ABSTRACT

Title of Dissertation:	BIOACTIVE COMPONENTS FROM BLACKBERRIES TO AUGMENT DIETARY APPROACHES TO OBESITY TREATMENT OR PREVENTION: INDIRECT CALORIMETRY STUDIES IN MAN AND TISSUE
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Rodent models of diet-induced obesity lead to the discovery of an anti-obesity and anti-diabetic effect of anthocyanins in several forms. In addition to improved body composition and insulin sensitivity, molecular research suggests the activation of PPAR pathways and corrected or improved mitochondrial function by way of AMPK activation. As the obesity epidemic in the United States progresses, cost effective and practical solutions are warranted, with an emphasis on improved dietary choices. Berries are a readily available source of anthocyanins and promising results on obesity demonstrated in rodents needs to be explored in humans.

A randomized, placebo-controlled, cross-over human feeding study was conducted at the Beltsville Human Nutrition Research Center. Twenty-four overweight or obese male subjects followed a 100% investigator controlled high fat diet for seven days that included 600 g of raw blackberries or an energy matched, anthocyanin free

control daily. In order to assess changes in the respiratory quotient, energy expenditure, and calculated substrate oxidation, the subjects completed a 24 hour stay in a room-size indirect calorimeter at the end of each diet period. To assess changes in insulin sensitivity, the subjects also participated in a 4-hour meal-based glucose tolerance test on the morning of day seven.

Significant findings include a reduction in the respiratory quotient and subsequent increases in the calculated amount of fat oxidized for energy across the 24 hour stay, as well as when calculated during shorter time intervals. Further, insulin sensitivity was increased as evidenced by a reduction in the area under the curve in response to the meal challenge as well as fasting concentrations. Future work should address dosing in longer studies.

Supporting cell culture work was conducted in order to determine changes in oxygen consumption at the cellular level. Adipocytes were treated with physiologically attainable levels of cyanidin-3-glucoside and mitochondrial respiration states were characterized with high-resolution respirometry on a fatty acid substrate. Findings include a significant increase in baseline (intact) cellular respiration as well as a marginal increase in the maximum respiratory capacity of the adipocytes, without significant changes in mitochondrial density, suggesting improved function of the adipocyte with fatty acid substrates.

BIOACTIVE COMPONENTS FROM BLACKBERRIES TO AUGMENT DIETARY APPROACHES TO OBESITY TREATMENT OR PREVENTION: INDIRECT CALORIMETRY STUDIES IN MAN AND TISSUE

by

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Preface

The most recent report from the CDC website indicates that adult obesity is at an all-time high: 37.7% – up 7.2 percentage points in 2013 compared to 1999. By comparison, the rate of increase in childhood obesity has been less dramatic, up 3.3 percentage points [1]. In addition, another one-third of American adults are overweight [2]. This is the new reality of our nation. How America has arrived at this state of obesity is multifaceted, but it is not mysterious. Studies have demonstrated that exposure to U.S. society causes obesity [3]. A palatable and affordable, calorically dense and shamelessly promoted food supply, a workforce paired with automation requiring less physical exertion, and a physical environment conducive to automobile transportation are all culprits in the case of the obesogenic state. Further, our society no longer operates around a 9-5, Monday through Friday, nuclear family lifestyle. Recent trends show Americans are sleeping less and working longer, irregular hours [4, 5]. With the advent of the information age and 24/7 business hours, coupled with a disparity between inflation and pay rate, time-saving, cheap, and delicious ready to eat foods fuel the midnight oil as much as they expand the waistline. The average American now spends so much of their time in front of a screen that software developers have gone to the length of creating games that encourage people to go outside for a walk [6].

The solution is obvious, yet remarkably unattainable. Lifestyle modification – more physical activity and fewer calories – remains the number one prescription to effectively combat obesity. No magic pill or silver bullet has been developed. Not in the depth of knowledge about the human genome, and the billions of dollars spent by

the pharmaceutical industry has their yet been a breakthrough to address the obesity crisis. That said, there may very well be a day where the solution to obesity is no less commonplace than iodized salt or folic acid fortified grains, but we are not there yet. In the meantime, it is advantageous for nutritionists to sift and winnow through food components that may potentiate the ability of diet to minimize or even alleviate adiposity, or dampen the effects of its downstream comorbidities, primarily cardiovascular disease and type-2 diabetes. This line of research is worthwhile because obesity comes with a cost: an estimated $147 billion was spent on medical care related to obesity in 2008. In addition, obesity dampens productivity and even perturbs recruitment into the nation's armed forces [7]. It is imperative that nutritionists aid in the discovery for a "cure" for obesity, which is likely to include as much nuance as the problem of obesity itself. Although obesity is grand in etiology and eventual remediation, it is not practical to discuss all its facets in a nutrition review, instead we fixate on only one nutrient packed foodstuff that has caught the attention of nutritionists operating in the realm of obesity research. The literature review will focus on one candidate, heterogeneous class of fruits rich in phytonutrients and implicated in the reduction of obesity-related chronic diseases: berries.

The review will survey the current state of nutrition research into berries' effects on obesity and related comorbidities with an emphasis on the biological responses to their consumption. Epidemiological studies will introduce the concept of berries as a health-promoting food, and primary research describing the protective effects of berries and/or their extracts as they relate to obesity will be described -

spanning rodent studies and tissue culture to controlled human trials. The review will not summarize the varying chemical and phenolic composition across various species of berries; for this information the reader should refer to a useful review by Nile et al. [8].

The second chapter will report on a controlled feeding study conducted at the Food Components and Health Laboratory of the Beltsville Human Nutrition Research Center in Beltsville, Maryland. The aim of the study was to translate the anti-obesity effects of berries observed in rodent models (discussed in the literature review) to humans using indirect calorimetry and a glucose tolerance test to discern possible benefits of berry consumption on fat oxidation and insulin sensitivity, respectively.

The third chapter will report on cell culture experiments also conducted at the Food Components and Health Laboratory. The literature review highlights mechanisms of action by anthocyanins in adipocyte cultures that may trigger a metabolic switch from energy storage to disposal, thus a metabolic sink to alleviate fatty acid overload as well as correct insulin resistance. This proposed action, combined with observations from the human feeding study in chapter two, was the basis for respirometry experiments conducted in adipocytes and skeletal muscle cells treated with anthocyanins from berry extracts or in purified form.

The research proposal from the PhD candidacy exam in February, 2016 was on a different topic: resveratrol administration in obese men with metabolic syndrome. Work from that proposal lead to the publication of a literature review. The literature review is included as the fourth chapter and detailed methods from that research proposal are attached in **Appendix 1** for the committee's reference.

Dedication

To my mom and dad, back in Viroqua, Wisconsin, for always believing in me and shaping me into the person I am today. Much love, always.

Acknowledgements

Woody Allen is credited for the quote “If you want to make God laugh, tell him about your plans”, and when I first fell on the Food Components and Health Laboratory’s doorstep in 2013, I certainly felt that someone was getting a kick out of my haphazard choices as a fledgling Nutritionist. Despite the rocky path that lead me to move cross-country to get involved with human subjects research, it did not take long for me to recognize the caliber of the research at the center as well as the great capabilities possessed by the staff.

The following dissertation would not have been possible without the coordinated efforts of the research team employed at the FCHL. Their dedication, grit, and camaraderie can make the most difficult of tasks seemingly less of a burden, and they made it a pure joy to come into the lab every morning. The list of names is too long, but you know who you are.

To our Dean’s representative, Dr. Seppo Iso-Ahola: I will forever challenge the validity of the methodologies used in whatever piece of literature I am perusing (nutrition or otherwise) and your push to challenge all facets of research design has made me a better scientist.

To our late comer, Dr. Qin Wang: for being so agreeable in a pinch to make the defense possible. I appreciate your flexibility and perhaps the research center could learn a thing or two from your microencapsulation techniques.

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To Dr. Castonguay: I value your encyclopedic knowledge of the world of nutrition. Thank you for taking on a lost kid when you really should have been more selfish at this point in your career. Your patience is unparalleled, as I discovered while sitting through your graduate course on energy balance. You have challenged my prose as well as the direction of this dissertation in an effort to improve both. I hope the results are works you are happy to take credit for.

Finally, to Dr. Novotny: for answering the knock at your door when no other lab would back in 2013. You provided endless opportunities so I could expand my knowledge of human subjects research with nutrition interventions. I cannot keep track of the new avenues you allowed me to go down in adopting protocols and trying out new tests at the center. You demonstrated honed diplomacy as I ran from lab suite to lab suite, disturbing your employees and doing whatever it took to make new assays work. Thank you for always encouraging new ideas and your trust in my ambition, which others may have perceived as frenetic and cavalier. I am truly proud of the work we have generated together the past four years.

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List of Abbreviations

IMT	Intima media thickness
PCC	Purple corn color
C3G	Cyanidin-3-O-β-glucoside
TNF-α	Tumor necrosis factor-alpha
AMPK	AMP-activated protein kinase
PPAR	Peroxisome proliferator activated receptor
HOMA-IR	Homeostasis model assessment of insulin resistance
IRS1	Insulin receptor substrate-1
PAI-1	Plasminogen activator inhibitor-1
BEE	Black elderberry extract
TGs	Triglycerides
NEFAs	Non-esterified fatty acids
LPL	Lipoprotein lipase
PCA	Protocatechuic acid
oxLDL	Oxidized LDL
siRNA	Small interfering RNA
C/EBPβ	CCAAT-enhancer binding protein-beta
SREBP1	Sterol regulatory element-binding protein 1
UCP-1	Uncoupling protein 1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFAT	Glutamine:fructose 6-phosphate aminotransferase
ATGL	Adipose triglyceride lipase
LPS	Lipopolysaccharide
HbA_{1c}	Glycated hemoglobin
BMI	Body mass index
CETP	Cholesterol ester transfer protein
LCAT	Lecithin cholesterol acyltransferase
FMD	Flow-mediated dilation
cGMP	Cyclic guanosine monophosphate
VCAM-1	Vascular cell adhesion molecule 1
PON1	Paraoxonase 1
hsCRP	High-sensitive c-reactive protein
metS	Metabolic syndrome
BB	Blackberries
RQ	Respiratory quotient
HOMA-B	Homeostasis model assessment of β-cell function
iAUC	incremental area under the curve
AUC	Area under the curve
CONSORT	Consolidated standards of reporting trials
MTT	Meal-based oral glucose tolerance test
EGCG	Epigallocatechin-3-gallate
BBE	Blackberry extract
SCR	Substrate control ratio

CCR	Coupled control ratio
CR	caloric restriction
CVD	cardiovascular disease
DXA	dual-energy x-ray absorptiometry
LDL	low density lipoprotein
RESV	resveratrol

Chapter 1: Literature Review

Epidemiological studies – fruits, vegetables, and berries

Several independent epidemiological studies have highlighted the protective effect of fruit and vegetable consumption against cardiovascular disease [9]. Further, a recent meta-analysis of sixteen prospective cohort studies was able to demonstrate a dose-response (4% reduction per serving) where as little as one serving of fruits and vegetables per day had a significant protective effect against all-cause mortality and added protection was negligible beyond five servings [10].

In addition to essential vitamins and minerals, fruits and vegetables provide a source of other phytonutrients that are an emerging area of nutritional sciences research, but whose health effects are less characterized relative to the classical view of nutritional deficiencies with vitamins and minerals. A diverse class of non-nutritive plant compounds are the polyphenols, appropriately named for their characteristic phenol moiety, which too have been identified as health-promoting by epidemiological studies. An example of their bioactive effect is demonstrated with a reanalysis of the PREDIMED study, a prospective trial which demonstrated the cardioprotective (fewer cardiovascular events or deaths related to cardiovascular causes) effect of a Mediterranean style diet, was able to estimate polyphenol intake by comparing food records to an online polyphenol database to determine that greater intake of polyphenols increased survival at six years of follow-up [11]. When parceled out into individual compounds, the investigators reported stilbenes, lignans, and isoflavones to be the most protective polyphenols against mortality, in this case [11].

Epidemiological studies set precedence for the contribution of berries towards the reduction of chronic disease and mortality. Two separate studies reexamined a prospective

cohort study of middle-aged men residing in eastern Finland (the Kuopio Ischaemic Heart Disease Risk Factor Study) to assess the effect of fruit, berries, and vegetables on both all-cause mortality and cardiovascular related death, or the development of type-2 diabetes [12, 13].

The first study, in-line with the studies mentioned above, found a significant 35% and 57% reduction in the relative risk of all-cause mortality and CVD-related death, respectively, in the subjects with the highest quintile of fruit, berries, and vegetable intake compared to the lowest at 12 years of follow-up. Interestingly, they found that the men who died in the first five years of follow-up consumed 41% fewer fruits, berries, and vegetables compared to men who survived during the study [12]. Associations between individual nutrients and mortality underscored vitamin C and folate as nutrients that explained the highest amount of the protective effect provided by fruit, berries, and vegetables. Unfortunately, this study did not separate out fruits, berries, and vegetables to determine individual contributions to all-cause and CVD related mortality. Further, the authors did not estimate consumption of polyphenols and their contribution to the protective effect, likely because phenolic databases did not exist at the time of the study, which would make the task burdensome.

The second study on this Finnish population of middle-aged men assessed risk of type-2 diabetes vs. quartiles of fruits, berries, and vegetables intake [13]. Their dataset included over 2300 men followed for an average of nineteen years, in which 432 cases of type- 2 diabetes were noted. Splitting intake into quartiles, the authors report a non-significant hazard ratio of 0.76 for development of type-2 diabetes when comparing the highest and lowest quartile using a model adjusted for type-2 diabetes risk factors [13]. However, they also separated out individual food groups, showing a significant 35% lower risk of type-2 diabetes in the group of men with the

highest berry intake (greater than 60 g of berries each day) [13]. Although a separate analysis for berries was possible, the authors were unable to estimate intake of specific varieties of berries.

A Norwegian study of 547 elderly men (average age 70 years) assessed fruit and berry intake as it related to cardiovascular risk, measured by intima media thickness (IMT) of the carotid artery, an independent risk factor in atherosclerosis leading to cardiovascular disease [14]. The assessment of dietary intake on IMT was determined from the reanalysis of an earlier intervention study involving diet counseling or fish oil administration for three years [15]. When intake of fruit and berries was divided into quartiles, the IMT of the highest quartile (greater than 255 g fruit and berries per day) was 5.5% less than the lowest group of intake (97 g or fewer of fruit and berries per day). Like the aforementioned study, berries were combined with intake of other fruits when estimating effects on IMT; the authors noted intake of berry consumption alone was not a significant predictor of IMT in their multivariate regression model, possibly due to low overall intake in this population caused by seasonal variation (9.8 g per day in the highest quartile) [14].

A large, prospective Norwegian study followed the lives of over nine-thousand men for forty years - a combination of two separate samplings conducted in the late 1960s – where dietary habits of the men were assessed with a rudimentary food frequency questionnaire [16-18]. Hjartaker et al. reanalyzed the dataset for the determination of the association of berries, fruits, and vegetables (grouped together or partitioned) and all-cause mortality as well as cancer and CVD related deaths [18]. At the conclusion of the study the average time of follow-up was twenty years where 92% of respondents died. Like previously described studies, higher quartiles of the combined fruit, vegetable, and berry intake yielded a protective effect against mortality – with all-cause mortality, a benefit was noted with as little as the second quartile of intake. In this

study, berries again demonstrated a protective effect against all-cause mortality, a marginal effect when assessing cancer-related mortality, but no effect with CVD-related deaths [18]. This differs from the observation of CVD protective effect of fruits, berries, and vegetables mentioned in the Finnish study. The authors note a limitation of the study is that the questionnaire assessed frequency of consumption of various food items, but did not estimate servings, therefore their regression analysis could not be adjusted for energy intake [18].

While these Scandinavian studies do not provide a definitive answer for the utility of berries in addressing the obesogenic state of America, what they do is provide evidence for its potential against chronic diseases related to or exacerbated by obesity. What's more, the PREDIMED reanalysis demonstrates the utility of polyphenols in increasing survival in a population at-risk for cardiovascular related mortality. It is well documented that berries are a rich source of a variety of phenolic compounds, including flavonoids, stilbenes, lignans, tannins, and phenolic acids [8]. Yet, what may put berries front and center in obesity related research could be their high anthocyanin content, as seminal work in a Japanese rodent study uncovered the anti-obesity effect of purple corn color (PCC), a rich source of one of the most aggressively studied anthocyanins, cyanidin-3-O-β-glucoside (C3G) [19].

Anti-obesity in rodents by berries and anthocyanin-rich foods

Purple corn color: seminal work on anthocyanins and obesity

Tsuda et al. spent the later part of the 1990s and early 2000s researching the antioxidant and cardioprotective effects of the anthocyanin C3G in vitro and in rodent models [20-26]. With the established obesity epidemic in 2003, Tsuda noted heavy use of PCC as a food colorant in the Japanese food supply, an additive rich in their compound of interest, and decided to research its applicability in a mouse model of obesity. Tsuda et al. fed C57BL/6J mice a standard or high-fat

diet (approximately 60% energy from fat) with or without supplementation of PCC (11 g per kg diet, or approximately 33-55 mg of PCC consumed per day) [19]. The reported density of C3G was 70 g per kg PCC, thus mice consumed approximately 2-4 mg of C3G per day. Four-week-old mice were fed one of four diet treatments for 12 weeks, during which time body weight and food intake were monitored [19]. Endpoints included gene expression of lipogenic enzymes in the liver and white adipose tissue as well as adipokines in white adipose tissue, adipose tissue morphology and fat pad weights, and circulating glucose and insulin.

Findings from Tsuda et al.'s study were mostly positive for the benefit of PCC administration vs. the diet-induced obese phenotype in normal mice. Their experiment demonstrated normalization of the growth curve of mice fed the high-fat diet in combination with PCC despite a level of food intake not different from the high-fat control treatment, although it should be noted that physical activity was not monitored [19]. Histology of white adipose tissue demonstrated a clear reduction in adipocyte size of the high-fat-PCC group, where they also observed a reduction in fat pat weights as well as mRNA expression of leptin (circulating levels normalized) and tumor necrosis factor-alpha (TNF-α). Gene expression of lipogenic enzymes in the liver and white adipose tissue (fatty acid synthase and sterol regulatory element binding protein) were reduced with a high-fat diet, but were lowered further by supplementation of PCC. Gene expression of two other lipogenic enzymes (acyl-CoA synthase 1 and glycerol-3-phosphate acyltransferase) was increased by the high-fat diet relative to the standard diet groups - characteristic of diet-induced obesity – was also abrogated by the PCC treatment [19]. Taken together these data suggest multi-tissue, anti-lipogenic effects of PCC. Worth noting, these enzymes were also reduced in the normal weight animals fed PCC, suggesting that decreased enzyme expression of lipogenic enzymes is independent of

hyperinsulinemia caused by a high-fat diet. The authors of the study postulate modulation of the four enzymes by activation of AMP-activated protein kinase (AMPK), a molecular switch that, when activated, signals consumption of stored energy substrates (glycolysis and β-oxidation) and inhibition of anabolic processes (glycogenesis and lipogenesis). Diet-induced obesity also elicited reduced insulin sensitivity and glucose intolerance in this experiment, which was reversed with PCC [19]. Tsuda elaborates on their proposed mechanism of action in a recent literature review [27], citing activation in AMPK in several tissues from a rodent study in diabetic mice treated with an anthocyanin-rich bilberry extract **Figure 1** [28]**.**

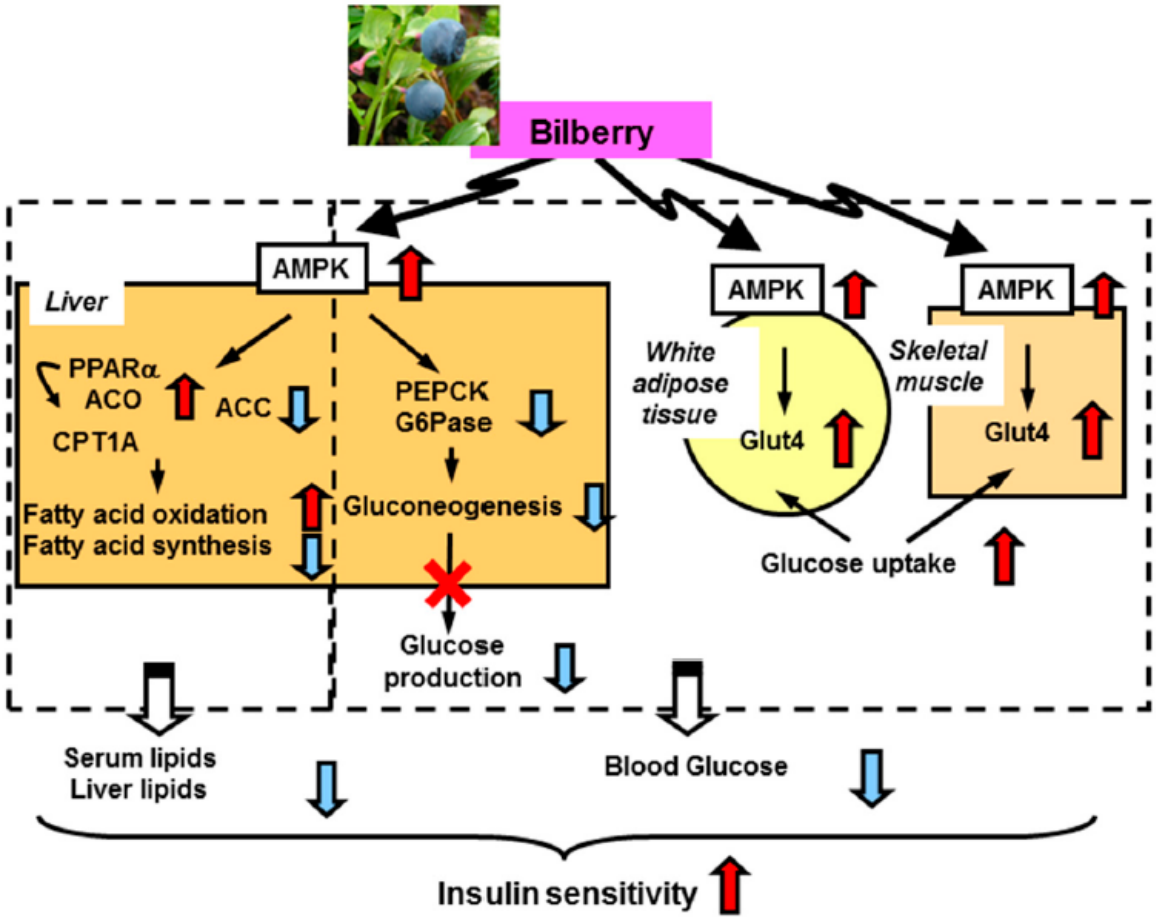

Figure 1. AMPK activation in several tissues by berry anthocyanins promotes anti-adipogenic and insulin sensitizing pathways. Figure reproduced from [27] under the terms of the Creative Commons license (https://creativecommons.org/licenses/by/4.0/).

Berries

The study by Tsuda et al. provides the first observations for the potential use of anthocyanin rich foods to ameliorate the obese phenotype. The study spearheaded a wave of experimental research in a variety of models to investigate purified anthocyanins, food extracts or whole food sources of anthocyanins to confirm their findings. Below, we will focus on studies that used berries or berry extracts to further elucidate the mechanisms by which they invoke biological activity in the resolution or prevention of diet-induced obesity.

Prior et al. conducted a series of experiments using whole berries, juices, or berry anthocyanin extracts in drinking water to determine any effect on high-fat diet induced obesity [29-31]. They describe two separate experiments in their first communication, where they fed freeze-dried blueberry or strawberry powder incorporated into a standard or high-fat diet in the first experiment, and added purified anthocyanins to drinking water, as well as increased the density of the high-fat diet from 45% to 60% in the second experiment [31]. The animals were weanling (21d) C57BL/6J mice. Outcome variables included body weight and body composition with MRI and tissue weights. The findings from their first report are in relative discord with the earlier findings by Tsuda et al.; while the freeze-dried strawberry powder treatment offered no protection to the high-fat fed mice, the blueberry treatment exacerbated the obese state as evidenced by a greater growth curve and higher body fat percentage [31]. By comparison, the results of the second experiment recapitulate the PCC study, in that anthocyanin extracts placed in the drinking water protected the mice weight gain when fed a high-fat (60% energy from fat) diet, although not to the extent that their weight was normalized to the low-fat control group [31]. As noted by a different communication discussed later [32] the formulation of the diets in the first experiment are cause for dismissing the findings of greater weight gain in the freeze-dried powder blueberry arm, as the mice in this group consumed 11% more energy relative to the high-fat control [31]. Further, in formulating the freeze-dried diets, the authors had to displace corn starch for incorporation of the respective fruit powder, likely higher in simple sugars (not reported). These discrepancies may partially explain the significant increase in body weight and body fat with the blueberry powder treatment.

Prior et al. performed two subsequent experiments with slight modification of the experiments in their first report, namely the manipulation of the mode of delivery and source of

berry anthocyanins [29, 30]. In one experiment they increased the concentration of purified anthocyanins in drinking water to include their original dose, 0.2 mg/mL, as well as a higher dose, 1.0 mg/mL, which added no observable benefit in protection against weight gain that was again observed with the low-dose [30]. However, a novel finding of purified anthocyanins in drinking water was the reduction in body fat percent even when mice were fed the standard fat diet. In this study they also included arms that were supplemented with blueberry juice in place of drinking water, which provided a nonsignificant reduction in cumulative weight gain and body fat as measured by fat pad weight compared to the control animals fed the high-fat (45%) diet [30].

A second and final follow-up experiment by Prior et al. used a similar experimental design with high-fat (60%) diets supplemented with anthocyanins in the drinking water, juice, or freeze-dried berry powder, except that black raspberries were chosen as the source of anthocyanins instead of blueberries or strawberries, to determine if source of anthocyanins resulted in altered effects on body weight and fat percentage in their model [29]. Ultimately the results were similar to their previous experiments, where purified anthocyanins in the drinking water yielded more of a benefit, and the fruit juice and freeze-dried powders actually exacerbated obesity [29]. Taken together, Prior et al.'s series of experiments with various preparations of anthocyanins from three berry sources highlights two separate points: anthocyanins can protect mice from diet-induced obesity; and mode of delivery needs to be taken into consideration, as freeze-dried powders, and sometimes juice forms, can further complicate diet-induced obesity likely due to concentration of sugar content or elimination of the fiber component. As the authors mention, there are likely differences in the magnitude of anti-obesity effects of anthocyanins derived from various berry sources as their anthocyanin profiles are drastically

different (table 2. of [31], and table 1. of [30]), and structural variation along with level of glycosylation likely determine biological activity [8].

A separate study also interested in the effects of freeze-dried blueberry powder in a rodent obesity model explored potential biomolecular mechanisms of action [32]. The group utilized the diabetic Zucker fatty rat, which due to a leptin receptor mutation presents spontaneous obesity and models type-2 diabetes, insulin resistance, dyslipidemia, and chronic inflammation. Seven-week-old fatty rats were fed a 45% high-fat diet supplemented with 2% blueberry powder or sugar control for 90 days [32]. In addition to monitoring weight change, body composition, and glucose tolerance, modulation of peroxisome proliferator activated receptor (PPAR) alpha and gamma activities and gene expression were assessed in both adipose and skeletal muscle tissues [32]. Modulation of these transcription factors by blueberry powder administration is of interest due to their known regulation on lipid metabolism.

After 90 days of feeding, body weights between control and blueberry fed groups did not differ, however visceral fat depots (retroperitoneal and epididymal) weighed significantly less in the blueberry fed group [32]. The authors connect this observation to the molecular level by demonstrating greater PPAR-α and PPAR-γ activities and gene expression in both adipose and skeletal muscle tissues. PGC1α, the master regulator of mitochondrial biogenesis, was also upregulated in these tissues with blueberry feeding, however changes in energy expenditure were not assessed [32]. As discussed by Seymour et al., thiazolidinediones are troublesome for their singular PPAR-γ activation, which, while remediating insulin resistance and dyslipidemia, are problematic in that they promote adipogenesis. What is attractive with their findings is pan-PPAR agonism: the removal of lipids from the circulation and disposal (oxidation) in peripheral tissues [32]. The diabetic state of the blueberry fed group was also improved, resulting in a 4%

reduction in glucose area under the curve, 20% reduction in fasting insulin, improved homeostasis model assessment of insulin resistance score (HOMA-IR), and 5% lower fasting triglycerides – supported molecularly by an increase in GLUT4 and insulin receptor substrate-1 (IRS1) gene expression in both tissues [32]. Although outside of the scope of the "berries" theme of the current review, the research group has performed similar experiments using anthocyanin rich tart cherry and discovered similar benefits in metabolic stress attributable to dual PPAR activation in several models [33, 34].

The positive results of the Seymour study compared to the mixed, sometimes complicating effects of blueberry powder in the Prior et al. studies begs the question of differences in design between the two research endeavors. Key differences include: dose of dietary blueberry powder, 2% vs. 10%; fat content, 45% vs. 45% and 60%; animal model, Zucker fatty rats (genetically obese) vs. C57BL/6J mice (genetically normal); and feeding regimen, 90% of ad lib vs. ad lib; Seymour et al. vs. Prior et al. studies, respectively. It is the opinion of this author that pair feeding and adjustment of the control diet for added sugar content of the blueberry powder maximized potential differences in measured outcomes attributable to anthocyanin content of the blueberry powder in the Seymour et al. study. As Seymour et al. pointed out, greater caloric intake in the blueberry arm vs. high-fat control could mask the benefit of berry anthocyanins in Prior et al.'s studies.

A Swedish study performed a comprehensive assessment of the anti-obesity and anti-diabetic potential of eight different berry varieties (lingonberry, blackcurrant, bilberry, raspberry, acai, crowberry, prune, or blackberry) in a C57BL/6J rodent model of diet-induced obesity [35]. Six-week-old mice were fed either a high fat (45%) control diet or high fat diet supplemented with 20% freeze dried berries for thirteen weeks ad libitum. Dependent variables included body,

organ, and fat pad weight, body composition, blood chemistry, and lipid analyses of liver and feces. Main study findings included a battery of improvements for three particular berries including lingonberry, blackcurrant, and bilberry [35]. Improved outcomes included reduced body weight and body fat percentage, fasting glucose, insulin and subsequent HOMA-IR score, liver weight and TGs, increased cecum weight and fecal TGs, and circulating plasminogen activator inhibitor-1 (PAI-1), a measure of systemic inflammation [35]. Interestingly, the authors reported an increase in energy intake with the blackcurrant and bilberry treatments; however they noted difficulty in food-intake assessment for the blackcurrant treatment. The most dramatic improvements were noted in the lingonberry group, whereas the acai berry exacerbated the increase in body weight and metabolic aberrations stemming from a high fat diet [35]. The authors mention the presence of quercetin in lingonberries that may potentiate the effects seen with the berries containing predominantly anthocyanins; but unfortunately only a qualitative analysis of flavonoid profiles for each berry was included [35]. The marked improvement in obesity phenotype attributable to berry treatment in this study is in stark contrast to the transient benefits observed in the Prior et al. experiments [29-31]. This is despite a nearly identical design: similar modes of diet incorporation and ad lib feeding, study duration, and identical mouse model. A convenient difference is berry source for anthocyanins, but better control of diet formulation in this study seems a more realistic possibility (see Table 2. of [35] vs. Table 1. of [31]). Both studies utilized the same diet manufacturer, but carbohydrate profiles are standardized across the different diets in the Heyman et al. report [35].

A different investigation by Farrell et al. performed a rodent feeding experiment with incorporation of black elderberry extract (BEE) with a high-fat (60% fat) diet to assess any potential of the berries to modulate the obese phenotype [36]. To determine dose-response, BEE

was fed to ten-week-old C57BL/6J mice at 0.25 and 1.25 percent of diet, spray-dried onto maltodextrin in order to maintain the macronutrient profile, which provided an estimated 20-200 mg of anthocyanins (mainly as C3G) per kg of bodyweight when fed for sixteen weeks. The authors considered these doses achievable by a normal human diet that incorporated 60 g of black elderberries daily [36]. At the end of the feeding period, serum biochemistry and inflammation, as well as hepatic and adipose tissue morphology and genetic markers of inflammation were measured to determine effects of BEE incorporation into high-fat treatments.

Farrell et al. report no change in body weight or food intake, but a significant 13% reduction in liver weight by both BEE treatments [36]. Serum triglycerides (TGs) were normalized by either BEE dose, while significant increases in cholesterol, non-esterified fatty acids (NEFAs), and alanine aminotransferase attributable to high-fat feeding were unaltered by BEE treatments. However, serum monocyte chemoattractant protein-1, TNFα, and fasting insulin concentrations, as well as HOMA-IR scores were significantly lowered by both doses of BEE compared to the high-fat control diet [36]. Histological examination of liver and adipose tissue indicated no benefit of BEE treatments against macrophage infiltration or fibrosis of both tissues by the high-fat diet treatment. Hepatic cholesterol content and fatty acid synthase gene expression were decreased by the higher dose and both doses, respectively. In adipose tissue, PPARγ and lipoprotein lipase (LPL) gene expression were significantly increased with the higher dose BEE treatment, which may help explain the reduction in circulating TGs. However, the higher dose treatment also had a greater staining for fibrosis and higher expression of transforming growth factor β compared to the other two high-fat diet treatments [36]. Thus, the findings from Farrell et al.'s feeding study with BEE are mixed; low dose anthocyanin treatment – achievable levels in a normal human diet – showed protection against a high-fat diet via

increased insulin sensitivity, moderate improvement in hepatosteatosis, and increased clearance of circulating TGs with improved function of adipose tissue, however the improvements may be at the cost of increased inflammation in the adipose tissue. A caveat of their study is the exceptionally high fat diet (60%) used to induce obesity. Their berry doses were set at a level that they calculated to be achievable by humans, which is especially useful for translational purposes, however, it would have then also been more informative to use a diet with a fat content more in line with human consumption, which is 34% on average [37]. Conversely, they could have included a pharmacological dose of BEE for proof of concept in protection against a very high-fat diet.

Other sources

Tangential from the berry theme, other C3G rich fruits have been tested for their ability to ward off diet-induced obesity [38]. A recent example of such work comes from an Australian study where ten-week-old rats were fed Queen Garnet (Japanese plum) juice or an equivalent amount of purified C3G alongside a high-fat (approximately 50% energy from fat), high-carbohydrate (25% fructose in drinking water) diet for sixteen weeks – flavonoid treatment was not started until week eight [38]. Dependent variables included glucose tolerance, body, fat pad and organ weights, body composition, histological staining of heart and liver for macrophage infiltration, and blood biochemistry. All parameters were improved by both modes of delivery while energy intake was not altered by flavonoid treatments [38]. This study provides further support of the bioactive effect of C3G and the notion that food matrices do not appear to inhibit absorption or activity. This work however was unable to detect circulating anthocyanins or metabolites in plasma, which may be due to improper handling of samples, as sample acidification was not mentioned in their methods for blood collection [38].

Finally, a Chinese study focused on anti-obesity effects of purified C3G in a different genetic model of rodent obesity and type-2 diabetes, the KK-Ay mouse model, which develop the phenotype independent of diet [39]. In addition to tracking changes in body weight and composition, similar to Seymour et al., Wei et al. also investigated the biomolecular route of action of anthocyanin feeding specifically with activated (phosphorylated) AMPK and LPL activities in skeletal and adipose tissues [39]. The authors fed six-week-old mice for 12 weeks with normal rodent chow or chow supplemented with 0.1% purified C3G. Significant findings of C3G supplementation included reduced body and visceral adipose tissue weights, reduced adipocyte size and lower hepatic steatosis scores. Molecularly, LPL activity was increased in skeletal muscle and decreased in visceral adipose tissues by C3G [39]. Further experimentation in culture demonstrated how the effects on LPL were dependent on activation of AMPK, as C3G dependent effects on LPL were abrogated with AMPK knockdown [39].

These studies using a variety of obese rodent models repeatedly describe a benefit in berries or purified C3G in preventing excess and ectopic body fat deposition. C3G is likely the favorite isolated anthocyanin for study due to its involvement in the seminal work by Tsuda et al. with PCC. However, as noted by the Heyman et al. study of several berries, various berry varieties contain a polypharmacy of anthocyanins as well as other flavonoids. Specifically, the greatest berry effect in their hands was the lingonberry, which also provides several isoforms of quercetin. An example of emerging knowledge in less popular berries comes from a comprehensive study by Rojo et al. where the researchers discovered insulin-like effects of the maqui berry, which grows wild and abundant in Chile [40]. They described positive effects in a type-2 diabetic (high-fat fed) mouse model as well as in both hepatic and muscle tissue which they attribute to the berries' most prominent (and less characterized) anthocyanin, delphinidin 3-

sambubioside-5-glucoside [40]. Similarly, a separate study found insulin-sensitizing and anti-inflammatory effects of four wild berry varieties traditionally consumed by Native American populations [41]. While work should continue to describe C3G's capabilities against diet-induced obesity, holistic (whole berry) approaches of flavonoid delivery may uncover synergistic actions between flavonoids in alleviating the obese phenotype and provides the greatest translatability compared to forms that aren't typically consumed by humans.

Berries and anthocyanins vs. obesity and metabolic aberrations: mechanisms of action in adipocytes

Mechanistic studies of anthocyanins and berries in vivo as well as culture systems will provide the idiosyncratic details needed to fully understand the biological pathways by which these phytonutrients alleviate obesity and metabolic dysfunction. Pertinent studies in several tissue models are discussed below. These discoveries not only validate the benefits by which berries may augment health vs. obesity, but these mechanisms can also be pursued by the pharmaceutical and medical industries, as the development of safe and effective anti-obesity treatments are aggressively sought after.

Molecular mechanisms, substrate trafficking, and cell switching

An elegant series of experiments were performed by Scazzocchio et al. to describe the mechanistic underpinnings of the insulin-like effects of C3G and its major metabolite, protocatechuic acid (PCA), in an adipocyte cell culture model of obesity [42]. To model insulin resistance, adipocytes were cultured in the presence of oxidized low-density lipoprotein (oxLDL), which, elevated in obesity, are known to alter normal insulin-stimulated glucose uptake. Both biopsied human omental adipocytes as well as the most commonly used rodent cell line, 3T3-L1s, were cultured with or without C3G or PCA and oxLDL both in basal and insulin

stimulated states to assess glucose uptake. The authors also measured the insulin-sensitive glucose transporter, GLUT4, protein and mRNA expression as well as location (presence in the plasma membrane or intracellular storage vesicle). Influence of PPAR-γ's role was ascertained by measuring its activity as well as applying experimental treatments when it is knocked-down by small interfering RNA (siRNA). Secretion of the anti-inflammatory adipokine, adiponectin, is associated with insulin sensitivity and this too was assessed for modulation by anthocyanins.

Incubation of human omental and rodent 3T3-L1 adipocytes with 100 mg/L of oxLDL led to an approximate 50% reduction in glucose uptake when stimulated with insulin (20 nmol/L insulin) [42]. Reduced glucose uptake was reversed with the addition of C3G or PCA, in fact both compounds had insulin-like effects in unstimulated cells, yielding a two-fold increase in glucose uptake. In serum starved unstimulated cells, addition of the anthocyanins also normalized glucose uptake, adiponectin secretion, and PPAR-γ activity in the presence of oxLDL. Glucose uptake was maximal with 50 and 100 uM C3G and PCA, respectively [42]. While these C3G concentrations were well above the nanomolar levels observed in human pharmacokinetic studies [43], Scazzocchio et al. argue the possibility of anthocyanins concentrating in peripheral tissues.

