Frequent-Episodic Alcohol Consumption

Impairs Macro-and Microvascular Function in Young Adults

BY

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THESIS

Submitted as partial fulfillment of the requirements
for the degree of Master of Science in Rehabilitation Sciences
in the Graduate College of the
University of Illinois at Chicago, 2012

Chicago, Illinois

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This thesis is dedicated to my father, Lawrence Goslawski, MD, who has inspired me all my life.

ACKNOWLEDGEMENTS

I would like to thank my thesis committee--Shane Phillips, Mariann Piano, and Tracy Baynard—for their wavering support and assistance. They provided guidance in all areas that helped me accomplish my research goals and enjoy myself in the process.

A number of individuals involved with data collection and analysis were extremely helpful to me during this time, and I would like to thank them as well—at the University of Illinois at Chicago Clinical Research Center, the nursing staff; Jing-Tan Bian, Ana Cavka, and Ivana Grizelj for assistance with isolated perfused microvessel experiments; Mary Szczurek for data collection.

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LIST OF ABBREVIATIONS

A	Abstainers
ACLS	Aerobics Center Longitudinal Study
ACh	Acetylcholine
Ad.CMV.ET-1	Recombinant adenovirus with human pre-proendothelin-1
ALP	Alkaline phosphate
ALT	Alanine transaminase
AST	Aspartate aminotransferase
BAL	Blood alcohol level
BD	Binge drinkers
BH_4	Tetrahydrobiopterin
BMI	Body mass index
CBC	Complete blood count
CBF	Coronary blood flow
CRP	C-reactive protein
CV	Cardiovascular
COV	Coefficient of Variation
ECE	Endothelin-converting enzyme
ED	Endothelial-dependent
EI	Endothelial-independent
eNOS	Endothelial nitric oxide synthase
ERE	Exhaustive resistance exercise
ET	Endothelin

LIST OF ABBREVIATIONS (continued)

ET_A	Endothelin-A receptor
ET_B	Endothelin-B receptor
ET-1	Endothelin-1
ETOH	Ethanol
FMD	Flow-mediated dilation
GSH	Glutathione
H_2O_2	Hydrogen peroxide
HDL	High-density lipoprotein
Hgb	Hemoglobin
HR	Heart rate
INDO	Indomethacin
LDL	Low-density lipoprotein
L-NAME	L-N^G-Nitroarginine methyl ester
MET	Metabolic equivalent
MnSOD	Manganese superoxide dismutase
NAD	Nicotinamide adenine dinucleotide
NIAAA	National Institute of Alcohol Abuse and Alcoholism
NO	Nitric oxide
NOS	Nitric oxide synthase
NTG	Nitroglycerin
ROS	Reactive oxygen species
SBP	Systolic blood pressure

LIST OF ABBREVIATIONS (continued)

SNP	Sodium nitroprusside

SUMMARY

A study investigating the vascular effects of frequent, episodic, alcohol consumption in young adults was carried out using a non-randomized, cross-sectional approach. Two clinical visits were conducted on 17 binge drinkers, and 19 current alcohol abstainers (consumption of less than 5 standard alcoholic drinks in the past year) served as controls. All participants were free of cardiovascular disease. Information on demographics, nutrition, physical activity, medical history, and alcohol consumption pattern was collected for both groups. In addition, endothelial-dependent and independent dilation was assessed through vascular ultrasonography through the method of flow-mediated dilation and nitroglycerin-induced dilation. Resistance arterioles dissected from gluteal fat biopsies were cannulated and underwent perfusion experiments to test dilation (endothelial-dependent and independent) and constriction responses to endothelin-1.

Binge drinkers were found to have significantly lower endothelial-dependent dilations through flow-mediated dilation of the brachial artery, and lower endothelial-independent dilations through nitroglycerin administration. Resistance arterioles of binge drinkers had preserved endothelial-independent dilation with endothelial-dependent dilation that may not be mediated through nitric oxide or prostaglandins. Constriction to endothelin-1 was enhanced in binge drinkers compared to abstainers. This may suggest that frequent binge drinking mediates lasting macro-and microvascular impairments in young adults without overt cardiovascular disease

I. INTRODUCTION

A. Background

Excessive alcohol consumption is the third greatest preventable cause of mortality in the United States[1, 2]. Between 1995 and 2001, there was a 35% increase in binge drinking among adults greater than 18 years of age[2]. Rates of binge drinking are highest on college campuses and among 18- to 25-year-olds; however, 73% of moderate drinkers (mean age 32 years) also report binge drinking[2]. In addition, a recent Centers for Disease Control (CDC) report reflects that those defined as "binge drinkers" reported an average number of eight drinks per binge drinking episode[3]. The cardiovascular (CV) effects of repeated episodes of binge drinking are poorly and incompletely understood. It is well established that ethanol's CV effects are dose-dependent; low-to-moderate levels of ethanol intake are associated with a reduced CV risk, whereas high intake levels are associated with increased risk of coronary artery disease, stroke, hypertension, and cardiomyopathy[4, 5]. Interestingly, in certain geographical areas where binge drinking is more frequently reported, investigators have failed to find a cardioprotective effect with any level of ethanol consumption[6]. These data highlight the need to evaluate the pattern of drinking, rather than amount of alcohol intake alone, and underscore the possibility that failure to differentiate between drinking patterns (daily vs. binge) may obscure the adverse associations between ethanol consumption and CV disease[7].

B. Main Objective

The main objective of this thesis was to examine the precursor of cardiovascular disease and blood vessel damage[8], endothelial function, in young college-age students, who had a history

of binge drinking. To our knowledge, no previous research has investigated the macro- and micro-vascular effects of frequent binge drinking in humans.

C. Hypothesis

Specifically we sought to determine, whether endothelial-dependent (ED) and -independent (EI) dilation was impaired in young adult binge drinkers through brachial artery flow-mediated dilation (FMD) and brachial artery NTG-induced dilation, respectively. Also, I evaluated the potential changes in vasodilator and vasoconstrictor responses to agonists, such acetylcholine (ACh) and endothelin-1 (ET-1) in binge drinkers and abstainers. The latter experiments were conducted using resistance arterioles isolated from gluteal fat biopsies. I hypothesize that binge drinkers will have a 1) reduced response to FMD, with 2) reduced EI dilation to NTG in the brachial artery; 3) Reduced ACh-induced dilations (ED dilation); 4) Preserved sodium nitroprusside (SNP) dilations (EI dilation); and 5) Enhanced ET-1 induced constriction in resistance arterioles.

D. Rationale

The rationale for investigating brachial artery FMD in young adult binge drinkers is from our previous laboratory studies showing intravariability between groups of weightlifters, cross trainers, runners, and inactive individuals, that may be attributed to binge drinking patterns in these groups. Understanding how binge drinking affects endothelial function clinically and physiologically in young adults may present novel findings on the extent of vascular damage that can occur with frequent binge drinking.

Since the developmental pathway of atherosclerosis and hypertension is complex and may vary in different pathological conditions, determining the onset and progression of cardiovascular disease by investigating the function of conduit and resistance arteries in humans who frequently binge drink may yield novel insights into therapeutic strategies for the prevention and treatment of cardiovascular disease induced by alcohol consumption.

Prior to reviewing the methods for this study, in the background section I will review the definitions of unhealthy and binge drinking, potential mechanisms underlying the adverse effects of binge drinking and other related literature related to binge drinking and endothelial dysfunction.

II. REVIEW OF BINGE DRINKING AND RELATED LITERATURE

A. Review of Binge Drinking Literature

1. Binge drinking: a public health concern

While benefits of moderate drinking (0.1 to 22.9 g ethanol per day) [9] exist, it is not recommended to begin due to the variable effects of alcohol on individuals.[10, 11] Further, the potential hazards of alcohol consumption may outweigh the benefits[12]. Heavy drinking, defined as more than 89 g of ethanol per day[9, 13], clearly shows the negative effects of alcohol consumption specified above, but recent attention has been given to heavy episodic drinking, or binge drinking.

Binge drinking is defined by the National Institute of Alcohol Abuse and Alcoholism (NIAAA) as a pattern of drinking alcohol that brings blood alcohol concentration to .08 percent or above, which for an average adult corresponds to 5 or more drinks for a male and 4 or more drinks for a female in a 2 hour period[14]. Frequent binge drinkers, defined as binging 3 or more times in the past month[15], represent about 22% of the college-age population. For female college students, alcohol problems and hangovers were almost three times more likely to occur as the result of drinking 7 or more times monthly. Frequent and infrequent binge drinking in college years for males that continue their heavy drinking are 6 times more likely to have dependence issues and over 9 times more likely to have alcohol abuse. College students are less likely to decline binge drinking or heavy drinking as they age[16]. In one study, women who continued to binge drink from college into their thirties had higher long-term odds for problematic drinking, and a high risk for alcohol abuse[15].

2. Binge Drinking and Cardiovascular Risk

Variability in drinking patterns compared to a consistent pattern in drinking can have heightened risk in cardiovascular events[17-19]. Binge drinking has shown heightened risk in cardiovascular events, like heavy drinking, but there is a reduction of risk for those drinking a similar amount per week spread over more days[17]. It has been demonstrated that intoxicating amounts of alcohol dilate conduit arteries, increase heart rate, and stimulate sympathetic nervous system activity, causing an increase in blood pressure that is sustained for hours after vasodilation dissipates[20]. Consuming 8 or more drinks at a sitting in the past year shows a significant relationship with the occurrence of chronic heart disease in both men and women, and applied to some degree for hypertension in men[21].

In a study investigating the French Paradox regarding blood pressure and alcohol consumption, individuals in France, who had homogenous alcohol consumption throughout the week with a slight increase on weekends had consistent blood pressure readings throughout the week[22]. However those in Northern Ireland, where Fridays and Saturdays account for 66% of total alcohol consumption, blood pressure measurements were highest on Monday, and decreased until Thursday[22]. This coincides with evidence in Moscow where young and early middle-aged men have a sudden increase in sudden cardiac death on weekends[23], when binge drinking is more likely[24]. Furthermore, coronary occlusion is more likely with variable drinking patterns, regardless of amounts consumed[25]. Men with a heavy binge pattern of alcohol drinking also show greater progression of carotid atherosclerosis compared to men with a more evenly distributed drinking habit[26].

