a possible fifteen, with only one percent of people experiencing a score of greater than nine.

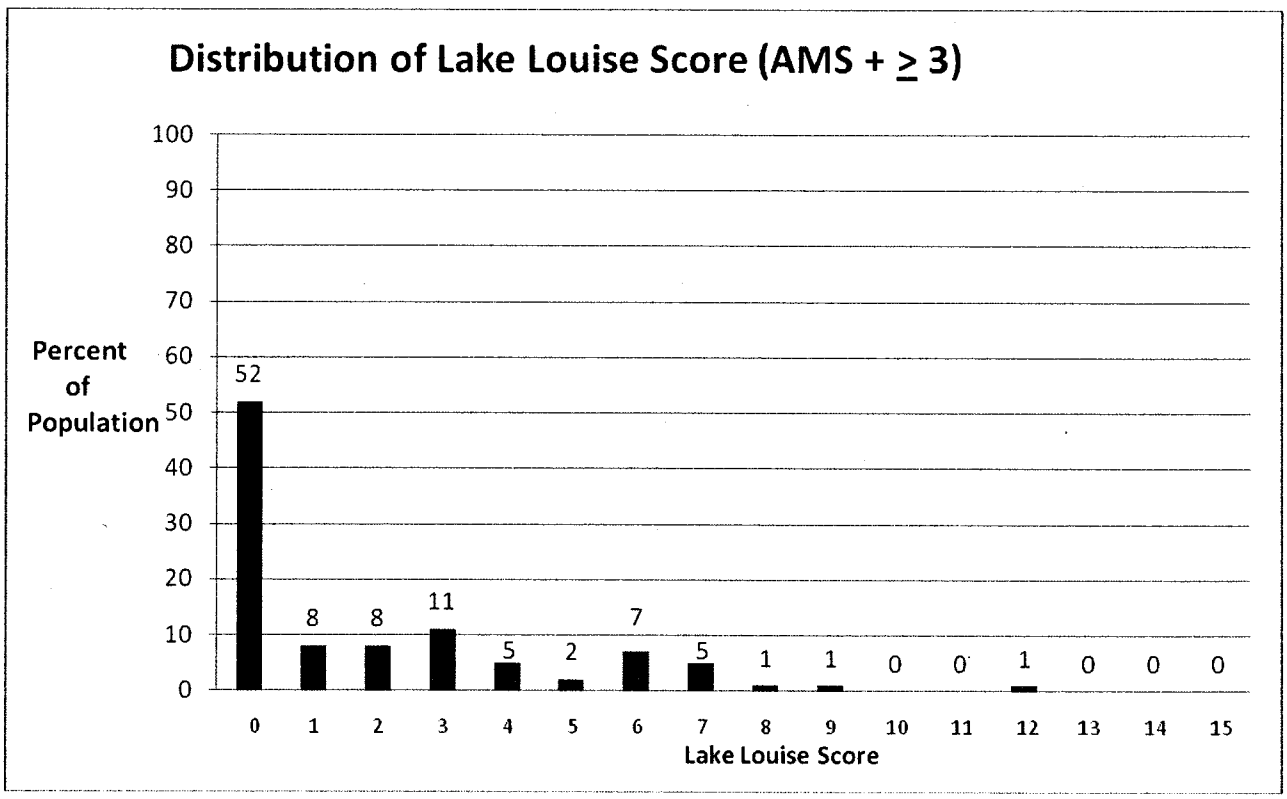

Figure 4.3.1 - *Percent Distribution of Lake Louise Score*

4.4 Frequency Information for New Lake Louise Score

Table 4.4.1 presents information for the New Lake Louise Score. Lake Louise Score was divided into two groups based on the presence (AMS $\geq$ 3), or absence (AMS < 3) of symptoms. Using a Lake Louise AMS score of 3 or greater to indicate the presence of AMS, the incidence of AMS was 32.71%. The incidence of participants who experienced AMS according to the Lake Louise Criteria was approximately one third of participants.

Table 4.4.1

Frequency Information for New Lake Louise Score

Variable	Frequency	Percent
New Lake Louise Score		
AMS-	72	67.28
AMS+	35	32.71

4.5 *Anthropometry and Physiologic Measurements for New Lake Louise Score*

Table 4.5.1 displays mean values for each continuous variable by New Lake Louise Score. The most notable differences in the data were age, systolic blood pressure, and diastolic blood pressure.

Table 4.5.1

Mean Values for Continuous Variables by New Lake Louise Score (AMS- vs. AMS+)

Variable	*AMS –*	*AMS+*	*P*	*N*
Age	30.07 ±10.44	39.55 ± 14.58	0.02*	103
BMI	22.40 ± 3.26	22.43 ± 3.48	0.63	103
HR	87.54 ± 16.77	90.92 ± 14.70	0.62	37
SBP	115.09 ± 11.37	125.27 ± 12.72	0.62	38
DBP	78.39 ± 9.63	88.33 ± 12.70	0.24	38
SpO_2	82.57 ± 6.70	78.88 ± 5.86	0.47	40

*Note: * = significant; BMI = body mass index; HR = heart rate; SBP = systolic blood pressure; DBP = diastolic blood pressure; SpO_2 = arterial oxygen saturation*

4.6 Pearson Correlations

Table 4.6.1 presents Pearson product moment correlation coefficients between all continuous variables (age, BMI, heart rate, systolic blood pressure, and SpO_2) for all participants. A p value of less than 0.05 was considered significant. BMI and heart rate were shown to be significantly related ($r = 0.35$, $p = 0.03$). Heart rate was also significantly related to SpO_2 ($r = -0.52$, $p = 0.00$). Systolic blood pressure was shown to be significantly correlated to diastolic blood pressure ($r = 0.71$, $p = 0.00$) and to SpO_2 ($r = -0.33$, $p = 0.05$).

Table 4.6.1

Pearson Correlations between Anthropometric and Physiologic (column heading) Data, and Physiologic and Anthropometric (row heading) Data for All Participants

Variable	Age	BMI	HR	SBP	DBP	SpO_2
Age	-	-	-	-	-	-
BMI	-	-	$r = 0.35$ $p < 0.03$	-	-	-
HR	-	$r = 0.35$ $p < 0.03$	-	-	-	$r = -0.52$ $p < 0.00$
SBP	-	-	-	-	$r = 0.71$ $p < 0.00$	$r = -0.33$ $p < 0.05$
DBP	-	$r = 0.39$ $p < 0.01$	-	$r = 0.71$ $p < 0.00$	-	-
SpO_2	-	-	$r = -0.52$ $p < 0.00$	$r = -0.33$ $p < 0.05$	-	-

Note: Correlations with $p > 0.05$ are not included; BMI = body mass index, HR = heart rate; SBP = systolic blood pressure; DBP = diastolic blood pressure; SpO_2 = arterial oxygen saturation

The results for Lake Louise Score were divided into two groups based on the presence (AMS+), or absence (AMS-) of symptoms using the Lake Louise guidelines (AMS+ ≥ 3, AMS- < 3) (Roach et. al., 1993).

Table 4.6.2 presents Pearson correlations between all continuous variables (age, BMI, heart rate, systolic blood pressure, and SpO_2) for participants found not to have AMS. A p value less than 0.05 was considered significant. Age was shown to be significantly correlated to BMI ($r = 0.33$, $p < 0.01$). Heart rate was significantly correlated to systolic blood pressure ($r = 0.47$, $p < 0.03$), and negatively correlated to SpO_2 ($r = -0.61$, $p < 0.00$). Systolic blood pressure and diastolic blood pressure were shown to be significantly correlated ($r = 0.77$, $p < 0.00$).

Table 4.6.2

Pearson Correlations between Anthropometric and Physiologic (column heading) Data, and Physiologic and Anthropometric (row heading) Data for AMS - Participants

Variable	Age	BMI	HR	SBP	DBP	SpO_2
Age	-	$r = 0.33$ $p < 0.01$	-	-	-	-
BMI	$r = 0.33$ $p < 0.01$	-	-	-	-	-
HR	-	-	-	$r = 0.47$ $p < 0.03$	-	$r = -0.61$ $p < 0.00$
SBP	-	-	$r = 0.47$ $p < 0.03$	-	$r = 0.77$ $p < 0.00$	-
DBP	-	-	-	$r = 0.77$ $p < 0.00$	-	-
SpO_2	-	-	$r = -0.61$ $p < 0.00$	-	-	-

Note: Correlations with $p > 0.05$ are not included; BMI = body mass index; HR = heart rate; SBP = systolic blood pressure; DBP = diastolic blood pressure; SpO_2 = arterial oxygen saturation

Table 4.6.3 presents Pearson correlations for significant relationships between all continuous variables (age, BMI, heart rate, systolic blood pressure, and SpO_2) for participants who presented with AMS (AMS+). A p value less than 0.05 was considered significant. Age was shown to be significantly correlated to heart rate ($r = 0.58$, $p < 0.02$). BMI was significantly correlated to diastolic blood pressure ($r = 0.57$, $p < 0.03$), and to SpO_2 ($r = -0.61$, $p < 0.01$). Systolic blood pressure was shown to be significantly correlated to diastolic blood pressure ($r = 0.53$, $p < 0.04$), and to SpO_2 (r=-0.57, p<0.03).