In a separate experiment Scazzochio et al. determined a positive effect of C3G and PCA on GLUT4 gene expression, and protein expression and location; however these cells were not inhibited with oxLDL [42]. They show increases in gene and protein expression of GLUT4 by both compounds, and that a larger GLUT4 content is incorporated into the plasma membrane vs. internal storage vesicles, similar to insulin stimulation. Silencing of PPAR-γ with siRNA ablated the positive effects of C3G and PCA on increased gene expression of adiponectin and GLUT4, further, increased translocation of GLUT4 to the plasma membrane was reversed, demonstrating

the causative role of PPAR-γ on the insulin-mimicking effects of C3G and PCA in adipose tissue [42]. The authors also mentioned the utility of 3T3-L1 adipocytes as a surrogate for mechanistic studies in human adipocytes as both cell models yielded the same results to their experimental conditions. Overall this study describes a mechanism by which anthocyanins can remedy insulin resistance in adipose tissue; a worthwhile application if it can be confirmed in vivo.

A study by Kowalska et al. assessed the anti-adipogenic ability of freeze-dried cranberry extract in differentiating 3T3-L1 adipocytes [44]. Cranberries are rich in flavonoids, including quercetin, anthocyanins, and procyanidins [8]. The expression of several genes related to lipid metabolism were measured in the cells after 6-8 days of exposure to 0, 2.5, 5, and 10 mg cranberry extract per mL of cells in media. The adipogenic genes fatty acid binding protein-4, LPL, fatty acid synthase, perilipin-1, hormone sensitive lipase, and leptin were all dose dependently inhibited by the addition of cranberry extract [44]. Adiponectin was dose-dependently increased with cranberry extract, except for the highest dose. The same trends were confirmed for the secretion of adiponectin and leptin. The study effectively demonstrated the inhibition of differentiating adipocytes by cranberry extract, and earlier work by the same group described inhibition of adipogenic transcription factors PPAR-γ, CCAAT-enhancer binding protein-beta (C/EBPβ), and sterol regulatory element-binding protein 1 (SREBP1) [45]. A few caveats of this study include polyphenol dose and cytotoxicity of cranberry extract. Connecting this study to others in the mechanisms of berry phenolics is difficult because the concentrations of the cranberry polyphenols cannot be verified. Their work cites downstream effects of PPAR-γ inhibition, whereas both animal and cell models described above with blueberries and C3G describe how its activation is what may help alleviate the metabolic dysregulation of obesity. Further, most genes measured as well as the two secreted proteins were not detectable at the

highest dose of treatment, suggesting a cytotoxic effect, but viability measurements were not reported. Although their concentrations may be questionable, the dose-dependent effects on leptin and adiponectin indicate the potential of cranberry extract to promote an insulin sensitive, anti-inflammatory adipocyte.

An elegant series of experiments by Matsukawa et al. pieced together a compelling biomolecular story of C3G's beige-inducing action on differentiating 3T3-L1 adipocytes [46]. Similar to Kowalska et al., treatment effects were assessed after cells were incubated for up to eight days in differentiation medium. Adipocytes were incubated with 20 – 100 μM C3G during differentiation. Afterwards cells were characterized for brown adipose tissue characteristics; although 3T3-L1s are considered white adipocytes, conversion to a brown or "brite" adipocyte is possible [46]. Key differences between the two include a higher mitochondrial content, lipid droplet morphology, and the conversion of energy substrates to heat instead of ATP via an increase in uncoupling protein 1 in brown adipocytes. Changes in mitochondrial density of differentiated adipocytes were assessed by staining mitochondrial membranes with rhodamine 123, as well as determining expression of several mitochondrial genes: mitochondrial transcription factor A, pyruvate dehydrogenase kinase isozyme 4, superoxide dismutase 2, uncoupling protein 1 (UCP-1), and UCP-2. Further, genes related to adipocyte differentiation (C/EBPβ and C/EBPα, PPARγ), specifically to brown adipocytes (T-box protein 1 and Cbp/p300-interacting transactivator 1), as well protein expression of AMPK and PGC-1α (regulators of mitochondrial biogenesis) were assessed [46].

Matsukawa et al. reported no effect on viability of differentiating adipocytes with 20 – 100 μM C3G, and that when inspecting morphological features, lipid droplets of adipocytes treated with 100 μM C3G were more characteristic of brown adipocytes with multilocular lipid

droplets [46]. Both rhodamine 123 staining and mitochondrial gene expression showed a dose dependent increase in mitochondrial content of 50 and 100 μM C3G treatments. Parallel to these observations, protein expression of activated (phosphorylated) AMPK, PGC-1α, and UCP-1 were significantly upregulated compared to control cells, providing additional evidence for an increase in mitochondrial density [46]. The genetic markers of adipocyte differentiation and browning listed above were also activated by C3G treatment. The increase in thermogenic potential vs. energy production was also evidenced by a reduction in intracellular ATP levels as well as a drop in reactive oxygen species. The authors conclude that C3G's browning effect stems from its ability to inhibit phosphodiesterases thus increasing levels cyclic-AMP thereby stimulating differentiation of adipocytes into brown adipocytes via activation of C/EBPβ ultimately leading to the increase in mitochondrial density equipped with thermogenic (vs energetic) membranes (see figure 7. of [46] for a proposed pathway of action). Conceptually, the browning of adipose tissue is an attractive application in terms of obesity as this would cause a greater inefficiency of energy substrates and create a metabolic sink for excess calorie intake as well as reduce adipocyte size and number, ultimately increasing insulin sensitivity. However, dose is likely to be a limitation for these effects in vivo, as maximal effect was noted at 100 μM dose of C3G, and the anthocyanin has been reported in picomolar concentrations in the adipose tissue from animal studies [47].

A separate study by the same research team investigated the effects of C3G derived from black soybeans on adipocyte differentiation and metabolism as well as effects of adipocyte conditioned medium on markers of mitochondrial regulation in C2C12 myotubes [48]. This model takes the work described above a step further by probing for the secondary effects of C3G on skeletal muscle tissue. Similar to the other study, differentiating adipocytes were incubated

with 20 or 100 μM C3G for eight days. Effects on adipocyte metabolism include adiponectin and TNF-α secretion, triglyceride accumulation and gene expression of adipogenesis (PPARγ and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression), as well as glucose uptake and markers of insulin signaling (C/EBPα, insulin receptor, Akt, and GLUT4). Influence of adipocyte conditioned medium (treated with C3G) on mitochondrial regulation in myotubes was determined by changes in gene expression of PGC-1α, sirtuin 1 (SIRT1) – thought to activate AMPK and/or PGC-1α, and UCP-3 [48].

Histological examination of differentiating adipocytes showed that C3G treatment yielded a smaller adipocyte, which is an attribute of increased insulin sensitivity [49-51]. The C3G treated cells also dose-dependently secreted more adiponectin (up to 425% with the 100 μM C3G treatment) and less TNF-α (57% less with the 20 μM C3G treatment) compared to untreated adipocytes. PPARγ gene expression, GAPDH activity, and intracellular triglycerides were all increased with C3G treatment. Regarding insulin sensitivity, C3G treatment resulted in up to a 3.4 fold increase in C/EBPα gene expression, as well as significant increases in both activation (phosphorylation) and protein content of both insulin receptor and Akt, as well as increased gene expression of GLUT4 [48]. These increases were confirmed by an increase in glucose uptake by up to 1.8 fold. When differentiated C2C12 myotubes were incubated with the conditioned medium of C3G treated adipocytes for 24 hours, all three markers of mitochondrial regulation listed above were significantly upregulated with the 100 μM C3G treatment. However, because of the research design, it's not clear whether these effects on the myotubes were due to adipocytes effects on the medium or because C3G was acting directly on the mytoubes, but Matsukawa et al. believe the increase in thermogenic genes in the myotubes can be explained by the increased secretion of adiponectin by the C3G treated adipocytes [48].

Insulin resistance and ectopic fat are hallmarks of obesity. Matsukawa et al.'s two studies on C3G's effects on differentiating adipocytes demonstrates the potential for remediation of metabolic dysregulation of adipose tissue by way of increased adipogenesis (PPARγ), insulin sensitivity, as well as the potential for increased thermogenesis in both beige-induced adipocytes and the correction of mitochondrial deficits in the skeletal muscle tissue of the obese [52]. Concentrations of the C3G used in these studies could pose a challenge, but the same pathways of activation are worth probing in human studies.

Studies of C3G action in adipocytes described above highlight the potential for C3G to increase adipogenesis, i.e., clearance of NEFAs from the circulation, to increase insulin sensitivity, and promote thermogenesis in both adipose and skeletal muscle tissues. Another angle in realignment of dysfunctional adipose tissue metabolism is introduced by a mechanistic study by Guo et al. which assessed the modulation of lipolysis by C3G in 3T3-L1 adipocytes in high glucose conditions [53]. Differentiated adipocytes were incubated with high-glucose (30.5 mM) media for 24 hours followed by treatment with 0-100 μM C3G for up to 48 hours. Lipolysis was assessed by measurement of NEFAs and glycerol in the culture media as well as triglyceride hydrolase activity. The glycosylation of transcription factor FoxO1 by glutamine:fructose 6-phosphate aminotransferase (GFAT) increases expression of adipose triglyceride lipase (ATGL) ultimately increasing lipolysis in adipocytes, thus effects of C3G on this pathway were also included in the study [53].

High glucose incubation of adipocytes increased secretions of NEFAs and glycerol by over three-fold compared to the control, low glucose medium without affecting cell viability [53]. The increase was dose-dependently attenuated with C3G by up to 40%. In addition, Guo et al. observed a two-fold increase in triglyceride hydrolase activity of the high glucose incubation,

which was also inhibited by a similar degree by addition of 50 µM C3G. The addition of a hormone sensitive lipase inhibitor in the triglyceride hydrolase assay still showed an inhibitory effect of C3G, suggesting inhibition on ATGL, the enzyme that performs the first step in the lipolysis of triglycerides [53]. High glucose incubation increased the glycosylating activity of GFAT by six-fold compared to low glucose, which also was attenuated by C3G, however when added directly to the GFAT activity assay, C3G did not reduce its activity, implying an indirect inhibition of the enzyme in culture. The study also demonstrated an increase in FoxO1 protein glycosylation as well as an increase in ATGL; both increases were attenuated by addition of C3G. Similar to the studies described above, Guo et al. also reported a normalization in activated AMPK as well as its activity with C3G in high glucose media [53]. Because C3G did not act directly on GFAT activity despite its ability to reduce glycosylation of FoxO1 and subsequent reduction in secreted NEFAs and glycerol, Guo et al. propose C3G acts at the level of AMPK activation which then elicits post-translational modification on GFAT to reduce glycosylation of FoxO1 (see figure 5. Of [53]).

Inflammation

Chronic, low-level systemic inflammation is a common feature of obesity [54]. Further, inflammation in the adipose tissue due to macrophage infiltration leads to an imbalance of adipokine secretion, insulin resistance, increased lipolysis and efflux of NEFAs into the circulation, which exacerbates insulin resistance in other tissues [54]. A study conducted by Garcia-Diaz et al. characterized the effects of anthocyanin enriched fractions from blackberry and blueberry wine on interactions between lipopolysaccharide (LPS) stimulated macrophages and 3T3-L1 adipocytes [55]. Three different anthocyanin mixtures were tested: 100% blackberry, 70% blackberry and 30% blueberry, or 30% blackberry and 70% blueberry where

dose was set at 100 µM C3G equivalents for each treatment. Experiments included level of activation and cytokine excretion of LPS-activated macrophages pretreated for 1 hour with the respective anthocyanin mixture, adipogenesis in differentiating adipocytes treated with anthocyanin mixtures, and lipolysis and insulin sensitivity assays in differentiated adipocytes treated with media conditioned from LPS-activated macrophages that were treated with the three different anthocyanin mixtures [55].

Measurement of nitric oxide and TNF-α in cell supernatants were reported as a means to describe activation of the macrophages by stimulation with 1 µg/mL LPS. Secretions of both nitric oxide and TNF-α showed the most dramatic reduction when the macrophages were pretreated with the 30% blackberry and 70% blueberry mixture, where the other two treatments demonstrated a trend in the same direction [55]. This observation was also supported by a significant reduction in the activation (phosphorylation) of p65 subunit of NFκB. In line with the adipogenic effect of C3G described in the above studies, Garcia-Diaz et al. reported an increase in intracellular triglyceride content and reduced glycerol secretion in differentiating adipocytes, where effects were again greatest (28% increase compared to control) with the 30% blackberry and 70% blueberry mixture [55]. Interestingly when the differentiated adipocytes were treated with macrophage conditioned media for 24 hours, it was the other two berry mixtures that showed a significant reduction on glycerol release (14% and 16% reduction, 100% blackberry and 70% blackberry and 30% blueberry mixtures, respectively) which may suggest differential effects of different anthocyanin profiles on lipolysis with or without inflammation. Finally, compared to positive control, both intermediate berry blends reduced the hindrance in glucose uptake by adipocytes treated with LPS-stimulated conditioned media by up to 11% [55].

These mechanistic studies demonstrating the potential of berry extracts or C3G in adipose tissue need to be confirmed in human feeding studies. The mechanisms described go one step further than the pan PPAR agonism proposed by Seymour with their rat feeding study in that adipose tissue would not only be a site of removal for FFAs from circulation, but they could also act as a metabolic sink to reverse or prevent the effects of excess caloric intake. If Matsukawa et al.'s proposed effects of increased adiponectin secretion by C3G treated adipocytes on skeletal muscle can be confirmed in vivo, this would also indicate an endocrine pathway of activation of anthocyanins to ameliorate obesity, or at least its secondary features, by way of increased thermogenesis. The dual culture experiments by Garcia-Diaz also point toward alleviation of the inflammation-provoked disturbances in lipolysis and insulin sensitivity. Despite the obvious challenge of obtaining similar C3G concentrations in human studies, these mechanistic studies describe pathways that could be explored by pharmaceutical research as well.

Berries and anthocyanins vs. obesity and metabolic aberrations: human studies

Meta-analyses

A recent meta-analysis was conducted by Huang et al. to determine if cardio-protective effects of berries existed based upon information from available controlled human trials [56]. Altogether, the study included twenty-two randomized controlled trails which included over 1200 subjects, spanning eight different berries as sources of phytochemicals: elderberry, cranberry, bilberry, black currant, lingonberry, blueberry, whortleberry, and black raspberry. Subjects ranged from healthy to type-2 diabetic/metabolic syndrome, or presenting with cardiovascular risk factors (excess weight, dyslipidemia, hypertension, and/or impaired glucose tolerance). The majority of the studies used a parallel-arm study design [56].

Key findings from Huang et al.'s study include significant reduction in LDL cholesterol in groups consuming berries vs. control treatments (weighted mean difference of 0.21 mmol/L, or 8 mg/dL lower with berries) [56]. Similarly, HDL was increased by berry treatment (weighted mean difference of 0.06 mmol/L, or 2.3 mg/dL higher with berries); however, this effect was marginal and the authors report that the required information size was not reached to definitely determine the effect of berry feeding on HDL, thus they call for more research on this variable. Further, in their secondary analyses they describe significant improvement in systolic blood pressure, fasting blood glucose, hemoglobin A_1C (HbA_1C), body mass index (BMI), and TNFα. They were also able to show in subgroup analyses that greater effects (more benefit of berries) was elicited by bilberry and whortleberry, with greater lengths of treatment (> 8 weeks), and in subjects with greater cardiovascular risk [56]. Although the authors report low risk of study bias, there was significant heterogeneity with the majority of dependent variables described, attributable to variability in research design, i.e. study populations, design and duration, and type of berry treatment as well as modality of delivery. Taken together, this meta-analysis reaffirms the benefits of berry consumption on cardiovascular health first described in epidemiological studies, however not all the dependent variables associated with cardiovascular risk can be definitively proven as modifiable with berry consumption with the current state of knowledge gleaned from the studies described by Huang et al.

A second meta-analysis by Guo et al. collected data from five prospective cohort studies to determine the effect of either berry or anthocyanin intake on the risk of developing type-2 diabetes [57]. Three of the prospective cohort studies were from the United States (the Nurse's Health Study I and II, and the Healthcare Practitioners Follow-Up study), while the other two were from Finland (the Finnish Mobile Clinic Health Examination Survey, and the Kuopio

Ischaemic Heart Disease Risk Factor Study) [13, 58-60]. The meta-analysis included over 190,000 participants in which approximately 13,000 cases of type-2 diabetes were identified at follow-up. The American studies used food frequency questionnaires to determine berry intake while the Finnish studies used either in-person interviews or 4-day food records. Guo et al. calculated the anthocyanin intake in the prospective studies using the USDA's flavonoid content database [61].

Relative risks were calculated for either berry or anthocyanin intakes to determine effect on type-2 diabetes development. Guo et al. describe a significant 15% reduction in risk when comparing highest vs. lowest intakes of berries, and a significant 18% reduction when comparing highest vs. lowest intake of anthocyanins [57]. Heterogeneity in the berry meta-analysis required a sub-analysis that described a protective effect of berries for females, but interestingly the same benefit was not clear in males. Further, older subjects (>50 years) and European cohorts demonstrated a greater benefit than younger or US counterparts, respectively, although all groups were reported to have a significant benefit from berry intake. Finally, the study also demonstrated a significant dose response of either berry or anthocyanin intake in protecting against type-2 diabetes: for every 17 g of berries or 7.5 mg of anthocyanins, there was an incremental 5% lower risk of developing type-2 diabetes (see figure 3 of [57]).

Berries and human obesity

The meta-analyses described above highlight modulation of biomarkers commonly affected by diet-induced obesity by berries or anthocyanins, from both experimental and observational research. The missing lynchpin connecting the rodent and tissue data described earlier to research on berry consumption in human data are clear and reproducible human studies demonstrating protective effects of berries against weight gain or a positive impact on weight

loss. However, if the changes described in the meta-analyses are any indication, there is likely influence by berries on how the body is storing and using fat. This lack of information on berries and adiposity in humans is the basis of this dissertation.

A variety of clinical research on several kinds of high anthocyanin berries and their effects on clinical biomarkers influenced by obesity exist. Here we will review pertinent randomized, controlled, dietary research trials investigating anti-obesity (clinical complications related to obesity) effects of different berry modalities. Publications will be separated out based on how the berry anthocyanins were administered: anthocyanin rich extracts, freeze-dried powders, or as whole berries.

Human studies with anthocyanin rich extracts

A randomized, double-blind, placebo controlled trial studied the effects of an anthocyanin extract in 120 community-dwelling, overweight subjects with dyslipidemia [62]. Subjects were given 320 mg of a bilberry/black currant extract (majority of anthocyanins were glycosylated variants of cyanidin or delphinidin) or placebo control daily for 12 weeks. In addition to determining changes in circulating TGs, LDL and HDL cholesterol, cholesterol ester transfer protein (CETP) and lecithin cholesterol acyltransferase (LCAT) concentrations and activities were assessed because of their role in reverse cholesterol transport. Findings include a significant 13.7% increase in HDL cholesterol, a 13.6% decrease in LDL cholesterol, as well as significant reductions in both concentration and activity of CETP in the group receiving anthocyanin capsules, where changes in both types of cholesterol had a significant association with changes in CETP [62]. These findings lead the authors to conclude a CETP-dependent effect on the positive changes in LDL and HDL cholesterol in dyslipidemic subjects consuming

anthocyanins from berries, and that these improvements translate to a 27% reduction in the risk heart disease [63].

In a separate study by the same group, a similar study design was used: anthocyanin rich extract from bilberry and black currant was administered (320 mg per day) for 12 weeks to investigate the effect of anthocyanins on flow-mediated dilation (FMD) of the brachial artery - a marker of endothelial function, inflammation, and cyclic guanosine monophosphate (cGMP) (an indicator of nitric-oxide related vasodilation in endothelial cells) in 146 hypercholesterolemic, overweight men and women [64]. The study was also randomized, double-blinded, and placebo controlled using a parallel-arm design. Results included a significant 12% increase in cGMP as well as a significant improvement in FMD from 8% to 11% with anthocyanin administration while no changes were observed in the placebo controlled group [64]. Vascular cell adhesion molecule 1 (VCAM-1), a marker of endothelial inflammation, was also improved with anthocyanin treatment. Similar to the aforementioned study, the anthocyanin treatment also elicited improvements in HDL and LDL cholesterol. Interestingly, the authors also administered a nitric-oxide synthase inhibitor to a subgroup of subjects with or without a simultaneous infusion of purified anthocyanin and discovered improvements in FMD by the anthocyanin infusion were blocked by the inhibitor, suggesting the dependence on nitric oxide synthase activity in anthocyanin related improvements in FMD [64]. The improvements in endothelial function, HDL and LDL cholesterols, and inflammation by anthocyanins in this study suggest a reduction in atherosclerotic progression via anthocyanins in hypercholesterolemic subjects.

Two subsequent research communications from this same feeding study were produced, probing further into the anthocyanin extract's effects on inflammation and paraoxonase 1 (PON1) activity of HDL [65, 66]. With regard to inflammation, the authors also found that the

anthocyanin extract significantly lowered circulating high-sensitive c-reactive protein (hsCRP) as well as IL-1β by 22 and 13%, respectively, providing further evidence of a systemic reduction in inflammation [66]. HDL-PON1 activity is responsible for protecting against oxidation of LDL and foam cells and delays the progression of atherosclerosis [67]. Zhu et al. found that the anthocyanin extract elicited a significant 17% increase in PON1 activity in the hypercholesterolemic subjects as well as a 21% reduction in oxidation of HDL particles in a dihydrorhodamine oxidation assay [65]. The authors propose an antioxidant effect of anthocyanins via PON1 on HDL particles allows for greater reverse cholesterol transport resulting in the observed decrease in LDL noted in the earlier study [62, 65].

In a study by a separate group, eight obese, type-2 diabetic males participated in an acute experiment consisting of a single 470 mg dose of bilberry extract (36% anthocyanins by weight) or placebo followed by a five hour oral glucose tolerance test [68]. The study was double-blind, randomized, and crossed-over. Significant findings from the study included a reduction in both glucose and insulin area under the curve, but no response in incretins (GIP and GLP-1) or inflammation via the measurement of monocyte chemoattractant protein 1. The authors hypothesize that the bioactive effects of the extract are a combination of increased insulin sensitivity as well as delayed and/or blocked absorption of sugar in the gut via the inhibition of digestive enzymes and transporters [68]. Their findings are supported by another study from Liu et al. where forty-eight overweight, type-2 diabetics were fed their 320 mg dose of bilberry and black currant extract or placebo for 12 weeks [69]. Although changes in serum lipids were not different like their work in the hypercholsterolemic studies, they did detect a 29% increase in serum adiponectin and 16% reduction in fasting serum insulin which demonstrate positive changes in glucose management in diabetics with chronic feeding of anthocyanin extracts [69].

Finally, Kianbakht et al. studied the effects of a whortleberry extract on blood lipids in a group of obese subjects with hyperlipidemia not being treated with pharmacotherapy [70]. In a parallel-arm, double-blind design, 105 randomized subjects consumed 1050 mg of encapsulated whortleberry extract or placebo daily for two months to assess changes on serum lipids. The treatment corresponded to 7.4 mg of anthocyanins each day. Despite a lower dose of anthocyanins compared to the doses used by the two research groups described above, this study observed dramatic improvements on blood lipids: 28, 19, and 26% reductions in total cholesterol, TGs, and LDL, respectively, and a 38% increase in HDL [70]. These dramatic improvements in serum lipids despite lower dose of anthocyanins compared to the other studies using extracts warrant further elucidation of bioactives present in whortleberry.

Taken together, these controlled trials on anthocyanin enriched extracts demonstrate the potential utility of anthocyanins from berries against common insults of obesity: dyslipidemia and impaired glucose tolerance. If these findings can be confirmed it would provide an alternative or complementary measure to ameliorate the clinical disturbances associated with obesity, particularly if a patient doesn't tolerate pharmacotherapy, thus future work on extracts is warranted. Lack of diversity is a limitation of this research area; five of the seven projects described above come from the same research group [62, 64-66, 69], and the interventions only include three different berry sources for anthocyanin extraction. Future work should include broader involvement by different research institutes as well as determine the efficacy of other high anthocyanin berries.

Human studies with freeze-dried powders

A double-blind, parallel arm study by Stull et al. determined the effect of blueberry smoothies on insulin sensitivity in obese adults with insulin resistance [71]. Thirty-two subjects

were fed a smoothie containing either 22.5 g of freeze-dried blueberry powder or a placebo twice daily for six weeks. Both groups also received nutritional counseling in order to accommodate the energy provided by either smoothie treatment. The 45 g of berry powder equated to two cups of blueberries, or over 600 mg of anthocyanins. Insulin sensitivity was assessed by a hyperinsulinemic-euglycemic clamp, where rate of glucose infusion required to maintain euglycemia was corrected by lean body mass. Stull et al. reported that subjects in the blueberry powder group had a greater improvement in percent increase in insulin sensitivity compared to placebo (22 vs. 5%, blueberries vs. placebo, respectively) [71]. Secondary analyses including body composition and markers of inflammation were unchanged.

The same research group performed an additional study using a similar study design, i.e., a placebo controlled, double-blind, parallel arm trial with the same blueberry smoothie, where they sought effects of blueberries on hypertension, insulin sensitivity, and endothelial function in subjects with metabolic syndrome [72]. The same dosing regimen was applied for six weeks in forty-four subjects. The major findings from the study include no effect of the blueberry smoothie on 24 hr ambulatory blood pressure or insulin sensitivity. Changes in blood pressure due to blueberries could have been masked by the use of antihypertensive medications, as their use was not exclusion for subject enrollment. A deviation of this study from the former was the use of an intravenous glucose tolerance test in place of the hyperinsulinemic-euglycemic clamp, which may have affected the ability to detect differences in insulin sensitivity. A positive finding from the study was an increase in endothelial function with the blueberry smoothie [72].

A similar study by Basu et al. performed a randomized, placebo controlled trial with forty-eight subjects with metabolic syndrome where subjects consumed 50 grams of blueberry powder reconstituted in 960 mL water or a water control for eight weeks [73]. Their blueberry

powder reportedly contained 742 mg of anthocyanins per 50 g dose. Study aims included measuring changes in the five possible determinants of metabolic syndrome (metS) (central adiposity, hypertension, TGs, impaired fasting glucose, or low HDL) as well as lipoprotein oxidation and inflammation. Key findings from the study include significant reductions in systolic and diastolic blood pressure by 6 and 4%, respectively, in subjects in the blueberry treatment group [73]. No other differences were found on criteria of the metS. Oxidation of LDL particles was also significantly lower with blueberry treatment, but markers of inflammation were unchanged [73]. This study's findings on blood pressure are in direct contention with the aforementioned study by Stull et al. Despite the obvious difference with a slight increase in the dose as well as a few weeks longer duration in the Basu et al. study, preparation of blueberry powder was different in that Stull et al. mixed their powder into a yogurt based smoothie while Basu et al. simply had subjects dissolve the powder in water. Interestingly, the intervention was well tolerated in the Stull et al. study while Basu et al. saw 27% attrition from their blueberry arm, notably due to gastric distress [73]. These differences in tolerability between the two studies may be an indicator of the potency of the different preparations of the blueberry powder. A useful measurement would have been plasma anthocyanins, which was not assessed in either study.

A study by Johnson et al. supports the notion of an antihypertensive effect of blueberries in at-risk populations [74]. Forty-eight postmenopausal, obese women with mild hypertension were randomized to receive 22 g of freeze-dried blueberries or an energy matched placebo for eight weeks in a double-blind, parallel arm study. Both treatments were reconstituted in water prior to consumption. Primary findings included significant reductions of systolic and diastolic blood pressures by 5 and 6%, respectively, as well as a reduction in arterial stiffness as measured

by pulse-wave velocity in the blueberry treatment group where no changes were observed with the placebo. Further, nitic oxide levels in the serum were increased by 68% with the blueberry treatment. Similar to the Basu et al. study, a 20% attrition rate was noted in the blueberry treatment with gastric distress cited as a main problem [74].

Another study by Basu et al. enrolled sixteen obese, female subjects with metS to determine the effect of freeze-dried strawberry powder on indices of the metS as well as inflammation [75]. The subjects had all indices of metS except for impaired fasting glucose. Subjects were fed 50 g of strawberry powder daily for four weeks. The powder was dissolved in water and provided 154 mg of anthocyanins each day. The study was not controlled or blinded. Key findings included significant reductions in total and LDL cholesterol as well as lipid peroxides by 5, 6, 14%, respectively [75]. Body weight, blood pressure, blood glucose, and inflammation were unchanged by the strawberry powder. Compared to the study on blueberry powder, the strawberry powder intervention in this study was well tolerated, as none of the subjects dropped out due to gastric issues caused by treatment.

The investigators conducted a follow-up study in twenty-seven obese subjects with metS where a control group was included as a parallel arm and the duration of study was extended to eight weeks utilizing the same daily dose of strawberry powder [76]. The control group was assigned to consume the amount of water required to dissolve the strawberry powder (960 mL) thus the study was not blinded. This study confirmed the effects observed in the uncontrolled study, where total and LDL cholesterols were significantly lowered by the strawberry powder treatment and to a greater extent than the previous work (10 and 11%, respectively) which could be explained by the longer study duration of eight weeks. An anti-atherosclerotic effect was also suggested with strawberry treatment by a reduction in circulating concentrations of VCAM-1.

A recent study by Park et al. focused on the acute effects of strawberry powder in twenty-five insulin resistant subjects with central obesity (waist circumference > 110 cm) [77]. Insulin resistance was defined as fasting insulin concentrations > 13 μIU/mL or a HOMA-IR score greater than 1. In a randomized, cross-over, single-blind study, subjects were given one of four doses of freeze-dried strawberry powder (0-40 g) incorporated into whole milk and provided up to 155 mg of anthocyanins. The treatments were given in combination with a high fat, high carbohydrate breakfast and postprandial changes on blood glucose, insulin, and lipids were assessed out to six hours. The main finding from the study was a significant 12% reduction in six hour averaged postprandial insulin concentrations with the highest strawberry powder treatment (40 g) [77]. Further, a dose response effect was suggested by a statistical trend for lower insulin to glucose ratios with the 10 and 20 g doses of strawberry powder compared to the control group. These observations on insulin sensitivity were observed while there were no differences between the postprandial glucose curves for all treatment groups [77]. Blood lipids and postprandial inflammation were not different between treatments. Postprandial plasma concentrations of pelargonidin (the major anthocyanin present in strawberries) and cyanidin conjugates confirmed the dose-response of the four treatments and changes in circulating pelargonidin were associated with changes in postprandial insulin with all strawberry powder treatments [77].

The noted improvements by strawberry powder on biomarkers in subjects with metS or insulin resistance are corroborated by a study with type-2 diabetic subjects [78]. In a randomized, double-blind, parallel arm study Moazen et al. administered 50 g of strawberry powder (providing 154 mg of anthocyanins) or placebo powder (both treatments dissolved in water) daily to thirty-six type-2 diabetic subjects for six weeks to assess changes on long-term glucose regulation and inflammation. The subjects consuming the strawberry powder benefited from a

6.5% reduction in HbA_1C where an increase was observed in the control arm. The group also saw a 20% reduction in hsCRP and lipid peroxides [78]. The findings demonstrate a cardioprotective benefit of strawberry powder in type-2 diabetics by both improved regulation of glucose metabolism leading to lower protein glycosylation as well as hindered atherogenicity by curbing chronic inflammation and lipid oxidation.

These studies highlight the therapeutic effects of two common and accessible berries on clinical features associated with obesity and metS. Noted in the discussion by Park et al., freeze-dried preparations are commonly studied as a surrogate delivery form for berries in order to ensure consistency of the anthocyanin content, and other bioactive components attributable to feeding the intact berry may be overlooked in these studies, namely, the positive health benefits associated with fiber [77]. Fortunately, the powder doses used in these studies are achievable from normal amounts of berry intake; between one and three servings (one to three cups) of berries contain an equivalent amount of anthocyanins.

Human studies with whole berries

Twenty-three healthy subjects were recruited by Alvarez-Suarez et al. in order to assess changes in blood lipids, oxidative stress, and platelet function after consuming 500 g of fresh strawberries (equivalent to approximately 300 mg of anthocyanins) daily for thirty days [79]. The study was not placebo controlled. Sample collections included baseline, post thirty days, as well as after a fifteen day washout to assess any long term changes by the intervention. Main findings include significant improvement of serum total cholesterol, LDL, and TGs, where concentrations were lowered by 9, 14, and 21%, respectively, after the thirty day strawberry treatment. Lipid peroxides, oxidized DNA, and urinary isoprostanes – all makers of oxidative stress – were also significantly reduced after the strawberry regimen. Blood platelet function was

also improved, as the amount of activated platelets (central clustered morphology) was reduced by 31% after the thirty day treatment [79]. Interestingly, following the fifteen day washout, all noted benefits due to the berry intervention were abrogated. Although it is attractive to conclude a cardiovascular benefit from high-dose berry consumption for one month, because the study was not controlled a confounding study effect on the subjects (motivated to eat healthier due to participation in a health study) cannot be ruled out. However, the noted differences are worth confirming in follow-up studies that utilize a stronger experimental design.

Finally, a study by Lehtonen et al. conducted a randomized, cross-over trial in eighty obese women fed one of four berry derived interventions: 100 g per day bilberries, 100 g per day sea buckthorn berries, or doses of either ethanol or oil extracts of sea buckthorn that are equivalent to a 100 g serving of sea buckthorn berries [80]. The aim of the study was to determine any effect of the two berries on biomarkers of metabolic disease. Each intervention period and washout period were approximately thirty days. Changes in the subjects' diets were monitored by food diaries. Key findings of the study include modest, yet significant reductions in weight (0.2 kg) and waist circumference (1.2 cm) with bilberries, while waist circumference was also reduced with sea buckthorn berries (1.1 cm) [80]. Improvements in inflammatory markers were also improved by the berry groups but were inconsistently reported. Paradoxically, insulin and/or HbA_1C measures deteriorated during all four berry interventions, however, the authors note significant deviations in the habitual diets of the subjects during both washout and intervention periods, which may explain these conflicting results [80]. While the study utilized the power of a crossover design to detect small differences in body weight and waist circumference, the authors chose not to include a control group and the inability to control the

basal diet of the subjects may have hindered the ability to accurately assess the health benefit attributable to the berry interventions.

These two studies using whole berries demonstrate the ability to modulate pertinent complications of obesity that are commonly targeted by healthcare practitioners: blood lipids and body weight. However, due to their poor study design [79] and noncompliant subject populations [80], protective effects of whole berries are inconclusive. There is a dearth of information on the effects of whole berry treatments in obese subjects; more research is needed with better (placebo-controlled) study designs. Further, none of the clinical trials discussed in this section controlled the diet of the subjects, thus the influence of background diets on the parameters reported in these trials are extraneous variables that can only be resolved by controlling every aspect of what the test subjects consume – both food items as well as their quantities.

Conclusions

Epidemiologic data indicates a protective effect of fruit and vegetable consumption against mortality. Specifically, epidemiological reports indicate a protective effect of polyphenols. Further, anthocyanins have been implicated for their health promoting effects. The obesity epidemic continues to challenge the American healthcare system, and an aging population means the comorbidities associated with the condition will begin to surface. This literature review has taken a tour through rodent, mechanistic, and human clinical trials investigating the effect of anthocyanins or berries – a rich and natural source of anthocyanins – against obesity and its secondary complications. Results from rodent studies using berry interventions suggest an anti-obesity effect. Adipose tissue studies highlight molecular pathways that, once activated, have the potential to ameliorate dyslipidemia and insulin resistance and, if

beige-inducing can be confirmed, may also convert adipose tissue into a metabolic sink while signaling muscle tissue to increase mitochondrial density. Human clinical trials of berry extracts and powders touch upon the potential protective effect of berry treatments on clinical parameters complicated by obesity, however, findings are inconsistent and have not been translated to a form of berry consumption in a typical diet, nor have researchers successfully controlled for the confounding effects of heterogeneous diet habits of subjects participating in berry intervention studies.

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Chapter 2: Seven Day Blackberry Feeding Lowers the Respiratory Quotient in Males and Improves Insulin Sensitivity

Introduction

Two-thirds of Americans can be classified as overweight or obese [1]. This statistic is the culmination of a multifaceted phenomenon that will be aggressively studied until its resolution and closely monitored so that it can never again tax the healthcare system an annual $147 billion [2]. The combination of overeating and physical inactivity is the primary driver in the development of the obesity epidemic. Labor automation, a car-centric, overworked society, the food science revolution, and the disparity in cost between healthy, nutrient dense foods and high-fat, nutritionally deficient food items are just a few of the factors thought to produce obesity [3]. Complications related to downstream morbidities of obesity will begin to surface as the American population ages, notably, type-2 diabetes and heart disease [2].

Lifestyle modification still proves to be the most effective means to combating obesity. Medical and pharmacological research up to this point has failed to produce an alternative to the gold standard treatment: improved/informed diet choices and increased physical activity [4]. Within the realm of diet choices, the protective effect of fruit and vegetable consumption against all-cause mortality and cardiovascular disease are notable [5, 6]. Most fruits and vegetables are rich sources of fiber, essential vitamins and minerals. An increasing body of evidence is uncovering the health-promoting effects offered by their phytonutrients, namely polyphenols. Polyphenols are a diverse class of phytonutrients including lignans, stilbenes, and flavonoids. As reported in the PREDIMED study, there is a protective effect of higher intakes of polyphenols against all-cause mortality [7]. Anthocyanins are one of the main classes of flavonoids garnished with substantial attention in regards to obesity. Anthocyanins are a class of phytochemicals responsible for the red, blue, and purple hues of fruits and vegetables. Of the fruits and

vegetables found in a typical American diet, berries provide one of the greatest sources for anthocyanin consumption [8].

A principal anthocyanin in obesity research is cyanidin-3-O-β-glucoside (C3G), which first received attention when delivered in the form of purple corn color in seminal work by Tsuda et al. [9]. They fed mice a high fat (60% energy from fat) diet for twelve weeks supplemented with or without the food colorant which provided 2-4 mg of C3G per day. Mice fed C3G had reduced body weight, adipocyte size, and reduced expression of lipogenic enzymes in hepatic and adipose tissues [9]. These findings encouraged berry anthocyanin research in rodent obesity. A series of experiments by Prior et al. using various berry anthocyanin preparations reported conflicting results in the capability of berries to protect against diet induced obesity [10-12]. Conversely, experiments by Seymour et al. demonstrate amelioration of the obese phenotype via a PPAR "pan-agonist" effect of blueberries and tart cherry in both adipose and skeletal muscle tissues [13-15]. Further, mechanistic studies of anthocyanins in culture experiments suggest an improvement in insulin signaling in adipose tissue with C3G as well as phenotype switching to characteristics of brown adipose tissue [16-19].

Translating anti-obesity effects of berries noted in rodents to humans has been less successful. Meta-analyses of human clinical studies demonstrate a reduction in several of obesity's secondary effects: dyslipidemia, hypertension, and risk of developing diabetes [20, 21]. However, most controlled trials involving berry treatments in obese or metabolic syndrome presenting subjects fail to note improvements in body weight [22-28]. We believe that this is likely due to most investigator's inability to control their subjects' diet, as most research centers are not outfitted with large-scale feeding facilities. It is our contention that the attractive findings of reduced adiposity with berry anthocyanins from rodent studies needs to be explored in human

clinical trials and that the improvements in cardio metabolic parameters noted with preliminary human trials should be confirmed. Thus, the aim of the current study is to determine the effect of berry feeding on measures of fat oxidation and insulin sensitivity in a group of overweight or obese male subjects fed a controlled high fat diet.

Methods

Hypotheses

Primary Hypothesis: Fat oxidation will be greater when healthy, overweight or obese adult volunteers consume a high fat diet with blackberries compared to fat oxidation when healthy, overweight or obese adult volunteers consume a high fat diet with an anthocyanin-free, calorie matched control food.

