

The influence of sugar sweetened beverage consumption on cerebrovascular function and postprandial health in adolescents

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ABSTRACT:

Cardiovascular diseases (CVD) are the leading cause of non-communicable diseases worldwide, with the underlying atherosclerotic process originating in youth. Children and adolescents with CVD risk factors have impaired endothelial function, which is implicated in the process of atherosclerosis. Habitual sugar sweetened beverage (SSB) consumption is associated with the progression of CVD risk factors in youth, and adolescents consume the highest quantities of SSBs. Acute SSB consumption results in vascular dysfunction in adults, though the effects in youth are unknown. It is thought that exposure to CVD risk factors in youth may impair cerebrovascular reactivity (CVR), possibly having implications for future CVD risk. It is also unknown whether the types of sugar in SSBs have different consequences on vascular function. This thesis aimed to investigate the effect of sugar moiety on cerebrovascular function in adolescents, following consumption of a sugary drink and subsequent meal. Data on the reliability of CVR in a paediatric population was needed to first establish if this was a reliable measure of endothelial function. The purpose of this thesis was to: 1) examine the within and between-day reliability of a breath-hold protocol to assess CVR in adolescents 2) examine the acute effect of sugar moiety (fructose, sucrose, glucose) on CVR and putative blood outcomes, and 3) examine the effects of SSB consumption on postprandial health in adolescents. Chapter 3 examined the reliability of a breath-hold protocol to assess CVR in youth, determined via transcranial Doppler ultrasonography of the middle cerebral artery (MCA). CVR was calculated as the percentage increase in MCAv mean following three breath-hold attempts. This outcome yielded acceptable levels of within and between-day reliability for use in multiple visit experiments to assess CVR in adolescents. Chapter 4 investigated the effect of sugar moiety on cerebrovascular function, measured through breath-hold induced CVR, in adolescents following SSB consumption and a subsequent challenge meal. This study found that the glucose and sucrose drinks resulted in elevated blood glucose levels compared to fructose and water. With consumption of fructose, elevations in uric acid were present, however the sugar moieties all presented similar increases in TAG concentrations following meal consumption. Despite these different metabolic responses, no significant impairments in CVR were present following the drink or challenge meal.

This thesis demonstrated that consumption of SSBs led to increases in glucose and uric acid concentrations, which have previously been shown to be atherogenic. This thesis also provided data on the reliability of CVR as a non-invasive and easy to administer tool for measurement of endothelial function in youth. This is the first study to demonstrate that breath-hold induced CVR can be reliably measured in youth, as a practical, affordable and non-invasive method. These findings provide valuable data that will inform the implementation and analysis of a breath-hold protocol for reliable CVR assessment in youth in future research. Having established that CVR was reliable within and between-day, it was not possible to determine if it was sensitive to change, with no effects seen on CVR following acute SSB consumption. To build on these findings, future research should explore the acute and chronic effects of SSB consumption, with consideration of measuring a range of different vascular outcomes such as changes in peripheral microvascular and macrovascular functions. As this thesis did not include another measure of peripheral endothelial function, it is not certain whether endothelial function was impaired, or if CVR was not sensitive to change in the present study. In order to determine if CVR is sensitive to change, future investigation is needed with established measures of peripheral endothelial function (i.e. flow mediated dilation) alongside measures of CVR.

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SYMBOLS AND ABBREVIATIONS:

AHA	American heart association
ANOVA	Analysis of variance
BHI	Breath-hold index
BMI	Body mass index
BP	Blood pressure
CO ₂	Carbon dioxide
CON	Control trail (Water)
CV	Coefficient of variation
CVD	Cardiovascular disease
CVR	Cerebrovascular reactivity
DBS	Disclosure and Barring service
ES	Effect size
FMD	Flow mediated dilation
FRU	Fructose
GLU	Glucose
iAUC	Incremental area under the curve
ICC	Intraclass correlation coefficient
LDL	Low density lipoproteins
MAP	Mean arterial pressure
MCA	Middle cerebral artery
MCA _v	Middle cerebral artery velocity
MCA _v _{mean}	Middle cerebral artery mean velocity
MMTT	Mixed meal tolerance test
NHS	National health service
PA	Physical activity
PaCO ₂	Arterial partial pressure of carbon dioxide
P _{ET} CO ₂	End-Tidal carbon dioxide
RPM	Revolutions per minute
SBP	Systolic blood pressure
SD	Standard deviation

SPSS	Statistical packages for social sciences
SSB	Sugar sweetened beverage
SUC	Sucrose
T2DM	Type 2 diabetes mellitus
TAG	Triglyceride
tAUC	Total area under the curve
TCD	Transcranial doppler ultrasonography
TE	Typical error
$\dot{V}CO_2$	Carbon dioxide production
$\dot{V}O_2$	Pulmonary oxygen uptake
$\dot{V}O_{2max}$	Maximal oxygen uptake
$\dot{V}O_{2peak}$	Peak oxygen uptake
WHO	World Health Organisation

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CHAPTER 1: Introduction and Literature Review

This section provides insight into the prevalence, morbidity and mortality relating to cardiovascular disease (CVD) and its origins in youth. This is followed by an overview of the relationship between sugar intake and the development of CVD in youth, specifically detailing the relationship between sugar moiety (glucose, fructose and sucrose) and their effects on cardiometabolic health. Finally, this section will end with a critical examination of the evidence relating to the acute effects of sugar consumption on cardiovascular health in youth.

Cardiovascular diseases

In England, the National Health Service (NHS) spends an estimated £7.4 million on CVD related healthcare (Wilkins et al, 2017), with 22% of all premature deaths caused by CVD (BHF, 2017). Accordingly, CVD is an important topic for both research and development of interventions to reduce the prevalence of disease. Despite advances in prevention and treatment, CVD's encompassing conditions such as heart attacks and strokes, remain the leading causes of non-communicable deaths worldwide (WHO, 2011). CVDs are responsible for one third of deaths globally (17.5 million deaths a year) (Deaton et al., 2011), with this predicted to increase to 23.3 million deaths a year by 2030 (Mathers & Loncar, 2006). This is attributable to the increased prevalence of risk factors for CVD, such as type 2 diabetes mellitus (T2DM), which more than doubles the risk of CVD occurrence (Sarwar et al., 2010). CVD risk factors are habits, behaviours or biological characteristics of an individual that precede a well-defined outcome of disease, predict that outcome, or are directly in the biological causal path. The Framingham Heart Study demonstrated that the presence and severity of known risk factors (see Table 1.1) can explain 75 to 90% of CVD events (Greenland et

al., 2003). As well as the increase in CVD risk factors and global CVD, the development of risk factors are increasingly occurring at younger ages. This is highlighted by evidence showing a growing number of children diagnosed with T2DM (Grundy *et al.*, 1999), which was typically seen in individuals over the age of 35 years (WHO, 2016). Multiple epidemiological studies have also demonstrated an increase in the prevalence of obesity beginning in childhood, with at least 18% of 5 to 19 year olds diagnosed as overweight or obese (WHO, 2016). This is of particular concern given the associations between obesity and CVD risk factors such as hypertension and dyslipidaemia beginning in childhood. Given the health and economic burden of CVD, the need for early intervention to reduce the occurrence and progression of CVD in youth is clearly important.

Table 2.1. Table of risk factors known to be related to CVD and atherosclerosis progression from the American Heart Association (AHA) (Kavey *et al.*, 2003).

Evaluated Risk Factors of CVD
Family History
Age
Gender
Nutrition/Diet
Physical Inactivity
Smoking
Hypertension
Hyperlipidaemia
Overweight/ Obesity
Impaired glucose tolerance, insulin resistance and diabetes
Inflammatory Markers
Perinatal Factors

Atherosclerosis is a slow, progressive, inflammatory disease with known origins in childhood, see Figure 1.1 (McGill *et al.*, 2000). The process of atherosclerosis is a pre-requisite to overt CVD, initially characterised by dysfunction of the vascular endothelial lining. This creates a pro-atherogenic environment, leading to the infiltration of lipids, cholesterol and cellular debris, forming a fatty streak in

the artery. With continued progression, this forms a fibrous plaque which, in the process of lipid deposition and proliferation of smooth muscle, causes enlargement and calcification of fibrous plaques. This may lead to vessel occlusion or plaque rupture, promoting a thrombotic occlusion and, in doing so, clinically overt CVD (Cai & Harrison, 2000; Landmesser et al., 2004; McGill et al., 2000).

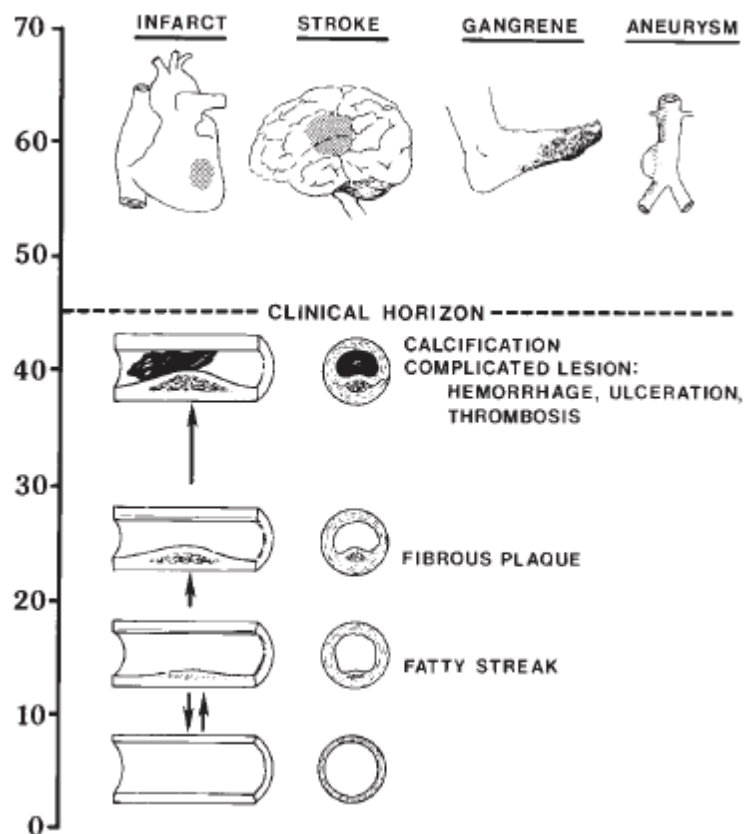


Figure 1.1. The progression of the atherosclerotic process. Reproduced from McGill et al., (2000).

Paediatric origins of cardiovascular disease

Whilst clinically overt CVD may not occur until the fifth decade of life, there is strong evidence that the process of atherosclerosis has its origins in youth (McGill et al., 2000). Evidence of the presence of atherosclerosis at a young age (15-34 years) was identified in Korean and Vietnam war casualties in the Pathological Determinants of Atherosclerosis in Youth study (PDAY), demonstrating the presence of atherosclerosis measured at autopsy after accidental death (Strong

et al., 1999). This has been supported by additional autopsy data demonstrating the presence of atherosclerotic lesions and fatty streaks in the arteries of children and adolescents (Enos et al., 1953; Strong & McGill, 1962; Wissler & Strong, 1998). More specifically, evidence has shown that 65% of 12-14 year olds have coronary atherosclerotic lesions, with a further 8% demonstrating advanced lesions (Stary, 1989), with the presence of fatty streaks in childhood associated with cardiovascular events in adulthood (Katz et al., 1976).

Risk factor status and progression in youth

Evidence shows that CVD risk factors tend to cluster in adolescence (Andersen et al., 2003), which are shown to track into adulthood (Andersen et al., 2004). Findings from Anderson *et al* (2004) show that adolescents with clustered CVD risk factors are six times more likely to have high risk factor status in adulthood. This is particularly important as evidence has shown that, although adult interventions can modify risk factor status, they do not eliminate the elevated CVD risk (McGill et al., 2008).

It has been found that the presence of CVD risk factors in childhood are the strongest predictors of the adult atherosclerotic processes (Kavey et al., 2006), with evidence showing that established risk factors such as elevated serum lipoproteins and high blood pressure (BP), track from youth into later life (Clarke et al., 1978; Webber, et al., 1983). Data from the Bogalusa Heart study of participants aged 6 to 30 years who died of accidents, suicides or homicides, identified that the progression of the atherosclerotic process was proportional to CVD risk factor status in youth (Berenson et al., 1992). Studies have also identified that elevated cholesterol levels in childhood are associated with a two-fold higher rate of CVD mortality in adulthood (Schrott et al., 1979). The

Cardiovascular Risk in Young Finns study concluded that childhood obesity, elevated BP, smoking, physical inactivity and insulin resistance are the strongest predictors of adult CVD risk factor status and atherosclerosis progression (Juonala et al., 2013).

Given the well-established paediatric origins of CVD, recent research has focussed on preventing or modifying risk factor status in youth, with particular interest in lifestyle and behaviour approaches, including physical activity and dietary interventions.

Using transcranial Doppler ultrasonography as a measure of vascular function

Measures of endothelial function are thought to be the earliest detectable manifestations of the atherosclerotic process (Juonala et al., 2004; Ross, 1999), and are associated with the presence of CVD risk factors (Celermajer et al., 1992). Non-invasive ultrasound techniques examining arterial health and endothelial dysfunction have been shown to be prognostic of, and associated with, increased cardiovascular events (Fernhall & Agiovlasis, 2008). The use of these techniques, including measures of carotid intima-media thickness (cIMT) and flow-mediated dilation (FMD), have become clinically important markers of atherosclerotic progression (Touboul et al., 2004) and are an independent predictor of future CVD in adolescents (Raitakari et al., 2003). The non-invasive and easy to administer protocol of endothelial function make this ideally suited for use in children and adolescents, where invasive procedures may be deemed unethical.

Previous research examining endothelial function has predominantly focussed on FMD in the peripheral vasculature (Bond et al., 2015b; Bond et al., 2015c;

Hopkins et al., 2012), but in recent years there has been a growing interest in cerebrovascular function as a diagnostic and screening tool in both research and clinical settings (Ainslie & McManus, 2016). As the brain has a limited ability to store energy, the maintenance of adequate cerebral blood flow is integral for normal brain functioning and survival.

Endothelial function of the cerebrovasculature can be determined via transcranial Doppler (TCD) ultrasonography, measuring the reactivity of the middle cerebral artery (MCA) to a hypo- or hyper- capnic stimulus. Cerebrovascular reactivity (CVR) refers to the ability to regulate cerebral blood flow in response to a vasoactive or vasodilatory stimulus. A hypercapnic stimulus causes the cerebrovascular vessels surrounding the MCA to dilate in response to changes in the partial pressure of carbon dioxide (CO₂). The hypercapnic stimulus was originally presented by CO₂ breathing (Kety & Schmidt, 1948), since this directly manipulates the concentration of CO₂ the participant is breathing, and therefore arterial CO₂ (PaCO₂) concentration. However, a simplified, easy to administer and more affordable method of a breath-hold test has been introduced and significantly correlates ($r=0.67$, $p<0.001$) with CO₂ induced CVR in adults (Markus & Harrison, 1992; Settakis et al., 2002). This method, used as a surrogate of CO₂ breathing, delivers a hypercapnic stimulus through a simple breath-hold, with the arterial partial pressure of CO₂ (PaCO₂) shown to contribute two thirds to the CVR response in adults, with one quarter attributed to increases in mean arterial pressure (MAP) (Przybylowski et al., 2003).

Whether the validity of this measure holds true in a paediatric population has not been explored. Several studies in adolescents however, have demonstrated blunted CVR in patients with increased CVD risk factors following a breath-hold protocol (Páll et al., 2011; Lande et al., 2012), similarly to studies using CO₂

breathing (Wong et al., 2011). This suggests that in paediatric groups, impairments in CVR using the breath-hold protocol have similar sensitivity to CVD risk and clinical outcomes to the CO₂ breathing method. However, a possible limitation to the breath-hold test is the individual variability and ability to appropriately perform the test, introducing potential errors. Compared to CO₂ breathing, this method is more dependent on participant adherence, with factors such as a Valsalva manoeuvre and different breath-holding lengths potentially confounding CVR results (Urback et al., 2017; Wu et al., 2015). In order to control for these differences in breath-holding length the breath-hold index (BHI) is often employed as an outcome, as it normalises the CVR response to breath-hold length. However, equal breath-hold lengths do not lead to an equal increase in PaCO₂, therefore this cannot be standardised, resulting in slightly different stimuli across and within participants. Importantly however, studies have found no mean differences in CVR values obtained from CO₂ breathing compared to breath-hold methods, with the two methods strongly correlated in adults ($r=0.67$, $p<0.001$), despite these potential sources of error (Kastrup et al., 2001; Markus & Harrison, 1992; Tancredi & Hoge., 2013). Evidence indicates that the breath-hold protocol is well tolerated in youth (Müller et al., 1995) though reliability data on this outcome in a paediatric population is needed to ensure that the protocol is appropriately adhered to, and ensure that any changes in CVR outcomes are not due to poor reproducibility of the protocol and its outcomes, in order that test validity is not compromised in a young population.

The breath-hold method gives a measure of the reactivity of the MCA, with impairments from this test predictive of stroke and neurocognitive decline in adults (Serrador et al., 2005; Xie et al., 2006), as well as being established as an independent predictor of future CVD events (Markus & Cullinane, 2001). The use

of CVR assessment as a clinical marker is supported by observations that, even in youth, CVR is impaired in the presence of risk factors for CVD, such as hypertension and white coat hypertension (Lande et al., 2012; Settakis et al., 2003). Furthermore, the use of CVR as an alternative measure of endothelial function is supported by findings demonstrating a common nitric oxide pathway in responses to systemic (FMD) and cerebrovascular (CVR) endothelial function (Ainslie et al., 2008; Lavi et al., 2006). FMD is a surrogate measure of coronary artery function ($r=0.79$, $P<0.001$) (Takase et al., 1998), shown to independently predict CVD events in populations at risk of CVD (Chan et al., 2003; Meyer et al., 2006; Wang et al., 2009), as well as demonstrating prognostic value in asymptomatic groups (Rossi et al., 2008; Shechter et al., 2009). Recent evidence demonstrates that CVR may have additional prognostic value, since it provides a more direct measure of vascular function in the brain, associated with dementia, stroke (Silvestrini et al., 2000), Alzheimer's disease (Keage et al., 2012) and cognitive decline (Wong, Evans, & Howe, 2016).

Given the easy to administer and non-invasive nature of this breath-hold protocol, CVR has utility as a research tool in paediatric populations. Before this can be made possible, protocol methods need to be made explicitly clear as current adult studies are difficult to interpret due to differing methods of assessing breath-hold induced CVR. Furthermore, there are no data available on the reliability of determining CVR via the breath-hold test in a paediatric population.