Table 4.6.3

Pearson Correlations between Anthropometric and Physiologic (column heading) Data, and Physiologic and Anthropometric (row heading) Data for AMS + Participants

Variable	Age	BMI	HR	SBP	DBP	SpO_2
Age	-	-	$r = -0.58$ $p < 0.02$	-	-	-
BMI	-	-	-	-	$r = 0.57$ $p < 0.03$	$r = -0.61$ $p < 0.01$
HR	$r = -0.58$ $p < 0.02$	-	-	-	-	-
SBP	-	-	-	-	$r = 0.53$ $p < 0.04$	$r = -0.57$ $p < 0.03$
DBP	-	$r = 0.57$ $p < 0.03$	-	$r = 0.53$ $p < 0.04$	-	-
SpO_2	-	$r = -0.61$ $p < 0.01$	-	$r = -0.57$ $p < 0.03$	-	-

Note: Correlations with $p > 0.05$ are not included; BMI = body mass index; HR = heart rate; SBP = systolic blood pressure; DBP = diastolic blood pressure; SpO_2 = arterial oxygen saturation

4.7 Chi Square Analysis

Table 4.7 presents a chi square analysis testing the uniformity across cells for sex, ACE, and NO. Sex was not found to be significant in relation to New Lake Louise Score. New Lake Louise Score was also not found to be significantly determined by the ACE gene. New Lake Louise Score was found to be significant in relation to NO, with the majority of individuals containing the GG homozygote. To further investigate this finding, and because the group sizes for TT and TG were small, the GG homozygote was isolated by collapsing the data into New NO containing two groups: GG and TT+TG. The chi square analysis was then repeated. Collapsing the data into New NO increased the significance, showing that the cells in the analysis were not uniform. This finding suggests that the GG homozygote may be protective against AMS, as most of the AMS- individuals were identified with the GG homozygote.

Table 4.7.1

Frequency and Chi Square Values for Categorical Variables by New Lake Louise Score

Variable	AMS-	AMS+	Chi Square	P
Sex				
Male	60	24	3.0413	0.0812
Female	12	11		
ACE				
DD	17	5	1.4	0.4947
ID	27	16		
II	26	12		
NO				
GG	48	14	8.2138	0.0165 *
TG	18	18		
TT	4	1		
NewNO				
GG	48	14	6.3992	0.0114 *
TG/TT	22	19		

Note: * = significant; *ACE = angiotensin converting enzyme (D = deletion, I = insertion); NO = nitric oxide (G = guanine, T = thymine)*

4.8 Generalized Linear Modeling

The generalized linear model (GLM) was used to measure the significance of the predictive ability of the genes ACE and NO across New Lake Louise Scores. Lake Louise Score was divided into two groups based on the presence, or absence of AMS in order to investigate the groups separately. The GLM procedure was used for all continuous variables (age, BMI, heart rate, systolic blood pressure, and SpO_2), none of which were found to be predicted by ACE or NO in either AMS- or AMS+ groups. The results of the GLM analysis for systolic blood pressure and diastolic blood pressure also proved to be negative, showing that neither ACE nor NO provided predictability for systolic blood pressure and diastolic blood pressure.

Table 4.8.1 shows the GLM procedure using SpO_2 as the dependent variable across both levels of New Lake Louise Score. ACE and NO were not found to be significant predictors of SpO_2 in the AMS- group ($p=0.53$). In the AMS+ group ACE and NO were found to be significant predictors of SpO_2, with ACE ($p=0.04$) being significant, and NO ($p=0.06$) lending itself towards significance. Table 4.8.1 presents the mean square (MS), the mean square error (MSE), the F value (F), and the probability for both the model and for the individual genes.

Table 4.8.1

The GLM procedure for SpO_2 by ACE, NO across New Lake Louise Scores

Group	Model	MS	F	P
AMS -	SpO_2 Model	38.46	0.76	0.53
	ACE	28.27	0.56	0.58
	NO	47.03	0.93	0.35
AMS +	SpO_2 Model	86.30	3.86	0.04 *
	ACE	86.57	3.87	0.05 *
	NO	93.34	4.17	0.06

*Note: * = significant; SpO_2 = arterial oxygen saturation; ACE = angiotensin converting; NO = nitric oxide*

ACE and NO predict SpO_2 for the AMS+ group because the variables are robust as the group is homogeneous. The larger AMS- group may have too much variability causing a more profound effect on the predictability of ACE and NO on SpO_2.

4.9 Regression Analysis

To determine the predictive capacity of ACE and NO on systolic blood pressure, diastolic blood pressure and SpO_2 across New Lake Louise Scores, a regression analysis was performed. The outcome produced by these analyses was that SpO_2 was the only variable that was significantly predicted by the genes ACE and NO in the AMS+ group. The model was found to be significant (p=0.0347) and the prediction equation is show below:

Where;

NewNO	NewACE
1 = GG	1 = DD
2 = TG or TT	2 = ID
	3 = II

For AMS+ group:

$SpO_2 = 92.8 - 4.4$ (NewNO) – 3.35 (newACE)

For example, if a person presents with a NewNO of TT, and they contain the II polymorphism for NewACE, his/her predictive equation will be:

$SpO_2 = 92.8 - 4.4\ (2) - 3.35\ (3)$

$= 79.4\%$

The results of this regression procedure indicate the ability of genetic markers (ACE and NO) to predict a significant difference in SpO_2 for individuals in the AMS+ group.

Chapter 5: Discussion

5.1 Conclusions of Findings

The purpose of the present study was to determine which variables are correlated to the incidence of AMS among individuals participating in the Janai Purnima Festival in Nepal. Exploratory analyses were used to investigate the relationships between several variables based on findings of previous research. As such, this research was an extension of earlier work completed by Wang, P., Koehle, M.S., and Rupert, J.L. in 2008 at the University of British Columbia. The previous study focussed on the association between NO and AMS based on the clinical diagnosis of AMS (Roach et al., 1993). We further investigated the relationship between NO and ACE on several physiologic variables based on the self-assessment portion of the Lake Louise Questionnaire (Roach et al., 1993).

Participants were divided into two groups based on the presence or absence of AMS, derived from data from Lake Louise Score. They were then collapsed into two groups thereby creating a New Lake Louise Score. According to Table 4.4.1 in the results section, the incidence rate of AMS was 33%, much lower than the finding of Basnyat et al. (2000) that was also recorded during the Janai Purnima festival. This discrepancy in incidence occurred despite a similar methodology of assessment (Roach et al, 1993). The results from the present study were consistent with those of previous findings (Maggiorini et al. 1993; Gallaher and Hackett, 2004).

A t-test determined that there was a significant difference ($p=0.02$) for the mean ages between the AMS- ($\bar{x}=30.1$) and the AMS+ ($\bar{x}=39.6$) groups. This finding is in contrast to the findings of Gallagher and Hackett (2004), Roach et al. (2002) and Vardy et al. (2005), and Honigman et al. (1993), who all indicated that advanced age may be preventive of AMS. It is

interesting to note that while the t-test for variables such as BMI, systolic blood pressure and diastolic blood pressure did not produce significant results, when looking at the data, the values for each are lower in the AMS- group, suggesting that increased fitness capacity may be preventive against AMS, but that this difference was not statistically significant is contrary to previous findings (Hackett and Roach, 2001; Jafarian et al., 2008; Roach et al, 2000; Honigman et al, 1995; Birnbaum and Plourde, 2007; Bircher et al., 1994; Bärtsch et al., 2001).

Pearson Product Moment Correlations displayed a fairly strong negative relationship between heart rate and SpO_2 (r=-0.61, P=0.00) indicating that as SpO_2 decreases, heart rate increases. This finding seems logical, as decreased oxygen availability should stimulate an increased heart rate to ensure adequate oxygen availability. As expected, systolic blood pressure and diastolic blood pressure were strongly correlated (r-0.71, p=0).

A chi square analysis of NO across New Lake Louise Scores provided significant results, indicating that the majority of individuals containing the GG homozygote did not suffer from AMS. This finding suggests that the GG homozygote may be protective against AMS. It should be noted that the TT and TG groups were small in comparison to the GG group, and an increased sample size containing a larger number of all genotypes would provide further insight into the significance of this finding.

The chi square analysis for ACE did not provide statistically significant results (p=0.49), indicating that there does not appear to be an allele (I or D) that is protective for the ACE gene. This contradicts the findings of Bigham et al., 2008, who found that the I-allele was associated with increased performance at high altitude. The Bigham et al., 2008, study, however, was conducted on high-altitude natives, while the present study examined predominantly lowlanders.