Secondary Hypothesis: Insulin response, as assessed by area under the plasma insulin concentration time curve during a meal-based glucose tolerance test, will be lower after healthy, overweight or obese adult volunteers consume a breakfast meal with blackberries compared to insulin response after healthy, overweight or obese adult volunteers consume a breakfast meal with an anthocyanin-free, calorie matched control food.

Subjects

Subjects were recruited from the Washington D.C. metropolitan area via email advertisements (**Appendix 3)**. Interested applicants were required to attend an information session detailing the time requirements, study dates, and potential risks of the study. Following the meeting potential subjects completed a study application (appendix 3**)**, provided informed consent (**Appendix 4)**, and scheduled an appointment for screening. Eligibility for the study was determined by review of a health history questionnaire (appendix 3) to assess personal and familial medical histories as well as collection of blood and urine samples to generate a routine

chemistry panel to rule out any unreported health conditions. Charts and questionnaires were reviewed by a registered nurse practitioner to determine eligibility. Women were not eligible for participation because of the known effects of menstrual cycling on substrate oxidation and energy expenditure, the primary measurements of the study. Male subjects were eligible if they were between the ages of 25 and 75 years old with a BMI of at least 25 kg/m^2. Subjects were excluded from participation if they used blood-thinning medication, had gastrointestinal or metabolic diseases, type-2 diabetes under pharmaceutical treatment or fasting glucose greater than 126 mg/dL, fasting triglycerides greater than 300 mg/dL, use of anti-obesity medication or supplements for the preceding six months before study initiation, cardiovascular disease, used tobacco products, took antibiotics in the preceding month, or experienced adverse reactions to blackberries. The study protocol was approved by Medstar Health Research Institute institutional review board (Hyattsville, MD, appendix 3) and a coauthorization agreement was established with the institutional review board at the University of Maryland, College Park, in order to present the findings as a part of this dissertation. Twelve subjects completed the study protocol in the summer and early fall of 2013 in order to generate pilot data on the effects of blackberry feeding on the respiratory quotient (RQ). Using the pilot data and a power calculation appropriate for crossover trials [29], it was estimated that significant changes in the 24 hour averaged RQ would require 15 subjects to reach 80% power with alpha=0.05. Further, technical complications lead to loss of data integrity with nine of the twelve original subjects, thus an additional fifteen subjects completed the study protocol during the fall of 2015 and spring of 2016. This trial was registered at clincaltrials.gov (NCT01932879).

Study design

A study design worksheet for the controlled feeding study is provided in **Appendix 2.** The study was designed to be a randomized, placebo controlled, cross-over trial with two treatments. Subjects and investigators were not blinded to the treatments due to feasibility. Subjects consumed either 600 g of blackberries (BBs) or a calorically matched amount of artificially flavored gelatin daily for one week. Treatment periods were separated by a one week washout where subjects were asked to avoid red and purple pigmented fruits and vegetables. Treatment doses were divided in two and consumed at breakfast and dinner, where they were under the observation of dietetic technicians. The 2015-2020 Dietary Guidelines for Americans recommends consuming 2 cups of fruit daily for adult men [30]. In an effort to maximize treatment differences, the subjects in the present study consumed twice the recommended amount of fruit intake as 4 cups or 600 g of blackberries. The blackberry treatment was estimated to provide over 1200 mg of flavonoids and 540 mg of total anthocyanins each day. Estimated flavonoid content is reported in **Table 1.**

Table 1. Flavonoid Content of Blackberries

Flavonoid	Per 300 g serving	Per 600 g daily dose
Cyanidin 3-glucoside	180.7 mg	361.3 mg
Catechin	32.9 mg	65.8 mg
Epicatechin	200.9 mg	401.7 mg
Kaempferol glucoside	283.7 mg	567.3 mg
Quercetin glucoside	39.7 mg	79.4 mg
Total measured flavonoids	737.9 mg	1475.5 mg

Diets

Subjects consumed a 100% investigator controlled diet with a macronutrient profile of 40% of energy from fat, 45% from carbohydrate, and 15% from protein sources. Menus were formulated around these definitions by an on-site registered dietician using the ESHA software program (Salem, OR), and meals were prepared by professional chefs at the on-site metabolic

kitchen. Energy needs for a subject were estimated by the Harris-Benedict equation which takes height, weight, age, gender, and physical activity level into consideration. Calorie levels (in increments of 200 calories) were assigned to subjects based upon this estimation. Each subject's weight was measured daily, Monday through Friday.

Meals were provided as a 2-day menu rotation using common American menu items (each day's menu is reported in **Appendix 5**). Subjects were instructed to eat only the food provided to them by the center. Monday through Friday, subjects were required to eat both breakfast and dinner at the center, while both lunch and all weekend meals were packed out for the subjects to consume off-site. Diet compliance was assessed by both changes in weight as well as subject observation by dietary technicians during meals that were eaten in the cafeteria on-site.

Assessments, sample collection and analysis

The final twenty-four hours of each diet period was completed while residing within a room-sized indirect calorimetry chamber to allow measurement of respiratory gasses in order to measure the respiratory quotient (RQ) (the quotient of CO_2 expired to O_2 inspired) as well as perform subsequent calculations for energy expenditure and substrate oxidation. A description for the technical assembly and performance of the specific chambers used for this study has been published previously by Seale et al. [31]. Air sampling occurred every 100 seconds and gaseous concentrations were measured simultaneously with a Perkin Elmer MGA 1200 multiple gas analyzer. Prior to the subjects' stay, each of three chambers was calibrated via combustion of ethanol lamps to determine expected versus observed O_2 and CO_2 recoveries. Raw data from the gas analyzer was recorded onto a personal computer with Labview software. The O_2 and CO_2 data were plotted for visual inspection and erroneous values were deleted and replaced by

averaging the preceding and succeeding concentrations. A regularized deconvolution algorithm was written in Matlab and was applied so as to reduce instrumental noise. The algorithm also interpolated the dataset so as to generate minute-by-minute estimates for CO_2 production and O_2 consumption. This is a unique aspect of the calorimetry dataset, as these variables are typically reported as twenty-four hour averages. A detailed description of the algorithm as well as its verification with an experimental dataset has been described by Gribok et al. [32]. The algorithm was used to produce corrected respiratory gasses datasets for each subject for both diet periods. Six subsequent different time isolations were used to calculate the RQ, energy expenditure, and substrate oxidation datasets: 24 hours, 4 hours starting with first bite of dinner meal, 2 hours at night (between 2-4 AM), 4 hours starting with first bite of breakfast meal, 2 hours starting with first bite of lunch meal, and 30 minutes during low-intensity (3 miles-per-hour) treadmill walking. Using the corrected, minute-by-minute calorimetry dataset, energy expenditure was calculated with the Weir equation (equation 1), and substrate oxidation (g of fat or carbohydrate used for energy production) was calculated using the equations of Livesey and Elia (equations 2-11) **Table 2** [33]. These equations factor out the volume of oxygen consumed and CO_2 produced dedicated for protein oxidation (equations 2-3) in order to estimate the remaining gaseous quantities (equations 4 and 5) partitioned into energy production via fatty acid (equations 6-9) or carbohydrate (equations 6-7 and 10-11) substrates. Because the subjects were weight stable, they were assumed to be in nitrogen balance, thus the rate of protein oxidation, required to calculate oxidation of fat and carbohydrate with Livesey equations, was determined by estimating protein intake from the week of controlled feeding.

(1) Energy expenditure (kcals) = (1.106*VCO2) + (3.941*VO2)
(2) VO2P=6.26*total urinary nitrogen (estimated from protein intake)
(3) VCO2P=VO2P*.835
(4) VO2NP=-VO2(measured)-VO2P

(5) VCO2NP=VCO2(measured)-VCO2P
(6) RQNP=VCO2NP/VO2NP
(7) HNP=(15.547*VO2NP+5.573*VCO2NP)
(8) PFAT=(19.50*(1.0-RQNP)/(19.50*(1-RQNP)+21.12*(RQNP-0.71)))
(9) GFAT=PFAT*HNP/39.5
(10) PCHO=(21.12*(RQNP-0.71)/(21.12*(RQNP-0.71)+19.50*(1-RQNP)))
(11) GCHO=PCHO*HNP/17.5

Table 2. Equations used to estimate energy expenditure as well as grams of fat and carbohydrate oxidized based on gaseous exchange after correction for protein oxidation. V, volume; P, protein; NP, non-protein; RQNP, respiratory quotient-non-protein; HNP, heat-non-protein; PFAT, percent fat; GFAT, grams fat; PCHO, percent carbohydrate; GCHO, grams carbohydtrate.

On day six of each diet period, subjects arrived at the center between 4 and 5 PM for their twenty-four hour chamber stay. For subject comfort, the chamber is equipped with the latest technologies for leisure activity as well as a fold-down bed, computer desk, and lavatory for personal hygiene. The subjects were introduced into their room and provided with instructions to operate the electronics as well as a list of pertinent investigator phone numbers in case of emergency. Each subject was instructed to not engage in any form of physical activity and was only allowed to sit or lay peacefully with the exception of thirty minutes of supervised treadmill walking in the early afternoon of day seven. The subjects were given meals and bottled water through an air lock and each chamber had a ceiling fan set at low to promote even mixing of respiratory gases. The chamber was climate controlled and the subjects were allowed to set a comfortable temperature as needed. The subjects were under the care of a calorimeter attendant overnight to provide their evening meal as well as in case they needed extra bottled water or needed to communicate an emergency. Following the twenty-four hour stay, each chamber was thoroughly disinfected.

In order to determine modulation of insulin sensitivity and glucose tolerance by BBs, a meal-based oral glucose tolerance test (MTT) was administered on the morning of day seven while the subjects were completing their twenty-four hour calorimetry chamber stay. Subjects

were awakened at approximately 6 AM, and two baseline blood draws were collected between 6:15 and 6:45 AM. Blood was sampled from an indwelling catheter which was placed by a registered nurse who then also performed all subsequent blood sample collections and resolved any positional or clotted catheter lines. If sample collection was burdensome or collected samples were hemolyzed, the nurse would place a second catheter in the opposing arm. Subjects were able to provide their arm to the nurse on the outside of the chamber by way of a small port that was only opened for catheter setting and subsequent blood collections. Blood sampling typically took no longer than one minute and when sealed the port did not allow chamber gasses to leak outside of the chamber. Subjects were provided with approximately 75 g of sugar in the form of toaster waffles and syrup in combination with 300 g of blackberries or calorically matched gelatin treatments **Table 3**.

	Quantity (g)	Protein (g)	CHO (g)	Sugar (g)	Fat (g)	Energy (kcal)
Blackberry treatment						
Waffles, Aunt Jemima	56.5	3.3	20.5	2.4	3.7	128.5
Pancake Syrup, Monarch	60	0	40.2	36.3	0	160.8
Blackberries, frozen, unsweetened	302	3.6	47.3	32.2	1.3	215.3
Total	**418.5**	**6.9**	**108**	**70.9**	**5**	**504.6**
Gelatin treatment						
Waffles, Aunt Jemima	56.5	3.3	20.5	2.4	3.7	128.5
Pancake Syrup, Monarch	60	0	40.2	36.3	0	160.8
Gelatin, strawberry	273	2.8	45.5	43.2	0	193.2
Total	**389.5**	**6.1**	**106.2**	**81.9**	**3.7**	**482.5**

Table 3. Macronutrients of the MTT.

The subjects had ten minutes to consume both the waffle meal and their respective treatment foods. Subsequent blood sampling was collected at 30, 60, 90, 120, 180, and 240 minutes after the first bite of the breakfast meal for a total of eight samplings, including the two baseline samples. Blood was collected in serum, plasma-EDTA, and sodium-fluoride tubes,

centrifuged, aliquoted, and stored at -80°C until analyzed. Serum glucose, non-esterified fatty acids (NEFAs), and triglycerides were analyzed using standard protocols on an automated clinical chemistry analyzer (Vitros 5,1 FS, www.orthoclinical.com). Serum or plasma insulin was measured using ELISA kits (EMD Millipore) per the manufacturer's instructions on an automated plating and spectrophotometry system (DSX workstation, Dynex Technologies, Chantilly, VA).

Statistics

Linear mixed models were written for both calorimetry and clinical variables to test for statistically significant differences between the blackberry and gelatin treatments using "proc mixed" repeated measures analysis of covariance with SAS version 9.4 (SAS institute, Cary, NC). Normality and homoscedasticity of residuals were determined by the Shapiro-Wilk test and visual inspection of residual plots, respectively. Non-normality of residuals was addressed by mathematical transformation and LSmeans were back calculated for reporting purposes. Model estimates of response variables from each treatment are repeated on subject fit with the best covariance structure which is determined by information criteria as well as visual inspection of residual plots for each covariance structure used in preliminary analyses. Main effect of treatment and covariates of subject BMI, age, and sequence order were included in the model statement. Interactions of BMI and age with treatment were determined with backward elimination of non-significant terms. Random side error effects are estimated for subject nested in sequence. Data are presented as least-squares means for each treatment and $p<0.05$ is considered statistically significant.

With respect to measurements and calculations for indirect calorimetry, each parameter (averaged RQ and summed fat oxidation, carbohydrate oxidation, and energy expenditure) is

reported for each of the six different time isolations (24 hr, dinner, nighttime, MTT, lunch, and exercise). With respect to the clinical measurements performed on the MTT blood samples, incremental area under the curve (iAUC) was calculated for glucose and insulin, and area under the curve (AUC) for NEFAs using central Riemann-sum. Fasting concentrations of glucose and insulin were used to calculate homeostasis model assessment of insulin resistance and β-cell function (HOMA-IR and HOMA-B), which are commonly used equations that provide a surrogate index of insulin sensitivity without administering a hyperinsulinemic-euglycemic clamp test [34] and are calculated as follows: HOMA-IR = (fasting serum insulin x fasting serum glucose) /22.5 and HOMA-B = (20 x fasting serum insulin)/(fasting serum glucose – 3.5), where fasting serum insulin concentrations are μU insulin/mL serum and fasting serum glucose concentrations are mmol glucose/L serum. Fasting triglyceride concentrations are also presented.

Results

Of the thirty-six potential subjects who provided informed consent, thirty-five subjects completed the screening and twenty-seven were selected for participation in the study (**Figure 1**). Two subjects withdrew from the study; one because of intolerance of the indwelling catheter and the other due to prescribed steroid use for a sinus infection. Regarding the calorimetry dataset, unexpected power outages and mass spectrometer failures of the gas analyzers lead to loss of data integrity for eight of the subjects from the pilot study. Thus, a total of seventeen subjects had successful calorimetry stays that provided reliable data of the gaseous exchanges that were used in the analysis (Figure 1). Subject characteristics of the two data sets are reported in **Table 4.**

		Meal Tolerance Test	Calorimetry
Treatment		N=24	N=17
Gelatin	Weight	95.7 ± 12.8	94.3 ± 13.6
	BMI	30.6 ± 3.8	30.6 ± 4.1
	Age	57.8 ± 10.5	61 ± 7.8
Blackberry	Weight	95.7 ± 12.9	94.4 ± 13.7
	BMI	30.6 ± 3.8	30.6 ± 4.2
	Age	57.8 ± 10.5	61 ± 7.8

Table 4. Subject characteristics measured on the day of calorimetry entry for each diet treatment. Data are dichotomized into meal tolerance test and calorimetry datasets due to different sample sizes, i.e., not all subjects who completed the meal tolerance test had calorimetry data. Data are means ± SE for each variable within each diet treatment across the two datasets.

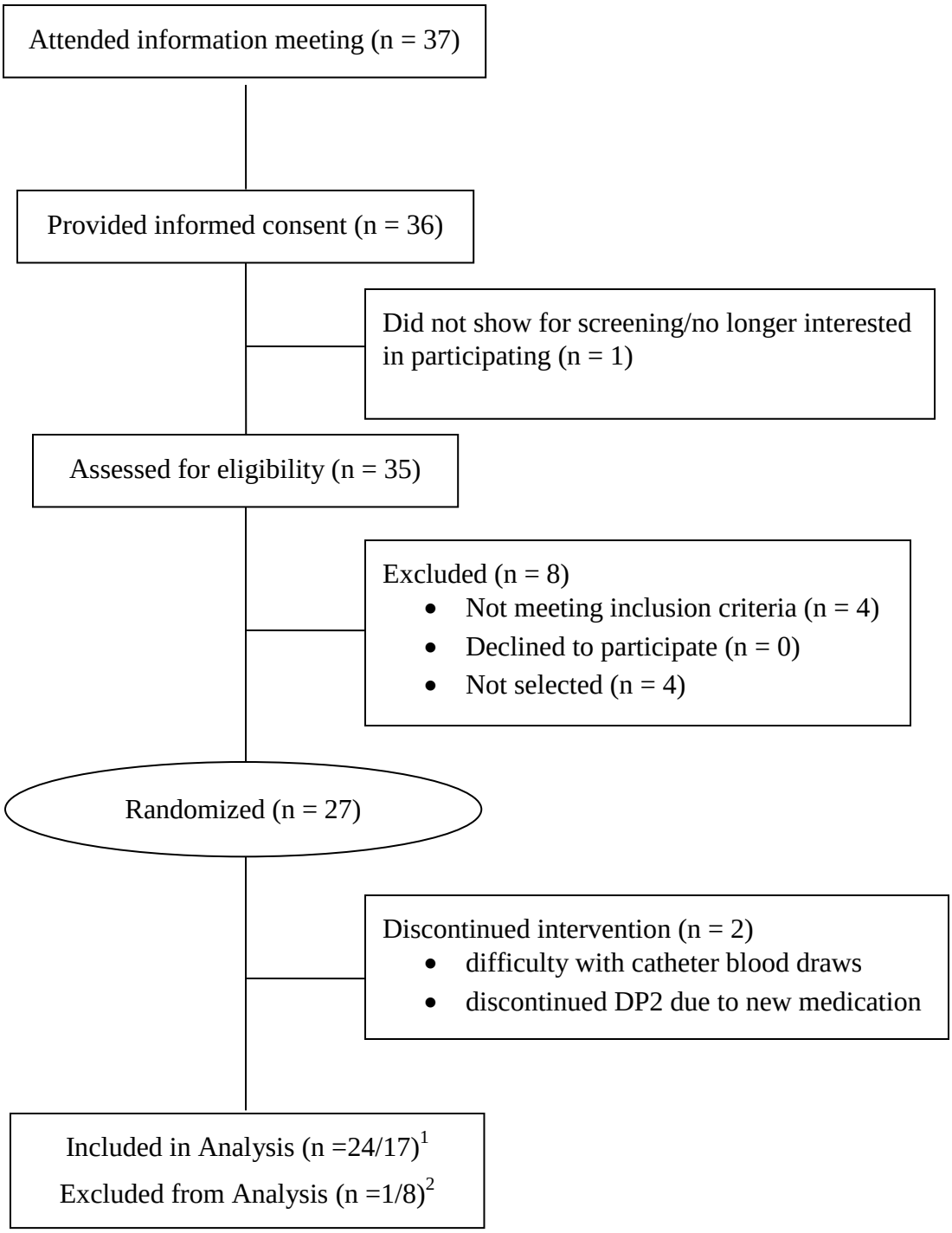


Figure 1. CONSORT (Consolidated Standards of Reporting Trials) diagram for the blackberry calorimetry study. [1]Analyzed for meal tolerance test parameters/analyzed for calorimetry dataset. [2] One subject was excluded from the meal tolerance analysis for noncompliance. Eight subjects were excluded from the calorimetry dataset due to data integrity.

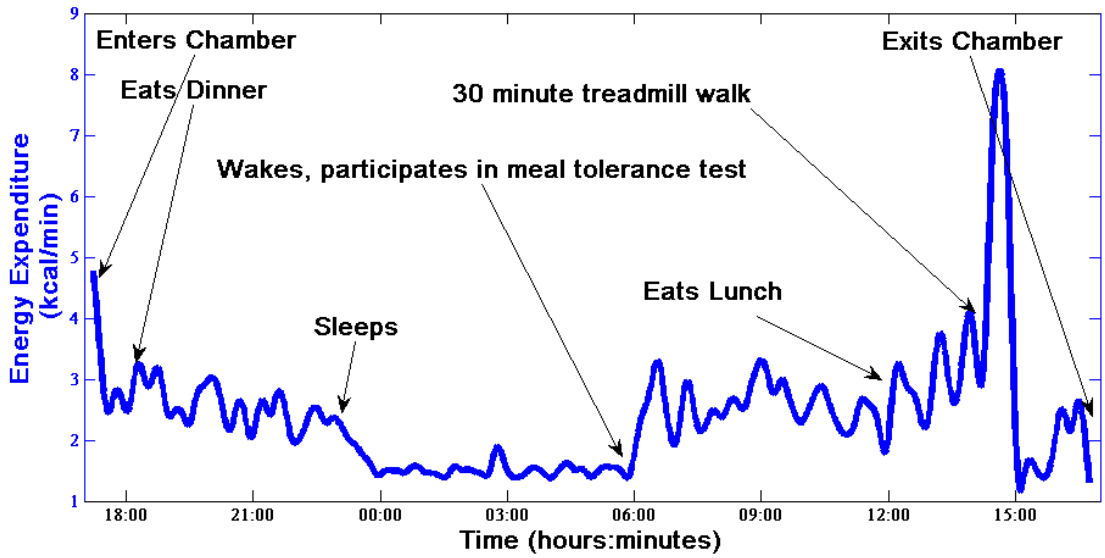


Figure 2. Energy expenditure trace of a study participant throughout the entire length of their stay in the room-sized calorimeter. Subjects Entered between 4:30 and 5:30 PM. Dinner was served to the subjects at 6:00 PM. The subjects were asked to turn out the lights and sleep or lay in their bed at 11 PM. The subjects were rousted shortly after 6:00 AM and the meal tolerance test was administered between 6:30 and 11:00 AM. Lunch was served at noon. A 30 minute treadmill walk began at 2:00 PM. The subjects were released from the chamber between 4:00 and 5:00 PM.

24 hr Energy Expenditure and Substrate Utilization

Results for all indirect calorimetry and MTT parameters and group-wise comparisons are reported in **Table 5.** 24 hr calculations for indices of indirect calorimetry are based upon the shortest time-span where data was available for every time point across all subjects. The shortest calorimeter run was performed between 5:32 PM on day 1 and 3:23 PM on day 2 for a total of 21 hours and 51 minutes of recorded data for each subject. Although we call this longest time-range our "24 hr" comparisons, we did not extrapolate the dataset out to 24 hr estimates, as energy expenditure and substrate oxidation are not constant across time, but are heavily influenced by different physical states (eating, sleeping, walking); see **Figure 2** for a representative trace of energy expenditure. There was a significant effect of diet treatment with 24 hr RQ where subjects, when fed BBs for seven days, had a significant reduction in 24 hr RQ compared to

when they were fed the gelatin control (0.8101 vs. 0.8171, BB vs. gelatin, respectively, p=0.0402, **Figure 3**). This corresponds to an estimated 24 hr fat oxidation of 141 g when fed BB compared to 132 g when fed gelatin (p=0.0420). BMI was a marginally significant covariate in the 24 hr fat oxidation model (p=0.0516) where estimated g of fat oxidized increased with BMI independent of treatment. There was also a marginal increase in CHO oxidation when subjects were fed the control gelatin treatment (183 vs. 173 g, gelatin vs. blackberry, respectively, p=0.0678). There was a nonsignificant 2% increase in 24 hr energy expenditure with the blackberry treatment (2485 vs. 2439 kcals, BB vs. gelatin treatments, respectively, p=0.1055). BMI was a significant covariate in the 24 hr energy expenditure model where energy expenditure increased with BMI independent of treatment (p=0.0399).

	Gelatin			Blackberry			Difference of LSmeans (Gelatin – Blackberry)			
		95% CI			95% CI			95% CI		
	LSmean	Lower	Upper	LSmean	Lower	Upper	Difference	Lower	Upper	P[3]
24 hour (5:32PM-3:23PM)										
RQ (CO2/O2)	0.8171	0.8079	0.8262	0.8101	0.8010	0.8192	0.0069	0.0004	0.0135	**0.0402**
Energy Expenditure (kcals)	2438.50	2254.16	2622.83	2485.40	2301.06	2669.75	-46.91	-104.86	11.05	0.1055
Fat Oxidation (g)	132.38	117.22	147.53	140.84	125.69	156.00	-8.47	-16.59	-0.35	**0.042**
Carbohydrate Oxidation (g)	182.71	166.19	199.24	172.95	156.42	189.47	9.77	-0.80	20.33	0.0678
Dinner (~6-10PM)										
RQ (CO2/O2)	0.8358	0.8229	0.8486	0.8178	0.8050	0.8306	0.0180	0.0059	0.0301	**0.0063**
Energy Expenditure (kcals)	483.38	449.84	516.91	493.29	459.75	526.82	-9.91	-27.96	8.15	0.2616
Fat Oxidation (g)	24.60	21.34	27.85	27.98	24.73	31.24	-3.39	-5.70	-1.07	**0.0069**
Carbohydrate Oxidation (g)	41.56	37.14	45.98	35.87	31.45	40.29	5.69	1.87	9.51	**0.0061**
Nighttime (2-4AM)										
RQ (CO2/O2)	0.7928	0.7790	0.8066	0.7960	0.7822	0.8098	-0.0031	-0.0135	0.0072	0.5289
Energy Expenditure (kcals)	155.71	143.59	167.83	159.63	147.51	171.75	-3.92	-10.72	2.88	0.2384
Fat Oxidation (g)	9.49	8.34	10.63	9.48	8.34	10.63	0.01	-1.61	1.62	0.9935
Carbohydrate Oxidation (g)	6.60	5.11	8.09	7.35	5.86	8.83	-0.75	-1.86	0.37	0.1727
MTT (~7-11AM)										
RQ (CO2/O2)	0.8512	0.8403	0.8620	0.8416	0.8308	0.8525	0.0095	0.0036	0.0155	**0.0036**
Energy Expenditure (kcals)	439.99	406.11	473.88	451.67	417.79	485.56	-11.68	-30.32	6.96	0.2028
Fat Oxidation (g)	19.35	16.58	22.12	21.30	18.53	24.07	-1.95	-3.43	-0.47	**0.0129**
Carbohydrate Oxidation (g)	43.11	39.80	46.42	41.13	37.82	44.44	1.98	-0.85	4.81	0.1567
Lunch (~12-1:50PM)										
RQ (CO2/O2)	0.8135	0.8003	0.8267	0.8098	0.7966	0.8230	0.0037	-0.0049	0.0123	0.3806
Energy Expenditure (kcals)	224.94	203.72	246.15	230.40	209.18	251.61	-5.46	-17.94	7.02	0.3674
Fat Oxidation (g)	13.30	11.37	15.23	13.63	11.70	15.56	-0.33	-1.08	0.42	0.3671
Carbohydrate Oxidation (g)	15.02	12.10	17.94	15.45	12.53	18.37	-0.43	-2.91	2.05	0.7185
Exercise (2-2:30PM)										
RQ (CO2/O2)	0.8708	0.8615	0.8801	0.8553	0.8460	0.8646	0.0155	0.0057	0.0253	**0.0041**
Energy Expenditure (kcals)	196.37	175.68	217.06	197.36	176.67	218.05	-1.00	-7.98	5.99	0.7667
Fat Oxidation (g)	8.32	7.06	9.59	9.35	8.08	10.62	-1.03	-1.68	-0.37	**0.0044**
Carbohydrate Oxidation (g)	25.71	23.07	28.35	23.46	20.82	26.10	2.24	0.00	4.49	**0.0497**
OGTT										
Glucose iAUC (mg*min/dL)[1]	8.31	8.05	8.57	8.16	7.90	8.41	0.15	-0.04	0.35	0.1151
Insulin iAUC (µU*min/mL)[1]	9.02	8.77	9.26	8.78	8.53	9.02	0.24	0.13	0.35	**0.0002**
HOMA-IR[1]	0.57	0.34	0.80	0.47	0.24	0.70	0.10	0.01	0.19	**0.0318**
HOMA-β	91.24	72.22	110.26	80.32	61.30	99.34	10.93	2.11	19.75	**0.0175**
NEFA AUC (meq*min/L)	39.13	34.09	44.17	47.67	42.63	52.71	-8.54	-13.15	-3.92	**0.0009**
Fasting Triglycerides (mg/dL)[2]	0.0107	0.0090	0.0123	0.0111	0.0094	0.0127	-0.0004	-0.0011	0.0003	0.2412

Table 5. LSmeans for every test and group-wise comparison (blackberry vs. gelatin diet treatments) from the controlled feeding study. [1]Dataset is ln transformed. [2]Dataset is reciprocal transformed. [3]P-Values for group-wise differences. All transformed data are back-calculated for graphical representation.

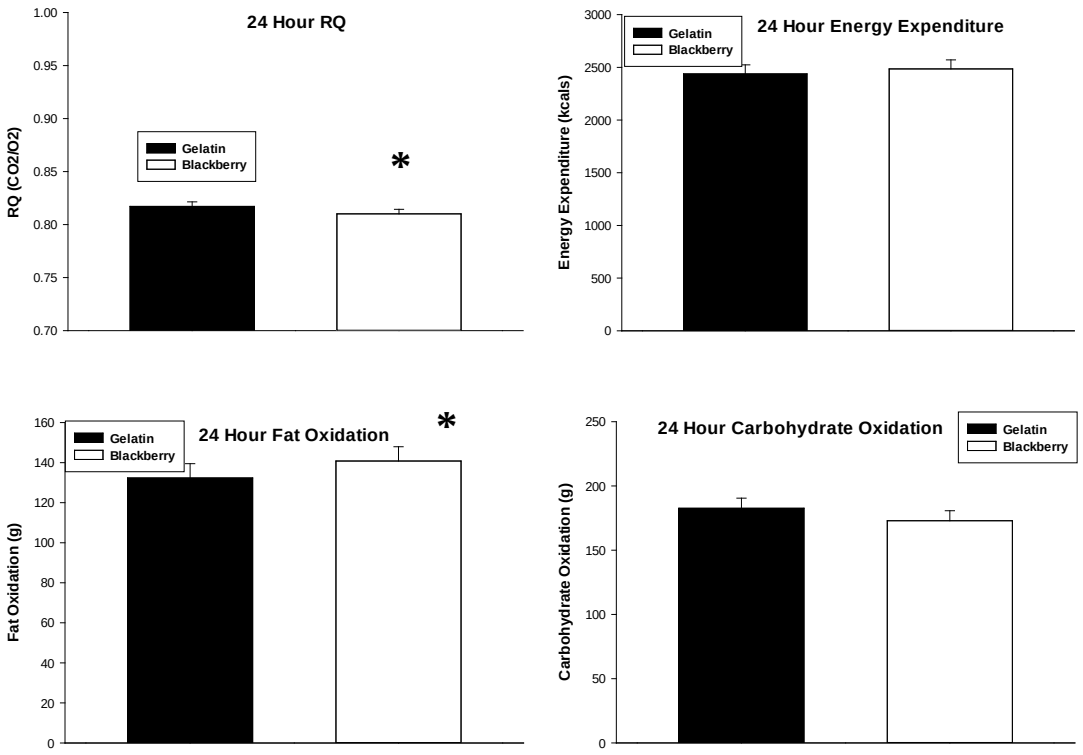


Figure 3. 24 hr Energy Expenditure and Substrate Utilization– Between 5:32 PM on day 1 and 3:23 PM on day 2. Asterisks indicate significant differences between the gelatin and blackberry diet treatments, $p < 0.05$. Data are LSmeans ± SE.

Evening Energy Expenditure and Substrate Utilization

The evening time isolation was the 4-hour post-prandial period after dinner on the evening of day 1 in the chamber that was initiated by the first bite of the subjects' evening meal, typically between 6:30 PM and 10:30 PM. There was a significant 2% reduction in the average RQ with the BB treatment during this time period compared to the gelatin control (0.8178 vs. 0.8358, BB vs. gelatin, respectively, p=0.0063, **Figure 4**). This change corresponded to a significant 14% increase in fat oxidation, where subjects oxidized 28 g of fat on average when administered the BB treatment, and approximately 25 g when administered the gelatin diet, p=0.0069. Similarly, when fed the gelatin treatment, subjects oxidized 16% more carbohydrates compared to the BB diet (42 vs. 36 g, gelatin vs. BB, respectively, p=0.0061). There was no

effect of diet on energy expenditure during the dinner time isolation (493 vs. 483 kcal, BB vs. gelatin, p=0.2616). BMI was a significant covariate in the dinner energy expenditure model where EE increased with increasing BMI independent of diet treatment, p=0.0229.

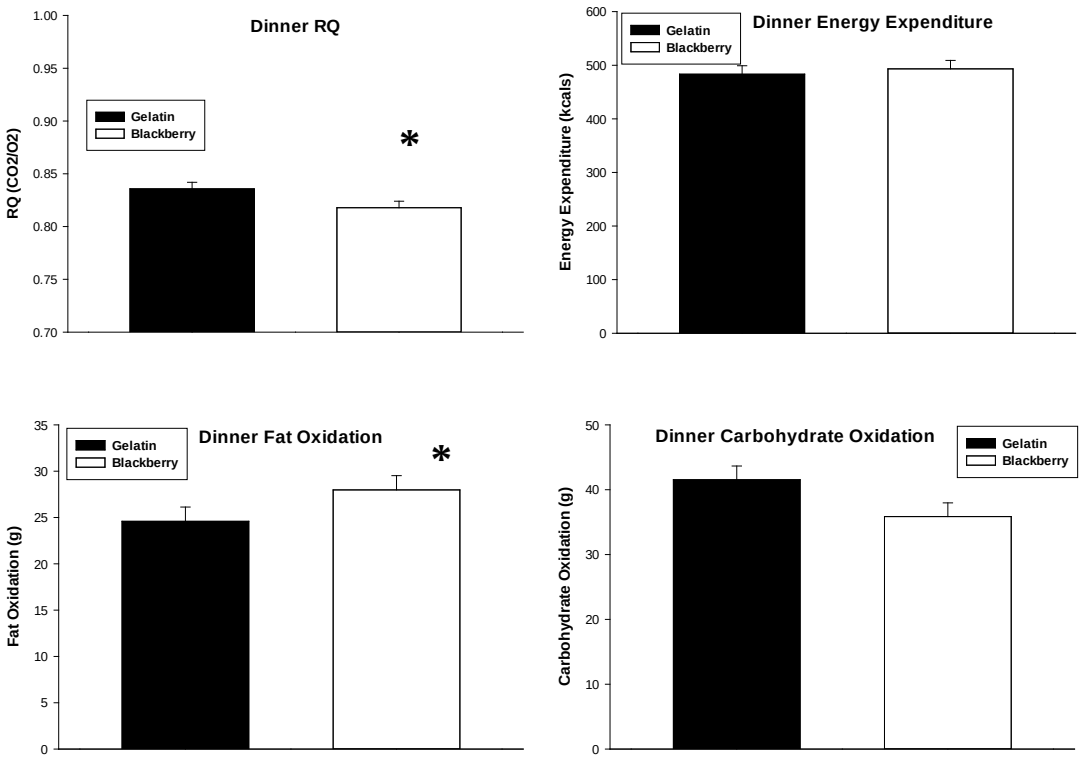


Figure 4. Evening Energy Expenditure and Substrate Utilization – between approximately 6:00 and 10:00 PM on day 1. Asterisks indicate significant differences between the gelatin and blackberry diet treatments, $p < 0.05$. Data are LSmeans ± SE.

Nighttime Energy Expenditure and Substrate Utilization

The nighttime time isolation was defined as the two hours between 2 AM and 4 AM on day 2 where subjects are presumed to be asleep and at or near their basal metabolic rate. There was no significant effect of diet treatment on average nighttime RQ (0.7960 vs. 0.7928, BB vs. gelatin, respectively, p=0.5289, **Figure 5**). Further, no treatment difference was observed on nighttime fat oxidation, where subjects burned approximately 9 g of fat on average when fed the gelatin diet, and 8 g when fed the BB diet, p=0.6813. However, a significant interaction of BMI and treatment shows an effect of BBs to increase EE in lower BMI subjects while diet effects are

less obvious in the majority of subjects with a BMI greater than 30 (p for interaction = 0.0436). Similarly, a marginal interaction between BMI and treatment terms were observed for CHO oxidation, where greater oxidation is observed in lower BMI subjects while administered the BB treatment and diet effects are less clear in subjects with BMIs at or around 35 (p for interaction = 0.0650). These interactions may suggest moderate increases in basal metabolic rate attributable to an increase in CHO, but not fat oxidation, in overweight but not obese subjects with BB feeding.

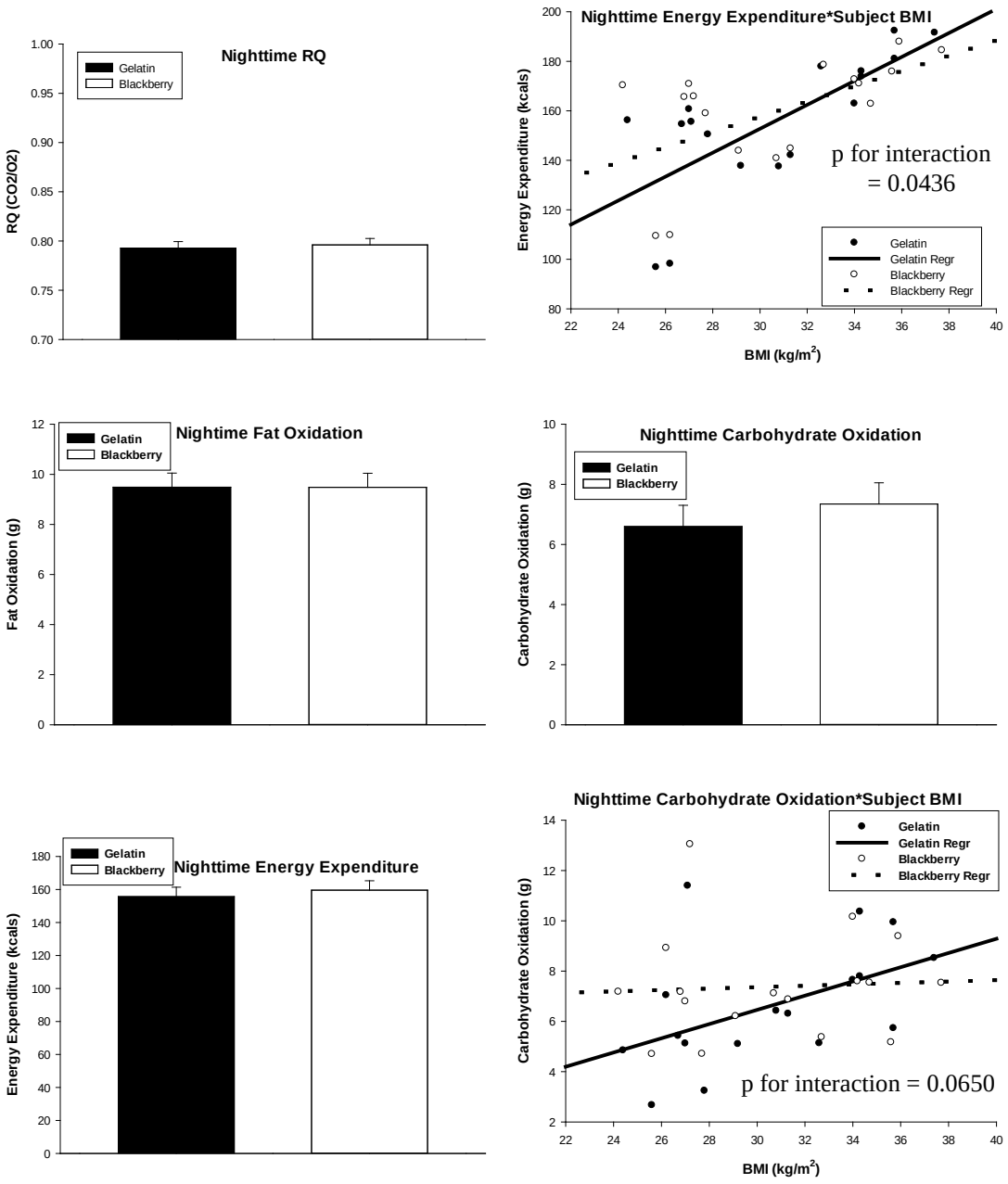


Figure 5. Nighttime Energy Expenditure and Substrate Utilization – between 2:00 and 4:00 AM on day 2. Bar graphs are LSmeans ± SE. Scatter plots are model predicted dependent variables vs. BMI separated by diet treatment.