3. Binge Drinking and Endothelial Dysfunction

The cardiovascular risk associated with binge drinking emphasizes the importance of studying endothelial dyfunction as a precursory event before CV symptoms arise. Endothelial dysfunction is an early indicator of blood vessel damage and atherosclerosis[8], and is closely related to coronary endothelial function[27]. Endothelial function can be assessed through a reliable, noninvasive method called flow-mediated dilation (FMD), which is an ultrasonic assessment of FMD in response to occlusion-induced hyperemia[28]. The assessment of endothelial function through FMD represents endothelium-derived NO availability in humans[29]. During the FMD test, vasodilation occurs following an acute increase in blood flow, typically induced by circulatory arrest in the arm (supra-systolic cuff occlusion) for a period of 4-5 minutes[28]. The hyperemia increases laminar shear forces parallel to the long axis of the vessel[30], which is transduced through luminal mechanoreceptors to the endothelial cell[28]. The increase in arterial diameter, as a consequence of reactive hyperemia, is compared to the baseline diameter and is expressed as % FMD. However, it is arguable whether NO is the only mediator of endothelium dependent vasodilation[31] since the mechanism of vasodilation depends on vessel type and its size and how FMD is induced [32, 33].

Impairment of endothelial function is apparent in many cardiovascular diseases such as hypertension, stroke, coronary heart disease, and atherosclerosis,[34] as well as in individuals who heavily drink alcohol[35, 36]. Using FMD techniques, others have examined a one-time or acute binge episode on endothelial function[20, 37, 38]. To our knowledge, there are no studies examining the repeated episodes of binge drinking on vascular or endothelial function. Hijmering et al. found that three standard drinks of red wine or a low polyphenolic alcoholic beverage resulted in

a significant decrease in FMD.[37] In addition, Bau et. al.[20] evaluated FMD and NTG-induced dilation 4 hours and 13 hours after ingestion of 60 g of ethanol. There was a significant decrease in FMD and NTG-induced dilation 4 hours after alcohol consumption compared to control subjects. However, 13 hours post alcohol consumption FMD and NTG-induced dilation approached baseline levels. While we see attenuation in endothelial dysfunction and smooth muscle function, this study did not document if the subjects participated in this drinking frequency consistently, and how these baseline FMD levels compare to those who abstain from alcohol.

In comparison to findings from a single binge episode, long-term, heavy alcohol consumption has demonstrated impaired endothelial function but preserved smooth muscle function. For instance, Maiorano et al. demonstrated that in withdrawing alcoholics (3 months) with no preexisting conditions of hypertension, smoking, or cholesterolemia, FMD was lower than controls [35]. Additionally, Di Gennaro et al. established that even after a longer alcoholic withdrawal between 6-144 months, FMD was still significantly lower in detoxified, normotensive alcoholics when compared to lifetime alcohol-abstaining controls.[36]

B. Review of Related Literature

1. Alcohol metabolism and oxidative stress

Oxidative stress may be a critical mechanism that underlies the adverse effects of alcohol on cells and tissues, eventually leading to cell and tissue damage and endothelial dysfunction. There is evidence in many other organ systems, especially the liver that alcohol may contribute to the generation of free radicals and therefore an oxidative stress environment. One mechanism may be related to ethanol metabolism and the generation of metabolic

byproducts and changes in reducing equivalents such as nicotinamide adenine dinucleotide (NAD) [39-43]. For example, ethanol metabolism influences the redox state of a cell, since conversion to acetaldehyde by alcohol dehydrogenase (ADH) involves NAD^+, and metabolism to acetate and NADH by aldehyde dehydrogenase 2 (ALDH2) is further coupled to oxidation by the electron transport chain[44]. Increased mitochondrial activity and NADH metabolism results in increased superoxide production[45]. Superoxide will bind to and cause destruction of cell membranes by enhancement of apoptosis by oxidized low-density lipoprotein (LDL)[43, 46] and damage to DNA, RNA, and proteins. This ultimately results in cell and organ damage[39, 46, 47].

Other oxidative pathways include the cytochrome P450 and catalase pathways. Cytochrome P450 isozyme CYP2E1, present predominantly in microsomes in the liver[47], is induced in chronic alcohol consumption and metabolizes ethanol to acetaldehyde at elevated ethanol concentrations (K_m=8-10 mM)[44]. Additionally, it produces reactive oxygen species (ROS), which increase the risk of tissue damage[44, 47]. CYP2E1 increases chemical damage done by ROS on lipid components of cell membranes in liver cells[43], and a strong correlation exists between the level of CYP2E1 in the liver and other tissues and the degree of alcohol-induced liver injury[47]. Catalase, located in peroxisomes, oxidizes ethanol *in vitro* in the presence of a hydrogen peroxide (H_2O_2)-generating system, such as NADPH oxidase or the enzyme xanthine oxidase[44].

2. Alcohol consumption, oxidative stress, and endothelial dysfunction

Early predictors of cardiovascular disease, such as reduced endothelial function, occur before intimal hyperplasia or lipid deposition where either increased degradation or impaired synthesis/ release of nitric oxide (NO) and have been shown to be associated with oxidative

stress[48]. Endothelial cells produce NO via endothelial nitric oxide synthase (eNOS), when L-arginine reacts with oxygen[48]. NO activates soluable guanylyl cyclase in vascular smooth muscle cells to produce cyclic GMP which elicits smooth muscle relaxation[34]. Endothelial NO mediates vasodilation, increases blood flow, lowers blood pressure, inhibits platelet aggregation and adhesion, inhibits leukocyte adhesion, and reduces smooth muscle proliferation, which all help to prevent atherosclerosis[34].

For enzymatic activity, eNOS protein must first bind to flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), then bind to its cofactors L-arginine and tetrahydrobiopterin (BH_4), along with a heme group and dimerize. Calmodulin must bind to the dimers upon increased intracellular calcium concentration[48]. An insufficiency or impaired metabolism of BH_4 may lead to uncoupling of the L-arginine –NO pathway, resulting in increased formation of superoxide anion and reduced NO production[49]. For example, in studies of alcohol-fed rats, impaired NOS-dependent dilation of pial arterioles was related to a deficiency in BH_4 while eNOS levels remained stable[49]. In human studies, upon topical administration of BH_4, NOS-dependent dilatation of the saphenous vein can be restored in smokers[50], as well as in coronary blood vessels with atheroscerlosis[51].

Antioxidant enzymes such as manganese superoxide dismutase (MnSOD), catalase (CAT), glutathione peroxidase (GSH-Px), and glutathione (GSH) help ameliorate the oxidative stress produced by free radicals[40,41,43,52]. Endogenous glutathione plays an important role in maintaining the integrity of mitochondria and cell membranes[43] along with cellular homeostasis[52]. Transgenic mice overexpressing MnSOD (MnSOD $^{+++}$) are shown to be protected against mitochondrial DNA (mtDNA) damage and depletion, which can cause liver disease, after an alcohol binge[41]. In mice that were heterozygote knockouts for the MnSOD gene

(MnSOD $^{+/-}$), there was increased mitochondrial ROS, decreased mtDNA levels, and lower mitochondrial GSH levels compared to wild-type (WT) mice. In alcohol abusers, it has been shown that plasma intracellular GSH concentration is lower than in nondrinkers, and gradually increases in concentration after alcohol withdrawal[40], demonstrating that alcohol decreases antioxidants, which could ameliorate oxidative stress.

3. Background on endothelin-1: a pathophysiological vasoconstrictor

Endothelin-1 (ET-1), a potent vasoconstrictor[53], may be an important peptide hormone mediating the adverse effects of binge drinking brought upon by an environment of oxidative stress. ET-1 potentiates other vasoconstrictors such as norepinephrine and serotonin[54]. Additionally, *in vitro* experiments have shown ET-1 stimulating the release of $PGF_{2\alpha}$, a vasoconstrictor, from proliferative human endometrium[55, 56]. Increased plasma ET-1 levels have been found in individuals with cardiovascular disease[57, 58] including atherosclerosis[59], diabetes[60], obesity[61], and in people who consume alcohol[62].

The biological precursor of ET-1, big ET-1 (or proendothelin-1) is converted to ET-1 through endothelin-converting enzyme (ECE)[63]. There are two distinct pathways that are known to secrete ET-1. A constitutive pathway involves a continuous release of ET-1 in the maintenance of vascular tone, and is modulated at the level of mRNA transcription[64]. This pathway involves a nonpolar transport of secretory vesicles to the cell surface[65]. Under normal physiological conditions, ET-1 and NO are constitutively released by the endothelium and provide a balance between vasoconstrictor and vasodilator activity[66]. Several *in vivo* findings indicate that physiological or pathophysiological stimuli such as hypoxia, thrombin, or shear stress[67] can lead to stimulated release of ET-1 via the regulated secretory pathway through

storage granules called Weibel-Palade bodies[65, 68, 69]. Weibel-Palade bodies store vasoactive compounds including histamine, von Willebrand factor, P-selectin, and calcitonin gene-related peptide.[68] Weibel-Palade bodies involve polar mobilization to the basolateral membrane of the endothelial cell, in which polarized release of ET-1 toward vascular smooth muscle cells produce rapid vasoconstriction[65]. Furthermore, ethanol and acetaldehyde have shown to stimulate ET-1 gene transcription by activating hypoxia-inducing factor (HIF-1)[70], which has been seen in a variety of cell types including endothelial cells[71].

Overproduction of ET-1 can decrease NO bioavailability, either by decreasing its production by upregulating cavelin-1, leading to inhibition of eNOS activity, or by increasing its degradation via formation of oxygen radicals[72]. Vascular injury can compromise the endothelium, causing reduced secretion and production of NO and overproduction of ET-1. It is hypothesized that release of ET-1 and von Willebrand factor via the regulated secretory pathway may provide an initial haemostatic response to vascular endothelial cell damage[73]. Interestingly, in patients with atherosclerosis, ET-1 seems to originate from the vascular lesion[59].