Physical Activity and Cardiovascular disease

Intervention studies to date have primarily focussed on protection of CVD risk through physical activity (PA) engagement and improvements in dietary habits (Andersen et al., 2006). Evidence has shown that increases in PA reduce the clustering of traditional CVD risk factors in children and adolescents such as

insulin insensitivity, BP, glucose, triglycerides (TAG) and cholesterol levels, independently of sedentary time (Ekelund et al., 2012). Despite the strong evidence for PA promotion and efforts to increase participation, UK data shows that only one fifth of youth are meeting the current PA recommendations for health (Hallal et al., 2012), and that these levels decline from childhood into adolescence (Townsend et al., 2015). It would therefore seem that adolescents are not performing enough PA to protect themselves from risk factors of CVD. This, combined with poor dietary habits, could be having detrimental effects on their overall CVD risk profile, with the World Health Organisation (2011) stating that unhealthy diet and a lack of PA are the leading global risks to health. Given that efforts to increase PA in children have been unsuccessful (Metcalf et al., 2012), investigation into nutritional interventions in this population is of clear importance.

Diet and Cardiovascular disease

The presence of CVD risk factors such as high BP, obesity and dyslipidaemia, have been associated with diet (Raitakari et al., 2003). Evidence shows that adolescents with a high-quality diet that is low in saturated fats, salt and added sugars have a lower risk of developing CVD risk factors and CVD in adulthood (Dahm et al., 2016). Impaired glucose tolerance, insulin resistance, increased BP and dyslipidaemia strongly predict the risk of CVD, T2DM and subclinical atherosclerosis in later adulthood, and are highly related to diet, independent of obesity (Yajnik et al., 2015). There is strong evidence on the importance of a healthy diet, with data demonstrating a reduction in CVD risk factors by one third when meeting dietary guidelines in youth (British Nutrition Foundation, 2016). Diet in youth is of particular importance given evidence that health and dietary behaviours established in early life track into adulthood (Kelder et al., 1994). This

occurs alongside the tracking of CVD risk factors from childhood to adulthood (Morrison et al., 2007). It would thus seem that interventions to promote healthy dietary behaviours should be established in youth.

Dietary fat and dyslipidaemia:

Dietary recommendations with regards to CVD prevention have historically focussed on the consumption of dietary fats, particularly saturated fats, because of their implication on TAG and cholesterol levels. Guidelines by the National Cholesterol Education Programme (NCEP) for normal and at-risk children recommend an intake of total fats be limited to 30% of total calories, with saturated fat limited to 7 to 10%. Following these guidelines has been shown to result in reductions in TAG levels in healthy adolescents and children (NCEP, 1992). This is important as increased fasting TAG concentrations have been positively associated with elevated CVD risk (Austin et al., 1998). Although this may be a useful risk factor in the detection of CVD risk, postprandial measures have been suggested as a more powerful measure of CVD risk (Patsch et al., 1992). In addition to this, postprandial TAG responses in adolescence is associated with CVD events in the fourth and fifth decades of life (Morrison et al., 2009), with postprandial hyperlipidaemia promoting transient endothelial dysfunction and oxidative stress (Bae et al., 2001). Elevations in fasting and postprandial TAG levels have been traditionally linked to high intake of dietary fat, though recent evidence indicates that added sugars may have a role in elevating postprandial and fasting TAG concentrations, particularly following sugar sweetened beverage (SSB) consumption (Stanhope et al., 2015). More research into SSB consumption and the metabolic consequences may be of importance for postprandial health, which is shown to be associated with CVD risk (Burns et al., 2012; Morrison et al., 2009).

Sugar consumption:

The consumption of SSBs has come under scrutiny for its potential role in CVD progression. SSBs include, but are not limited to, sodas, fruit juice, and sports and energy drinks. SSBs are comprised of different types of sugars, termed sugar moiety. The consumption of such drinks has increased in both the USA and Europe over the last three decades (Nielsen & Popkin, 2004). This is problematic, especially given that associations have been made with SSB intake and increased CVD risk factors present at levels far below current consumption levels in US children (Vos et al., 2017). Despite American Heart Association (AHA) statements to reduce the intake of added sugars, with recommendations that adolescents consume less than 25 g of added sugars a day, adolescents in England consume 210 g of SSBs daily (Public Health England, 2013-2014). Adolescents appear an important target group for interventions aiming to reduce SSB consumption, since they consume 60% more calories from SSBs than children (Public Health England, 2013-2014). In addition, these statistics are from dietary self-report data, and therefore estimates may be conservatively low in comparison to actual intake levels, since self-reported dietary assessments often underreport dietary intake (Freedman et al., 2014; Trumbo et al., 2002). Although this consumption problem of SSBs is predominantly in adolescence, this problem tracks from childhood to adulthood, with early introduction of added sugars in the diet of infants and toddlers thought to promote sweet taste preference later in life (Morrison et al., 2012). Therefore, SSBs likely establish a future dietary preference for sweet things in early life.

Sugar intake and cardiovascular disease risk:

It is well established that adolescents consume substantially more than the recommended guidelines for daily sugar intake (Reedy et al., 2014),

predominantly through SSBs. However, limited data are available regarding the effects of SSB consumption in these years on CVD risk factors (Chan et al., 2014). Existing data demonstrates that increased habitual sugar intake is positively associated with increased risk factors such as adiposity, dyslipidaemia, elevated BP and diabetes in adolescents (Vos et al., 2017). Cross-sectional evidence has also demonstrated that each additional SSB consumed daily by youth is associated with a 5% increase in insulin resistance, a 0.2 mmHg increase in systolic BP, a 0.47 cm increase in waist circumference, a 0.9 percentile increase in BMI for age, and a 0.48 mg/dL decrease in high density lipoprotein cholesterol (HDL-C) concentrations (Bremer et al., 2009).

Habitual consumption of SSB induces frequent episodes of acute hyperglycaemia, which is associated with increased risk of CVD risk factors such as obesity, T2DM (Imamura et al., 2015) and development of the metabolic syndrome (Huang et al, 2014). Recent cross-sectional data suggests that increases in TAG and uric acid concentrations are also evident following consumption of SSBs, which are shown to be associated with increased CVD risk (Vos et al., 2017), as demonstrated in Figure 1.2.

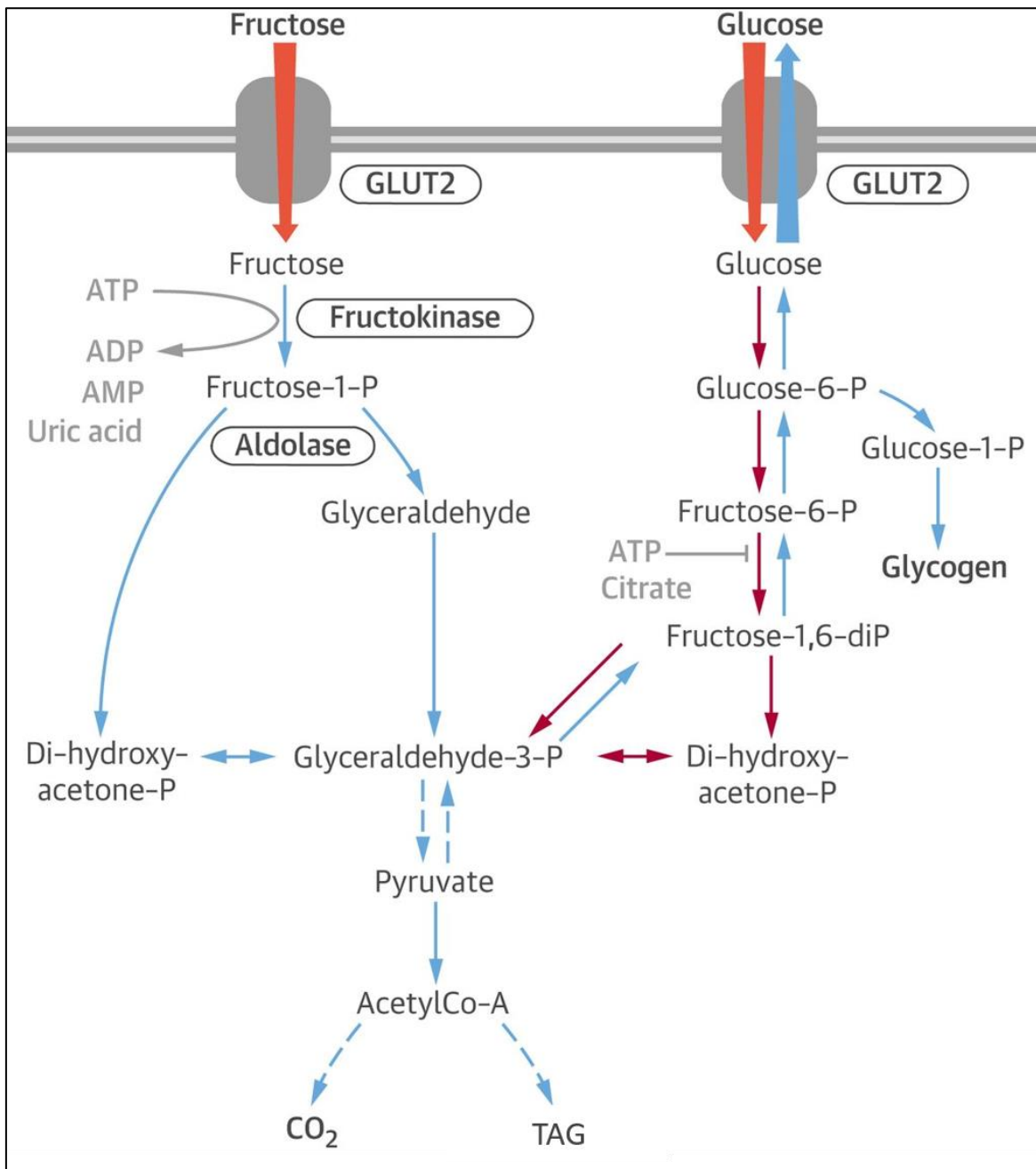


Figure 1.2. Metabolism of SSB consumption demonstrating the differential mechanisms underlying fructose and glucose metabolism in the liver. Fructose metabolism depicted by the red arrows, differs from glucose (blue arrows) due to nearly complete hepatic extraction different enzyme and reactions for its initial metabolic steps. Fructose taken up by the liver can be oxidized to CO₂ and then converted into lactate and glucose; glucose and lactate are subsequently either released into the circulation for extrahepatic metabolism or converted into hepatic glycogen or fat. The massive uptake and phosphorylation of fructose in the liver can lead to a large depletion of ATP to AMP and uric acid. AcetylCo-A = acetyl coenzyme A; ADP = adenosine diphosphate; AMP = adenosine monophosphate; ATP = adenosine triphosphate; diP = diphosphate; P = phosphate; TAG = triglyceride.

Despite limited research on the metabolic consequences of SSB intake in youth, evidence from cross-sectional and longitudinal studies demonstrates lowered

TAG concentrations in children with low consumption of added sugars (Vos et al., 2017). Research has shown that fasting TAG concentrations in youth are related to future atherosclerosis (Raitakari et al., 2003), with postprandial TAG concentrations significantly associated with CVD events in the fourth and fifth decades of life (Morrison et al., 2009).

Increases in uric acid may be associated with CVD risk, with evidence indicating a strong positive association between uric acid concentrations and systolic BP, independently of obesity (Jalal et al., 2015; Vos et al., 2017). Data in adolescents with a high habitual SSB intake have also demonstrated elevated uric acid levels and increased systolic BP (Nguyen et al., 2009). Data from the Bogalusa Heart study offers further evidence for the link between uric acid and CVD risk, demonstrating uric acid levels in youth are predictive of future hypertension in adulthood (Alper et al., 2005).

It is apparent that there is an association between SSB intake and some traditional CVD risk factors, however, there is a lack of evidence on the relationship between SSB intake and endothelial function.

The role of different types of sugars

A criticism of the limited existing literature on the effects of SSBs on endothelial function is that these have often focused solely on a glucose load. The main sugars in SSBs are sucrose or high fructose containing sugars. Sucrose is a disaccharide made of the monosaccharides glucose and fructose in equal proportion. These have unique metabolic pathways in the body and may therefore have different effects on endothelial function and CVD risk.

Glucose metabolism

Repeated exposure to hyperglycaemia following glucose consumption has been implicated in the progression of endothelial dysfunction and CVD risk factors (Reusch & Wang, 2011). Acute hyperglycaemia as a result of an oral glucose tolerance test or consumption of a SSB has been associated with transient endothelial dysfunction due to increased oxidative stress and reduced nitric oxide bioavailability (Kawano et al., 1999; Tominaga et al., 1999). The high glycaemic load presented by glucose ingestion may lead to β -cell dysfunction and insulin resistance (Malik et al., 2010), also implicated in CVD progression.

Fructose metabolism

Fructose metabolism differs to glucose metabolism in two main ways: via complete hepatic extraction of fructose, and via different enzymatic reactions. Fructose is absorbed into the portal vein and is metabolised in the liver where it is converted into fructose-1-phosphate. Further metabolic reactions result in the production of glyceraldehyde, which is metabolised by many different pathways (seen in Figure 1.2), yielding end products of glucose, glycogen, CO₂, lactate and fatty acids. This occurs independently of insulin secretion. These metabolic pathways result in production of hepatic TAG via very low-density lipoproteins (VLDL) from *de novo* lipogenesis (Malik & Hu, 2015). As a result of this, a high intake of fructose (generally accepted as 10-25% of total energy) can promote elevated fasting TAG concentrations, and significant increases in postprandial TAG, which is not seen following glucose ingestion (Stanhope et al., 2011). This may be detrimental to vascular health, with evidence linking postprandial hypertriglyceridemia with the atherosclerotic process (Hyson et al., 2003; Karpe, 1999; Nordestgaard et al., 2007) resulting in impaired FMD in adults and children (Bae et al., 2001).

In addition to elevated TAG concentrations following fructose metabolism, the phosphorylation of fructose in the liver leads to increases in uric acid production. This is a result of ATP depletion, and has been shown to induce metabolic complications and promote an atherogenic environment (Malik & Hu, 2015). Fructose consumption may be associated with reduced endothelial function alongside increased CVD risk, through a reduction in endothelial nitric oxide bioavailability and increased inflammation, with elevated uric acid thought to be a contributing mechanism (Nakagawa et al., 2006; Roglans et al., 2007).

Sucrose metabolism

Sucrose is the most common form of dietary sugar and is referred to as “table sugar”. As a disaccharide, sucrose is digested into its component monosaccharides of both glucose and fructose via the enzyme sucrase, which are metabolised separately. These combined responses may together result in elevated blood glucose and insulin concentrations (from glucose metabolism), alongside elevated TAG and uric acid concentrations (from fructose metabolism). Therefore, it could be suggested that sucrose may have the most negative effect on cardiovascular function, through the combined consequences of glucose and fructose metabolism. This has not been previously investigated, and given the high quantities of sucrose consumption, clearly warrants research in this area.

Critique of available studies on sugar sweetened beverage consumption

Currently, there is no data on the effects of SSB consumption on CVR, though a recent meta-analysis has highlighted the effects of hyperglycaemia following SSB consumption on peripheral endothelial function. This review reported impairments in peripheral endothelial function based on 39 studies, hypothesised to be due to increased oxidative stress and reduced nitric oxide bioavailability (Loader et al., 2015). Only three of these studies, however, were conducted in a

paediatric population, with one study in adolescents with type 1 diabetes (Dye et al., 2012) and one study in obese adolescents (Dengel et al., 2007). Previous research in healthy adolescents is therefore limited to a single study investigating the effects of a 75 g glucose drink on endothelial function in healthy and overweight adolescents (Dengel et al., 2007). This study reported no effect of the glucose SSB on peripheral macrovascular function (FMD), though the dose and sugar provided are not representative of a commercially available SSB, typically containing 60 g of sucrose. However, recent evidence in a rat model suggests that the type of sugar may have implications for acute changes in endothelial function, with negative effects on blood vessel function seen following fructose, but not glucose, ingestion (Sanguesa et al., 2017). Recent observations in humans support this, demonstrating increased fasting plasma insulin and glucose, as well as increased *de novo* lipogenesis and adiposity in individuals who habitually consumed fructose SSBs compared to subjects who consumed glucose SSBs (Stanhope et al., 2009). Research should therefore focus on investigating the acute effects of these different sugars to explore the relationship between SSB consumption and CVD risk. It is not known whether the combined or independent effects of these sugars alter CVD risk (Baena et al., 2016).

Taken collectively, there is a paucity of data on the acute endothelial response following sugar consumption, and in particular, in healthy adolescents. This is important as the endothelial response to sugar consumption in healthy adolescents is not well established, and may differ from that observed in healthy adults. Furthermore, no studies exist examining the effects of SSB consumption on CVR, which is shown to share the same nitric-oxide dependent pathway as peripheral measures (Lavi et al., 2006).

Postprandial lipaemia following sugar sweetened beverage consumption

In addition to the mechanisms of fructose metabolism having the potential to cause acute endothelial dysfunction, there is also evidence that fructose is responsible for an exaggerated postprandial lipaemic response following a high fat meal (Cohen & Schall, 1988). This is important given evidence that elevations in postprandial TAG concentrations in adolescents are associated with future CVD events and an increased CVD risk (Morrison et al., 2009). This offers one possible mechanism through which SSB consumption is related to CVD risk. Exaggerated postprandial hyperlipidaemia may result in transient endothelial dysfunction and oxidative stress, which if repeated frequently could contribute to chronic endothelial dysfunction.

Thesis Aims

There are three aims to this thesis. The first aim of this thesis is to examine the within and between-day reliability of the primary outcome, cerebrovascular reactivity. The experimental aims of this study are to investigate: 1) The acute impact of sugar moiety in SSBs (glucose, fructose, sucrose) on cerebrovascular function in adolescents, and 2) The impact of sugar moiety on postprandial cerebrovascular function and metabolic blood outcomes in adolescents.

The following research questions and hypotheses will be addressed:

- 1.) What is the best way to analyse and administer a breath-hold protocol for measures of CVR?
- 2.) What is the within and between-day reliability of CVR via a breath-hold stimulus? It was hypothesised that there will be an appropriate level of reliability for measures of within and between-day CVR.

- 3.) What are the acute effects of sugar moiety on CVR and metabolic blood outcomes? It was hypothesised that different types of sugars found in SSBs (sucrose, fructose, glucose) will impair CVR when compared to water.
- 4.) What are the postprandial effects of sugar moiety on CVR and metabolic blood outcomes? It was hypothesised that intake of fructose will result in an elevated postprandial lipaemic and uric acid response with impaired CVR.

CHAPTER 2: General Methods

General experimental procedures

Ethics and informed consent

Ethics for this study was approved by the Sport and Health Sciences Ethics Committee (171206/B/07), University of Exeter, prior to the onset of data collection. All participants were provided with participant information sheets, outlining the study aims, experimental procedures, and potential benefits and risks of taking part in the study. Following this, participants and their parents were given a week-long period to ask any questions/clarify their understanding and decide if they wanted to take part in the study.