The major finding of the present study was the apparent predictive capacity of the genes ACE and NO on SpO_2 across New Lake Louise levels. The implications of the ability of ACE and NO to predict SpO_2 levels in the AMS+ group suggests that there is potential for genetic markers for SpO_2. While the relationship between AMS and SpO_2 remains inconclusive, it seems logical that as SpO_2 level declines, exposure to a more hypoxic condition increases.

5.2 Limitations

The Janai Purnima festival is a religious pilgrimage undertaken by thousands. The altitude attained is consistent; however ascent rates, and therefore exertion levels, may vary among participants. As increased exertion has been found to increase incidence of AMS (Roach et al., 2000), and thus an individual's level of exertion on the trek may influence the susceptibility to AMS.

Other events influence physiologic factors such as sympathetic activity. In addition to altitude and hypoxia, the participants may also be exposed to cold, wind, radiation, hypoglycaemia, or dehydration. Psychological factors, expectations, and excitement may also influence the sympathetic nervous system, especially when investigations are carried out in the field (Rostrup, 1998).

Only participants who made it to the emergency medical tent were assessed, possibly excluding those who may not have reached the tent for various reasons. This limitation may influence the results, as those affected by high-altitude illness may have been amongst those who did not reach the medical tent.

The participants of the present study were Nepali pilgrims, and as such the findings of the present study may not be generalizable to other populations.

5.3 Delimitations

The present study examines retrospective non-invasive easily measured physiologic and anthropological data. The parameters for the present study were limited to the data that were collected by researchers in 2005, and may have excluded variables that might contribute to the predictability of an individual's susceptibility.

5.4 Significance of Findings

The findings of the present study suggest that the GG homozygote for the NO gene may offer protective qualities against AMS. If further research supports this finding, it means that individuals could be tested for their susceptibility to AMS, and strategies could be created (e.g. alternative ascent plans) to prevent AMS.

In addition, the fact that SpO_2 can be predicted by the ACE and NO genes has a potentially more profound significance. In addition to being significant for those who ascend to high altitudes, this information could also be useful in the clinical setting, as hypoxia is found in many common cardiovascular and respiratory illnesses.

5.5 Recommendations for Future Research

As this was a retrospective analysis, variables were limited to those measured when the primary research was collected in 2005. A prospective analysis using similar variables could increase the significance of this research.

Further research investigating the significant findings of the present study would determine the potential of ACE and NO as genetic markers for SpO_2 levels in AMS susceptible individuals, and of NO's GG homozygote's potential protective capacity.

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LAKE LOUISE ACUTE MOUNTAIN SICKNESS SCORING SYSTEM

The Lake Louise Acute Mountain Sickness (AMS) score provides a system for both self-assessment and clinical assessment of the symptoms and signs of AMS. The self-assessment score can also be administered by a healthcare provider or researcher. A person is considered to have AMS *only* if he or she has a headache score of at least mild severity and one additional symptom. The person's total points (maximum 15) are summed to become his or her AMS score.

1. Self-Assessment

a. Headache
- 0 No headache
- 1 Mild headache
- 2 Moderate headache
- 3 Severe headache (incapacitating)

b. Gastrointestinal symptoms
- 0 No gastrointestinal symptoms
- 1 Poor appetite or nausea
- 2 Moderate nausea or vomiting
- 3 Severe nausea or vomiting (incapacitating)

c. Fatigue, weakness, or both
- 0 Not tired or weak
- 1 Mild fatigue or weakness
- 2 Moderate fatigue or weakness
- 3 Severe fatigue or weakness (incapacitating)

d. Dizziness or Lightheadedness
- 0 Not dizzy or lightheaded
- 1 Mild dizziness or lightheadedness
- 2 Moderate dizziness or lightheadedness
- 3 Severe dizziness or lightheadedness (incapacitating)

e. Difficulty Sleeping
- 0 Slept as well as usual
- 1 Did not sleep as well as usual
- 2 Woke many times, poor night's sleep
- 3 Could not sleep at all

2. Clinical Assessment

a. Change in mental status
- 0 No change in mental status
- 1 Lethargy or lassitude
- 2 Disoriented or confused
- 3 Stupor or unconsciousness

b. Ataxia (heel to toe walking)
- 0 No ataxia
- 1 Maneuvers to maintain balance
- 2 Falls down
- 3 Can't stand

c. Peripheral edema
- 1 No peripheral edema
- 2 Peripheral edema at one location
- 3 Peripheral edema at two or more locations

3. Functional Score

Overall, if you had any symptoms, how did they affect your activity?
- 0 No reduction in activity
- 1 Mild reduction in activity
- 2 Moderate reduction in activity
- 3 Severe reduction in activity

Adapted with permission from Roach RC, Bärtsch P, Hackett PH, Oelz O, and the Lake Louise AMS Scoring Consensus committee. The Lake Louise Acute Mountain Sickness scoring system. In: Sutton JR, Houston CS, Coates G, eds. *Hypoxia and Molecular Medicine*. Burlington, Vt: Queen City Press; 1993: 273–274.

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No Association Between Variants in the ACE and Angiotensin II Receptor 1 Genes and Acute Mountain Sickness in Nepalese Pilgrims to the Janai Purnima Festival at 4380 m

MICHAEL S. KOEHLE,[1,2] PEI WANG,[2] JORDAN A. GUENETTE,[2] and JIM L. RUPERT[2]

ABSTRACT

Koehle, Michael S., Pei Wang, Jordan A. Guenette, and Jim L. Rupert. No association between variants in the ACE and angiotensin II receptor 1 genes and acute mountain sickness in Nepalese pilgrims to the Janai Purnima Festival at 4380 m. *High Alt. Med. Biol.* 7:281–289, 2006.—Acute mountain sickness (AMS) causes significant morbidity among visitors to altitude. The primary contributors to developing AMS are altitude and rate of ascent; however, the substantial variation in susceptibility between individuals has led a number of investigators to propose that there may be genetic predilection to the disease. The ACE I/D polymorphism has been shown to predict performance among elite mountaineers. This study compares genotype and allele frequencies at the ACE I/D locus, two other loci in the ACE gene, and one locus in the angiotensin-2 receptor gene between individuals who did, or did not, express signs of AMS while attending a high altitude religious festival in Nepal (4380 m). Subjects (80 males, 23 females) were recruited and genotyped. All subjects were Nepalese. Forty-four of the subjects had been diagnosed with AMS by physicians at a high altitude health camp; the rest were free from altitude illness. All subjects were genotyped at polymorphic loci in the genes encoding angiotensin converting enzyme (*ACE*) and angiotensin II receptor type 1 gene (*AGTR1*). The polymorphisms examined were two single nucleotide polymorphisms (SNPs) in *ACE* ($ACE^{A\text{-}240T}$, dbSNP rs4291; and ACE^{A2350G}, dbSNP rs4343), the intronic *Alu* insertion in *ACE* (ACE I/D), and the SNP ATR^{A1166C}, (dbSNP rs17231380) in *AGTR1*d. All polymorphisms in *ACE* were found to be in linkage disequilibrium. No significant associations were found between AMS incidence and any of the alleles, suggesting that variants at these loci do not contribute to susceptibility to AMS in this population.

Key Words: altitude-related illness; altitude; hypoxia; Nepalese; angiotensin; acute mountain sickness (AMS)

INTRODUCTION

ACUTE MOUNTAIN SICKNESS (AMS) is a widespread condition affecting sojourners to high altitude. The symptoms of AMS include headache, nausea, anorexia, fatigue, dizziness, and insomnia. The clinical presentation of AMS can range from very mild to severe (Hackett and Roach, 2001; West, 2004). As altitude-related illness often occurs in remote areas re-

[1]Allan McGavin Sport Medicine Centre, Department of Family Practice, University of British Columbia, Vancouver, Canada.
[2]School of Human Kinetics, University of British Columbia, Vancouver, Canada.

quiring risky and expensive evacuations and subsequent medical care, prevention is important to reduce morbidity, mortality, and healthcare costs. Unfortunately, acclimatization to altitude in humans is extremely variable. Some individuals are able to acclimatize very quickly; others have much more difficulty. Evidence indicates a genetic contribution to the pathophysiology of these conditions (Rupert and Koehle, 2006) and that identifying specific genotypes that increase susceptibility or severity would both enhance our understanding of the pathophysiology of the disease and improve our ability to develop interventions.

Fluid retention is linked to AMS (Hackett et al., 1982); consequently, there has been substantial interest in the association of variants in the gene-encoding components of the renin–angiotensin system (RAS), which plays a role in the regulation of plasma volume and vascular tone, and susceptibility to or history of the condition. Many of these studies involve the presence (or insertion, I) or absence (or deletion, D) of an intronic *Alu* segment in the gene-encoding angiotensin converting enzyme (ACE) (Rigat et al., 1990). The potential impact of the ACE variants on altitude acclimatization was first identified in a landmark study of European mountaineers in which the insertion allele, which is associated with lower plasma ACE activity, was shown to be overrepresented in successful climbers (Montgomery et al., 1998). Hypothesizing that susceptibility to AMS may preclude an individual from becoming an elite mountaineer, the investigators conducted two prospective studies of the ACE I/D polymorphism in European mountaineers; however, they were unable to find a convincing link between genotype at this locus and AMS incidence (Tsianos et al., 2005).

