Investigating the effectiveness of New Zealand Blackcurrant extract to modulate postprandial glycaemia in overweight/obese individuals

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Abstract

Obesity and type 2 diabetes mellitus (T2DM) has now reached epidemic proportions, and therefore strategies to prevent and treat these conditions are required. Long-term adherence to current strategies that primarily revolve around increasing energy expenditure or reducing caloric intake is poor, and therefore alternative therapies need to be investigated. A major component surrounding the progression of obesity to diabetes and further co-morbidities is sustained periods of postprandial hyperglycaemia. Current epidemiological evidence has suggested that habitual anthocyanin intake is linked to lower T2DM risk. The aim of this thesis was to investigate whether anthocyanin-rich New Zealand blackcurrant (NZBC) extract could be an effective nutritional strategy to reduce postprandial glycaemia in overweight and obese individuals. **Chapter 2** provides evidence that an acute bolus of NZBC extract is unable to mediate postprandial glucose or triglyceride responses to a carbohydrate-fat test meal, irrespective of the dose used. Therefore, **Chapter 3** investigated whether 8 days NZBC extract supplementation is effective at reducing postprandial glucose responses under free-living conditions using continuous glucose monitoring. **Chapter 3** showed that NZBC extract supplementation reduced postprandial glucose responses to breakfast and dinner (-9% and -8%, respectively), as well as improving insulin sensitivity (+22%) in overweight/obese individuals. In conclusion, this thesis provides evidence that short-term supplementation with an anthocyanin-rich blackcurrant extract is effective in reducing postprandial glucose responses under free-living conditions, thereby highlighting the potential for anthocyanins to be an effective strategy in mediating postprandial glycaemia and improving insulin sensitivity in individuals at risk of developing type 2 diabetes.

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Chapter 1 General Introduction

1.1 Obesity and type 2 diabetes

The most recent estimates from Public Health England suggest that nearly two thirds of adults (63%) are now classified as being overweight or obese and this is expected to continue to rise (Public Health England 2017). This can be primarily attributed to the evolution of an environment in which sedentary working time has increased, leaving less opportunities and desire to undertake physical activity. Combined with an environment saturated with readily available high energy-low effort foods, this has resulted in an epidemic of obesity and related pathologies, such as type 2 diabetes mellitus (T2DM). Inevitably, this has created a burden on the health care system, with recent estimates suggesting that current costs to the NHS are set to be around £6 billion per year (Public Health England 2017).

Obesity is the result of an imbalance between energy intake and expenditure and is therefore considered a consequence of physical inactivity coupled with the consumption of an energy dense diet. Physical inactivity and obesity is also associated with insulin resistance, which is characterised by an inability of insulin to effectively mediate glucose transport into peripheral tissues. In healthy individuals, insulin is secreted from the pancreas in response to carbohydrate ingestion, and is responsible for the clearance of glucose from the plasma into peripheral tissues, such as skeletal muscle. Importantly, glucose uptake into peripheral tissue is precisely matched by the rate of endogenous glucose production to tightly maintain plasma glucose concentration between 3.9-5.5 $mmol.L^{-1}$ (Abdul-Ghani, Lyssenko et al. 2009). However, in the insulin resistant state physiological insulin secretion becomes insufficient to stimulate glucose uptake into skeletal muscle, resulting in greater

insulin secretion in order to return to a state of euglycemia. Further progression of insulin resistance to T2DM is characterised by prolonged periods of postprandial hyperglycaemia and hyperinsulinaemia, where high levels of insulin are inadequate to reduce plasma glucose concentrations following a meal. Ultimately, this leads to tissue damage and a myriad of secondary complications including cardiovascular disease, nephropathy, microvascular damage and retinopathy (Nathan, Genuth et al. 1993). Notably, the progression to T2DM correlates strongly with BMI (Edelstein, Knowler et al. 1997), indicating that obesity (and physical inactivity) is a primary risk factor for the development of T2DM.

1.2 Glucose homeostasis

1.2.1 Carbohydrate digestion and absorption

Carbohydrate metabolism and storage is tightly regulated through the actions of hormones and digestive enzymes. Carbohydrate digestion begins in the mouth, where α-amylase is released and hydrolyses the α (1,4)-glycosidic bonds of polysaccharides. These are then broken down into the peptides; amylose and amylopectin. Once the carbohydrate reaches the small intestine, additional pancreatic α-amylase is secreted alongside α-glucosidase which acts on sucrose and maltose, breaking them down into glucose and fructose ready for absorption and transportation into the circulation. The surface of the small intestine consists of microvilli which extend into the unstirred water-layer phase of the intestinal lumen. This microvillus membrane is known as the brush border and contains transporters that take up these monosaccharides into the

circulation. Glucose enters the blood through the actions of sodium-dependant glucose transporter 1 (SGLT1) and glucose transporter 2 (GLUT2), the latter of which is predominantly active during periods in which luminal glucose concentrations are high. Sodium-dependant glucose transport is initiated when sodium is pumped from the cell to create a sodium gradient between the intestinal lumen and the interior of the cell. The resultant sodium gradient drives the co-transporter (SGLT1) so that one molecule of sodium and one molecule glucose or galactose are transported into the cytoplasm of the enterocyte. Sodium-independent transport works through the action of glucose transporters in which glucose is pumped out of the enterocyte and into the intracellular space through the actions of GLUT2.

A rise in plasma glucose levels (due to feeding) causes a compensatory increase in insulin secretion from the pancreas. Insulin is secreted by the β-cells of the islets of Langerhans in the pancreas and binds to the plasma membrane of skeletal muscle, liver and adipose tissue by facilitating the removal of glucose out of the circulation through enhancing the activity of tissue-specific glucose transporters. This process allows plasma glucose levels to remain at around 5-6 $mmol.L^{-1}$ 60-90 mins post-absorption, and these levels are tightly maintained with slight reductions only being observed during long-term starvation (Owen, Reichard et al. 1974). Upon entry into tissue, glucose can then be stored as glycogen or used to provide energy via glycolytic processes (or in adipose tissue converted to triacylglycerol via *de novo* lipogenesis). Importantly, skeletal muscle is the primary site of glucose uptake, with skeletal muscle being responsible for ~80% of glucose removal from the circulation in healthy, lean insulin sensitive individuals during a hyperinsulinaemic euglycaemic clamp (DeFronzo, Jacot et al. 1981, Baron, Brechtel et al. 1988).

1.2.2 Insulin-mediated vasodilation

Despite being a key signalling hormone in the uptake of glucose into the peripheral tissue via glucose transporters, the pleiotropic properties of insulin involve other important homeostatic processes including vascular control. The vasodilatory properties of insulin are primarily categorised by its ability to upregulate the production of nitric oxide (NO) from the vascular endothelium, independent of other classical calcium-dependant mechanisms used by vasodilators such as acetylcholine. NO is a vasodilator which acts on the smooth muscle layer of terminal arterioles leading to a decrease in vascular resistance and a subsequent increase in microvascular perfusion. The process in which insulin stimulates NO production involves the phosphatidylinositol-3-kinase (PI3-K) signalling pathway in which activation of the insulin receptor tyrosine kinase leads to a phosphorylation of insulin receptor substrate 1 (IRS-1) leading to a subsequent binding and activation of PI3-K. This then causes a further activation of Akt which directly phosphorylates and activates endothelial nitric oxide synthase (eNOS), leading to an increased production of NO within minutes (Fleming and Busse 2003). Insulin is also capable of promoting vasoconstriction through the synthesis of endothelin-1 (ET-1) using the mitogen-activated protein kinase (MAPK)-dependant signalling pathway.

This increase in insulin-mediated vasodilation can lead to a subsequent increase in peripheral blood flow, with studies demonstrating leg blood flow is increased following consumption of an oral glucose load (Baron, Laakso et al. 1990). Furthermore, microvascular blood volume has been shown to increase 15-30 min after

the start of a hyperinsulinemic euglycemic clamp and is related to increases in glucose uptake (Vincent, Clerk et al. 2004). Conversely, individuals with insulin resistance (such as obese and diabetic populations), have a simultaneous impairment in insulin-induced vasodilation (Baron, Laakso et al. 1991, Baron, Laakso et al. 1991, Clerk, Vincent et al. 2006). Ultimately, the role of insulin-mediated vasodilation is to allow an increase in microvascular perfusion which subsequently leads to a greater delivery of insulin and glucose to the myocyte, allowing for efficient glucose storage.

1.2.3 Insulin-mediated glucose uptake into skeletal muscle

The process in which glucose enters skeletal muscle is initiated by insulin binding to the extracellular α-subunit of the insulin receptor (IR) on the plasma membrane, thereby stimulating tyrosine autophosphorylation of the transmembrane β-subunit. This leads to activation of the intrinsic tyrosine kinase causing a downstream tyrosine phosphorylation of insulin receptor substrate proteins (IRS), leading to two signalling cascades; the MAPK and PI3-K pathways. The PI3-K pathway involves first the conversion of phosphatidylinositol (4,5)-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-triphosphate (PIP3). PIP3 then triggers the activation of Akt (specifically Akt2) through the actions of intermediate protein kinases, PDK1 and mTOR. Phosphorylation of Akt on threonine^{308} and ser^{473} residues results in its activation and activated Akt downstream targets include glycogen synthase kinase-3 (leading to glycogen synthesis) and TBC 1 domain family member 4 (TBC1D4, also known as AS160). Under basal conditions, the GTPase-activating protein domain of TBC1D4 retains RAB proteins in an inactive (GDP-bound) state. Insulin stimulation leads to

activated Akt phosphorylating TBC1D4, which in turn suppresses the GTPase activity, and Rab proteins subsequently become GTP-loaded. This is critical because it permits a reorganisation of the cytoskeleton, which is required for glucose transporter mobilisation and translocation. While there are 14 glucose transporter isoforms (Uldry and Thorens 2004), glucose transporter 4 (GLUT4) is the predominant insulin responsive isoform required for skeletal muscle glucose uptake (Watson and Pessin 2001). GLUT4 facilitates diffusion-mediated uptake of glucose across the plasma membrane.

1.3 Characteristics of obesity and insulin resistance

One of the major mechanisms underpinning the pathogenesis of obesity and its progression to further co-morbidities (including T2DM) is insulin resistance. Insulin resistance is defined as an inability for insulin to efficiently mediate glucose entry into peripheral tissues, and predominantly skeletal muscle. While insulin resistance can occur during the normal life cycle such as in puberty, pregnancy and with aging, prolonged hyperglycaemia (such as seen in obesity/T2DM) can lead to tissue toxicity and additional complications (Defronzo 1979, Buchanan, Metzger et al. 1990, Moran, Jacobs et al. 1999). Furthermore, studies have demonstrated that individuals suffering from T2DM can spend as much as 38% of the day (~9 hours 10 min) in a hyperglycemic state (van Dijk, Manders et al. 2011).

The development of obesity-mediated insulin resistance is complex and defies explanation by a single etiological pathway. An increase in visceral and abdominal adiposity has been shown to correlate with waist circumference, a known clinical

marker of metabolic disease risk (Zhu, Wang et al. 2002) and metabolic syndrome (Grundy 2004). Moreover, lipolysis of visceral adipose tissue triglyceride stores releases free fatty acids (FFA) directly into the portal vein, which is then further transported into the liver and can contribute to the development of fatty liver. The portal vein is responsible for ~80% of total liver blood flow (Schenk, Mc et al. 1962) and studies in obese individuals suggest that plasma IL-6 concentrations were much greater in the portal vein than in peripheral arterial blood (Fontana, Eagon et al. 2007), indicating the importance of visceral fat in the development of chronic low-grade systemic inflammation and the disruption of normal glucose kinetics.

Furthermore, an impairment in the capacity of adipose tissue to store plasma-derived triglyceride (TAG) results in an elevated plasma TAG concentration, thereby leading to a 'spillover' of fatty acids into the skeletal muscle; this has been proposed to interfere with skeletal muscle glucose uptake. Additionally, insulin resistance has been characterised as a pro-inflammatory condition in which normal inflammatory pathways are disrupted leading to chronic low-grade inflammation. Interestingly however, the exact mechanisms surrounding this prolonged inflammatory response are unclear. It has been postulated that perhaps obesity not only lends itself to an increase in total adipose tissue mass but also adipocyte hypertrophy. This creates microvascular complications as an inefficient vascular system is unable to provide adequate oxygen supply to the adipocytes leading to hypoxia and ultimately cell death. This 'micro-hypoxia' may then lead to a recruitment of macrophages into the adipose tissue and increase the expression of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α. Alternatively, increased endoplasmic reticulum stress caused by

an obesity-mediated increase in synthetic demand, may lead itself to an increase in the activation of pro-inflammatory pathways.

Interestingly, while increased levels of obesity are associated with macrophage infiltration into the adipose tissue there is also a distinct shift in macrophage phenotypes. Macrophages come in two distinct subtypes; the 'classically activated macrophage' phenotype, termed M1, which secretes pro-inflammatory cytokines and the 'alternatively activated macrophage' termed M2, which secretes anti-inflammatory cytokines. Obesity is associated with a shift from the M2 to M1 phenotypes which generates a greater inflammatory response and is associated with insulin resistance. Ultimately, it is the combination of these mechanisms which explain the pathogenesis of insulin resistance and its physiological implications on metabolic health.

1.3.1 Insulin resistance and the microvascular system

The microcirculation encompasses vessels <150 μm in diameter and is responsible for nutrient delivery to peripheral tissues, removal of cellular waste products and maintenance of capillary hydrostatic pressure (Verdant and De Backer 2005). The metabolic action of insulin to stimulate glucose uptake in skeletal muscle is mediated through stimulation of PI3-kinase-dependent signalling pathways within the endothelium leading to an upregulation of NO production that is required for insulin to facilitate its own entry into skeletal muscle. Obesity however, is characterised by an increased production of reactive oxygen species (ROS) and NO scavenging by superoxide (O_2^-) (Laight, Kengatharan et al. 1998, Landmesser and Drexler 2006). An

elevation of free fatty acids increases ROS production and inflammation in the microvasculature, leading to the induction of NAD(P)H-oxidase and the production of superoxide anions which in turn will scavenge NO resulting in a reduction in NO bioavailability. Exposure of the vasculature to high levels of free fatty acids also leads to the accumulation of diacylglycerol (DAG) and ceramides (Symons and Abel 2013), which induces serine phosphorylation of IRS-1 in the endothelium and ultimately a reduction in insulin-stimulated Akt phosphorylation and eNOS activation (Naruse, Rask-Madsen et al. 2006). Conversely, insulin-mediated vasoconstriction pathways seem mostly intact in obesity, with studies showing that the MAPK pathways in obese individuals maintain ET-1 production, leading to an imbalance between NO and ET-1 production, leading to a preference for vasoconstriction (Mather, Mirzamohammadi et al. 2002). Moreover, eNOS activity and abundance have also been shown to be decreased in obese and diabetic subjects leading to a reduced capacity for NO production (Higashi, Sasaki et al. 2001, Gruber, Mayer et al. 2008).