As participants were minors under the age of 18, bespoke considerations included requirement for parents/guardians to fill in an informed consent form and participants to complete an assent form (Jago et al., 2011). Consent and assent forms highlighted participants' right to withdraw from the study at any point without consequence. Participants were also required to fill out a health screening form with help of their guardians, and a contact details form. During all experimental procedures, health and safety guidelines established in the Sport and Health Science department were abided by and all researchers were Disclosure and Baring Service (DBS) checked, with two DBS checked adults present at all times throughout the experimental visits and driving of participants to and from the school. Emergency contact details were also kept on hand at all times and researchers were first aid trained.

Participants

Sample size for the study was estimated using G* Power (3.9.1.2) calculation, based upon a power of 80%, alpha level of 0.05, repeatability of peripheral

vascular function (FMD) of 0.78 (Bond et al., 2015) and a partial eta squared (η_p^2) effect size of 0.05 (moderate effect), a sample size of 32 participants was required for this study. This effect size was based on a moderate effect size which we have based directly upon previous research using measures of peripheral vascular function (Loader et al., 2015). This systematic review examining the effects of hyperglycaemia on vascular function (Loader et al, 2015) found a large effect size. However, this effect was predominantly based on a wide population of adult studies, with only three studies in adolescents, and only one study with healthy adolescents examining the effect of glucose on vascular function (Dengel et al. 2007). As adolescents are characterised by augmented vascular function compared to adults this study was powered to detect a moderate effect size.

All participants were recruited from a local school in Devon. After initial discussion with the teachers describing the study requirements, potential participants were approached through an assembly talk to ~ 400 year nine students (aged 13-14 years), outlining the study aims and procedures and what was required during participation in this project. After the assembly, students were given the opportunity to ask any further questions, and potential participants who were interested in taking part were provided with a study information pack (~100 participants) and asked to discuss the project with their guardians. Of the 100 information packs handed out, 31 packs were returned, indicating a desire to take part in the study. From these 31, three participants withdrew and four participants were excluded due to school behaviour issues, with a further two not included due to time restraints or no longer wanting to take part in the study. Adolescents who agreed to take part were asked to return all completed forms to a designated school contact. Following return of these forms, parents/guardians were then contacted to discuss any further questions and ensure they agreed to all study

procedures and arrangements. Participants were recruited onto the study if they were ostensibly healthy and aged between 12-15 years. Participants were excluded from the study if they had any known cardiometabolic diseases, such as diabetes, or contraindications to exercise, such as recent injury or illness, or the use of any supplement or medication known to influence blood vessel function, glucose or fat metabolism. Participants with food allergies to the test meal were also excluded from the study, as well as any participants who did not understand the protocol.

Experimental overview

All participants completed five visits to the laboratory in total, one preliminary familiarisation visit, and four subsequent experimental visits over a six-week period. Each visit was separated by approximately one week. On all visits to the laboratory, participants were instructed to attend the laboratory in a 12 hour fasted state, having not performed any vigorous exercise in the 24 hours preceding each experimental visit. The study followed a double-blinded, repeated measures design, in order to compare the effects of (1) glucose (GLU), (2) fructose (FRU), (3) Sucrose (SUC), on vascular function compared to a control condition of (4) Water (CON), both before and after a high fat, high sugar challenge meal. All sugar conditions were representative of the amount of sugar in a typical SSB containing 60 g of sugar (e.g. Coca-Cola®) mixed with 300 mL of water

Visit 1: Preliminary measures

Prior to the experimental visits, all participants completed a preliminary visit in order to familiarise them to the testing procedures and for measurement of descriptive variables including cardiorespiratory fitness and anthropometric measures.

Participants were collected from school and transported to the laboratory by car following a 12 hour overnight fast. Body mass (Hampel XWM-150K, Hampel Electronics Co. Taiwan) and stature (Seca stadiometer SEC-225, Seca, Hamburg, Germany) were measured to the nearest 0.1 kg and 0.1 cm, respectively, using standard procedures. Body mass index (BMI) was calculated using the following equation:

$$\text{Equation 1: BMI} = \frac{\text{weight (kg)}}{\text{height}^2 (\text{m}^2)}$$

Centiles for overweight and obesity thresholds were used to define body weight status (Cole, Bellizzi, Flegal, & Dietz, 2000).

Percentage body fat was measured using the gold standard (Lowry & Tomiyama, 2015) method of air displacement plethysmography (BodPod®, Body Composition System, Life Measurement Instruments, Concord, California, USA). Prior to testing, the system was calibrated following the manufacturer's guidelines using a cylinder of known volume (49.887 L). Participants were required to wear a swimming costume and cap before being instructed to remain still in the chamber to calculate body volume. This measurement was taken twice and if the difference between the first two measures was more than ± 75 mL, a third measurement was taken. The mean of the two closest measurements was then used for calculating body density. Lung volume was estimated using age and sex specific prediction equations provided as part of the software, and used to estimate body composition using the Siri equation (Siri, 1993). Maturity status was determined for each participant by self-assessment of secondary sex characteristics according to the five stages of pubic hair development (Morris & Udry, 1980).

Following anthropometric measures, participants were habituated to the cerebrovascular measure and familiarised with all testing procedures. Participants were coached to perform a breath-hold on their preliminary visit as part of the CVR protocol, with the breath-hold following a normal inspiration. Participants were then required to complete a ramp incremental cycle test (Lode, Excaliber Sport, Groninger, The Netherlands) to exhaustion to determine their maximal oxygen consumption ($\dot{V}O_{2max}$) (Barker et al., 2011). Age- and sex-appropriate $\dot{V}O_{2max}$ (Adegboye et al. 2011) cut points for increased cardiometabolic risk were used to define fitness and characterise the sample. The test consisted of 3 minutes of unloaded pedalling followed by an incremental ramp rate of 25 W.min⁻¹, during which participants were required to maintain a cadence of 70-80 revolutions per minute (rpm). Exhaustion was defined as the point where participants experienced a drop in cadence below 60 rpm for 5 consecutive seconds, despite strong verbal encouragement. Beat-to-beat heart rate was measured (Polar M400, Polar Electro, Finland) throughout the ramp test, with maximal heart rate taken as the peak heart rate obtained during the ramp test. Ventilation and gas exchange variables were monitored using a metabolic cart (MedGraphics, UK, Ltd), which was calibrated prior to each measurement using standard calibration gas (15.1% O₂, 5% CO₂) and a 3.0 L calibration syringe (Hans Rudolph, USA). Peak power output was defined as the highest work rate achieved during the ramp test. $\dot{V}O_{2peak}$ was determined as the highest 10 second average in $\dot{V}O_2$ during the ramp test. Although no supramaximal verification test was used in the current study, the ramp test to exhaustion is known to provide a 'true' $\dot{V}O_{2max}$ measure in ~90% of adolescent participants in our laboratory (Barker et al., 2011).

Visit 2-5: Experimental visits

An overview of the experimental protocol is given in Figure 2.1. Following a 12 hour overnight fast, participants were collected from school and driven to the laboratory for 08:00 am. Participants rested in the supine position in a darkened and temperature-controlled room for 30 minutes before a baseline measurement of CVR was taken, and a fasting capillary blood sample collected. Following the first blood sample, participants were given 10 minutes to consume one of the drink conditions (GLU, FRU, SUC or CON). All sugar drinks consisted of 60 g of either GLU, SUC or FRU mixed with 300 mL of water, in order to replicate a commercially available SSB. The control condition involved consumption of 300 mL of water. Blood samples for glucose and uric acid were taken at 30 minute intervals for the following two hours, with the final blood sample also analysed for TAG. Sixty minutes following drink consumption, CVR was reassessed. 120 minutes following drink consumption, participants consumed a mixed meal tolerance test (MMTT, providing 60 g fat, 45 g of sugar, 1316 kcal), consisting of pizza (Chicago Town®, four cheese pizza) (310 g), ice cream (Essential Waitrose®, soft scoop vanilla ice cream) (125 g) and a chocolate pudding (Cadburys®, milk chocolate sticky puds) (95 g). The macronutrient composition of the MMTT has previously been shown to impair vascular function in adolescents (Bond et al., 2015c). Capillary blood samples were assessed at 60 minute intervals during the three hour postprandial period for glucose and uric acid, with the final blood sample also analysed for TAG. Three hours following the MMTT, measures of CVR were repeated to coincide with the elevation in TAG and the fall in peripheral vascular function reported previously in adolescents (Bond et al., 2015c). Participants consumed no other food during the experimental visits and were required to remain inactive throughout.

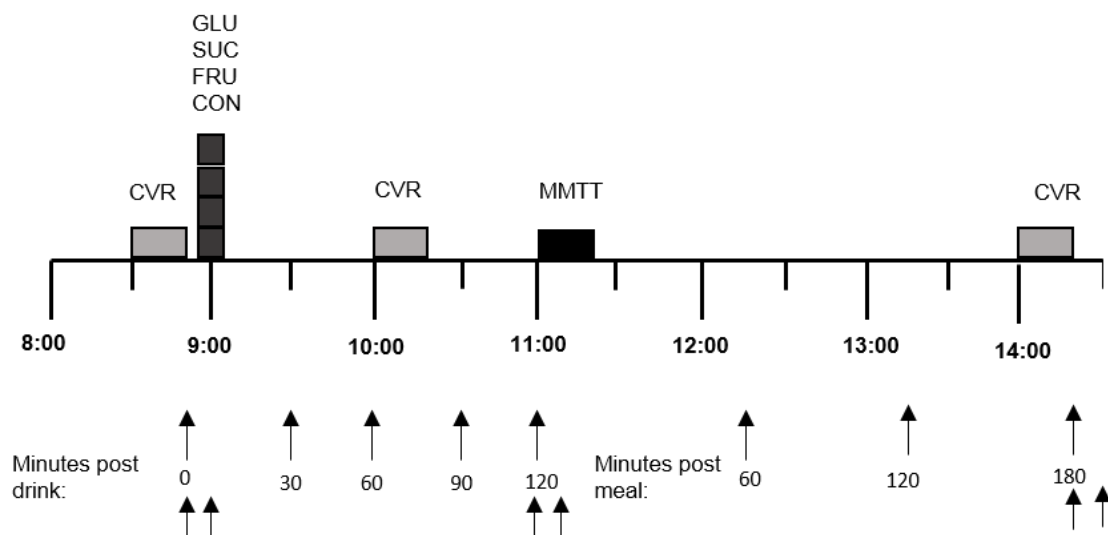


Figure 2.1. Protocol schematic for the four experimental visits which participants completed. The single arrows represent collection of capillary blood samples for plasma glucose and uric acid. The double arrows represent addition blood samples for triglyceride. CVR = cerebrovascular reactivity; MMTT = mixed meal tolerance test; GLU = glucose, SUC = sucrose, FRU = fructose, CON = water.

Experimental measures:

Maturity status

Maturity status was determined for each participant using self-assessment according to the five stages of development for secondary sex characteristics (Tanner, 1962). Following a verbal explanation, pubertal stage was determined via self-assessment of pubic hair development. This required participants to look at scientific drawings depicting five stages of development of pubic hair, and identify and circle the stage which best described their development. This self-assessment method was chosen because of its simplicity and practicality, along with being used as a valid method in the paediatric literature, with correlations of ≥ 0.6 with physicians observations (Morris & Udry, 1980).

Blood outcomes

For each capillary blood sample, ~ 600 μL of blood was collected into lithium-heparin coated (TAG) and heparin-fluorine coated (glucose) Microvettes (CB 300

Tubes, Saerstedt, Ltd, Leicester, UK). All samples were centrifuged immediately at 13,000 *g* for 15 minutes. Plasma was then aliquoted and either stored at -80 degrees Celsius for TAG analysis or analysed immediately from a single sample for glucose (YSI 2300 Stat Plus Glucose analyser, Yellow Springs, OH, USA). Plasma TAG was quantified in duplicate by enzymatic, calorimetric methods using an assay kit according to the manufacturer's guidelines (Cayman Chemical Company, MI, USA). The coefficient of variation (CV) for the inter error assay plasma TAG was 5.8%. Uric acid was analysed in capillary whole blood from a single sample using a portable uric acid meter (UASure, Apex Biotechnology Corp., Hsinchu, Taiwan). The intra-assay coefficients of variation (CVs) of the UASure® have been reported as 4.8% at UA levels of 5.8 mg/dl. The uric acid concentrations tested by this portable meter are strongly correlated with invasive venous sampling methods ($r=0.87$, $P<0.001$) (Kuo et al., 2002). The time points for the collection of each blood outcome are summarised in Table 2.1.

Table 3.1. Capillary blood analysis for each experimental visit.

	Acute SSB observation				MMTT observation				
Minutes	0	30	60	90	120 (0)	60	120	180	Total samples
Glucose	X	X	X	X	X	X	X	X	8
Uric acid	X	X	X	X	X	X	X	X	8
TAG	X				X			X	3

SSB = Sugar sweetened beverage; MMTT = Mixed meal tolerance test

Cerebrovascular reactivity

CVR was determined as the increase in cerebral blood flow velocity in the middle cerebral artery (MCA) by transcranial Doppler ultrasonography using a 2 MHz pulsed Doppler ultrasound system (DWL ®, Doppler-Box™X, Compumedics, Germany). Insonation of the MCA was initiated at a depth of ~ 50 mm, and then

optimised prior to locking the probe in place using a size adjustable headset for unilateral measurement of the MCA (DWL ®, DiaMon ®, Compumedics, Germany, GmbH). Attempts were made to ensure replication of the same insonation angle for within-day measurements. The headset was not kept on during within-day measurements as this resulted in movement and poorer replication of the angle of insonation. However, by keeping the same adjustments of the headset and attachment arm to the probe, and using an anatomical marker to mark the position of the probe, this resulted in improved replication of the same angle of insonation. Beat-by-beat mean blood flow velocity of the MCA ($MCAV_{mean}$) was exported for analysis as the primary outcome. Participants wore a leak-free facemask (Hans Rudolph, Kansas City, USA) during the protocol to sample end-tidal CO_2 ($P_{ET}CO_2$) through a gas analyser (ADInstruments, Gas analyser, ML206, Colorado Springs, CO, USA). Beat-by-beat BP was continuously measured by finger plethysmography (Finometer PRO, Netherlands). All data were acquired continuously at 200 Hz using an analog-to-digital converter (Powerlab; model - 8/30, ADInstruments, Colorado Springs, CO, USA) interfaced with a laptop computer. The input from the gas analyser was time shifted by minus 2 seconds to account for a time delay due to the length and diameter of the sample. Data were stored at 200 Hz for subsequent analysis using commercially available software (Lab Chart version 8, ADInstruments).

Baseline $MCAV_{mean}$ readings were obtained over a one-minute period and then averaged. Participants then performed a maximal breath-hold for up to 30 seconds following a normal inspiration. Participants were asked to avoid a Valsalva manoeuvre, with this protocol being practiced in visit 1 to coach participants how to perform the breath-hold. A one-minute recovery period followed the breath-hold. This whole protocol consisting of baseline-breath-hold-

recovery (as seen in Figure 2.2) was repeated three times (Bright & Murphy, 2013). The protocol to assess CVR lasted 7 minutes 30 seconds. CVR was determined as the greatest increase in mean $MCA_{V_{mean}}$ in the 10 seconds following the breath-hold, expressed as the percentage increase from baseline $MCA_{V_{mean}}$, for each of the three breath-holds. CVR was then taken as an average of the CVR% for each of the three breath-hold attempts. MAP was recorded, and the change from baseline during the last five seconds of the breath-hold was calculated, to account for the presence of any Valsalva manoeuvre. This increase was analysed visually by two researchers, and if MAP was substantially elevated (>15 mmHg) following the breath-hold, this breath-hold was removed. The within-day reliability of our CVR% was calculated using the data pre and post drink from the water condition and yielded a CV of 10.8%. Between-day reliability was calculated from the baseline scans of the different conditions, yielding a CV of 15.3% (See Chapter 3).

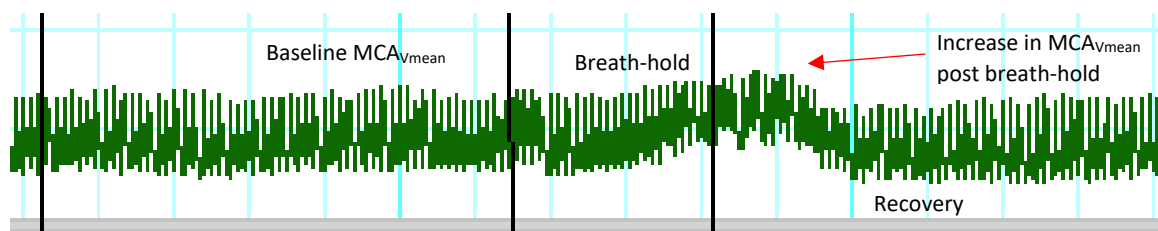


Figure 2.2. Cerebral blood flow of the middle cerebral artery (MCA) during one cycle of the breath-hold protocol, demonstrating the increase in $MCA_{V_{mean}}$ following maximal breath-hold following a normal inspiration.

Beat by beat BP was monitored (Finometer PRO, Netherlands) throughout the breath-hold test. A BP cuff was placed on the finger (finger plethysmography) and held at chest height throughout recording during the cerebrovascular measurement. Brachial BP was measured to calibrate the Finometer fingertip BP measurement (Guelen et al., 2008), which has been validated in paediatric groups (Tanaka et al., 1994).

Statistical analyses:

Statistical analyses were conducted using SPSS (version 25, Chicago, USA) and data are presented as a mean \pm SD. Statistical significance was accepted at an alpha 0.05. For the experimental trials, analysis of baseline and peak $MCAV_{mean}$, CVR, plasma TAG and glucose concentrations and blood uric acid concentrations were performed using a repeated measures ANOVA with condition (GLU, SUC, CON, FRU) and time (Baseline, Post Drink, Post meal) as the main effects. Total area under the curve (tAUC) and incremental area under the curve (iAUC) analyses were used to characterise metabolic outcomes of glucose and uric acid responses following both the drink and the MMTT. The tAUC and iAUC analyses were performed using the time point immediately before the drink for the acute response, and the time point immediately before the MMTT for the postprandial response. All AUC analyses were calculated using the trapezoid rule (GraphPad Prism, GraphPad Software, San Diego, CA) and mean differences between conditions analysed using one-way repeated measures ANOVA. Homogeneity of variance was determined using the Mauchly's test of sphericity and the degrees of freedom were corrected using the Greenhouse-Geisser correction if required. Effect Sizes for the ANOVA model were displayed as partial eta squared (η_p^2), and interpreted as <0.06 = small, $0.06-0.14$ = moderate and ≥ 0.14 = large. In order to locate significant differences between conditions, post hoc analyses were run as pairwise comparisons between means and interpreted using the P value and standardised effect sizes (d) to document the magnitude of the effect using the following thresholds: small (0.2), moderate (0.5), and large (0.8) (Cohen, 1992).