The ACE I/D polymorphism is only one of several polymorphisms of the ACE gene (*ACE*) that can modulate circulating ACE levels. The $ACE^{A\text{-}240T}$ (dbSNP rs4291; ACE-4 in Zhu et al., 2001) and ACE^{A2350G} (dbSNP rs4343; ACE-8 in Zhu et al., 2001) polymorphisms have been shown to modulate serum ACE levels, accounting for 6% and 19% of the variability, respectively (Zhu et al., 2001). Furthermore, ACE is only one component of the RAS pathway. Angiotensin II (the product of ACE proteolysis of angiotensin 1) plays several roles in the control of water and salt retention (either directly or through the release of aldosterone); thus variants in the angiotensin II receptor type 1 gene (*AGTR1*) might also play a role in AMS susceptibility. The most frequently studied of these is an A to C transversion at base 1166 (ATR^{A1166C}, dbSNP rs17231380) that has been associated with hypertension (Szombathy et al., 1998) and risk of myocardial infarct (Castellano et al., 1996).

The purpose of the current study is to compare genotypes of functional polymorphic loci in the ACE gene (I/D, A-240T, A2350G) and the AT2 receptor gene (A1166C) in a cohort of Nepalese pilgrims at risk of AMS while undertaking a pilgrimage to the Janai Purnima Festival at Lake Gosain Kunda at 4380 m.

MATERIALS AND METHODS

Subjects

Data collection was performed at the 2005 Himalayan Rescue Association Gosain Kunda Temporary Health Camp. This was an emergency medical clinic set up to provide medical care to the pilgrims at the Janai Purnima Festival at Lake Gosain Kunda at 4380 m in Lang Tang, Nepal. Approximately 12,000 pilgrims traveled from their lowland homes (<1800 m) to the festival over a period of approximately 36 h. Such a rapid ascent in lowlanders leads to a high incidence of AMS (reported as high as 68% in previous research at this event; Basnyat et al., 2000). In 48 h, the team treated approximately 350 patients with a variety of conditions, approximately half of which were related to altitude.

A temporary laboratory was set up at the clinic for data collection. Treating physicians identified subjects with clinical AMS and then determined their Lake Louise scores (Roach et al., 1993), and then the subjects were referred to the researchers prior to administration of treatment. An interpreter explained the purpose of the study, and if prospective subjects were interested, informed consent was obtained. Healthy, non-AMS participants were contacted by researchers outside the clinic. Once subjects were recruited and consented,

anthropometric and historical data were determined, including age, height, weight, and personal and family history of altitude illness. Both males and females were included in the study. There is little evidence that susceptibility to AMS is influenced by sex (reviewed in Rupert and Koehle, 2006) or that there is a confounding effect of menstrual timing on assessment (Riboni et al., 1999).

Buccal mucosal scrapings were taken from four locations in the mouth using an endocervical sampling cytobrush (CooperSurgical Inc., Trumbull, CT, USA) and stored dry in envelopes for transport (King et al., 2002).

DNA samples and genotyping

On return to Canada, genomic DNA was extracted from the sample brushes (Saftlas et al., 2004). Briefly, the brush heads were incubated in lysis buffer containing 0.5% SDS and 6-mg proteinase K overnight at 55°C. The brush heads were removed and the remaining solution was RNAse treated (2 μg) for 60 min at 55°C. The proteins were precipitated with 0.5 vol. 5 M potassium acetate and removed by centrifugation. The DNA was precipitated from the supernatant, dried, and resuspended in TE buffer. The DNA samples were PCR amplified and genotyped for the ACE I/D, ACE^{A240T} and ACE^{A2350G} polymorphism in the ACE gene and the ATR^{A1166C} polymorphism in the angiotensin-2 receptor type 1 (AT_2R_1) gene. PCR conditions were as follows: DNA (100 ng) was amplified in a PTC-0148 MJ Mini Cycler (Bio-Rad Laboratories, Hercules, CA, USA) using 0.033 nmole of each primer and 0.625 units Taq polymerase (Invitrogen Corp., Carlsbad, CA, USA). The final reaction mixture (25 μL) consisted of 0.2 mM dNTPs, 1.0 mM $MgCl_2$ (0.66 mM $MgCl_2$ for the ATR^{A1166C} primers), 20 mM Tris/Cl pH 8.4, and 50 mM KCl. Amplification conditions were 35 to 40 cycles at 94°C for 20 sec; 94°C for 20 sec; 58°C (ACE I/D and ACE^{A2350G}) or 55°C (ACE^{A-240T} and ATR^{A1166C}) for 30 sec (ATR^{A1166C} for 1 min); 72°C for 10 sec (all ACE) or 2 min (AT_2R_1). Primer sequences, diagnostic restriction enzyme, and product sizes are given in Table 1. Amplified product (10 μL) was then electrophoresed on an 8% acrylamide gel in TBE buffer, either immediately (ACE I/D) or following digestion with the diagnostic restriction endonuclease. Gels were stained with ethidium bromide and recorded using a Polaroid photodocumentation system.

Statistics

Allele frequencies were established by gene counting and compared by 2×2 contingency tables (available from <http://faculty.vassar.edu/lowry/VassarStats.html>). Tests for Hardy–Weinberg equilibrium and the significance of genotype distributions in different groups were done by chi-square analysis. When observed or expected values included a cell with a value less than 5, Fisher's exact test was used. In all cases, significance was accepted at $p < 0.05$. The values required for minimum detectable significant frequency change were estimated by 2×2 contingency tables and based on hypothetical changes from the control (non-AMS) allele frequencies.

RESULTS

DNA was obtained from 103 subjects (80 males, 23 females). Mean age was 33.5 (range 18 to 76). For the diagnosis of AMS, two separate criteria were used: physician clinical diagnosis and Lake Louise score. All physicians that took part in the clinic had training in high-altitude medicine and diagnoses were based on history and physical and global clinical impression. Two separate diagnostic criteria were used for AMS. Subjects who had been diagnosed as having AMS by a physician were considered clinical AMS. All subjects were also assigned a Lake Louise score rating by the physicians. The Lake Louise score is a validated measure to assess the severity of AMS. A score greater than 3 with a headache and recent gain in altitude is considered diagnostic. Therefore, patients with scores greater than 3 and a headache were considered AMS by Lake Louise score. Average Lake Louise AMS score for the entire cohort was 1.85 ± 2.60 (SD), with a range from 0 to 12 (out of a possible 15). Mean score was 0.11 (+0.37) in those not diagnosed with AMS and 4.33 (+2.40) for those with AMS. The AMS cohort was significantly older than the

TABLE 1. PRIMER SEQUENCES USED FOR PCR AMPLIFICATION OF POLYMORPHIC REGIONS IN THE ACE AND AGTR1 GENES

Gene product	*Primer name*	*Sequence*	*Product size (bp)*	*Polymorphic site (diagnostic enzyme)*
Angiotensin-converting enzyme (ACE)	ACE-1[a]	5′ CAT CCT TTC TCC CAT TTC TC 3′	Depends on genotype	287 bp Alu insertion in intron 16
	ACE-2[a]	5′ TGG GAT TAC AGG CGT GAT ACA G 3′		
	ACE-3[a]	5′ ATT TCA GAG CTG GAA TAA AAT T 3′		
ACE	ACE-8-F[b]	5′ CTG ACG AAT GTG ATG GCC GC 3′	122 bp	G/A^{2350} (*Bst*UI)
	ACE-8-R[b]	5′ TTG ATG AGT TCC ACG TAT TTC G 3′		
ACE	ACE-4-F[b]	5′ TCG GGC TGG GAA GAT CGA GC 3′	137 bp	A/T^{-240} (*Xba*I)
	ACE-4-R[b]	5′ GAG AAA GGG CCT CCT CTC TCT 3′		
Angiotensin-II receptor type 1	AT2R1-F	5′ ATA ATG TAA GCT CAT CCA CC 3′	300 bp	C/A^{1166} (*Dde*I)
	AT2R1-R	5′ GAG ATT GCA TTT CTG TCA GT 3′		

[a]Previously described in Evans et al., 1994.

[b]Previously described in (Keavney et al., 1998). The underlined base in the ACE-8-F primer is a mismatch that replaces an A to create a diagnostic *Bst* UI recognition site in the presence of the G allele at G/A^{2350}. The underlined base in ACE-4-R replaces an A to create a diagnostic *Xba* I recognition site in the presence of the T allele at A/T^{-240}.

non-AMS cohort (average 38.4 and 28.4 years, respectively), although the oldest subject in the study did not have AMS. Age does not seem to contribute to AMS susceptibility (Yardy et al., 2006)

Allele and genotype frequencies for the polymorphisms tested are shown in Table 2. All genotype frequencies are consistent with the populations being in Hardy–Weinberg equilibrium. No significant differences of the genotype or allele distributions at any of the four loci were detected between the AMS and non-AMS groups (either clinical AMS or AMS by Lake Louise score) (Table 3). Even after the removal of the potentially confounding "mild" cases of AMS (LL score 1 to 2), no significant associations were found.