1.3.2 Insulin resistance in the muscle

In obese individuals with large subcutaneous and visceral adiposity, there is considerable evidence to suggest that the ability of adipose tissue to buffer the lipid flux is compromised (Lewis, Carpentier et al. 2002, Bays, Mandarino et al. 2004). This creates an impairment of insulin mediated adipose tissue lipolysis which subsequently results in increased plasma FFAs (Hickner, Racette et al. 1999). Interestingly, it seems that the increase in plasma FFA may not directly interfere with insulin action, as rates of FFA release in obese individuals are relatively normal when adjusted to total fat

mass (Campbell, Carlson et al. 1994). However, the capacity of adipose tissue to store plasma-derived TAG is impaired, resulting in an elevated plasma TAG concentration. Consequently, these TAG remnants of chylomicron-TAG are then stored within the liver and provide substrate to produce very low-density lipoproteins containing TAG (VLDL-TAG). The circulating FFAs (generated from the hydrolysis of VLDL-TAG) then 'spillover' into peripheral tissues (including skeletal muscle) and ultimately accumulate as intramuscular triglyceride (IMTG). Elevated IMTG content is negatively associated with insulin-mediated skeletal muscle uptake in humans and provides a risk factor for the development of insulin resistance and/or T2DM (Pan, Lillioja et al. 1997). However, endurance-trained athletes also exhibit elevated IMTG stores, but this is combined with high levels of insulin sensitivity in the 'athlete's paradox' phenomenon (Goodpaster, He et al. 2001). As such, the consensus in the literature is that an accumulation of lipid metabolites, such as long chain fatty acyl-CoA's, diacylglycerol (DAG) and ceramides, rather than IMTG per se, are responsible for reducing insulin sensitivity. Indeed, DAGs and ceramides have been shown to accumulate within skeletal muscle of obese individuals and T2DM patients and appear to contribute to the activation of the inflammatory serine threonine kinases such as conventional PKC's, IKK-β and JNK (Hotamisligil 2006). Specifically, diacylglycerol (DAG) (an intermediate in both IMTG synthesis and hydrolysis) can increase serine phosphorylation of IRS-1, while ceramides (which require long chain saturated FA for their biosynthesis) can dephosphorylate Akt (Itani, Ruderman et al. 2002). Ultimately, this leads to downregulation of the insulin signalling cascade and a reduction in GLUT4 translocation to the plasma membrane, limiting glucose entry into the skeletal muscle.

During periods of chronic hyperglycaemia, the pancreatic β-cells will attempt to compensate through increased insulin secretion. However, a chronic physiologic increase in plasma insulin concentration has a detrimental effect on insulin sensitivity as an increase in plasma insulin concentrations in healthy individuals for as little as 72-96 hrs is enough to reduce insulin-mediated glucose disposal (Del Prato, Leonetti et al. 1994). Insulin stimulation of the PI 3-kinase pathway is dramatically reduced in obese nondiabetics and is virtually absent in T2DM, primarily through a reduction in insulin receptor and IRS-1 phosphorylation alongside a reduction in IRS protein association with p85 and PI3-K (Cusi, Maezono et al. 2000). Unfortunately, however this can then lead to a plethora of deleterious conditions including dyslipidemia, hypertension, systematic inflammation, β-cell dysfunction, endothelial dysfunction and cardiovascular disease.

The development and implementation of strategies to combat these conditions has received considerable attention in the past decade. Exercise is one therapeutic strategy suggested to improve insulin resistance and ultimately support individuals living with metabolic syndrome and T2DM (Poehlman, Dvorak et al. 2000). However, exercise is not always an effective solution as long-term adherence alongside inadequate exercise prescription can prove difficult in many populations leading to ineffective long-term intervention retention (Dishman 1988). Furthermore, weight loss is an important strategy to reduce the complications associated with obesity and T2DM (Knowler 2006). It is important to note that many individuals with T2DM will use medications such as metformin and acarbose, however these medications often come with undesirable side effects. To that end, one such strategy that is growing in

interest is the use of functional foods as there is expanding evidence in their potential to improve metabolic health and insulin sensitivity.

1.4 Polyphenols and anthocyanins

Polyphenols are a large heterogeneous group of phytochemicals found within plant-based foods that include flavonoids, proanthocyanidins, phenolic acids and resveratrol. Anthocyanins are a major subgroup of the flavonoid class and are one of the more recognisable phytonutrients due to their bright blue/purple/red pigmentations visible on the outside of the plant (Galvano, La Fauci et al. 2004). Anthocyanins are water soluble glycosides of 2-phenylbenzopyrylium or flavylium salts with the 6 most commonly found anthocyanins being cyanidin, delphinidin, peonidin, pelargonidin, malvidin and petunidin located within dark coloured fruits/vegetables including blackcurrants, blueberries, blackberries, bilberries, pomegranate, and strawberries. Despite the occurrence of anthocyanin in many plants, the total quantity can vary considerably between different fruits and vegetables with the total anthocyanin content ranging from 0.28 to 1480mg/100g. Berries however, have the highest estimated anthocyanin content in the range of 160-1300mg/100g fresh weight (Wu, Beecher et al. 2006, Scalzo, Currie et al. 2008, Fang 2014). Despite the known benefits of a varied a balanced diet, the Look AHEAD research group cohort found that only 36% and 38% of individuals with T2DM met the recommended daily fruit and vegetable intake, respectively (*n*=2757) (Vitolins, Anderson et al. 2009). Furthermore, dietary intake of anthocyanins can vary depending on global location, with Mediterranean countries (Greece, Spain, Italy,

south of France) having higher habitual intakes than other non-Mediterranean countries (north-east and north-west of France, Germany, Netherlands, UK, Denmark, Swedan and Norway) (Zamora-Ros, Knaze et al. 2016). Finally, intake may also vary between sexes given that a Finnish cohort study into total polyphenol consumption found habitual daily anthocyanin intake to be higher in females than males (53 ± 76 and 43 ± 82 respectively) (Ovaskainen, Torronen et al. 2008).

1.4.1 Epidemiological studies

It has been well documented that polyphenol intake is related to a wide range of positive health benefits including reduced risk of cardiovascular disease (Mink, Scrafford et al. 2007), hypertension (Cassidy, O'Reilly et al. 2011), and diabetes (Jayaprakasam, Vareed et al. 2005). Jennings, Welch et al. (2012) measured habitual polyphenol intake of 1898 women aged 18-75 y from the TwinsUK registry and found that polyphenol consumption was associated with reduced central systolic blood pressure (-3.0 ± 1.4 mmHg), lower mean arterial pressure (-2.3 ± 1.2 mmHg) and improved pulse wave velocity. Additionally, berries have been shown to positively influence T2DM risk with a Finnish cohort study of 10,054 men and women showing an inverse relationship between berry consumption and T2DM risk (Knekt, Kumpulainen et al. 2002). Similar findings were also reported in the population-based Kuopio ischaemic heart disease risk factor study (*n*= 2332) which found that consumption of >59.7 g berries per day compared with <1.3 g reduced the risk of T2DM (Mursu, Virtanen et al. 2014). Interestingly though, in this study total fruit or

vegetable consumption was not associated with reduced T2DM risk, indicating a potential important role of berries in the mediation of disease risk (Mursu *et al*., 2014).

When considering anthocyanins alone, a large prospective study consisting of 3 cohorts (70,359 women, 89,201 women and 41,334 men) found that higher habitual intake of anthocyanins was associated with a lower risk of T2DM when adjusted for several covariates (BMI, smoking, physical activity, multivitamin use and family history of diabetes) (Wedick, Pan et al. 2012). Importantly though, there were no significant associations with T2DM risk found for total flavonoid intake or other flavonoid subclasses (Wedick, Pan et al. 2012), highlighting that anthocyanins are perhaps the key polyphenol linked with reduced T2DM risk. In the same cohort, Muraki, Imamura et al. (2013) found that anthocyanin-rich foods were associated with a reduced T2DM risk, with blueberries having the greatest modulating effect. It should be noted, however, that these positive findings are not universal, since results from a Framingham Offspring cohort (*n*= 2,915) found a modulating effect of flavonol and flavan-3-ol, but not anthocyanin while adjusting for time-dependant covariates (BMI, smoking and prevalent CVD) (Jacques, Cassidy et al. 2013). Furthermore, the Iowa Woman's health study prospective cohort (*n*= 35,816 postmenopausal women) also found no association between total flavonoid or anthocyanin intake and risk of T2DM (Nettleton, Harnack et al. 2006). Recently, however, in a cohort of 1,997 women, a high anthocyanin intake was found to be associated with increased insulin sensitivity (measured using the homeostatic model for assessment of insulin resistance; HOMA-IR), while women reporting a higher habitual intake of anthocyanidins via food frequency questionnaires had lower HOMA-IR scores and lower fasting insulin levels following adjustment for BMI, medication, medical history and physical activity

(Jennings, Welch et al. 2014). Taken together, these observations suggest a possible relationship between anthocyanin intake and reduced T2DM risk exists, which occurs by anthocyanins modulating insulin sensitivity independent of BMI and other factors.

1.4.2 Bioavailability and enzyme inhibition

While the link between anthocyanin intake and health has been shown in prospective cohort studies, the exact mechanisms that influence the beneficial response are unclear. This is partly due to the poor bioavailability of anthocyanins compared to other polyphenols *in vivo*, in which only 1.4-12.4% of total content is detected in the plasma post-ingestion (Czank, Cassidy et al. 2013). However, it has been shown that anthocyanin metabolites do remain within the blood up to 48 h post-ingestion, indicating that it is likely that chronic ingestion of anthocyanin-rich foods will lead to an accumulation of anthocyanin metabolites in the blood over time. Furthermore, there may be potential synergistic effects with anthocyanins and other compounds, as previously it has been shown that quercetin and resveratrol interact with ethanol in their stimulation of the nitric oxide pathway (Chan, Mattiacci et al. 2000), and therefore significant effects of polyphenols may require broad combinations of phytonutrients.

Fundamentally, it is proposed that anthocyanins can mediate the digestion and absorption of glucose from the gut. Anthocyanin has been shown to inhibit α-amylase, which can be secreted by the salivary glands and is responsible for initiating the breakdown process of glucose for further digestion (McDougall, Shpiro et al. 2005). Cyanidin-3-rutinoside (commonly found within blackcurrant) is capable of inhibiting

pancreatic α-amylase, the effect of which was further increased with the addition of acarbose (a popular diabetic drug for the management of hyperglycaemic episodes) (Akkarachiyasit, Yibchok-Anun et al. 2011). It should be noted that data on anthocyanins ability to influence α-amylase is limited, and therefore further clarification is warranted. However, the greatest effect of anthocyanins on digestion and absorption appears to be on intestinal α-glucosidase, with an α-glucosidase inhibitory assay demonstrating a significant inhibition of maltase activity by 12 natural pigmented anthocyanins, interestingly sucrase activity was unaffected (Matsui, Ueda et al. 2001). Furthermore, four diacylated pelargonidin, cyanidin and peonidin 3-sophoroside-5-glucosides were also subjected to an α-glucosidase inhibitory assay and found significant maltase inhibitory activities with no sucrase inhibition (Matsui, Ueda et al. 2001). Similarly, Tadera (2006) found that anthocyanin potently inhibited yeast α-glucosidase and had a weak inhibition of rat intestinal α-glucosidase. Moreover, blackcurrant and rowanberry extracts were both found to inhibit intestinal α-glucosidase activity, but despite these berries having significantly different polyphenolic structures, α-glucosidase activity inhibition was not increased when the extracts were combined (Boath, Stewart et al. 2012). Additionally, examination of cyanidin-3-rutinoside found that baker's yeast α-glucosidase inhibition occurred in a dose-response manner (Adisakwattana, Ngamrojanavanich et al. 2004) highlighting the importance of dosing strategies.

Studies into the effectiveness of cyanidin-3-galactoside, which are found in high concentrations in blueberries and cranberries, showed inhibition of sucrase and maltase enzyme activity (Adisakwattana, Charoenlertkul et al. 2009). However, later research by the same author found that cyanidin-3-rutinoside largely inhibited

intestinal sucrase and maltase activity 30-90 min after loading and was especially effective when combined with acarbose (Adisakwattana, Yibchok-Anun et al. 2011). Aglycone cyanidin has also been shown to inhibit sucrase enzyme activity but to a much lesser extent than its glycosides, and cyanidin 3,5- diglucoside showed no inhibition (Akkarachiyasit, Charoenlertkul et al. 2010). Diacylated anthocyanins from the purple sweet potato were found to be potent maltase inhibitors reducing blood glucose 30 min post-ingestion in rat models, but its effect on sucrase and glucose transport was limited (Matsui, Ebuchi et al. 2002). Additionally, a study into the polyphenols found in acerola determined that cyanidin-3-alpha-O-rhamnoside and pelargonidin-3-alpha-O-rhamnoside were found to be ineffective inhibitors of α-glucosidase, with only quercetin providing an inhibitory response (Hanamura, Hagiwara et al. 2005). Furthermore, when polyphenol-rich extracts from a variety of fruits were tested on their ability to inhibit both α-amylase and α-glucosidase, it was shown that while all tested fruits had some ability to inhibit α-amylase, α-glucosidase was more potently inhibited by fruits with higher total anthocyanin contents including blueberry and blackcurrant extracts (McDougall, Shpiro et al. 2005). To that end, the effects on digestive enzymes seem to be dependent on the type of anthocyanin present within the foodstuff. Moreover, it is plausible that foods with greatest quantities/types of anthocyanin may provide the largest inhibitory effect.

1.4.3 Glucose transport and uptake from the gut

The human epithelial cell line Caco-2 has been widely used as an *in vitro* model of the small intestine, and anthocyanin is known to be absorbed through Caco-2 intestinal

cells and may interfere with glucose uptake (Faria, Pestana et al. 2009). It is proposed that anthocyanins suppress the activity of sodium dependant glucose transporter-1 (SGTL-1) and GLUT2 transporters. Indeed, Manzano and Williamson (2010) found that anthocyanin-rich strawberry extract was capable of inhibiting glucose uptake and transport via inhibition of SGTL-1 and GLUT2. Furthermore, acute exposure (15 min) to a mixed berry extract and individual anthocyanin (cyanidin, cyanidin glucoside, cyanidin rutinoside) all significantly decreased both sodium-dependent (total uptake) and sodium-independent (facilitated uptake) glucose uptake (Alzaid, Cheung et al. 2013). The importance of SGTL-1 and GLUT2 for facilitating anthocyanin entry into circulation is reinforced through studies in which admistration of pharmacological agents phlorizin (an inhibitor of SGTL-1) and phloretin (an inhibitor of GLUT2) to Caco-2 cells resulted in a marked reduction in cyanidin-3-0-β-glucoside transport within the cell (Zou, Feng et al. 2014). Furthermore, Hanamura, Mayama et al. (2006) found a reduction in glucose transport across Caco-2 cells, which was attributed to a suppression of intestinal glucose absorption alongside an inhibition of α-glucosidase activity, indicating the mediation of glucose uptake is likely a combination of both these factors. However, while *in vitro* studies have provided an insight into the ability of anthocyanins to influence carbohydrate uptake, they do not account for the complexities of *in vivo* metabolism, especially given the poor bioavailability of some polyphenols. Additionally, due to the biodiverse nature of fruits, there may be multiple bioactive compounds working synergistically, and therefore it is perhaps difficult to elucidate whether the transport inhibition is more effective when combined with other bioactives.