Morning Energy Expenditure and Substrate Utilization

The morning time isolation was the 4-hour period on the morning of calorimeter day 2 that was initiated by the first bite of the subject's meal-based oral glucose tolerance test, typically between 7 AM and 11 AM. There was a significant 1% reduction in average RQ when the subjects were assigned the BB diet treatment compared to the gelatin control (0.8416 vs. 0.8512, BB vs. gelatin, respectively, p=0.0036, **Figure 6**). This corresponded with a significant 2 g increase in fat oxidation with the BB diet treatment (21 vs. 19 g, BB vs. gelatin, p=0.0129). No differences were observed between diet treatments with respect to CHO oxidation where subjects oxidized 43 g on average with assignment to the gelatin diet and 41 g on average when consuming the BB diet, p=0.1567. Total EE during the morning was also not different between treatments (452 vs. 440 kcals, BB vs. gelatin, respectively, p=0.2028) but again BMI was a significant covariate. There was an increase in EE with increasing BMI independent of diet assignment, p=0.0366.

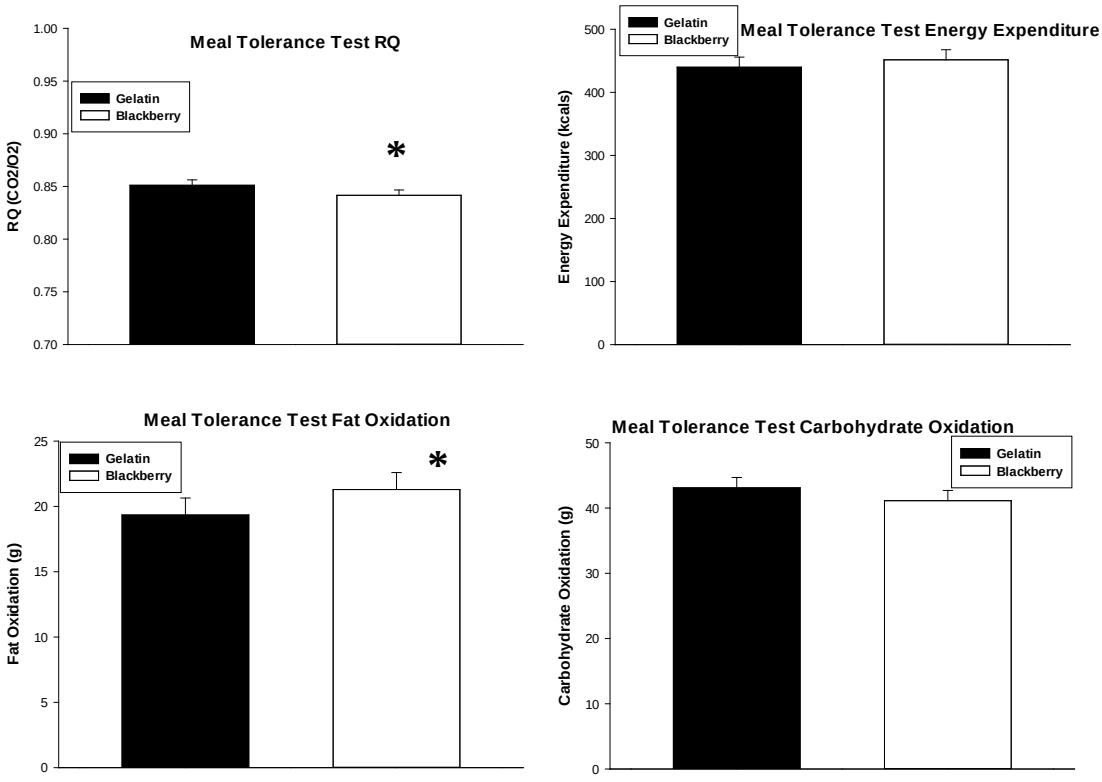


Figure 6. Morning Energy Expenditure and Substrate Utilization – between approximately 7:00 and 11:00 AM on day 2. Asterisks indicate significant differences between the gelatin and blackberry diet treatments, $p < 0.05$. Data are LSmeans ± SE.

Afternoon Energy Expenditure and Substrate Oxidation

The afternoon time isolation was a 1-hour, 50-minute period around midday of calorimeter day 2 that was initiated by the first bite of the subjects' lunch meal, typically between noon and 1:50 PM. Compared to the other meal time isolations, this period was truncated due to the exercise period which started around 2 PM, thus the afternoon time isolation ended ten minutes preceding the exercise session in order to exclude light activity that may have occurred, i.e., changing clothes, while the subject prepared for the exercise bout. There was no significant difference in average RQ for the afternoon isolation between the two treatments (0.8098 vs. 0.8135, BB vs. gelatin, respectively, p=0.3806, **Figure 7**). However, there was a significant interaction between subject age and treatment that suggests younger subjects oxidized

more fat while prescribed the blackberry diet (between eight and twenty-two g) compared to when they followed the gelatin diet (between eight and twenty g fat) where this effect was not noted in older subjects (p for interaction = 0.0480). BMI was a marginally significant covariate in the model (p=0.0692) which again describes increasing fat oxidation with increasing BMI independent of diet treatment. Carbohydrate oxidation was not different between diet treatments where subjects oxidized an average of 15.4 g when fed the BB diet, and 15.0 g when fed the gelatin control (p=0.7185). There was no difference between diet treatments on energy expenditure during the afternoon isolation (230 vs. 225 kcals, BB vs. gelatin, respectively, p=0.3674).

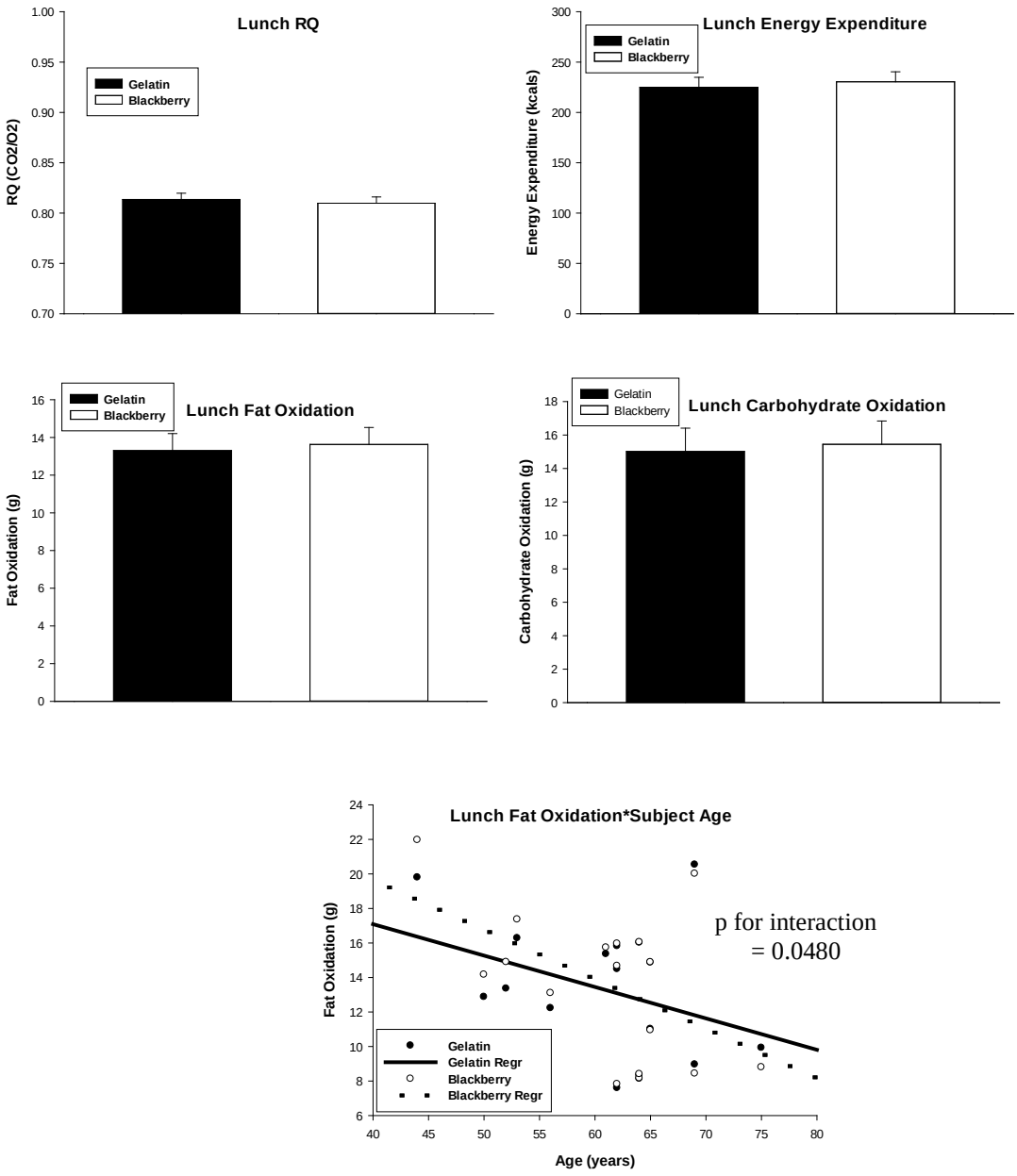


Figure 7. Afternoon Energy Expenditure and Substrate Oxidation – between noon and 1:50 PM on day 2. Bar graphs are LSmeans ± SE. Scatter plot is model predicted fat oxidation vs. age separated by diet treatment.

Exercise Energy Expenditure and Substrate Utilization

The exercise time isolation was a 30-minute period in the early afternoon of day 2. It was initiated by reaching the walking speed setting of 3 miles-per-hour on a treadmill inside the

calorimeter set to zero incline, typically around 2 PM. There was a significant 2% reduction in the 30-minute averaged RQ with the BB treatment (0.8553) compared to the gelatin control (0.8708), p=0.0041, **Figure 8**. This was reflected by a significant 12% increase in fat oxidation with the BB diet treatment (9.4 g) compared to the gelatin control (8.3 g), p=0.0044. Further, subjects oxidized 10% more carbohydrates when fed the gelatin control (25.7 g) compared to the BB diet treatment (23.5 g). This diet effect was significant (p=0.0497) with a significant covariate of BMI (p=0.0281). There was no significant effect of diet treatment on energy expenditure during the exercise bout (197 vs. 196 kcals, BB vs. gelatin, respectively, p=0.7667) and a significant BMI covariate (p=0.0286) demonstrated higher energy expenditure in subjects with higher BMI independent of diet. Thus, these differences in subjects' RQ and subsequent calculations on fat oxidation, either calculated as a 24 average, or reduced to shorter time intervals of post-prandial, basal, or moderate exercise activities, suggest modulation by BBs which may indeed indicate the preferential oxidation of fatty acid substrates.

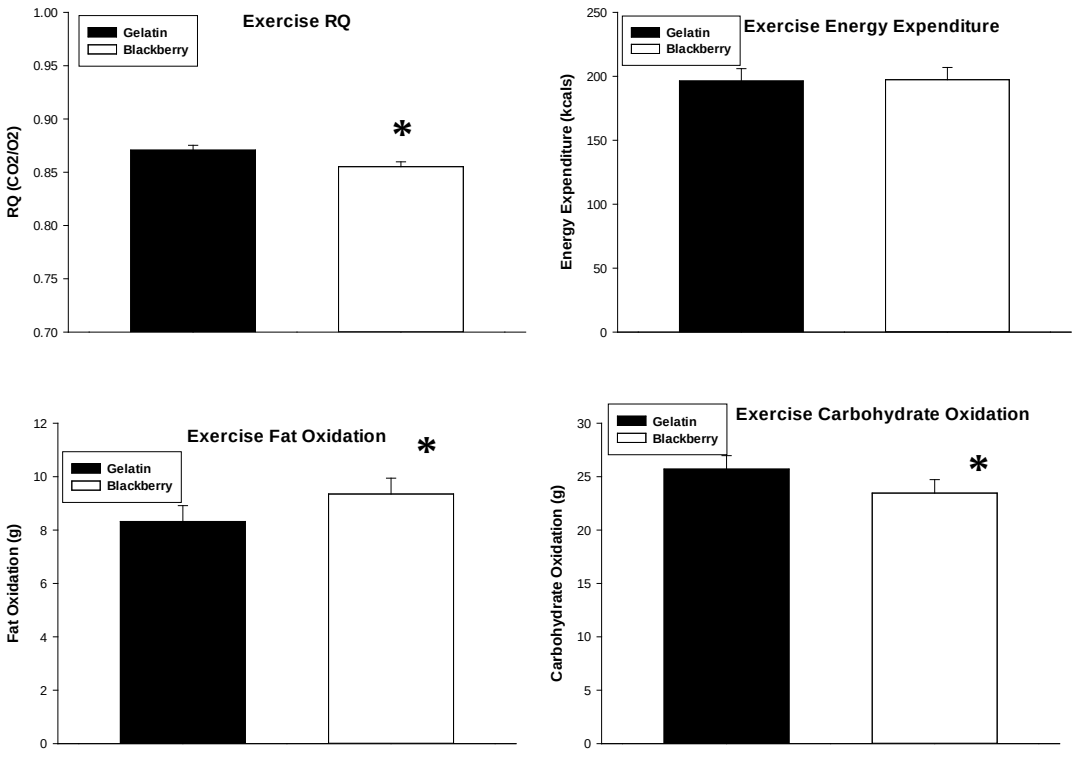


Figure 8. Exercise Energy Expenditure and Substrate Utilization – between 2:00 and 2:30 PM on day 2. Asterisks indicate significant differences between the gelatin and blackberry diet treatments, $p < 0.05$. Data are LSmeans ± SE.

Glucose metabolism and insulin sensitivity

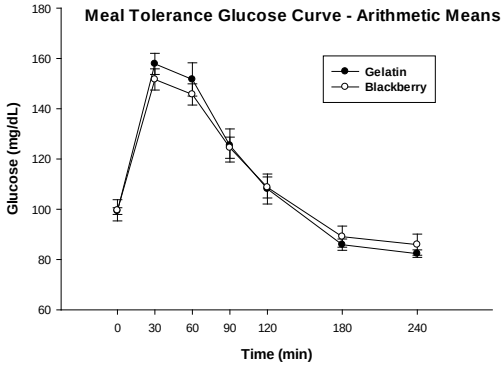
Both the glucose and insulin curves required ln transformation prior to parametric analyses. One subject's MTT measurements were excluded from analyses due to suspected noncompliance. The difference between diet treatments for glucose iAUC was not significantly different (4070 vs. 3488 mg*min/dL, gelatin vs. BB, respectively, p=0.1151, **Figure 9**). However, the difference between treatments in insulin iAUC was significant, where subjects had significantly lower iAUC when fed the BB diet compared to gelatin control (6485 vs. 8245 µU*min/mL, BB vs. gelatin, respectively, p=0.0002). BMI was a significant covariate (p=0.0002) where insulin iAUC increased with increasing BMI. Additionally, a significant sequence effect of the model indicates subjects who ate gelatin and crossed-over to the

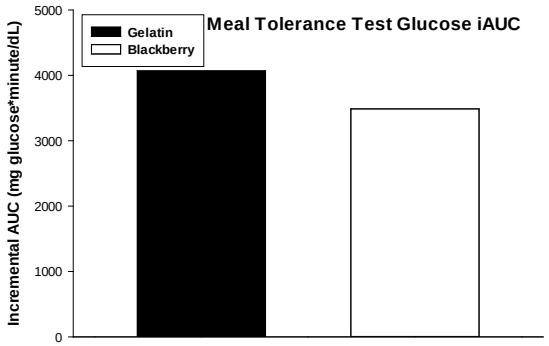
blackberry treatment had a higher insulin iAUC on average (iAUC = 9589 µU*min/mL) compared to subjects who were assigned the BB diet, then crossed-over onto the gelatin treatment (iAUC = 5576 µU*min/mL), p=0.0284. This interaction reveals that a there was a potential carryover effect of BBs on insulin sensitivity. However this effect is only present with the inclusion of two subjects with high residuals, i.e., a sequence effect is not observed and ln transformation of the dataset are not required if the two outliers are removed. Conversely, if this data were treated as having a carryover effect and only sequence 1 subjects (gelatin then blackberries) were analyzed, the same conclusion would remain, where subjects had significantly lower insulin iAUC when fed BBs (8528 µU*min/mL) compared to gelatin (10841 µU*min/mL), p=0.0143.

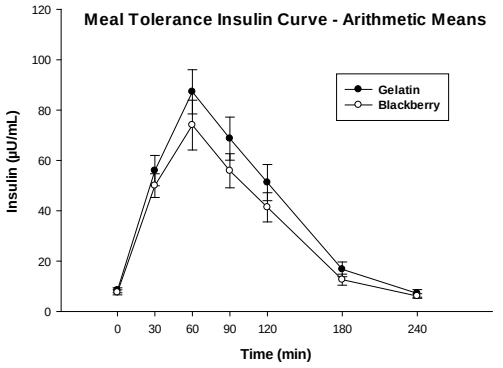
The HOMA-IR dataset required ln transformation whereas the HOMA-B was normal. There was a significant 9% reduction in HOMA-IR scores when subjects were fed the BB diet (LSmean = 1.5979) compared to the gelatin control (LSmean = 1.7630), p=0.0120, Figure 9. Further, there was a significant diet treatment by age interaction (p for interaction = 0.0269) which suggests that a reduction in HOMA-IR scores is more prominent in lower age subjects (aged less than sixty years) whereas improvement in score due to the BB diet are less dramatic in older subjects. BMI was a significant covariate in the model (p=0.0002) where HOMA-IR scores increased with increasing BMI independent of diet treatment. Mirroring the 9% improvement in HOMA-IR was a significant 11% reduction in β-cell function (HOMA-B scores), p for diet treatment=0.0048, where subjects' scores were 91.2% on average with the gelatin control, and 80.3% with the BB diet, Figure 9. Again there was a significant diet treatment by age interaction (p for interaction = 0.0122) where marked improvements occurred in subjects aged less than 60 years and improvements are less dramatic in older subjects. BMI is

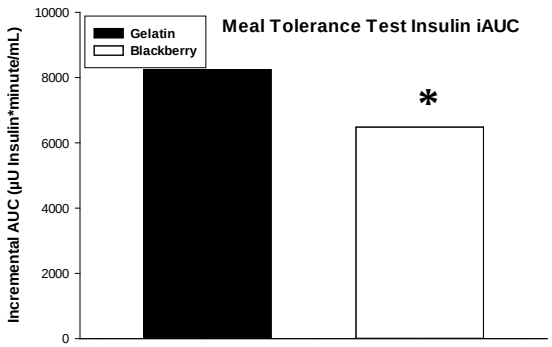
also a significant covariate in the HOMA-B model (p=0.0025) demonstrating the same effect of increased HOMA-B score with increasing BMI.

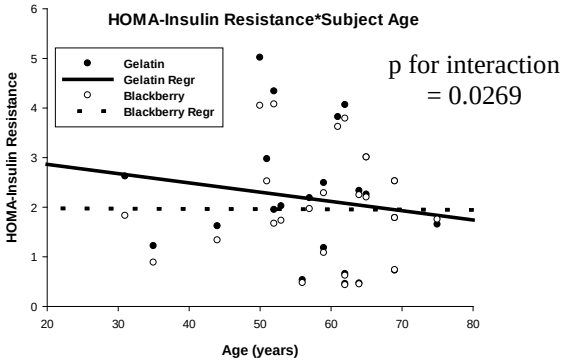
Serum NEFA AUC was significantly higher on average when the subjects were fed the BB diet treatment (LSmean= 48 meq*min/L) compared to when they consumed the gelatin control (LSmean= 39 meq*min/L), p=0.0009. Finally, the analysis for fasting triglycerides required a reciprocal transformation for parametric analysis. There was no significant difference between diet treatments when comparing fasting triglycerides (90 vs. 94 mg/dL, BB vs. gelatin, respectively, p=0.2707). BMI was a significant covariate in the model which revealed higher fasting triglycerides with increasing BMI.



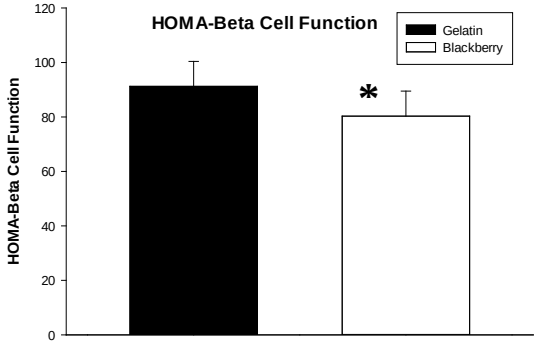


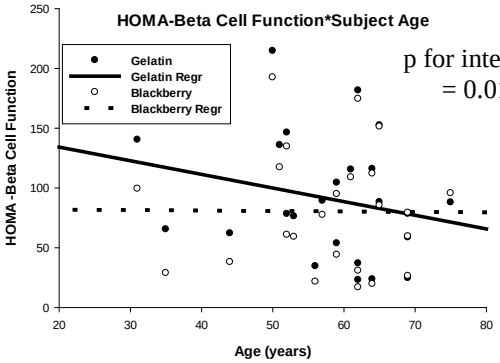






p for interaction = 0.0269





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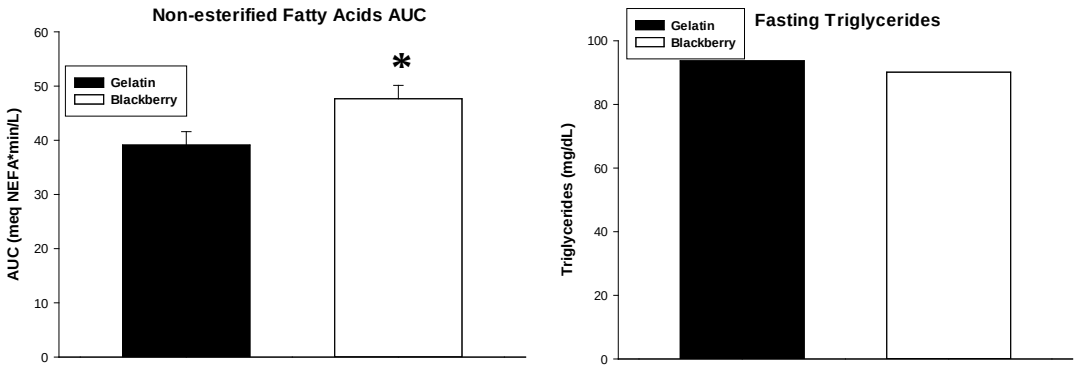

Figure 9. Glucose metabolism and insulin sensitivity. Asterisks indicate significant differences between the gelatin and blackberry diet treatments, $p < 0.05$. Bar graphs are LSmeans ± SE where available; bar graphs without error bars are back-calculated values from transformed statistical analyses. Scatter plots are model predicted dependent variables vs. age separated by diet treatment. Glucose and Insulin time-response curves are arithmetic means ± SE.

Discussion

Augmentation of lifestyle modifications with functional foods as a means to promote weight loss is an attractive approach for dieticians, physicians, and the consumer because of their relative low cost and easy access when compared to pharmacotherapies or invasive surgeries. In order to improve the chances of increased weight loss, or curbed weight gain, nutritionists must be able to demonstrate which foods promote this activity in order to equip dieticians with the most effective dietary approaches to combat the obesity epidemic.

Rodent studies have successfully demonstrated the anti-obesity effects of anthocyanins either from berries or other sources, stimulated by seminal work on purple corn color [9, 15, 35-38]. Alongside reductions in weight gain attributable to high fat diets, these same studies observe an increase in insulin sensitivity. Human studies using freeze-dried berries or purified anthocyanin extracts have reported less consistent results, but improvement of clinical markers often complicated by obesity have been observed [20-24, 26, 39-42]. A major limiting factor of the human research to date is the lack of control on the diet of the study participants, which

provides a great source of confounding for dependent variables often assessed in human clinical trials. To our knowledge this is the first study to assess modulation of RQ and substrate oxidation by BB consumption in parallel with a meal-based oral glucose tolerance test using a 100% controlled diet and a crossover, placebo controlled design. Unfortunately, due to the nature of the intervention (whole blackberries vs. gelatin) blinding was not possible.

Weight loss intervention studies are difficult to execute due to the time required to observe significant weight loss, typically on the order of months, and combined with controlled feeding would be costly. Another issue with any weight-loss study would be compliance, whereas two weeks of controlled feeding at weight maintenance is achievable by study participants, months on a hypocaloric diet would introduce significant emotional burden and increase the risk of compromised results due to deviation from prescribed diets. Fortunately, the use of indirect calorimetry to determine changes on substrates used for energy production allows for more timely comparisons between diet interventions and provides proof of concept in early investigations of candidate foods that may augment fat oxidation. In this study, a week of controlled feeding was sufficient to detect differences between diet treatments on measures of indirect calorimetry. Specifically, this study shows a significant increase in fat oxidation with BBs compared to a polyphenol-free, energy matched control as measured by group differences in RQ and as calculated using the equations of Livesey. Utilization of a deconvolution algorithm allowed for detection of sub-24hr differences in these measures between interventions [32, 43]. Treatment differences included lowered RQ (increased fat oxidation) in several post-prandial states as well as during a short bout of low intensity physical activity. The logical next steps for the positive effects of blackberries on increased fat oxidation described here would be to determine effectiveness in augmenting weight loss or preventing weight gain in subsequent free-

living studies. Further, high impact findings leading to the development of recommendations for berry and/or polyphenol intake would be gleaned from free-living studies that continue to show a positive effect of berries on body composition.

Although we are the first to describe an increase in fat oxidation due to consumption of blackberries, many others have studied the effects of flavonoid containing ingredients on fat oxidation as measured by indirect calorimetry, primarily from oolong or green tea sources. Seminal work by Rumpler et al. investigated the effects of oolong tea on measures of indirect calorimetry [44]. Noting weight loss interventions with oolong tea [45], Rumpler et al. fed twelve men 1.5 L per day of full-strength oolong tea, a caffeine-matched positive water control, or a placebo water control in a crossover, randomized design for three days with a 23 hr stay in a room-sized calorimetry chamber on the third day. The investigators found that subjects expended 280 more calories when consuming oolong tea and that there was a 12% increase in 24 hour fat oxidation when compared to placebo water [45]. By comparison, subjects in this study saw a statistically insignificant 2% increase in energy expenditure and a significant 7% increase in fat oxidation when consuming BB diet treatment compared to the gelatin control. Interestingly, the Rumpler et al. study reported greater differences with a shorter time of consumption of their test beverage (four days) compared to this study's protocol (seven days). However, their observations of increased energy expenditure and fat oxidation with oolong tea were not different compared to the caffeine-matched water control, thus effects of the oolong tea treatment may be due to the thermogenic effect of caffeine present in the beverage.

Green tea is another beverage that received substantial attention due to its potential to augment fat oxidation along with its main flavonoid, epigallocatcechin-3-gallate (EGCG) [46]. In a double-blind crossover study, twelve overweight men showed an increase in postprandial fat

oxidation, measured using a ventilated hood, after consuming a low dose capsule (300 mg) of EGCG for three days compared to placebo, which was comparable to the effect of 200 mg of caffeine [47]. Interestingly, a higher dose of EGCG (600 mg) abolished this effect. Similar to the lack of difference between the oolong tea and caffeine-matched control groups mentioned earlier, the investigators reported no synergistic effect of combination EGCG and caffeine on postprandial fat oxidation [47]. A 12-week, placebo controlled weight-loss intervention study in obese women found null results on rates of fat oxidation where the intervention group was supplemented with 300 mg of EGCG per day [48]. Further, none of the other outcome variables pertinent to a weight loss study (body composition, cardiovascular and metabolic profiles) were enhanced by ECGC, suggesting no added benefit to a hypocaloric diet regimen. Conversely, a different study examining combination green tea extract and sprinting exercises indicates an increase in fat oxidation in healthy weight, untrained young females both before and shortly after exercise by increments of 24% and 29%, respectively, with addition of the green tea extract [49]. Earlier studies investigating combination treatments of green tea extracts with guarana, bitter orange, or capsaicin yield mixed but slightly positive effects on indirect calorimetry [50-52].

Strengths of the present study include the incorporation of a 100% investigator controlled diet. Day-to-day variation in habitual diets can have great influence on short-term dietary intervention studies and this control allowed us to maximize the possibility of detecting calorimetric and glucoregulatory differences attributable to the BB treatment. Other studies examining the health modulating effects of whole berries did not control the diet, and one even noted the possibility of significant changes in the subjects' macronutrient intake could have interfered with study objectives [53, 54]. Another advantage of the present study is that the intervention was from an intact food source, thus translating some of the earlier studies positive

effects that were noted when feeding participants freeze-dried powders to a form that is more commonly consumed and readily available.

Major limitations of this study include population, dose, and duration. Since this was a pilot study, the design only included male subjects due to the added variation that would be introduced with the inclusion of females, particularly with indirect calorimetry. The positive findings of the study warrant future research into BBs and fat oxidation/energy expenditure. In future studies the effects described here need to be confirmed in female subjects. The dose of BB treatment was 600 g per day. While the dose was tolerable for the subjects, and was chosen to maximize the possibility of observing significant effects; it does not reflect habitual intake; the average American eats approximately 30 g of citrus, berries, or melons daily [55]. Further, it is estimated that Americans eat an average of 214 mg of total flavonoids (9 mg of anthocyanins) daily [56]. Thus, if our positive findings are attributable to flavonoid bioactivity, then other modes of delivery, or the development of high flavonoid berry crops, should be considered to allow easy and long-term achievement of the doses fed here. Finally, duration of treatment is a limitation as it is unknown whether the improvements observed in the study would continue with prolonged intake of BBs. Future studies with lower doses of BBs fed for extended periods could address these concerns and determine if the benefits can be translated to habitual consumption.

In conclusion, BB feeding significantly increased fat oxidation and insulin sensitivity in overweight or obese men fed a high-fat diet including 600 g BB per day for one week compared to an energy matched control treatment in a randomized, cross-over study. This study was proof-of-concept, and is the first to translate observations of the anti-obesity effects of berries in rodent models to humans by means of indirect calorimetry. Future work in male and female obese subjects utilizing lower doses and longer durations would be of benefit to determine if berry

consumption can indeed augment fat oxidation as to provide a simple, accessible, and affordable option to enhance weight loss or prevent weight gain.

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Chapter 3: A Physiologic dose of Cyanidin-3-Glucoside from a Blackberry Extract Increases Oxygen Consumption in Differentiated 3T3-L1 Adipocytes but the Purified Form Has No Effect in Skeletal Muscle Cells

Introduction

Untreated, metabolic complications of obesity are not only taxing to the healthcare system, but are also detrimental to perceived quality of life for the one-third of Americans classified as obese [1-4]. Trends in the prevalence of obesity since the early 2000s show no sign of improvement, despite intense approaches towards remediation by several regulatory agencies [4]. Ecological studies of obesity demonstrate how Americans with a genetic predisposition for the obese state are setup for failure by their physical environment as well as how Western society approaches food availability and pricing [5-7]. Obesity will persist in the United States until systemic changes at the societal level address this multifaceted disease. Despite this moored challenge and unanswered call for societal-level intervention, it is still advantageous for nutritionists to probe the obesity-modulating capabilities of functional foods, as the characterization of bioactive food components and their role in obesity may lead to affordable, primary actions that would be readily accessible to the afflicted [8, 9].

As discussed in the literature review (Chapter 1) diets rich in berries have favorable effects on all-cause and cardiovascular disease related mortality as well as development of type-2 diabetes in epidemiological investigations [10, 11]. Several berry cultivars typically consumed in Western countries are a rich source of anthocyanins, a diverse class of flavonoids responsible for the dark blue and purple color of the fruits and vegetables where they are present [12]. The anthocyanin cyanidin-3-O-β-glucoside (C3G) has received a lot of attention in the area of obesity research due to its anti-obesity effects first described in a diet-induced model of rodent obesity,

where it was delivered in the form of a heavily used food colorant (purple corn color) that normalized weight gain, insulin sensitivity, and gene-expression of lipogenic enzymes in mice after twelve weeks of feeding [13]. Rodent studies of anthocyanins delivered in the form of berries or isolated compounds also had a protective effect against diet-induced obesity [14-17].

Investigations into anthocyanin effects at the tissue level have also uncovered mechanisms that may suggest anti-obesity action. In one study, human omental cells and the mouse adipocyte cell line 3T3-L1 were exposed to oxidized low density lipoprotein in order to model insulin resistance, then subsequently exposed to C3G and its metabolite, protocatechuic acid [18]. The authors described normalization in glucose uptake and adiponectin secretion, and reported that these changes were dependent on PPAR-γ activity when the cells were exposed to C3G or its metabolite [18]. An intriguing concept described in one study is the conversion of white adipocytes to a brown adipocyte phenotype with C3G administration [19]. The potential implications of white to brown switching is an adipocyte with higher mitochondrial density and uncoupler protein-1 (UCP-1), thus in place of energy storage that is characteristic of white adipose tissue, a greater proportion of brown-like adipose tissue would conceivably lead to increased thermogenesis and excess energy would be lost as heat, thereby transforming the adipocyte from having a role in energy storage to having a role in energy disposal. The same research group demonstrated how these changes in adipocytes via anthocyanin treatment could have positive downstream effects on the mitochondrial density of skeletal muscle tissue [20].

In the previous chapter, the positive effects of blackberry feeding on insulin sensitivity and fat oxidation in overweight and obese males fed 600 g of blackberries each day for one week were described. Measurement of the respiratory quotient via indirect calorimetry demonstrated a significant reduction at several time-points with the blackberry treatment compared to control.

Such a reduction indicates the preferential oxidation of fat for energy production. Similar measurements on oxygen consumption at the tissue level are possible [21]. To our knowledge there are no published studies that connect the anthocyanin-induced molecular changes in adipocyte and skeletal muscle tissues described above to potential changes in cellular respiration. Thus, the aim of this study was to characterize the effect of blackberry extract (BBE) on cellular respiration and mitochondrial phenotyping in 3T3-L1 adipocytes and primary skeletal muscle tissues in an effort to support the observed changes in the human feeding study.

Methods

Hypotheses

Six days of cyanidin-3-glucoside treatment using a blackberry extract will increase oxygen consumption on a fatty acid substrate in a dose-dependent manner in differentiated 3T3-L1 adipocytes.

24 hours of purified cyanidin-3-glucoside treatment will increase oxygen consumption on a fatty acid substrate in a dose-dependent manner in primary skeletal muscle tissue.

Blackberry extract

Anthocyanin-rich extract from blackberries was prepared according to the method of Dai et al. [22]. Frozen blackberries were thawed overnight and approximately 100 g was homogenized in a commercial blender for approximately one minute. The puree was pushed through a cheese-cloth-lined metal sieve with a plastic paddle in order to remove the majority of the skins and seeds. 10 g of blackberry puree was combined with 25 mL of extraction solvent (ethanol with 0.01% HCl) and sonicated for 30 minutes. The mixture was centrifuged at 416 x g for 15 minutes and the supernatant was vacuum-filtered using Whatmann #1 filter paper (approximately 11 microns). The filtered supernatant was divided into 4 mL aliquots and dried

down in a centrifuge under vacuum at 40°C for approximately 8 hours. Dried samples were resuspended with 4 mL of sterile DI water, combined, filtered a second time, and frozen at -80°C. Frozen samples were placed in a freeze-drier for 3-4 days. Lyophilized samples were resuspended with sterile DI water at 140 mg extract/mL, aliquoted, and stored at -80°C. C3G concentration of the extract was determined using liquid chromatography-mass-spectrometry and an external standard curve.

Cell culture

3T3-L1 preadipocytes were purchased from Zenbio (Research Triangle Park, North Carolina). Preadipocytes were seeded at 3000 cells/cm^2 in T-75 flasks until they reached 70-80% confluence. Cultures were sub-cultured using 0.25% trypsin until adequate flask numbers were reached to perform five independent repetitions of BBE administration experiments. Cells were received at passage 8 and per manufacturer instructions cells were not expanded beyond passage 12. Cells were maintained in preadipocyte media (15 mL per T-75, changed every other day) until they reached confluence (media compositions are described in **Appendix 6**). Confluent flasks in groups of 4 were differentiated with the addition of 20 mL of differentiation media and incubated for 3 days. Following differentiation, 12 mL of differentiation media was removed and 16 mL of maintenance media was added with 0 (control), 150 nM, 50 µM, or 100 µM C3G via BBE. 150 nM was chosen as the physiologic dose based upon peak C3G plasma concentrations in human pharmacokinetic studies (Novotny, unpublished data), and the two higher doses were chosen in order to match doses used in the glucose trafficking and mechanistic experiments of Scazzochio et al. as well as the adipocyte browning experiments of Matsukawa et al. [18, 19]. Differentiated adipocytes were incubated in maintenance media +/- BBE for 6 days. On day 3, 16 mL of media was removed and replaced with an equal volume of fresh maintenance media

with the respective BBE treatment for a total of 2 extract doses over the course of the 6 days. Cell respiration was assessed on day 6.

Primary skeletal muscle tissue was purchased from Lonza (Walkersville, MD). Cells were seeded at 3500 cells/cm^2 in 2 T-75 flasks and grown to 70-80% confluence. Cells underwent one sub-culture to generate adequate flask numbers to perform three independent repetitions of C3G administration experiments. Purified C3G was used in place of BBE following the discovery of cytotoxicity of the two higher doses in the 3T3-L1 experiments as well as confirmation of cytotoxic effects in skeletal muscle cells. Flasks were grown to 100% confluence in skeletal muscle growth media-2 supplemented with gentamicin-amphotericin, recombinant-human epidermal growth factor, dexamethasone, L-glutamine, and fetal bovine serum. Once flasks reached confluence, they were treated with 0 (control), 150 nM, or 50 µM C3G for 24 hours. Cell respiration was assessed after the 24 hour incubation +/- C3G.

Cell respiration

Tissue oxygen consumption was determined with an oxygraph (Oroboros, Austria) which measures the concentration of oxygen in solutions with a clark-type electrode and is capable of measuring rate of cellular consumption in real-time [21]. The instrument is equipped with two oxygen chambers, thus each sample was measured in duplicate and oxygen consumption rate was averaged between the two chambers. The rate of oxygen consumption is normalized to one million cells. Prior to the oxygraph protocol, cells were trypsinized with 3-4 mL of trypsin for 15-30 minutes (adipocytes) or 5 minutes (skeletal muscle cells) at 37°C without agitation. Once the majority of cells were detached from the flask, the trypsin was neutralized with 12 mL of culture media and transferred to a 50 mL falcon tube. The flask was rinsed with 12 mL of Hank's balanced salt solution and collected in the same falcon tube. Cells were pelleted by

centrifugation at 125 x G for 10 minutes, resuspended in culture media, and pelleted a second time before resuspension and cell counting in MiRO5 respiration media. The intermediary wash step was included due to difficulty obtaining a single-cell suspension when cells were resuspended directly in respiration media. Cells were counted with a Neubauer chamber and viability was determined with erythrosin B staining. MiRO5 media was prepared following the protocol of the oxygraph manufacturer [23]. Highly oxidative samples deplete the oxygen content of the media, and before the start of the respiratory protocol 560 units of catalase were added to each chamber. When samples depleted oxygen from the media, 200 mM H_2O_2 were added to each well in 3 µL increments until the oxygen concentration was restored to 160-200 nmol/mL.