ACE I/D allele frequency in the Nepalese was consistent with previous reports in Asians of mixed Indian descent (Kidd, 2006) and Nepalese (Umemura et al., 1998). No allele frequency data for $ACE^{A\text{-}240T}$, ACE^{A2350G}, or the ATR^{A1166C} polymorphisms in this population could be found in the literature. There was near complete concordance between the ACE I/D I allele and the ACE^{A2350G} A allele, suggesting that they were in strong linkage disequilibrium. This has been seen in a number of other populations, including Andean natives (Rupert et al., 2003), African (Zhu et al., 2001) and Europeans (Keavney et al., 1998). The frequencies of genotype combinations for the $ACE^{A\text{-}240T}$ and ACE^{A2350G} polymorphisms were consistent with some degree of linkage disequilibrium between these loci, with the $ACE^{A\text{-}240T}$ A allele usually segregating with the ACE^{A2350G} A allele, and the $ACE^{A\text{-}240T}$ T allele usually segregating with the ACE^{A2350G} G allele. These correlations are consistent with the common haplotypes reported in Europeans (Keavney et al., 1998). There are currently no data for $ACE^{A\text{-}240T}$ or ATR^{A1166C} (or for the non-SNP ACE I/D polymorphism) in HapMap (http://www.hapmap.org).

DISCUSSION

Allele frequencies for four polymorphisms in two gene-encoding components of the renin–angiotensin pathway were compared between Nepalese pilgrims with and without AMS who had recently traveled to a high-altitude (4380 m) religious festival in the Nepal Himalaya. No significant differences were detected in genotype or allele frequencies for any of the polymorphisms tested between the two populations.

Two previous studies examined the association of AMS incidence and the ACE I/D polymorphisms in Europeans. Tsianos and colleagues (2005) reported a significant association between heterozygosity at the locus and reduced incidence of AMS; however, this apparent protective effect was not observed on day 2 of the study. Dehnert and colleagues (2002) examined genotype in 83 visitors to the Capanna Regina Margherita hut at 4559 m. No

TABLE 2. GENOTYPE AND ALLELE FREQUENCIES IN NEPALESE ATTENDEES AT THE JANAI PURNIMA FESTIVAL

Polymorphism	*Genotypes (n = 103)*		*Allele frequency*	
ACE I/D	I/I	38	I	0.58
	I/D	43	D	0.42
	D/D	22		
$ACE^{A\text{-}240T}$ (ACE-4[a])	A/A	33	A	0.56
rs4291[b]	A/T	50	T	0.44
	T/T	20		
ACE^{G2350A} (ACE-8[a])	A/A	37	A	0.57
rs4343[b]	G/A	44	G	0.43
	G/G	22		
ATR^{A1166C}	A/A	91	A	0.94
rs17231380[b]	A/C	12	C	0.06
	C/C	0		

[a]Nomenclature from Zhu et al., 2001.
[b]dbSNP identification number (http://www.ncbi.nlm.nih.gov/projects).

TABLE 3. GENOTYPE AND ALLELE FREQUENCIES IN 103 UNRELATED NEPALESE WITH AND WITHOUT ACUTE MOUNTAIN SICKNESS (AMS) DIAGNOSED BY TWO DIFFERENT CRITERIA (CLINICAL EVALUATION AND LAKE LOUISE SCORE OF >3)

	Clinical evaluation					*Lake Louise score >3*				
	AMS (n = 44)		*Non-AMS (n = 59)*			*AMS (n = 22)*		*Non-AMS (n = 81)*		
Polymorphism	*Genotypes*	*Allele frequency*	*Genotypes*	*Allele frequency*	p *Values*[a]	*Genotypes*	*Allele frequency*	*Genotypes*	*Allele frequency*	p *Values*[a]
ACE I/D	19 I/I 19 I/D 6 D/D	0.65 I 0.35 D	19 I/I 24 I/D 16 D/D	0.53 I 0.47 D	0.22; 0.11	9 I/I 8 I/D 5 D/D	0.59 I 0.41 D	29 I/I 35 I/D 17 D/D	0.57 I 0.43 D	0.84; 1.0
ACE^{A-240T}	10 A/A 29 A/T 5 T/T	0.56 A 0.44 T	20 A/A 26 A/T 13 T/T	0.56 A 0.44 T	0.08; 0.92	5 A/A 12 A/T 5 T/T	0.50 A 0.50 T	25 A/A 43 A/T 13 T/T	0.57 A 0.43 T	0.66; 0.48
ACE^{A2350G}	18 A/A 20 A/G 6 G/G	0.64 A 0.36 G	19 A/A 24 A/G 16 G/G	0.53 A 0.47 G	0.25; 0.15	9 A/A 8 A/G 5 G/G	0.59 A 0.41 G	28 A/A 36 A/G 17 G/G	0.57 A 0.43 G	0.79; 0.92
ATR^{A1166C}	40 A/A 4 A/C 0 C/C	0.95 A 0.05 C	51 A/A 8 A/C 0 C/C	0.93 A 0.07 C	0.55; 0.79	20 A/A 2 A/C 0 C/C	0.95 A 0.05 C	71 A/A 10 A/C 0 C/C	0.94 A 0.06 C	1.00; 1.00

[a]No significant difference (at $p < 0.05$) was found between genotype or allele frequencies for any of the groups compared.
First value compares genotype frequencies; second value compares allele frequencies.

association was found between AMS incidence and genotype in this group. The current study is the first to assess this relationship in a non-European population. Nepal is located between Tibet and India, encompassing a wide variety of geographical ecosystems, including regions of the Tibetan plateau (average elevation 4500 m) and the Ganges floodplain (as low as 67 m in Biratnagar); therefore, the Nepalese inhabit a range of altitudes. The population of Nepal is primarily of Mongoloid descent, with some Caucasoid admixture and features from the Indian subcontinent (Roychoudhury and Nei, 1985). Individuals from higher altitudes (closer to Tibet) tend to have more Mongoloid ancestry than those from the lower-altitude regions (closer to India), who were the subjects in this research.

The lack of association for the I/D locus in this population is consistent with the aforementioned studies in European climbers. The only comparison to approach significance was between genotype distributions for the $ACE^{A\text{-}240T}$ polymorphism in clinical versus nonclinical AMS ($p = 0.08$), which resulted from an overrepresentation of heterozygotes among the AMS cases. This was not seen at the other ACE loci. Curiously, a similar overrepresentation of I/D heterozygotes was mentioned earlier (Tsianos et al., 2005).

This is the first study to look at the association between AMS and the three other polymorphisms ($ACE^{A\text{-}240T}$, ACE^{A2350G}, and ATR^{A1166C}) in any population. Genotypes at each of these loci can potentially affect fluid status, vascular tone, or both. Again no associations were demonstrated. Many genes are involved in the control of the RAS. These genes have been studied extensively and linked to cardiovascular disease (reviewed in Bleumink et al., 2004) and exercise performance (reviewed in Jones et al., 2002). Angiotensin-converting enzyme (ACE) levels are potentially affected by the three ACE polymorphisms measured in this study (Zhu et al., 2001). In linkage and association analysis of a Nigerian population, Zhu and colleagues found that ACE^{A2350G} and $ACE^{A\text{-}240T}$ contributed 19% and 6% to the variation in serum levels of ACE. They also found that the ACE I/D polymorphism was in linkage disequilibrium with the ACE^{A2350G} gene and was not contributing directly to serum ACE levels. Linkage between the various ACE polymorphisms in the Nepalese population has not been studied. In fact, this is the first study to report genotype distributions of the ACE^{A2350G} and $ACE^{A\text{-}240T}$ polymorphisms in Nepalese.

This study is also the first to look at the role of the angiotensin II receptor (type 1) gene in AMS. This gene codes for a receptor to which circulating angiotensin II binds, triggering vascular smooth muscle contraction and aldosterone secretion from the adrenal cortex. The ATR^{A1166C} genotypes were in Hardy–Weinberg equilibrium in the entire sample, as well as in both the AMS and non-AMS cohorts. No association was found between AMS incidence and genotype at the ATR^{A1166C} locus; however, as the C allele is relatively uncommon, the association would have to have been quite pronounced to be detected in this study.