1.4.4 Glucose transport and uptake into peripheral tissues

The ability to effectively transport nutrients and hormones to peripheral tissues is an important component in the maintenance of glucose homeostasis. To this end, the release of vasodilators (such as nitric oxide) is instrumental in supporting nutrient transport to peripheral tissues for utilisation or storage. Blackcurrant concentrate has been found to cause endothelium-dependant vasorelaxation in noradrenaline pre-contracted rat aorta (Nakamura, Matsumoto et al. 2002). Similarly, red wine polyphenols (delphinidin) have been found to relax rat aortic rings via an increased stimulation of Ca^{2+}-dependant nitric oxide (Martin, Andriambeloson et al. 2002). It is postulated that bilitranslocase (an endothelial plasma carrier that transports flavonoids) mediates an important step in the ability for anthocyanin to increase nitric oxide synthase and in turn nitric oxide production, as inhibition of bilitranslocase decreases anthocyanin-mediated vasodilation (Ziberna, Lunder et al. 2013). Furthermore, *in vivo* human trials have demonstrated that a single acute bolus of 320 mg of anthocyanin significantly increased flow mediated dilation (FMD) in hypercholesterolic individuals (Zhu, Xia et al. 2011). Similarly, acute anthocyanin ingestion has been shown to increase peripheral blood flow at rest and prevent the decrease in oxygenation that occurs during typing work (Matsumoto, Takenami et al. 2005). Interestingly however, the increase in nitric oxide-mediated vasodilation is not solely responsible for the increase in skeletal muscle nutrient uptake. Cell based bioassays of Canadian lowbrush blueberrys determined root, stem and leaf extracts significantly enhanced glucose uptake in C2C12 myocyte cells by 15-25% in the

presence and absence of insulin after 20 h of exposure (Martineau, Couture et al. 2006). Because C2C12 myotubes lack a microvascular component, the increased glucose uptake is independent of the vasodilatory effects shown in previous research. This highlights that anthocyanin-mediated increases in glucose transport and uptake into peripheral tissues may occur through a combination of mechanisms.

1.4.5 Glycaemic control and insulin sensitivity

Animal studies

While anthocyanin research in humans has increased dramatically in the past decade, previously, animal studies have instead been used to investigate the chronic effects of anthocyanins. A marked increase in adipose tissue mass is often associated with increased low-grade systemic inflammation, which can lead to the development of insulin resistance. Moreover, it is well documented that improvements in low-grade systemic inflammation can offset (and even reverse) the negative cascade caused by insulin resistance and restore normal glucose dynamics. Studies in mice fed a typical chow or high fat diet combined with or without cyanidin-3-glucoside-rich purple corn colour for 12 weeks found dietary purple corn colour significantly suppressed the high fat diet-induced weight gain as well as increases in white and brown adipose tissue mass (Tsuda, Horio et al. 2003). Furthermore, the high fat diet-induced hyperglycaemia, hyperinsulinemia and hyperleptinemia were completely normalised in the group who were supplemented with purple corn colour. In another study, male C57BL/6J mice who were fed a modified diet containing high fat/cholesterol diet supplemented with blackcurrant extract displayed significantly lower adipocyte size

and reduced inflammatory markers compared to high fat/cholesterol diet control group (Benn, Kim et al. 2014). Alongside the potential effect on adipose tissue mass and inflammation, cyanidin-3 glucoside and delphinidin-glucoside were found to be effective insulin secretagogues, while pelargonidin-3-galactoside caused a 1.4-fold increase in insulin secretion (Jayaprakasam, Vareed et al. 2005). This was expanded further by Tani, Nishikawa et al. (2017) who orally administered a blackcurrant extract high in delphinidin-3-glucoside to Sprague-Dawley rats before a intraperitoneal glucose injection, and demonstrated a suppressed rise in plasma glucose after 30 and 60 min, and an increase in plasma insulin at 15 and 30 min which was attributed to an increase in glucagon-like peptide-1 (GLP-1). Interestingly, delphinidin-3-glucoside did not significantly degrade in the intestinal tract for at least 45-60 min post blackcurrant ingestion, indicating that the GLP-1 mediating effect was likely due to delphinidin-3-glucoside and not its degradation products.

AMPK is a metabolic sensor which upregulates catabolic pathways and regulates GLUT4 expression or translocation to the plasma membrane through an insulin independent mechanism. Furthermore, pharmacological activators of AMPK (such as metformin) have widely been used as a means of upregulating skeletal muscle transport into peripheral tissue without a concomitant increase in insulin. Takikawa, Inoue et al. (2010) administered dietary bilberry extract for 5 weeks to male KK-A^y mice and found an increase in total AMPKα and phosphorylation of AMPKα at Thr172 in white adipose tissue (WAT) and skeletal muscle, along with a 2.1-fold increase in GLUT4 protein expression. These findings were repeated in Iizuka, Ozeki et al. (2018) in which 7 weeks blackcurrant extract in mice was capable of both upregulating and increasing phosphorylation of AMPKα at Thr172 leading to an increase in GLUT4

protein expression in the plasma membrane. Therefore, it is becoming evident that anthocyanin may be able to influence glucose kinetics through an increase in insulin secretion alongside an increase in glucose uptake through both insulin dependent and independent pathways.

1.4.6 Acute studies in humans

In the past decade, there has been a substantial increase in the number of studies investigating the acute effects on anthocyanin on glucose control. In this respect, Torronen, Sarkkinen et al. (2010) administered a 150 g berry/35 g sucrose (containing 37.5 g blueberry, cranberry and strawberry) puree or a placebo control load to 12 healthy participants and found a reduction in glucose concentrations at 15 and 30 min post-ingestion. However, in this study blood glucose concentrations were greater at 150 min post-consumption, meaning that the anthocyanin-rich puree did not affect the overall postprandial glycaemic response. Similarly, when men and postmenopausal women were provided a low sugar fruit drink containing blackcurrant extract providing 150 mg, 300 mg, 600 mg anthocyanins or a no blackcurrant control, early plasma glucose concentrations were significantly reduced following the highest anthocyanin dose (600 mg) relative to the control, but again this did not manifest as an overall decrease in postprandial glucose response (Castro-Acosta, Smith et al. 2016). Additionally, plasma insulin, and the incretins glucose-dependent insulinotropic polypeptide (GIP) and GLP-1, were also reduced following the high anthocyanin dose at 90 min post-ingestion (Castro-Acosta, Smith et al. 2016). The same research team then tested a 1200 mg apple polyphenol and 600 mg apple

polyphenol/600 mg blackcurrant anthocyanin test drink to a high carbohydrate meal and found both drinks improved area under the curve (AUC) glucose, $AUC_{insulin}$, C-peptide concentrations and lowered plasma GIP concentration relative to a control (Castro-Acosta, Stone et al. 2017). Interestingly, while $AUC_{glucose}$ was reduced in both apple polyphenol and apple polyphenol/blackcurrant anthocyanin at 0-30 min, only the latter combined test drink caused a further reduction at 0-120 min indicating a potential synergistic effect between polyphenols in mediating glucose absorption. A limitation of these studies is the use of an oral glucose tolerance test to investigate the effectiveness of anthocyanin on glucose control, because while an oral glucose tolerance test is a convenient clinical measure, it does not reflect a typical mixed meal, which includes carbohydrate, fat and protein. To address this, Edirisinghe, Banaszewski et al. (2011) provided a high carbohydrate-moderate fat meal accompanied by either a strawberry or placebo beverage to overweight men and women and found that while plasma glucose concentrations were unchanged, postprandial inflammatory markers (IL-6 and C-reactive protein) were reduced and this was associated with a reduction in postprandial insulin response. Therefore, while initial research indicates a potential beneficial effect of acute anthocyanin supplementation on postprandial glycaemia, further research is warranted particularly in determining optimal dosing strategies.

1.4.7 Chronic studies in humans

Compared with the number of studies investigating the effect of anthocyanin on glucose control in humans, there are a limited number of studies investigating the

chronic effects of anthocyanin supplementation. Due to the known accumulation of anthocyanin metabolites 48 h following ingestion of anthocyanin-rich foods/extracts (Kay, Mazza et al. 2005), it is possible that the positive glycaemic effects of anthocyanin supplementation are increased following chronic intake. Zhu, Ling et al. (2013) investigated the effect of 320 mg purified anthocyanin (cyanidin-3-O-β-glucoside and delphinidin-3-O-β-glucoside) capsule (two 80 mg capsule twice daily) on 150 subjects with hypercholesterolemia and found significant reductions in C-reactive protein and plasma IL-1β, both inflammatory markers associated with insulin resistance. Currently, evidence surrounding chronic anthocyanin supplementation is limited with Stull, Cash et al. (2010) determining that 6 weeks ingestion of a twice daily high anthocyanin blueberry smoothie improved insulin sensitivity (measure via a hyperinsulinaemic euglycemic clamp) with no concomitant change in adiposity, indicating an improvement in insulin dependent or independent mechanisms. Furthermore, ingestion of 7 days of 6 g/day blackcurrant powder dissolved in water before undertaking an oral glucose tolerance test improved both plasma glucose (8% lower at 60 min) and insulin (18% lower at 30 min and 39% at 60 min), which resulted in a decrease in AUC for both glucose and insulin (Willems, Silva et al. 2017). Whilst this study provides proof-of-principle evidence for a positive effect of blackcurrant extract on postprandial glucose control, it was only conducted in relatively healthy individuals. Furthermore, the product itself has a limitation in its current form, given the requirement of dissolving the blackcurrant powder in water to consume it. Therefore, more convenient methods of supplementation that are more amenable should be investigated, especially due to current difficulties in long-term participant retention to supplementation interventions.

1.5 Thesis aims

While epidemiological data alongside *in vitro* studies have helped to determine the potential benefits of anthocyanin supplementation, alongside determining some of the potential mechanisms surrounding this effect, current research into the effectiveness of anthocyanin supplementation on at-risk populations, particularly under 'free-living' conditions are limited. Using a high-anthocyanin supplement (New Zealand blackcurrant [NZBC] extract), the aim of this thesis is to 1) determine the effect of NZBC at different doses on acute blood glucose control after consumption of a mixed meal, and 2) examine whether NZBCs supplementation is a viable means of modulating postprandial hyperglycaemia in free-living conditions. Given the link between overweightness/obesity and the development of T2D, these studies will be conducted in overweight/obese sedentary individuals. Ultimately, it is the aim that these studies will provide the basis for NZBC supplementation to become one new strategy with which to improve glycaemic control and therefore limit the progression of obesity to T2DM.

Chapter 2

A single bolus of New Zealand blackcurrant extract does not improve postprandial blood glucose and triglyceride responses to a carbohydrate-fat meal in sedentary, overweight individuals.

2.1 Abstract

Consumption of low-quality, high-energy diets in combination with a sedentary lifestyle have made obesity and type 2 diabetes mellitus (T2DM) into worldwide epidemics. Regular consumption of flavonoids (such as anthocyanins) is associated with reduced T2D risk. 7 day high-anthocyanin blackcurrant extract supplementation has been shown to improve glycaemic responses to a glucose challenge in healthy individuals. We examined whether a single bolus of New Zealand blackcurrant extract (NZBC) can improve glucose and triglyceride responses to a mixed-meal in sedentary overweight individuals and whether a dose-response relationship exists. In a double-blind, randomised, cross-over, placebo-controlled design, 13 sedentary overweight office workers (10 male, 3 female, 30±10 y, BMI: 27.6±1.3 $kg \cdot m^{-2}$, ingested a single dose of NZBC extract (300, 600, 900 mg) or a visibly identical placebo (PLC) 30 minutes prior to consuming a mixed-meal test drink (75g carbohydrate, 50g fat). Intermittent blood sampling was undertaken for 3 h postprandial, with samples analysed for both glucose and triglyceride concentrations. Postprandial fasting glucose and triglyceride concentrations were similar between conditions, and peaked at 90 min and 120 min, respectively. There was no significant difference for glucose AUC (P=0.918) or triglyceride AUC (P= 0.834) between doses or conditions. A single bolus of NZBC extract is unable to positively influence postprandial blood glucose or triglyceride responses to a carbohydrate-fat meal in overweight and obese individuals, irrespective of anthocyanin dose. The inclusion of fat in the meal tolerance test alongside the limited bioavailability of anthocyanins may have blunted any potential postprandial benefits of acute NZBC extract ingestion, therefore more chronic doses of NZBC extract may be more beneficial.

2.2 Introduction

Recent estimates suggest that nearly two-thirds of adults (63%) in England are currently classified as overweight or obese (Public Health England 2017). This can be attributed to lifestyles in which sedentary behaviour and overconsumption of hypercaloric foods are commonplace, leading to increased levels of obesity. Although sedentary behaviour and obesity are often interlinked, prolonged sitting is associated with premature cardiovascular and all-cause mortality independent of physical activity and adiposity (Tremblay, Colley et al. 2010). Therefore, office-workers are particularly at risk due to the large periods of sedentarism present throughout the working day. Both sedentary and overweight/obese individuals exhibit elevated postprandial blood glucose and triglyceride responses (Mekki, Christofilis et al. 1999, Dunstan, Kingwell et al. 2012), which increases the risk of developing insulin resistance (Cavalot, Petrelli et al. 2006). Indeed, a characteristic of individuals with T2DM is an elevated postprandial glycaemic response to each meal, with some estimates suggesting they may spend as much as 38% of the day in a hyperglycaemic state (9 h 10 min) (van Dijk, Manders et al. 2011). Therefore, the development of novel strategies to improve the postprandial glycaemic response remains an important area of research.