Cellular respiration was assessed using a modified respirometry protocol for permeabilized cells as described by Boyle et al. [24] in order to determine any changes in oxygen consumption on fatty acid substrates due to BBE treatment in 3T3-L1 adipocytes or C3G treatment in primary skeletal muscle cells. 1 x 10^6 adipocytes or 2 x 10^6 primary skeletal muscle cells were added to each 2 mL respiratory chamber and allowed to stabilize before protocol initiation i.e. baseline, intact respiration was assessed before permeabilization. Subsequent addition of 9 substrates phenotypes mitochondrial respiration (rate of oxygen consumption in picomoles per second·million cells) in mitochondrial respiration states 2 through 4 [25] as described in **Table 1**.

Table 1. Mitochondrial Phenotyping Protocol

Step	Substrate	Purpose/Respiration state
1	10 µg digitonin	cell permeabilization
2	5 µM palmitoyl-carnitine	energy substrate
3	2 mM malate	state 4
4	2.5 mM adenosine diphosphate	state 3
5	10 µM cytochrome c	Mito membrane integrity
6	10 mM glutamate	state 3, complex 1 coupling
7	10 mM succinate	state 3, complex 2 coupling
8	5 µg oligomycin	state 2
9	0.5 µM steps of FCCP	uncoupled, max respiration state

Mito, mitochondrial; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone.

After addition of each substrate, time was allowed (approximately 30 seconds to 1 minute) for cells to reach a new respiration steady-state before progression to the next substrate. Maximum oxygen consumption for each steady-state was recorded using the Datlab program that interfaces with the oxygraph instrument.

Mitochondrial content

Respiration rates are corrected by cellular density in the oxygraph chamber. Respiration rates are also reported after correction for mitochondrial content. Mitochondrial content was determined by RT-PCR using the methods described by both Venegas et al. and Rooney et al. for skeletal muscle cells [26, 27] and primer sequences and thermocycler settings for 3T3-L1 adipocytes were described by Hao et al. [28]. Primers for mitochondrial DNA amplify a tRNA that is conserved in the displacement-loop of the mitochondrial genome [29]. Mitochondrial DNA amplification is corrected by amplification of nuclear DNA encoding β2 microglobulin for skeletal muscle samples or 18S rRNA for 3T3-L1 adipocyte samples. Relative mitochondrial content is calculated as described by Venegas: ΔCt = (nuclear DNA average Ct) – (mitochondrial DNA average Ct) and mitochondrial DNA content = 2 x 2^(ΔCt). This additional correction allows for the explanation of respiratory changes due to either modulation of mitochondrial density or function as described by Boyle et al. [24]. After cells were treated with their respective

doses, counted, and resuspended in MiRO5, a subsample of cells (approximately 250,000 to 1 million cells) was collected prior to the respiration protocol, pelleted at 10,000 x g for ten minutes at 4°C, and stored at -80°C until all samples for each tissue could undergo DNA extraction on the same day. DNA was extracted from the cell pellet using a DNeasy blood and tissue kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. Total DNA was quantified using a nanodrop spectrophotometer (Thermofisher, Wilmington, DE). DNA content was normalized to 3 ng per µL and 6 ng of DNA was added to each PCR reaction in triplicate for each sample.

Data processing and statistics

Adipocytes and skeletal muscle cell respiration rates were assessed for differences due to BBE or C3G treatments, respectively, during baseline (intact cells), state 4 (step 3), state 3 (step 7), and uncoupled (step 9) respiration states. Treatment differences were also assessed for each sample's coupled control ratio (CCR) which is defined as the ratio of uncoupled to state 3 respiration states, as well as each sample's substrate control ratio (SCR) which is defined as the ratio of step 4 (presence of palmitoyl-carnitine, malate, and ADP) to step 3 (presence of palmitoyl-carnitine and malate) respiration. The CCR is an assessment of the electron transport capacity relative to the maximal state of oxidative phosphorylation, and the SCR an assessment of oxidative phosphorylation capacity on palmitoyl-carnitine - by the addition of ADP - while holding coupling state constant (prior to addition of glutamate or succinate) [24, 30]. Each of these six respiration states or ratios were then corrected for mitochondrial content for each tissue and reanalyzed to explore possible effects of mitochondrial density or function on the observed respiration states for each sample.

Main effect of treatment was determined with one-way analysis of variance using "proc mixed" in SAS version 9.4 (SAS institute, Cary, NC). Normality was formally tested with the Shapiro-Wilk test and equal variance of residuals by inspection of residual plots. Data are presented as least-squares means for each treatment for each respiration state described above for both tissues before or after correction for mitochondrial content. Mitochondrial content was also tested for treatment differences. $P<0.05$ is considered statistically significant.

Results

Differentiated adipocytes

The oxygraph protocol was successfully conducted and data collected from two independent experiments for untreated adipocytes and from three independent experiments for adipocytes treated with 150 nM of C3G from BBE. Adequate sample was not available for one of the three 150 nM from BBE samples to reserve for mitochondrial content assessment, and it was discovered that post-oxygraph sample mitochondrial estimates are different from the same samples that have not undergone oxygraph phenotyping, thus, mitochondrial correction was only available for two of the three 150 nM BBE samples. The 50 µM and 100 µM doses of C3G from BBE were cytotoxic to the adipocytes and oxygraph phenotyping was not possible.

All data from the oxygraph experiment with differentiated adipocytes are reported in **Table 2**. Baseline respiration was significantly higher in adipocytes treated with 150 nM C3G from BBE (147 vs. 123 pmol O_2/second·million cells, BBE vs. control, respectively, p=0.0362, **Figure 1**). State 4 respiration was not significantly different between the two treatments, but the direction was higher oxygen consumption with BBE treated cells compared to control (105 vs. 86 pmol O_2/second·million cells, p=0.0946, Figure 1). The difference between treated and untreated cells for state 3 respiration was marginally significant, where oxygen consumption was

higher in BBE treated cells (830 vs. 656 pmol O_2/second·million cells, BBE vs. control, respectively, p=0.0712, Figure 1). The difference for uncoupled, maximum respiration was also marginally significant in the same direction as state 3 (1497 vs. 1201 pmol O_2/second·million cells, BBE vs. control, respectively, p=0.0614, Figure 1). CCRs and SCRs were not significantly different between the two treatments. Directionality of the treatment effects persisted after correction for mitochondrial content, but statistical significance was lost for the effect observed with uncorrected baseline respiration, and marginally significant effects became weaker (table 1). The difference for uncoupled respiration remained marginally significant, where electron transport capacity was greater in adipocytes treated with BBE (141 vs. 64 pmol O_2/second·million cells/mitochondrial content, BBE vs. control, respectively, p=0.0726). Relative mitochondrial content was not significantly different between the two treatments (19.8 vs. 10.4 2x2^(ΔCt), control vs. BBE, respectively, p=0.2182). CCR trended towards treatment differences after correction for mitochondrial content where cells treated with BBE had a higher corrected ratio compared to control cells (0.1747 vs. 0.0995 uncoupled/state3/mitochondrial content, BBE vs. control, respectively, p=0.1316).

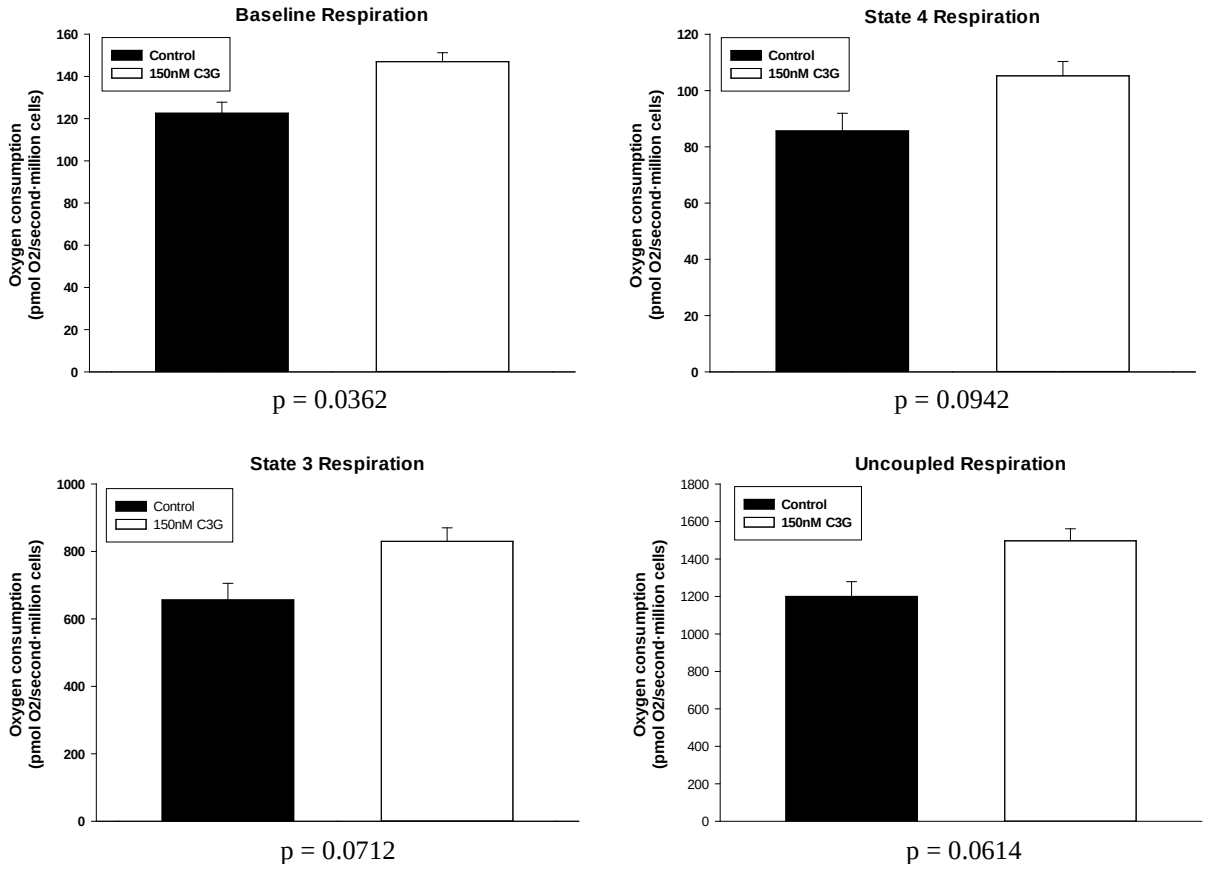

Figure 1. Oxygen consumption rates in different mitochondrial respiration rates from differentiated 3T3-L1 adipocytes with or without six-day treatment of 150 nM C3G from blackberry extract. Values are LSmeans ±SE.

Primary skeletal muscle cells

The discovery of cytotoxicity with high dose BBE in the adipocyte studies led to the use of purified C3G for investigation into effects on respiration states in skeletal muscle, which allowed for the inclusion of a supraphysiologic dose (50 µM) of C3G without inhibiting cell viability. The oxygraph protocol was successfully conducted and data collected on three independent experiments of C3G treatment in primary skeletal muscle tissue. Mitochondrial density was successfully measured in all samples.

All data from the oxygraph experiments with skeletal muscle cells are reported in **Table 2**. The response to C3G treatment across the uncorrected respiration states was a slight increase in oxygen consumption with the physiologic dose of C3G (150 nM) and a slight inhibitory effect of the 50 µM dose compared to untreated skeletal muscle cells, but all comparisons were nowhere near statistical significance. After correction for mitochondrial content, these responses were nullified, with the exception of state 3 respiration. Mitochondrial content was not significantly different between the three treatments, however the directionality was an increase with the C3G treatments (527 vs. 623 vs. 626 2x2^(ΔCt), control vs. 150 nM C3G vs. 50 µM C3G, respectively, p=0.4313).

	Control			150 nM C3G			Difference of LSmeans (Control - 150 nM)			
		95% CI			95% CI			95% CI		
	LSmean	Lower	Upper	LSmean	Lower	Upper	Difference	Lower	Upper	P[3]
Baseline[1]	122.6	106.0	139.2	147.0	133.4	160.6	-24.4	-45.8	-3.0	**0.036**
State 4[1]	85.7	65.7	105.6	105.2	88.9	121.5	-19.6	-45.4	6.2	0.095
State 3[1]	656.5	500.4	812.5	830.1	702.6	957.5	-173.6	-375.1	27.9	0.071
Uncoupled[1]	1200.7	950.2	1451.1	1497.4	1292.9	1701.9	-296.8	-620.1	26.6	0.061
CCR (Uncoupled/State 3)	1.83	1.53	2.14	1.81	1.56	2.06	0.02	-0.37	0.42	0.859
SCR (PCMD/PCM)	1.79	0.95	2.64	1.51	0.82	2.20	0.28	-0.81	1.37	0.470
Baseline[2]	6.5	-2.8	15.9	14.7	5.3	24.0	-8.2	-21.4	5.1	0.117
State 4[2]	4.5	-1.3	10.3	9.9	4.1	15.7	-5.4	-13.6	2.8	0.105
State 3[2]	34.6	-20.9	90.2	80.4	24.9	135.9	-45.8	-124.3	32.8	0.129
Uncoupled[2]	63.7	-3.7	131.1	141.4	74.0	208.8	-77.7	-173.0	17.7	0.073
CCR[2]	0.099	0.007	0.192	0.175	0.082	0.267	-0.075	-0.206	0.056	0.132
SCR[2]	0.100	-0.102	0.302	0.169	-0.033	0.370	-0.068	-0.354	0.217	0.412
mitochondrial content (2*2^ΔCt)	19.8	3.6	36.1	10.4	-5.9	26.6	9.5	-13.5	32.5	0.218

Table 2. Respiration rates (LSmeans ± 95% CI) from untreated differentiated adipocytes or after treatment with 150 nM C3G from BBE for six days. [1]Values are steady state oxygen consumption rates corrected to one million cells (pmol O_2/second·million cells) from two independent experiments for the control group and from three independent experiments for the 150 nM C3G treated cells. [2]Values are the same steady states after correction for mitochondrial content (pmol O_2/second·million cells/mitochondrial content) from two independent experiments for both treatment groups. [3]P-Values for group-wise differences.

	Control			150 nM C3G			50 µM C3G		
		95% CI			95% CI			95% CI	
	LSmean	Lower	Upper	LSmean	Lower	Upper	LSmean	Lower	Upper
Baseline[1]	19.03	13.68	24.39	21.74	16.39	27.10	17.06	11.71	22.42
State 4[1]	9.42	7.13	11.70	10.74	8.45	13.02	8.20	5.91	10.49
State 3[1]	26.70	9.48	43.92	37.21	19.99	54.43	27.52	10.30	44.73
Uncoupled[1]	71.39	48.45	94.34	82.18	59.23	105.12	66.69	43.75	89.64
CCR (Uncoupled/State 3)	3.03	1.08	4.98	2.46	0.51	4.41	2.84	0.89	4.78
SCR (PCMD/PCM)	1.93	1.20	2.66	2.20	1.47	2.93	2.30	1.57	3.02
Baseline[2]	0.0372	0.0242	0.0501	0.0351	0.0221	0.0480	0.0277	0.0148	0.0406
State 4[2]	0.0185	0.0121	0.0249	0.0175	0.0111	0.0238	0.0134	0.0070	0.0197
State 3[2]	0.0523	0.0214	0.0832	0.0621	0.0312	0.0930	0.0424	0.0116	0.0733
Uncoupled[2]	0.1399	0.0848	0.1949	0.1326	0.0776	0.1877	0.1089	0.0539	0.1639
CCR[2]	0.0059	0.0021	0.0097	0.0038	0.00001	0.0077	0.0048	0.0010	0.0086
SCR[2]	0.0037	0.0025	0.0050	0.0036	0.0024	0.0049	0.0036	0.0024	0.0049
mitochondrial content (2*2^ΔCt)	527	374	680	623	470	777	626	473	779

	Diff of Lsmeans (Control - 150 nM)				Difference of Lsmeans (0 - 50 µM)				Difference of Lsmeans (150 nM - 50 µM)			
		95% CI				95% CI				95% CI		
	Difference	Lower	Upper	P[3]	Difference	Lower	Upper	P[3]	Difference	Lower	Upper	P[3]
Baseline[1]	-2.71	-10.28	4.86	0.415	1.97	-5.60	9.54	0.548	4.68	-2.89	12.25	0.181
State 4[1]	-1.32	-4.56	1.91	0.356	1.22	-2.02	4.45	0.393	2.54	-0.70	5.77	0.103
State 3[1]	-10.51	-34.86	13.84	0.332	-0.82	-25.17	23.53	0.937	9.69	-14.66	34.04	0.368
Uncoupled[1]	-10.79	-43.23	21.66	0.447	4.70	-27.75	37.15	0.735	15.49	-16.96	47.93	0.287
CCR (Uncoupled/State 3)	0.57	-2.18	3.32	0.631	0.19	-2.56	2.95	0.870	-0.38	-3.13	2.38	0.749
SCR (PCMD/PCM)	-0.27	-1.30	0.76	0.547	-0.37	-1.40	0.66	0.419	-0.10	-1.13	0.93	0.826
Baseline[2]	0.0021	-0.0162	0.0204	0.788	0.0094	-0.0088	0.0277	0.254	0.0073	-0.0109	0.0256	0.364
State 4[2]	0.0010	-0.0080	0.0101	0.792	0.0051	-0.0039	0.0141	0.215	0.0041	-0.0049	0.0131	0.310
State 3[2]	-0.0098	-0.0534	0.0339	0.603	0.0099	-0.0338	0.0535	0.600	0.0197	-0.0240	0.0633	0.313
Uncoupled[2]	0.0072	-0.0706	0.0850	0.828	0.0309	-0.0469	0.1088	0.368	0.0237	-0.0541	0.1016	0.484
CCR[2]	0.0021	-0.0034	0.0075	0.388	0.0011	-0.0043	0.0065	0.632	-0.0010	-0.0064	0.0045	0.684
SCR[2]	0.0001	-0.0017	0.0019	0.914	0.0001	-0.0017	0.0019	0.899	0.00002	-0.0018	0.0018	0.985
mitochondrial content (2*2^ΔCt)	-96	-313	120	0.284	-99	-316	118	0.273	-3	-219	214	0.975

Table 3. Respiration rates (LSmeans ± 95% CI) from untreated skeletal muscle cells or after treatment with 150 nM or 50 µM purified C3G for 24 hours. [1]Values are steady state oxygen consumption rates corrected to one million cells (pmol O_2/second·million cells). [2]Values are the same steady states after correction for mitochondrial content (pmol O_2/second·million cells/mitochondrial content). [3]P-Values for respective group-wise differences. All values are from three independent experiments.

Discussion

To our knowledge, this is the first study to explore the effects of anthocyanins from berry extracts or purified C3G on measures of respiration at the cellular level. This work stemmed from the significant findings of berry consumption on indirect calorimetry from a controlled human feeding study (chapter 2) where blackberries significantly lowered the respiratory quotient, indicative of an increase in fat oxidation, and also increased insulin sensitivity, as measured by decreases in fasting insulin as well as a reduction in the insulin 4-hour area under the curve after a high-sugar meal challenge. There was also a marginal increase in energy expenditure with the berry treatment. These differences measured at the whole-body level suggest modulation of substrate trafficking and oxygen consumption at the tissue level, therefore we adopted the protocol described by Boyle et al. which successfully demonstrated differences in respiration rates in muscle biopsy from lean and obese subjects on a fatty acid substrate, palmitoyl-carnitine, using a versatile instrument designed solely for respirometry in tissues [21, 24].

The observed increases in oxygen consumption noted in the differentiated adipocytes treated with physiologic amounts of C3G via a BBE preparation corroborate the "beiging" molecular changes noted by Matsukawa in the same 3T3-L1 adiopocyte cell line [19]. Matsukawa et al. noted increases in molecular targets and morphological features that indicate an increase in mitochondrial density with high dose C3G treatments during differentiation [19]. Despite the failed investigation of high dose C3G via BBE in this experiment, adipocyte treatment with physiological amounts of C3G resulted in significant increases in baseline oxygen consumption. Intact cells had a higher respiration rate prior to manipulation to characterize different respiration states. Our findings from the respirometry protocol indicate a greater

oxidative capacity of adipocytes treated with BBE on the fatty acid substrate palmitoyl-carnitine. Respiration rates were higher during state 3 and uncoupled respiration states. Although marginal, the persistence of these differences after mitochondrial correction suggests enhanced mitochondrial function. These differences cannot be explained solely by mitochondrial density.

The increased respiration of BBE treated adipocytes supports the therapeutic potential of anthocyanins against obesity, as brown adipocytes offer a metabolic sink for excess fatty acids with a greater proportion fated for oxidation as opposed to storage. Further, this study suggests effects at doses achievable from dietary consumption of anthocyanin containing foods. Beyond a greater potential for fatty acid disposal, beiging adipocytes with anthocyanins, if it could be demonstrated *in vivo*, could elicit positive changes on a neuroendocrine basis, i.e., adipose tissue adipokine signaling pathways are accepted as a modulators of energy balance beyond energy storage/disposal, intervening in neural circuitry related to hunger/satiety as well as insulin sensitivity [31]. Future work investigating berry anthocyanins and augmentation of brown adipose tissue should determine effects *in vivo*, as imaging technology that quantifies brown adipose tissue in humans is available [32].

Other groups have investigated high-resolution respirometry in adipose tissues [33-35]. Hirzel et al. characterized the antioxidant capacity and possible changes to cellular respiration by the antioxidants mitoQ (a synthetic antioxidant), resveratrol, and curcumin in human adipocytes from mesenchymal stem cells after 24 hours of the respective treatment [33]. All three reagents demonstrated antioxidant properties. Resveratrol and curcumin had no inhibitory effect on cellular respiration, while mitoQ had an inhibitory effect on baseline and uncoupled respiration [33]. A unique study by Sidossis et al. demonstrated the browning effect of severe burn trauma in subcutaneous adipose tissue in human patients which they cite as threshold adrenergic stress.

Cold exposure did not have the same effect (10 fold, chronic vs 1.5 fold, acute increases in norepinephrine, respectively) [34]. Morphological examination demonstrated increases in UCP-1 expression as well as increased mitochondrial content, plus multilocular lipid droplets characteristic of brown adipose tissue [34]. These morphological changes were supported by significant increases in the uncoupled respiration state as well as an increase in the CCR. The authors speculated that they had observed an increase in thermogenic potential of the tissue [34]. A study by Kraunsoe et al. used high-resolution respirometry to phenotype both subcutaneous and visceral adipose tissues from morbidly obese patients [35]. Significant findings included higher respiration rates in state 3 and uncoupled states in visceral compared to subcutaneous adipose tissues, and that this is explained by a greater mitochondrial density, as correction for mitochondrial density showed higher rates of oxygen consumption in the subcutaneous adipose tissue [35]. A different study in Zucker diabetic fatty rats supports the notion of depot specific responses to treatment, where rats treated with 200 mg/kg bodyweight resveratrol for six weeks saw a significant increase in state 3 and uncoupled respiration in subcutaneous and retroperitoneal fat pads, but no response in the epididymal fat pad [36]. These studies support the utility of metabolic phenotyping in adipose tissues. Of particular interest, the observed increase in respiration by the browning of subcutaneous adipose tissue described by Sidossis et al. [34] coupled with the demonstrated browning effect of C3G by Matsukowa et al. [19], further combined with the observed increases in baseline, state 3, and uncoupled respiration states by physiologic amounts of C3G from BBE in adipocytes from this study highlight a promising avenue of nutrition research as it relates to the challenge of obesity.

Beneficial effects of C3G administration in primary skeletal muscle cells were less obvious than the more discernable differences in the adipocyte model. This difference could have

been due to responsiveness of the tissue donor to C3G, as both Boyle et al. and Gnaiger report differences in respiration rates between lean and obese subjects [24, 30] and the anthropometric data of our donors were not provided by the commercial supplier, only that they were generally healthy. Shortened incubation with C3G (24 hours) relative to differentiating adipocytes (6 days) may account for dampened effects, or other anthocyanins and phytochemicals present in the BBE may have potentiated the greater differences observed in the adipocytes, where we compromised this potential synergism in an effort to achieve higher C3G doses like those described in Matsukawa et al. without disturbing cell viability [19]. The attractiveness of primary skeletal muscle cells was the potential to demonstrate increased respiration on a fatty acid substrate after anthocyanin treatment in an in vitro model in greater proximity to human subjects compared to the same experiment in an established cell line, albeit translational. Future work should instead use myotubes, which will allow for greater incubation times with C3G due to requisite differentiation prior to respiratory phenotyping, making treatment duration similar to the adipocyte model, and others have shown the utility of myotubes in detecting treatment differences with oxygraph protocols [37]. Others have also shown their utility in studies of fatty acid metabolism and insulin resistance with high lipid incubations [38]. Interestingly, other groups have corroborated findings from dietary interventions observed in humans assessed by indirect calorimetry to tissue models of differentiated myotubes treated directly with the compound of interest; Larsen et al. were able to demonstrate that dietary nitrate lowered resting metabolic rate in their human subjects, and 25 μM nitrite administration reduced baseline respiration in differentiated myotubes [39].

In conclusion, this is the first study to investigate the ability of anthocyanins from a BBE or purified C3G to affect cellular respiration in adipose or skeletal muscle cells, respectively.

Positive findings include increased respiration on a fatty acid substrate as well as maximal respiratory capacity in adipocytes after treatment with a physiologic dose of BBE that cannot be explained by increases in mitochondrial density, suggesting enhanced function. Future work could characterize UCP-1 expression and cellular morphology to determine if the adipocytes are presenting features characteristic of brown adipose tissue. Data from respirometry studies using primary skeletal muscle tissue were less compelling, although exhibiting the same directionality as the adipocytes. Future work in myotubes would allow for increased incubation times to determine if longer exposure elicits stronger responses like those observed in the adipose tissue.

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Chapter 4

Resveratrol and Metabolic Syndrome in Obese Men – a Review

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Key Facts

- Physical inactivity in combination with a hypercaloric diet is a root cause of the obesity epidemic.
- Excess adipose tissue invokes metabolic dysregulation, leading to metS, type-2 diabetes, and their associated comorbidities.
- Pharmacological interventions target the clinical complications of metS but do not provide a long term solution, as none are proven to reduce excess adipose tissue. Lifestyle interventions (diet and exercise) have been shown to be more effective in reducing metS.
- Epidemiological evidence shows a protective effect of polyphenol intake (non-nutritive compounds found primarily in fruits and vegetables) against development of metS, more so than pharmacological treatment. Several classes of polyphenols are being studied for their capacity as a nutraceutical for metS treatment.
- Resveratrol, a stilbene found mostly in grapes and peanuts, shares a route of action similar to that of caloric restriction. Preliminary research shows a protective effect against diet-induced obesity, where increased mitochondrial density could target excess adipose tissue, thus reversing downstream metabolic dysregulation and preventing metS.

Summary Points

- 35% of American adults are obese. As of 2011, 35% also have a form of metS – defined by three of five possible criteria. Lifestyle modifications to target obesity have been unsuccessful because recommendations are not realistic or socially acceptable.
- Excess visceral fat leads to elevated circulating free fatty acids which in turn leads to the clinical complications of obesity and metS: insulin resistance and increased cardiovascular health risks.
- Diet and lifestyle therapy remain the most effective measures to combat metS, yet challenges with recidivism and compliance appear to be unsurmountable.
- Polyphenols are a diverse grouping of compounds in fruits and vegetables believed to elicit positive health benefits that include longevity and reduction of chronic disease.
- Certain polyphenols, such as flavonoids from green tea and berries, may prove a useful tool to combat obesity and metS.
- RESV, a stilbene found in grapes and peanuts, activates the same sirtuin pathways that have been implicated in caloric restriction, which is believed to promote longevity.
- Animal studies show RESV increases lifespan in lower level organisms. Further, RESV is protective against a high-fat diet in mice: alleviating criteria of metS and improving survival.
- Human studies of RESV and obesity are more ambiguous due to variations in study design and subject population. Despite this, the same pathways implicated in animal studies have been translated to humans.

- In both animal and human studies RESV shows the potential to alleviate the criteria used to define metS, although human studies involving RESV administration to subjects with metS are lacking.
- This review provides support for the study of RESV in humans with metS with the use of a controlled feeding experiment to provide further information to this important research question.

Abstract
There is currently an unprecedented need for the development of safe and effective methods to treat obesity and its comorbidities. One such promising method is the use of dietary supplements that have the potential of diminishing the incidence of hypertension, excess fat accumulation, insulin resistance and the other components of what researchers refer to as “metabolic syndrome”. Among these potential dietary supplements is the compound Resveratrol, a stilbene first identified in 1978 that has been examined as a potential therapeutic agent in the treatment of hypertension and some forms of cancer. This short review will attempt to highlight studies that have examined how resveratrol might be implicated in not only extending lifespan, but also how it may have significant and beneficial impact on excessive fat deposition, insulin sensitivity, dyslipidemia and hypertension.

Abbreviations

CR	caloric restriction
CVD	cardiovascular disease
DXA	dual-energy x-ray absorptiometry
EGCG	Green tea/Epigallocatechin-3-gallate
LDL	low density lipoprotein
metS	metabolic syndrome
RESV	resveratrol
TG	triglycerides

Overview

A recent query in Pubmed that was restricted to using only the term “Resveratrol” returned 8776 papers. The oldest paper included in the search results was one dated 1978. Adding the term “review” resulted in 1204 papers. Clearly, it is beyond the scope of this review (or for that matter any review) to comprehensively survey the literature that has developed around RESV. Given this limitation, the present chapter attempts to highlight several important lines of research that chronicle the effect of RESV, a stilbenoid found in grapes, blueberries, raspberries and peanuts, and its influence on the Metabolic syndrome (metS). Included in our review are several examinations of how RESV affects several components of the metS. These include papers focused on the effects of RESV on adipose tissue, on insulin sensitivity, on glucoregulation, on dyslipidemia and on hypertension. We conclude this chapter with a call for more research on how RESV might contribute to the clinician’s tools in the treatment of metS. We propose that, under the appropriate conditions, significant improvements might be observed in insulin resistance, fasting glucose and lipids, and blood pressure with RESV administration. Further, enhanced activation of molecular targets in skeletal muscle and subsequent modulation of energy balance may be possible with RESV in obese men with metS.

Introduction

According to a 2013 report by the CDC, heart disease, stroke, diabetes, liver disease, and kidney disease are numbers 1, 5, 7, 12, and 13, respectively (CDC website) in their rank of diseases afflicting Americans. Poor nutrition and physical inactivity leading to excessive weight gain are the leading modifiable risk factors for these conditions (World Health Organization, 2003). The CDC also reports that as of 2012, 69% of American adults over 20 years of age are overweight or obese (35% are obese). This staggering increase in the prevalence of overweight and obesity is a 25 year old story in the United States with no sign of improvement. However, based on current trends some experts believe the prevalence to have stabilized or that the curve has flattened out since 2011 (Ogden *et al.*, 2014).

The same behaviors leading to the current prevalence of overweight and obesity in the nation further complicate health and longevity by way of the metabolic syndrome. MetS, as defined by the National Cholesterol Education Program's Adult Treatment Panel II, is a condition in which a individual/patient meets at least 3 of the 5 following criteria: waist circumference greater than 102 cm for men or 88 cm for women; serum triglycerides (TG) above 150 mg/dL; HDL cholesterol less than 40 mg/dL for men or less than 50 mg/dL for women; blood pressure above 130/85 mm Hg or prescribed hypertension medication; and/or fasting plasma glucose above 100 mg/dL or prescribed diabetic medication (National Cholesterol Education Program Expert Panel on Detection and Treatment of High Blood Cholesterol in, 2002). Aguilar et al. report the overall prevalence of metS in the United States between 2011-2012 to be 34.7% with a greater prevalence in women vs. men (36% vs. 30%) as well as old vs. young (46.7% vs 18.3%) (Aguilar *et al.*, 2015). These statistics highlight the relationship between obesity and metabolic function: the complications of inactivity and poor diet choices. However, different criteria can

satisfy the classification of metS with ten possible different combinations of three risk factors to describe the same syndrome (see figure 1 from Lim et al. 2014) (Lim and Eckel, 2014). Different combinations have an influence on morbidity. To illustrate this point, a person with only obesity and hyperglycemia (not technically considered metS) has a 2 fold greater chance of becoming type 2 diabetic compared to a person who has all the criteria for metS except hyperglycemia (Nichols and Moler, 2010). Different combinations of criteria call for individualized approaches to yield effective therapy, but a staple for the majority of cases will involve a reduction in fat mass.

Doctors and dieticians who counsel overweight/obese patients about remedial approaches typically include recommendations that their patients eat less calorically dense foods and increase their physical activity. Further, announcements to the public to adopt an active lifestyle (for example, the former First Lady's "Let's move" campaign) have gone to great lengths to bring the issue of this health crisis to the public's attention. Unfortunately, the advantages of an energy dense, nutrient poor diet – affordable, convenient, and palatable – are more captivating to the average consumer compared to the encouragement of health officials to adopt healthier eating behaviors (Drewnowski and Eichelsdoerfer, 2010). Indeed, nutritious meal plans designed to be conducive to low-income family budgets do not consider social acceptability or consumer meal patterns and are an unrealistic and ineffective solution. Nutritious meal plans, when constrained to the meal pattern of the majority of a population, more than double their cost (Maillot *et al.*, 2010). Clearly, the health community must approach this dilemma through different avenues. It is not enough to prescribe medications to treat the biochemical aberrations elicited by the obese state. Rather, researchers must discover and address the source of the dysfunctional cascade that leads to downstream comorbidities of excess weight.

Mechanistically, the complications of metS are attributable to excess body fat which results in abnormally high concentrations of non-esterified fatty acids (NEFAs) which then interfere with regulatory (insulin) signals in liver and skeletal muscle (Vega *et al.*, 2011). This results in a futile cycle of peripheral insulin resistance and increased hepatic release of glucose. The distribution of excess body fat determines the magnitude of this effect; excess adipose tissue surrounding the vital organs (visceral adipose tissue) is a primary insult. Excess adipose tissue also releases cytokines and plasminogen activator inhibitor-1 (PAI-1) resulting in a chronic, low-grade inflammation and increased risk of thrombosis (Vega *et al.*, 2011). Ultimately, effective strategies designed to combat metS (nutritional or otherwise) must address the source of the problem; an effective therapy is one that will reduce excess adipose tissue. Please refer to Figure 1 for a summary illustration of metS and the interaction of factors that promote excess adiposity.

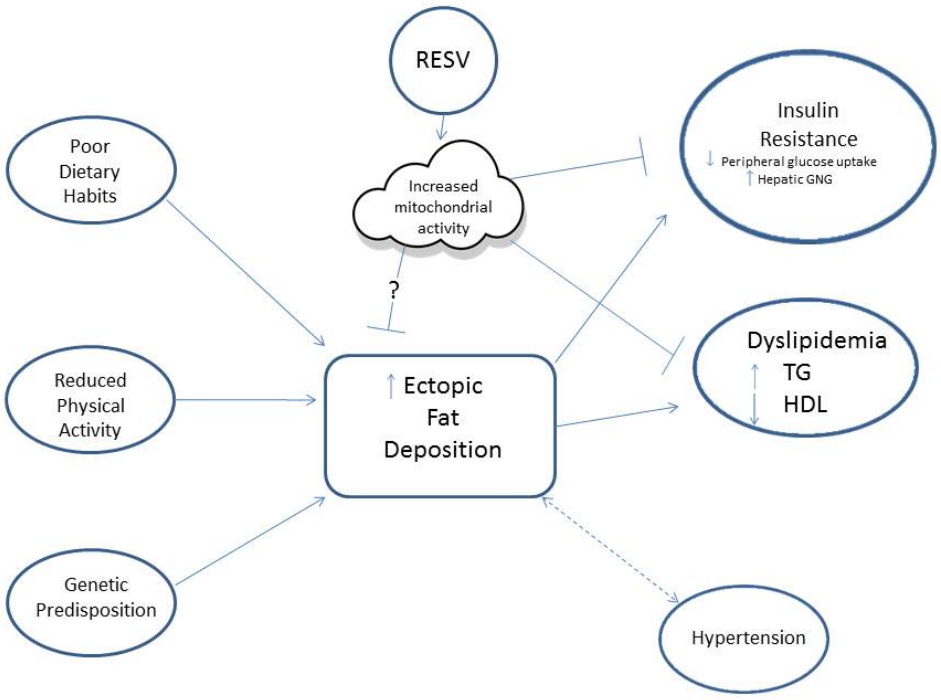

Figure 1. Ectopic fat interrupts normal metabolic function.

Excess calories in combination with a sedentary lifestyle and a genetic predisposition (inability to store all excess calories as adipose tissue) lead to ectopic fat deposition. Fat storage in lean tissues (skeletal muscle, liver, and heart) in combination with an increase in circulating free fatty acids is believed to impede normal signaling pathways of carbohydrate and fatty acid metabolism; cornerstones of the metabolic syndrome. RESV, discussed in this review, has the potential to address the complications of metS via increased mitochondrial density and/or activity.

Diet therapy and metabolic syndrome

Pharmacological intervention is a standard approach to treating the clinical signs of metS: thiazolidinedione or metformin for hyperglycemia, statins for dyslipidemia, and ACE inhibitors

for high blood pressure. Anti-obesity drugs are being developed as well, but are not as commonly used because of issues with side-effects (Lim and Eckel, 2014). Thus pharmacological treatments attempt to reduce the risk of comorbidities introduced by metS, namely, type-2 diabetes and cardiovascular disease, but safely targeting fat loss remains a challenge; lifestyle modifications (diet/exercise) remain the primary solutions. Recidivism and translation of promising lifestyle modification experiments to clinical practice are common challenges to health practitioners who are tasked with the treatment of the obese/metabolically stressed.

The potency of lifestyle over pharmacological intervention has been illustrated best in a meta-analysis by Dunkley et al. (Dunkley *et al.*, 2012). They identified 13 longitudinal studies that described the effects of lifestyle, drug, or combination treatments on subjects with metS, most of whom had not developed advanced comorbidities. Lifestyle intervention resulted in a fourfold reduction in the incidence of metS (reversal of metS from baseline) compared to control, whereas drug therapy alone yielded a comparably modest, yet significant 60% reduction in the incidence of metS. The results of the analysis also showed the effectiveness of anti-diabetic drugs in reversing metS. Dunkley et al. noted that the heterogeneity amongst the trials prevented firm conclusions to be drawn. Although this study highlights the effectiveness of lifestyle modification, the benefit of drug therapy shouldn't be overlooked: in a sub-set of the studies with an appreciably long amount of time to follow-up, beneficial effects of metformin and statin drugs reduced incidence of diabetes and cardiovascular-related mortality at 3 and 10 year follow-up, respectively (Clearfield *et al.*, 2005; Geluk *et al.*, 2005; Ramachandran *et al.*, 2007).

Despite what may be superior effects of lifestyle treatment described above, recidivism is the greatest weakness of this intervention. For this reason, difficultly lies in translating promising

experimental findings into clinical practice. Dietary approaches to promote weight loss or prevent weight gain are ubiquitous, and unfortunately there is still no consensus on what are the most effective forms of dietary treatment for this at-risk population (Brown *et al.*, 2009; Meydani, 2005; Tarantino *et al.*, 2015). Conversely, diet adherence ensuring caloric restriction is more critical to success, thus comparisons regarding effectiveness of various diet compositions should be secondary and selected according to patient preference; individualized diet plans that maximize compliance will be most useful for weight loss (Dansinger and Schaefer, 2006). However, it is beneficial to determine what diet components lend to the success of certain diet patterns in managing body weight in at-risk populations, as this knowledge can then be utilized in advancing therapies that maximize the potential for weight loss or maintenance.