For the reliability trials, parameters of CVR (Baseline $MCAV_{mean}$, CVR%, BHI, breath-hold duration, time to peak), MAP and End-tidal CO_2 ($P_{ET}CO_2$) were

initially analysed using a mixed model ANOVA with visit (between-day) or assessment (within-day) as the main effects. Effect Sizes for the ANOVA model were displayed as partial eta squared (η_p^2) using the previously stated thresholds. The reproducibility of these outcomes was explored using the typical error (TE), the typical error expressed as a coefficient of variation (CV) and Pearson's correlation coefficient (r) or intra class correlation coefficient (ICC) (Hopkins, 2000).

CHAPTER 3: The reliability of a breath-hold protocol to determine cerebrovascular reactivity of the middle cerebral artery in adolescents

ABSTRACT

Purpose: Impairments in cerebrovascular function are present in adolescents with cardiovascular disease risk factors. The breath-hold method is proposed as an easy to administer and non-invasive method of assessing CVR in youth, yet there are no data on the reliability of this outcome in a paediatric population. This study aimed to identify the within-day and between-day reliability of a breath-hold protocol to measure CVR in adolescents. *Methods:* Twenty-one 12-15 year olds visited the laboratory in a fasted state on two separate occasions, within a six week period. CVR was assessed non-invasively via a breath-hold protocol to quantify changes in MCA blood flow velocity via transcranial Doppler ultrasonography. For within-day reliability, participants repeated the breath-hold protocol 60 minutes later. CVR was then calculated in two ways for subsequent analyses; as the percentage increase in mean MCA velocity ($MCAV_{mean}$) from baseline to peak following breath-hold, or as breath-hold index (BHI), where this value was normalised for breath-hold length. *Results:* The within and between-day coefficients of variation for CVR outcomes were as follows: Baseline $MCAV$: 4.5% and 6.6%, peak $MCAV$: 5.8% and 7.6%, CVR%: 10.8% and 15.3%, BHI: 14.0% and 12.5%, respectively. *Conclusions:* CVR assessed via a simple breath-hold protocol can be reliably measured in adolescents, yielding similar within and between-day reliability. In the present study it was demonstrated that breath-hold length and CVR% were unrelated and therefore BHI was deemed an unnecessary analysis.

Key words: endothelial function, reproducibility, transcranial Doppler ultrasonography, cerebral blood flow, adolescents

INTRODUCTION

Although clinically overt CVD is not typically apparent until adulthood, the atherosclerotic manifestations of the disease processes originate in childhood (Stary, 1989). An impairment in endothelial function is a sentinel event in the progression of atherosclerosis and a prerequisite to structural changes to the vessel wall (Juonala et al., 2004). Consequently, the ability to non-invasively determine endothelial function in paediatric groups is important to develop strategies aimed at the primary prevention of CVD.

Previous paediatric research has predominantly focussed on FMD as a measure of endothelial function in the peripheral vasculature (Bond et al., 2015; Hopkins et al., 2012). In recent adult and paediatric studies, however, there has been a growing interest in cerebrovascular in both research and clinical settings (Ainslie & McManus, 2016; Willie et al., 2011). CVR provides a direct measure of the cerebrovasculature, offering additional insight to peripheral measures, and shares a common nitric-oxide dependent pathway with systemic endothelial function (Ainslie et al., 2008; Lavi et al., 2006). CVR in adults is associated with Alzheimer's disease (Keage et al., 2012), neurocognitive decline (Wong et al., 2016), stroke (Silvestrini et al., 2000; Yonas et al., 1993) and independently predicts future CVD events in patients with CVD risk factors (Markus & Cullinane, 2001). Impairments in CVR are present in youth with CVD risk factors, such as hypertension (Lande et al., 2012; Settakis et al., 2002) and white coat hypertension (Páll et al., 2011), supporting its sensitivity to risk factor status.

Endothelial function of the cerebrovasculature can be determined via transcranial Doppler ultrasonography, measuring the reactivity of the MCA to a hypo/hypercapnic stimulus. CVR refers to the ability to regulate cerebral blood flow in response to a vasoactive or vasodilatory stimulus. A hypercapnic stimulus

causes the cerebrovascular vessels surrounding the MCA to dilate in response to changes in the partial pressure of CO₂. CO₂ breathing was used as the original hypercapnic stimulus (Kety & Schmidt, 1948), however a more simplified, cheaper and easier to administer method of a breath-hold has been introduced. This method delivers a hypercapnic stimulus through a simple breath-hold, with changes in arterial CO₂ concentrations shown to reflect two thirds of the CVR response in adults, with one quarter attributed to increases in mean arterial pressure (MAP) (Przybylowski et al., 2003). CVR determined from the breath-hold test correlates highly ($r=0.67$, $p<0.001$) with CO₂ induced CVR in adults (Markus & Harrison, 1992).

Given the easy to administer and non-invasive protocol of the breath-hold, with clinical applications of CVR in early risk factor detection, this method may have merit for intervention studies in children and adolescents to assess CVR. Before this can be made possible, protocol methods need to be made explicitly clear as current adult studies are difficult to interpret due to differing methods of assessing breath-hold induced CVR. Furthermore, there are no data available on the reliability of determining CVR via the breath-hold test in a paediatric population.

Breath-hold induced CVR is commonly assessed using the parameter of the breath-hold index (BHI) (Markus & Harrison, 1992; Müller et al., 1995). This index is defined as the maximum percentage increase in cerebral blood flow velocity divided by the breath-hold length. The BHI has been reported to have appropriate short-term reliability in some adult studies, with an intra class correlation coefficient of 0.41 to 0.50 (Totaro et al., 1999). However, its between-day (24 hour) reliability is poor, with an intraclass correlation coefficient of 0.17 (Totaro et al., 1999). This questions the appropriateness of the BHI as a measure of CVR for studies involving multiple visits on separate days. Furthermore, no studies

have explored the relationship between breath-hold length and increase in $MCAV_{mean}$, to determine the validity of normalising increases in $MCAV_{mean}$ to breath-hold length. There is a lack of detail on the reliability of factors related to the hypercapnic stimulus and breath-hold execution, such as the breath-hold length, partial pressures of CO_2 (reflected as End-Tidal CO_2 ($P_{ET}CO_2$)) and BP during the breath-hold protocol. Consequently, it may be important to measure these factors to assess test compliance, to examine if changes are consistent between and within individuals, as differences have been shown to directly affect vessel reactivity (Willie et al., 2011).

In addition to a lack of evidence examining the reliability of the breath-hold test as a measure of CVR in youth, previous work has also failed to identify the most reliable method of analysis of the $MCAV$ response within a test protocol. There are discrepancies between studies in whether they report CVR as a BHI (Müller et al., 1995) or a CVR% (Settakias et al., 2002). In addition, there is a lack of standardisation in the number of breath-holds performed, ranging from six (Bright & Murphy, 2013) to two (Markus & Harrison, 1992), and it is often unclear how these are averaged. Many studies also fail to report when the peak $MCAV_{mean}$ is recorded following the breath-hold (Markus & Harrison, 1992; Przybylowski et al., 2003), whilst others record the percentage increase during the breath-hold (Silvestrini et al., 2000). In order to determine how best to administer and analyse CVR, measures of within-test reliability are needed.

The purpose of this study was to identify the within-test, and within and between-day reliability of a CVR breath-hold protocol in an adolescent population consisting of three breath-hold attempts. This study will also identify the different analysis outcomes used in order to improve the reliability of the breath-hold test

to determine CVR in youth, and establish the most reliable method of analysis of CVR.

METHODS

Participants

Twenty-one 12 to 15 year olds volunteered to take part in this study, with a mean \pm SD age, height and body weight of 14.4 ± 0.4 years, 164.5 ± 8.2 cm and 58.8 ± 11.0 kg, respectively. Participant assent and parental consent were obtained prior to participation in the study, for which ethical approval was obtained from the University of Exeter Sport and Health Sciences Ethics committee (171206/B/07). These participants were recruited as part of the study in Chapter 4 with this chapter derived from the baseline and control day of this experiment. One participant was removed from analyses due to an inability to regularly perform the breath-holds without a Valsalva manoeuvre.

Experimental procedures

Participants visited the laboratory a total of three times, with visits two and three as experimental visits, separated by ~ 1 week. Visit one involved collection of participant descriptive data, as well as to provide a familiarisation to the testing procedures.

Visit 1

Participants were transported to the laboratory by car, following a 12 hour overnight fast. Body mass, stature, percentage body fat, maturity status and BMI status were measured as outlined in Chapter 2.

Following anthropometric measures, participants then completed a maximal ramp-incremental test to exhaustion on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, the Netherlands) to determine their peak

$\dot{V}O_{2peak}$. Age and sex specific $\dot{V}O_2$ cut points were used to characterise the sample for increased cardiometabolic risk (Adegboye et al. 2011).

Visit 2 and 3

Participants completed two experimental visits to the laboratory, separated by approximately one week, for assessment of CVR. The visits were a part of a larger trial in which four visits were completed in a randomised order, of which the control condition was used as the within-test and within-day reliability for the present study. With parental supervision, participants were asked to replicate their evening meal prior to each laboratory visit, and avoid any vigorous exercise during the 24 hours preceding each laboratory visit.

Following a 12 hour overnight fast, participants were transported by car to the laboratory for 08:00. Participants then rested in a darkened and temperature-controlled room in the supine position for 30 minutes prior to assessment of CVR. Subsequently, CVR was assessed as described in Chapter 2.

To examine the within-day reliability of CVR, participants repeated these measures 60 minutes following their first assessment, after the consumption of 300 mL of water. During this 60 minutes, participants consumed no food and were required to remain inactive throughout, permitted to sit at a desk and work, watch TV or play board games. Participants were allowed to drink water *ad libitum* during all visits.

Assessment of cerebrovascular function

CVR was assessed as described in Chapter 2. Data from the three breath-holds were used to identify the within-test reproducibility, and how to appropriately analyse the CVR outcome. Analysis of within-test outcomes from the three

breath-holds was used to inform the analysis of the within and between-day CVR analysis.

End-Tidal CO₂ (P_{ET}CO₂) was measured following the breath-hold, as a surrogate measure of arterial partial pressures of CO₂, to reflect the changes in CO₂ at the end of the breath-hold. Participants were required to wear a leak-free facemask (Hans Ruolph, Kansas City, USA) during the protocol in order to sample P_{ET}CO₂ through a gas analyser, which was calibrated via known concentrations of O₂ and CO₂ (15.1%, 5.0%, respectively) (ADInstruments, Gas analyser, ML206, Colorado Springs, CO, USA). During the protocol, beat-by-beat BP was non-invasively measured (Finometer PRO, Netherlands). MAP was recorded, and the change from baseline during the last five seconds of the breath-hold was calculated, to account for the presence of any Valsalva manoeuvre. This increase was analysed visually by two researchers, and if MAP was substantially elevated (>15 mmHg) following the breath-hold, this breath-hold was removed.

Data analyses

To explore changes in the ratio between MAP and MCAV_{mean}, the cerebrovascular conductance index (CVCi) and cerebrovascular resistance index (CVRi) were calculated as follows:

$$\text{CVCi} = \text{MCAV}_{\text{mean}}/\text{MAP}$$

$$\text{CVRi} = \text{MAP}/\text{MCAV}_{\text{mean}}$$

Where MCAV_{mean} and MAP are taken as the average during the baseline preceding each breath-hold attempt.

Statistical analyses were conducted using SPSS (version 25, Chicago, USA) and data are presented as a mean ± SD. Statistical significance was accepted at an

alpha 0.05. Parameters of CVR (Baseline and peak $MCAV_{mean}$, CVR%, BHI, breath-hold duration, time to peak), MAP and P_{ETCO_2} were initially analysed using a mixed model ANOVA with visit (between-day) or assessment (within-day) as the main effects. For within-test data, the relationship between mean breath-hold length and CVR% was explored using Pearson's correlation. Effect sizes for the ANOVA model were displayed as partial eta squared (η_p^2), and interpreted as <0.06 = small, $0.06-0.14$ = moderate and >0.14 = large effect size. For within-test analyses where three breath-holds were analysed, significant difference between breath-hold attempts were located using pairwise comparisons and interpreted using the P-value and standardised effect sizes (d) to document the magnitude of the effect using the following thresholds: <0.5 = small, <0.8 = moderate and ≥ 0.8 = large (Cohen, 1992). The reproducibility of these outcomes was explored using the typical error (TE), the TE expressed as a CV and intraclass correlation coefficient for within-test analyses, and Pearson's correlation coefficient (r) for within and between-day analyses (Hopkins, 2000)

RESULTS

Participant characteristics

Participant characteristics are presented in Table 3.1. The maturation status for boys ($n = 11$) and girls ($n = 10$) is as follows: (Stage 2: $n=2$, Stage 3: $n=2$, Stage 4: $n=15$, Stage 5: $n=2$).

Table 3.1. Participant characteristics

	Mean (\pm SD)	Range
Age (y)	14.3 (\pm 0.4)	13.7-15.3
Body Mass (kg)	55.1 (\pm 11.0)	34.0-73.9
Stature (cm)	164.5 (\pm 8.2)	149.0-183.5
BMI (kg/m ²)	16.7 (\pm 2.8)	11.2-22.2
Body fat (%)	22.0 (\pm 9.0)	6.9-36.2
$\dot{V}O_{2\text{ peak}}$ (L.min ⁻¹)	2.1 (\pm 0.6)	0.5-3.4
$\dot{V}O_{2\text{ peak}}$ (mL.min ⁻¹ .kg ⁻¹)	40 (\pm 9)	10-51
Fasting plasma TAG (mmol.L ⁻¹)	0.63 (\pm 0.04)	0.19-1.46
Fasting plasma glucose (mmol.L ⁻¹)	4.67 (\pm 0.11)	2.14-6.27
Fasting plasma uric acid (μ mol.L ⁻¹)	5.28 (\pm 0.14)	3.00-9.20

Results expressed as mean \pm SD. BMI, body mass index; $\dot{V}O_2$, oxygen uptake; TAG, triglyceride.

Within-test reliability

The within test reproducibility for parameters of interest are presented in Table 3.2. Baseline $MCA_{V_{\text{mean}}}$ declined across the three breath-holds ($P=0.002$, $\eta p^2=0.29$), with a significantly lower baseline $MCA_{V_{\text{mean}}}$ in breath-hold 3 than 1 ($P=0.001$, $d=0.4$) and 2 ($P=0.034$, $d=0.2$). Peak $MCA_{V_{\text{mean}}}$ systematically declined across the three breath-holds, with breath-hold 3 lower than 1 and 2 ($P<0.001$, $\eta p^2=0.27$; 1 vs 3: $P=0.003$ $d=0.3$, 2 vs 3: $P=0.02$ $d=0.2$). No significant mean differences were apparent between breath-holds for all other outcomes ($P>0.05$, $\eta p^2\leq 0.10$). Significant correlations were observed between breath-holds for all outcomes except time to peak ($0.64\leq r\leq 0.95$) ($p<0.01$). Mean breath-hold duration was not significantly correlated with CVR% ($r=0.35$, $P=0.13$). The TE expressed as a CV for all other outcomes ranged from 3.2 to 119.7%. These outcomes informed the analysis of breath-hold data for within and between-day analysis, with it deemed appropriate to take an average of the three breath-hold attempts within the protocol.

Table 3.2. Within-test reliability for outcomes of interest.

Variable	Breath-hold			Change in Mean		P	Typical error	CV (%)	r
	1	2	3	(1-2)	(2-3)				
Baseline MCA_{Vmean} (cm/s)	89.6 ± 14.9	86.9 ± 11.4	84.8 ± 12.5 *	-2.7	-2.1	0.002	0.28	4.6	0.92
Peak MCA_{Vmean} (cm/s)	130.5 ± 19.1	128.2 ± 17.6	124.5 ± 19.8 *	-2.3	-3.7	0.003	0.25	3.2	0.95
Recovery MCA_v mean (cm/s)	82.7 ± 13.2	81.11 ± 12.2	80.4 ± 11.2	-1.6	-0.8	0.14	0.28	3.9	0.93
BH length (s)	25.5 ± 4.8	26.0 ± 4.4	25.0 ± 5.3	0.5	-1.9	0.42	0.54	13.5	0.73
Time to peak (s)	4.7 ± 2.6	4.2 ± 2.9	3.9 ± 2.9	-0.5	-0.3	0.67	2.71	65.3	0.07
CVR (% increase)	46.7 ± 12.0	47.5 ± 11.5	47.4 ± 14.5	0.8	-0.1	0.88	0.49	15.2	0.77
BHI (s ⁻¹)	1.88 ± 0.48	1.85 ± 0.43	1.94 ± 0.60	-0.1	0.1	0.62	0.62	16.2	0.64
MAP baseline (mmHg)	81.47 ± 13.5	81.48 ± 14.8	81.47 ± 14.7	-0.01	-0.02	0.99	0.2	3.8	0.97
MAP Δ during BH (mmHg)	8.8 ± 8.6	9.9 ± 8.4	8.8 ± 8.6	1.2	-1.1	0.53	0.48	119.7	0.78
MAP peak (mmHg)	97.34 ± 10.9	97.79 ± 11.30	99.20 ± 10.93	0.45	1.42	0.46	0.4	4.4	0.84
End-Tidal CO ₂	40 ± (3.9)	39.8 ± 3.8	40.5 ± 4.7	-0.2	0.7	0.44	0.41	4.1	0.85
CVRi (mmHg cm s ⁻¹)	1.11 ± 0.2	0.96 ± 0.1	0.97 ± 0.2	-0.15	0.01	0.10	0.2	28.5	0.91
CVCi (cm s ⁻¹ mmHg ²)	0.94 ± 0.2	1.10 ± 0.2	1.07 ± 0.2)	0.14	-0.03	0.10	0.2	28.5	0.91

Data presented as mean ± SD. P-values indicate ANOVA main effect, with significant effects highlighted in **bold**. * indicates P<0.05 compared to other breath-holds. MCA_{Vmean} , mean middle cerebral artery velocity; BH, breath-hold; CVR, cerebrovascular reactivity; BHI, breath-hold index; MAP, mean arterial pressure, CO₂, carbon dioxide.

Within-day reliability

The within-day reliability for parameters of interest are presented in Table 3.3.

Between assessment 1 and 2, a significant decline in baseline (P=0.02, $\eta p^2=0.24$), peak (P=0.02, $\eta p^2=0.24$) and recovery (P=0.03, $\eta p^2=0.22$) MCA_{Vmean} was observed. P_{ETCO_2} also significantly declined between assessments (P=0.01, $\eta p^2=0.30$). No significant mean differences were apparent between assessments

1 and 2 for all other outcomes ($P \geq 0.12$, $\eta p^2 \leq 0.12$). Significant correlations were observed between assessments 1 and 2 for all outcomes ($0.71 < r < 0.92$) ($p < 0.01$). Typical error ranged from 2.1% to 14.0%, except for changes in MAP which had a CV of 150.7%.

Table 3.3. Within-day reliability for outcomes of interest.