Although there is evidence for a genetic predisposition to AMS (Rupert and Koehle, 2006), the absence of any clear inheritance pattern or obvious familial clustering suggests that more than one gene is involved. In a polygenic model, multiple alleles can contribute to the phenotype, and any of these alleles can be present in phenotypically normal individuals and/or absent in affected individuals, depending on their genotype at other loci. Overrepresentation of alleles in the affected population may indicate that the allele contributes to the phenotype even if the allele is present in the unaffected population as well. Association studies can only exclude an overrepresentation within the limits of the study's power to detect frequency variation. For the ACE polymorphisms, the sample size in this study has sufficient power to detect an increased allele prevalence of 33% (ACE I/D, ACE^{A2350G}) or 35% ($ACE^{A\text{-}240T}$) in the AMS cases ($n = 44$) compared to the non-AMS cohort ($n = 59$). As the presumptive risk allele is relatively rare (7%) at the ATR^{A1166C} loci, only a relatively large overrepresentation (>2.5 fold) of this allele in the AMS cases would have been detected in this study.

Limitations to the study

The Janai Purnima festival is a religious pilgrimage undertaken by thousands. The route

to the Lake is relatively uniform; however, this study does not account for pilgrims who had faster or slower ascent rates. Theoretically, some pilgrims could have gained 3000 m in 24 h; others could have taken as long as 72 h for the same ascent and, as rapid rate of ascent potentiates altitude-related pathologies, not all subjects in the study faced equivalent AMS risk (which is compounded by rapid ascent rate). The itinerary that most subjects likely followed was to ascend from the Kathmandu Valley (1320 m) via Dhunche (1950 m) over a period of 36 to 48 h. Furthermore, because the participants were not asked about recent history of altitude exposure, residual acclimatization in some subjects could be a confounding variable in this study.

Some patients diagnosed by a physician as having clinical AMS received lower scores than would be diagnostic by Lake Louise score (less than 3). This was because the Lake Louise score gives points for nonspecific symptoms such as insomnia or gastrointestinal upset common at this festival due to the crowds, poor hygiene, noise, and other factors. Physicians may have given patients a lower score in situations when they were unsure whether the symptom was due to external factors other than the altitude. Data were therefore analyzed using both diagnostic criteria with equivalent results. It should be noted that the AMS cases were self-selected (in the sense that they chose to go to the clinic) and that this may have introduced some selection bias into the study if the more mildly, or conversely the more severely, affected individuals were underrepresented in the AMS cohort.

CONCLUSIONS

Allele frequencies at four polymorphic loci in two genes that encode components of the renin–angiotensin system (ACE-I/D, ACE^{A-240T}, ACE^{A2350G}, ATR^{A1166C}) were compared between cohorts of Nepalese individuals who did or did not develop symptoms of AMS after arriving at the Janai Purnima Festival at 4380 m. No associations were found between the ACE alleles and AMS incidence, suggesting variants at these loci do not contribute substantially to susceptibility to AMS in this population (although moderate effects cannot be excluded by these data alone). There was also no association between the alleles in the AT2 receptor gene polymorphism and AMS; however, the sample size was sufficient only to identify a large change in allele frequency.

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Address reprint requests to:
Jim Rupert, PhD
School of Human Kinetics
University of British Columbia
6081 University Boulevard
Vancouver, BC, Canada V6T 1Z1

E-mail: rupertj@interchange.ubc.ca

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Scientific Article

Genotype at the Missense G894T Polymorphism (Glu298Asp) in the *NOS3* Gene Is Associated with Susceptibility to Acute Mountain Sickness

Pei Wang,[1] Michael S. Koehle,[1,2] and Jim L. Rupert[1]

Abstract

Wang, Pei, Michael S. Koehle, and Jim L. Rupert. Genotype at the Missense G894T Polymorphism (Glu298Asp) in the *NOS3* Gene Is Associatd with Susceptibility to Acute Mountain Sickness. *High Alt. Med. Biol.* 10:261–267, 2009.—Acute mountain sickness (AMS) is a potentially serious affliction that frequently occurs in travelers to altitudes above 2500 m. The probability of developing AMS depends on environmental factors such as rate of ascent and altitude attained; however, familial clustering and recurrence rates suggest that there may be a genetic contribution to the etiology of the condition. The underlying pathophysiology of AMS is unknown, but it may involve vasogenic edema secondary to hypoxia-induced sympathetic response and endothelial dysfunction. Nitric oxide is a potent vasomodulator, and variants in the gene that encodes endothelial nitric oxide synthase (*NOS3*) have been shown to affect blood pressure. We tested the hypothesis that haplotypes, as determined by tagSNPs, in *NOS3* would be differentially represented in individuals with and without AMS sampled at the Janai Purnima Festival at Lake Gosain Kunda, Nepal, at 4380 m. Seven SNPs were tested, and a highly significant association ($p = 0.004$) was found for genotypes of the commonly studied missense polymorphism Glu298Asp (rs 1799983; G/T transversion at base 894). The T allele, which previously has been associated with hypertension, was overrepresented in individuals with AMS (0.30 vs. 0.10), but not significantly when the data were corrected for multiple testing ($p = 0.024$). These data suggest that a variant in a gene involved in nitric oxide synthesis is a risk factor for developing AMS.

Key Words: nitric oxide; endothelial nitric oxide synthase; cerebral hypoxia; altitude sickness; physiological adaptation; acclimatization

Introduction

EVERY YEAR MILLIONS OF PEOPLE ascend to high altitudes (above 2500 m) for personal, recreational, and professional reasons and, due to time constraints and improvements in travel facilities, often do so rapidly. This can result in unforeseen medical complications, because too rapid ascent is a risk factor for developing altitude-related illnesses such as acute mountain sickness (AMS), high altitude cerebral edema (HACE), and high altitude pulmonary edema (HAPE). Like the physiological responses to hypoxia, these pathological consequences of high altitude exposure are not fully understood, but they likely result from the interactions between genetic factors and the hypoxic environment (Hackett and Roach, 2001).

Acute mountain sickness often occurs 6 to 12 h after rapid ascent. The probability of developing AMS depends on rate of ascent and the height reached; depending on these conditions, the frequency of affliction ranges from 10% to 93% (Honigman et al., 1993; Basnyat et al., 2000; Gertsch et al., 2002). The symptoms, which include headache, anorexia, nausea, vomiting, fatigue, malaise, and disturbed sleep, can resolve in a few days at a constant altitude; however, in some individuals, the condition may develop into life-threatening HACE. The etiology of AMS is unclear, but vascular homeostasis may play a role through its involvement in cerebral

[1]School of Human Kinetics, University of British Columbia, Vancouver, Canada.
[2]Allan McGavin Sport Medicine Centre, Department of Family Practice, University of British Columbia, Vancouver, Canada.

hemodynamics, pulmonary vascular activity, and fluid retention (see reviews by Hackett and Roach, 2001; Hackett and Roach, 2004).

The severity of and susceptibility to AMS are highly variable, and predicting who will or will not suffer from AMS at a given altitude and ascent rate is challenging. The condition occurs in both sexes and all age ranges, with physical fitness affording no protection (Schneider et al., 2002); however, one predictor is previous history (Robinson et al., 1971). In a study done at the Capanna Margherita (4559 m), the susceptibility of individuals with a history of AMS was three times higher, an odds ratio of 3.0 (95% CI, 2.1–4.1), compared with those without history (Schneider et al., 2002). Whether this predilection to developing the condition is genetic is unknown. A number of gene association studies have been designed to identify specific genetic variants as risk factors (Zhou et al., 2005; Koehle et al., 2006; Rupert and Koehle, 2006; Wang et al., 2007), but strong evidence for such an AMS susceptibility gene has not yet been found.

Nitric oxide (NO) is a strong vasodilator. NO-dependent endothelial function plays an important role in vascular homeostasis, and it may be instrumental in individual acclimatization to acute hypoxia and adaptation to chronic hypoxia through its action on cerebral blood flow and pulmonary vascular tone (Scherrer et al., 1996; Van Mil et al., 2002). The exhaled NO level of highlanders was 25% to 200% greater than that of lowlanders (Beall et al., 2001), prompting investigators to postulate that changes in NO-mediated hypoxia responses play a role in evolutionary adaptation to high altitude (Hoit et al., 2005; Ahsan et al., 2005). In contrast, NO levels decrease when lowlanders are exposed to acute hypoxia, which may be a maladaptive response related to the development of high altitude illnesses (Busch et al., 2001; Ahsan et al., 2006). Several studies have demonstrated a relationship between NO and high-altitude pulmonary edema (e.g., Duplain et al., 2000; Droma et al., 2002); however, no correlation was seen between exhaled levels of NO and self-reported AMS symptoms (Brown et al., 2006).

The biosynthesis of NO occurs in a reaction involving the conversion of L-arginine to L-citrulline by a family of enzymes called nitric oxide synthases. Endothelial-derived nitric oxide synthase (eNOS) is the main source of NO in blood vessels. The gene (*NOS3*) encoding the 1153 amino acid eNOS protein is on chromosome 7 (7q36) and comprises 26 exons spread over approximately 21 kbp of DNA. Several functional polymorphisms T−786C, G/T894 (Glu298Asp, rs1799983) and 4b/4a] in the gene have been shown to affect expression, enzyme activity, and concentration of exhaled NO (Ahsan et al., 2005; Ahsan et al., 2006; Dosenko et al., 2006).