4. Endothelin receptors and pathogenic role in cardiovascular disease

To date, two ET-1 receptors have been discovered: ET_A and ET_B. Each receptor plays a distinctive role in maintaining vascular tone, where altering the ET_A/ET_B receptor ratio in various tissue beds has a severe impact on CV health[74]. Both ET-1 receptors are G protein coupled, consisting of seven transmembrane helices, linked to the generation of rises in cytoplasmic calcium[75-77]. The disulfide interchange in the endothelin (ET) receptor-ligand complex may be responsible for the irreversible binding of ET[78], while in isolated rat thoracic aortas, ET receptor externalization may be responsible for the sustained contractile responses

induced by ET-1[79]. Additionally, potency of ET-1 has been shown to be independent of vessel size[80]. Most ET-1 is thought to be synthesized and released from endothelial cells to bind and activate vasoconstrictor ET_A receptors, which predominate on the smooth muscle[81]. The ET_A receptor, located on smooth muscle, mediates vasoconstriction in humans mainly in coronary[82, 83] and renal arteries[84]. Vasoconstrictor sensitivity of ET_B is less than that of ET_A[85]. In humans, vasoconstrictor ET_B receptors have been described in small resistance arteries[80] and in pulmonary arteries of normotensive rats[86] . However, ET_B agonist studies have shown that with concurrent antagonism of ET_A receptors, contraction still occurs in human mammary arteries and veins, as well as in porcine coronary arteries, suggesting ET_B receptors have a role in vascular smooth muscle contraction in some species and vascular beds[85].

The ET_B receptor on the smooth muscle seems to be functionally distinct from the ET_B receptor on the vascular endothelium, which mediates vasorelaxation via the release of NO[87] and/or prostacyclin[88], most notably in small human arteries[89]. However, whether the ET_B receptor creates endothelial-dependent dilation in human arteries is unknown. Endothelium-dependent relaxation to ET-1 or to ET_B-selective agonists IRL1620 or sarafotoxin S6c was not observed in pre- contracted human isolated mammary arteries[85] or in human radial arteries[77]. Additionally, radio-ligand binding of ET-1 was not observed in sections of luminal endothelial cells isolated from human coronary arteries or endothelial cells lining small blood vessels in the adventitia of patients with congestive heart failure,[82] indicating that no ET_B clearance receptors on the endothelium were observed in diseased conduit or resistance arteries. However, this does not validate whether ET_B clearance receptors exist elsewhere, or in non-pathogenic tissues.

Through *in vivo* and *in vitro* studies, ET_B receptors have been shown to play a role in clearance of ET-1 from the circulation by endothelial cells[90] in various tissues, most notably the

lung, kidney medulla, and liver[91, 92]. Ligand-induced receptor internalization has been suggested to be an important mechanism for peptide clearance, where rapid internalization of the ET-1/ET_B receptor complex is followed by transportation to the lysosomes for degradation[93] and down regulation of ET-1 gene expression[94]. In a previous study, 81% of [^{125}I] ET-1 was internalized within the first 10 minutes of incubation in human astrocytoma cells[95]. On the other hand, in pericardium smooth muscle cells, which have predominantly ET_A receptors, only 35% of ET-1 internalization occurred after 30 minutes, suggesting that a lower amount of ET-1 was internalized due to fewer ET_B receptors[96].

In vitro autoradiography has revealed the adaptation of the clearance role of ET_B receptors in pathological conditions where high levels of labeled ET_B agonist [^{18}F]-BQ3020 are found in macrophages within atherosclerotic coronary arteries[91]. Additionally, within human coronary arteries containing atherosclerotic lesions, ECE-1b and ECE-2 antisera stained infiltrating macrophages. ECE-2-like immunoreactivity has been thought to have a pathophysiological role during intracellular acidosis associated with ischemia[97] and be localized to vesicles in the constitutive pathway[55]. This suggests that in pathological conditions where ET-1 synthesis is higher, upregulation of ET_B receptors occurs as an adaptive mechanism to clear excess ET-1 from the circulation and surrounding tissues.

Unfortunately, ET_A receptors can increase ROS production and eventually result in endothelial dysfunction through oxidation of BH_4 by ROS[72]. Studies have discovered that BH_4 levels can be restored by inhibiting nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity, a major source of free radicals, or by ET-1 receptor antagonists[72]. Both low and oscillatory shear stress increase ET-1 expression, which may lead to vascular inflammation[98]

and expression of adhesion molecules on isolated endothelial cells[99], while decreasing eNOS expression[100].

5. Endothelin-1 and blood pressure regulation

The association with ET-1 and blood pressure regulation, including hypertension[101-103] has been a well-studied topic across several species, including mice, rats, and humans. Significant increases in mean arterial pressure (MAP) accompanied with a fivefold increase in plasma ET-1 levels has been shown in male Wistar rats injected with recombinant adenovirus (Ad.CMV.ET-1) containing the human pre-proendothelin-1[101]. Additionally, the density of both ET_A and ET_B receptors was significantly reduced in Ad.CMV.ET-1 rats within the liver, an ET-1 clearance organ, with a reduction in B_{max} values for both receptors. Similar ET_A receptor downregulation was seen in Ad.CMV.ET-1 rats within the heart, but no change in the kidney, showing that during an acute response, down regulation of tissue specific ET receptors may play an adaptive role to elevated plasma ET-1 levels.

Along with vascular tone, ET-1 is a mediator of blood pressure modulation. Administration of exogenous ET-1 to an intact animal produces transient hypotension and vasodilation that is mediated through ET_B receptors[104, 105]. Vasodilation occurs through enhanced generation of NO and prostaglandin, which is a response that precedes ET_A -mediated vasoconstriction[104, 105]. Functional studies in normal, conscious mice, show that ET_A and ET_B receptors play a physiological role in regulation of basal blood pressure, where ET_B receptor blockade produces sustained hypertension that is absent in mice pretreated with an ET_A antagonist. This suggests that ET_A receptors maintain hypertension produced through ET_B receptor blockade[103].

Common to studies in rats and mice, humans with severe hypertension[106, 107] or target organ damage[108] are more likely to have elevated plasma ET-1 concentrations. This can be explained additionally through patients with atherosclerosis, who have higher circulating levels of ET-1 irrespective of blood pressure[59]. Another study demonstrated an increase in ET-1 gene expression in resistance arteries that was confined to patients with severe hypertension, with only plasma ET-1 elevation approaching significance[109]. This suggests that increases in ET-1 may be seen abluminally within the resistance vessel, rather than in the plasma.

Under normal physiological conditions, plasma endothelin-like immunoreactivity is said to compromise around 60% big ET-1 and 30% ET-1[106]. Most of the focus within plasma has been on ET-1 because it is the major active isoform in blood, although it is possible that big ET-1 could act as a circulating hormone, which is processed to ET-1 at its site of action[102]. Most ET-1, about 90%, is released from endothelial cells abluminally, where ET-1 concentration in the plasma is likely to reflect a balance between production/overspill and its clearance[102]. Under normal circumstances, ET-1 is rapidly cleared from the circulation by ET_B receptors and neural endopeptidases, and has a plasma half-life in healthy humans of approximately one minute[110]. Reduction in ET_B receptors, enzymatic degradation, or renal function, may reduce this clearance and increase plasma ET-1 half-life[102].

6. Endothelin-1 and the microcirculation

The endothelium of resistance arteries, vessels critically involved in generating resistance to flow, play an important role in hypertension[111]. ET-1 release mediated by cyclooxygenase products, causes constriction and hypertrophy of resistance arteries and may play a role in the development and complications of moderate to severe hypertension in humans[112, 113]. In the

microcirculation, hypertension leads to abnormalities that promote tissue and target organ damage[114].

Several studies have investigated the detrimental effects of ET-1 and changes in ET-1 receptor density within the microcirculation[115-117]. Pierre and Davenport demonstrated through microvascular reactivity experiments involving ET_A receptors that in human pial arterioles, the ET_A receptor is key in maintaining vasoconstriction[116]. Similarly in another study investigating the effects an ET_A antagonist, BQ-123, and a dual ET_A/ET_B antagonist, bostenan, on coronary flow and release of ET-1 into coronary effluent in male Porton rats, BQ-123 progressively decreased perfusion rate compared to control hearts, even after wash-out[117]. This indicates that ET_A receptor antagonism within small arteries has a lasting vasodilatory effect. Furthermore, in male Wistar rats after Ad.CMV.ET-1 injection, the density of ET_A receptors reduced significantly for the AD.CMV.ET-1 group, with no change in ET_B receptor density, followed by a decrease in potency of ET-1 to constrict aortas from the AD.CMV.ET-1 group[115]. This suggests that during an acute phase of ET-1 overload (approximately 7 days), adaptations occur systemically that reduce constriction, such as down regulation of ET_A receptors, which may not occur under chronic elevated ET-1 exposure[74].

In some instances, ET-1 induced constriction is not augmented, but constriction may be enhanced through big-ET-1[118]. In endothelium-denuded human coronary arteries, the constriction response to big ET-1, the precursor to the mature peptide, ET-1, was significantly enhanced in atherosclerotic arteries when compared to nondiseased arteries, and the enhanced response was abolished by an ECE inhibitor. With no difference in ET-1 constriction-responses in both groups, increased ET via enhanced ECE activity may have led to the enhanced

constriction to big ET-1, suggesting not only the complexity of ET-1 function within different types of tissues, but also through different pathological conditions.

ET-1 and its modulation of the NO pathway for vasodilation have been thoroughly studied across tissue beds, species, and pathological conditions[82, 119-124]. Specific experiments in coronary arteries have demonstrated suppression of NO mediates ET-1 induced vasoconstriction[120, 124]. It is hypothesized that NO inhibits ET release, whereas inhibition of NO accelerates ET release, where NO plays a role in the physiological termination of ET-1 signaling at the receptor level and at the post receptor level[125]. This emphasizes the homeostatic relationship between the vasoconstrictor and vasodilator properties of ET-1[120]. *In vivo* experiments of male Japanese white rabbits' beating hearts show that when NO release is impaired, the ischemic myocardium-derived vasoactive signal that produces coronary microvascular constriction is unmasked, and is mediated by ET_A receptors[120]. Additionally, an ET_A receptor blocker enhances ischemia-induced coronary microvascular dilation, suggesting that ischemic myocardium simultaneously releases signals activating NO release and those activating ET release[120]. Within resistance coronary vessels in conscious dogs, an ET-1-dependent process magnified the inhibitory effects of L-N^G-Nitroarginine methyl ester (L-NAME), a NOS blocker, on ACh-induced coronary blood flow (CBF) responses, but ET-1-dependent effects failed to attenuate ACh-induced CBF responses when NO formation was intact[124]. Common to other studies involving coronary arteries, the ET_A receptor was the only receptor to limit CBF responses to ACh after L-NAME.