Variable	Assessment 1	Assessment 2	Change in mean	P value	Typical error	CV (%)	r
Baseline MCA_{vmean} (cm/s)	85.9 ± 11.9	82.9 ± 13.7	-2.9	0.021	0.31	4.5	0.92
Peak MCA_{vmean} (cm/s)	126.8 ± 15.5	122.3 ± 21.0	-5.5	0.020	0.42	5.8	0.89
Recovery MCA_{vmean} (cm/s)	81.0 ± 11.6	77.7 ± 13.6	-3.3	0.027	0.38	5.7	0.89
BH length (s)	25.2 ± 4.3	26.0 ± 4.2	0.8	0.21	0.46	8.1	0.79
CVR (%)	47.3 ± 11.7	46.2 ± 10.4	-1.1	0.48	0.52	10.8	0.79
BHI (s^{-1})	1.9 ± 0.5	1.8 ± 0.4	-0.1	0.12	0.69	14.0	0.71
MAP baseline (mmHg)	81.5 ± 14.2	79.0 ± 12.1	-1.5	0.64	0.7	13.1	0.49
MAP Δ during BH (mmHg)	10.0 ± 6.7	9.1 ± 7.5	0.5	0.77	1.09	150.7	0.74
MAP peak (mmHg)	92.8 ± 16.0	91.2 ± 16.7	-1.9	0.58	0.9	14.8	0.57
End-Tidal CO_2	40.1 ± 3.9	39.3 ± 3.5	-0.2	0.01	0.24	2.1	0.95
CVRi (mmHg $cm s^{-1}$)	0.95 ± 0.2	0.98 ± 0.2	0.03	0.34	0.1	12.4	0.67
CVCi ($cm s^{-1} mmHg^2$)	1.08 ± 0.2	1.06 ± 0.2	-0.11	0.65	0.3	30.6	0.72

Bold indicates significant mean difference between assessment 1 and 2. Data presented as mean ± SD. MCA_{vmean} , mean middle cerebral artery velocity; BH, breath-hold; CVR, cerebrovascular reactivity; BHI, breath-hold index; MAP, mean arterial pressure, CO_2 , carbon dioxide.

Between-day reliability

The between-day reliability for parameters of interest are presented in Table 3.4. Significant mean differences were observed for BHI with a decline between assessments 1 and 2 ($P=0.005$, $\eta p^2=0.34$). No significant mean differences were apparent between assessments 1 and 2 for all other outcomes ($P > 0.11$, $\eta p^2 \leq 0.12$). Significant correlations were observed between assessments 1 and 2 for all variables ($0.48 < r < 0.83$; $p < 0.01$) except CVRi and CVCi. Typical error expressed as a CV ranged from 3.7% to 15.3%, with changes in MAP having a CV of 100.2%.

Table 3.4. Between-day reliability for parameters of interest.

Variable	Assessment 1	Assessment 2	Change in mean	P value	Typical error	Typical error as CV (%)	r
Baseline MCA_{Vmean} (cm/s)	84.1 ± 14.7	87.1 ± 12.0	3.0	0.11	0.43	6.6	0.83
Peak MCA_{Vmean} (cm/s)	125.9 ± 22.4	127.0 ± 17.5	1.1	0.73	0.50	7.6	0.78
Recovery MCA_{Vmean} (cm/s)	78.2 ± 14.8	80.8 ± 11.6	2.7	0.16	5.98	7.5	0.82
BH length (s)	24.2 ± 5.0	25.5 ± 4.6	1.3	0.11	0.51	11.5	0.74
CVR (%)	49.4 ± 12.0	46.3 ± 12.0	-3.1	0.17	0.74	15.3	0.64
BHI (s^{-1})	2.1 ± 0.5	1.9 ± 0.4	-0.2	0.005	0.61	12.5	0.74
MAP baseline (mmHg)	82.0 ± 13.5	85.4 ± 7.2	3.4	0.30	1.0	15.2	0.11
MAP Δ during BH (mmHg)	9.4 ± 9.4	8.0 ± 7.4	-1.5	0.45	6.20	100.2	0.48
MAP peak (mmHg)	96.4 ± 41	98.1 ± 10.2	1.7	0.59	1.12	12.9	0.46
End Tidal CO_2	39.1 ± 2.6	39.6 ± 3.2	0.6	0.23	0.49	3.7	0.78
CVRi ($mmHg.cm^{-1}$)	1.0 ± 0.2	1.0 ± 0.2	0.0	0.96	0.1	16	0.35
CVCi ($cm s^{-1}mmHg^2$)	1.0 ± 0.2	1.0 ± 0.2	0.0	0.72	0.2	16	0.39

Bold indicates significant mean difference between assessment 1 and 2. Data presented as mean ± SD. MCA_{Vmean} , mean middle cerebral artery velocity; BH, breath-hold; CVR, cerebrovascular reactivity; BHI, breath-hold index; MAP, mean arterial pressure, CO_2 , carbon dioxide.

DISCUSSION

The main findings of this study were that using the percentage increase in $MCAV_{mean}$ in the 10 seconds following the breath-hold was reliable within the three breath-holds performed in the protocol. Therefore, it was deemed appropriate to average the score from the three breath-hold attempts. Using these analyses, CVR% yielded similar levels of reliability within-day (10.8%) and between-day (15.3%).

Within-test

The most widely used outcome of reporting breath-hold induced CVR as a BHI, yielded a CV of 16.2% for within-test reliability. This is in line with a within-test CV of 11.4% previously reported in adults (Alwatban et al., 2018). Nevertheless, there are concerns with its application (Urback et al., 2017), as the relationship between breath-hold length and the $PaCO_2$ stimulus remains unclear, with the stimulus influenced by many other factors (Fierstra et al., 2013). This method was first employed to account for differences in breath-hold length and its possible influence on CVR (Markus & Harrison, 1992). This was considered to have merit in elderly patients who could not hold their breath for longer than 15 seconds (Settakias et al., 2002). The present study found that breath-hold length was not associated with the increase in $MCAV_{mean}$ ($r=0.35$, $P=0.13$), and therefore the normalisation of CVR% to breath-hold length appears unnecessary. This is in addition to evidence indicating that breath-hold length is not strongly related to changes in $PaCO_2$ following a breath-hold (Sasse et al., 1996). Collectively, these data indicate that it is not necessary to normalise the MCA response to breath-hold length, as it does not alter the vasoactive stimulus of increases in $PaCO_2$.

There is a lack of consistency in how previous studies have handled breath-hold data, and it is evident that standardisation of CVR is needed across the literature.

The time during the breath-hold protocol in which the increase in $MCA_{V_{mean}}$ is taken is inconsistent between studies, with some using the 4 seconds following the breath-hold (Markus & Harrison, 1992), and others analysing the peak during the breath-hold (Alwatban et al., 2018). In addition, some studies take the increase in $MCA_{V_{mean}}$ “immediately following” the breath-hold, though when this occurs has not been made clear (Settakias et al., 2002). The time taken to peak $MCA_{V_{mean}}$ was variable between (4.1 ± 1.8 seconds) and within ($CV = 65.3\%$) individuals in the present study. This finding indicates that using a predefined point of 4 seconds following the breath-hold is unlikely to capture the increase in $MCA_{V_{mean}}$ consistently within and between individuals. This could therefore underestimate CVR. In the present study, peak $MCA_{V_{mean}}$ always occurred in the 10 seconds following the breath-hold, which informed our subsequent analyses for within and between-day outcomes, and is therefore recommended for use in future work in this population.

It has not been made clear in previous studies whether breath-hold data are reported as an average across breath-holds, or whether the highest or lowest values have been reported or removed. In addition, it is not clear or consistent how many breath-holds are performed in study protocols with some reporting six breath-holds (Bright & Murphy, 2013) and others only two (Markus & Harrison, 1992) or three breath-holds (van Niftrik et al., 2016), whilst other fail to report this (Alwatban et al., 2018). From the three breath-hold protocol used in the current study, baseline and peak $MCA_{V_{mean}}$ systematically declined between breath-holds. However, when CVR was expressed as a percentage increase, there was no significant difference across breath-holds, with breath-hold one to three being strongly correlated ($r=0.77$) with a within participant CV of 15.2%. It therefore seems appropriate to take an average of the three breath-holds for analysis, and

also suggests that one breath-hold may be sufficient if utilised in a time sensitive protocol. When analysed by these methods, the proposed outcome of CVR% yields a marginally higher level of reliability, shown through a lower CV, than the original BHI method (CV = 15.3% vs 16.2%). These initial findings informed subsequent within and between-day analyses.

Within-day and between-day

Evidence of within and between-day reliability of breath-hold-induced CVR protocols is essential when conducting intervention and observational experiments. In this study, similarly to the within-test reproducibility, there was a systematic decline in baseline and peak $MCAV_{mean}$ from assessments 1 to 2. This is in line with the decline observed in P_{ETCO_2} , supporting suggestions that CVR is mostly related to changes in CO_2 (Fisher et al., 2017). Previous literature has reported variation in $MCAV_{mean}$ due to variations in MAP (Demolis, 1993). However, in the present study measures of CVC_i and CVR_i demonstrated no differences between assessments both within and between-days. This suggests that, although there was a high individual variation in MAP, when baseline MAP was accounted for, MAP did not influence the $MCAV_{mean}$ response. This lends supports to the use of a breath-hold protocol as a measure of CO_2 -induced vessel reactivity. The one hour within-day variation of $MCAV_{mean}$ and P_{ETCO_2} highlights the time sensitivity of this measure and the importance of conducting measures at the same time of day to minimise variation. Despite this, CVR was not significantly different within-day and evidenced a CV of 10.8%. This indicates that the responsiveness of the vessel is not altered through the day despite different baseline $MCAV_{mean}$. The reliability of CVR may be considered as acceptable when compared to the within-day CV following CO_2 breathing tests in adults ranging

from 4.8 to 40.6% (Goode et al., 2009; Leontiev & Buxton., 2007; Tancredi et al., 2015).

In the present study, between-day variability of CVR% was 15.3%, with the between day tests significantly largely correlated with each other ($r=0.64$, $P=0.002$) (Mukaka, 2012). This is consistent with CVR data from CO₂ breathing in adults, with a between-day intraclass CV of 0.73 (McDonnell et al., 2013). The magnitude of the change in MCAV_{mean} following the breath-hold stimulus (34-62%) is in line with previous reports of normal variation in a paediatric population between 40-69% (Settakris et al., 2002).

Variability in CVR between and within days could be attributed to a number of potential sources of error in the breath-hold method. It is important that the breath-hold is completed following a normal inspiration, as to standardise the PaCO₂ concentrations. It is also important to avoid a Valsalva manoeuvre throughout the protocol. For this reason, it is of interest to measure MAP simultaneously, to make attempts to assess compliance with the protocol and cooperation of the participant, particularly when working in a paediatric population. However, in this study and previous literature (Müller et al., 1995), it is evident that this protocol is well tolerated in youth. In the present study, MAP baseline and peak were reliable within a participant, both within (CV: Baseline=13.1% and Peak=14.8%) and between-day (CV: Baseline=15.2% and Peak=12.9%). However, the change in MAP during the breath-hold was highly variable with both within-day (CV=150.7%) and between-day (CV=100.1%). Although this variation is large, this is summative of the variation of MAP at both baseline and peak, and when expressed as a percentage this variability becomes amplified. Despite this seemingly large variation, there were no resultant changes in CVR, supporting these changes in MAP as being acceptable ranges and not

having an influence on the subsequent $MCAv_{mean}$ response. Measurement of both MAP and P_{ETCO_2} are of importance to ensure that any changes in CVR are attributable to changes in responsiveness in the blood vessel, and not breath-hold execution. In the current study, P_{ETCO_2} was reliable within a participant, both within-day (CV=2.1%) and between-day (CV=3.7%), and therefore any influence on the variability on outcomes of CVR is unlikely to be from variability in P_{ETCO_2} .

Conclusions

The present investigation sought to address the within-test reproducibility for the breath-hold protocol. Analyses revealed normalisation of the BHI was not statistically supported, as BH length and CVR% were unrelated in the present study. Within-test analyses indicated that CVR% was reproducible within a protocol, and thus it was deemed acceptable to average the outcome of the three breath-holds. Using these methods, this study addressed the within and between-day reliability of a single protocol to non-invasively measure cerebrovascular function at the MCA. The present study demonstrated that, when CVR was expressed as a percentage increase in $MCAv_{mean}$ in the 10 seconds following the breath-hold, it was a reliable method of assessing CVR in adolescents. Importantly, this supports the use of this outcome in future studies investigating changes in CVR that utilise measures between and within visits. Future analyses, however, need to be conducted to establish whether this outcome of CVR% correlates with other measures of peripheral vascular function such as flow mediated dilation (FMD), and furthermore, the associations with clinical outcomes to support this as a valuable predictor of future health outcomes.

CHAPTER 4: The acute and postprandial effects of sugar moiety on cerebrovascular function in adolescents

ABSTRACT:

Introduction: The process of CVD may originate in youth, with evidence indicating an association between SSB consumption and progression of CVD risk factors. This study aimed to investigate the effect of sugar moiety on cerebrovascular function in adolescents following a sugary drink and subsequent meal. *Methods:* Twenty one adolescents (14.3 ± 0.4 years) performed four conditions in a randomised order, consuming the following drinks on separate visits: (1) glucose (GLU); (2) fructose (FRU); (3) sucrose (SUC); and (4) water (control; CON). Cerebrovascular reactivity ($n=20$) (CVR) was measured using the breath-hold test via transcranial Doppler ultrasonography at baseline and 60 minutes following drink consumption, and 180 minutes following a mixed meal tolerance test (MMTT: 60 g fat, 45 g sugar). Capillary blood samples for glucose, uric acid and TAG were taken throughout. *Results:* CVR did not differ between conditions ($P=0.26$, $\eta_p^2=0.07$) or across time ($P=0.39$, $\eta_p^2=0.05$). Blood analyses for TAG revealed no significant condition x time interaction ($P=0.18$, $\eta_p^2=0.07$), with TAG elevated in all conditions following meal consumption ($P<0.01$, $d=2.0$). Blood glucose had a significant condition x time interaction effect ($P<0.001$, $\eta_p^2=0.59$), with blood glucose significantly elevated in GLU compared to all conditions at all time points following drink consumption ($P\leq 0.002$, $d \geq 0.8$). Post hoc analyses revealed that uric acid concentrations were elevated in FRU compared to all conditions 60 and 120 minutes following drink consumption ($P\leq 0.02$, $d=0.7$). *Conclusion:* No changes in CVR were present following SSB and MMTT consumption, despite sugar moiety resulting in different metabolic responses following SSB consumption.

Key words: sugar-sweetened beverage, endothelial function, cardiovascular health, acute, postprandial

INTRODUCTION

Although clinically overt CVD typically presents in adulthood, sub-clinical manifestations of the disease process occur in childhood (McGill et al., 2000), highlighting the importance of CVD risk factor modification in the first two decades of life. Children and adolescents who present with CVD risk factors have impaired endothelial function (Celermajer et al., 1992), which is a pre-requisite for structural changes to the vessel wall (Fernhall & Agiovlasitis, 2008). Previous studies examining endothelial function in youth have predominantly focused on peripheral arterial function via FMD (Celermajer et al., 1992). Recently, however, there has been growing interest in measuring cerebrovascular function, which has been shown to share the same nitric oxide-dependent pathway as FMD (Lavi et al., 2006). Evidence indicates that impairments in CVR are already seen in children who present CVD risk factors, such as hypertension (Lande et al., 2012). These findings highlight that exposure to CVD risk factors in youth may have deleterious consequences on the cerebrovasculature, which may have implications for future cerebrovascular disease and stroke (Keage et al., 2012; Silvestrini et al., 2000; Wong et al., 2016; Yonas et al., 1993).

The consumption of SSBs has received growing interest for their potential role in elevating CVD risk. Adolescents in the UK consume 60% more calories from SSBs than children, averaging 210 g of SSB intake per day (Public Health England, 2013-2014), exceeding the recommended maximum intake of 25 g of sugar per day. The consumption of SSBs is associated with increased CVD risk factors in youth, such as decreased insulin sensitivity (Basu et al., 2013),

hypertension (Chan et al., 2014a), dyslipidaemia (Vos et al., 2017) and future weight gain (Ludwig et al., 2001).

The influence of SSBs on CVR is currently unknown, though there are data on peripheral arterial function (Lavi et al., 2006). Data from a meta-analysis of 39 studies in healthy and diseased adults and paediatric groups demonstrated that hyperglycaemia following SSB consumption acutely impairs peripheral endothelial function, likely via increased oxidative stress and reduced nitric oxide bioavailability (Loader et al., 2015). Despite this finding, the three available studies in adolescents report no impairments in peripheral endothelial function following a glucose load in healthy or obese adolescents (Dengel et al., 2007), and adolescents with type 1 diabetes (Dye et al., 2012). However, a glucose load is not representative of typical SSBs, in which the main sugar is sucrose (constituting of equal parts glucose and fructose) or high fructose containing sugars. Unlike glucose, fructose is metabolised in the liver independently of insulin, which has been shown to increase *de novo* lipogenesis (Cohen & Schall, 1988) and uric acid concentrations (Malik & Hu, 2015). The different metabolic fate of fructose may play an integral role in the detrimental effects of SSB consumption and CVD risk (Malik & Hu, 2015). The impacts of fructose consumption on vascular function are currently unknown, with no study measuring CVR following a fructose load. It is also not known whether the combined or independent effects of these sugar moieties alter vascular function and CVD risk (Stanhope et al., 2009).

A limitation of previous studies investigating the effects of SSB consumption on cardiometabolic health in adolescents is the absence of postprandial measures, which may be more insightful and representative of day to day living (Morrison et al., 2009). Elevated TAG concentrations from *de novo* lipogenesis may increase

CVD risk, with evidence demonstrating that elevations in TAG coincide with acute impairments in peripheral endothelial function in youth (Bond et al., 2015a). Associations between TAG and future disease risk are supported by evidence showing that elevations in fasting and postprandial TAG in youth are an independent predictor of CVD outcomes later in life (Morrison et al., 2009). Furthermore, data demonstrates TAG as a strong CVD risk factor with postprandial TAG (and glucose concentrations) shown as more powerful predictors of CVD risk than fasting concentrations (Freiberg et al., 2008; Morrison et al., 2009). Elevated production of hepatic uric acid following fructose consumption may be associated with reduced endothelial function alongside increased CVD risk, through a reduction in endothelial nitric oxide bioavailability and increased inflammation (Nakagawa et al., 2006; Roglans et al., 2007).

The primary aim of this study was to investigate the effects of sugar moiety (sucrose, glucose, and fructose) on CVR and blood markers of glucose, TAG and uric acid concentrations. A secondary aim was to investigate whether the consumption of different types of sugar found in SSBs influences CVR and postprandial metabolic outcomes following a MMTT.

METHODOLOGY

Participants

Twenty one healthy 12 to 15-year old adolescents (11 males) took part in this study. Sample size was estimated based upon a power calculation (G*Power) based upon the primary outcome of CVR, and resulted in a sample size of 24 participants to detect a power of 80% ($\eta_p^2 = 0.12$; $\alpha = 0.05$). Participants were recruited from a local school in Devon, for which ethics approval was obtained from the Sport and Health Sciences Ethics Committee, University of Exeter

(171206/B/07). Exclusion criteria were any contraindications to exercise or use of any medication known to influence the study outcomes. Participants for this study were the same as those recruited in Chapter 3.