Anthocyanin is a flavonoid subclass commonly characterised by its bright blue/red/purple pigmentations and is therefore found in high concentrations in blackcurrant, blueberry, blackberry and cherries. Epidemiological studies demonstrate that higher habitual anthocyanin intakes are associated with a reduced

risk of cardiovascular disease, hypertension, cancer and T2DM (Knekt, Kumpulainen et al. 2002, Lala, Malik et al. 2006, Wallace 2011, Jennings, Welch et al. 2012). While all fruits containing anthocyanin show some ability to suppress the activity of enzymes regulating carbohydrate digestion and uptake from the gut, fruits with the highest anthocyanin concentration exhibit the largest suppressive effect (McDougall, Shpiro et al. 2005), indicating a potential dose-response relationship exists. The mechanisms surrounding this modulating effect are suggested to be due to both an inhibition of pancreatic α-amylase (Akkarachiyasit, Yibchok-Anun et al. 2011) and intestinal α-glucosidase enzymes (McDougall, Shpiro et al. 2005), alongside a disruption of sodium-dependent glucose transporter-1 (SGLT1) and glucose transporter-2 (GLUT-2)-mediated intestinal glucose transport (Alzaid, Cheung et al. 2013). While *in vitro* studies highlight the potential mechanistic effects of anthocyanins on carbohydrate digestion and absorption, few studies have investigated its effectiveness *in vivo*. Two studies have reported that acute consumption of mixed berry purees (containing bilberries, blackcurrants, cranberries and strawberries) high in anthocyanins are effective in delaying the early postprandial glucose spike (30-45 min post-carbohydrate ingestion), although the overall postprandial response was not affected (Torronen, Sarkkinen et al. 2010, Torronen, Kolehmainen et al. 2012). Blackcurrant (*Ribes nigrum*) has one the highest known concentrations of the anthocyanins delphinidin-3-rutinoside, delphinidin-3-glucoside, cyanidin-3-rutinoside and cyanidin-3-glucoside, equal to approximately 160-1300 mg.100g^{-1} fresh weight (Scalzo, Currie et al. 2008). In this regard, Castro-Acosta, Smith et al. (2016) reported that ingestion of a sugar free drink containing a blackcurrant extract prior to consuming a carbohydrate-rich meal improved the postprandial glucose, insulin and incretin

responses in a dose-dependent manner, with the highest blackcurrant dose (600 mg anthocyanin) showing a greater modulating effect than a medium or low dose (300 and 150 mg anthocyanin, respectively). Therefore, while there is evidence that acute blackcurrant extract consumption is capable of modulating glycaemic responses to carbohydrate ingestion (Castro-Acosta, Smith et al. 2016), it is less clear whether improved glycaemic control continues with the inclusion of protein and/or fat in the meal. This is important, because a mixed meal is more reflective of what is usually consumed under 'real-world' conditions, in which dietary fat delays the digestion and absorption of carbohydrate within the meal. If including fat in a meal tolerance test, the triglyceride response of a meal is then also an important factor to consider given that postprandial triglyceride handling is directly related to cardiovascular disease risk (Trombold, Christmas et al. 2013).

To this end, the primary aim of this study was to investigate whether a single bolus of New Zealand blackcurrant (NZBC) extract (high in anthocyanins) can improve postprandial glucose and triglyceride responses to a carbohydrate-fat meal. A secondary aim was to determine whether a dose-response relationship existed in the postprandial glucose and triglyceride responses. We hypothesised that NZBC extract would improve both glucose and triglyceride postprandial responses to a carbohydrate-fat meal, with the largest dose showing the greatest glucose and triglyceride-lowering effect.

2.3 Methods

Subjects

13 sedentary, overweight participants (see Table 1 for subject characteristics) volunteered to take part in the study, which was approved by the Liverpool John Moores University Research Ethics Committee. Written, informed consent was obtained following an explanation of the experimental procedures. Participants were deemed to be inactive if they undertook <1 h structured physical activity per week (in the preceding 6 months). All participants were absent of any other metabolic co-morbidities and cardiovascular disease.

Table 1 Participant characteristics (*n* = 13)

M/F	10/3
Age (y)	30 ± 10
Height (m)	1.75 ± 0.10
Weight (kg)	83.6 ± 6.4
BMI ($kg \cdot m^{-2}$)	27.6 ± 3.3
Lean mass (kg)	64.0 ± 7.2
Fat mass (kg)	25.5 ± 5.6
Daily anthocyanin intake ($mg \cdot day^{-1}$)	30 ± 25

Values are means ± SD

Experimental design and protocol

Participants initially visited the University laboratory where height and weight were measured, alongside body composition using electrical bio-impedance (Tanita BC 418 MA Segmental Body Composition Analyser, Tanita, Japan). Participants then visited the laboratory to undergo an experimental trial on 4 separate occasions. Twenty-four hours prior to each experimental trial participants consumed a standardised diet (50% carbohydrate, 30% fat, 20% protein) that was otherwise matched to habitual intake.

On the morning of each experimental trial, participants attended the laboratory following an overnight fast (> 10 h) and first consumed a standardised breakfast (25% daily caloric intake) before working at a computer or sitting quietly for 2-3 hours. In a randomised, double-blind crossover design, participants then ingested either NZBC extract (300, 600, 900 mg) or a visibly identical placebo with water 30 min prior to lunch. Each 300 mg NZBC capsule contained 105 mg of anthocyanins, consisting of 35-50% delphinidin-3-rutinoside, 5-20% delphinidin-3-glucoside, 30-45% cyanidin-3-rutinoside, and 3-10% cyanidin-3-glucoside (CurraNZ™, Health Currancy Ltd, Surrey, UK). Each placebo capsule contained 300 mg microcrystalline cellulose. Following ingestion of NZBC or placebo, an indwelling cannula was placed into the antecubital vein of one arm and a blood sample was obtained. Thirty min following ingestion of NZBC or placebo, participants then consumed a carbohydrate-fat liquid test meal consisting of 75 g maltodextrin (MyProtein™, The Hut Group, Cheshire, UK) and 50 g unsaturated fatty acids (Calogen, Nutricia, Amsterdam, NL) (see Table 2 for nutritional breakdown of test drink). Blood samples were subsequently collected at 15 min intervals for the first hour and 30 min intervals for the remaining two hours. Once the testing procedure was completed the cannula was removed and participants were able to leave the laboratory. All experimental trials were separated with a washout period of 7 days.

Table 2 Nutritional information of test drink

Average contents	Per 100 ml
Energy (Kj)	1850
Protein (g)	0
Carbohydrate (g)	0.1
of which sugars (g)	75.1
Fat (g)	50
Saturates (g)	5.3
MUFA (g)	30.4
PUFA (g)	14.3
%LCT (%)	100
*n*6: *n*3 (ratio)	5:1
Dietary fibre (g)	0

monosaturated fatty acid (MUFA), polyunsaturated fatty acid (PUFA), % long chain triglyceride (%LCT), omega 6: omega 3 (*n*6: *n*3).

Habitual dietary intake and anthocyanin consumption

Habitual dietary intake was assessed using a written diary for 72 h (see table 3 for macronutrient and energy intake). Food diaries were analysed for total energy intake and macronutrient composition of the diet. At the first visit, participants also competed a food frequency questionnaire which listed the quantity and frequency of anthocyanin-containing foods and drinks compiled from the Phenol Explorer database (Neveu, Perez-Jimenez et al. 2010). By multiplying the anthocyanin content of the portion size by the total consumption frequency of each food, daily anthocyanin intake was calculated.

Table 3 Daily absolute and relative macronutrient and energy intake via 72 h self-reported diet diary

Carbohydrate	
G	242 ± 42
g kg body mass^{-1}	2.6 ± 0.6
Protein	
g	95 ± 12
g kg body mass^{-1}	1.0 ± 0.1
Fat	
g	98 ± 27
g kg body mass^{-1}	1.0 ± 0.2
Total energy intake	
kJ	9306 ± 1248
kJ kg body mass^{-1}	98.6 ± 12.2

Values are means ± SD

Blood sample analysis

Plasma samples were obtained through centrifugation (10 min at 3000g at 4°C) and stored at –80°C for subsequent analysis. Plasma glucose and triglyceride concentrations were determined spectrophotometrically using a semi-automatic analyser in combination with commercially available kits (Randox Laboratories, Antrim, UK). Each sample was analysed in duplicate.

Data and statistical analysis

All data are expressed as means ± SD. Statistical significance was set at the 0.05 level of confidence. The area under the curve (AUC) for glucose and triglyceride was calculated using the trapezoid method. AUC and time-dependent changes were investigated using a two-way within-subjects ANOVA, with the factors 'time' and 'condition'. Significant main effects or interactions were assessed using Bonferroni adjustment *post hoc* analysis.

2.4 Results

Plasma glucose

There was a main effect of time for glucose concentration during the carbohydrate-fat tolerance test (P<0.001). Glucose peaked at 60 min in the placebo and 300 mg trial, and peaked at 90 min in the 600 and 900 mg, before decreasing to baseline levels by 150 min, with no difference between trials (P=0.872). Similarly, there was no difference in baseline (P=0.471) or peak glucose concentration (P=0.495) between conditions. There was also no interaction effect between time and condition (P= 0.843). $AUC_{glucose}$ was not significantly different between conditions (P= 0.982).

Plasma triglyceride

There was no main effect of time for triglyceride concentration during the carbohydrate-fat tolerance test across all doses (P=0.168). In all trials, triglyceride concentration peaked at 120 min, with no difference between trials (P= 0.210). Additionally, there was no difference in triglyceride baseline (P= 0.550) or peak (P= 0.808). There was also no interaction effect between time and condition (P= 0.513). $AUC_{triglyceride}$ was not significantly different between doses (P=0.834).

Bivariate correlation analysis was performed to investigate whether a relationship between habitual anthocyanin intake and $AUC_{glucose}$ (300 mg: r= -0.135, P= 0.660; 600 mg: r= -0.421, P= 0.152; 900 mg: r= -0.156, P= 0.612) and $AUC_{triglyceride}$ (300 mg: r= -0.072, P= 0.833; 600 mg: r= -0.286, P= 0.395; 900 mg: r= -0.169, P= 0.620), but no relationships were observed for either variable.

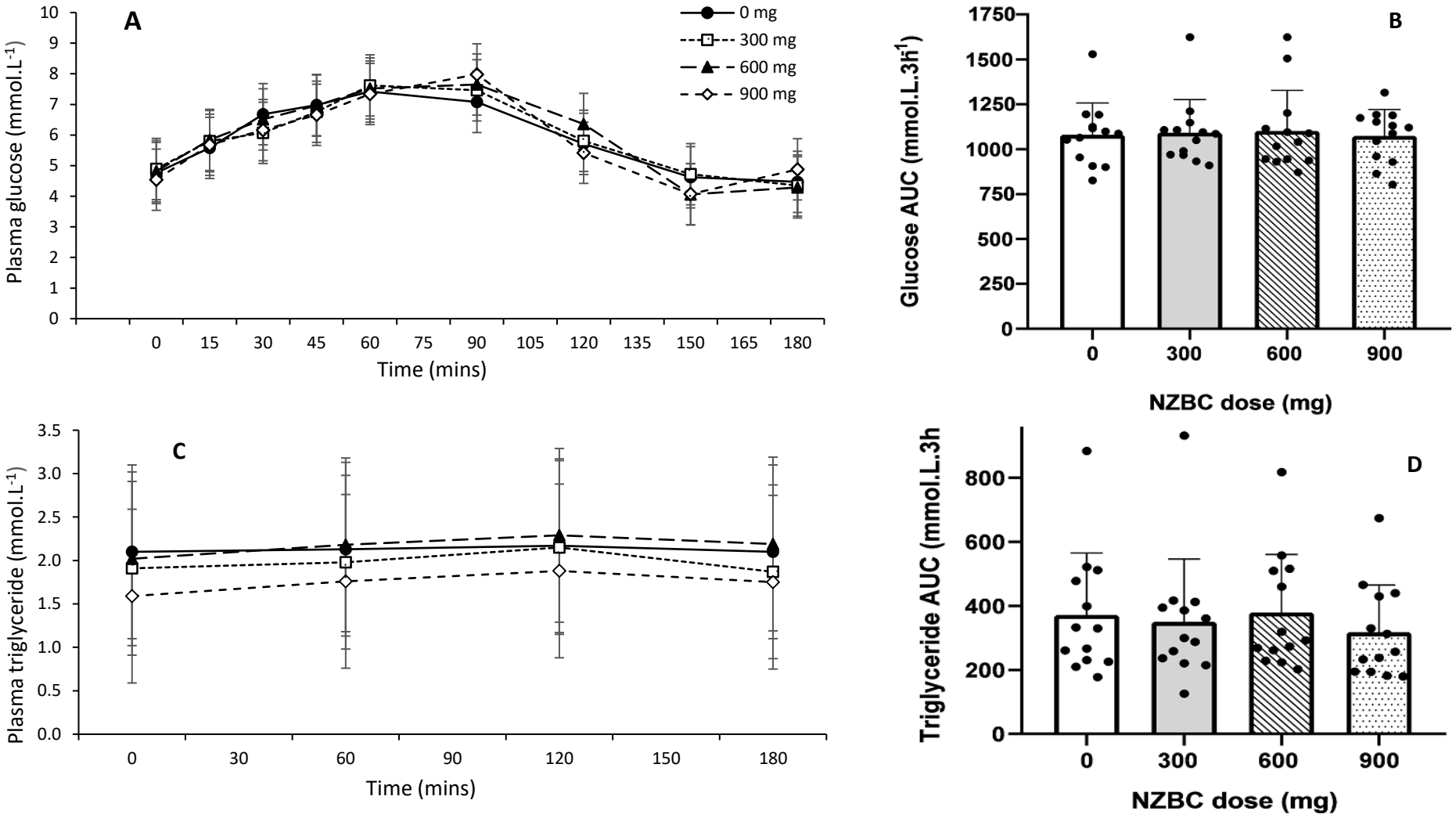

Figure 1. (A) Plasma glucose responses over 3 h postprandial period. (B) Overall postprandial glucose AUC response. (C) Plasma triglyceride responses over 3 h postprandial period. (D) Overall postprandial triglyceride AUC response.

2.5 Discussion

The aims of this study were two-fold; 1) to determine whether ingestion of a single bolus of NZBC extract could improve postprandial glucose and triglyceride responses to a carbohydrate-fat liquid test meal, and 2) whether a dose-response relationship existed. The novel finding from this study was that a single bolus of NZBC extract in overweight, sedentary participants did not alter the postprandial glucose and triglyceride responses to a carbohydrate-fat test meal. Moreover, this remained true across three NZBC doses with increasing anthocyanin content.