Furthermore, the clearance role of ET_B receptors on the endothelium has been shown to affect ED dilation via NO[126]. Endothelial cell ET_B knockout mice showed an increase in plasma ET-1, with impaired ACh-induced vasodilation in aortic rings, with no impairment in EI dilation

via the vasodilator diethylenetriamine NONOate (DETA-NO) or an increase in blood pressure[126]. However, EI vasodilation has been shown to be impaired in anesthetized goats during reperfusion after 1 hour of partial ischemia[127] or 15 minutes total ischemia[128], with coronary effects of ACh being preserved. Both ED and EI dilation was impaired in goats during reperfusion after 1 hour of total ischaemia[128]. This suggests that under ischemic conditions, milder conditions affect endothelial-independent dilation, but more severe conditions affect both pathways.

NO donors have been tested *in vitro* to reverse the effects of ET-1 induced contraction[123]. NO donor S-Nitroso-N-Acetyl-D,L-Penicillamine (SNAP) completely reversed ET-1 mediated constriction in conductance coronary arteries, with additional relaxation of basal tone with only partial reversal in resistance coronary arteries from human explanted hearts[123]. Previous studies have emphasized that ET-1 receptors are more sensitive in the distal coronary artery tree[129], suggesting that the greater potency of ET-1 in resistance arteries may exacerbate the effects of increased circulating levels of the peptide in disease[123]. Studies validating abnormalities in vascular smooth muscle cell tone in either peripheral resistance vessels or the renal vasculature directly causing hypertension[130] emphasizes the pivotal importance of endothelial function in resistance arteries, and the predisposition of hypertension with elevations of ET-1 and endothelial dysfunction.

7. Endothelin-1 and alcohol consumption

There have been several studies performed examining the effects of acute and chronic ethanol consumption/exposure on ET-1 physiology[42, 62, 74, 131-133]. These studies have been conducted primarily using animal models and cell culture systems due to the lethal effects of ET-

1 at high concentrations *in vivo*. In human dermal microvascular endothelial cells in cell culture, acute treatment with ethanol (25-100mM) increased ET-1 mRNA expression and ET-1 release[42]. Ethanol also increases ET-1 production in human cultured umbilical veins in a dose-dependent manner between 25-100 mM [132]. Levels between 25-100mM of ethanol correspond to intoxicating and lethal concentrations; not moderate consumption.

Chronic intake of ethanol in the rat enhances ET-1 induced contraction in isolated rat carotid arteries, with a decreased expression of vasodilator endothelial ET_B receptors[133]. Furthermore, rats chronically treated with ethanol after 2 weeks exhibit an enhanced, ET_A-dependent, pressor response to ET-1, increased ET_A receptor protein levels in cardiac tissues and in resistance vessels, and reduced ET_B receptors in aortic and renal tissue homogenates[74]. Additionally, rats treated with ethanol for 2 weeks exhibited elevate plasma ET-1 levels[131]. Cultured human endothelial cells with ethanol were shown to activate endothelial production of endothelin and nitric oxide, an effect that was shown to be associated with increased oxidative stress[62].

When investigating blood pressure and plasma ET-1 levels after alcohol consumption, using aortic rings investigators found that chronic ethanol ingestion increased systolic blood pressure significantly. Conversely, the latter pressor response was not related to increased vascular responsiveness to vasoconstrictors like ET-1[134]. Endothelium-dependent reactions, with increased relaxation to acetylcholine but not endothelium-independent reactions in aortic rings were observed[134], which could have been due to the biphasic effects of ethanol and NO release. In cultured human umbilical vein endothelial cells (HUVEC), ethanol (10 and 50mM) stimulated increases in NO levels and HUVEC proliferation, with high concentrations (100-150mM) reducing NO synthesis and endothelial proliferation[135], along with mitochondrial apoptosis[136].

These data support the idea that lower concentrations of ethanol may exert beneficial effects, while higher concentrations can be detrimental.

III. METHODS

A. Study design and Subjects

This was a non-randomized prospective, cross-sectional study. A convenience sample of men and women (n=37) were recruited from an urban university setting. Binge drinkers (BD) (male, n=11; female, n=8) were defined as those that consumed 5 standard drinks (12 oz beer, 5 oz table wine, 1.5 oz of 80-proof spirits, 8-9 oz of malt liquor) or more in a 2-hour period in the last 2 weeks if male, and 4 or more standard drinks in a 2-hour period in the last 2 weeks if female[14]. Also recruited were alcohol abstainers (A) (n=18) and were defined as those that consumed no more than 1-5 drinks standard drinks in the last year (male=10; female=9). Alcohol abstainers had to not be abstaining alcohol due to a medical illness or prior alcohol abuse. All BD and A subjects recruited through their response to an advertisement, and were further screened through email and/or telephone. Exclusion criteria included no history of diabetes, hypertension, pregnancy, tobacco use in the past 6 months, illicit drug use, cardiovascular disease or events, thyroid disease, pituitary tumor, a genetic disease causing disability, gout and a body mass index (BMI) $\geq$30 kg/m^2. All subjects agreed to not take any vasoactive medications within the study. The study was approved by the Office of Protection of Research Subjects and Institutional Review Board (IRB) at the University of Illinois at Chicago.

B. Overview of Study Protocol

All data were collected in the Clinical Research Center (CRC) (University of Illinois-Chicago, Chicago, Illinois, USA) and subjects were required to complete two separate CRC visits. The second visit was 7-10 days within the first visit where all subjects fasted (8 hours) and abstained from exercise (8 hours) before each study visit. Subjects completed medical

history questionnaires, diet and alcohol beverage consumption questionnaires (see below). Sitting blood pressure, heart rate (HR), oxygen saturation, and temperature were measured (Welch Allen 53ST0). Urine samples were collected from female subjects to verify that they were not pregnant. Venous blood samples were obtained for the measurement of total cholesterol, high-density lipoproteins (HDLs), LDLs, triglycerides, insulin, and glucose through Alverno Clinical Laboratories (Hammond, Indiana).

Subjects completed a Block Brief 2000 Nutrition Questionnaire[137] (NutritionQuest: Berkeley, California, USA). Questionnaires were sent to NutritionQuest (Berkeley, California, USA) for analysis of average daily macro- and micronutrient consumption for the past year. A modified 6 Question Set on Binge Drinking Pattern (NIAAA)[138] was also completed by BD and A participants. All participants were educated on what constituted a standard drink of alcohol before subjects recalled the last episode of alcohol consumption, the time elapsed since last drink, along with the number of standard drinks during a certain period of time was documented. Data from the modified 6 Question Set on Binge Drinking Pattern was used to determine binge drinking frequency in the past year and duration of binge drinking. The questions asked were based on recommendations from NIAAA's task force to address the pattern of alcohol consumption in a research setting[138]. Addressing the pattern of alcohol consumption is consistent with recommended questions for alcohol research from the World Health's Organization (WHO) International Guide for Monitoring Alcohol Consumption and Related Harm [139] . Moreover, it is based on recent epidemiological studies on alcohol intake and risks which have demonstrated that for estimating risk of mortality, morbidity (including injuries) and other problems including drunk driving and social harms that it is essential to assess heavy quantity consumption in addition to usual frequency and quantity of consumption[138]. In addition,

the rate of alcohol consumption (grams/hour) was computed for prior alcohol consumption based on how many standard drinks per hour was consumed before each study visit. An average of 14 grams of alcohol was used as an equivalent to one standard drink of alcohol[140].

Rate= Total grams of alcohol consumed/Time spent consuming alcohol

Average physical activity in the participants was assessed by a metabolic activity questionnaire for the last two months [Aerobics Center Longitudinal Study (ACLS)]. Previous studies validate the ACLS questionnaire for associations between physical activity levels and health, as well as fitness[141, 142]. Reported activity was later computed to determine total metabolic equivalent (MET) hrs/week, moderate MET hrs/week, and vigorous MET hrs/week. Moderate METs were considered 4-6 MET equivalents, while vigorous METs were considered >6 MET equivalents[143]. Dual X-Ray Absorbitometry (DXA) (Hologic 1500) was performed all subjects for total fat analysis. At the end of the visit, gluteal fat pad biopsies were performed, and resistance arteries were later dissected for isolated perfused microvessel experiments.

During the second visit, binge drinking activity was re-evaluated if the subject was a BD. Venous blood was obtained for a complete blood count (CBC) differential, C-reactive protein (CRP), liver enzymes [albumin, aspartate aminotransferase (AST), alkaline phosphate (ALP), alanine transaminase (ALT), total and direct bilirubin, and protein], and blood alcohol level (BAL) levels (Alverno Clinical Laboratories, Hammond, Indiana, USA).

C. Measurement of Blood Analytes

Venous samples were centrifuged and plasma removed. The measurement of LDLs, HDLs, triglycerides, insulin, glucose, CRP, BAL, CBC with differential, and liver enzymes were performed by Alverno Clinical Laboratories (Hammond, Indiana, USA) for analysis. LDLs were

measured through colorimetric direct assays [Roche-P Moduler; coefficient of variation (COV) ≤2.5], HDLs through direct photometric PEG assays (Roche-P Moduler; COV< 3 %), triglycerides through colorimetric enzymatic endpoint assays (Roche-P Moduler; COV≤ 2%), insulin through electrochemiluminescence immunoassay (ECLIA) (Roche-P Moduler; COV< 3.2%), and glucose through colorimetric hexokinase assay (Roche-P Modular; COV≤ 1.5%).

CRP levels were measured through kinetic immunonephelometry (Beckman Coulter-Immage; COV<3%) and BAL levels through the ultraviolet (UV) enzymatic method with ADH (Roche-Integra 800; COV< 1.2%).

For the individual liver enzymes, albumin was analyzed through the bromocresol green colorimetric method (Roche-P Modular; COV<2.2%), total bilirubin through a colorimetric assay with diazonium ion, blanked (Roche-P Modular, COV ≤ 2.5%), direct bilirubin through a colorimetric diazotization assay (Roche-P Modular; COV≤ 4%), total protein through a colorimetric assay with the biuret, serum blank, and endpoint method (Roche-P Modular; COV≤ 1.2%), ALT through a UV enzymatic rate assay without pyridoxal-5'-phosphate (P5P) (Roche-P Modular; COV < 2%), AST through a UV photometric assay without P5P (Roche-P Modular; COV ≤ 2%), and ALP through enzymatic rate of p-nitrophenyl phosphate (PNPP) hydrolysis (Roche-P Modular; COV < 2 %).

CBC with differential was measured through pulse measurement technology, hydrodynamic focusing, radio-frequency detection, and direct current detection (Beckman/Coulter LH750).