Experimental procedures

Participants completed a total of five visits to the laboratory over a six-week period, with each visit separated by approximately one week. Visit one was a preliminary visit, with visits 2-5 as experimental visits, completed in a randomised order. The four experimental visits consisted of a different drink to compare the effects of: (1) glucose (GLU); (2) fructose (FRU); (3) sucrose (SUC); and (4) water (control; CON) on CVR and metabolic outcomes of TAG, glucose and uric acid.

Visit 1: Preliminary anthropometric and familiarisation visit

Participants were collected from school and transported to the laboratory by car following a 12 hour overnight fast. Body mass, stature, percentage body fat, maturity status and BMI status were measured as outlined in Chapter 2.

Following anthropometric measures, participants were familiarised with all testing procedures. Participants then completed a maximal ramp-incremental test to exhaustion on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, the Netherlands) to determine their peak $\dot{V}O_{2\text{peak}}$ as outlined in Chapter 2.

Visits 2-5: Experimental visits

An overview of the experimental protocol is illustrated in Figure 4.1. Following a 12 hour overnight fast, participants were collected from school and driven to the laboratory for 08:00 am. Participants then completed all testing procedures as

outlined in Chapter 2 with measures of CVR taken 60 minutes post drink consumption and 180 minutes post meal consumption.

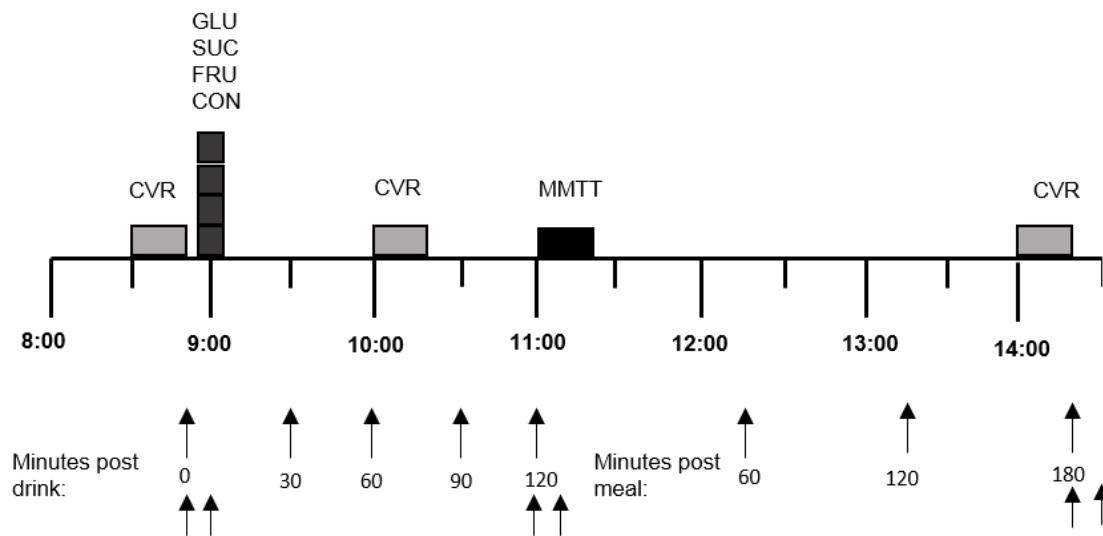


Figure 4.1. Protocol schematic for the four experimental visits. The single arrows represent collection of capillary blood samples for plasma glucose and uric acid. The double arrows represent addition blood samples for plasma triglyceride. CVR = cerebrovascular reactivity; MMTT = mixed meal tolerance test; GLU = glucose, SUC = sucrose, FRU = fructose, CON = control (water).

Cerebrovascular reactivity

CVR was determined as the increase in cerebral blood flow velocity in the MCA by transcranial Doppler ultrasonography using a 2 MHz pulsed Doppler ultrasound system (DWL®, Doppler-Box™X, Compumedics, Germany). CVR was assessed as outlined in Chapter 2.

The within-day reliability of CVR% was calculated using the data pre and post drink from the water condition and yielded a CV of 10.8% (see Chapter 3). Between-day reliability was calculated from the baseline scans of visits two and three and demonstrated a CV of 15.3% (See Chapter 3).

Blood outcomes

Collection of capillary blood samples and subsequent blood analyses were conducted as outlined in Chapter 2.

Total area under the curve (tAUC) and incremental area under the curve (iAUC) analyses were used to characterise metabolic outcomes of glucose and uric acid responses following both the drink and the MMTT. The tAUC and iAUC analyses were performed using the time point immediately before the drink to for the acute response, and the time point immediately before the MMTT for the postprandial response. All AUC analyses were calculated using the trapezoid rule (GraphPad Prism, GraphPad Software, San Diego, CA).

Statistical analyses

Statistical analyses were conducted using SPSS (version 25, Chicago, USA) and data are presented as a mean \pm SD. Analysis of baseline and peak MCAV_{mean}, CVR, plasma TAG and glucose concentrations and blood uric acid concentrations were performed using a repeated measures ANOVA with condition (GLU, SUC, CON, FRU) and time (baseline, post drink, post meal) as the main effects. Differences in the AUC responses for glucose and uric acid following each drink condition were explored using one-way repeated measures ANOVA. Homogeneity of variance was determined using the Mauchly's test of sphericity, with the Greenhouse-Geisser correction performed if required. Effect sizes for the ANOVA model were displayed as partial eta squared (η_p^2), and interpreted as <0.06 = small, <0.14 = moderate and ≥ 0.14 = large effect size. In order to locate significant differences between conditions, post hoc analyses were run as pairwise comparisons between means and interpreted using the P value and standardised effect sizes (d) to document the magnitude of the effect using the following thresholds: small (0.2), moderate (0.5), and large (0.8) (Cohen, 1992). Statistical significance was accepted at an alpha of $P < 0.05$.

RESULTS

Participant's descriptive characteristics are as presented in Chapter 3 (see Table 3.1). Pubertal status ranged from stage 2 to 5 (stage 2, n=2, stage 3: n=2 stage 4: n=15, stage 5: n=2). Participants were all defined as normal weight according to BMI centile classifications. According to VO_{2max} cut points 6 participants were classified as low fit (boys n=3), with the remaining (n=15) above the cut off for low fit. One participant was removed from the CVR analysis due to substantial Valsalva manoeuvre during the breath-hold protocol. Therefore, CVR data are presented with a sample of n=20.

Cerebrovascular function

Baseline and peak $MCAV_{mean}$ data and the CVR% response to the drink conditions and subsequent MMTT are shown in Table 3.5. Baseline $MCAV_{mean}$ did not differ between conditions ($P=0.26$, $\eta_p^2=0.07$). Similarly, peak $MCAV_{mean}$ following the breath-holds did not differ between conditions ($P=0.27$, $\eta_p^2=0.07$). There was a main effect of time for both baseline $MCAV_{mean}$ ($P=0.016$, $\eta_p^2=0.23$) and peak $MCAV_{mean}$ ($P=0.02$, $\eta_p^2=0.24$). Baseline $MCAV_{mean}$ was not significantly different from pre to post drink ($P=0.2$, $d=0.1$), but a decrease was evident from post drink to post MMTT ($P=0.05$, $d=0.3$). Peak $MCAV_{mean}$ significantly decreased pre to post drink ($P=0.005$, $d=0.1$) with no significant change from post drink to post MMTT ($P=0.07$, $d=0.4$). CVR% was not different between conditions ($p=0.84$, $\eta_p^2=0.01$) or time ($p=0.39$, $\eta_p^2=0.05$), and no condition by time interaction was present ($p=0.82$, $\eta_p^2=0.02$).

Table 4.5. Cerebrovascular function data at baseline and following drink conditions and subsequent meal consumption.

		Baseline (0 minutes)	Post drink (60 minutes)	Post MMTT (300 minutes)
Baseline MCAv_{mean} (cm/s)	Sucrose	86.1 (±12.0)	86.9 (±13.9)	82.2 (±9.9)
	Fructose	86.6 (±12.3)	83.0 (±10.0)	81.4 (±10.8)
	Water	86.3 (±11.7)	83.4 (±13.9)	80.7 (±10.2)
	Glucose	84.7 (14.0)	86.8 (±13.0)	85.4 (±10.4)
Peak MCAv_{mean} (cm/s)	Sucrose	126.5 (±21.6)	129.0 (±22.8)	121.5 (±18.3)
	Fructose	125.9 (±16.0)	122.5 (±17.1)	118.5 (±16.0)
	Water	125.6 (±15.8)	120.8 (±21.3)	118.1 (±17.7)
	Glucose	124.0 (±18.7)	125.6 (±18.5)	122.1 (±16.4)
CVR (%)	Sucrose	47.3 (±11.4)	47.5 (±12.6)	44.5 (±11.6)
	Fructose	45.6 (±10.8)	46.9 (±12.0)	45.3 (±9.4)
	Water	46.3 (±11.0)	44.5 (±7.0)	44.2 (±9.2)
	Glucose	46.5 (±8.4)	46.9 (±7.9)	44.8 (±9.6)

Data presented as mean (±SD) for Baseline and Peak MCAv mean and CVR%. MCAv, Middle cerebral artery velocity; CVR, cerebrovascular reactivity; MMTT, mixed meal tolerance test.

Blood outcomes

Plasma TAG, plasma glucose and whole blood uric acid concentrations are shown in Figure 4.2. Plasma TAG was not significantly different between conditions ($P=0.17$ $\eta_p^2=0.08$), nor was there a significant condition by time interaction effect (Figure 4.2A, $P=0.18$ $\eta_p^2=0.07$). However, there was a significant effect of time ($P<0.001$ $\eta_p^2=0.82$), with no change from baseline to post drink ($P=0.81$, $d=0.1$), but an increase from post drink to post MMTT ($P<0.01$, $d=2.0$).

There was a significant condition by time interaction for plasma glucose (Figure 4.2B, $p<0.001$ $\eta_p^2=0.59$). Post hoc analyses revealed that plasma glucose was higher in GLU than all other conditions at all time points post drink (30–120 minutes post drink consumption) ($P\leq 0.002$, $d\geq 0.8$). Plasma glucose was higher

in SUC compared to FRU 30 minutes following drink consumption ($P<0.001$) and CON ($P<0.001$, $d\geq 2.4$). SUC remained higher than CON at 60 minutes post drink consumption ($P=0.01$, $d=0.8$). Following the MMTT, plasma glucose for FRU, GLU and SUC were significantly elevated from the CON condition for the following two hours ($P<0.001$, $d\geq 0.6$), with no significant differences at 3 hours following MMTT consumption between any conditions.

The tAUC and iAUC for glucose are presented in Figure 4.3 for each condition following drink and meal consumption, separately. Post drink glucose tAUC (Figure 4.3A) was significantly greater following GLU (744 ± 64 mmol.L⁻¹.120min) compared to CON, FRU and SUC (573 ± 40 , 577 ± 60 and 664 ± 61 mmol.L⁻¹.120min, respectively, all $P<0.01$, $\eta_p^2\geq 0.68$). In addition, post drink glucose tAUC following SUC ingestion was significantly greater compared to CON and FRU ($P<0.01$). Post-drink iAUC (Figure 4.3B) was significantly lower following CON (20 ± 12 mmol.L⁻¹.120min) compared to GLU (186 ± 90 mmol.L⁻¹.120min), FRU (133 ± 76 mmol.L⁻¹.min) and SUC (150 ± 99 mmol.L⁻¹.120min, all $P<0.01$, $\eta_p^2>0.48$).

Post meal tAUC for glucose (Figure 4.3C) was significantly greater following CON (1013 ± 76 mmol.L⁻¹.180min) compared to GLU, FRU and SUC (961 ± 68 , 951 ± 70 and 946 ± 65 mmol.L⁻¹.180min, respectively, all $P<0.01$, $\eta_p^2>0.21$). Post meal glucose iAUC (Figure 4.3D) was significantly lower following FRU (63 ± 61 mmol.L⁻¹.180min) compared to CON, GLU and SUC drink conditions (172 ± 105 , 132 ± 67 and 120 ± 66 mmol.L⁻¹.180min, respectively, all $P<0.01$, $\eta_p^2>0.27$).

There was a significant condition by time interaction for blood uric acid concentrations following drink and MMTT consumption (Figure 4.2C, $p=0.03$, $\eta_p^2=0.10$). Post hoc analyses revealed that uric acid was elevated in FRU

compared to all other conditions 60 and 120 minutes following FRU consumption ($P \leq 0.02$, $d = 0.7$). Additionally, uric acid was greater in FRU compared to CON 30 minutes ($P = 0.009$, $d = 0.6$) and 90 minutes ($P = 0.002$, $d = 0.6$) following drink consumption. An elevated response with FRU consumption was evident compared to GLU 180 mins following drink consumption ($P < 0.001$, $d = 0.6$), and 180 minutes following MMTT ($P = 0.04$, $d = 0.5$). The FRU condition had a significantly elevated response compared to SUC 90 mins following drink consumption ($P = 0.003$, $d = 0.6$), and 60 and 120 minutes following MMTT ($P \leq 0.01$, $d \geq 0.4$). Figure 4.4 shows the total and incremental AUC for uric acid following the drink and MMTT in each condition. tAUC post drink and MMTT were not significantly different between conditions ($P = 0.11$ and 0.10 , respectively), neither was iAUC post drink ($P = 0.19$) or post MMTT ($P = 0.32$).

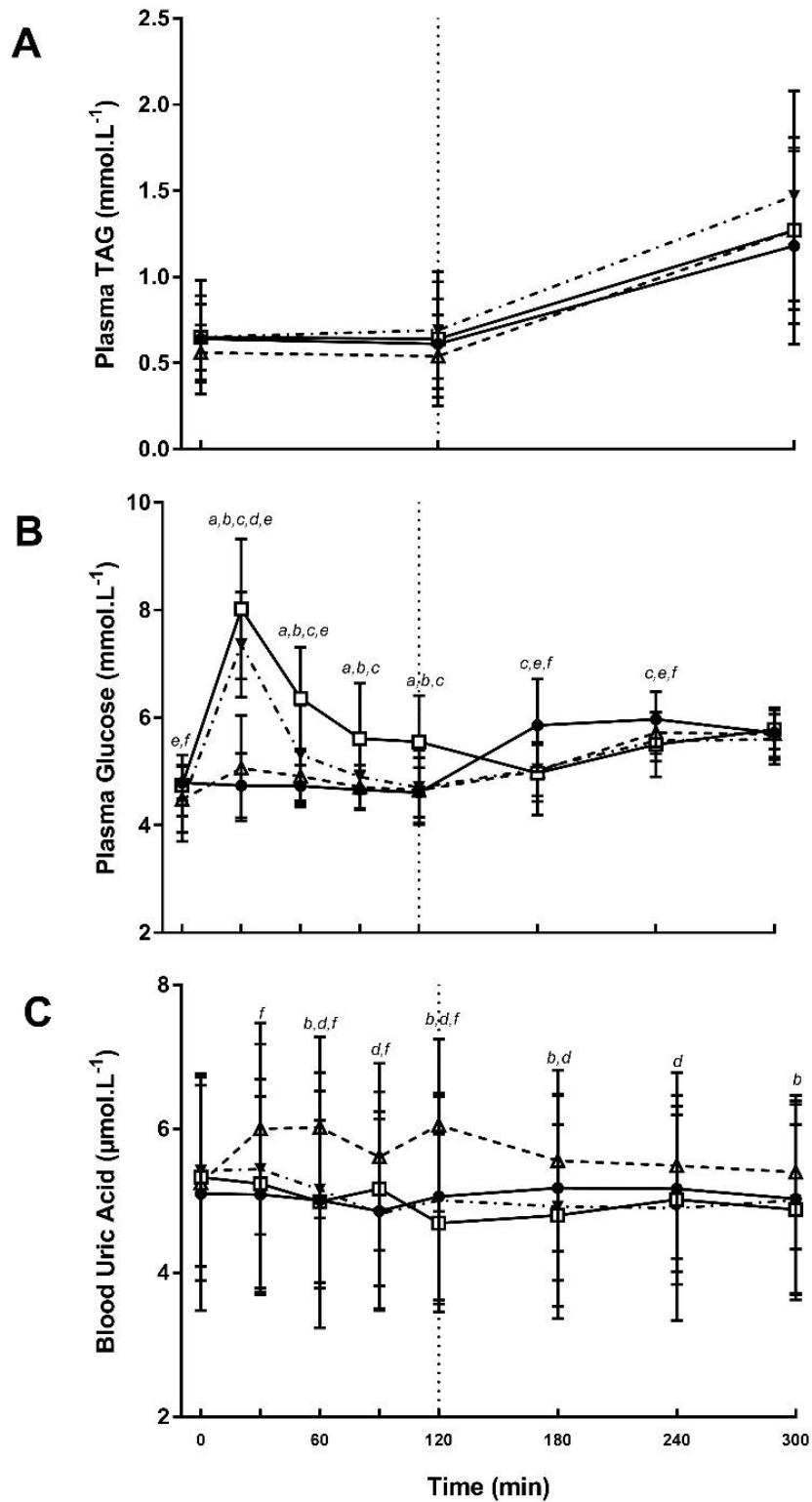


Figure 4.2. Plasma triacylglycerol (TAG) (A), plasma glucose (B) and uric acid (C) responses for each condition following drink consumption (0 min). Dashed line indicates mixed meal tolerance test. Data are shown as mean \pm SD.