Our observation that acute ingestion of an anthocyanin-rich extract/foodstuff is unable to influence postprandial glucose responses could be considered to run contrary to several other studies. For example, (Torronen, Sarkkinen et al. 2010) provided healthy participants with a drink containing 35g sucrose in combination with or without 150g of berry puree (containing bilberries, blackcurrants, cranberries and strawberries), and found that inclusion of the berry puree reduced plasma glucose concentrations at 15 and 30 min, which is indicative of a delayed postprandial response. However, in the same study plasma glucose concentrations were significantly higher at 150 min following ingestion of the sucrose and berry puree drink meaning that overall there was no reduction in glucose AUC between conditions (Torronen, Sarkkinen et al. 2010). Similarly, when men and postmenopausal women were provided with blackcurrant extract providing either 150mg, 300mg and 600mg total anthocyanin's or placebo, early plasma glucose concentrations (10-30 min) to glucose ingestion were significantly reduced following the high anthocyanin dose relative to placebo, but this was followed by a rebound (at 75 min) meaning that no

overall difference in glucose AUC was observed (Castro-Acosta, Smith et al. 2016). In the present study, we found that peak glucose occurred much later in the 600 and 900 mg (90 min) than the placebo and 300 mg condition, however this difference was non significant. Furthermore, the peak at 90 min was much later than previously reported in the literature, which is likely due to the inclusion of fat in the liquid test meal. Indeed, it is well documented that fat ingestion inhibits gastric emptying and digestion and subsequent diffusion across the intestinal membrane via a contraction of the pyloric sphincter region (Quigley 1941). Moreover, this effect likely explains why the results of this study do not support a glucose-lowering effect of anthocyanins previously reported in the literature. Considering the mechanisms surrounding the ability of anthocyanins to regulate glucose metabolism, it is believed that anthocyanins delay glucose digestion via a reduction in α-amylase (Akkarachiyasit, Yibchok-Anun et al. 2011) and α-glucosidase activity (Matsui, Ueda et al. 2001, McDougall, Shpiro et al. 2005, Tadera 2006), alongside an inhibition of glucose transport along the intestinal membrane through SGTL1 and GLUT-2 (Faria, Pestana et al. 2009, Alzaid, Cheung et al. 2013). Interestingly however, despite some evidence demonstrating an ability for anthocyanins to improve blood flow responses via increased glucose delivery to the peripheral tissue and improvements in blood pressure responses (Martineau, Couture et al. 2006, Asgary, Sahebkar et al. 2014), this did not seem to influence glycaemic control in this instance. Therefore, the observed delay in postprandial peak glucose concentrations in this study are likely related to the inclusion of fat in the test meal, and as a result mask any anthocyanin-mediated improvements which tend to occur in the early-postprandial phase. It is also possible that the suppression of glucose digestion and absorption by fat simply overrides the

reported glucose-lowering effects of anthocyanin, which also may explain why no effect of NZBC extract on postprandial glucose responses was observed.

It should be noted that some studies have demonstrated that anthocyanin supplementation is capable of influencing time-dependent changes in postprandial insulin, like that observed for plasma glucose (reduction at 15-45 min with a rebound at ~90 min) (Torronen, Sarkkinen et al. 2010, Torronen, Kolehmainen et al. 2012). Interestingly however, Edirisinghe, Banaszewski et al. (2011) provided a high carbohydrate, medium fat meal accompanied by either a strawberry anthocyanin beverage or a placebo and while there was no difference in postprandial serum glucose responses there was an improvement in insulin concentrations. According to the authors, this was perhaps due to a reduction in inflammatory markers (high sensitive C-reactive protein and IL-6) as the insulin signalling cascade is highly sensitive to the redox balance of the cell. Importantly though, these results suggest that anthocyanin is potentially capable of lowering insulin secretion independent of a decrease in plasma glucose.

Currently, there is limited evidence on the effects of anthocyanins on postprandial triglyceride responses, largely since a direct mechanism for anthocyanins to mediate triglyceride digestion and absorption is yet to be identified. However, the postprandial triglyceride response is a predictor for cardiovascular disease incidence, nonfatal myocardial infarction, ischemic stroke and fatal cardiovascular events (Zilversmit 1979, Bansal, Buring et al. 2007), and therefore it is important to investigate whether strategies that target a reduction in postprandial glucose responses also attenuate the

transient postprandial increases in plasma triglyceride concentrations. Pancreatic lipase is an important enzyme in the breakdown of dietary fat and is responsible for 50-70% of the hydrolysis of dietary triglyceride (Mukherjee 2003). There is emerging evidence that anthocyanin (particularly cyanidin) may competitively inhibit pancreatic lipase and cause a reduction in dietary fat absorption (You, Chen et al. 2011, Fabroni, Ballistreri et al. 2016). However, this is not ubiquitous as McDougall (2009) found anthocyanin was unlikely to inhibit pancreatic lipase due to fruits high in anthocyanin (including blackcurrant) showing no inhibitory effect and that perhaps the inhibition displayed in other fruits (including cloudberry, raspberry and strawberry) involved a synergistic interaction between phytonutrients (such as tannins and ellagitannins) due to the use of whole plant extracts. Furthermore, some studies have demonstrated that a high polyphenol intake does not increase faecal lipid excretion, as would be expected if lipase was inhibited and that perhaps any inhibition is offset via a compensatory increase in lipase secretion (Griffiths 1986, Tsuda 2008). Therefore, further work is required to confirm if bioactives can inhibit lipase *in vivo* and whether they are a viable method of preventing weight gain.

While epidemiological evidence suggests that polyphenol consumption is associated with improved arterial health (Jennings, Welch et al. 2012), reduced cardiovascular disease risk (Cassidy, O'Reilly et al. 2011) and reduced T2DM risk (Knekt, Kumpulainen et al. 2002, Wedick, Pan et al. 2012, Mursu, Virtanen et al. 2014), these effects may manifest after prolonged anthocyanin exposure, thereby making acute doses less effective. Kay, Mazza et al. (2005) found that anthocyanin metabolites were present in the blood 48 h following ingestion of cyanidin 3-glycosides from chokeberry extract,

highlighting that there is potential for a residual increase in serum anthocyanin with habitual intake that is not observed with acute ingestion. Furthermore, while *in vitro* experiments have allowed us to determine the potential mechanisms by which anthocyanin can modulate digestion and absorption of carbohydrate, it should be noted that these responses may not manifest *in vivo* since anthocyanin bioavailability is very low (~12%) (Czank, Cassidy et al. 2013). However, the metabolism of anthocyanin is suggested to involve a number of biotransformations with studies indicating cyanidin-3-glucoside (C-3-G) is associated with 17 compounds in the serum during digestion, including protocatechuic acid (PCA), phloroglucinaldehyde (PGA), 13 downstream metabolites of PCA and 1 metabolite derived from PGA (de Ferrars, Czank et al. 2014). Furthermore, the half life of the anthocyanin metabolites are much greater than parent anthocyanin with ferulic acid (metabolite derived from PGA) displaying a much greater serum half life (~96 hr) compared to the parent anthocyanin C-3-G (0.4 hr). Interestingly, anthocyanin metabolites have been shown to directly interfere with mechanisms of vascular function with vanillic acid (metabolite derived from PCA) being shown to NO synthesis (Edwards, Czank et al. 2015). Taken together this suggests that the magnitude and duration of exposure is important, with some evidence suggesting duration is more important than dose (Hassellund, Flaa et al. 2013), further highlighting the potential benefits of chronic supplementation. In support, Willems, Silva et al. (2017) recently investigated the effect of 7 days supplementation with NZBC powder (6 $g.day^{-1}$ NZBC powder containing 138.6 mg anthocyanin) on plasma glucose and insulin response to an oral glucose tolerance test. They reported that after 7 days, NZBC powder lowered plasma glucose at 60 min post-glucose ingestion, and plasma insulin at 30 and 60 min, as well as reducing both

$AUC_{glucose}$ and $AUC_{insulin}$ over the 2 h postprandial period. While a single bolus of NZBC extract may only delay the postprandial glycaemic response, more chronic supplementation may well alter the overall response, thereby highlighting the potential for NZBC to improve insulin sensitivity.

The strength of this study relates to its design which included the use of a carbohydrate-fat test drink which is more indicative of what would be habitually consumed under free-living conditions. Furthermore, the inclusion of overweight/obese individuals is important due to their obesity related changes in glucose kinetics as well as being an important target demographic for health and lifestyle interventions. A limitation of this study was that anthocyanin intake was determined by a food frequency questionnaire and self-reporting errors may well have occurred, causing a discrepancy. However, calculated habitual anthocyanin intake was found to be ~30 mg per day, indicating that habitual intake was well below even the lowest anthocyanin dose (105 mg) provided in this study.

In conclusion, acute NZBC ingestion is unable to alter postprandial glucose or triglyceride responses to a carbohydrate-fat meal in sedentary, overweight individuals. In this instance, the inclusion of dietary fat in the test meal likely overrides the previously reported glucose-lowering effect of anthocyanins. Future studies should now aim to determine the effects of postprandial insulin responses to a mixed meal as 600 mg anthocyanin has been shown to influence insulin dynamics via a reduced plasma insulin, plasma GIP and plasma GLP-1 to an OGTT (Castro-Acosta, Smith et al. 2016). Therefore, whether these responses manifest in mixed meals may

highlight whether anthocyanin can be a viable supplement in improving insulin secretion. Furthermore, more chronic supplementation of NZBC extract on postprandial glucose and triglyceride responses in free-living conditions may determine the usefulness of anthocyanin as a dietary intervention.

Chapter 3

8 days supplementation with New Zealand blackcurrant extract improves free-living glycaemic control and insulin sensitivity in sedentary, overweight individuals

3.1 Abstract

Prolonged periods of postprandial hyperglycaemia are shown to be an independent risk factor for the development of T2DM. Short-term anthocyanin supplementation has been shown to improve glycaemic control, however currently all evidence has been conducted using laboratory-based control drinks. In a double-blind, randomised, placebo-controlled design, 12 sedentary, overweight, office workers (6 male, 6 female, 28 ± 9 yr, BMI 29.9 ± 4.8), ingested 8 days of NZBC extract (600 $mg.d^{-1}$) or a visibly matched placebo before undertaking a 2 h OGTT where glucose and insulin concentrations were determining via intermittent blood sampling. Participants also wore a continuous glucose monitoring system (CGMS) and consumed a 24 h standardised diet under free-living conditions where interstitial glucose excursions were determined. Postprandial glucose and insulin were similar between conditions. Following NZBC ingestion plasma glucose was lower at 45, 60 and 90 min with an 8% reduction in $AUC_{glucose}$ (P<0.001) There was no time effect for insulin (P=0.226), however $AUC_{insulin}$ was 14% lower (P=0.018). Free-living glucose excursions were lower during breakfast (9%; P=0.010) and lunch (8%; P=0.02), with no difference at dinner (P=0.643). There was no difference in HOMA-IR (P=0.413), hepatic (P=0.430) or peripheral insulin resistance (P=0.426), however Matsuda index was 22% higher following NZBC ingestion (P=0.011). Short-term NZBC extract supplementation can enhance postprandial glucose and insulin responses to a glucose challenge and whole-body insulin sensitivity, as well as improving free-living glycaemic responses under standardised dietary conditions. Future work should establish the mechanism(s) by which these effects are induced by NZBC extract.

3.2 Introduction

In **chapter 1** it was explained that obese individuals and T2DM patients exhibit lower insulin sensitivity alongside greater postprandial glucose excursions, when compared to lean, healthy individuals. It was also highlighted that epidemiological evidence suggests higher anthocyanin intake is associated with lower T2DM risk (Jennings, Welch et al. 2012). Several studies have been able to demonstrate that acute ingestion of anthocyanin-rich foods or extracts improve the early postprandial glucose response to a glucose challenge (Torronen, Sarkkinen et al. 2010, Torronen, Kolehmainen et al. 2012). However, this is often offset by a later postprandial rise in glucose concentrations which means that the overall postprandial glucose response is unaffected (Torronen, Sarkkinen et al. 2010). Furthermore, in **chapter 2** it was observed that a single bolus of an anthocyanin-rich New Zealand blackcurrant (NZBC) extract was unable to mediate blood glucose and triglyceride responses to a carbohydrate-fat meal, independent of the dose used. As such, more chronic periods of anthocyanin supplementation may be required to convey desirable effects on postprandial glucose responses and insulin sensitivity. In support, Stull, Cash et al. (2010) found that 6 weeks of blueberry supplementation in obese, insulin resistant individuals improved insulin sensitivity during a hyperinsulinaemic euglycaemic clamp with no concomitant change in adiposity . More recently, Willems, Silva et al. (2017) reported that short-term (7 days) supplementation with NZBC powder reduces both postprandial glucose and insulin responses to a glucose challenge. However, this study was only conducted in healthy individuals, and therefore the effects of short-term

supplementation on individuals with lower insulin sensitivity, such as those who are overweight/obese, is currently unknown.

The effectiveness of most interventions aimed at improving glycaemic control were evaluated by using an OGTT. However, this is a clinical test which is undertaken in a controlled environment using a purely carbohydrate challenge. This is far removed from what is habitually consumed during meals under 'real-world' situations, whereby meals consist of a more mixed macronutrient content. Continuous glucose monitoring systems (CGMS) provide an opportunity to evaluate the effectiveness of an intervention on postprandial glucose responses under free-living conditions. Furthermore, continuous glucose monitoring systems (CGMS) have been found to be an effective and reliable way of measuring daily glucose excursions under free-living conditions (Rodbard 2017). To that end, we aimed to investigate whether 8-day supplementation of NZBC would improve glycaemic control in overweight/obese participants in response to an OGTT and under free-living conditions. We hypothesised that NZBC supplementation would improve both glucose and insulin responses to and OGTT and would improve glycaemic control in free-living conditions.

3.3 Methods

Subjects

12 sedentary, overweight participants (see Table 1 for subject characteristics) volunteered to take part in the study, which was approved by the Liverpool John Moores University Research Ethics Committee. Written, informed consent was obtained following an explanation of the experimental procedures. Participants were deemed to be inactive if they undertook <1 h structured physical activity per week (in the preceding 6 months). All participants were absent of any other metabolic co-morbidities and cardiovascular disease.

Table 1 Participant characteristics (*n* = 12)

M/F	6/6
Age (y)	28 ± 9
Height (m)	1.73 ± 0.10
Weight (kg)	88.9 ± 16.1
BMI ($kg \cdot m^{-2}$)	29.9 ± 4.8
Lean mass (kg)	62.1 ± 14.2
Fat mass (kg)	25.5 ± 12.8
Daily anthocyanin intake ($mg \cdot day^{-1}$)	15.3 ± 16.3

Values are means ± SD.

Experimental design

Participants initially visited the University laboratory where height and weight were measured, alongside body composition using electrical bio-impedance (Tanita BC 418 MA Segmental Body Composition Analyser, Tanita, Japan). After this initial visit, participants then undertook two supplementation periods in a randomised order, where they ingested either NZBC extract (two 300 mg capsules) or a visibly-identical

placebo with water, twice a day (one prior to breakfast and one before dinner) for 8 days. Each 300 mg NZBC capsule contained 105 mg of anthocyanins, consisting of 35-50% delphinidin-3-rutinoside, 5-20% delphinidin-3-glucoside, 30-45% cyanidin-3-rutinoside, and 3-10% cyanidin-3-glucoside (CurraNZ^{TM}, Health Currancy Ltd, Surrey, UK). Each placebo capsule contained 300 mg microcrystalline cellulose. Each supplementation period was separated by 14 days, which acted as a washout. Both participants and investigators were blinded to the condition. Participants were inserted with a Dexcom G4 platinum (Dexcom, San Diego, USA) and instructed to consume a standardised diet. Participants returned to the laboratory on day 7 following an overnight fast to undergo an oral glucose tolerance test (OGTT). Following collection of a fasted blood sample from an indwelling cannula placed in an antecubital forearm vein, participants consumed 75 g maltodextrin (MyProtein^{TM}, The Hut Group, Cheshire, UK) diluted in 220 ml of water. Further blood samples were collected at 15 min intervals for the first hour and 30 min intervals for the second hour. On day 8 participants undertook their usual daily activities, and thus postprandial glucose responses to breakfast, lunch and dinner were examined under free-living conditions using continuous glucose monitoring.