Because NO is associated with acclimatization to high altitude, and eNOS is the main source of NO in blood vessels, we decided to test if variants in *NOS3* are associated with susceptibility to AMS by comparing allele and genotype distributions between individuals with and without AMS. The subjects were all Nepalese attending the Janai Purnima Festival at Lake Gosain Kunda at 4380 m in Lang Tang, Nepal. We used the HapMap tagSNP picking program (Thorisson et al., 2005) to identify maximally informative SNPs in the eNOS gene. TagSNPs are single nucleotide polymorphisms (SNPs) at which alleles are most representative of the haplotypes that represent the gene in the population (haplotypes are segments of DNA that tend to be transmitted intact between generations). An allele at a tagSNP is statistically likely to be a marker for all the other alleles on the same haplotype. Using tagSNPs allows investigating associations across the entire gene with a minimum number of tests (Zhang et al., 2002)

Materials and Methods

Subjects

One hundred and three Nepalese lowlanders provided informed consent to participate in the study. The recruitment of subjects was performed at the 2005 Himalayan Rescue Association Gosain Kunda Temporary Health Camp, where emergency medical care was provided for the pilgrims to the Janai Purnima Festival at Lake Gosain Kunda (4380 m). Approximately 12,000 pilgrims traveled on foot from their lowland homes (<1800 m) to the festival. Ascent time was not recorded for this study cohort, but typically attendees take 24 to 36 h to reach the festival. Such rapid ascent in lowlanders leads to a high prevalence of AMS, reported as high as 68% in previous research at this event (Basnyat et al., 2000). Treating physicians identified the subjects with clinical AMS and then determined their Lake Louise Score (LLS) (Roach et al., 1993). Control subjects were recruited from pilgrims attending the festival who were not symptomatic for AMS. Clinical diagnosis was performed by physicians experienced in high altitude medicine. Diagnoses were based on history and physical and global clinical impression. The LLS questionnaire was also administered by the treating physician. Subjects who were diagnosed as having AMS by a physician were assigned clinical AMS, and those who had LLS >3 were assigned LLS AMS. To increase the rigor of our phenotype characterization, we eliminated the 11 subjects for whom clinical evaluation and Lake Louise evaluation were discordant due to low LLS (i.e., they had been diagnosed as AMS by a physician, but their LLS was <3). The final cohort used for association analysis was: 33 AMS^+ and 59 AMS^- ($n = 92$).

Buccal (cheek) cells were collected using an endocervical sampling cytobrush (CooperSurgical, Inc., Trumbull, CT, USA), stored dry in envelopes for transport (King et al., 2002), and returned to Canada for DNA extraction (Saftlas et al., 2004). All procedures were approved by the University of British Columbia Clinical Research Ethics Board.

DNA sampling and genotyping

Sampling and DNA preparation have been described previously (Koehle et al., 2006). The HapMap (http://www.hapmap.org) tagSNP picker identified seven maximally informative SNPs in the 23.53-kbp region encompassing the ~21 kb *NOS3* gene (from 35 SNPs present in the database). The loci identified were rs1808593 (T/G), rs7830 (C/A), rs743507 (A/G), rs3918188 (A/C), rs3918186 (A/T), rs1800781 (G/A), and rs 1799983 (G/T at base 894), the commonly studied missense polymorphism (Glu298Asp). The HapMap data source was Rel 21a/phaseII Jan07, NCBI B35 assembly, db- SNP b125, and the tagSNP picker parameters were pairwise tagger, $r^2 = 0.95$, minor allele frequency (MAF) >0.1, CHB sample. To ensure that the most common haplotypes were assayed, a high r^2 value (0.95) was used and, since AMS is not uncommon, polymorphisms in which the MAF was <10% were excluded (i.e., MAF was set to 0.1).

Polymerase chain reaction was performed in a G-Storm Thermal Cycler (AlphaMetrix Biotech GmbH, Rödermark, Germany) using the following protocol: DNA (100 ng) was

TABLE 1. POLYMORPHISMS IN THE eNOS GENES AND ASSAYS USED TO GENOTYPE THESE LOCI

Polymorphism[a]	*PCR primers*	*Annealing temperature* (°C)[b]	*Genotyping assay*	
			Restriction enzyme	*Digestion products*
rs1800781 G/A	5′ GAGCATCACCTATGACACCCT 3′ 5′ CATCTGAGGCCAGGCCTTAGGCAC 3′	60	*Ban*I	G allele: 106 bp → 83 + 23 bp A allele: 106 bp
rs1799983 G/T (Glu298Asp)	5′ AAGGCAGGAGACAGTGGATGGA 3′ 5′ CCCAGTCAATCCCTTTGGTGCTCA 3′	59	*Ban*II	G allele: 248 bp → 163 + 85 bp T allele: 248 bp
rs3918186 A/T	5′ ATTTACAACATGTGTGCACCTCTGGAC 3′ 5′ GGGAAGGAAGCTGGAAGGAACTTGATC 3′	60	*Bcl*I	A allele: 160 bp T allele: 160 bp → 133 + 27 bp
rs3918188 A/C	5′ AGCAGCAAGGCACACGTACAAGCG 3′ 5′ ATTGTACTTCACTGAGACTGA 3′	55	*Bst*UI	A allele: 83 bp C allele: 83 bp → 62 + 21 bp
rs743507 A/G	5′ ACCTGGAGAATCCAGCCATGAAT 3′ 5′ GCCACCCCAATGAGGCACAGG 3′	60	*Hae*III	G allele: 170 bp → 104 + 66 bp A allele: 170 bp
rs1808593 T/G	5′ GGCTAAGCTTTGGCTCTCTCATT 3′ 5′ GATTTAGTGACTGTAGTTCCCA 3′	54	*Dpn*II	T allele: 187 bp G allele: 187 bp → 110 + 77 bp
rs7830 C/A	5′ CCTTCAGGCAGTCCT TTAGGC 3′ 5′ GGGTCCAGGCACTGGCATTGC 3′	60	*Hae*III	C allele: 103 bp → 81 + 22 bp A allele: 103 bp

[a]dbSNP identifying numbers (http://www.ncbi.nlm.nih.gov/projects/SNP/) and base change.
[b]annealing temperature for PCR amplification.

TABLE 2. POLYMORPHISMS TESTED AND GENOTYPE AND ALLELE FREQUENCIES IN NEPALESE ATTENDEES AT THE JANAI PURNIMA FESTIVAL

Polymorphism	*Function and location*[a]	*Genotypes*	*Allele frequency*
rs1800781 G/A	Intronic, intron 3	G/G = 84 G/A = 19 A/A = 0	G = 0.91 A = 0.09
rs1799983 G/T	Missense (Glu298Asp), exon 7	G/G = 62 G/T = 36 T/T = 5	G = 0.78 T = 0.22
rs3918186 A/T	Intronic, intron 14	A/A = 85 A/T = 18 T/T = 0	C = 0.92 G = 0.08
rs3918188 A/C	Intronic, intron 14	A/A = 14 A/C = 47 C/C = 42	C = 0.64 A = 0.36
rs743507 A/G	Intronic, intron 21	A/A = 57 A/G = 43 G/G = 3	C = 0.76 G = 0.24
rs1808593 T/G	Intronic, intron 23	T/T = 58 T/G = 40 G/G = 5	G = 0.76 T = 0.24
rs7830 C/A	Intronic, intron 24	C/C = 32 C/A = 59 A/A = 12	C = 0.60 A = 0.40

[a]From dbSNP GeneView and Ensemble ENSG00000164867 (*NOS3*) $n = 103$.

amplified in a 25-μL reaction buffer containing 0.2 mmol/L dNTPs, 1.0 mmol/L $MgCl_2$, 20 mmol/L tris/Cl pH 8.4, 50 mmol/L KCl, 0.015 nmol of each primer, and 0.5 unit *Taq* polymerase (Invitrogen Corporation, Carlsbad, CA, USA) for 40 cycles of 1 min at 94°C, 30 sec at 54° to 60°C (depending on primers; see Table 1), and 15 sec at 72°C, followed by a 5-min soak at 72°C. The amplification products were cut with diagnostic restriction endonucleases (Table 1), separated by gel electrophoresis (8% PAGE gels run in TBE buffer), and visualized using ethidium bromide staining.

Statistics

Allele frequencies were established by gene counting and compared using 2 by 2 contingency tables. Because no cells were below 5, the Pearson p value was used. Genotype frequencies were compared using 2 by 3 contingency tables and Fisher's exact test. Allele and genotype analysis was done using tools available through VassarStats (http://faculty.vassar.edu/lowry/VassarStats.html). Tests for Hardy–Weinberg equilibrium were done by χ^2 analysis. When observed or expected values included a cell with a value less than 5, Fisher's exact test was used. When the Bonferroni method was used to address the issue of multiple testing (Bland and Altman, 1995), significance was accepted at $p < 0.0071$ (i.e., 0.05/7).