▼ Sucrose ▲ Fructose ● Water □ Glucose

a, $P < 0.05$ GLU v SUC. b, $P < 0.05$ GLU v FRU. c, $P < 0.05$ GLU vs CON. d, $P < 0.05$ SUC vs FRU. e, $P < 0.05$ SUC vs CON. f, FRU vs CON

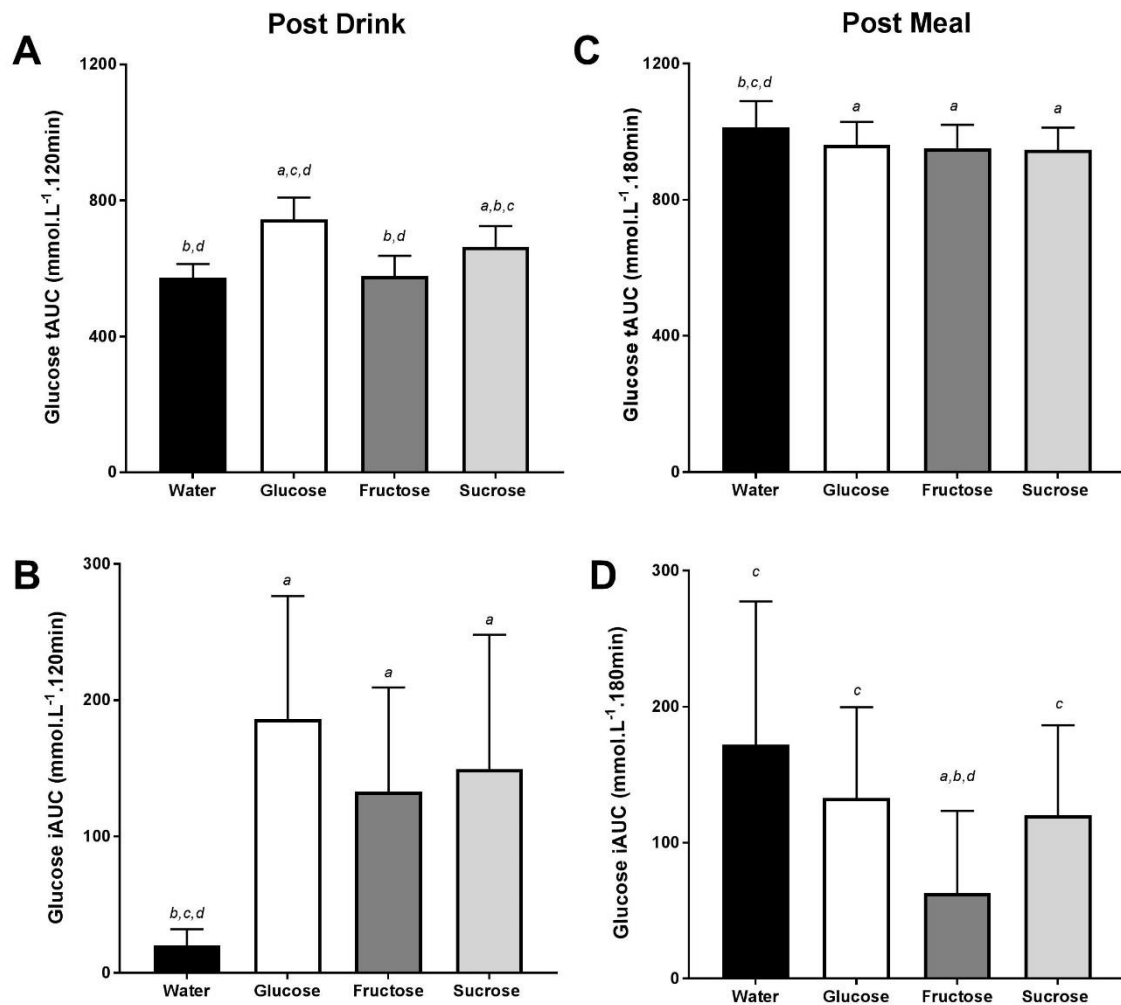


Figure 4.3. Total area under the curve (tAUC, A,C) and incremental area under the curve (iAUC, B,D) of plasma glucose following consumption of each different drink and subsequent MMTT. Data shown as mean \pm SD.

a, $P < 0.05$ vs water. b, $P < 0.05$ vs glucose. c, $P < 0.05$ vs fructose. d, $P < 0.05$ vs sucrose.

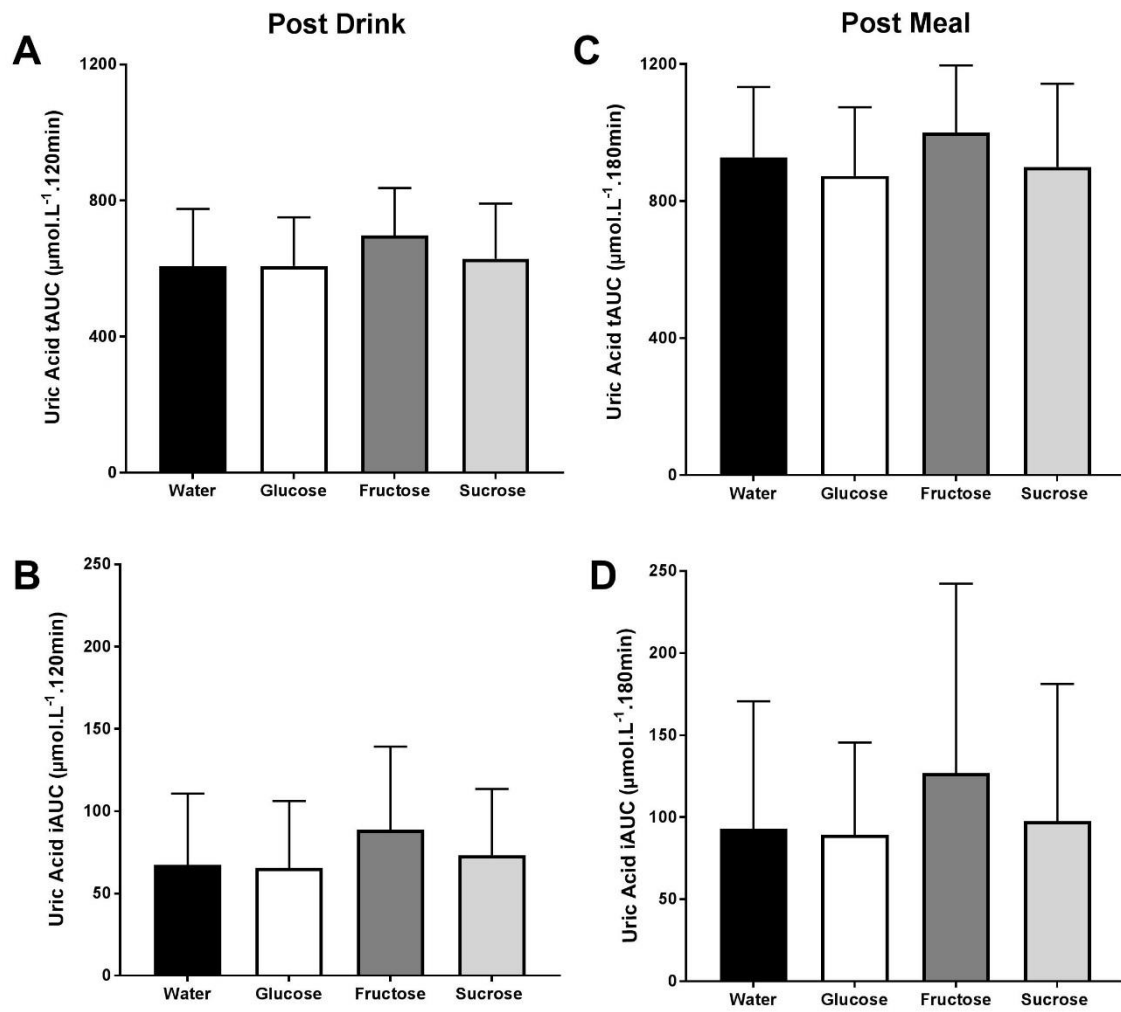


Figure 4.4. Total area under the curve (tAUC, A,C) and incremental area under the curve (iAUC, B,D) of blood uric acid following consumption of each different drink and subsequent MMTT. Data shown as mean \pm SD.

DISCUSSION:

The main finding of this study was that following consumption of 60 g of glucose, fructose or sucrose, CVR of the middle cerebral artery was preserved in a healthy adolescent population. CVR was also not influenced following a MMTT, despite elevations in TAG in all conditions, and elevations in uric acid following the fructose condition.

In support of the rationale, looking at the independent and combined effect of sugar moieties, the metabolic responses (blood glucose and uric acid) significantly differed following SUC, GLU and FRU consumption in the present study. Following the FRU condition, blood glucose concentration was not elevated and responded similarly to CON. The FRU condition produced an elevated uric acid response at all time points following drink consumption, however there were no observed differences between conditions for TAG levels, with similar increases following the MMTT in all conditions.

Hyperglycaemia following SSB consumption, as presented by the GLU and SUC conditions in the present study, have been associated with impaired endothelial function in previous studies in healthy and clinical populations (Loader et al., 2015). The systematic review of 39 studies found impaired peripheral macrovascular function in 30 studies, which may be attributed to increased oxidative stress and decreased nitric oxide bioavailability (Loader et al., 2015). In the present study, there were no effects on CVR following hyperglycaemia induced by SSB consumption (SUC or GLU). Although other studies have found impairments in endothelial function, these were present when measured using peripheral endothelial function (FMD) (Akbari et al., 1998; Kawano et al., 1999). It may be hypothesised that the contrasting results are due to this discrepancy in measurement of endothelial function. This is evidenced by data in an adolescent

population demonstrating impairments in FMD following a MMTT containing the same composition as the current study (Bond et al, 2015b). In the present study, however, there were no significant impairments in endothelial function measured by CVR following MMTT for any of the SSB drink conditions (SUC, FRU or GLUC), despite elevations in TAG. From this present investigation, it appears that the MCA endothelium in adolescents is preserved following the challenge presented by a high sugar and fat load.

The systematic review by Loader et al. (2015) included three studies in a paediatric population, with only one study including healthy adolescents (Dengel et al., 2007). The authors reported no differences in endothelial function measured using FMD following a 75 g glucose load, similarly to results from the present study using CVR. This suggests that endothelial function following a sugar load in adolescents is protected compared to adults, whether measured via CVR or FMD. This supports evidence demonstrating associations between measures of FMD and CVR (Lavi et al., 2006). Discrepancies between the present study and conclusions from Loader et al. (2015) may be hypothesised to be due to the differences in population, with majority of evidence from the systematic review focussing on an adult population (36 of the 39 studies).

There is conflicting evidence in the adult literature, with some studies reporting no effects of hyperglycaemia on endothelial function (Akbari et al., 1998; Kawano et al., 1999; Reed et al., 2004). Despite this, conclusions seem clear demonstrating acute impairments in endothelial function following SSB consumption in adults (Loader et al., 2015). In the present population, it was hypothesised that vascular function following SSB consumption declines over more prolonged periods and may not occur until later in life with repeated exposure to acute sugar loads. Therefore, this may need to be repeated over time

for any significant impairments in cerebrovascular function to be seen, reflective of a high habitual SSB intake. Evidence on the associations between habitual SSB consumption and cardiovascular health are clear, with a meta-analysis from 310,819 participants demonstrating that individuals in the highest quartile of SSB consumption (1-2 servings per day) had a 26% higher risk of developing T2DM than individuals in the lowest quartile (Malik et al., 2010). It is also known that individuals with T2DM have impaired vascular function (Shah & Urbina., 2017). This suggests that the metabolic responses observed in this study of hyperglycaemia and raised uric acid may be detrimental to health in adolescents, however it may be a cumulative effect over time rather than an acute response following one drink (60 g load) that impacts CVR.

Evidence from the systematic review by Loader et al., (2015) highlights that individuals who express reduced endothelial function following SSB consumption may have at risk CVD profiles, due to habitual intake of SSBs or metabolic complications such as obesity, impaired glucose tolerance or hypertension. On review of the evidence, it seems a much clearer conclusion that studies performed in clinical populations with obesity (Kawano et al., 1999; Lavi et al., 2009), Type 1 (Dye et al., 2012) and Type 2 (Ceriello et al., 2008) diabetes and hypertension (Zhang et al., 2012) consistently display impairments in endothelial function following hyperglycaemia from acute SSB ingestion. The sample in the present study were all normal weight (Cole et al., 2000), with fasting TAG below the 50th percentile (Tamir et al., 1981). Since the population were healthy and did not present early CVD risk factors, endothelial function would appear protected from acute dysfunction from hyperglycaemia or hyperuricemia in the present study. This further highlights that discrepancies in findings may not be due to the age of the population, but the presence of clinical CVD risk factors and early

manifestations of disease, which are likely elevated with age and disease progression. This however, remains a speculation until this protocol is repeated in at risk teenagers.

Previous studies have administered a glucose load, which is not representative of a SSB, which contains glucose and fructose in equal proportion. In the current study, the effect of fructose on CVR was similar to that of glucose, in that endothelial function was preserved. Fructose ingestion has been shown to contribute to increased CVD risk, associated with hepatic production of uric acid occurring within 30-60 minutes following oral ingestion (Stirpe et al., 1970). In the present study, uric acid concentrations were shown to be significantly elevated in the FRU condition, though this occurred in the absence of any changes in CVR. Post-drink and post-meal AUC responses for uric acid were not significantly different between drink conditions, suggesting the present study may not have delivered a large enough dose, or over a long enough time period, to influence CVR measured post meal and post drink.

Any negative impacts of fructose (or sucrose) consumption on endothelial function could be more closely associated with fasting and postprandial TAG concentrations than uric acid (Rutledge & Adeli, 2007). In addition, negative health implications of SSBs may be related to habitual intake over longer periods of time, with previous research demonstrating elevated fasting TAG levels associated with a high chronic intake of fructose-rich SSBs in adolescents (Chan et al., 2014b). Other studies investigating the metabolic impacts of fructose have administered two week trials or longer (Bantle et al., 2000). Although these studies did not measure CVR, they support suggestions that the effects of fructose and the potential mechanism of SSB consumption influencing CVR is a cumulative effect over time, rather than an acute response following one drink.

This is reinforced by evidence that SSB intake increases the risk of CVD-related complications such as hypertension, dyslipidaemia, inflammation, stroke and diabetes (Malik & Hu, 2015)

Considerations and limitations

A key strength of the present study is the replication of a typical SSB, containing 60 g of sucrose, providing ecological validity of the acute effects of SSB consumption. Furthermore, the present study compares the combined and independent effects of the sugars present in an SSB to determine which sugars influence CVR. The inclusion of a postprandial observation is of key importance in the present study, given that postprandial metabolic outcomes are more predictive of CVD risk than fasted concentrations (Morrison et al., 2009), as well as being representative of everyday life. This therefore provides insightful information and should be included in future research into the effects of SSB consumption. The present study also demonstrated a high level of reproducibility for the primary outcome of CVR for both within and between-day (See chapter 3).

However, it is important to consider the limitations of the present work. These include not accounting for participant's habitual sugar intake, with many studies to date focussing on adverse health outcomes with habitual SSB intake (Chan et al., 2014a). However, the present study controlled for any acute effects of diet, through the replication of participant's diet in the 24 hours preceding their experimental visits. The small sample size in the present study should be considered, with post-hoc power analyses based on the observed effect sizes of 0.12, and the repeatability of CVR, demonstrated that a sample size of 24 participants was required to detect a power of 80% ($\eta_p^2 = 0.12$) ($\alpha = 0.05$). The

lack of consideration of habitual PA is a further limitation of the present study. A further methodological concern in the present study is that CVR measurements were only taken at two-time points, with the post meal measurement three hours following meal consumption. This was based upon previous postprandial studies in adolescents demonstrating impairments in peripheral endothelial function three hours following a MMTT (Bond et al, 2015b), however, this remains an assumption of the present work with regards to CVR%. In addition, this measurement time point did not align with elevations in uric acid in the present study following FRU ingestion, which occurred in the first 30-60 minutes following drink consumption. Therefore, any acute impairments in CVR from elevated uric acid concentrations may have been missed in the present study. Future research is needed to investigate the time course of the response of SSB consumption on CVR. Other key areas that warrant future investigation include studies exploring which participant characteristics are predictive or associated with the response to an acute sugar load, to understand what sub-groups may present the greatest risk.

Conclusions

This study demonstrates that the metabolic responses following consumption of the three sugar moieties differ, with glucose and sucrose drinks resulting in elevated blood glucose levels compared to fructose and water. With consumption of fructose, elevations in uric acid were present, however, the sugar moieties all presented similar increases in TAG concentrations following MMTT consumption. Despite these different metabolic environments, which have previously been shown to be atherogenic (Gleissner et al., 2007; Kang et al., 2004; Morrison et al., 2009), no acute impairments in CVR were seen in the present study following the drink or MMTT. Nevertheless, evidence indicates that elevations in

postprandial glucose and TAG concentrations are still seen as independent risk factors for CVD (Nordestgaard et al., 2007; Zilversmit, 1979). Further investigation into SSB consumption is needed, focusing on dose-response relationships, time course of the cardiovascular effects following a SSB, and the important effects of chronic intake.

CHAPTER 5: Summary, Future Directions and Conclusions

The aim of this thesis was to determine the within and between-day reliability of a breath-hold protocol for measures of CVR in adolescents. The second aim of this thesis was to investigate the acute and postprandial effects of sugar moiety (sucrose, glucose and fructose) on CVR and metabolic blood outcomes of TAG, uric acid and glucose. Due to the increase in investigations using CVR as a measure endothelial function, shown to be related to future disease risk (Keage et al., 2012; Silvestrini et al., 2000; Wong et al., 2016; Yonas et al., 1993), alongside an absence of data on the reliability of this outcome, this was essential to ensure that the experimental study utilised a measure with an acceptable level of reliability. To achieve this, two studies were undertaken:

- 1) To assess the between and within-day reliability of a breath-hold protocol to non-invasively measure CVR.
- 2) Investigate the acute and postprandial effect of sugar moiety on cerebrovascular function and metabolic outcomes.

Summary of the present thesis

Investigation into the acute effects of SSB consumption is of vital importance for preventative healthcare. This is of particular importance given the high intake of SSBs in adolescent populations (Public Health England 2013-14), paired with evidence that the presence of CVD risk factors in youth are the strongest predictors of the adult atherosclerotic processes (Juonala et al., 2004; Juonala et al., 2013; Kavey et al., 2006). This highlights the need for research into the acute and chronic effects of SSBs in order to firstly understand the impacts of SSB consumption on CVD risk, and subsequently make informed interventions and

recommendations that provide protective benefits against any adverse effects of SSB consumption, or that reduce their consumption.

A key strength of the present thesis is the replication of a commercially available SSB containing 60 g of sucrose. This provides ecological validity on the acute effects of SSB consumption which adolescents frequently encounter. A limitation of the existing data in this population is that the majority of studies only examine the acute effects from a glucose load, which is not representative of a SSB. This current thesis therefore adds to existing literature by investigating both the independent and combined effects of sugar moiety on CVR and metabolic blood outcomes of uric acid, TAG and glucose. A further novelty of the present study, which previous literature may have overlooked, is the inclusion of a subsequent meal and follow-up observation. This provides additional ecological validity, with most of the day spent in the postprandial period, alongside evidence indicating that postprandial metabolic outcomes are more predictive of CVD risk than fasted measures (Morrison et al., 2009).

This inclusion of a reliability study on the primary outcome of CVR in the present thesis offers a key strength to this thesis. Despite its clinical applications as a valid measure, with correlations between breath-hold induced CVR measures and the gold standard full range vasodilatory method (Ringelstein et al., 1988), the use of the breath-hold as a hypercapnic surrogate for CO₂ breathing is still an emerging area of research. There are no methodological studies offering protocol guidelines, and few studies investigating the reliability of this outcome, with none conducted in adolescents. This thesis therefore addressed this by conducting a separate study examining the within-test and within and between-day reliability of this outcome. This provided evidence that the measures of endothelial function in the experimental chapter were reliable, and between and within-day

comparisons could be made to an appropriate level of reproducibility (See Chapter 3).