Continuous glucose monitoring

Participants were required to enter the laboratory on day 5 of supplementation where a Dexcom G4 Platinum continuous glucose monitoring (CGM) device (Dexcom, San Diego, USA) was inserted subcutaneously into the lower abdominal region. Participants were then instructed on how to use the device efficiently and then

allowed to exit the laboratory and the monitor would record glucose excursions under 'free-living' conditions. The monitor remained in place for the next 4 days, during which participants were provided with a standardised diet to consume (50% carbohydrate, 30% fat, 20% protein) that was otherwise matched to habitual energy intake and the remainder of day 5 and 6 served as the 'bedding in' period for the monitor. Participants were allowed to start consuming breakfast between 7-9 am, however there was a minimum 3 hr period between meals ensuring an uninterrupted postprandial period. Furthermore, the standardised diet was to be consumed in 3 complete meals (see table 2). Participants were required to enter the laboratory on day 7 however food consumption remained the same as that of the free-living days.

Table 2 Relative macronutrient intake over free-living day

Breakfast CHO/FAT/PRO %	(73%/11%/14%)
Carbohydrate (g.kg body mass^{-1})	6.28 ± 1.13
Fat (g.kg body mass^{-1})	0.95 ± 0.17
Protein (g.kg body mass^{-1})	1.22 ± 0.22
Lunch CHO/FAT/PRO%	(54%/22%/22%)
Carbohydrate (g.kg body mass^{-1})	4.62 ± 0.83
Fat (g.kg body mass^{-1})	1.84 ± 0.33
Protein (g.kg body mass^{-1})	1.88 ± 0.33
Dinner CHO/FAT/PRO	(35%/45%/18%)
Carbohydrate (g.kg body mass^{-1})	3.98 ± 0.71
Fat (g.kg body mass^{-1})	5.06 ± 0.9
Protein (g.kg body mass^{-1})	1.88± 0.55

Values are means ± SD

Habitual dietary intake and anthocyanin consumption

Habitual dietary intake was assessed using a written diary for 72 h (see table 3 for macronutrient and energy intake). Food diaries were analysed for total energy intake and macronutrient composition of the diet. At the first visit, participants also competed a food frequency questionnaire which listed the quantity and frequency of anthocyanin-containing foods and drinks compiled from the Phenol Explorer database (Neveu, Perez-Jimenez et al. 2010). By multiplying the anthocyanin content of the portion size by the total consumption frequency of each food, daily anthocyanin intake was calculated.

Table 3 Daily absolute and relative macronutrient and energy intake via 72 h self-reported diet diary

Carbohydrate	
g	238 ± 71
g kg body mass^{-1}	1.8 ± 0.8
Protein	
g	96 ± 38
g kg body mass^{-1}	1.1 ± 0.4
Fat	
g	86 ± 36
g kg body mass^{-1}	1.0 ± 0.3
Total energy intake	
kJ	8991 ± 2204
kJ kg body mass^{-1}	101.7 ± 19.3

Values are means ± SD

Blood sample analysis

Plasma samples were obtained through centrifugation (10 min at 3000g at 4°C) and stored at –80°C for subsequent analysis. Plasma glucose concentrations were

determined spectrophotometrically using a semi-automatic analyser in combination with commercially available kits (Randox Laboratories, Antrim, UK). Plasma insulin concentrations were determined via a commercially available enzyme linked-immuno-sorbent-assay (ThermoFisher Scientific, UK). Each sample was analysed in duplicate.

Data and statistical analysis

All data are expressed as means ± SD. Statistical significance was set at the 0.05 level of confidence. The area under the curve (AUC) was calculated using the trapezoid method. From the CGMS data obtained on day 8 the 3 h AUC was calculated for each meal. Additionally, average glucose was calculated for the entire 24 h period alongside average glucose during the day (0600-0000) and during the nocturnal period (0000-0600).

Glucose and insulin AUC was calculated during the OGTT using HOMA IR:

Fasting glucose x fasting insulin / 22.5

Additionally, Matsuda index of whole-body insulin sensitivity was also calculated (Matsuda and DeFronzo 1999) using the following equation:

(10,000/√[fasting glucose x fasting insulin] x [mean glucose x mean insulin during OGTT])

Using the equations proposed by Abdul-Ghani et al. (2007), we also estimated hepatic and peripheral insulin sensitivity, as follows:

Hepatic insulin sensitivity was calculated as:

$$glucose_{0–30[AUC]} \times insulin_{0–30[AUC]}$$

Peripheral insulin sensitivity was calculated as:

$$(dG/dt)/ \text{mean plasma insulin}$$

where dG/dt is the rate of decline in plasma glucose concentration and is calculated as the slope of the least square fit to the decline in plasma glucose concentration from peak to nadir.

Time-dependent changes were determined using a two-way within-subjects ANOVA, using the factors 'time' and 'condition'. Differences in AUC between conditions were investigated using a paired samples T-test. Significant main effects or interactions were assessed using Bonferroni adjustment *post hoc* analysis. One oral glucose tolerance response did not generate the typical 'bell-shaped' time-course and therefore this participant was omitted from the OGTT analysis. Therefore, all OGTT data is presented for n=11.

3.4 Results

Oral glucose tolerance test

Plasma glucose

Fasting plasma glucose concentrations were not different between conditions (P=0.634) (see figure 1). There was a main effect of time for glucose during the OGTT (P<0.001), with glucose concentrations peaking after 45 min in both conditions and returning to baseline by 120 min. Moreover, a significant interaction was observed

(*P*=0.005), such that glucose concentrations were significantly lower after NZBC supplementation, with post hoc analysis revealing significant reductions at 45 min (*P*=0.003), 60 min (*P*=0.001) and 90 min (*P*=0.008). $AUC_{glucose}$ was 8% lower after NZBC supplementation compared to placebo (*P*<0.001).

Plasma insulin

Fasting plasma insulin concentrations were not different between conditions (*P*=0.226) (see figure 1). There was a main effect of time for insulin during the OGTT (*P*=0.002), with insulin concentrations peaking at 60 min in both conditions. However, no significant interaction was observed (*P*=0.696). $AUC_{insulin}$ was 14% lower after NZBC supplementation compared to placebo (*P*=0.018).

Markers of insulin sensitivity

There was no significant difference in HOMA-IR between NZBC and placebo conditions (*P*=0.413). Matsuda insulin sensitivity index was 22% higher following NZBC supplementation compared to placebo (*P*=0.011). Hepatic insulin sensitivity was not significantly different between conditions (*P*=0.430), nor was there a difference in peripheral insulin sensitivity(*P*=0.426). Participants were also screened on their level of insulin resistance and 1 participant displayed normal insulin sensitivity, 2 showed

early insulin resistance (HOMA > 1.9) and the other 9 displayed significant insulin resistance (HOMA >2.9).

Free living glucose excursions

3 h $AUC_{glucose}$ concentrations were 9% lower at breakfast (*P*=0.010) and 8% lower at lunch on the free-living day (*P*=0.02) following NZBC supplementation compared to placebo. However, 3 h $AUC_{glucose}$ during dinner was not significantly different between conditions (*P*=0.643). Furthermore, there was no significant difference in 24 h average glucose (*P*=0.444), daytime average glucose (*P*=0.328) or nocturnal average glucose concentrations (*P*=0.959) was observed between conditions.

Table 4 CGMS time-course glucose concentrations

	Placebo	NZBC
24 h average glucose ($mmol \cdot L^{-1}$)	5.25 ± 0.36	5.12 ± 0.38
Daytime glucose ($mmol \cdot L^{-1}$)	5.32 ± 0.33	5.18 ± 0.33
Nocturnal glucose ($mmol \cdot L^{-1}$)	5.00 ± 0.56	5.02 ± 0.64

Values are means ± SD

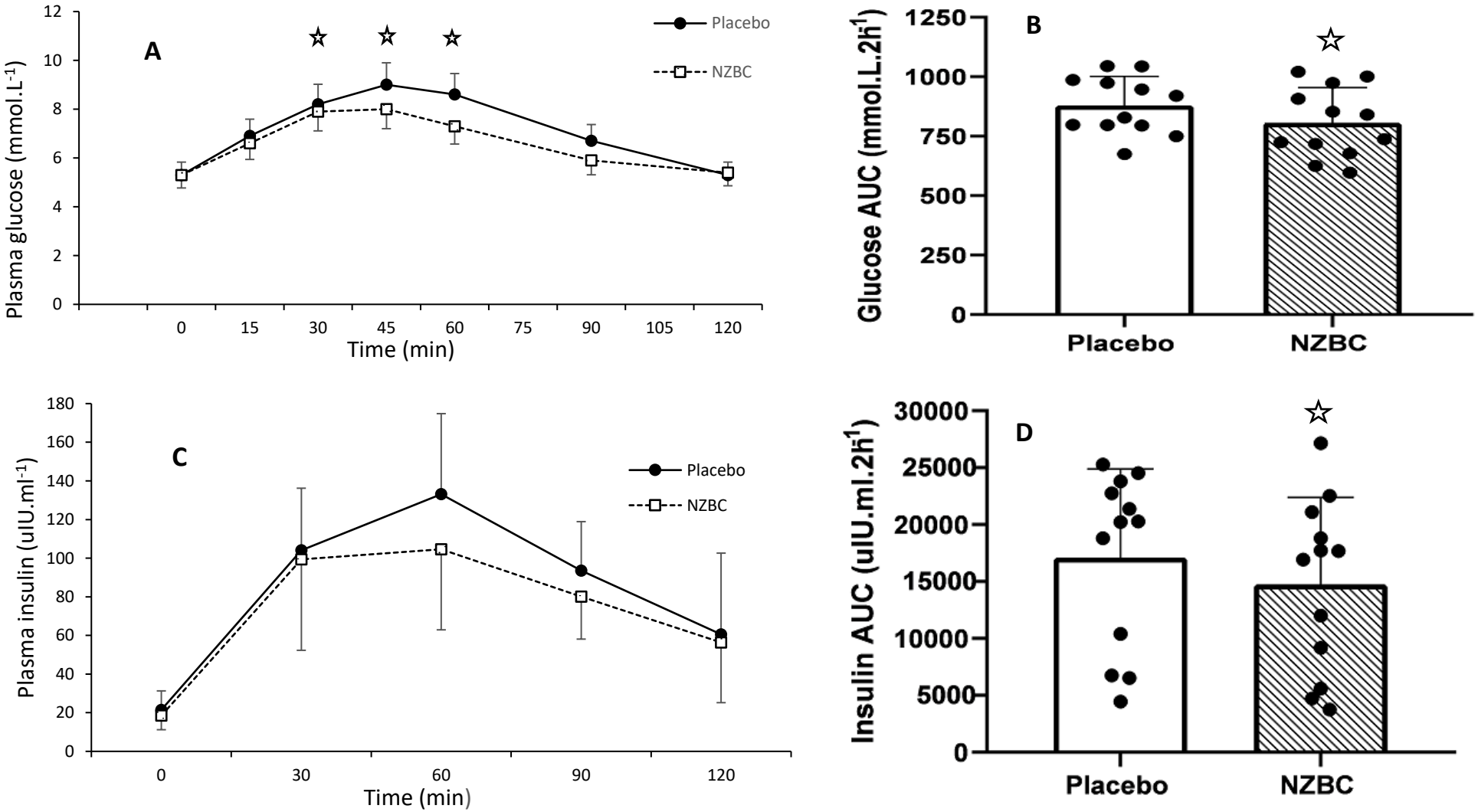

Figure 1. Time-course of plasma glucose (A) and insulin (C) concentrations during oral glucose tolerance test, and subsequent glucose (B) and insulin AUC (D). *P<0.05 vs. placebo.

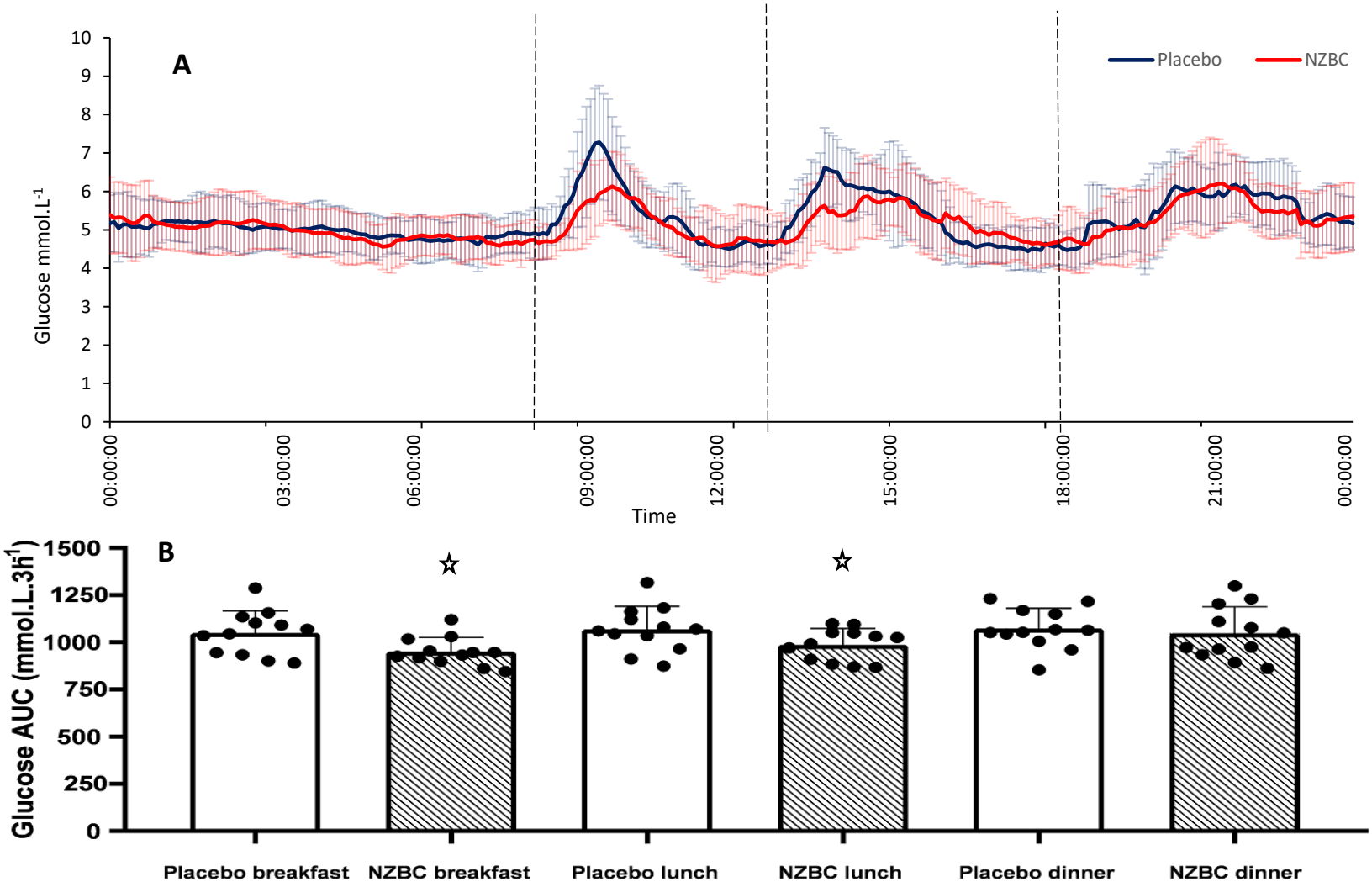

Figure 2. (A) 24 h time-course CGMS data from 00:00 to 00:00 following NZBC and placebo supplementation. Dashed lines indicate the time of meal consumption. (B) 3 h postprandial glucose response to breakfast, lunch and dinner on day 8 (free-living day) following NZBC and placebo supplementation. *P<0.05 vs. placebo.