D. Brachial Artery Flow-Mediated Dilation

Prior to FMD and blood pressure measurement, subjects were supine on a hospital bed (at least 10 minutes) in a temperature controlled room. Ultrasound imaging was conducted using MicroMAxx ultrasound (Sonosite; Seattle, WA). Imaging of the brachial artery was performed in a longitudinal plane, at approximately 5 cm proximal to the antecubital fossa of the right arm, abducted approximately 80° from the body, with the forearm supinated. The ultrasound probe (11 MHz) was positioned at a 60° insonation angle to visualize the anterior and posterior lumen-intima interfaces to measure diameter or central flow velocity (pulsed Doppler). Baseline images and blood pressure readings of the opposite arm with an automated sphygmomanometer were recorded. After baseline ultrasound imaging, Doppler readings of peak flow and average flow were performed for at least 5 seconds. A blood pressure cuff was placed on the forearm, distal to the antecubital fossa on the right arm being imaged and inflated 60 mmHg above baseline systolic blood pressure (SBP) for 5 minutes. Once the cuff was released, blood pressure and HR measurements in the opposite arm were taken, along with Doppler readings of the first 10 seconds after cuff release. The brachial artery was then imaged continuously to capture 30 seconds, 1 min, 2 min, and 3 min post cuff release.

In accordance to Dr. Phillips' previous study design of "The Mechanism of Exertional Hypertension and Vascular Dysfunction,"[137, 144] following the brachial artery ultrasound, most subjects underwent acute exhaustive resistance exercise (ERE) through a single bout of bilateral lower body weight lifting. This was accomplished using an isotonic variable resistance leg press machine (Hoist HD-1610 Selectorized Leg Press; Hoist Fitness Systems; San Diego, California), with an additional blood draw and brachial artery FMD post-ERE. Ultrasound assessment of NTG-induced vasodilation was performed at least 10 minutes after cuff release from the previous

FMD. Baseline diameter before NTG administration was not significantly different than baseline diameter prior to FMD testing (p=.164), with previous studies in our lab showing that within subjects, NTG-induced dilation did not differ significantly after acute ERE when compared to a lack of acute ERE. This suggests that acute ERE does not significantly impact NTG-induced dilation. NTG was administered to subjects that had a resting SBP above 90 mmHg in the supine position. The brachial artery was continuously imaged 2, 3, 4, and 5 minutes after NTG administration. An additional subcutaneous gluteal fat pad biopsy was performed on the opposite hip from the previous week.

Images were digitally recorded using Brachial Imager (Medical Imaging, Iowa City, Iowa, USA) and analyzed as previously described[28]. 450 frames (7.5 frames per second for 10 seconds) were captured, digitized, and analyzed from the M-line (border between intima and media of brachial artery) of the same location of blood vessel using visible landmarks through edge detection software. Approximately 75 frames were analyzed for each baseline and time point measurement through an average of brachial artery diameters over the entire R-R interval. Electrocardiogram (ECG) gating was not performed for all subjects during ultrasound analysis. Previous research demonstrated that when comparing FMD and NTG-induced dilation analyses through QRS gating or an average of brachial diameters over the entire R-R interval, a strong agreement was found between both methods for FMD and NTG-induced dilation, with measurements based on average diameter not reducing accuracy[145]. Percent FMD and the response to NTG were calculated using the averaged minimum mean brachial artery diameter at baseline compared with the largest mean values obtained after release of the forearm occlusion or administration of NTG. COV for intraobserver variability was 1.5% for baseline brachial

artery diameter for FMD, 6.3% for maximum dilation to hyperemic flow (FMD %), 1.7% for baseline brachial artery NTG diameter, and 3.2% for NTG-induced dilation. The peak flow velocity was observed from 5 seconds of baseline diameter Doppler readings and 10 seconds of post-cuff release Doppler readings were recorded for shear rate calculations. Shear rate was calculated as blood velocity (cm/s) divided by vessel diameter (cm)[146].

E. Experiments on Isolated Perfused Microvessels

After the subcutaneous gluteal fat pad biopsy was performed, fat tissue was stored in cold (4°C) HEPES solution and was returned to our vascular physiology lab for vessel isolation. Resistance arterioles (60-300 μM) were isolated from fat tissue, where the adipose tissue was stored at -80°C. Resistance arterioles were then cannulated in an organ chamber with glass micropipettes (external tip diameter ~40μm) filled with KREBS solution adjusted to pH= 7.40 (mmol L^{-1}: NaCl 123, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 16.0, EDTA 0.026, Glucose 11.0, KH_2PO_4 1.2). Both ends of the vessel were secured with 10-0 nylon Ethilon monofilament suture, and the vessel was maintained at an intraluminal pressure of 20 mmHg for 30 minutes, where the intraluminal pressure was then increased to 60mmHg for an additional 30 minutes. Each preparation was transferred on a stage of an inverted microscope (magnification x 200) attached to a video camera, video monitor, and a video measuring device (Boeckeler model VIA-100: Tuscon, AZ). The external bathing medium was continuously superfused with heated buffer solution (pH= 7.4, P_{O2}= 140± 10 mmHg) aerated with a gas mixture of 21% O_2-5% CO_2-74% N_2 made fresh daily, and maintained at 37°C. After resistance arterioles equilibrated for 30 minutes at 60mmHg, vessels were constricted 30-50% with ET-1 (100-200 pM final concentration) before each dose response to dilators ACh and SNP. Vessels that did not constrict ~30% were excluded from analysis.

The responses of resistance arterioles to the ED vasodilator ACh (10^{-9}-10^{-4} M) (A: n=10; BD: n=12) and nitric oxide donor SNP (10^{-9}-10^{-4} M) (A: n=8; BD: n=8) were performed within 100 mL of circulation through the organ chamber. Resistance arterioles were monitored continuously and measured at the maximum diameter after the addition of each dilator concentration. Dose responses with ACh and the NOS inhibitor, L-NAME (100 μM) (A: n=9; BD: n=11), were measured 10 minutes following L-NAME. Dose responses with ACh and coinhibition of L-NAME with cyclooxygenase inhibitor indomethacin (INDO; 10μM) (BD: n=5) were obtained after 30 minutes of inhibitor incubation at 60 mmHg. Inhibition with INDO was only performed in bingers as a post-hoc test to help determine mechanism of resistance artery vasodilation. At the end of each dose response to each specific dilator, or inhibitor and dilator, a maximal response to papaverin (PAP; 10^{-4} M) was performed, followed by a wash with 150-200 mL of KREBS solution. Resistance arterioles were allowed to equilibrate back to resting baseline diameter before the next dose response. At the end of the experiment, a dose response to ET-1 (10^{-11}-10^{-7} M) (A: n=9; BD: n=10) was performed in 20mL within the organ chamber.

F. Drugs

L-NAME, ACh, SNP, ET-1, INDO were obtained from Sigma-Aldrich Corporation (St. Louis, Missouri, USA). Reagents (NaCl, KCl, $MgSO_4$, $CaCl_2$, $NaHCO_3$, EDTA, Glucose, KH_2PO_4) were purchased from Sigma Aldrich Corporation (St. Louis, Missouri, USA) and Fisher Scientific (San Jose, California, USA).

G. Statistical Analysis

All data are reported as mean± SE, with P <.05 as significant unless otherwise noted. FMD % and NTG upon Visit 2 (or first ultrasound), and other cardio-metabolic and descriptive

characteristics that were continuous variables were assessed using Student's t-test for between-group comparisons. Categorical variables were analyzed through a Chi-square test for independence for between group comparisons. Data underwent the Shapiro-Wilkinson test for normality and Mann-Whitney U test for between-group comparisons with nonparametric data. Dilation in dose response data is expressed as a percentage, with 100% representing the change from constricted diameter to the maximal diameter at 60 cmH_20 intraluminal pressure (usually in the presence of papaverine). Constriction responses to ET-1 were represented as a percentage, with the vessel diameter before the ET-1 dose response subtracted from the minimum diameter for each dose, divided by the pre-ET-1 dose response diameter. A closed lumen with no visible dilation represented (-) 100%. Dose responses to ET-1, SNP and ACh with and without INDO and L-NAME were assessed with a two-factor, repeated-measures analysis of variance (ANOVA) to determine the effect frequent binge drinking on this response. For correlation analyses, Pearson product-moment was used for parametric variables, and Spearman rho for ordinal or nonparametric variables. An analysis of covariance (ANCOVA) was completed to correct for correlates of FMD and NTG that were not the grouping variable. Analyses were run with IBM SPSS Statistics software (Version 19.0, SPSS Inc., Chicago, Illionis, USA). ET-1 EC_{50} values were computed in SigmaPlot Version 12.0 (San Jose, CA, USA) with the Dynamic Fit Wizard with the following equation for XY pair data.

$$Y= \text{min}+ \text{max-min}/ 1+ 10^{(\log EC_{50}-x)} \text{Hillslope}$$

IV. RESULTS

A. Demographics

Age, gender, % Fat, BMI, SBP (mmHg), diastolic blood pressure (DBP) and HR(bpm) were not significantly different between BD and A (TABLE I). Binge drinkers were more likely to be white ($p=.004$)[147] and report greater levels of moderate ($p=.024$), vigorous ($p=.022$), and total MET hrs/wk ($p=.018$). Macro and micronutrient content were not significantly different between groups (data not shown). On average, BDs drank alcohol at a rate 34±7 g/hr 65±11 hrs before each study visit. BDs had a binge drinking episode 6±1 times per month, consistently for the last 4± 0.6 years.

TABLE I

DEMOGRAPHIC CHARACTERISITCS[a]

Characteristics	Abstainers (n = 19)	Binge Drinkers (n =17)	*p* Value
Age, y	25 ± 1	25 ±1	.925
Male, %	53	65	.463
Race, % white	21	76*	.001
White (n)	4	13	
Black (n)	3	0	
Asian (n)	7	1	
Hispanic (n)	2	2	
Native American (n)	1	0	
Other (n)	2	1	
Body Mass Index (BMI), kg/m^2	23.7 ± 0.9	25.0 ± (1)	.370
Fat, %	24 ± (2)	21 ± (2)	.303
Moderate MET hrs/wk	28 ± (7)	42* ± (6)	.024
Vigorous MET hrs/wk	24 ± (16)	28* ± (7)	.022
Total MET hrs/wk	52 ± (21)	70* ± (11)	.018
Grams of EtOH/hr	---	34 ± (7)	---
Time since last Binge Session, hrs	---	65 ± (11)	---
Frequency of Binge Drinking, episodes per month	---	6 ± (1)	---
Years Binge Drinking	---	4 ± (0.6)	---
Systolic Blood Pressure(SBP), mm Hg	114 (3)	120 ± (2)	.092
Diastolic Blood Pressure (DBP), mm Hg	67 ± (1)	70 ± (2)	.163
Heart Rate (HR), bpm	64 ± 2	65 ± 2	.800

[a] All data expressed as mean ± (SE)

* Signifcance compared to A, $p<.05$

All lipid levels, CRP levels, and liver enzymes were similar among BD and A (TABLE II). There was a trend for higher triglyceride and AST levels among BDs.