In Chapter 3, the reliability of a breath-hold induced CVR protocol was assessed, focusing on within-test, and within and between-day measures of reliability for cerebrovascular outcomes. Within-test reliability data then informed the subsequent analyses of within and between-day CVR. Key findings from this were that the BHI, although originally used to account for differences in breath-hold length (Markus & Harrison, 1992), was not associated with the subsequent increase in $MCAV_{mean}$, and therefore was not justified as a necessary normalisation. Instead, CVR was expressed as a percentage increase from baseline to the peak velocity in the 10 seconds following the breath-hold in the present thesis (CVR%). Another finding from the present data was that there was no significant difference in CVR when expressed as a percentage between the three breath-hold attempts, and thus it was deemed appropriate to take an average of the three. From these findings, it would appear that performing one breath-hold attempt would be appropriate if used in a time sensitive protocol. The reliability of the within and between-day outcome of CVR was in line with that reported in the literature for similar endothelial function measures, providing important information for informing data analysis within these protocols to yield an appropriate level of reliability. These findings are important in providing researchers data on the reliability of this non-invasive measure for interpreting future experimental data and informing sample size calculations. Furthermore, the analyses provide information on the most reliable and appropriate methods of reporting breath-hold data, in order to prescribe consistent reporting methods across the literature, particularly in an adolescent population.

In Chapter 4, the findings demonstrated that the metabolic blood responses following consumption of the three sugar moieties was different, with glucose and sucrose drinks resulting in elevated blood glucose levels compared to fructose and water, in line with the study rationale. With consumption of fructose, elevations in uric acid were present, however the sugar moieties all presented similar increases in TAG concentrations following meal consumption. Despite these different metabolic challenges, no significant impairments in CVR were seen in the present study following the drink or MMTT in the sample of healthy adolescents. Having established that CVR% is a reliable tool of measurement in Chapter 3, Chapter 4 was unable to determine whether this outcome is sensitive to change. As this study did not include another measure of global endothelial function (i.e. FMD), it is not certain whether endothelial function did not change, or if CVR was not sensitive to change in the present study. Acute elevations in glucose and uric acid following drink consumption, however, may be associated with future disease risk factors, with elevations in uric acid concentrations associated with increased BP (Nguyen et al., 2009), and increases in glucose concentrations associated with impaired glucose tolerance and insulin resistance (Loader et al., 2017).

Future implications/directions

This thesis provides valuable data on the implementation and analysis of non-invasive assessment of CVR in youth through the breath-hold test, for use in future studies. These methods are shown to have good levels of reliability both within and between-days in adolescents, and indicate that a single breath-hold can be utilised in time-sensitive protocols.

Given the pilot nature of the present study, the experimental findings from this thesis were primarily speculative and highlight important areas that warrant future investigation. These include examining the acute response to SSBs in adolescents who present CVD risk factors (such as obesity), to determine whether current CVD risk alters the acute response to a SSB. Given that participants in the present study may have been protected from impairments in CVR, due to a healthy cardiometabolic risk profile, highlighted by descriptive characteristics of their VO_{2max} , body composition and fasting blood markers. In addition, studies investigating which participant characteristics are predictive or associated with the response to an acute sugar load presents an important area of future research, to understand what sub-groups may present the greatest risk. This includes characteristics such as age, pubertal status, sex, body composition and cardiorespiratory fitness. Furthermore, SSBs often include caffeine or stimulants, which may influence endothelial function, and should therefore be included in future research to identify the effect of a typical SSB on endothelial function. Investigation of chronic SSB consumption also warrants investigation, especially given clear evidence that habitual SSB intake increases the risk of CVD-related complications. A meta-analysis from 310,819 participants demonstrated individuals in the highest quartile of SSB consumption (1-2 servings per day) had a 26% higher risk of developing diabetes than individuals in the lowest quartile (Malik et al., 2010). This suggests that the metabolic responses observed in this study of hyperglycaemia and raised uric acid may be detrimental to health in adolescents, however it may be a cumulative effect over time rather than an acute response following one drink. This highlights the need for future studies to investigate the effects of SSB consumption over larger doses

and prolonged periods of intake, to examine what effects these metabolic responses have on the vasculature over repeated episodes.

A limitation of existing paediatric studies, and the present study, is that only macrovascular endothelial function of the large conduit arteries has been measured following an acute sugar load, with no studies investigating the effects on capillaries and arteriole beds via microvascular endothelial function. This may be of importance for future research into the consumption of SSBs in adolescent populations, as changes in microvascular function precede any changes in macrovascular function (Pinkney et al, 1997). Previous studies have demonstrated no associations between these measures of macro- and micro-vascular function (Dhindsa et al, 2008), and thus investigations into microcirculatory health should be carried out in future studies. Furthermore, in the present study, although CVR% was established as a reliable measure, it was not possible to identify if it is subject to change. In order to do this, future investigation is needed with established measures of endothelial function (i.e. FMD) alongside measures of CVR to identify if this outcome is sensitive to change. From our current findings we cannot discern whether there are effects on endothelial function following SSB consumption. This highlights the need for studies to investigate the responses to SSB consumption in other vascular beds to fully understand the effects of SSB consumption on CVD.

Scientific investigations into the effects of sugar consumption are a continuing area of research. The merit of this thesis was to inform future research and implications for continued development of the literature on this area, given the high sugar consumption levels of adolescents in England, in addition to a lack of research. The present findings were therefore predominantly of a pilot study

nature, which is of huge importance for the progression of research in this area to develop.

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APPENDIX

Appendix 1: Certificate of ethical approval



College of Life and Environmental Sciences
SPORT AND HEALTH SCIENCES

St. Luke's Campus
University of Exeter
Heavitree Road
Exeter
EX1 2LU
United Kingdom

Certificate of Ethical Approval

Proposal Ref No: 171206/B/07

Title: To examine whether the consumption of different types of sugar (fructose, glucose and sucrose) Impairs blood vessel health in adolescents when compared to the consumption of water

Applicants: Jodie Koep, Rohit Banger MSc, Rhys Banks PhD, Dr Bert Bond, Dr Alan Barker, Kate Sansum Placement student (UG), Ricardo Oliveira PhD, Sascha Kranen PhD, Chloe Bland MSc, Dimitris Vlachopoulos, Owen Tomlinson

The proposal was reviewed by the Sport and Health Sciences Ethics Committee.

Decision: *This proposal has been approved until July 2018*

Signature:

A handwritten signature in black ink, appearing to read 'Melvyn Hillsdon'.

Date: 7/2/2018

Name/Title of Ethics Committee Reviewer: Dr Melvyn Hillsdon

Your attention is drawn to the attached paper which reminds the researcher of information that needs to be observed when Ethics Committee approval is given.

Appendix 2: Health Screening Questionnaire

HEALTH SCREEN FOR CHILD VOLUNTEERS (PARENTAL FORM)

Name:

It is important that volunteers participating in research studies are currently in good health and have had no significant medical problems in the past. This is:

- i) To ensure their own continuing well-being
- ii) To avoid the possibility of individual health issues confounding study outcomes

Your answers to the questions in this questionnaire, on behalf of your child, are strictly **confidential**.

Please complete this brief questionnaire to confirm your child's fitness to participate:

1. **At present**, does your child have any health problem for which they are:

(a) On medication, prescribed or otherwiseYES NO

(b) Attending a general practitioner YES NO

(c) On a hospital waiting list YES NO

2. **In the past two years**, has your child had any illness that required them to:

(a) Consult your family GP..... YES NO

(b) Attend a hospital outpatient department YES NO

(c) Be admitted to hospital..... YES NO

3. **Has your child ever** had any of the following:

(a) Convulsions/epilepsy YES NO

(b) Asthma YES NO

(c) Eczema YES NO

(d) Diabetes YES NO

(e) A blood disorder YES NO

(f) Head injury YES NO

(g) Digestive problems YES NO

(h) Heart problems YES NO

- (i) Lung problems YES NO
- (j) Problems with bones or joints YES NO
- (k) Disturbance of balance/coordination YES NO
- (l) Numbness in hands or feet YES NO
- (m) Disturbance of vision YES NO
- (n) Ear/hearing problems YES NO
- (o) Thyroid problems YES NO
- (p) Kidney or liver problems YES NO
- (q) Allergy to nuts YES NO
- (r) Eating disorder YES NO

4. Do you know of any other reason why your child should not engage in physical activity?

YES NO

If **YES** to any question, please describe briefly (for example, to confirm the problem was/is short-lived, insignificant or well controlled).

A member of our research team may contact you if we have any further questions.

Thank you for your cooperation

Appendix 3: Parent/Guardian consent form



**Sport and Health Sciences
College of Life and Environmental
Sciences**

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Telephone: +44 (0)1392 26
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Web: www.ex.ac.uk

Study: To examine whether the consumption of different types of sugar (fructose, glucose and sucrose) impairs blood vessel health in adolescents when compared to the consumption of water.

Researcher: Jodie Koep (MSc)

Organisation: The University of Exeter

Version: #2 14/01/18: reviewed by The University of Exeter ethics committee

Participant Identification Number: ID no.

Informed consent form for parent/guardian

**Please
initial
box**

I confirm that I have read and understand the information sheet version #2 14/01/2018 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.

I understand that my child's participation is voluntary and that I am free to withdraw them at any time, without giving any reason.

I understand that any information given by me may be used in future reports, articles or presentations by the research team.

I understand that my child's name will not appear in any reports, articles or presentations.

I understand that my child will perform an incremental cycle tests to exhaustion on their first visit.

I understand that my child will be required to drink three high sugar drinks or water on four separate occasions.

Ultrasound will be used to determine changes in the width of the arm artery. A laser will also be placed onto the forearm to quantify skin blood flow. All of these techniques are routinely used with children for research purposes and are considered to be non-invasive.

I can confirm the absence of any food allergies related to this study.

I understand that ultrasound will be used to measure the speed of blood flow at the side of the head during a 30 second breath hold and when performing 5 minutes of repetitive sitting and standing.

I understand that my child will be asked to record dietary information and wear an accelerometer to measure their physical activity. My child will consume the same meal no later than 8:00 pm before each visit to the laboratory.

I understand that my child will be required to assess their pubertal status according to five drawings of secondary sex characteristics. The purpose of this has been made clear to me.

I understand that on each of the four test visits five capillary blood samples will then be taken from the fingertip (<1 mL each time) in order to measure fat, sugar, insulin in the blood.

I understand that on each of the four test visits my child will have to consume a meal provided consisting of a pizza, ice cream and chocolate pudding. My child will not have eaten beforehand

I understand that on each visit my child will have their blood pressure measured by placing a cuff around their bicep which will be inflated during measurement.

I agree for my child to take part in the above study.

Name of
Parent/Guardian

Date

Signature

Name of Researcher

Date

Signature

Appendix 4: Example of information sheets handed to participants



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College of Life and
Environmental Sciences**

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To examine whether the consumption of different types of sugar (fructose, glucose and sucrose) impairs blood vessel health in adolescents when compared to the consumption of water.

Invitation and brief summary

We would like to invite your child to take part in a research study into the effects of sugar sweetened drink consumption on blood vessel health in adolescents. Taking part in the study is entirely up to you and your child so before you decide, it is important for you to understand why the research is being done and what it will involve. Please take the time to read the following information and to discuss it with other people to decide whether you wish for your child to take part or not. Thank you for taking the time to read this information. Please be aware that participation in this study means that your child will be required to spend approximately five school days outside of school/the classroom.

What's involved?

High sugar consumption in youth is linked with poor blood vessel and metabolic health and obesity. Worryingly, teenagers in the UK are known to consume eight times the recommended maximum amount of added sugar from sugary drinks alone. However, the effect of sugary drinks on blood vessel function in adolescents is poorly understood. This study will investigate the effect of different sugar types found in sugary drinks on blood vessel health. We will do this by using a range of non-invasive techniques (including the use of ultrasound) and also by taking a series of fingertip capillary blood samples (<1 mL each) to measure the response to different sugary drinks. We will also see if sugary drink consumption influences the blood vessel response to a subsequent meal (Pizza, ice cream and a chocolate pudding).

What would taking part involve?

We have invited your child to take part because we are looking for healthy participants between the ages of 12-15 years old. We will be inviting 30-35 male and female participants to take part. If you would like your child to take part they will be asked to attend a laboratory at the University of Exeter's St. Luke's campus on five separate occasions. You will be asked to visit our laboratory in a group of two or three for all visit.

Preliminary testing visit (visit 1) - This will take four hours. Having completed and handed in participant assent, parental consent, health screening and contact details forms, we will collect your child from school at 7.30 am and drive them to

the University for their familiarisation visit. This visit will initially provide your child with an opportunity to discuss with the investigators any questions they may have regarding any aspect of the study's objectives, procedures or results.

If your child wishes to continue to take part, the rest of this visit will involve some preliminary measurements including, height, sitting height, body weight and body fat percentage (using a machine in which you sit inside a chamber). We will also take a resting measure of your child's blood pressure. We will then familiarise your child with all testing procedures and what to expect on future visits, so that they can make sure that they would like to take part. This includes non-invasive measures of blood vessel function at three different sites; the arm, forearm and head. We will also demonstrate how we take small capillary blood samples from the fingertip as this will be done on future visits.

After we have shown all the techniques, and providing that your child is still happy to take part, we will measure blood vessel function in the arm. This involves sticking a small laser probe to your child's forearm, and scanning the large blood vessel in the bicep using ultrasound. We will then pump up a blood pressure cuff around the forearm of the same arm for 5 minutes. During this time, your child may experience pins and needles in their hand, but this is normal and short lived. We often use this technique for our research with teenagers, and it is well tolerated. Following this we will also measure blood vessel function within the head by placing a small ultrasound probe at the temple, and asking your child to hold their breath for 30 seconds. Blood vessel function at the head will also be measured during a 5-minute period where we ask your child to sit and stand repeatedly.

Your child will then be asked to perform a cycling test to maximal effort cycle test. This test will feel like cycling up a hill as it gets steeper and steeper until your child can no longer carry on. It will only feel very hard at the end. During this cycle test your child will be wearing a heart rate monitor and a face mask in order to determine their aerobic fitness. We will also monitor blood flow to the head using the ultrasound probe at the temple as used above, monitoring your child throughout this time.

After the exercise test, measures of blood vessel health in the arm and head will be repeated as above using non-invasive ultrasound. Measures of blood pressure will also be routinely assessed following the exercise bout.

Before we drive your child back to school (at ~ 12.30 pm) they will be given an instruction pack for the remainder of the study. Your child will be required to;

1. Wear an accelerometer (a small activity monitor which looks like a wrist watch) for 7 days prior to the first visit and then 2 days prior to each subsequent visit. They will hand this in to our research team during the subsequent visits.
2. Record their food intake on the two days prior to each subsequent visit.
3. Avoid any structured moderate or vigorous intensity physical activity (other than day to day tasks) for 48 hrs prior to their next visit.
4. Refrain from eating or drinking anything apart from water after 8 pm the night before all laboratory visits.
5. Take home a set of scientific drawings showing five stages of pubic hair development and circle the picture that best describes them. They will seal this in an envelope and return it to us on their next visit.

Trials 1-4 (visits 2, 3, 4, 5) - Each trial day will last from 8.00 am to 2.40 pm. As for visit 1, we will collect your child from school at 7.30 am having not had any breakfast. The following procedures will be included in these visits:

- 8 am - We will assess the health of your child's blood vessels by scanning the artery in the arm and side of the head as described in visit 1.
- 9 am – Your child will then be given one of three sugary drinks or water (to act as a comparison), receiving a different one on each visit.
- A small sample of blood (less than 1mL, or about the size of a pin head) will be taken from your child's fingertip every 30 mins for the first 2 hours and then every hour following for the next 3 hours.
- Hunger levels will be assessed requiring your child to report their hunger on a visual scale, 60 and 120 minutes after sugary drink consumption, and 180 minutes after test meal consumption.
- 10 am - Blood vessel health will then be reassessed after 35-75 minutes. As well as measures of blood pressure.
- 11am – Your child will then be given a meal of a pizza, ice cream and a chocolate pudding.
- During this time, we will discuss with your child aspects of sports science, medicine and nutrition. Your child will also be able to watch a film, play on the Playstation® or do some school work.
- 2 pm - Three hours after the breakfast we will measure your child's blood vessel health and blood pressure. After this measurement, we will drive you child back to school for 3.30 pm.

What are the possible benefits of taking part?

The main benefits of the proposed research are educational and there will be limited personal benefit to your child. However, the results will increase our understanding of the risks associated with sugar intake and the different types of sugars found in sugary drinks. The study will hopefully be enjoyable and interesting for your child and allow them to learn about exercise physiology and nutrition in a fun and interactive way. By taking part your child will get to spend time in a University Laboratory, and we will give you access to full fitness and nutrition assessment from our report. This study will give your child first-hand experience about what it's like to be involved in science at a higher level.

What are the possible disadvantages and risks of taking part?

Blood sampling can cause some temporary discomfort if participants are not comfortable with blood. However, this technique is used extensively in physiological testing. The investigators are trained and experienced in all aspects of these procedures to ensure that they are performed safely and with the minimum possible discomfort.

Does my child have to take part?

Please remember that participation in this study is entirely voluntary. It is up to you and your child to discuss and decide whether you would like them to take part or not. If you decide for them to take part they free to leave the study at any time without giving a reason as to why they wish to do so.

If you and your child do agree for them to participate in this study then please complete the following:

- Sign the parent/guardian consent form
- Sign the child assent form
- Complete the contact details form
- Complete the health questionnaire

Please return these documents to your child's school in the brown envelope provided, so we can collect them. A member of the research team will then be in contact to arrange your child's involvement in the study. You will also be given a copy of the forms and this information sheet for your own records.

Are my results confidential?

If you consent to take part in this study you have a right to privacy. Your child's name will be linked to an ID number on a password protected database and only these IDs will be used as labels during blood and data analysis.

What will happen to the results of this study?

The results will increase our understanding of the risks associated with different types of sugars found in sugary drinks. We will aim to publish the findings as a masters by research project, in research journals and to present them at conferences in the UK or abroad. Your data will always remain anonymous and your name will not appear on any results. However, we will explain all your results of the study to you at the end if you would like to know them.

Who has reviewed this study?

All research activity at the University of Exeter is examined and approved by an ethics committee to protect your interests. This study has been approved by the Ethics Committee of Sport and Health Sciences, College of Life and Environmental Sciences, University of Exeter.

Who is funding/sponsoring this study?

This study will be funded by the University of Exeter.

Contacts for further information

If you would like more information or if you have any further questions about the study please contact the investigators using the details below:

Ms Jodie Koep	Dr Alan Barker	Dr Bert Bond
Sport and Health Sciences	Sport and Health Sciences	Sport and Health Sciences
Baring Court	Baring Court	Baring Court
St. Lukes Campus	St. Lukes Campus	St Lukes Campus
Exeter University	Exeter University	Exeter University
EX12LU	EX12LU	EX12LU
Tel: 07769226211	Tel: 01392 722766	Tel: 01392 724903
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