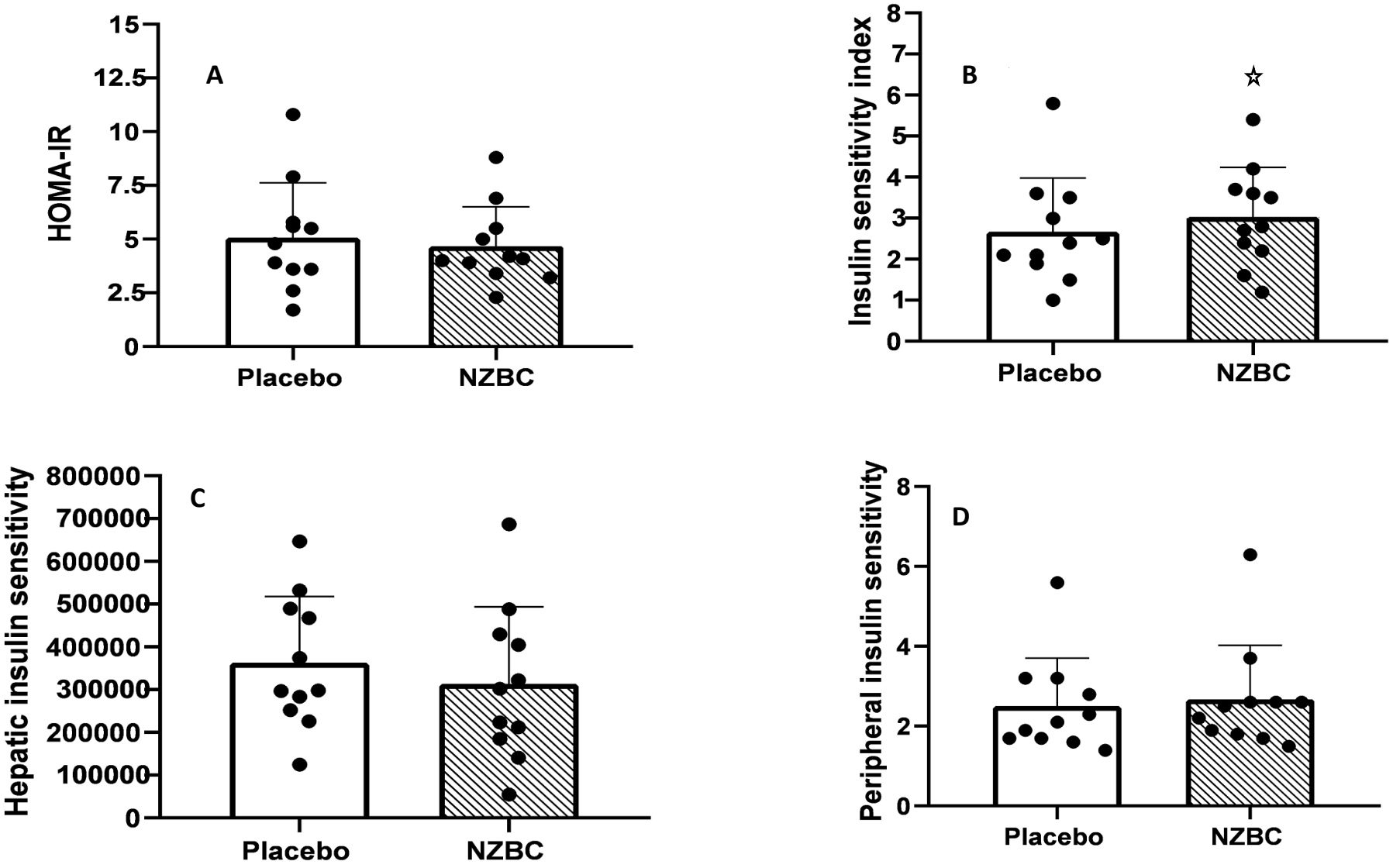

Figure 3. (A) HOMA-IR insulin sensitivity index, (B) Matsuda insulin sensitivity index, (C) Hepatic insulin sensitivity, (D) Peripheral insulin sensitivity.
*P<0.05 vs. placebo.

3.5 Discussion

The overall aim of this study was to investigate whether short-term supplementation with NZBC extract can improve glycaemic control and insulin sensitivity in sedentary, overweight individuals. The novel observations are that short-term NZBC extract supplementation can 1) improve glucose and insulin responses to a glucose challenge, resulting in enhanced whole-body insulin sensitivity, and 2) improve postprandial glucose control under free-living conditions.

Our data from the OGTT agrees with that reported by Willems, Silva et al. (2017), who found that after 7 days supplementation with NZBC blackcurrant powder (providing 138.6 mg anthocyanins) both plasma glucose (8% lower at 60 min) and plasma insulin (18% at 30 min and 39% at 60 min) responses to a glucose challenge were reduced in healthy individuals. We now extend these observations to sedentary, overweight individuals, and importantly we report that both glucose and insulin AUC were reduced following 7 days supplementation with NZBC extract. Interestingly, chronic supplementation of anthocyanin extract seems to have a more profound effect on glycaemic control when compared to single, acute doses. When a single bolus of anthocyanin-rich puree or extract is administered prior to a glucose challenge there only seems to be a reduction in the early postprandial glucose response (15-45 min), which is followed by a rebound at ~90 min resulting in no overall change in AUC (Torronen, Sarkkinen et al. 2010, Torronen, Kolehmainen et al. 2012). Furthermore, any apparent effect of acute anthocyanin intake was absent when we investigated the glucose and triglyceride responses to a carbohydrate-fat meal **(chapter 2)**, indicating that the benefits of acute anthocyanin intake are limited to the early postprandial

period following the ingestion of carbohydrate only. The beneficial effects of acute anthocyanin doses are also likely to be limited due to the very low bioavailability of anthocyanins *in vivo* (~12%). Importantly, Kay, Mazza et al. (2005) found that anthocyanin metabolites were present within the circulation 48 h following ingestion of cyanidin 3-glycosides from chokeberry extract, indicating that chronic ingestion will lead to a residual increase in anthocyanin (metabolite) concentration that perhaps lends itself to greater postprandial benefits *in vivo.*

A limitation of the previous literature is the measurement of glycaemic control using controlled test drinks (such as an OGTT) which are clinical tests, undertaken under controlled laboratory-based conditions and may not be reflective of actual improvements in daily glycaemic control. To overcome these issues, we used CGMS to investigate changes in free-living postprandial glucose excursions following anthocyanin supplementation. We report for the first time that postprandial glucose responses to both breakfast and lunch were improved following NZBC supplementation compared to placebo in our sedentary, overweight participants. Notably, however, there was no difference in the postprandial glucose response to dinner. This is likely explained by the macronutrient content of dinner being higher in fat than carbohydrate (Table 2), especially when compared to both breakfast and lunch which had a higher proportion of calories from carbohydrate sources. It is likely that the high fat content within the dinner meal masked any postprandial benefit of anthocyanin ingestion. This has been previously suggested by us **(chapter 2),** and Edirisinghe, Banaszewski et al. (2011) who found that overweight adults who consumed a strawberry anthocyanin beverage alongside consumption of a high-carbohydrate/moderate-fat meal had no changes in postprandial glucose

concentrations. It should also be noted that average 24 h, nocturnal and daytime glucose concentrations were all similar between doses indicating that the potential benefit of anthocyanin supplementation may reside solely in mediating the postprandial glycaemic response. This effect is important as elevated postprandial glucose responses are linked to both macro and micro vascular complications (Qiao, Tuomilehto et al. 2003, van Genugten, Serne et al. 2013). Fundamentally, NZBC extract supplementation was still capable of improving postprandial glycaemic responses to mixed-meals when administered under free-living conditions, highlighting the potential for an anthocyanin-rich product(s) to be used in individuals at risk of hyperglycaemic complications.

As well as improving postprandial glucose and insulin responses, our results demonstrate that short-term NZBC extract supplementation also improved whole-body insulin sensitivity, as assessed through the Matsuda insulin sensitivity index. This is in line with the observations of Stull, Cash et al. (2010) who determined that 6 weeks ingestion of a high anthocyanin blueberry smoothie twice daily improved insulin sensitivity. We also calculated hepatic and peripheral insulin sensitivity based on the equations of Abdul-Ghani, Matsuda et al. (2007), but no differences in hepatic or peripheral insulin sensitivity were demonstrated to exist. Therefore, it is not currently possible to determine whether the improvements in the postprandial glucose response and whole-body insulin sensitivity is related to beneficial adaptations to skeletal muscle or the liver. However, given that skeletal muscle is believed to be the primary site for glucose disposal following a meal, it could be speculated that adaptations to support greater delivery and uptake of glucose into muscle will underpin these improvements. In support, administration of blackcurrant juice

concentrate to human umbilical vein endothelial cells (HUVECs) significantly increased Akt and eNOS phosphorylation (Edirisinghe, Banaszewski et al. 2011). Furthermore, treatment of bovine artery endothelial cells (BAECs) with a comparable dose of 0.1 μmol.L cyanidin-3-glucoside significantly enhanced eNOS protein expression (Xu, Ikeda et al. 2004). In addition, Matsumoto, Takenami et al. (2005) demonstrated that acute anthocyanin intake can increase peripheral blood flow at rest. Therefore, it is possible that an increase in peripheral blood flow could potentially lead to an increase in peripheral glucose disposal, particularly as insulin is known to facilitate its own glucose disposal into the skeletal muscle via increased microvascular perfusion. Alternatively, anthocyanin may be able to impact on the myocyte itself as it has been shown that root, stem and leaf extracts from Canadian lowbrush blueberry (containing high anthocyanin levels) significantly enhanced glucose uptake in C2C12 myocyte cells by 15-25% in the presence and absence of insulin after 20 h of exposure (Martineau, Couture et al. 2006). Importantly, because C2C12 myotubes lack a microvascular component, this indicates that anthocyanins could also directly mediate adaptations in muscle that would lead to improved glucose uptake. In support, 3T3-L1 adipocytes cells which were treated with cyanidin-3-glucoside demonstrated a significant upregulation of GLUT4 gene expression (Inaguma, Han et al. 2011). However, caution should be taken as the cyanidin-3-glucoside dose provided (20 and 100 μM) is much larger than what would commonly be experienced *in vivo*. Therefore, whether anthocyanin has the potential to increase GLUT4 expression and translocation and whether this adaptation occurs *in vivo* is yet to be determined.

It is well documented that an overproduction of pro-inflammatory cytokines leading to low-grade systemic inflammation is an important marker for the development of

insulin resistance (Xu, Barnes et al. 2003). Furthermore, Interventions which reduce plasma cytokine levels (such as weight loss) are associated with a concurrent decrease in insulin resistance (Ryan and Nicklas 2004). Therefore, positive improvements in markers of inflammation may lend itself to an increase in peripheral glucose uptake. Anthocyanin has previously been shown to influence markers of inflammation as 320 mg purified anthocyanin (cyanidin-3-O-β-glucoside and delphinidin-3-O-β-glucoside) on 150 subjects with hypercholesteremia was capable of markedly reducing both C-reactive protein and plasma IL-6 (Zhu, Ling et al. 2013). Taken together, it is possible therefore that anthocyanins lead to improvements in insulin sensitivity through a synergistic interaction between multiple mechanisms, although future research is warranted to determine this.

A strength of this study is the use of continuous glucose monitoring systems to measure postprandial glycaemic responses under free-living conditions. Continuous glucose monitoring has been previously shown to be an effective measure in determining instantaneous real-time displays of glucose levels and is an effective tool in the management of diabetes (Rodbard 2017). There are variations in accuracy of CGMS monitors, however the Dexcom G4 sensor used in the present study has been shown to be one of the most accurate widely available CGMS devices with a mean absolute relative difference of ~14%, (Matuleviciene, Joseph et al. 2014). Another strength of this study was the very low self-reported habitual intake which provided an opportunity to highlight the effects of increasing anthocyanin intake through supplementation as habitual intake was almost non-existent. A limitation of this study was that due to the free-living element of the study we cannot say for certain whether participants followed the standardised diet provided, however fluctuations in plasma

glucose coincided with meal times therefore it seems unlikely that this had a detrimental effect.

In conclusion, 8 days NZBC supplementation can improve postprandial glucose responses under dietary controlled but otherwise free-living conditions. Furthermore, 8-day NZBC supplementation can improve postprandial glucose and insulin responses, alongside an improvement in ISI when subjected to an OGTT. Future studies should aim to determine the effects of longer duration supplementation, and whether NZBC can improve markers of inflammation in overweight/obese populations.

Chapter 4 General discussion

4.1 Thesis overview

The rise in global obesity crisis can now only be described as an epidemic, with >650 million adults worldwide classified as obese (BMI ≥30 $kg \cdot m^{-2}$), which is expected to rise to 1.12 billion by 2030 (Kelly, Yang et al. 2008, WHO 2018). The Public Health England 'All Our Health' resource, which aims to guide health professionals in promoting health and wellbeing, describes low fruit and vegetable intake, low physical activity and consumption of high energetic foods as major components of obesity (Public Health England 2018). Furthermore, in 2016 overweight and obesity-related ill-health was estimated to have cost the NHS £6.1 billion, with the wider economic development cost much higher at £27 billion (Public Health England 2017). Interventions aimed at reducing obesity levels through dietary modification or physical activity promotion have been found to be effective in reducing levels of obesity and its comorbidities (Poirier, Giles et al. 2006, Colberg, Sigal et al. 2016), however long-term adherence to these lifestyle changes is often poor (Curioni and Lourenco 2005).