TABLE II

BLOOD METABOLIC CHARACTERISICS[a]

Characteristics	Abstainers (n = 19)	Binge Drinkers (n = 17)	*p* Value
Total Cholesterol, mmol/L	4.20 ± 0.23	4.30 ± 0.16	.689
Low-density Lipoproteins (LDL), mmol/L	2.36 ± 0.21	2.23 ± 0.13	.573
High-density Lipoproteins (HDL), mmol/L	1.42 ± 0.08	1.6317 ± 0.13	.201
Triglycerides, mmol/L	0.84 ± 0.11	0.97 ± 0.09	.107
Glucose, mmol/L	4.72 ± 0.11	4.94 ± 0.11	.167
Insulin, pmol/L	45.1 ± 7.6	32.6 ± 4.9	.245
Albumin, g/L	45 ± 1	46 ± 1	.371
Aspartate aminotransferase (AST), U/L	19 ± 1	23 ± 2	.058
Alkaline Phosphate (ALP), U/L	55 ± 4	55 ± 3	.959
Alanine transanimase (ALT), U/L	9 ± 1	11 ± 1	.249
Total Bilirubin, μmol/L	6.84 ± 1.71	5.13 ± 1.71	.798
Direct Bilirubin, μmol/L	3.42 ± 0.34	1.71 ± 0.34	.798
Protein, g/L	69 ± 1	70 ± 1	.324
Blood Alcohol Level (BAL), gram%	0.01 ± 0.00	0.01 ± 0.002	.762
C-Reactive Protein (CRP), nmol/L	12.38 ± 5.71	15.24 ± 6.67	.421

[a] All data expressed as mean ± SE

For the majority, WBC and RBC values were not significantly different between BD and A (TABLE III). However, hemoglobin (Hgb) levels (p=.046) and platelet values (p=.045) were significantly higher in BD compared to A.

TABLE III

WHITE BLOOD CELL AND RED BLOOD CELL CHARACTERISTICS[a]

Characteristics	Abstainers (n = 19)	Binge Drinkers (n =17)	*p* Value
White Blood Cells (WBC), $x10^9$/L	5.3 ± 0.3	6.1 ± 0.5	.158
Red Blood Cells (RBC), x 10^{12}/L	4.5 ± 0.1	4.6 ± 0.1	.306
Hemoglobin (Hgb), g/L	132 ± 3	142* ± 3	.046
Hematocrit (HCT), proportion of 1.0	0.39 ± 0.001	0.42 ± 0.01	.132
Mean Corpuscular Volume (MCV), fL	87.8 ± 2	89.9 ± 1	.397
Mean Corpuscular Hemoglobin (MCH), pg/cell	28.0 ± 2.0	30.6 ± 0.6	.240
Mean Corpuscular Hemoglobin Concentration (MCHC), g/L	338 ± 2	334 ± 7	.602
Red Blood Cell Distribution Width (RDW), proportion of 1.0	0.138 ± 0.003	0.132 ± 0.002	.114
Platelets (Plt), x 10^9/L	198 ± 8	224* ± 9	.045
Mean Platelet Volume, fL	9.7 ± 3.0	9.1 ± 0.2	.106
Neutrophils , proportion of 1.0	0.51 ± 0.02	0.55 ± 0.04	.325
Lymphocytes , proportion of 1.0	0.37 ± 0.02	0.34 ± 0.03	.377
Monocytes , proportion of 1.0	0.08 ± 0.01	0.07 ± 0.01	.279
Eosinophils , proportion of 1.0	0.032 ± 0.004	0.03 ± 0.01	.788
Basophils , proportion of 1.0	0.004 ± 0.001	0.004 ± 0.0004	.900

[a] All data expressed as mean ± SE

* Significance compared to A, $p<.05$

B. Brachial Artery: Endothelial-Dependent and Independent Dilation

Blood vessel characteristics were not similar among BD and A (TABLE IV). Baseline brachial artery FMD diameter (p=.049) and baseline brachial artery NTG diameter (p=.017) were significantly higher in BDs compared to A. Not surprisingly, peak shear rate (p<.005) was significantly higher in abstainers compared to BDs. Both FMD % (A: 11.0 ±0.7, BD: 8.4±0.7; power= .646 p=.022) and NTG-induced dilation (A: 27.9± 2.0, BD: 19.7± 1.8; power=.774, p=.009) was significantly lower in BDs compared to A (Figure 1).

TABLE IV

BRACHIAL ARTERY CHARACTERISTICS[a]

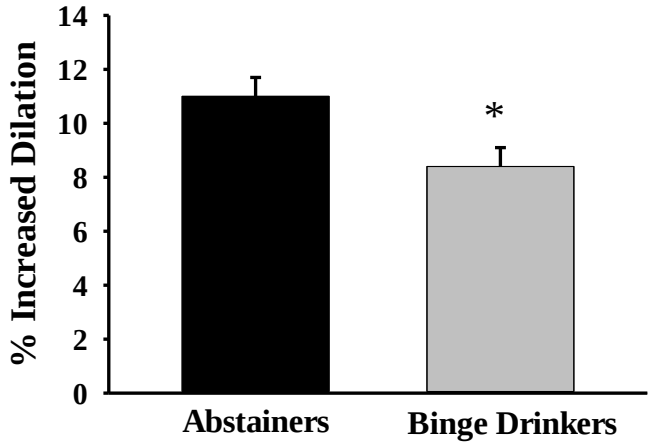
Characteristics	Abstainers (n = 19)	Binge Drinkers (n =17)	*p* Value
Baseline Brachial FMD Diameter, mm	3.5 ± 0.1	3.9* ± 0.2	.049
Maximum Brachial FMD Diameter, mm	3.9 ± 0.1	4.3 ± 0.2	.053
Δ Flow, cm/s	98 ± 12	80 ± 10	.176
Peak flow, cm/s	142 ± 7	128 ± 9	.142
Δ Shear rate, ¯sec	98 ± 12	95 ± 15	.433
Peak shear rate, ¯sec	412 ± 18	328* ± 20	<.005
Baseline Brachial NTG diameter, mm	3.5 ± 0.1	4.00* ± 0.2	.017
Maximum Brachial NTG diameter, mm	4.5 ± 0.1	4.8 ± 0.2	.153

[a] All data expressed as mean ± SE

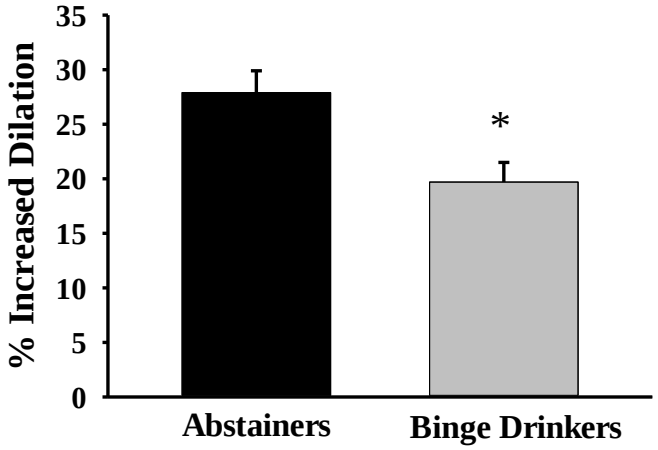
* Significance compared to A, p<.05

Figure 1. (A) FMD, a method of ED dilation, was significantly different between abstainers and late BD (P=.022) and (B) NTG-induced dilation, a method of EI dilation, was significantly different between groups (P=.009). Results are shown in mean± SE.

A



B



FMD % was negatively correlated with Hgb (ρ=-.390, p=.028). NTG was negatively correlated with vigorous MET hrs/wk (ρ=-.451, p=.011) and race (ρ=-.447, p=.007). FMD % and NTG were corrected for covariates (total MET hrs/week and race). Among binge drinkers, there was no significant correlation between moderate, vigorous, or total MET hrs/week and FMD or NTG responses.

C. Resistance Arterioles: Endothelial-Dependent and Independent Dilation

ACh-induced dilation in resistance arterioles was not significantly different between BD and A (Figure 2) ($p=.365$). In BD, ACh-induced dilation was not significantly different to ACh-induced dilation with the NOS-inhibitor L-NAME ($p=.155$). However L-NAME significantly blocked ACh-induced dilation in A ($p=.002$). With SNP-induced dilation, no significant difference was found between BD and A ($p=.948$) (Figure 3).

Figure 2. ACh-induced dilation, a method of endothelial-dependent dilation, was not significantly different between BD and A (p=.365). L-NAME, a NOS inhibitor, significantly blocked ACh-induced dilation in A (p=.007) but not in BD (p=.155).

* Denotes significance, p≤.05 between ACh-induced dilation and ACh+L-NAME-induced dilation in A.

† Denotes significance, p≤.05, between respective doses of ACh in control conditions and inhibition of L-NAME in A.