Results

The genotype distributions of the seven tagSNPs in the total cohort ($n = 103$) (Table 2) were in Hardy–Weinberg equilibrium. Allele frequencies differed significantly (at $p < 0.05$) between Nepalese and the HapMap Chinese population (data not shown) for three of the SNPs (rs1799983, $p = 0.02$; rs1808593, $p = 0.03$; and rs3918188, $p = 0.02$) and between Nepalese and the HapMap Caucasian population for one SNP (rs1799983, $p = 0.01$); however, there were no differences when the Bonferroni correction was applied.

The T allele at rs1799983 (which encodes an aspartic acid at eNOS residue 298) was overrepresented in the AMS group when compared to the non-AMS group (30.0% vs. 16.0%, $p = 0.024$) (Table 3). Genotype frequencies also differed ($p = 0.004$) and remained significant when the data were corrected for multiple testing using the Bonferroni method.

Discussion

Our study found that genotype frequencies at SNP rs1799983, a functional mutation in the gene encoding the enzyme endothelial nitric oxide synthase, differed significantly between the AMS cohort and unaffected cohort, even when a very conservative α value was used. This was due to an overrepresentation of G/T and T/T genotypes in the affected group. Allele frequencies differed as well when α was set at 0.05, but not after correction. The Bonferroni correction is very conservative when applied to multiple tests that may not be completely independent. *NOS3* is over 20 kb in length and, although the tagSNPs were selected to best represent all common haplotypes, there will still be some degree of linkage disequilibrium between the markers; therefore, the tests for association will not be totally independent. We applied the strict correction to minimize the possibility of a type I error and, although association studies do not test causality, these data are consistent with G/T and T/T genotypes at rs1799983 or with genotypes that coassort with them due to linkage disequilibrium, thus contributing to the susceptibility to develop AMS at moderate altitude (4380 m).

The SNP rs1799983 is a missense transversion (G→T) in exon 7. The T allele, which causes a conservative change in the protein (an aspartic acid is substituted for a glutamic acid) at amino acid 298, was more common in the AMS^+ cohort (at $p < 0.05$), although not significantly when a strict correction factor was applied. A number of studies have shown an association between the T allele at rs1799983 and hypertension (Sawada et al., 2008; Srivastava et al., 2008). The mechanism by which this occurs is unknown; however, Veldman and colleagues (2002) showed a blunted response to L-NMMA in individuals carrying the T allele and concluded that the allele was associated with lower basal NO production. NO synthesis is normally inhibited by hypoxia. This response may be counteracclimatory, since an adaptive role at altitude for NO (and for variants in the eNOS gene, including rs1799983) is suggested by observations that high altitude-adapted populations (native Tibetans) have higher levels of exhaled NO (Beall et al., 2001) and a differential representation of eNOS alleles (Ahsan et al., 2006). The capacity of artificial NO synthesis stimulation to alleviate the symptoms of AMS is unclear. Administration of L-arginine (a NO precursor) at altitude increased arterial O_2 saturation and slightly improved AMS symptoms (Schneider et al., 2001), despite the fact that a well-known side effect of exogenously administered NO donors is headache (e.g., glyceryl trinitrate, Ashina et al., 2000). Whether this apparent discrepancy is due to altitude of administration or the method of stimulation is unknown. Differential responses to NO may also contribute to the variation in response. Appenzeller and colleagues (2006) demonstrated population differences in vascular response to

TABLE 3. GENOTYPE AND ALLELE FREQUENCIES IN SUBJECTS WITH AND WITHOUT ACUTE MOUNTAIN SICKNESS

	AMS (n = 33)		*Non-AMS (n = 59)*		
Polymorphism	*Genotypes*	*Allele frequency*	*Genotypes*	*Allele frequency*	P *values*[a]
rs1800781	26 G/G 7 G/A 0 A/A	0.89 G 0.11 A	49 G/G 10 G/A 0 A/A	0.92 G 0.08 A	0.780; 0.632
rs1799983 (Glu298Asp)	14 G/G 18 G/T 1 T/T	0.70 G 0.30 T	43 G/G 13 G/T 3 T/T	0.84 G 0.16 T	**0.004**[b]; 0.024[c]
rs3918186	23 A/A 10 A/T 0 T/T	0.85 A 0.15 T	51 A/A 8 A/T 0 T/T	0.93 A 0.07 T	0.061; 0.067
rs3918188	3 A/A 16 A/C 14 C/C	0.33 A 0.67 C	8 A/A 28 A/C 23 C/C	0.37 A 0.63 C	0.908; 0.594
rs743507	15 A/A 18 A/G 0 G/G	0.73 A 0.27 G	36 A/A 20 A/G 3 G/G	0.78 A 0.22 G	0.113; 0.424
rs1808593	16 T/T 17 T/G 0 G/G	0.74 T 0.26 G	36 T/T 18 T/G 5 G/G	0.76 T 0.23 G	0.051; 0.764
rs7830	7 C/C 21 A/C 5 A/A	0.53 C 0.47 A	20 C/C 32 A/C 7 A/A	0.61 C 0.39 A	0.432; 0.292

[a]The first *P* value compares genotype frequencies; second *P* value compares allele frequencies.
[b]Significant at $P < 0.0071$ (Bonferroni correction applied).
[c]significant at $P < 0.05$ (but not significant if Bonferroni correction applied).
AMS diagnosis based on a positive clinical evaluation and a Lake Louise score of >3.

exogenous NO treatment and suggested that an enhanced NO response at altitude was an adaptive trait.

Our genetic data are not consistent with the physiological data of Brown and colleagues (2006), who measured exhaled NO following rapid (~2 h) ascent to 4200 m and reported no correlation between exhaled NO and AMS symptoms. Exposure time at 4200 m was short (3 h), and the relatively low incidence of AMS (19%) following such a rapid ascent limits the robustness of the findings. Also, the authors acknowledged that the small sample size (13 AMS cases) limits the strength of their conclusions about the relationship between exhaled NO and AMS.

Although the rs1799983 polymorphism affects NO production, whether this in turn affects vasodilation is unclear. Studies by Schneider and colleagues (2000) and Sofowora and colleagues (2001) reported no effect of genotype on endothelium-dependent vasodilation; however, an allelic effect on NO synthesis was observed in the latter study, prompting the authors to conclude that the allele may have a pathological effect if the endothelium is dysfunctional. Endothelial dysfunction has been seen in AMS (Palma et al., 2006; Strapazzon et al., 2008).

Although the chance of developing AMS is influenced by altitude attained and rate of ascent (Hultgren and Marticorena, 1978), some families and individuals are predisposed to developing the condition at moderate altitudes (Bartsch et al., 2004; Schoene, 2004), and previous history is a reliable predictor of subsequent affliction (Honigman et al., 1993; Jiang et al., 2005). Innate susceptibility and familial clustering (but lack of simple Mendelian patterns of inheritance) are consistent with a polygenic, environmentally mediated trait involving the interaction of a number of genes (reviewed in Rupert and Koehle, 2006). Our data support a role for variants in *NOS3* in susceptibility to AMS, but suggest that the presence of the rs1799983 T allele is, in itself, insufficient to cause the condition (several of our unaffected cohort were T/T homozygotes). The T allele phenotype may be interacting with other, variable, endothelial responses or exacerbating preexisting endothelial dysfunction. The observation that genotype frequencies differed between cohorts while allele frequencies did not (when the data were strictly corrected) may result from the overrepresentation of G/T heterozygote's among the AMS cases (54% vs. 22%) and/or the small number of T/T homozygotes in both cohorts. When the G/T and T/T genotypes are combined, they are more common in the AMS cohort than in the unaffected cohort after correction ($p = 0.0039$).

NOS3 genotype may be a risk factor or a resistance factor for AMS; however, further studies on the impact of this genotype at altitude and on the role of eNOS and NO in acute altitude acclimatization are needed before the magnitude, and clinical value, of this relationship can be fully appreciated.

Limitations to the study

The sample size in this study lacks the power to detect small effects and, although we corrected for multiple testing, the possibility of a type I error still exists. Thus the association(s) that we observed should be corroborated, either in an independent AMS^+/AMS^- sample or by demonstration of a causal phenotype for the 298Asp variant. We also do not have data on the ascent rate for this particular cohort; however, data from the 2008 festival suggest that most individuals take between 24 and 36 h to climb to the site. In the absence of Nepalese haplotype data, we used the HapMap Chinese (CHB) database to select tagSNPs, which could affect extrapolation of our data to other polymorphic loci if the two populations differed in haplotype structure.

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Disclosures

Authors Wang, Koehle, and Rupert have no financial ties or conflicts of interest to disclose.

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Address correspondence to:
Jim L. Rupert, Ph.D.
School of Human Kinetics
University of British Columbia
6081 University Boulevard
Vancouver, BC, Canada V6T 1Z1

E-mail: rupertj@interchange.ubc.ca

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