A major component in the progression of obesity and T2DM to further comorbidities is sustained periods of postprandial hyperglycaemia throughout the day (Bonora and Muggeo 2001). Indeed, postprandial glucose has been shown to be an independent risk factor for cardiovascular disease, with therapies aimed at reducing postprandial glucose found to be more effective than those that target improved fasting glucose at managing diabetic complications (Bastyr, Stuart et al. 2000). The inclusion of 'functional foods' in the diet has been shown to improve outcomes associated with a wide range of diseases including cardiovascular disease, T2DM, cancer and

neurodegenerative diseases (Pandey and Rizvi 2009). It is possible that the inclusion of easy-to-consume dietary supplements may be an effective method of administering the health promoting benefits of these plant-based phytonutrients. As such, the overall aim of this MPhil was to investigate whether an anthocyanin-rich blackcurrant extract could be used to help mediate the postprandial glucose response in overweight/obese individuals. **Chapter 2** first aimed to investigate whether a single bolus of NZBC extract could improve postprandial glucose and triglyceride excursions, and if this was dose-dependent. **Chapter 3** then attempted to determine the ecological validity and effectiveness of short-term NZBC extract supplementation using CGM alongside more typical glucose tolerance testing.

4.2 Key findings

4.2.1 Chronic supplementation with NZBC extract is required to mediate the postprandial glucose response

Chapter 2 demonstrated that a single, acute dose of NZBC extract was unable to alter postprandial glucose or triglyceride responses to a carbohydrate-fat meal. The lack of response found is likely linked to the inclusion of dietary fat in the test drink, as other research showing positive effects have only used glucose/sucrose control loads (Torronen, Sarkkinen et al. 2010, Torronen, Kolehmainen et al. 2012, Castro-Acosta, Stone et al. 2017). Moreover, Edirisinghe, Banaszewski et al. (2011) also used a combined carbohydrate-fat test meal and found no beneficial effect on postprandial glucose responses. Interestingly however, they did find an improvement in plasma insulin concentrations, leading to speculation that perhaps acute doses can increase

insulin secretion with no change in glycaemic responses. It is also possible that acutely there is an inability for NZBC extract to mediate the high-fat meal indicating a potential fat percentage threshold within the meal at which NZBC supplementation becomes ineffective. It could be argued that more chronic supplementation is required to induce a glucose-lowering effect to a mixed-meal, and in this respect **chapter 3** provides some of the first data to support this notion. Importantly, the use of CGMS in **chapter 3** provided an insight into glycaemic responses to mixed-meals under free-living conditions, rather than in laboratory settings as seen in the previous literature (Willems et al., 2017). To our understanding this was the first investigation into the effect of anthocyanin supplementation on glycaemic control using CGM systems. Here, we were able to demonstrate that some of the masking effect fat has on glucose responses to a mixed-meal are overcome when more chronic periods of supplementation are undertaken, as evidenced by the 9% and 8% reduction in glucose responses to breakfast and lunch, respectively. This builds on data from others showing that glucose and insulin responses to a glucose challenge are improved following short-term blackcurrant extract supplementation (Willems et al., 2017). Moreover, we provide novel evidence for the efficacy of NZBC supplementation in overweight/obese individuals to improve postprandial responses under both scientifically rigid and ecologically valid conditions.

4.2.2 Chronic supplementation with NZBC extract improves whole-body insulin sensitivity

Through the measurement of insulin during the OGTT in **chapter 3,** it was possible to estimate insulin sensitivity. While HOMA-IR was similar between doses, it should be noted that it only takes into consideration fasting glucose and insulin concentrations. Moreover, the effects of anthocyanin supplementation only seem to influence postprandial glycaemia, with minimal effects on fasting concentrations. Furthermore, Willems, Silva et al. (2017) also demonstrated an improvement in glycaemic responses with no change in HOMA-IR. Interestingly, in **chapter 3** we observed a 22% improvement in Matsuda insulin sensitivity index, highlighting an improvement in whole-body insulin sensitivity. Importantly, this is the first data to demonstrate an improvement in insulin sensitivity following NZBC extract supplementation. This improvement is similar to that reported in other lifestyle interventions such as drastic weight loss (39.5%) and exercise training (23%). However, the inclusion of a supplement into the diet requires less motivation and lifestyle change than exercise or weight loss interventions, and may therefore be more applicable to many individuals (Barwell, Malkova et al. 2008, Rabol, Svendsen et al. 2009). Given that no changes in hepatic or peripheral insulin resistance were observed though in chapter 3, it remains to be determined what the precise mechanism(s) are that underpin the improvement in whole-body insulin sensitivity.

Taken together, the evidence provided in **chapter 2 and 3** helps provide insights into how acute and chronic NZBC supplementation influences postprandial glucose responses under more ecologically valid conditions.

4.3 Lipid metabolism

In **chapter 2** blood samples were analysed spectrophotometrically to quantify plasma triglyceride concentrations. This analysis aimed to determine the effect of NZBC supplementation on fat metabolism and specifically whether NZBC extract was effective in reducing postprandial triglyceride concentrations. Increased fasting and postprandial triglyceride concentrations are commonly found within obese subjects (Couillard, Bergeron et al. 1998). The effect of anthocyanin on postprandial triglycerides has so far gone unreported in the literature, however previous research into acute anthocyanin doses found an inability to influence substrate metabolism (Edirisinghe, Banaszewski et al. 2011). This could be explained through the limited ability for anthocyanin to interact with lipid digestion compared to the carbohydrate digestive pathways. Digested fat begins its entry into the circulation via hydrolyzation in the small intestinal lumen through the action of pancreatic lipase. While some evidence suggests anthocyanin may be capable of competitively inhibiting pancreatic lipase (Fabroni, Ballistreri et al. 2016), this in controversial due to the lack of increased faecal lipids that would be associated with a reduction in lipid digestion (Griffiths 1986, Tsuda 2008). Furthermore, fatty acid digestion is much slower than glucose due to a contraction of the pyloric sphincter region to allow for bile production for lipid digestion (Quigley 1941). The ability for fatty acid to enter the circulation differs from carbohydrate fat passing into the plasma in the form of chylomicron triacylgleride (TAG) and due to its hydrophobic nature, is required to be bound to albumin for transport thereby ignoring intestinal sugar transporters.

Ultimately, this helps explain why anthocyanin may be unable to interact with lipid digestion processes and instead may only be able to interfere with carbohydrate pathways.

4.4 Directions for future research

4.4.1 What are the mechanisms by which anthocyanins lead to improvements in insulin sensitivity

While our understanding into the mechanisms surrounding the ability for anthocyanin to mediate glycaemic control have improved over the last few years, current evidence surrounding how anthocyanin mediates microvascular perfusion and glucose uptake are limited. Evidence currently suggests that anthocyanin is capable of endothelium-dependant vasorelaxation, alongside stimulation of Ca^{2+} -dependant nitric oxide (Martin, Andriambeloson et al. 2002, Nakamura, Matsumoto et al. 2002). To fully investigate this, *in vitro* trials exposing human endothelial cells to anthocyanin extract may help to determine the effect anthocyanin has on eNOS expression and subsequent NO activity within the endothelium and to what extend anthocyanin has on increasing microvascular perfusion. Furthermore, currently only one study has attempted to investigate the effects of anthocyanin extract on peripheral blood flow in humans (Matsumoto, Takenami et al. 2005). Therefore, future studies in humans consisting of short term NZBC supplementation and its effects on microvascular function, including NO activity and peripheral blood flow.

Furthermore, anthocyanin has been shown to be capable of increasing glucose uptake in C2C12 myocyte cells by up to 15-25% (Martineau, Couture et al. 2006). Interestingly, the lack of a microvascular component within the C2C12 myocytes indicates a potential ability to increase GLUT4 translocation independent of an increase in microvascular perfusion. Therefore, the next would be to investigate the effect of anthocyanin on C2C12 myocyte cells to see whether anthocyanin can increase GLUT4 expression and/or insulin-induced translocation. Furthermore, these mechanisms could be investigated in human supplementation studies, by obtaining muscle biopsies and employing immunofluorescence microscopy assays to investigate endothelium-specific eNOS expression and phosphorylation (Cocks, Shaw et al. 2013) as well as GLUT4 translocation (Bradley, Shaw et al. 2015).

4.4.2 What is the optimal strategy to improve insulin sensitivity in T2DM

Short-term supplementation can help mediate the postprandial glycaemic responses associated with carbohydrate-rich control meals and more ecologically valid mixed-meals. Currently our understanding into the effects of long term supplementation is limited with our current knowledge being limited to the effects of 8-day supplementation. While epidemiological evidence has helped suggest that long-term supplementation may well be effective in the mediation of some metabolic disorders (Jayaprakasam, Vareed et al. 2005, Mink, Scrafford et al. 2007, Cassidy, O'Reilly et al. 2011), it is so far unknown whether these effects manifest early in the supplemental period or whether these effects continue to manifest over time (6+ weeks). This is particularly important as while we are aware that anthocyanin metabolites can be

present within the circulation ~48 hours post ingestion (Czank, Cassidy et al. 2013), previous evidence has suggested that supplemental duration has the most profound effect on anthocyanin modulating responses (Hassellund, Flaa et al. 2013). Therefore, NZBC supplementation for a period of 6-8 weeks in overweight/obese individuals would help further our understanding surrounding the long-term beneficial effects of anthocyanin for the treatment of metabolic disorders, particularly surrounding whether the improvements in glycaemic control continue to increase over time or whether there is in fact a plateau or even a decrease glycaemic control.

Furthermore, dosing strategies also need to be developed, particularly surrounding washout periods and the effects of halting NZBC supplementation alongside determining the minimum effective dose. While we now understand that short-term NZBC supplementation is more effective at modulating glycaemic responses than a single acute bolus, determining whether the beneficial effects on insulin sensitivity are also lost upon cessation of supplementation and returning to baseline need to be investigated. To determine this a short-term supplemental period (7 days) followed by a washout period in which regular blood samples are taken over the course of the washout period, quantifying serum anthocyanin content could be undertaken. Alongside this, intermittent tolerance tests could additionally be undertaken, helping to determine insulin sensitivity changes (if any) over the course of the washout period. Additionally, a comparison of weekly, equal doses of daily NZBC ingestion vs. sporadic ingestion (such as 2 or 3 times weekly) would determine whether daily ingestion is necessary in mediating postprandial responses. Additionally, varying doses of anthocyanin **(like chapter 2)** taken for extended periods of time (7+ days) would help

determine whether the glycaemic benefits of NZBC supplementation continue to increase or whether the effects reach a dosing limit.

Individuals suffering from T2DM regularly take medication designed to alleviate extended periods of hyperglycaemia. These medications can vary from reducing glucose entry in the gut, increasing insulin sensitivity and even insulin secretion. Metformin is the most commonly taken first-line, diabetic medication and works to increase glucose entry into the peripheral tissue through the increase in AMPK mediated glucose uptake without a concomitant increase in insulin secretion. A major downside to Metformin however, is the associated side-effects which may range from diarrhoea, nausea to severe abdominal pain. Therefore, determining whether anthocyanin ingestion may supplement or even alleviate the need for glucose lowering medication may help improve the lives of individuals currently suffering from T2DM. To test this, an investigation into the effects of NZBC supplementation on individuals suffering from T2DM but who are not currently taking any anti-diabetic medication with tolerance tests performed at the start and conclusion of supplementation would help to determine whether the effects of NZBC supplementation are effective enough at improving glycaemic control.

4.4.3 The effect of blackcurrant extract on mixed diets

The combined evidence from **chapter 2 and 3** indicates an attenuation in the positive postprandial effects of NZBC supplementation. The data provided gives an insight into the potential positive effects on carbohydrate digestion after consumption of high carbohydrate meals. However, under free-living conditions meals often consist of

varying macronutrient combinations, therefore more insight into the effects of NZBC supplementation on mixed and high fat meals needs to be undertaken before the complimentary effects of NZBC on glycaemic control are properly supplementation on mixed-meal or high fat tolerance tests. To date no study has administered a high-fat or mixed meal tolerance test after chronic anthocyanin supplementation and as previous found, chronic supplementation has a greater benefit on overall glycaemic responses when tested via an OGTT (Willems, Silva et al. 2017). However, mixed meal tolerance tests are likely more reflective of the normal postprandial response (when compared to an OGTT), due to an impairment of microvascular perfusion which occurs during periods of acute hyperglycaemia (Russell, Hu et al. 2018). Therefore, by using a mixed meal tolerance test, this may provide a greater indication as to the effect NZBC supplementation has in increasing skeletal muscle insulin sensitivity and whether this explains the reported increase of whole body insulin sensitivity.

Additionally, **chapter 3** helped provide evidence that NZBC extract can improve glycaemic responses to high-carbohydrate meals, however further research should aim to determine whether individuals consuming high-fat vs. high carbohydrate meals still receive a postprandial benefit from NZBC supplementation. This may help determine whether certain individuals subscribing to dietary restrictions (in this case high-fat) can still benefit from NZBC supplementation or whether the free-living benefits are primarily restricted to individuals consuming high carbohydrate diets.

4.4.4 NZBC on T2DM

While **chapters 2 and 3** aimed to quantify the effects of NZBC ingestion in individuals at risk of developing T2DM, due to the deterioration of insulin signalling present within T2DM it would be useful to determine whether these metabolic improvements are present within this population. A major issue in the progression of T2DM to further complications such as CV disease is the sustained elevated hyperglycaemia (Laakso 1999). While **chapter 3** helped demonstrate the increase in whole body insulin sensitivity associated with short term NZBC supplementation. This increase of 22% is similar to that reported for other interventions such as exercise and weight loss (Barwell, Malkova et al. 2008, Rabol, Svendsen et al. 2009), but requires far less lifestyle mediation. A large percentage of individuals suffering from T2DM are aged 65 years or over (Center for Disease Control and Prevention 2011). Due to the difficulty this demographic would have undertaking exercise interventions, perhaps nutritional interventions aimed at improving diabetic outcomes would be more effective. A chronic supplemental period (4-6 weeks) of NZBC on individuals suffering from T2DM would help provide an insight into the potential usefulness of bioactives on treating T2DM.

4.5 Final conclusions

The work provided within this MPhil provides solid evidence for the use of NZBC supplementation in the mediation of postprandial hyperglycaemia in overweight/obese individuals. **Chapter 2** determines that a single bolus of NZBC extract is ineffective at improving postprandial glucose and triglyceride responses to

a carbohydrate-fat tolerance test. **Chapter 3** demonstrates that 8-day supplementation is capable of improving postprandial glucose, insulin and insulin sensitivity to an OGTT, as well as improving daily glucose excursions under free-living conditions using CGMS equipment.

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