Polyphenols and metabolic syndrome

In a segment of nutrition research, scientists are exploring the capabilities of bioactive food components to quell the insult to human health caused by today's obesogenic environment. Bioactive food components are believed to be one of the main effectors of a diet high in fruit and vegetables. It has repeatedly been found that a diet high in fruits and vegetables positively effects biomarkers of human health and reduces risk of chronic disease as outlined in epidemiologic studies (Hung *et al.*, 2004; Knekt *et al.*, 2002; Liu, 2003). The term polyphenol is used to describe a diverse spectrum of plant metabolites that can be categorized into several classes dependent on a unique chemical structure; examples include stilbenes, lignans, and flavonoids. What all of the various classes have in common is the presence of phenolic structures derived from the phenylpropanoid pathway (see Figure 1 of Hollman et al) (Hollman, 2014). The vast array of polyphenols characterized and isolated combined with the task to elucidate the effects of the high fruit and vegetable diet pattern resulted in a new facet of research for

nutritionists and biochemists to investigate the effect of polyphenols on health and function. Polyphenols are being studied for their nutraceutical capabilities in the context of the metabolic dysregulation of obesity (Sae-tan *et al.*, 2011b). To demonstrate the potential for polyphenol sources to improve metS, here we will briefly review two particularly intriguing and well-studied sources of polyphenols: catechins from tea and flavonoids from berries.

Green tea/Epigallocatechin-3-gallate (EGCG)

Green tea has a rich cultural history, and has long been thought to positively contribute towards health (Wolfram *et al.*, 2006; Yang *et al.*, 2007). Polyphenols constitute approximately 40% of green tea, primarily as flavonoids, of which flavan-3-ols (sometimes referred to as catechins, though catechin is actually a specific compound itself) are the main flavonoid subgroup (Cabrera *et al.*, 2006; Cabrera *et al.*, 2003; Del Rio *et al.*, 2004; Graham, 1992). EGCG is the most abundant flavan-3-ol in green tea and has high antioxidant activity; its abilities to chelate metals, scavenge free radicals, and induce superoxide dismutase have been noted (An *et al.*, 2014; Meng *et al.*, 2008; Rice-Evans, 1999). Conversely, increased apoptosis by production of reactive oxygen species by EGCG at high doses has also been described in cancer models (Li *et al.*, 2010; Min *et al.*, 2012) as well as in adipocytes (Lin *et al.*, 2005).

Green tea or green tea polyphenol administration to animal models of obesity show a protective effect against body weight gain as well as decreases specifically to fat mass (Bose *et al.*, 2008; Choo, 2003; Fiorini *et al.*, 2005; Hasegawa *et al.*, 2003; Park *et al.*, 2011). Studies in rodent models of obesity were also able to demonstrate recapitulation of green tea's beneficial effects on fat mass after it had been decaffeinated, as confounding results attributed to caffeine were a concern (Ikeda *et al.*, 2005; Richard *et al.*, 2009). One mechanism by which green tea or EGCG are thought to reduce obesity in rodents is decreased digestibility and subsequent excretion of

lipids in the feces (Bose *et al.*, 2008; Muramatsu *et al.*, 1986; Raederstorff *et al.*, 2003; Yang *et al.*, 2001), an effect that may be facilitated by EGCG's observed inhibition of pancreatic lipase *in vitro* (Grove *et al.*, 2012; Juhel *et al.*, 2000; Shishikura *et al.*, 2006; Wang *et al.*, 2014). Parallel to this intestinal effect, EGCG can increase fat oxidation and reduce activity of fatty acid synthase, of which expression of genes involved in the pathway support the findings (Friedrich *et al.*, 2012; Huang *et al.*, 2015; Ikeda *et al.*, 2005; Klaus *et al.*, 2005; Sae-Tan *et al.*, 2011a; Wolfram *et al.*, 2005).

Preliminary studies in obese or metS human subjects have been conducted with promising findings regarding body weight and outcomes related to obesity and metS (see table 3 of (Legeay *et al.*, 2015)). For instance, a parallel-arm study of 35 obese subjects with metS found significant reductions in body weight (-2.5 kg vs control), as well as reduced LDL and lipid oxidation after 870 mg of EGCG per day either as green tea or green tea extract for 8 weeks (Basu *et al.*, 2010). In contrast, one follow-up study, Mielgo-Ayuso et al. were unable to reproduce EGCG's effects in obese women fed 300 mg/d for 12 weeks, however, both the EGCG intervention group and the control group were following a diet intervention to lose weight (Mielgo-Ayuso *et al.*, 2014). In addition to the anti-obesity effects of green tea or EGCG, a plethora of work describing its ability to modulate the other criteria of the metS exists (for useful reviews on the topic, see Sae-tan et al. and Legeay et al.) (Legeay *et al.*, 2015; Sae-tan *et al.*, 2011b).

Berries/flavonoids

Dark and red pigmented berries are a good source of vitamin C, ellagic acid/ellagitannins, and flavonoids. The high density of phytochemicals found in berries results in a food source with a high antioxidant capacity. Of all of the frequently consumed beverages in the United States,

blueberry and cranberry juices were shown to have the greatest antioxidant potential (Basu and Lyons, 2012; Seeram *et al.,* 2008). Because the constituents of berries are thought to elicit positive health benefits to humans beyond nutritional value i.e. by promoting normal function or preventing chronic disease, they are classified as functional foods (Brown *et al.*, 2015; Ozen *et al.*, 2012). Here we are interested in their ability to reduce adiposity or alleviate factors related to metS.

Blueberries have high anthocyanin content; anthocyanins comprise the majority of the polyphenol profile of blueberries (Gavrilova *et al.*, 2011). Animal models of metS that studied blueberry juice, extract, or isolated anthocyanins found varied effects on markers of metS. In one study, anthocyanin extract didn't modulate weight gain in the presence of high-fat diet fed in mice, but still had a positive effect on insulin sensitivity (Guo *et al.*, 2012). A different study found a protective effect of blueberry juice or purified anthocyanins on fat mass, where purified anthocyanins also increased insulin sensitivity, but the juice did not (Prior *et al.*, 2010). The mechanisms by which the anthocyanins protect against a high-fat diet are not fully understood, but studies using high-fat or genetic mouse models highlight the involvement of the inhibition of sterol regulatory element binding protein 1, and down regulation of enzymes involved in fatty acid synthesis and inflammation (Tsuda *et al.*, 2003; Wei *et al.*, 2011).

In human experiments, a method to deliver a greater quantity of polyphenols from berries is to feed them as a freeze-dried powder, which concentrates the polyphenol content by approximately seven fold (Basu and Lyons, 2012). Basu et al. highlight several studies that have fed various freeze-dried berries, purees, cocktails, or juices to subjects with metS (see Table 1) (Basu and Lyons, 2012). The major findings of these human trials involve decreases in blood pressure, lipid oxidation, and LDL cholesterol concentrations. Although berry consumption may not prove

applicable to reduce adipose tissue, its antioxidant and anti-atherosclerotic observations in humans prove berries a useful tool in augmenting a diet that reduces metabolic stress.

The description above of studies utilizing tea or berry sources for polyphenols to impede metS is simply to give the reader an appreciation for how polyphenol-rich foods may improve outcome for subjects with this cluster of metabolic complications of obesity. It is not exhaustive. Other sources of polyphenols, such as apples (quercetin), coffee (chlorogenic acid), and cinnamon may prove useful in addressing the complications of metS, although their effects on metS specifically are not as established (Cherniack, 2011). The remainder of our discussion will focus on one candidate polyphenol, RESV, and the studies that describe its potential as a nutraceutical therapy for metS. RESV provides a unique approach to the challenges of metS in that it aims to alleviate the source of the dysfunction: excess adipose tissue.

Resveratrol: encouraging research points toward a nutraceutical application for the metabolic syndrome

Resveratrol (RESV), a polyphenol abundant in red grapes, was first identified for its anti-cancer and anti-atherogenic properties *in vitro* (Jang *et al.*, 1997; Pace-Asciak *et al.*, 1995). It was not until 2003 in the seminal work by Howitz et al. where RESV was identified in a small molecule screening. RESV was described for its ability to activate the histone deacetylase SIRT1 in an elegant series of experiments describing its effects *in vitro* as well as *in vivo* via yeast and cell culture models (Howitz *et al.*, 2003). The implication of SIRT1 activation by RESV is above and beyond the prospect of promoting heart health and preventing cancer, as SIRT1 has been implicated in mediating calorie restriction extension of lifespan (Mercken *et al.*, 2014). Howitz et al. describes the ability of RESV to increase SIRT1 activity by 13 fold and increase the average lifespan of yeast by 70% compared to control conditions. The authors also demonstrate

RESV's ability to increase survival of cell cultures exposed to ionizing radiation via the attenuation of the tumor suppressor gene p53, a downstream target of SIRT1. These findings led the authors to call for future work investigating RESV's ability to act as a caloric restriction (CR) mimetic, as CR is also known to act through similar pathways to extend lifespan in a hormetic manner (Masoro, 2000; Mattson, 2008).

CR's observed benefits on longevity date back to rat models in the 1930s, and an extensive amount of work has been completed on long term trials in non-human primates that highlight the powerful benefits of CR on extending lifespan (Colman *et al.*, 2009; Kemnitz, 2011; McCay *et al.*, 1935). Seminal work by McCay et al. laid the framework for the elucidation of caloric restriction without malnutrition in a series of longitudinal experiments involving rodents and dogs (McCay, 1947; McCay *et al.*, 1935; McCay *et al.*, 1939). Their work also included the CR effects on the intestinal microflora via parabiosis of young vs. old, and fed vs. calorically restricted rats reared from the same parents. The authors found the CR animals (both rats and beagles) to be smaller in size with an increase in bone fragility, but the animals lives were substantially extended; in the case of the rat, as much as a two-fold increase in lifespan was observed (McCay *et al.*, 1939). McCay, clear in his (current-day controversial) views in how the discoveries needed to be translated to humans, was a pioneer in the topic of CR. As it stands today, the application of CR to humans is still not definitive (McCay *et al.*, 1956).

The benefits of CR are in the process of being translated to humans (Civitarese *et al.*, 2007; Heilbronn *et al.*, 2006) and long term studies investigating the translation of its benefit are underway (Stewart *et al.*, 2013). An excellent review by Mercken et al. addresses the challenges of connecting translational findings to humans; namely, even if a benefit of CR is identified the idea of the general public adhering to CR is optimistic at best, as the western world is steeped in

its poor diet and sedentary lifestyle (Mercken *et al.*, 2012). Mercken also summarizes the benefit of CR in the presence of obesity and metS referencing studies that confirm the widely accepted belief that reduced food intake leads to a reduction in visceral adiposity and the alleviation of metabolic abnormalities which prevents or even reverses the morbidities listed above (Fontana, 2008). Although CR investigations in humans are warranted, a place still stands for the assessment of RESV to tap into these same mechanisms in humans, as a plant extract may be an easier pill to swallow than a 30% reduction in energy intake.

The CR-like effect of RESV discovered by Howitz et al. spurred on other researchers to confirm its effect in other short lived animal models. Several review papers have done an excellent job summarizing the connection between CR and RESV, the animal models studied, the tissue specific molecular mechanisms activated and their postulated effects on overt physiological and psychological states, as well as the physiologic conditions under which RESV is most effective (Ingram and Roth, 2015; Mercken *et al.*, 2012; Ramis *et al.*, 2015; Wright, 2014). The biggest questions one is left with after coming to appreciate the many different ways RESV elicits benefits in these translational studies are the following: Can some or any of these be translated to the human condition? Which ones? And at what dose does RESV yield such benefits and when in life should a regimen commence?

Life extension

Investigators have demonstrated the ability of RESV to modulate gene expression in a CR like manner: Barger et al. calls RESV a “partial CR mimic” when fed low dose and started in middle age in control mice – see Figure 1 for gene expression comparison between RESV and CR (Barger *et al.*, 2008). Based on their experiment, SIRT1 protein expression is actually hindered by CR and RESV in heart and skeletal muscle tissue compared to control mice of a similar age

(Figure 3) (Barger *et al.*, 2008). A study by Pearson et al. also showed similarities in gene expression between CR and RESV (Figures 1 and 2) but report no life extending ability in control mice (mice fed a standard diet with the addition of RESV), although several markers of health deterioration were retarded (Figure 3) (Pearson *et al.*, 2008). Conversely, life extension was increased by 25% with the addition of RESV in the rodent groups fed a high-fat (60% fat) diet to the degree where life span was not different from the standard diet groups (Pearson *et al.*, 2008). In addition to the work by Howitz et al. where RESV extends lifespan in yeast, other scientists have found the same result in other animal models including worms, fruit flies, and a short-lived species of fish (Bauer *et al.*, 2004; Valenzano *et al.*, 2006; Wood *et al.*, 2004). However, conflicting studies exist (Bass *et al.*, 2007; Pearson *et al.*, 2008). Perhaps one of the most critical reviews of RESV's life extending capabilities in animal models comes from a meta-analysis by Hector et al. (Hector *et al.*, 2012). A hazard ratio was calculated for effect size allowing the investigators to compare species with different lifespans resulting in a comparison of 6 species from 19 studies. The authors concluded that a marked increase in longevity in lower order species exists, while beneficial effects in mice and Mexican flies were less obvious; however, a prominent effect was noted in turquoise killifish (mentioned above), which the authors attribute to their exceptionally short lifespan. The authors justly concluded that RESV should not be touted as a lifespan promoter in humans. It should be noted that one failure of this meta-analysis was to not include where in the lifespan of the animals RESV treatment was initiated, as this would be a useful inclusion of the other moderators described (see Figure 2 of that communication).

Colman et al. mention in the last line of their 2009 communication on the effects of CR in rhesus monkeys that its (CR's) effects in humans may never be known, but that will not dissuade their

group from continuing to determine the effect in their model (they've come too far!) (Colman *et al.*, 2009). Likewise, the "ultimate truth" of RESV's effects in these same avenues will likely never result in a definitive yes or no answer for life extension in humans, but as discussed below, other beneficial effects of RESV on human health can be gleaned along the way. As postulated by Mercken et al., metabolic stress may be a prerequisite condition for RESV to elicit a health benefit. A good demonstration of this is the comparison between two mice studies by Sinclair et al. in which one study found no age extension in adult mice on a normal diet +RESV (despite its impact on gene expression mentioned above), but the amelioration of metabolic dysregulation and increased survival were observed when adult mice were placed on a high-fat diet concurrent to RESV administration independent of weight gain (Baur *et al.*, 2006; Pearson *et al.*, 2008). A final point on longevity, CR itself has not definitively increased lifespan in all circumstances (for an excellent, exhaustive review of CR mimetics and their targets, see Ingram 2015).

Metabolic dysregulation and molecular pathways

Studies mentioned above paint a murky picture on the ability of RESV to extend lifespan, especially in higher order animals as these types of studies become laborious due to the time commitment. However, mounting evidence exists for its health span promotion i.e. the alleviation of chronic morbidity. Highlighted by Ingram and Roth, broad-reaching beneficial effects of RESV include changes in stroke, heart failure, seizures, Parkinson's, and Alzheimer's disease (Ingram and Roth, 2015). As nutritionists, our interest lays in its ability to alleviate the insults of poor dietary habits, namely a high-fat, high calorie diet. Alleviation of metabolic stress as indicated by the remediation of disturbed clinical markers by RESV, independent of a poor diet, would be a powerful, simple, and inexpensive solution to an overburdened health care

system in this country. If findings from animal studies can be confirmed in humans this would make RESV an attractive therapeutic tool.

A plethora of translational work exists on the mechanisms by which RESV alleviates metabolic dysfunction due to high-fat feeding (see pg 394 of Mercken et al, pg 32 of Ramis et al, pg 112-113 of Wright et al, and pg 54-55 of Ingram et al) (Ingram and Roth, 2015; Mercken *et al.*, 2012; Ramis *et al.*, 2015; Wright, 2014). The key findings include improvements in insulin sensitivity and blood glucose regulation. Regulatory metabolic pathways of adipose and skeletal muscle are described with a central theme on mitochondrial biogenesis. The authors highlight RESV's activation effect on the SIRT1 cascade, which includes AMPK and PPAR-γ's transcription cofactor PGC-1α, although there is debate about where in this pathway RESV exerts its effect (Hoeks and Schrauwen, 2012).

These molecular pathways and their affected clinical measurements have been confirmed in mice, rats, monkeys, and humans (Baur *et al.*, 2006; Beaudoin *et al.*, 2013; Jimenez-Gomez *et al.*, 2013; Lagouge *et al.*, 2006; Timmers *et al.*, 2011). Baur et al. fed RESV (0.4 g · kg diet^{-1} or approximately 22 mg · kg body weight^{-1} · day^{-1}) in combination with a high-fat (60% fat) diet to 1 year-old mice for 1 year and demonstrated a significant increase in activated (phosphorylated) AMPK protein content, activated (deacetylated) PGC1-α protein content, and mitochondria per cell in liver tissue compared to mice on the high-fat diet. The mice fed RESV also had improved insulin sensitivity and glucose tolerance. Lagouge et al. fed RESV (4 g · kg diet^{-1} or approximately 400 mg · kg body weight^{-1} · day^{-1}) in combination with a high-fat (40% fat) diet to growing (4 to 8 week-old) mice for fifteen weeks. The investigators observed an increase in mitochondrial size, number, and activity (via succinate dehydrogenase staining and citrate synthase activity) in the gastrocnemius of the mice fed RESV. Mitochondria size and number

were also increased in the brown adipose tissue. This study also observed a significant increase in the protein content of PGC-1α in the gastrocnemius, which confirms the molecular mechanism of the mitochondrial adaptation by RESV. To demonstrate the pivotal role of SIRT1 expression in subsequent effects on mitochondria, SIRT1 null mouse embryonic fibroblasts did not show increased mitochondrial gene expression (PGC-1α, cytochrome C, and medium-chain acetyl-CoA dehydrogenase) like that observed in positive controls when treated with RESV. Cardiac muscle PGC-1α acetylation was unchanged, likewise, a long-term feeding study of rhesus monkeys performed a comprehensive characterization of mitochondria content and activity in visceral white adipose tissue and showed no differences between treatments (a high-fat, high sugar diet with or without 480 mg RESV $\cdot$ day^{-1}), which may indicate the tissue specific action of SIRT1 on PGC1α and subsequent effects on mitochondria. Conversely, Zucker diabetic fatty rats fed a standard diet with or without RESV (200 mg $\cdot$ kg body weight^{-1} $\cdot$ day^{-1}) for six weeks demonstrated increased *ex vivo* oxygen consumption and mitochondrial protein content in subcutaneous and retroperitoneal white adipose tissue with RESV treatment (Beaudoin *et al.*, 2013). This discrepancy highlights differential effects of RESV on tissue compartments across animal models which are likely also affected by study duration, different diet treatments, and RESV doses.

Timmers et al. (Timmers *et al.*, 2011) translated the mechanistic effects of RESV to humans. They observed the activation of the same molecular pathways (increases in SIRT1, AMPK, and PGC1α protein content of the vastus lateralis) and subsequent effects on mitochondrial activity that were implicated in the translational studies described above. Eleven obese, but healthy male subjects were administered 150 mg of RESV or placebo every day for four weeks in a cross-over fashion with a four week wash-out between treatments. Here, mitochondrial density was not

different between RESV and placebo groups, however, *ex vivo* characterization of mitochondrial activity in permeabilized vastus lateralis muscle fibers (citrate synthase activity, oxygen flux in state 3 respiration using fatty acid substrates, and maximum capacity, uncoupled respiration) demonstrated an increase in mitochondrial efficiency with RESV treatment compared to placebo. This is the only human study to confirm the biochemical effects of RESV, thus additional studies are needed to confirm these findings. It should be noted that the review by Wright et al. incorrectly identified the obese healthy subjects in the Timmers et al. study as having type-2 diabetes, and the review by Ramis et al. overlooked the study entirely, thus the Ramis et al. conclusion that there was a failure to translate RESV's effects in animals to humans is unjustified.

RESV's impact on specific factors of the metabolic syndrome

We have introduced RESV as a potential life/health-extending, partial CR mimetic. These exciting claims are supported by empirical evidence involving mechanisms accepted as health promoting: the activation of a sirtuin, AMPK, PGC1-α axis that is believed to increase mitochondrial density and ultimately lead to the preferential use of fatty acids as energy substrate. The exact mechanism by which RESV exerts an effect on this pathway is not fully understood, but what is clear is that somehow it has a beneficial effect on energy homeostasis (Park *et al.*, 2012; Um *et al.*, 2010). Described below are studies that indicate RESV's utility in a hypercaloric environment. If this proposed pathway is activated in human subjects with metS, this would offer the possibility of a cost-effective, simple approach that can be added to the standard of care for patients with diet-induced metabolic stress, and may lead to other research focused on expanding the knowledge of this pathway, as well as other ways in which it could be

activated. Here we will review the effectiveness of RESV administration as it relates to each factor of the metS.

RESV and adipose tissue

Beyond the initial discovery of sirtuin activation in yeast, another exciting development of RESV and health was the anti-obesity effects described in rodents fed a high-fat diet. In growing mice fed RESV, a dose of 4 g · kg diet^{-1} (estimated as 400 mg · kg body weight^{-1} · day^{-1}) was protective against a high-fat diet over fifteen weeks of feeding (Lagouge *et al.*, 2006). The mice fed RESV in combination with the high-fat diet had a significant reduction in fat-pad mass as well as percent body fat as measured by DXA. These differences were observed despite similar calorie consumption between the two treatments. The authors also reported differences in energy expenditure independent of physical activity. They were also able to demonstrate increases in mitochondrial density in skeletal, hepatic, and brown adipose tissues with commensurate increase in PGC1-α protein levels in the gastrocnemius, which was less acetylated than control high-fat fed mice, suggesting activation of the molecular axis by RESV described above (Lagouge *et al.*, 2006). The increase in energy expenditure may be explained by the increase in mitochondria in the rodents' brown adipose tissue and subsequent increase on non-shivering thermogenesis, as uncoupling protein 3 gene expression was increased (in skeletal muscle) along with core temperature. The effects on thermogenesis and molecular targets in brown adipose tissue were confirmed in a related study, and technology is now available to assess RESV's effects on BAT in humans *in vivo* (Andrade *et al.*, 2014; van Marken Lichtenbelt *et al.*, 2009). A similar study using the same design as Lagouge et al. was able to reproduce the protective effect of RESV against weight-gain in mice fed a high-fat diet for 10 weeks (Kim *et al.*, 2011). In addition, this group demonstrated a reduction in inflammatory signaling in epididymal adipose

tissue and proposed a model of RESV's actions on galanin and toll-like receptor-mediated pathways of inflammation based on changes in gene expression (see Figure 4 of Kim et al) (Kim *et al.*, 2011).

As summarized in de Ligt et al. (de Ligt *et al.*, 2015), the protection by RESV to counteract increases in fat mass has not been translated to humans (see table 1 of that communication for a summary of relevant human studies) (Bhatt *et al.*, 2012; Crandall *et al.*, 2012; de Ligt *et al.*, 2015; Movahed *et al.*, 2013; Poulsen *et al.*, 2013; Timmers *et al.*, 2011; Yoshino *et al.*, 2012). De Ligt et al. emphasize the high-fat feeding of the mice, dose, and duration of treatment as main differences between the mice and human studies (de Ligt *et al.*, 2015). Age is also likely a point of disparity between animal and human studies. Indeed, the nature in which RESV has an anti-obesity effect in mice is in the context of young, growing mice fed a high-fat diet, whereas the human studies are performed with middle-aged subjects that are already obese, and none of the studies are controlling the subjects' diet. Similarly, in one longitudinal rodent study that fed RESV and a high-fat diet to middle-aged (12 months of age) mice, the investigators saw an initial difference in weight trajectory in the first few months of feeding, but differences were not observed between the two diet treatments in older mice (18-24 months of age) (Baur *et al.*, 2006). However, the survival curve of high-fat+RESV mice was normalized to the control, chow fed group (Baur *et al.*, 2006). The differences in body weight noted in other rodent studies could be transient and specific to the growing, young mouse. To recapitulate this same environment in humans is ethically impossible. Translating the appropriate dose from rodents to humans has also proven to be difficult. The metabolism of RESV is likely different between mouse and human, as Timmers et al. was able to achieve similar plasma concentrations of RESV using a 250-fold lower dose (150 mg $\cdot$ day^{-1}) than the rodent studies (Timmers *et al.*, 2011). The dose of RESV in

human studies of overweight/obese subjects ranges from 10 mg to 5 g daily (de Ligt *et al.*, 2015). Despite the disconnect from rodent to human studies regarding observable changes in fat mass, human studies have still demonstrated alleviations to the metabolic phenotype characteristic of overeating/metabolic stress (described below), as well as activation of molecular targets that indicate the potential for preferential use of fatty acids as a substrate. Well-designed studies of appropriate RESV dose and duration in the future may one day be able to address this question.

RESV and insulin sensitivity/glucoregulation

Impaired fasting glucose in metS is an indicator of excess adipose tissue stores in the liver and skeletal muscle and subsequent reduction in insulin sensitivity which puts the subject at risk for developing type-2 diabetes (McGarry, 2002). Like the rationale for reduction of adiposity mentioned above, increased or normalized mitochondrial density in hepatic and skeletal muscle tissue may be one approach to alleviating metabolic stress i.e. more mitochondria available to remove the excess fatty acids and restore normal insulin signaling. Research conducted on cardiovascular training in type-2 diabetics demonstrates an increased mitochondrial capacity parallel to increased insulin sensitivity (Meex *et al.*, 2010). Likewise, Timmers et al. was able to demonstrate comparable effects in healthy obese men fed 150 mg RESV for four weeks, demonstrating both reductions in fasting glucose and insulin sensitivity in addition to an increase in respiration using fatty acids in mitochondria isolated from the vastus lateralis (Timmers *et al.*, 2011). RESV has also been shown to preserve mitochondrial content and insulin sensitivity in a rat model of muscle atrophy (Momken *et al.*, 2011).

The rodent studies of RESV in combination with a high-fat diet found positive effects of RESV on insulin sensitivity. Lagouge et al. assessed insulin sensitivity with both the clamp method in C57Bl/6J mice fed respective diets for sixteen weeks as well as a meal tolerance test in KKAy

mice (a rodent model of obesity and diabetes) fed respective diets for eight weeks (Lagouge *et al.*, 2006). The C57Bl/6J mice fed high-fat + RESV had significantly lower fasting insulin concentrations and were able to tolerate a higher glucose infusion rate over the course of two hours compared to the high-fat fed mice. Similarly, the genetically obese/diabetic mice had lower fasting blood glucose as well as a trend for lower glucose area under the curve (AUC) out to 2.5 hours from the oral glucose bolus. These changes in the genetic mouse were noted without any changes on bodyweight/adiposity which, as Lagouge et al. conclude, exemplifies RESV's intrinsic anti-diabetic effects (Lagouge *et al.*, 2006). The longitudinal study by Baur et al. supports this claim, where their mice also responded to RESV with significant reductions in both insulin and glucose AUC after the same oral glucose bolus despite no appreciable changes in body weight after one year adherence to treatment diets (Baur *et al.*, 2006).

These *in vivo* experiments of RESV and increased insulin sensitivity have also been translated to non-human primates (Jimenez-Gomez *et al.*, 2013; Marchal *et al.*, 2012). In one study, Jimenez-Gomez et al. fed rhesus monkeys a high-fat, high sugar diet for two years with or without RESV (80 mg $\cdot$ day^{-1} the first year, and 480 mg $\cdot$ day^{-1} the second) and assessed inflammation, size, and insulin sensitivity of subcutaneous and visceral adipose tissue. The authors used microarray and subsequent gene-set enrichment analysis to demonstrate a protective effect of RESV against the increased gene expression related to inflammatory pathways caused by the high-fat, high sugar feeding. Specific to the visceral white adipose tissue, they discovered a reduction in adipocyte size, and increases in SIRT1, insulin receptor substrate 1, and GLUT4 glucose transporter protein expressions. The positive effects of RESV in the visceral adipose tissue extend the beneficial effects of RESV demonstrated in diet-stressed rodents to primates, and provide further evidence of a mechanism that addresses a primary cause of the metS. Unfortunately, this study did not

perform the comparison of systemic insulin sensitivity and glucose tolerance between the two experimental animal groups after two years of feeding the respective diets. A separate study did answer this question in a longitudinal study of grey mouse lemurs, albeit the animals were not under stress of a high-fat, high sugar diet. Control animals were fed a standard diet, a second group was calorically restricted (30% less kcals than control), and the third group was fed the standard diet supplemented with 400 mg RESV · kg bodyweight^{-1} · day^{-1}. Compared to the control animals, both the CR and RESV groups had a lower glucose AUC after three years of treatment, but not two. Similarly, the RESV fed animals' fasting insulin and HOMA-IR scores were not different from control lemurs after two years, but were significantly lower after three. Human studies of insulin sensitivity and glucose tolerance in response to RESV administration are mixed; for an informative, current review on the topic refer to (de Ligt *et al.*, 2015). Three studies have performed the clamp method to assess changes in insulin sensitivity with RESV treatment with doses ranging from 75 mg to 1.5 g per day for up to twelve weeks in overweight/obese men or non-obese postmenopausal women. All three studies found no effect of RESV on insulin sensitivity or glucose infusion rates (Chachay *et al.*, 2014; Poulsen *et al.*, 2013; Yoshino *et al.*, 2012). In fact, only the Timmers et al. study found a significant effect of 150 mg · day^{-1} RESV treatment on fasting insulin in normoglycemic, overweight subjects after 30 days (Timmers *et al.*, 2011). Conversely, beneficial effects of RESV are observed in subjects presenting advanced complications in glucose homeostasis: in three different clinical studies of overweight, type-2 diabetics, dose of RESV ranged from 10 mg to 1 g per day and trial duration varied from four weeks to three months (all parallel-arm) (Bhatt *et al.*, 2012; Brasnyo *et al.*, 2011; Movahed *et al.*, 2013). Significant outcomes include improvements in HOMA-IR, fasting glucose and insulin, and hemoglobin-A1c by RESV treatment. An informative meta-analysis

conducted by Liu et al. effectively illustrates this dichotomy in glucose homeostasis response to RESV treatment between obese-healthy vs type-2 diabetics: the overall weighted mean differences of two experimental studies on subjects with type-2 diabetes demonstrates reduced fasting glucose and insulin, hemoglobin-A1c, and HOMA-IR with RESV treatment, whereas the overall weighted mean differences of five studies on overweight or obese, nondiabetic subjects does not exhibit these same improvements (Liu *et al.*, 2015).

RESV and dyslipidemia

Elevated TG and LDL are major modifiable risk factors in the pathogenesis of atherosclerosis and resultant cardiac and cerebrovascular diseases, the number 1 and 5 leading causes of death, respectively. Zordoky et al. provides an exhaustive summary of RESV's effects on the components of CVD (Zordoky *et al.*, 2015). They provide a useful report of RESV action in a variety of rodent models of CVD (see Table 2). Studies range from two to twenty weeks in duration and RESV dose ranges from 1 to 500 mg $\cdot$ kg^{-1} bodyweight either directly by oral gavage, IP injection, or incorporated into diet. CVD induction included high-fat/cholesterol/fructose diets and/or genetic models (apo-e deficient mice, New Zealand rabbits). Overall, the studies show a lipid and cholesterol lowering effect of RESV supporting its ability to impede HMG-CoA reductase (Cho *et al.*, 2008; Do *et al.*, 2008; Penumathsa *et al.*, 2007). Interestingly, when lipids are not affected by treatment, anti-atherosclerotic and anti-inflammatory effects are still observed which is indicative of RESV's antioxidant properties, as one study found a reduction in oxidized LDL despite no overall changes in total lipids (Rocha *et al.*, 2009). These translational findings identify at least two or three different mechanisms by which RESV could have a positive effect on atherosclerosis and that improved outcome may exist independent of effects on dyslipidemia. Further, mechanistic work describes effects of RESV on vascular

smooth muscle cell migration as well as anti-platelet activity (Gocmen *et al.*, 2011; Lin *et al.*, 2014).

Effects of RESV on dyslipidemia in human clinical trials are less consistent than the aforementioned animal experiments. Like the other outcomes described in this communication, variability exists in subject population, dosing, duration, and design of the clinical trials making it difficult to confirm or refute RESV as an alleviator of metabolic dysregulation at the human level. However, improvements have been observed. In overweight or obese men with hypertriglyceridemia fed 1 to 2 g RESV for two weeks there was a significant 27% reduction in apoB-100, an indication of the reduced output of VLDL by the liver (Dash *et al.*, 2013). In a study by Magyar et al. 10 mg per day RESV for three months lowered LDL cholesterol by 14% in 20 patients with coronary artery disease (Magyar *et al.*, 2012). In type-2 diabetics, 1 g per day RESV for 45 days significantly increased HDL by 11% while TGs were marginally reduced (Movahed *et al.*, 2013). Alternatively, other human studies found no significant difference in circulating lipids in subjects fed RESV (Poulsen *et al.*, 2013; Yoshino *et al.*, 2012). In these studies, subjects tended to be healthier relative to the subjects in which a positive effect was noted. This is an indication of the principle described by Smoliga et al., where beneficial effects of RESV described in mechanistic and animal studies are most likely to be translated to a population of subjects that have the potential to benefit from the intervention; to correctly determine the action of RESV on the clinical signs of a given morbidity, the patients being studied should present said morbidity (Smoliga *et al.*, 2013).

RESV and hypertension

The association between RESV and cardiovascular health was first described indirectly via the association between red wine consumption and a reduction in risk of mortality, and was thought

to partially explain the “French paradox”: the consumption of a high-fat diet, but without an observable increase in mortality due to coronary complications (Kopp, 1998; Siemann and Creasy, 1992; Wang *et al.*, 2005). Subsequent research over the past 20 years from molecular pathways to clinical studies has sought to confirm and elucidate this relationship. The proposed cardio-protective mechanisms of RESV include modulation of hypertension, ischemia, angiogenesis, and atherosclerosis.

In a variety of rodent models of hypertension including surgical, chemical, and diet-induced hypertension, a range of RESV (1-800 mg · kg bodyweight^{-1}) was effective in reducing systolic blood pressure when administered for two to ten weeks (see table 1 of Zordoky et al.) (Zordoky *et al.*, 2015). According to Hamza et al., the inter-organ mechanisms by which RESV elicits its benefit on blood pressure includes central and peripheral vascular remodeling as well as improved renal function (figure 1 of (Hamza and Dyck, 2014)).

Several meta-analyses have assessed the effect of RESV on blood pressure (Hausenblas *et al.*, 2015; Liu *et al.*, 2015; Sahebkar *et al.*, 2015). Despite the same, limited collection of human clinical studies available for analysis, each author had a unique subset of studies in which to calculate an overall effect size, which is due to the main objective of each analysis: Liu et al. focused solely on blood pressure, Hausenblas et al. assessed RESV’s effects specifically in type-2 diabetics, and Sahebkar et al. addressed inflammation and a broader examination of its cardiovascular effects. In two of the three analyses, the authors reported a weighted mean difference that favored the effect of RESV in reducing systolic blood pressure while diastolic blood pressure appeared unaffected (Hausenblas *et al.*, 2015; Liu *et al.*, 2015). Liu et al. also performed sub-analyses where studies were grouped by dose, duration of treatment, and BMI of study subjects. Greater effects of RESV on systolic blood pressure were observed in studies

using doses of at least 150 mg per day, of duration no greater than three months, and in overweight or obese subjects. Two of these meta-analyses (Liu *et al.*, 2015; Sahebkar *et al.*, 2015) included a study that used a modified, microencapsulated form of RESV, "longevinex", where the authors did not report circulating levels of RESV, therefore this study may be confounding the subsequent meta-analyses as less is known about this form of RESV administration (Fujitaka *et al.*, 2011).

Conclusion

Substantial evidence exists to suggest that RESV can ameliorate several risk factors for chronic diseases facing adults, including cardiovascular disease, stroke, and diabetes. Moreover, RESV, embedded in empirical research that details a potential for correcting metabolic disturbances, could prove an effective treatment for individuals with metS. Based on its effects of each component of the metS detailed above, research into the efficacy of RESV on these subjects is warranted. However, there is disagreement in RESV's ability to fend off metabolic stress in the human condition. Work from Timmers et al. found positive effects on energy metabolism in obese men when fed 150 mg per day for four weeks (Timmers *et al.*, 2011). Their elegant work bridged the gap between what has been thoroughly examined in animal models and what every nutritionist who studies the polyphenol wants to know: will this compound have similar effects in humans? Poulsen et al., in an attempt to recapitulate the work by Timmers et al., found no significant effect of RESV despite a 10-fold increase in the daily dose (Poulsen *et al.*, 2013). Discrepancies exist between the two research designs that may account for an observable effect by the Timmers group, namely the advantage of a cross-over design instead of a parallel-arm. An additional issue with both of these studies is a failure to control the subject's food intake, which may also partially account for differences in study outcomes. Nonetheless, the number of studies

demonstrating positive outcomes after RESV administration warrants further research on this potentially healthful dietary component and supplement.

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Chapter 5: Summary and Future Directions

This dissertation has made the case for berry consumption as a tool within the realm of nutritional approaches towards weight management or obesity reduction. Obesity in the United States is a complex issue that requires intervention on societal, local, and individual levels. The expansion of the obese state through the 1990s and into the present harbors unprecedented medical costs, loss of productivity due to obesity related illness, and perhaps most importantly a reduction in the quality of life for the American public afflicted by the disease. In an aging population, in an era of medical intervention that has maximized the average lifespan, the consequences of obesity related diseases in the following decades will seep into myriad aspects of American society. Efforts to address the issue are present, from the directives of governing agencies to primary school classrooms, yet their ineffectiveness, as evidenced by recent National Health and Nutrition Examination Survey data, is cause for persistence and ingenuity. Nutritional approaches to curb obesity are the absolute ground floor towards effective, healthy, and sustainable weight management. Pharmaceuticals can combat obesity's secondary effects, treating its complications and even ameliorating cardiovascular disease and reducing mortality. Invasive surgeries can force patients into reduced food consumption, removing accountability at the individual level. Yet, only once obesity-prone citizens recognize their genetic susceptibility in an environment that fosters effective nutritional education and promotes nutrient packed (instead of calorie packed), affordable food items, can we then expect sustainable weight management on the societal level. Effective nutritional approaches will struggle to exist alongside a car-centric society, working in cubicles for fifty hours every week, with heavily promoted and affordable high-fat/high-sugar/high-addiction foods at their fingertips. The study of anthocyanins from berries as a means to promote normalized energy balance is an

infinitesimally small contribution to the larger need to characterize candidate nutrients present in readily accessible food items to improve the current obese state. Work of this nature must continue on other candidate foods if nutritionists hope to identify, characterize, and promote an effective dietary approach to reverse or prevent the consequences of excess calorie consumption.

The highlight of this dissertation is the observation of a significant reduction in the respiratory quotient in overweight or obese males consuming 600 g of blackberries daily for one week. The justification for the human feeding study is grounded in translational work on mice, where researchers observed anti-obesity and anti-diabetic effects of anthocyanins delivered in several forms, from food colorants, purified extracts, or in various berry powders incorporated into the diet. The human studies described in the literature review highlight the potential of berry consumption to positively affect the development of type-2 diabetes, dyslipidemia, and hypertension. However, human studies addressing the ability of berries to modulate obesity are lacking, and modulation of the respiratory quotient in the controlled feeding study described in chapter two provide a physiologic basis for this potential, coupled with an improvement in insulin sensitivity. Future work studying berry consumption in overweight or obese subjects can build off this study: a free-living study with berry supplementation followed by respiratory measurement could identify if this observation persists in subjects consuming an unrestricted diet. Further, methods of measuring substrate oxidation with stable-isotope tracers exist, which could complement the measurements of indirect calorimetry. Future work in females is warranted, as they were not enrolled in this study. Finally, other (lower) doses fed for extended periods should be pursued to determine effectiveness over longer periods with doses of berries that are easily achievable on a daily basis. Alternatively, high-anthocyanin berry cultivars could

be developed in collaboration with plant breeders in order to obtain the same dose of anthocyanins while reducing the gross consumption of berries to more realistic servings.