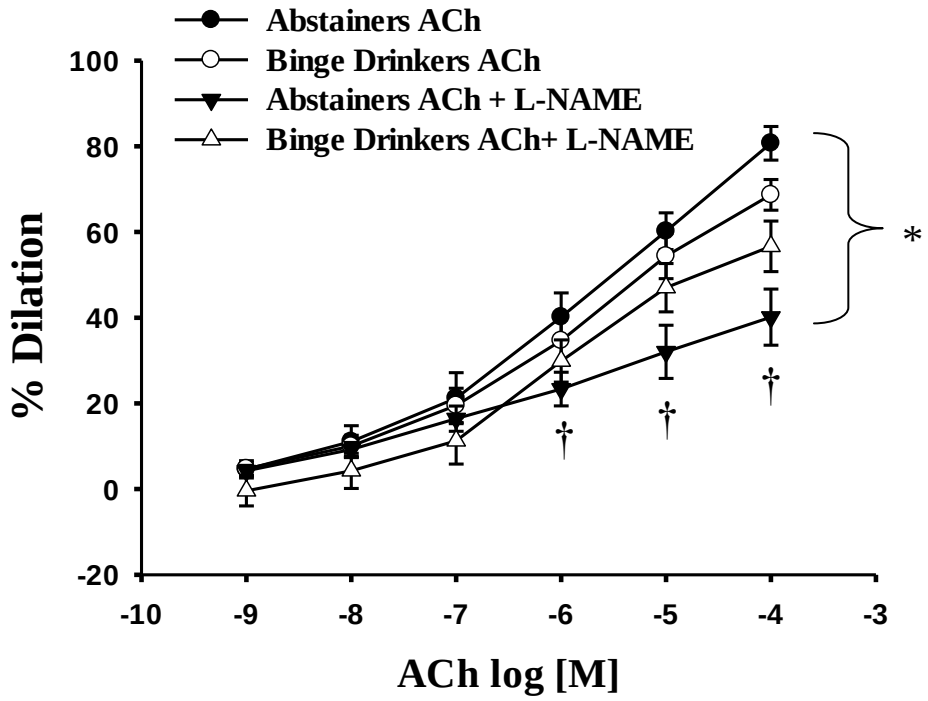
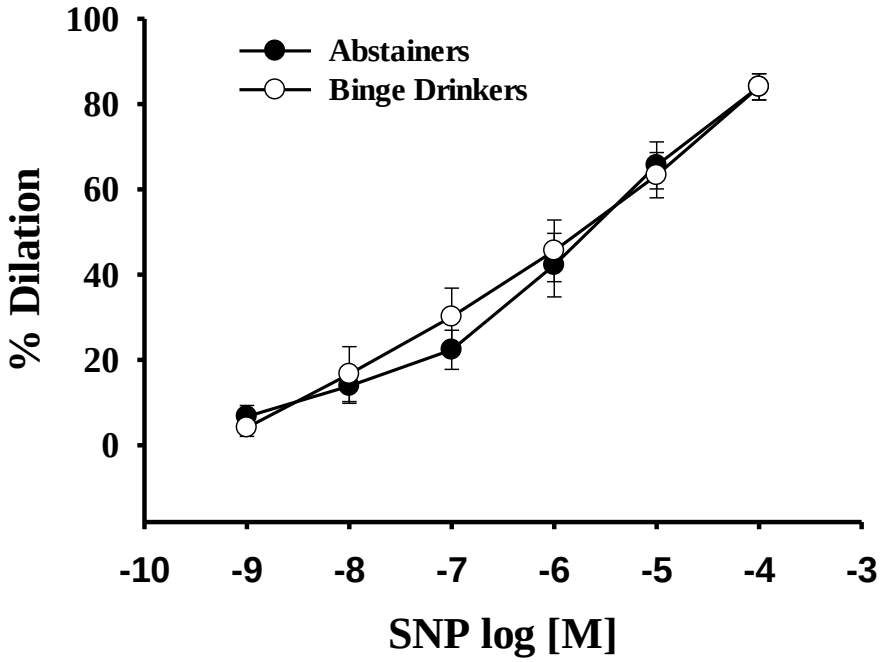
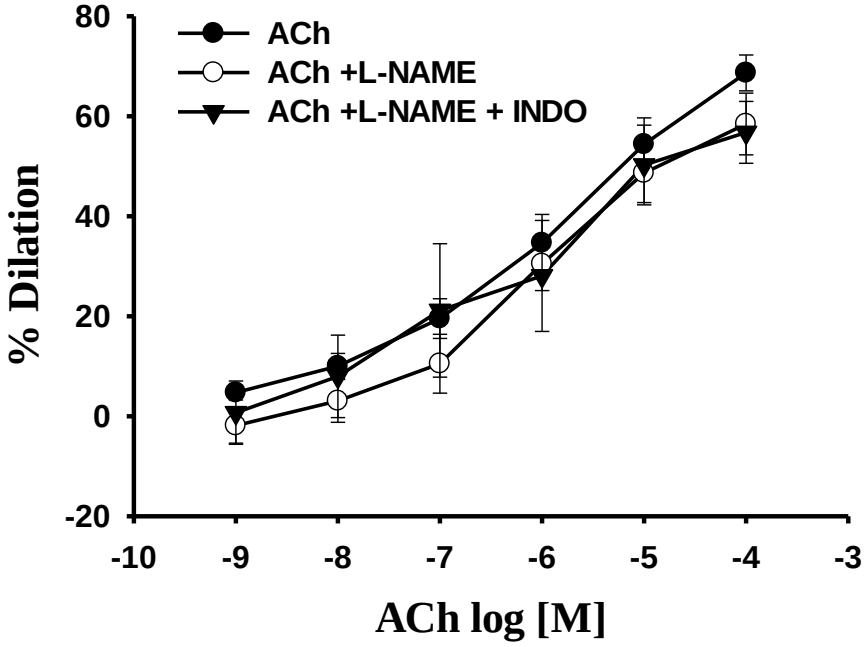

Abstainers ACh
Binge Drinkers ACh
Abstainers ACh + L-NAME
Binge Drinkers ACh+ L-NAME
% Dilation
100
80
60
40
20
0
-20
-10
-9
-8
-7
-6
-5
-4
-3
ACh log [M]
*
†
†
†

Figure 3. SNP-induced dilation was not significantly different between BD and A (p=.948).



Inhibition with L-NAME and coinhibition with L-NAME and INDO did not block ACh-induced dilation in BD (Figure 4) (p=.435), with 10% of dilation attributed to NO and 12% of dilation due to prostaglandin synthesis. In A, NO-induced dilation attributed to 46% of dilation.

Figure 4. ACh +L-NAME and ACh+ L-NAME+ INDO did not significantly block ACh-induced dilation in BD (p=.435).

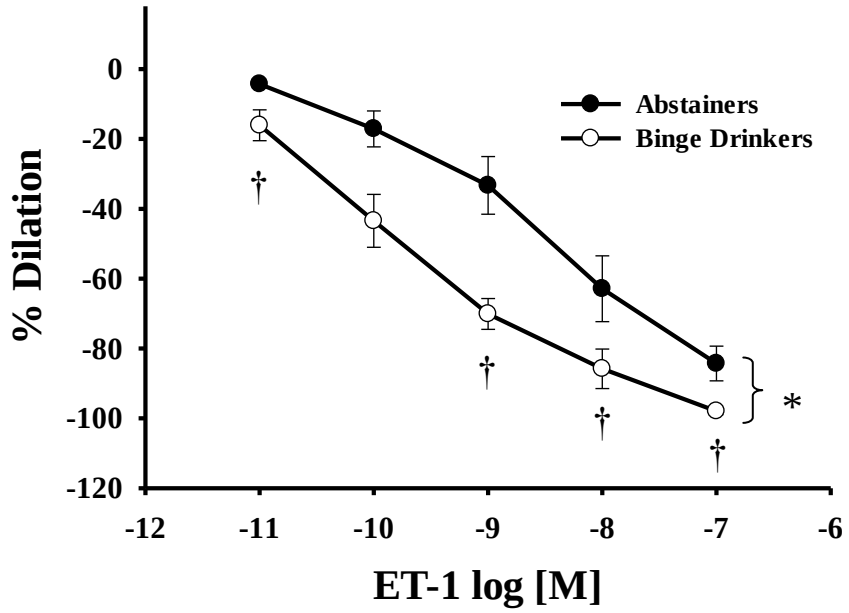


ET-1 induced constriction was significantly enhanced in BD compared to A (Figure 5) ($p=.002$). There was no significant difference between groups in ET-1 exposure (M) from previous experiments involving preconstriction with ET-1 (A: $1.6 \times 10^{-8} \pm 1.5 \times 10^{-8}$, BD: $1.4 \times 10^{-8} \pm 1.4 \times 10^{-8}$; $p=.442$)

Figure 5. ET-1 induced constriction was significantly greater in BD compared to A ($p=.002$).

* Denotes significant difference, $p \leq .05$, in dose response curve to ET-1 between BD and A.

†Denotes significant difference, $p \leq .05$, in ET-1 dose compared to A.



EC_{50} for ET-1 was significantly more potent in BD than A (A: -8.7 ±0.3, BD: -9.4 ± 0.1; p=.032). EC_{50} for BD underwent correlation analysis with FMD, NTG, vigorous, moderate, and total MET hrs/week, liver enzymes, CRP, years spent binge drinking, and frequency of binge drinking per month. Correlation analyses were also performed for ET-1 EC_{50} against the last alcoholic drinking episode in regards to hours prior to last drink, grams of ETOH consumed per hour, and total number of standard alcoholic drinks consumed before the subject underwent their first study visit and underwent a biopsy. There was a significant positive correlation between years spent frequently binge drinking (r=.688, p=.028), and a negative correlation between total bilirubin (r=-.673, p=.047) and EC_{50} of ET-1 in BD.

D. Correlation Analyses

Correlation analyses in binge drinkers between metabolic, cytokine, blood, lifestyle, vascular variables and FMD and NTG responses reveal several interesting findings. For variables related to binge drinking patterns, frequency of binge drinking was negatively correlated with protein levels (r=-.651, p=.006), while grams ETOH/hr consumed prior to Visit 1 was positively correlated with triglyceride levels (r=.762, p=.001). Additionally, years spent binge drinking was negatively correlated with triglyceride levels (r=-.531, p=.028) and insulin levels (r=-.500, p=.049).

Concerning brachial artery variables, peak shear rate was negatively correlated with triglycerides(r=-.500, p=.041). FMD responses in binge drinkers were negatively correlated with protein levels (r=-.566, p=.022) and ALP levels (r=-.498, p=.05).

V. DISCUSSION

This study presents many novel findings involving the effects of frequent binge drinking and the effects of EI and ED dilation. No previous studies have examined the macro and micro-vascular effects of frequent binge drinking in a young, healthy human population.