Mechanistic studies of cyanidin-3-glucoside's action in adipocytes were discussed in the literature review. According to the highlighted research, the anthocyanin has the capability of enhancing thermogenesis in adipocytes by activation of molecular pathways that regulate mitochondrial biogenesis and activity, i.e., proposed morphological transition from white to brown adipocyte phenotypes. The third chapter of the dissertation provides novel work in this model by the assessment of respirometric phenotyping of adipocytes after treatment of a physiologic dose of blackberry extract. The observed changes in different respiration states suggests increased respiratory capacity on a fatty acid substrate and electron transport capacity that cannot be explained by increases in mitochondrial density, hence, improved function. Future mechanistic work could couple characterization of the proposed molecular pathways, respirometry, and substrate trafficking.

In summary, this dissertation extends the benefits of berry consumption on metabolic complications related to obesity by describing the potential to increase fat oxidation, observed both in a controlled feeding study as well as supporting cell culture experiments. These encouraging findings should be explored further in an effort to confirm the effects, and these experiments provide a framework by which other candidate foods and their active components can be investigated for health-promoting effects related to the nutritional approaches towards remediation of obesity.

Appendix 1: Research Proposal for a Controlled Feeding Study of Resveratrol Administration - Methods and Expected Findings

I. Methods

Subjects

Subjects will be recruited from the Baltimore/Washington metro area. A mass mailing of fliers will be delivered to households in the zip codes surrounding the Beltsville Human Nutrition Research Center advertising the time and location of information meetings that will be held at the center. Additionally, the study will be advertised on the research center's homepage on the internet. At these information meetings, a presentation detailing the study's inclusion/exclusion criteria, method of controlled feeding, risks involved in participation, and sample collection dates will be communicated to potentially interested subjects. Following the presentation, interested applicants have the option to complete a study application and provide informed consent. Applicants will be scheduled for a screening date and provide a health history questionnaire detailing any medical complications in their experience as well as their immediate family. At screening, anthropometric measures will be collected and blood and urine samples will be collected in order to obtain a complete clinical chemistry profile in order to detect any unreported/unknown pathology.

Inclusion criteria: potential subjects must present three of the five following criteria to fit the metabolic syndrome definition: central obesity (waist circumference greater than 40 inches), blood pressure above 130/85 mm mercury (systolic/diastolic), fasting blood glucose above 110 mg/dl, fasting serum triglycerides above 150 mg/dl, and/or high-density lipoprotein below 40 mg/dl. Subjects must also be males between the ages of 25 and 60 as well as obese (BMI greater than 30 kg/m^2).

Exclusion criteria: the presentation of any disease outside of the metabolic syndrome definition (cardiovascular/kidney/liver/thyroid diseases, etc), a family history of early-onset (less than age 50) cardiovascular diseases or cancers, fasting blood sugar > 126 mg/dL, food allergies/intolerances or special diet restrictions, gastric bypass or weight loss surgery, any malabsorption disorders, a history of alcohol/substance abuse, a criminal record, age outside of the specified range, no reliable mode of transportation to the research center, or a general disagreement to follow a completely controlled diet for a one month period with an additional two week wash-out period where diet restrictions will apply, will disqualify an interested applicant from participation in the study.

From the pool of potential subjects who meet the inclusion criteria, subjects will be randomly selected for participation based upon number assignment and the use of a random number generator and randomly assigned to one of two treatment sequences.

Design

This study will utilize a randomized, double-blind, placebo controlled, cross-over design (a pretest-posttest control group design with cross-over). The selection of subjects with metabolic syndrome reflects the animal work described in the introduction where resveratrol's effects appear to be pronounced with concurrent metabolic stress. Further, this sample reflects the population of Americans with the most to benefit from resveratrol if its effects can indeed be confirmed.

Sample size/groups: Power calculations (80% power, alpha = 0.05) were performed using "proc power" in SAS with consultation by the equation of Senn [1] for AB/BA crossover designs. We do not have pilot data for the proposed experiment, thus power calculations were performed using affected clinical markers from the Timmers et al. study as well as reference to

clinical and calorimetry data from a previous study at the food components and health laboratory (FCHL), where subjects consumed a controlled, high fat diet with or without 600 g of blackberries for one week using the same cross-over design. Fasting insulin, glucose, and systolic blood pressure differences in the Timmers et al. study all suggest ≥80% power to detect differences can be achieved with between five and ten subjects. Pilot data from the blackberry study at FCHL had minimal differences in fasting glucose and insulin between treatments. However, differences in insulin (and a trend for glucose) AUC from a 4 hour meal tolerance test (MTT) was observed. Power calculations from those differences indicate ≥80% power can be achieved with approximately 20 subjects. Finally, calorimetry data was also utilized from the same blackberry study to perform power calculations. 24 hour average respiratory quotient and 24 hour energy expenditure suggest differences (≥80% power) can be detected with 15 and 20 subjects, respectively. The author puts faith in the more conservative estimates of sample size from the data generated from FCHL. The blackberry study trends or significant effects were observed during a high fat, 100% controlled diet, which was also of short duration (one week). Dissimilarities between the proposed study and the Timmers et al. study, principally duration and absence of controlled feeding, make selection of the smaller sample size calculation a risk for not detecting differences.

Each group will have a unique treatment sequence consisting of two diet periods; group 1, 300/placebo; group 2, placebo/300 (mg resveratrol per day). Each diet period, where a subject will follow controlled feeding and resveratrol/placebo treatment, will last 14 days and a 14 day wash-out period will separate each diet period. During the wash-out period, subjects will be instructed to avoid resveratrol containing foods (grapes, berries, wine, peanuts, and supplements).

Diet: Subjects will consume a diet with a macronutrient profile of 40% of energy from fat, 45% from carbohydrate, and 15% from protein sources. Menus will be formulated around these definitions by an on-site registered dietician using the ESHA software program (Salem, OR), and meals will be prepared by professional chefs at the on-site metabolic kitchen. Energy needs for a subject will be estimated by the Harris-Benedict equation which takes height, weight, age, gender, and physical activity level into consideration. Calorie levels (in increments of 200 calories) will be assigned to subjects based upon this estimation. Subjects' weight will be monitored daily, Monday through Friday, and any deviation from weight maintenance will be offset by adjustments made to a subject's calorie level. Subjects will be instructed to eat only the food provided to them by the center. Monday through Friday, subjects are required to eat both breakfast and dinner at the center, while both lunches and all weekend meals are packed out for the subjects to consume off-site. Diet compliance will be assessed by both changes in weight as well as subject monitoring by dietary technicians during meals that are eaten in the cafeteria on-site.

Treatment: 300 mg capsules of all-trans resveratrol will be purchased from a nutritional supplement company (resvida, DSM Nutritional Products, Columbia MD). Placebo capsules will be made in-house to mock the appearance of the supplement. Investigators and research personnel in charge of treatment distribution will not know the sequence of each group until the completion of data analysis in order to achieve double blinding. On weekdays when the subjects come to the center for breakfast, treatments will be taken in the presence of the same diet technician in charge of monitoring their meal consumption. On weekends, subjects must send a text message to the investigator by 10 AM confirming that they took their treatment dose. If a subject doesn't have the ability to text they can call the investigator using their landline. Failure

to contact the investigator by 10:30 AM will result in a phone call reminding the subject to take their dose.

Instrumentation

All serum/plasma biochemistry markers (except insulin) will be determined using a "Vitros 5,1 FS" clinical chemistry analyzer (www.orthoclinical.com).

Indirect calorimetry – substrate oxidation

Subjects will complete two 24 hour stays in room-sized calorimetry chambers: on the last (thirteenth) day of each treatment period. The calorimetry chambers measure respiratory gasses i.e. the amount of O_2 inhalation and the exhalation of CO_2 by means of measuring the composition of the air exiting the chamber using a multiple gas analyzer. An explanation of the functionality of these specific chambers has been described by Seale et al. [2]. Air sampling occurs every 100 seconds over the course of the 24 hour stay in the chamber. Subsequent calculations are performed using O_2 consumption and CO_2 production to estimate energy expenditure, respiratory quotient, and substrate level utilization of macronutrients for energy production. Energy expenditure is estimated using the Weir equation; the respiratory quotient, the amount of CO_2 produced divided by the O_2 consumed, is also an indicator of macronutrient utilization where 0.7 equates to fat oxidation, 1.0 equates to carbohydrate oxidation, and values in-between indicate a mixture of substrates; substrate level oxidation (in grams) is calculated via the equations of Livesey and Elia [3]. In order to correct for protein oxidation, 24 hour urine will be collected by the subjects to determine urinary nitrogen excretion.

Validity – Prior to each chamber stay by the subject, a known quantity of 100% ethanol is combusted in the chamber to compare the recovery of CO2 and O_2relative to their expected

volumes. As described in Table 4 of Seale et al., 30 trials of ethanol combustion experiments demonstrate high reproducibility of 100% recovery [2].

The measurement of respiratory gasses and subsequent energy substrate calculations are typically reported as 24 hour averages (RQ and EE), or as 24 hour totals (EE and substrate level oxidation) as the gas analyzers are not sensitive enough to detect differences on smaller time scales. To address this issue, Gribok et al. determined the variance in O_2 and CO_2 measurements reported by the gas analyzers from a series of trials where the chambers were empty, and a constant rate of a known gas mixture was infused [4]. The variance of the measurement allowed the authors to construct an algorithm (regularized deconvolution) which reduces the noise and allows for the comparison of smaller time intervals – on the order of tens of minutes. They also established concurrent criterion validity of this algorithm by comparing it to a regularized differential algorithm.

Reliability- Gribok et al. were able to demonstrate reliability of their developed algorithms from a 43 hour chamber stay by a test subject where the average point-wise standard error was below 10% for both gasses as well as the calculations of EE and RQ [4].

Dual-energy x-ray absorptiometry (DXA)

Body composition (fat mass, lean mass, and bone mass) will be evaluated by DXA (Hologic, Bedford, MA). Subjects will be scanned at the beginning and end of each diet period. We do not anticipate detectable changes in lean or fat mass: each intervention is only two weeks long and calorie levels are set at weight maintenance. However, both fat mass and lean mass measurements from DXA will be included as covariates in the statistical analyses for the other outcome variables described, as fat and lean masses influence metabolic rate, insulin sensitivity, and blood pressure.

Validity – Concurrent criterion validity has been described in a study by Glickman et al. [5]. Abdominal adiposity of 27 subjects was measured by DXA and multi-slice computed tomography (CT). Although DXA significantly underestimated abdominal fat mass compared to CT, the investigators describe moderate agreement between the two technologies (see figures 1 and 2 of [5] for Bland-Altman plots of total abdominal tissue and fat tissue as measured by both instruments). Despite this significant underestimation of the fat mass by DXA, it has high predictive ability of the variance in CT measurements, as it accounts for 80% of the variance in visceral adipose tissue measured by CT in a multiple regression model. Construct validity was also demonstrated by adding 0.5 to 1 kg of porcine lard to the subjects' abdominal area and performing a subsequent DXA scan: DXA detected 78% of the added fat.

Reliability – The same study also addressed inter-observer variation in DXA measurements and found the instrument to be reliable: 43 DXA scans were analyzed by three different operators and no significant differences in measurements of body composition were detected between the three operators [5]. In our study, the DXA scanner will be calibrated to a "spine phantom", provided by the manufacturer, on the morning of each scanning day.

Glucose

Plasma glucose concentrations will be determined as milligrams glucose per deciliter plasma using the Vitros 5,1FS platform. Briefly, this test uses technology first described by Trinder [6]. Glucose reacts with oxygen in the presence of glucose oxidase to form gluconic acid and hydrogen peroxide. In a second reaction, the formed hydrogen peroxide reacts with dye precursors, aminoantipyrine and dihydroxyaphthalene, in the presence of peroxidase to form a red dye. The intensity of the red dye is quantified colorimetrically following Beer's law (A=klc, where the absorbance of light (the optical density) is equal to a known constant (k), the length of

the cuvette where the sample is contained (l), and the concentration of the dye (c).) The formation of the red dye is linearly proportional to the amount of glucose present in the blood.

Validity: Trinder et al. demonstrated the validity of this assay using both construct and criterion validity [6]. Construct validity was achieved by performing a spike recovery experiment (Table 2 of [6]) where several different levels of a known quantity of glucose were added to glucose free plasma (glucose is decomposed after sitting at room temperature for 48 hours) and then the samples are quantified using his manual method. Second, concurrent criterion validity was achieved by comparing both his manual and automated methods against a gold-standard assay developed 22 years before his communication [6]. Table 3 of his communication shows good agreement across the three methods. The instructions booklet from the manufacturer (Vitros glucose protocol) establishes construct validity by showing excellent convergence of reported glucose concentrations in serum across the several platforms they offer (pg 10 of Vitros glucose protocol), where reliability for each platform is also assessed by the reported. Discriminability is established by the reported specificity where several different substances were shown to not cross react with urinary glucose in this method (page 11 of Vitros glucose protocol). The operator is to assume that these substances will also not interfere in the other matrices capable of glucose quantification by this method. Our instrument will be calibrated by ordering 2 different levels of plasma samples from orthoclinical (they maintain lots of calibrators to send out to customers who have purchased their equipment) where we can cross check that our system is in agreement with the concentrations reported by the manufacturer. If our instrument fails to report the appropriate concentrations +/-10% then the instrument will be serviced.

Reliability: Reliability of our instrument to quantify blood glucose will be achieved by the test-retest method. All samples (including calibrators) will be measured in duplicate. In any

sample where the CV is greater than 10% a third test will be performed on the sample. Samples failing a third test will not be included in the analysis and a new aliquot of plasma from the same subject will be tested in its place.

Insulin

Plasma insulin concentrations will be determined as microunits insulin per milliliter plasma using a sandwich ELISA (enzyme-linked immuno-substrate assay) kit which is commercially available by Millipore (Millipore Insulin ELISA protocol). In a sandwich ELISA, the sample analyte is captured by a primary (or capture) antibody that lines the wells of a 96-well plate where the reaction takes place; in this case, a mouse anti-human insulin antibody. With the sample analyte immobilized to the plate, a second binding anti-body is added to the solution (the sandwich). This second anti-body is biotinylated, which conjugates to an enzyme (here, horseradish peroxidase) added in the next step so that when its substrate is added (tetramethylbenzidine), product formation is proportional to the amount of enzyme bound to the well. Product formation is then dependent upon the presence of insulin in the sample. Here, a four-parameter fit best describes the relationship between product formation and the concentration of insulin in plasma.

Validity – As discussed by Sapin, radio-immuno-assay (RIA) was the first developed method for quantification of insulin in plasma samples [7]. This assay involved the addition of radio-labelled bovine insulin, the subject's sample, and polyclonal guinea-pig anti-bodies to the same tube, where radio-labelled insulin and sample insulin compete for the same anti-bodies, and binding results in a precipitate where radioactive counts can be measured, which is inversely proportional to a sample's insulin concentration. RIA was considered the gold standard in insulin quantification for the next 40 years, despite its flaws. Sapin calls for greater standardization of

immunometric assays, but acknowledges their improved accuracy with monoclonal antibodies (those used in sandwich ELISAs). This Millipore assay establishes criterion-related validity by comparing its sample insulin concentrations measured with the ELISA to concentrations measured with RIA in 97 subjects (r=0.978, pg 13 of Millipore Insulin ELISA protocol). Construct validity was demonstrated by spike-recovery and dilution-linearity experiments (pg 11-12, Millipore Insulin ELISA protocol). We will achieve construct validity in our lab by cross-referencing our obtained values against the reported values of a low and high quality control sample provided in the assay kit by the manufacturer.

Reliability – Reliability will be assessed by the test-retest approach as described for glucose. Additionally, the manufacturer reports the intra-assay and inter-assay precision of the method in their hands (pg 10, Millipore Insulin ELISA protocol).

Triglycerides

Serum triglycerides will be determined as milligrams triglyceride per deciliter serum using the Vitros 5,1FS platform. The enzymatic process for this method was first described by Fossati in 1982 [8]. The complete reaction couples four different enzymatic steps where the formation of dye is dependent on the formation of hydrogen peroxide whose presence is the result of a product of free glycerol (pg 1 of [8], pg 2 of Vitros triglyceride protocol). The amount of dye produced is linearly proportional to the amount of triglyceride in a serum sample (up to 1200 mg/dl as reported by [8]). Fossati et al.'s protocol specifically had in mind the development of an accurate and precise method that would be conducive for automated sample processing.

Validity – The method of Fossati et al. successfully demonstrates criterion-related validity by the comparison of their method to a more laborious, yet conventional method at the time and their data shows excellent agreement across 260 serum samples (r=0.995, figure 1 of

[8]). Construct validity was achieved by cross-referencing the reported values of 13 control sera against the concentrations achieved with their method, which they also compared to the conventional method. Precision of their assay as well as a list of interfering substances is provided in the communication. The Vitros triglyceride protocol, incorporating technology to assemble this multistep assay onto a microslide, then demonstrates their own criterion validity by comparing the data generated by their automated method to the method of Fossati et al.. Construct validity is achieved by comparing the values produced by the several platforms they offer. Our platform will be calibrated to the control samples provided by the manufacturer.

Reliability – Reliability for this assay is reported by Fossati (table 2 of [8]), the manufacturer (pg 7, Vitros triglyceride protocol), and will be determined on each of our samples and standards by the test-retest method described above in the glucose section.

Total cholesterol

Serum total cholesterol will be determined as milligrams cholesterol per deciliter serum using the Vitros 5,1FS platform. This method was optimized and first described by Allain in 1974 (adapted here with the use of a different chromagen: leuco dye in place of 4-aminoantipyrine and phenol) [9]. A surfactant and cholesterol ester hydrolase are used to convert all cholesterol into its free form, where it reacts in a third reaction to produce hydrogen peroxide which forms a dye in the presence of peroxidase, similar to the methods described above. The enzymatic production of dye is linearly proportional to the amount of free cholesterol in the serum sample up to 325 mg/dl (pg 4 Vitros total cholesterol protocol).

Validity – The Allain et al. communication demonstrates criterion validity of their developed method by comparing their results to three different conventional methods to quantify serum cholesterol (figures 10-12 of [9]). They also report specificity of the assay by cross-

reactivity with other sterol containing compounds and show that the method has a lower rate of error compared to one of the conventional methods (table 3 of [9]). The assay also has good resistance to the presence of interfering compounds found in serum (table 2 of [9]). The Vitros total cholesterol protocol establishes criterion related validity (pg 6, Vitros total cholesterol protocol) by comparing concentrations generated by the platform to those generated using one of the reference methods (the Abell-Kendall method) which was also the reference method from table 3 and figure 12 of the Allain et al. communication. Construct validity is achieved by comparing values generated on their different platforms (pg 7, Vitros total cholesterol protocol). Specificity is also established by the demonstration of no interference of an exhaustive list of compounds that can be found in serum up to the concentrations reported (pg 7, Vitros total cholesterol protocol). We will establish construct validity of our instrument using the quality control samples (standards) provided by the manufacturer.

Reliability – Acceptable within day and between day precision is reported by both Allain – CVs less than 1% for both within and between day (pg 474 of [9]) and the Vitros protocol - CVs less than 1 and 2%, respectively (pg 7, Vitros total cholesterol protocol). We will monitor our reliability for each sample and standard using the test-retest method described in the glucose section above.

High Density Lipoprotein cholesterol (HDL)

Serum HDL will be determined in milligrams HDL cholesterol per deciliter serum using the Vitros 5,1FS platform. This method is similar to the one described for total cholesterol in that production of hydrogen peroxide in a cholesterol dependent step reacts with a dye substrate which in turn leads to the formation of a measurable dye in the presence of the peroxidase enzyme as first described by Allain et al. [9]. However, isolation of HDL must first take place,

which requires the removal of other lipoproteins that contain cholesterol (LDL and VLDL). This isolation step was first described by Burstein et al. in 1970, where the additions of phosphotungstic acid and magnesium chloride selectively precipitate non-HDL lipoproteins from human serum [10]. Once the HDL is isolated, the cholesterol contained in the lipoprotein is disassociated with the use of a surfactant (emulgen B-66, pg 1, Vitros HDL protocol).

Validity – Burstein establishes construct validity for their isolation of HDL where they present four different methods of electrophoresis on their prepared (HDL isolated) serum compared to normal human serum (no lipoprotein isolation) which shows discriminability between treated and untreated samples (figures 5-9 of [10]). This discriminability is further exemplified in figure 10 where they show the staining band specific for HDL compared to untreated serum in immunoelectrophoresis. The validity for the remainder of the steps involved in the assay are the same as discussed above for the determination of total cholesterol in serum using the method reported by Allain et al. [9]. The Vitros HDL cholesterol protocol establishes criterion validity for their assay by comparing it to a sulfate/enzymatic method used by the CDC and outlined by the Clinical Laboratory Standards Institute protocol (pg 7, Vitros HDL protocol) which shows good agreement between the two methods. The protocol also demonstrates construct validity by showing convergence across their several platforms and specificity by an exhaustive list of compounds that do not interfere with the assay (pg 8, Vitros HDL protocol). We will establish construct validity of our instrument using the quality control samples (standards) provided by the manufacturer.

Reliability – Precision of the instrument is reported on pg 8 of the Vitros HDL protocol. We will also establish reliability of our own instrument using the test-retest method on samples and standards as described in the glucose section above.

Aortic blood pressure and stiffness

Pulse wave reflection (augmentation index) and aortic stiffness are independent predictors of cardiovascular complications and mortality [11, 12]. We will determine augmentation index and aortic stiffness by measuring pulse wave analysis and pulse wave velocity, respectively, with the sphygmocor xcel (Atcor medical, Sydney, Australia). The augmentation index is defined as the aortic augmentation pressure divided by pulse pressure, which are measurements estimated by the application of a transfer function to brachial pulse wave forms which are measured directly by the instrument. Pulse wave velocity is defined as the distance between carotid and femoral pulse waves divided by the time lag between the two pulse waves. Both femoral and carotid pulse waves are measured directly by the instrument. We are interested in determining any changes to these predictive markers of cardiovascular health caused by resveratrol administration.

Validity – A recent, independent study performed by Hwang et al. established concurrent criterion validity of the sphygmocor xcel by comparing its reproducibility of the same measurements recorded by an older, previously validated device from the same manufacturer (Table 2 of [13]). Further, they compared pulse wave velocity to two other measures of aortic stiffness and found good agreement between the different tests [13].

Reliability – Hwang et al. also demonstrated intratest and day-to-day reliability of the instrument for both pulse wave velocity and augmentation index (Table 3 and 4 of [13]). We will perform test-retest (2 reps) of both measurements on the same subject. Large deviations in recordings between reps will result in a third measurement.

Blood pressure

Systolic and diastolic blood pressure measurements will be determined by the calculation of millimeters of mercury by an oscillometric device, which removes human error from the determination of auscultatory blood pressure. Despite the plethora of new technologies created to measure blood pressure, the mercury manometer is still considered the gold standard in blood pressure measurement [14]. In order to avoid a mercury containing instrument, as well as to minimize operator error, an oscillometric device is the best option.

Validity – The validity of the oscillator device to calculate systolic and diastolic blood pressures was established by Graves et al. [15]. In their experiment they demonstrated construct validity by the convergence of the oscillator and trained hypertension nurse specialists on the diagnosis of hypertension with 92% agreement between the two methods (figure 1 of [15]). The authors also included Bland-Altman plots of agreement for both systolic and diastolic pressure to visually demonstrate the good agreement between the methods (figures 2-3 of [15]). In order to ensure that our measurements are precise, we will follow the same calibration step using the Digimano device described by Graves et al..

Reliability - We will establish stability of our instrument using the test-retest approach comparable to Graves et al.: we will perform four blood pressure measurements at 3 minute intervals, discarding the first measurement. If the CV from the three retained measurements is greater than 10% then 2 subsequent measurements will be performed. If problems persist the machine will be recalibrated.

Mitochondrial characterization – High-resolution respirometry (HRR)

Similar to the report of Timmers et al., mitochondrial characterization of permiabilized muscle fibers of the vastus lateralis will be investigated using an oxygraph (Oroboros instruments, Austria). Different states of oxidative phosphorylation will be evaluated by different

combinations of substrates added to the muscle fibers as described in the substrate-uncoupler-inhibitor titration (SUIT) protocols of Pesta et al. 2012 (pg 21 of [16]). For example, supplying the muscle fibers with malate and glutamate (to generate reducing equivalents from TCA for complex I), and octanoylcarnitine (a product of β-oxidation of fatty acids), and recording the subsequent oxygen consumption is an indicator of state 3 respiration activity, specifically with the activation of complex I, utilizing fatty acid substrates. Further characterization includes activation of complex II with the addition of succinate as well as a measure of maximal capacity of the electron transport chain (ETC) by uncoupling respiration to ATP synthesis with the addition of a chemical agent (a protonophore) that permeabilizes the inner mitochondrial membrane (removing the proton gradient,inhibiting ATP synthesis) to allow for maximum proton cycling of the ETC complexes I-III. We're interested in determining differences in these reactions that could be caused by resveratrol treatment.

Validity – Standards or calibrators, or reference to older technologies are not available for this instrument making validity more ambiguous. Regardless, relative comparisons between treatment groups can still be performed. Also, as noted by Pesta et al., experimental characterization of permeabilized cells/isolated mitochondria using the oxygraph are performed under temperature control (alterations in proton permeability of the mitochondrial membrane occur with temperature change) and the SUIT protocols utilize physiological concentrations of substrates to measure activity [16]. Construct validity can be proposed based on the instruments ability to describe dichotomies of mitochondrial function in various disease states including polycystic ovary syndrome, statin-induced myopathy, and non-alcoholic fatty liver disease [17-19].

Reliability – In a personal communication with a senior scientist from the oxygraph manufacturer, the scientist provided data from a recent study where they assessed reproducibility of the oxygraph via repeated measures on the respiration of peripheral blood mononuclear cells from the same subject. Two blood samples were drawn three hours apart, and the same respiration protocol was performed on each sample (test-retest reliability). All measurements on intact cells yielded CVs <20%, and measurements on permeabilized cells yielded CVs ≤10%. These data indicate the requisite permeabilization of the muscle fibers from our study may improve the reliability of the HRR measurements.

Molecular targets in skeletal muscle tissue

Protein expression of pAMPK/AMPK, PGC-1α, and SIRT1, thought to be activated by caloric restriction and resveratrol, will be assessed by gel electrophoresis and western immunoblotting. The density of PGC-1α and SIRT1, as measured by an imaging station, will be normalized to the loading controls actin and tubulin, respectively. Despite its wide use in the life sciences since its inception over forty years ago, using gel electrophoresis coupled with western blotting to assess protein abundance in target tissues remains a flimsy business. This is due in part to the wide variety of methodologies that exist; there is no one gold standard approach. Also, outlined by Taylor et al., the images produced from western blotting are easy to manipulate and constitute a majority of the inappropriately reported figures that exist in the literature [20]. Fortunately, Taylor et al. describe a series of steps researchers can take to standardize their western blotting protocol so they can have confidence in the images coming out of their lab.

Validity – The principle of western blotting is to correct the density, captured by film or an imaging station, of your protein of interest to the density of a loading control: a protein that is ubiquitously expressed in the cell, such as actin or tubulin. After correction by the loading

control, one can make comparisons of the relative densities between treatments. Construct validity can be achieved by the determination of the linear dynamic ranges described by Taylor et al. for protein loading, dilutions of primary and secondary antibodies, and loading controls [20]. The relative density produced by the imaging station should be proportional to the known amount of protein loaded onto a gel, achieved by spiking the tissue lysate with the protein of interest (figure 2b of [20]). Dilutions for antibodies will be determined by the linear range of detection for each protein of interest, where the appropriate dilution will correspond to the mid-point on the curve (figure 3a of [20]). Finally, an appropriate quantity of protein loaded onto the gel will be further validated by the linearity of the loading control (actin or tubulin) where the selected protein load will have good agreement for the loading control between the density measured by the imaging station, and the known amount of tissue lysate added to the well (figure 6 of [20]).

Reliability – Western blotting will be tested for stability with test-retest where each of the three proteins of interest will be tested in triplicate. CVs of less than 20% will be considered reliable.

Sample collection procedures

Sample collection will occur at baseline (the first morning of each diet period) as well as the end of each treatment period (The morning of the 13th day of each diet period). The subjects will be instructed to be 12 hours fasted as to not confound the biochemical indices of metabolic syndrome. Upon arrival, subjects will first have their blood pressure and pulse-wave measurements as the subsequent sampling could affect these outcome variables. Second, a DXA scan will be perfomed for body composition. Next, 10 ml serum and plasma EDTA tubes will be collected, aliquoted into 1 ml cryovials, and stored at -80° C until the day of analysis for

biochemical indices. Finally, skeletal tissue biopsies will be collected from the vastus lateralus muscle before and 1 hour after breakfast. Under local anesthetic, a small incision (2-3 cm) will be made and 50-100 mg of muscle tissue will be collected using a Bergstrom needle connected to a vacuum manifold. After removal of fatty and connective tissues, the majority of the sample will be snap frozen in cryovials using liquid nitrogen and then stored at -80°C until determination of protein expression by western blot. A small portion (10 mg) of the sample will be set aside for HRR analysis the same day of biopsy following the SUIT protocols described by Pesta et al. [16].

The subject will enter the calorimetry chamber on the afternoon of the 13th day for a 24 hour stay for the measurement of respiratory gases. On the morning of the 14th day, the subject will participate in a MTT, where they will be fed a standard breakfast of waffles and syrup + treatment which will provide approximately 75 g of simple carbohydrates. Blood collection into 8.5 ml Serum, 10 ml EDTA, and 2 ml NaF tubes will occur at -15, 0, 30, 60, 90, 120, 180, and 240 minutes, then aliquoted and stored at -80°C until analysis for insulin, glucose, and lipid response to a meal challenge.

Statistical analysis

Statistics will be performed using the “proc mixed” function of SAS version 9.4 (SAS institute, Cary, NC) for repeated measures, mixed models (fixed and random effects) ANCOVA. Mixed models use a restricted maximum likelihood algorithm to iteratively compute unique solutions to the design matrices of fixed and random effects. In our study, main effects (independent variables) of treatment and sequence in the model statement test for differences between resveratrol and placebo treatments and assess carryover, respectively. Where appropriate, covariate terms will be included in the model statement, which include:

anthropometric measurements (waist circumference, body mass index), body composition (fat mass and lean mass), baseline measurements from the beginning of each diet period, and age, as well as their interaction terms with treatment. Non-significant interaction and covariate terms will be removed from the final model. Between-subject variance within each sequence is treated as a random effect. Several covariance structures are available in the SAS package to estimate the covariance of fixed and random effects within each subject across several measurements. The appropriate covariance structure will be selected based on information criteria (functions of the log-likelihood, such as Akaike's criteria) and residual plots for homoscedasticity will be examined. The model will be repeated on subject for treatment. Data will be assessed for normality by Shapiro-Wilk. If data are not normally distributed, data transformations will be performed until the Shapiro-Wilk test is satisfied, or parametric analysis will not be performed. $P<0.05$ will be considered statistically significant. Effect sizes and confidence intervals will be reported for meta-analysis purposes.

II. Expected Findings

The primary objective of this study is to confirm the findings of Timmers et al. who demonstrated a significant effect of 150 mg a day of resveratrol on biochemical markers influenced by obesity [21]. They also provided supportive findings on the mechanism by which resveratrol exerts its effects by demonstrating modulation of regulatory proteins of energy metabolism (AMPK), lipid metabolism (PGC-1α), and longevity (SIRT1) in skeletal tissue, confirming similar findings in animal models. Interestingly, Poulsen et al. couldn't reproduce the findings of Timmers et al. with a 10 fold increase in resveratrol dose; therefore, it is appropriate to revisit the lower dose in a similar sample of subjects utilizing a powerful clinical design and controlling the subjects' dietary intake [22]. The advanced metabolic complications of the

subjects in the proposed study, relative to the studies mentioned above, may increase the likelihood of observing a benefit of resveratrol.

We expect to confirm the findings of resveratrol's action as described by the Timmers group. With an insult to homeostatic mechanisms of energy metabolism elicited by a high fat diet in overweight subjects with metabolic syndrome, we believe it is possible to reproduce the modulation of glucoregulation, blood pressure, and molecular mechanisms by resveratrol. We don't anticipate confirming changes in fasting insulin or glucose, as similar short term feeding studies conducted in obese men by our facility failed to show changes in those variables. However, changes in insulin sensitivity were noted with a MTT, and we expect to detect similar differences in this study. Animal studies show a positive effect of resveratrol treatment particularly when the animals are under metabolic stress due to a high fat diet [23, 24]. Intuitively, bioactive compounds thought to elicit positive health modulation will have the greatest chance of action in trials studying populations with the most to benefit from an intervention; an overweight, metabolically sick phenotype has the most to gain from a diet component intervention. By contrast, observing any effects of bioactives in healthy populations will be less obvious, as a healthy phenotype has less to gain (no need to change what is already working) from an intervention. Despite this obvious rationale, investigators study resveratrol alongside gold standards such as caloric restriction and cardiovascular training in order to assess its ability to mock those interventions in healthy populations. When, not surprisingly, no effect of resveratrol is found it is written off as another molecule that showed promise in translational work that has no benefit in human subjects. This is in error. Smoliga et al.. have an excellent communication describing this phenomenon (see figures 1a and 1b of [25]). They also provide helpful alternatives to study resveratrol in healthy populations (see figure 3 of [25]). We believe

Poulsen et al..'s design failed to detect a benefit of resveratrol because 1) the subjects, albeit obese, were healthy 2) they used a parallel arm design, thus we don't know how subjects would have responded to the alternative intervention (who were the non-responders?) and 3) they did not control the subjects' diet, which has a great ability to confound the effect of a dietary intervention. It would be advantageous to see if there is any benefit in the dose of resveratrol used by the Poulsen et al. study, but investigating a dose-response goes beyond the primary objective of reproducibility and statistical power would be sacrificed. A demonstrated ability of resveratrol to alleviate metabolic stress due to high fat feeding could have real world implications in western civilization, where obesity and its associated comorbidities require a cost-effective solution.

Limitations of the study must also be discussed. Failure to detect significant changes in the outcome variables described could be due to an erroneous power calculation using MTT and calorimetry data from a different study. Although there is a theoretical basis to determine sample sizes based on these variables, there is a chance that mediating variables of SIRT1 responsible for the primary biochemical endpoints may not be sufficiently influenced by upregulation of SIRT1 protein expression, leading to null results. Alternatively, SIRT1 may itself be a mediator influenced by an upstream target where resveratrol first signals its biochemical cascade [26, 27]. Also, in an attempt to create a model sample of subjects under metabolic stress, the design could conceivably push the phenotype beyond the brink of a therapeutic effect of resveratrol; the molecular pathways in charge of regulating energy metabolism may be so disturbed by the overabundance of fatty acids that the switches may be inaccessible by resveratrol! Other reasons for no observed benefit include a bad lot of the resveratrol supplement from the manufacturer, interference of resveratrol's intestinal absorption, a prevalence of non-responders, or a

heterogeneous sample of obese males with metabolic syndrome. Study duration and resveratrol dose also play a factor. The majority of resveratrol study in humans is typically on the order of a month or more, but what we sacrifice in duration we gain in complete control of diet interventions. There is no coherent basis for resveratrol dosage, which makes guesswork out of choosing a single dose. Depending on the results, these reasons for no significant effect of resveratrol can be systematically investigated.

Appendix 2: Study design worksheet

Chapter 2: Blackberry human feeding study

R X1 O_1 1 week wash-out P O_3

R P O_2 1 week wash-out X1 O_4

R = Randomized

O = Observation (Indirect calorimetry and 4 hour meal tolerance test)

X1 = 600 g blackberries per day for 7 days.

P = Placebo (energy matched gelatin) for 7 days.

Study design: Randomized, placebo controlled, cross-over posttest.

Independent variables: Treatment (blackberries – 600 g per day, or placebo), sequence (1-2),

Covariates: age, body mass index.

Dependent variables: Metabolic rate and substrate oxidation, and Fasting and meal tolerance test blood biochemistry (glucose, insulin, free fatty acids, and triglycerides).

Chapter 3: Adipocytes and skeletal muscle cells treated with C3G

3T3-L1 Adipocytes:

X1 O_1

O_2

Skeletal muscle cells:

Y1 O_1

Y2 O_2

O_3

O = Observation (oxygen consumption in oxygraph chamber)

X1 = 150nM C3G from blackberry extract for 6 days.

Y1= 150nM purified C3G for 24 hours.

Y2= 50µM purified C3G for 24 hours.

Control = no treatment.

Study design: Controlled, posttest only.

Independent variables: Treatment- C3G via blackberry extract for adipocytes or in purified form for skeletal muscle cells.

Mediator: Mitochondrial content.

Dependent variables: Oxygen consumption in different mitochondrial respiration states.

Appendix 3: Blackberry Feeding Study IRB Protocol

BLACKBERRY POLYPHENOL INTAKE AND FUEL MANAGEMENT

1. OBJECTIVES AND AIMS

A. Research question to be addressed (Objectives)

How does consumption of blackberries affect the body's fuel management?

B. Importance of proposed work (Aim)

The most recent statistics from the World Health Organization show that 1.5 billion adults are overweight, with 500 million of these adults being classified as obese. Worldwide, obesity has more than doubled in the last 30 years. The aim of this work is to seek dietary influences that can alter the body's fuel management to reduce body fatness.

2. BACKGROUND AND SIGNIFICANCE

A. Summary of related research (Background)

Berries are a rich source of flavonoids, especially the bright pigments called anthocyanins. Animal studies have suggested that berry preparations or anthocyanin-rich berry extracts can reduce body fatness in animal studies. In one study, freeze-dried blueberry powder added to a high fat diet and fed to rats resulted in lower fasting insulin, lower post-prandial glucose response, and less abdominal fat (1). In another study, mice fed purified blueberry anthocyanins had lower weight gains and body fatness than mice receiving no anthocyanins (2). Blueberry anthocyanins have been shown to decrease adiposity in mice fed a high fat diet (3) and tart red cherry intake by rats decreased adiposity and markers of inflammation (4). Previous studies with tea catechins, which belong to the flavonoid class of chemicals as do anthocyanins, suggest that these compounds can alter fat oxidation, and this may be the mechanism by which body fatness is influenced by anthocyanin intake.

B. Motivation for research (Significance)

Obesity leads to increased risk for a number of diseases, including cancer, heart disease, and diabetes. This reduces quality of life and adds significant cost and burden to the health care system. It would be advantageous to identify feasible, low cost ways to reduce obesity. Incorporation of berry products into the diet would be both low cost and practical.

3. STUDY DESIGN

The study will be a randomized cross-over intervention with two 1-week controlled feeding periods separated by a 1-4 week break. All volunteers will consume the same base diet, with the treatment food or control food item being the only difference. Total food intake will be adjusted to meet individual calorie needs. The treatment food will be blackberries daily or a calorie & fiber matched control food, such as jello. Treatment sequences will be randomly assigned.