A. Brachial Artery: Endothelial-Dependent and Independent Dilation

In this study, ED and EI in conduit arteries, such as the brachial artery, were impaired in frequent binge drinkers when compared to abstainers through the methods of brachial artery FMD and brachial artery NTG-induced dilation. Our results compare to other findings in previous studies of acute binge drinking in humans, where both ED and EI dilation are compromised[20]. Yet, Bau et al. found that FMD and NTG-induced dilation were impaired only after 4 hours, and returned to baseline levels after 13 hours of intoxication of alcohol. This differs from our study where our participants were frequent binge drinkers and time since last drink was not correlated to FMD % and NTG values. Previous studies involving chronic heavy alcohol abusers have shown impaired FMD but preserved NTG when compared to teetotalers[35, 36]. This suggests that heavy, episodic alcohol consumption may impair the macro-vasculature differently than heavy, consistent alcohol consumption where just the endothelium is compromised.

Higher baseline brachial artery diameter and lower peak shear rate in binge drinkers may have had an influence on the lower FMD % observed in binge drinkers[33]. Previous studies demonstrated that baseline brachial artery diameter increases after an acute binge drinking session[20], which may restrict additional dilation through reactive hyperemia and NTG[148].

FMD studies concerning exercise and physical activity demonstrate that baseline diameter and peak shear rate have a profound impact on the FMD response, due to the vessel wall adapting to the shear stress stimulus during exercise[149]. NTG responses are also decreased, hypothesized as desensitivity to NO[149]. Therefore, FMD responses and NTG responses tend to be lower in athletes, or those who perform consistent exercise. Based on physical activity questionnaires (ACLS), binge drinkers in our cohort were more active than abstainers, with significantly higher MET hrs per week in moderate and vigorous activity. Peak shear rate and baseline diameter might have an impact on the significantly lower FMD and NTG responses seen in this study, but this is not likely the case. Total MET hrs/wk were corrected for in FMD and NTG responses which still led to significant differences in FMD and NTG dilation between binge drinkers and abstainers. This clearly demonstrates that binge drinking has a more profound effect on decreased FMD responses, and that the decreased FMD observed was due to endothelial dysfunction and not vascular adaptation through physical activity.

Correlation analyses in binge drinkers also revealed no significant correlation with moderate and/or vigorous activity and FMD, suggesting that habitual moderate and vigorous physical activity is not protective against endothelial dysfunction in binge drinking. While there are studies showing that exercise interventions in people with cardiovascular disease[150, 151, 152] or post -MI[153] show enhanced FMD responses post exercise intervention, it seems that this enhancement is predominant in older, less-trained, and patients with current cardiovascular disease, where the effects of exercise might be more pronounced. In our population, our participants were young and free of cardiovascular disease and cardiovascular risk factors, so the effects of physical activity might not have a profound effect on FMD since these patients were young and healthy.

What was interesting in our binge drinking population was that time since last alcoholic drink, frequency of binge drinking, duration of binge drinking in years, grams of ETOH/ hour consumed and total standard drinks consumed all before FMD and NTG testing were not correlated in any way with FMD and NTG responses in binge drinkers, suggesting that the impaired FMD and NTG responses may be due to chronic impairments, not the acute effects of binge drinking or alcohol consumption. In acute binge drinking studies[37], FMD was impaired up to 4 hours after consumption[20], but no studies demonstrated the lasting effects of binge drinking on FMD and NTG administration. Likewise in previous studies involving chronic heavy alcohol use and FMD and NTG responses, FMD responses were still significantly lower in alcoholics after a 3-month[35] or 6-144[36] month period of abstinence. This suggests that alcohol consumption, whether in the form of binge drinking or daily, heavy alcohol consumption has lasting and detrimental effects on the endothelium.

Frequent binge drinking's chronic impairment of the endothelium and smooth muscle are novel findings, and the association between pro-inflammatory components of alcohol consumption and lower FMD[154, 155] and NTG [167] are strong. ALP when analyzed in serum, reflects released ALP from tissues, mainly from liver and bone, but is also a ubiquitous enzyme found in many cell types[156]. ALP has been shown in elevated levels in various processes of wound healing, but mainly during the acute phase, preceding high levels of type III collagen, where it is inducted oftentimes by the extracellular matrix, ascorbic acid, or IL-6. However, when chronic lesions or scars are in the process of healing, ALP activity is still present[156]. ALP was found to be negatively correlated with FMD responses in bingers. Further studies need to investigate the pro-inflammatory effects of ALP and its role in reduced FMD.

Likewise, total protein levels were negatively correlated with FMD, where higher protein levels (albumin and globulin), may be indicators of excessive inflammation in the liver or the immune system. Triglyceride levels in binge drinkers had a significant positive correlation with baseline brachial artery diameter, where previous studies involving triglycerides show association with YKL-40, a molecule secreted by macrophages in atherosclerotic lesions, involved in plaque rupture, and vascular remodeling[157]. In addition, asymptomatic individuals with hypertriglyceridemia had higher values of common carotid artery- inter-medial thickness (CCA-IMT) compared to matched controls[158]. Future mechanism studies need to investigate how the pro-inflammatory effects of protein and triglycerides impact endothelial function in humans who frequently binge drink.

An inverse relationship between Hgb and FMD has been established in Type II Diabetes and chronic kidney disease[159]. Hgb acts as buffer to NO, modulating its bioavailabity in different tissue beds[160]. Disturbed regulation of endothelial function, secondary to changes in Hgb concentration, appears to be a relevant pathophysiological pathway, where changes in red blood cell mass may exert adverse cardiovascular events[161]. No known studies have investigated the relationship between Hgb levels and endothelial function in those who consume alcohol heavily or episodically. Hgb levels were higher in binge drinkers compared to abstainers, with an inverse relationship existing between Hgb levels and FMD %. Additional analyses to determine the extent of variability in FMD that may be accounted for by Hgb in FMD responses may be required.

B. Resistance Arterioles: Endothelial-Dependent and Independent Dilation

In resistance arterioles, dose responses to ED dilator ACh and EI dilator SNP were not significantly different among binge drinkers and abstainers. However, NOS inhibitor L-NAME significantly attenuated ACh-induced dilation in abstainers, but not in binge drinkers. This suggests that in RAs of binge drinkers vasodilation is not mediated by nitric oxide. In normal physiological conditions, nitric oxide and prostaglandins are the primary vasodilator in resistance arterioles[162, 163], and eNOS may increase ACh-induced dilation through exercise training[162]. But in pathological conditions, other vasodilators such as hydrogen peroxide (H_2O_2)[164] may predominate due to ROS[164]. Incubation with the inhibitors L-NAME and INDO reveal that in binge drinkers, 10% of dilation is attributed to NO and 12% is due to prostaglandins. 78% of dilation cannot be elucidated, suggesting that H_2O_2 may be the primary vasodilator in the microcirculation of binge drinkers, but still needs to be confirmed in future experiments with flow-induced-dilation and Catalase (inhibitor of H_2O_2 production) inhibition. Previous studies in alcohol-fed rats show impaired ACh-induced dilation in pial arterioles[49], which under physiologically normal conditions dilate in response to NO, but no known studies on resistance arteries have investigated the effects of alcohol consumption whether heavy, daily consumption or binge drinking on H_2O_2-mediated vasodilation, which should be investigated in the future.

C. Resistance Arterioles: Vasoconstriction to Endothelin-1

ET-1 constriction determined through ET-1 dose responses in resistance arteries was found to be significantly enhanced in binge drinkers compared to abstainers, which confirms similar findings from previous studies in chronic alcohol-fed rats [133,134]. This is a novel finding,

given that this particular binge drinking population was young and healthy, and free of cardiovascular risk factors. This also suggests that enhanced ET-1 constriction is not reserved strictly for chronic, heavy alcohol consumption, but also for other forms of hazardous drinking that is more socially acceptable in younger populations yet yields similar cardiovascular risk factors.

Correlation analyses with ET-1 EC_{50} values positively correlate to years spent binge drinking. Potentially, chronic exposure to elevated ET-1 may have created an adapative mechanism to limit enhanced vasoconstriction. For instance, a previous study with rats has demonstrated down regulation of ET-1 vasoconstrictor receptors in the hearts of rats as an adaptation to elevated plasma ET-1 levels[101]. Future studies evaluating circulating ET-1 levels and receptor densities within resistance arterioles would help determine if adaptation to elevated ET-1 levels exist within the microcirculation of frequent binge drinkers.

Impaired ED dilation was found in the macro-and microcirculation of binge drinkers, while these impairments were not associated between conduit and resistance arteries. This is supported through previous studies[114, 152] looking at the macro-and microcirculation with cardiovascular risk factors, where impairments or improvements are observed in both circulating beds but are not related. Through correlation analyses of various inflammatory markers and metabolic variables, it seems that ED dilation in conduit arteries are impaired through different inflammatory pathways compared to the inflammatory pathways correlated with enhanced constriction in the microcirculation. Thus, the complexity of circulating vascular beds and endothelial dysfunction is maintained in this particular study.

D. Limitations

Several limitations exist within this study. This study was nonrandomized and cross-sectional, which introduces enhanced variability within this particular cohort. Additionally, further regression analyses need to be performed to correct for other covariates of FMD and NTG responses. Also, the study was not blinded by the researcher for ultrasound analysis, which may introduce bias to the FMD analysis. However, the intraobserver COV for baseline diameter, FMD, and NTG-induced dilation was within range of previous studies[165, 166]. Additional limitations include relying on recall of prior alcohol consumption as opposed to distribution of alcoholic beverages in a clinical setting, where amount of alcohol could be accurately documented.

E. Conclusions

In conclusion, frequent and heavy episodic alcohol consumption or binge drinking in young adults leads to 1) impaired ED and EI dilation in the brachial artery; 2) NO-mediated vasodilation in resistance arterioles of abstainers, but not binge drinkers and 4) enhanced constriction in resistance arterioles of binge drinkers.

Macro-and micro-vascular dysfunction in young adults who engage in frequent, episodic alcohol consumption emphasizes binge drinking as an ongoing public health concern. Ultrasound assessment of the brachial artery through FMD is a noninvasive and reliable method that could help with prevention of binge drinking, and detection of cardiovascular disease onset and progression within clinical settings.

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ABSTRACTS: Bian JT, Goldufsky J, Goslawski M, Franklin N, Phillips SA. Reduced catalase and increased hydrogen peroxide contribute to the protective effect of chronic exercise against microvascular dysfunction induced by acute exertion. *FASEB J.* 2011.
Ref Type: Abstract

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Goslawski MM, Bian JT, Szczurek M, Piano MR, Phillips SA. Frequent, Episodic Alcohol Consumption Alters Macro-and Micro-Vascular Reactivity in Young Adults. Circulation 124. 11-15-2011.
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