4. METHODS

A. Procedures

Base Diet. Participants will be provided a controlled diet during the entirety of each treatment period. Meals will be prepared using traditional American foods with a macronutrient composition representative of a high fat American diet (~40% of calories from fat). A high fat diet will be fed for this study based on the beneficial berry effect seen previously with rodents when consuming a high fat diet. After the first treatment period, there will be a break of 1-3 weeks prior to the start of the next treatment period. Participants will be instructed to eat all foods and only foods provided to them, with the exception of water, coffee, tea, and diet soda. Coffee and tea intake will be limited to 2 cups per day. Unlimited diet soda will be permitted. Consumption of coffee, tea, and soda will be recorded by study participants. Alcohol consumption will not be allowed during the intervention. Participants will be asked to abstain from vitamin, mineral, and herbal supplements beginning 2 weeks prior to the study and continue to abstain for the duration of the study. Participants will be asked to complete a daily questionnaire with questions regarding general health, over-the-counter or prescription medications, exercise, and amount of coffee, tea, and diet soda consumed in the previous 24-hours.

Study participants will be fed at weight maintenance. Body weight will be measured before breakfast, Monday through Friday, and patterns of weight loss or weight gain will be identified. To maintain body weight, portion size will be adjusted for all foods (in 200 or 400 kcal increments) to adjust energy intake such that nutrient content of the diet will be the same for all participants regardless of energy intake.

Volunteers will consume breakfast and dinner Monday through Friday in the dining facility of the Beltsville Human Nutrition Research Center (BHNRC) Human Studies Facility (HSF) under the supervision of a dietitian, study investigator, or study personnel. Monday through Friday, participants will be required to arrive at the Center between the hours of 6:30 a.m. and 8:30 a.m. for breakfast and return to the Center for dinner between 4:30 p.m. and 6:30 p.m. At each meal, they will collect their food and it will be checked by study personnel to ensure that the correct amounts are consumed. After their food is reviewed, the participants will prepare it for consumption (all food is precooked and only needs to be heated). Lunch will be provided for carry-out and will be available when the participants arrive at the Center for their breakfast. Meals for the weekend will be prepared for off-site consumption. The weekend meals will be prepared and packaged in a large cooler packed with ice which will be taken with the participants after they consume their dinner on Friday.

Dietary Treatments. The dietary treatments will include a blackberry diet period and a control diet period. During the blackberry diet period, participants will receive a high-fat American diet including 600 grams (4 servings) of blackberries daily, expected to provide 540 mg cyanidin-based anthocyanins (purple pigments) daily. During the control treatment period, participants will consume the same high-fat base diet, with the blackberries replaced with a control food, such as jello (matched for calories and fiber). Treatment order will be randomly assigned, with half the participants receiving blackberries first and half the participants receiving the control

food first. At the end of each diet period, participants will enter a room calorimeter for a period of 23.5 hours.

Body Weight and Anthropometry. Participants will be weighed daily (Monday through Friday) when they come to the Center for breakfast. Once during the study, body composition will be measured by air displacement plethysmography. Body volume and density will be measured using a dual-chambered, fiberglass plethysmograph that determines body volume by measuring changes in pressure within a closed chamber (The BOD POD, Life Measurement, Inc., Concord, CA).

Determination of substrate oxidation in calorimeters. On day 4 of each diet period, participants will report to the BHNRC at 4:00PM. Height and weight will be measured as well as blood pressure and heart rate.

Next, subjects will proceed to the Energy Metabolism Unit (EMU). The EMU staff will instruct the volunteer as to calorimeter procedures including operation of the air lock, entertainment equipment (computer, TV, DVD player, radio), treadmill, air conditioning, telephone system and bathroom and sleeping facilities. In addition, emergency procedures, meal times, and blood sampling will be reviewed with the subject. At 5:30 PM the calorimeter door will be closed (not locked) and subjects will reside in the calorimeter for the next 23.5 hours. Dinner will be provided at 6:00 PM. Subjects are expected to completely consume all food within 30 minutes. The remainder of the evening will be spent by the subject in leisure activities. Calorimeter lights are extinguished at 11 PM and the subject will be instructed to lie quietly in bed until awaken in the morning.

At approximately 6:30AM, utilizing a blood sampling port in the wall of the calorimeter, an indwelling catheter will be placed, by a trained phlebotomist. A high-fat breakfast, containing foods such as eggs, buttered English muffins, and donuts (either with blackberries or control), will be provided at 7:00AM and subjects will be expected to consume each meal within 30 minutes. Blood samples (10 cc each) will be collected, by a trained phlebotomist, prior to (-15, 0) and 30-, 60-, 90-, 120-, 180- and 240-min following the start of breakfast, for the determination of post-meal responses in glucose and triglycerides.

The doors of the calorimeter are closed during the measurements, but they are not locked. The calorimeters are staffed 24 hours a day when subjects are present. The trained staff members support the subjects and ensure that they adhere to the prescribed schedule for the study. Trained phlebotomists collect blood at the prescribed times. In the event of a medical emergency, staff members will notify the study investigators, including the PI, and will follow standard operating procedures for handling medical emergencies for the particular situation, including calling 911 if necessary. Any unanticipated adverse events will be documented and reported to the IRB. All non-medical emergency events are handled according to standard operating procedures which identify an appropriate senior staff contact, including the PI, for the particular situation. At any time the subject can request assistance from the on-duty staff, and can choose to exit the calorimeter prior to the end of the measurement, thereby choosing to terminate their participation in the study.

In the event of equipment failure, due to loss of power or other unforeseen circumstances, participants will be given the option to participate in a third diet period (1 week of a controlled diet) and a third calorimeter run with collections of blood, feces, and urine.

Sample Collection.

Blood collection. Blood will be collected at the end of each diet period, at the following times before and after breakfast: -15, 0, 30, 60, 90, 120, 180, 240 minutes. Plasma (collected at every time point) and serum (fasted sample) will be collected in vacutainers. After centrifugation, samples will be aliquotted into cryovials and stored at -80^{o}C until analysis. The total amount of blood taken on each testing day will be 224 mL, for a total of 448 mL (less than 1 pint).

Urine collection. While volunteers are resident in the room sized calorimeters, all urine will be collected for analysis. Urine will be collected in 4 L pre-weighed plastic containers which contain 20 ml of 6 N HCl. The acid is necessary to lower the pH of the urine to prevent release of ammonia. The date and time of the sample collection will be recorded in a laboratory notebook, the filled container will be weighed, and the urine will be sub-sampled and stored in a -80^{o}C freezer for subsequent analyses.

Fecal collection. On day 2 or 3, each subject will consume a gelatin capsule containing 30 mg of brilliant blue. Starting with their next bowel movement, subjects will collect all of their feces voided. At the beginning of each fecal collection period, each subject will be provided with a large Styrofoam cooler containing approximately 12 kg of dry ice. Additional dry ice will be available to all subjects. Subjects also will be provided with 12 plastic 6-mil bags, a waterproof marker, and a Plexiglas holder designed to fit on the toilet. Subjects will be instructed to place the Plexiglas holder over their toilet and place a plastic bag into the holder when they defecate. They will be asked to record the date and time of the event on the plastic bag and to place the plastic bag in the cooler with dry ice. At their next visit to the Center, they will deliver all samples collected during the intervening time. When delivered to the Center, the date and time of the sample collection will be recorded in a laboratory notebook and the samples will be stored in a -20 C freezer. Two to three days later, subjects will be given another capsule of 30 mg brilliant blue and they will be instructed to continue to collect their feces until the marker appears (usually 12 to 48 hours later). After all samples are collected, they will be placed in a freeze-drier and dried. Once they are freeze-dried, they will be crushed with various tools including hammers and rolling pins. Fecal samples will be analyzed for macronutrients and urine samples will be analyzed for nitrogen and energy.

Diet collection. Diet samples will be collected during the feeding phase. Trays of food identical to that consumed by the participants will be prepared as though they are to be consumed (e.g., eggs will be cooked, bread will be toasted). These foods will be blended with water and ice in a large Waring blender to obtain a homogenous mixture. This mixture will be freeze-dried. After they are dry, the samples will be crushed for chemical analyses.

Analyses.

Blood will be analyzed for appearance of blackberry nutrients and their metabolites, as well as for plasma glucose, insulin, and triglycerides. Urine will be analyzed for nitrogen (for protein determination) and for blackberry nutrients and metabolites.

B. **Study sample and recruitment plan**

Population. Adult males and females will be recruited from the greater Baltimore-Washington DC area by mailed fliers, newspaper ads, and direct calling and emailing. There are several thousand government and privately employed workers and several residential communities located within a 10-mile radius of the BHNRC from which a cohort of volunteers will be recruited. An equal number of males and females will be recruited. Recruitment will focus on nonsmoking individuals who are in good health.

Potential subjects will be required to attend an informational meeting during which the requirements of participating in the study along with the risks and benefits will be described in detail. Interested subjects will be required to complete and sign a consent form (attached) and complete a Study Application (attached). During this process, a study investigator and other professional staff are available to answer all questions that may arise.

Subjects will be informed that the study is completely voluntary and they may withdraw from the study at any time.

Screening. Healthy men and women from the Beltsville, MD area will be medically evaluated for underlying disease by a routine urinalysis and blood screening. Potential subjects' height, weight, and blood pressure will be measured, and each study applicant will complete a health and lifestyle questionnaire (Study Questionnaire). A physician or nurse practitioner will review the screening charts and will determine whether study eligibility criteria have been met. If a sufficient number of subjects cannot be identified in the first screening, additional subjects will be screened.

Individuals with active disease (peripheral vascular disease, degenerative kidney disease, degenerative liver disease, cancer, or endocrine disorders that may interfere with the study) will be excluded. Individuals with gastrointestinal disorders will also be excluded. Diagnosis of disease will be based on self-reported medical history.

Up to thirty three healthy adult males will be recruited to participate in the study, with recruitment closing after a complete data set is obtained for 18 individuals. Males will be selected because of the difficulty in scheduling calorimeter runs around female menstrual cycle, which has a significant impact on energy expenditure and variability in results. Volunteers will be selected to have high BMI (over 25 BMI indicating overweight status). Subjects with high BMI are preferred for this study based on the beneficial berry effect seen previously with rodents tending to become obese. If more than 33 eligible volunteers apply, we will preferentially select individuals based on high BMI, glucose, and fewer medications.

Exclusion Criteria:

- Younger than 25 years old or older than 75 years old
- Female
- Use of blood-thinning medications such as Coumadin (warfarin), Dicumarol (dicumarol), or Miradon (anisinidione)
- Presence of any gastrointestinal disease, metabolic disease, or malabsorption syndromes that may interfere with the study goals
- Have been pregnant during the previous 12 months, are currently pregnant or lactating, or plan to become pregnant during the study
- Type 2 diabetes requiring the use of oral antidiabetic agents or insulin
- Fasting triglycerides greater than 300 mg/dL
- Fasting glucose greater than 126 mg/dL
- History of eating disorders or other dietary patterns which are not consistent with the dietary intervention (e.g., vegetarians, very low fat diets, high protein diets)
- Use of prescription or over-the-counter antiobesity medications or supplements (e.g., phenylpropanalamine, ephedrine, caffeine) during and for at least 6 months prior to the start of the study or a history of a surgical intervention for obesity
- Active cardiovascular disease (such as a heart attack or procedure within the past three months or participation in a cardiac rehabilitation program within the last three months, stroke, or history/treatment for transient ischemic attacks in the past three months, or documented history of pulmonary embolus in the past six months).
- Use of any tobacco products in past 3 months
- Use of oral or IV antibiotics during the month preceding the study
- Unwillingness to abstain from herbal supplements for two weeks prior to the study and during the study
- Known (self-reported) allergy or adverse reaction to blackberries
- Unable or unwilling to give informed consent or communicate with study staff
- Self-report of alcohol or substance abuse within the past twelve months and/or current acute treatment or rehabilitation program for these problems (Long-term participation in Alcoholics Anonymous is not an exclusion)
- Other medical, psychiatric, or behavioral factors that in the judgment of the Principal Investigator may interfere with study participation or the ability to follow the intervention protocol

MedStar and USDA employee participation in the study is completely voluntary and will have no effect on their employment status. MedStar and USDA employees will not be encouraged to apply for the study, though they will not be excluded solely based on their employment with these institutions if they would like to be considered for the study.

C. **Data analysis and sample size justification**

This is a pilot study. This study is intended to generate data to determine whether further studies are warranted and to produce variance data on which a follow-up study can be powered. Eighteen individuals completing all measurements should give sufficient variance data to perform a power calculation if a trend appears with respect to anthocyanin intake and fat deposition. In the event of data loss from equipment failure, due to loss of power or other unforeseen circumstances, up to 33 individuals will be enrolled, with enrollment ceasing as soon as 18 individuals successfully complete all measurements.

D. **Facilities**

The study will be conducted at the USDA BHNRC in Beltsville, MD. The BHNRC's HSF is primarily designed to conduct feeding studies in free-living healthy adult volunteers. The HSF occupies 11,000 square feet including dining rooms, food storage, kitchens, and rooms for study recruitment, subject interviews, and sample collection, processing and long-term storage. Biological samples are stored in rooms with freezers on 24-hour power and temperature monitoring and are serviced by emergency generators in event of power interruption. Dr. Novotny maintains approximately 900 square feet of laboratory space which is equipped to measure a variety of biochemical properties of food and physiologic samples. In addition, approximately 300 square feet of office space is available. Personal computers are linked via a local area network and IT support is available to maintain computer services.

E. **Project organization**

The research project will be conducted within the BHNRC drawing on experienced research staff from the Food Components and Health Laboratory. The principal investigator, Dr. Janet Novotny, is a research physiologist in the Food Components and Health Laboratory. She has demonstrated proficiency in conducting human feeding studies. Past recent accomplishments include conducting feeding studies to determine: 1) bioavailability of healthful plant pigments from strawberries, carrots, kale, and red cabbage, 2) anticancer benefits of cabbage and mustard, 3) healthful effects of almonds on inflammation, 4) anticancer effects of garlic, and 5) the effect of macronutrient composition on energy content of foods. These successfully completed studies represent investigations with several hundred volunteers.

Other positions directly involved include:

William Rumpler, PhD, is a Research Physiologist in the Food Components and Health Laboratory and will participate in conducting the study and data interpretation.

Jayme Leger, PhD, is a Research Associate in the Food Components and Health Laboratory and will participate in recruitment, scheduling, logistics, sample collection, laboratory analysis, and data interpretation.

Patrick Solverson, MS, is a Research Associate in the Food Components and Health Laboratory and will participate in recruitment, scheduling, logistics, sample collection, laboratory analysis, and data interpretation.

Roman Mirecki, BS, is a Research Associate in the Food Components and Health Laboratory and will participate in recruitment, scheduling, logistics, and sample collection.

David Baer, PhD, is a Supervisory Research Physiologist in the Food Components and Health Laboratory and will participate in recruitment and data interpretation, and will be responsible for database management.

Vanita Aroda, MD, is the principal investigator from MedStar Health Research Institute who will be responsible for screening subjects and evaluating health records.

Deborah Wells, NP, is a nurse practitioner who will be responsible for screening subjects and evaluating health records.

Dawn Harrison, MS, is a Chemist in the Food Components and Health Laboratory and will be responsible for biological sample analysis for absorption of phytonutrients.

Theresa Henderson, MS, is a Chemist and a certified phlebotomist in the Food Components and Health Laboratory, and will be responsible for developing protocols for processing and analyzing biological samples, managing study supplies, and sample collection.

Medical services (nurse practitioner and physician) will be provided by MedStar Health Research Institute through a cooperative agreement.

F. **Project schedule**
February 2013 – February 2016: Subject recruitment/screening/selection of volunteers
March 2013 – March 2016: Study treatment period
June 2013 – August 2016: Analysis of biological samples and calorimeter data
September - October 2016: Data interpretation and manuscript preparation

G. **Problems / weaknesses**
If the variance in the data is much higher than expected, it may be difficult to determine a trend toward a positive effect of the berry intervention with 18 subjects.

5. RISK / BENEFIT ASSESSMENT

This study has a minimal level of risk. This assessment is based on the fact that study participants will be fed foods that are normally consumed by the general population. Biological samples collected include urine, feces, and blood. Urine and feces is collected by the subjects themselves and is thus considered minimally invasive. The risk associated with blood drawing is the possible development of a slight bruise at the site of needle puncture. Total blood collected during the study will be 473 mL (25 mL for screening and 224 mL for each of two blood draw days), which should not pose a risk to a healthy adult. Upon request, participants will be sent copies of scientific publications resulting from this study. The investigators will communicate the potential risks to the volunteers during the information meeting during recruitment as well as during screening.

Compensation. Study participants will be paid $400 ($200 for the completion of each treatment period). Payment will be delivered in a single check at the end of the study after all measurements have been taken. Participants will not be paid for the screening visits or the informational meeting. If participants choose to participate in a third treatment period, in the event of equipment failure, they will be compensated an additional $200 for completion of the third treatment period.

6. STUDY POPULATION – GENDER / ETHNIC INCLUSION

a) Rationale for research subject selection: The population from which subjects will be recruited consists of individuals living within commuting distance of the study site at Beltsville, MD. The population includes an ethnically diverse group of subjects. It is expected that treatments will have similar effects across ethnic groups.

b) Strategies / procedures for recruitment: These procedures will be the same as have been routinely used to recruit subjects for diet studies at the Beltsville facility in previous studies.

c) Rationale and justification for exclusions: Selection of subjects will be done without regard to ethnicity. No ethnic group will be excluded.

7. RECRUITMENT PLAN

The detailed recruitment plan is described above in the Methods section (4B).

8. VERTEBRATE ANIMALS

Not applicable.

9. CONSULTANTS

Not applicable.

10. CURRICULUM VITAE

11. LITERATURE CITED

1. Seymour EM, Tanone, II, Urcuyo-Llanes DE, Lewis SK, Kirakosyan A, Kondoleon MG, Kaufman PB, Bolling SF. Blueberry intake alters skeletal muscle and adipose tissue peroxisome proliferator-activated receptor activity and reduces insulin resistance in obese rats. J Med Food. 2011;14(12):1511-8.
2. Prior RL, Wu X, Gu L, Hager TJ, Hager A, Howard LR. Whole berries versus berry anthocyanins: interactions with dietary fat levels in the C57BL/6J mouse model of obesity. J Agric Food Chem. 2008;56(3):647-53.
3. Prior RL, S EW, T RR, Khanal RC, Wu X, Howard LR. Purified blueberry anthocyanins and blueberry juice alter development of obesity in mice fed an obesogenic high-fat diet. J Agric Food Chem. 2010;58(7):3970-6.
4. Seymour EM, Lewis SK, Urcuyo-Llanes DE, Tanone, II, Kirakosyan A, Kaufman PB, Bolling SF. Regular tart cherry intake alters abdominal adiposity, adipose gene transcription, and inflammation in obesity-prone rats fed a high fat diet. J Med Food. 2009;12(5):935-42.

12. COMPLIANCE:

This research will be conducted in compliance with the protocol, HHS, and all applicable institutional, state, and local requirements. Reporting of adverse events will adhere to MedStar Institutional Review Board requirements.

13. APPENDICES:

1) Study Application
2) Daily Questionnaire
3) Medical History
4) Email Advertisement – for approval
5) Newspaper Advertisement – for approval

STUDY APPLICATION

NAME: _____

PLEASE CIRCLE ONE: MALE FEMALE

PHONE NUMBER Home:__ _____ Work:_____

Cell:_____

HOME ADDRESS: _____

_____

Email: _____

1. Date of Birth_____ Age_____
2. Weight_____Height_____
3. Do you eat a special diet (including vegetarian)? Yes_____ No_____
4. Do you take vitamins, herbals or other nutritional supplements? Yes_____ No_____

   If so, please describe: _____

   Are you willing to discontinue use of supplements for this study? Yes_____ No_____
5. Are you willing to abstain from donating blood during the study? Yes_____ No_____
6. Have you ever experienced weakness or fainting during or after giving blood? Yes_____ No_____
7. Are there any foods which you absolutely cannot eat? Yes_____ No_____

   If so, please list:_____
8. Are there foods which you strongly prefer not to eat? Yes_____ No_____

   If so, please list: _____
9. Do you have any food or nut allergies? Yes_____ No_____

   If so, please list and indicate severity: _____

10. During the controlled diet phases of the study are you willing to consume the diets prepared for participants in the study and nothing else? Yes_____ No_____

    Do you understand that this includes beverages, and that alcoholic drinks are not allowed? Yes_____ No_____

11. If selected as a participant in this study, will you be able to remain in this area throughout the study, so that you can eat your meals at the Beltsville Center on weekdays? Yes_____ No_____

    If no, explain any problems or exceptions to the above, in detail:_____

    _____

12. Are there any house hold situations (for example, poor health) or other situations you can imagine which might require you to be away during any part of the study? Yes_____ No_____

13. Are you responsible for meal preparation for other people now or will you be during the study? Yes_____ No_____

    Will this present a problem with your own adherence to the protocol where you eat only what is provided? Yes_____ No_____

14. This study requires that we maintain your body weight during the entire study. Are you willing to maintain your current weight for the study period, and to weigh-in each morning before breakfast? Yes_____ No_____

15. Do you smoke? Yes_____ No_____

    If so, how many packs or cigarettes, cigars, pipes per day? _____

16. Have you previously participated in a diet study? Yes_____ No_____

    Which study?_____

17. Do you consider yourself in good health? Yes_____ No_____

18. Are you under routine physician's care for any medical condition? Yes_____ No_____

19. Do you regularly take medicine prescribed by a physician? Yes_____ No_____

If yes, describe:_____

20. Do you routinely take over-the-counter medicines such as aspirin, Tums, antihistamines, Tylenol, Advil, etc? Yes_____ No_____

    If yes, describe:_____

21. Are you willing to complete the "Daily Questionnaire" each day? Yes_____ No_____

22. Have you ever been diagnosed by a physician as having any of the following medical conditions?

	Yes	No
Diabetes	____	____
Thyroid disease	____	____
Gall bladder disease	____	____
Ulcers	____	____
Malaria	____	____
Hepatitis	____	____
Other liver disease	____	____
Prostate disease	____	____
High blood pressure	____	____
Heart disease	____	____
Kidney disease	____	____
Cancer (specify site: _____)	____	____
Benign breast disease	____	____
Gout	____	____
Food or nut allergies	____	____
Allergies of any kind	____	____
Anemia	____	____
Any other medical conditions (specify)	____	____

_____

DAILY QUESTIONNAIRE

Name: _____ ID Number: _____ Today's Date: _____

Please provide the following information covering THE PAST 24 HOURS

HEALTH:

Have you been sick or had medical treatment? YES_____ NO_____
If yes, describe briefly:_____

Have you taken any medications? YES_____ NO_____
If yes, please record the total amount taken for the day of the following:

Aspirin _____ Tylenol _____ Advil _____ Antacids _____

Other over-the-counter medicines: Name _____ Amount _____

Prescription medications: Name _____ Amount _____
Name _____ Amount _____
Name _____ Amount _____

DIET:

Please record **any food or beverages** (other than water) and the amounts which you ate or drank during the past 24 hours **other than those provided by the study**.

Food eaten and amount: _____

Record the amounts (in ounces) of the following beverages that you consumed:

Diet sodas: _____ Regular coffee: _____ Decaf coffee: _____ Tea: _____

Other beverages consumed: Name _____ Amount _____

EXERCISE:

Did you engage in any vigorous physical exercise? YES_____ NO_____

Is this in excess of your usual daily or regular exercise? YES_____ NO_____

If in excess of usual, please describe briefly:

What type of exercise: _____ Length of exercise _____

Today's Date: _____/_____/_____

HEALTH HISTORY QUESTIONNAIRE

All answers to questions contained in this questionnaire are strictly confidential.

Name: *(Last, First, M.I.)*	☐ **M** ☐ **F**	**DOB** _____/_____/_____ **Marital Status:** _____
Current Doctor:	**Phone #:**	**Date of Last Physical Exam:** _____

CURRENT HEALTH HISTORY

List Any Current Medical Problems That Are Being Evaluated and/or Treated by Your Doctor:

List Your Prescribed Drugs and Over-the-Counter Drugs, Such as Vitamins and Inhalers:

Name the Drug	Strength	Frequency Taken

Allergies to Medications, Foods, Pollens, etc:

Name the Drug or Item	Reaction You Had (e.g. rash, hives, itching, runny nose, respiratory difficulty, swelling)

PAST HEALTH HISTORY

Childhood Illness: ☐ Measles ☐ Mumps ☐ Rubella ☐ Chickenpox ☐ Rheumatic Fever ☐ Polio

List Any Past Medical Problems That Doctors Have Diagnosed:

Surgeries:

Year	Reason	Hospital

Other Hospitalizations:

Year	Reason	Hospital

Have you ever had a blood transfusion? ..... ☐ Yes ☐ No

LIST OF DISEASES/PROBLEMS

Check if you have, or have had, any symptoms in the following areas to a significant degree and briefly explain.

☐ Skin_____
☐ Head/Neck _____
☐ Ears _____
☐ Nose _____
☐ Throat _____
☐ Lungs _____
☐ Back _____
☐ Bone disease _____
☐ Bone fracture _____
☐ Osteoporosis _____

☐ Chest/Heart _____
☐ Circulation _____
☐ Intestinal _____ _____
☐ Bladder _____
☐ Bowel _____
☐ Arthritis_____
☐ Thyroid disease_____
☐ Diabetes ____ _____
☐ Abnormal glucose tolerance-____
☐ Abnormal blood sugar ___

☐ Elevated blood cholesterol_____
☐ Abnormal blood pressure_____

Recent Changes In:
☐ Weight _____
☐ Energy Level _____
☐ Appetite _____
☐ Ability to Sleep _____

Other Pain/Discomfort:
_____
_____

FAMILY HEALTH HISTORY

List all diseases which tend to occur in your blood relatives (parents, brothers, sisters, children). Especially note such illnesses as diabetes, high blood pressure, heart disease, high cholesterol levels, thyroid problems.

PARENTS:

SIBLINGS:

CHILDREN:
GRANDPARENTS:

HEALTH HABITS AND PERSONAL SAFETY

Exercise and Activity Level:	Are you able to do light chores, meal preparation, or climb 10 steps without developing shortness of breath or chest pain and without assistance of others? ☐ Yes ☐ No What is your usual activity level (check one): ☐ Sedentary (No exercise) ☐ Mild Exercise (i.e., climb stairs, walk 3 blocks, golf) ☐ Occasional Vigorous Exercise (i.e., work or recreation, < 4 times/week for 30 min.) ☐ Regular Vigorous Exercise (i.e., work or recreation 4 or more times/week for 30 min) Do you participate in exercise or a sports program? ..... ☐ Yes ☐ No Please describe:_____ Describe activities during typical day_____
Diet:	Are you dieting?..... ☐ Yes ☐ No If yes, are you on a physician prescribed medical diet? ..... ☐ Yes ☐ No Have you lost weight? How much weight_____ over what time period_____ # of meals you eat in an average day?_____ # Snack/day_____
Caffeine:	☐ None ☐ Coffee # of Cups/day? _____ ☐ Tea # of Cups/Cans per day? _____ ☐ Cola # of Cups/Cans per day? _____
Alcohol:	Do you drink alcohol? ..... ☐ Yes ☐ No If yes, what kind?_____ How many drinks per week? _____
Tobacco:	Do you currently use tobacco? ..... ☐ Yes ☐ No ☐ Cigarettes - Pks/day_____ ☐ Chew - #/day _____ ☐ Pipe - #/day _____ ☐ Cigars - #/day _____ ☐ # of Years _____ Have you used tobacco in the past? ..... ☐ Yes ☐ No ☐ # Years used _____ ☐ # Years since you quit _____
Education and Occupation:	Please indicate highest education level. ☐ < High School ☐ High School Graduate ☐ Technical/Trade School ☐ College Graduate ☐ Postgraduate or Professional School What is your occupation?_____

WOMEN ONLY

NUTRITONAL SUPPLEMENTS

During the last two months, have you taken any vitamins, minerals, or other nutritional supplements? ☐ Yes ☐ No

Age at onset of menstruation: _____ Date of last cycle's menstruation: _____/_____/_____
Age at onset of menopause:_____ Date of last menstruation: _____/_____/_____
How long is your cycle? _____ days. What is the range of your cycle length? _____
Have you had 1 cycle per month for the past 12 months? ..... ☐ Yes ☐ No
Do you consider your cycle to be regular? ..... ☐ Yes ☐ No
Do you have heavy periods, irregularity, spotting, pain or discharge? ..... ☐ Yes ☐ No
Number of pregnancies _____ Number of live births _____
Are you pregnant or breastfeeding? ..... ☐ Yes ☐ No
Do you take any birth control or estrogen medication? (please list in medication section) ☐ Yes ☐ No
Have you had a D&C, hysterectomy or cesarean? ..... ☐ Yes ☐ No
Any urinary tract, bladder or kidney infections within the last year? ..... ☐ Yes ☐ No
Any blood in your urine? ..... ☐ Yes ☐ No
Any hot flashes or sweating at night? ..... ☐ Yes ☐ No
Do you have menstrual tension, pain, bloating, or irritability at or around time of period? ☐ Yes ☐ No
Experienced any recent breast tenderness, lumps, or nipple discharge? ..... ☐ Yes ☐ No
Date of last pap? _____/_____/_____ Date of last rectal exam? _____/_____/_____

MEN ONLY

Do you usually get up to urinate during the night? ..... ☐ Yes ☐ No If yes, # of times _____
Do you feel pain or burning with urination? ..... ☐ Yes ☐ No
Any blood in your urine? ..... ☐ Yes ☐ No
Have you had any kidney, bladder or prostate infections within the last 12 months? .. ☐ Yes ☐ No
Do you have any problems emptying your bladder completely? ..... ☐ Yes ☐ No
Any testicle pain or swelling? ..... ☐ Yes ☐ No
Date of last prostate exam? _____/_____/_____ Date of last rectal exam? _____/_____/_____

If Yes, place a check for the Number of Tablets and How Long Taken:

Nutritional Supplement	Number of Tablets					How Long Taken		
	1 - 3 per week	4 - 6 per week	1 per day	2 per day	3 + per day	0 - 3 mo	3 -12 mo	1 + yr
Multiple Vitamins One-a-day type								
Stress-tab type								
Therapeutic, Theragram								
Other: _____								
Other Supplements								
Vitamin A								
Beta - carotene								
Vitamin C								
Vitamin E								
Vitamin B6								
Calcium or Tums								
Fish Oil								
Calcium								
Soy protein								
Whey protein								
Protein supplement								
Other, including Herbals (specify)								
Other Antioxidants (specify)								

EMAIL AD:

The Beltsville Human Nutrition Research Center, Beltsville, MD is recruiting

NONSMOKING

MALE AND FEMALE VOLUNTEERS

who are

25 - 75 YEARS OLD

for a nutrition study.

If you meet all of the above criteria, you may be eligible for this study to determine the effect of whole grains on heart disease.

Participants may be compensated up to $400.

The Principal Investigator is Janet Novotny, PhD.

FOR MORE INFORMATION:

- CALL (301) 504-5454
- EMAIL volunteers@ars.usda.gov
- Visit our website at www.usdanutritioncenter.us and click on “Blackberry & Fat Oxidation Study”

NEWSPAPER AD:

VOLUNTEERS NEEDED

The Beltsville Human Nutrition Research Center, Beltsville, MD

IS RECRUITING NONSMOKING MALE AND FEMALE VOLUNTEERS WHO ARE

25 - 75 YEARS OLD

FOR A NUTRITION STUDY

If you meet all of the above criteria, you may be eligible for this study to determine the effect of whole grains on heart disease.

PARTICIPANTS MAY BE COMPENSATED UP TO $400.

The Principal Investigator is Janet Novotny, PhD.

For more information, call the USDA at (301) 504-5454, or email volunteers@ars.usda.gov, or visit our website www.usdanutritioncenter.us and click on "Blackberry & Fat Oxidation Study"

IRB number:	2013-037	**Clinical Site IC Version:**	Version 3
Project Title:	Blackberry Polyphenol Intake and Fuel Management		
Principal Investigator:	Janet A. Novotny	**Institution:**	USDA

Appendix 4: Informed Consent Form

MedStar Health Research Institute Informed Consent for Clinical Research

INTRODUCTION

We invite you to take part in a research study called "*Blackberry Polyphenol Intake and Fuel Management*." You were selected as a possible participant in this study because you applied for the study and you met the inclusion criteria. Please take your time to read this form, ask any questions you may have and make your decision. We encourage you to discuss your decision with your family, friends and your doctor(s).

WHAT IS THE PURPOSE OF THIS STUDY?

This study is being done to learn about how blackberry nutrients affect the body's fuel management.

WHAT ELSE SHOULD I KNOW ABOUT THIS RESEARCH STUDY?

It is important that you read and understand several points that apply to all who take part in our studies:

- Taking part in the study is entirely voluntary and refusal to participate will not affect any rights or benefits you normally have;
- You may or may not benefit from taking part in the study, but knowledge may be gained from your participation that may help others; and
  - You may stop being in the study at any time without any penalty or losing any of the benefits you would have normally received.

The nature of the study, the benefits, risks, discomforts and other information about the study are discussed further below. If any new information is learned, at any time during the research, which might affect your participation in the study, we will tell you. We urge you to ask any questions you have about this study with the staff members who explain it to you and with your own advisors prior to agreeing to participate.

WHO IS IN CHARGE OF THIS STUDY?

IRB number:	2013-037	**Clinical Site IC Version:**	Version 3
Project Title:	Blackberry Polyphenol Intake and Fuel Management		
Principal Investigator:	Janet A. Novotny	**Institution:**	USDA

The investigator is Janet Novotny, PhD. The research is being sponsored by the United States Department of Agriculture. MedStar Health Research Institute is being paid by the U.S. Department of Agriculture to conduct this study with Janet Novotny, PhD, as the primary investigator.

WHO CANNOT PARTICIPATE IN THIS STUDY?

You cannot be in this study if any of the following apply to you:

- Younger than 25 years old or older than 75 years old
- Female
- Use of blood-thinning medications such as Coumadin (warfarin), Dicumarol (dicumarol), or Miradon (anisinidione)
- Presence of any gastrointestinal disease, metabolic disease, or malabsorption syndromes that may interfere with the study goals
- Type 2 diabetes requiring the use of oral antidiabetic agents or insulin
- Fasting triglycerides greater than 300 mg/dL
- Fasting glucose greater than 126 mg/dL
- History of eating disorders or other dietary patterns which are not consistent with the dietary intervention (e.g., vegetarians, very low fat diets, high protein diets)
- Use of prescription or over-the-counter antiobesity medications or supplements (e.g., phenylpropanalamine, ephedrine, caffeine) during and for at least 6 months prior to the start of the study or a history of a surgical intervention for obesity
- Active cardiovascular disease (such as a heart attack or procedure within the past three months or participation in a cardiac rehabilitation program within the last three months, stroke, or history/treatment for transient ischemic attacks in the past three months, or documented history of pulmonary embolus in the past six months).
- Use of any tobacco products in past 3 months
- Use of oral or IV antibiotics during the month preceding the study
- Unwillingness to abstain from herbal supplements for two weeks prior to the study and during the study
- Known (self-reported) allergy or adverse reaction to blackberries
- Unable or unwilling to give informed consent or communicate with study staff
- Are unwilling or unable to make scheduled appointments at clinical site as required by study protocol
- Self-report of alcohol or substance abuse within the past twelve months and/or current acute treatment or rehabilitation program for these problems (Long-term participation in Alcoholics Anonymous is not an exclusion)
- Other medical, psychiatric, or behavioral factors that in the judgment of the Principal Investigator may interfere with study participation or the ability to follow the intervention protocol

IRB number:	2013-037	**Clinical Site IC Version:**	Version 3
Project Title:	Blackberry Polyphenol Intake and Fuel Management		
Principal Investigator:	Janet A. Novotny	**Institution:**	USDA

WHAT IF I AM PRESENTLY PARTICIPATING IN ANOTHER RESEARCH STUDY?

Are you presently participating in any other research studies? Yes ☐ No ☐

If yes, please state which study(ies)_____

While participating in this study, you should not take part in any other research project without approval from the people in charge of each study. This is to protect you from possible injury arising from such things as extra blood drawing, extra x-rays, interaction of research drugs, or similar hazards.

HOW MANY PEOPLE WILL TAKE PART IN THE STUDY?

Up to 33 people will take part in this study, worldwide. Up to 33 people will be recruited at this site.

WHAT HAPPENS IF I AGREE TO BE IN THE STUDY?

If you agree to take part in this study, you will be "randomized" into one of the study groups. Randomization means that you are put into a group by chance. It is like flipping a coin. Neither you nor the investigators will choose what group you will be in. You will have a one in two chance of being placed in any group. Your group assignment determines the order in which you will participate in the two parts of the study.

Screening Procedures:

You will be scheduled for a brief screening at the Beltsville Human Nutrition Research Center (the Center). We will collect a blood sample of 25 ml (about 2 tablespoons) and a small urine sample. The blood sample will be analyzed for chemical compounds and for numbers of different types of blood cells. These analyses will include red and white blood cell counts, platelets, sugar, insulin, liver enzymes, bilirubin, cholesterol, and triglycerides. The urine sample will be used for routine laboratory analysis that includes testing for the presence of blood cells, sugar, or proteins. These are the same tests that a doctor uses to see if someone is healthy. You will also have your blood pressure tested. These tests and the physical exam will determine whether you are eligible for the study. You will be notified about any abnormal blood or urine test results and we advise that you follow up these results with your primary physician. These samples will be discarded after completion of the lab analyses. These results will be provided to you whether you are eligible for the study or not.

You will be selected to join based on health status, weight, body mass index, normal range of blood and urine analysis values, and availability to participate in the complete study. Your blood values for cholesterol, blood triglycerides, blood sugar, and blood pressure will also be used for diet selection. If more people apply for the study than there are openings

for participants, then participants will be selected based on body mass index, cholesterol, blood triglycerides, blood sugar, blood pressure, and medications.

Study Procedures:

You will participate in two controlled feeding periods that will be 1 week each in length, separated by a 1-4 week break. During these periods, you will consume traditional American foods representative of a high fat American diet provided by the USDA Nutrition Center. During one of the feeding periods, you will consume blackberries (4 servings daily) as part of the diet. During the other feeding period, blackberries will be replaced with a control food, such as jello.

For the controlled feeding, you will consume breakfast and dinner daily at the Center, and an early breakfast on the fourth and eighth day of the study. The dining facility is open from 6:30 to 8:30 AM for breakfast and from 4:30 to 6:30 PM for dinner. Lunch on all other days will be provided for carry-out. Meals will be prepared using traditional American foods. You will be expected to eat everything we provide for you, and not consume any other foods or beverages without approval from our staff. You will be expected to avoid food and beverages containing caffeine, except those provided by the Center. Alcohol consumption will not be allowed during the treatment period.

You are asked to limit intake of coffee to 2 cups per day or less during the treatment periods and you are asked to keep your coffee consumption constant. You also are asked to abstain from vitamin, mineral, and herbal supplements for the duration of the study.

You will be fed enough food to keep your body weight the same during the study. Each day we will weigh you before breakfast. If your body weight begins to change, we will adjust the amount of food that you are eating.

The study consists of staying in a room calorimeter (described below) on 2 separate occasions for approximately 23.5 hours each time (on the last day of each diet period). We will provide your meals during your stay in the calorimeter. One of your meals will consist of high-fat test meal either with blackberries or control, such as jello.

You will report to the Center the night before each calorimeter stay to consume dinner and pick up your breakfast and lunch. You will report to the Center for a 23.5-hour stay on the evening you are scheduled to enter the room calorimeter. You will do this 2 different times. While in the room calorimeter, blood, urine, and fecal samples will be collected.

The doors of the calorimeter are closed during the measurements, but they are not locked. The calorimeters are staffed 24 hours a day during your stay. The trained staff members will monitor that you receive your meals, get your blood drawn, and exercise at the appropriate times. In the event of a medical emergency, the staff member will call 911. All non-medical emergencies are handled according to standard operating procedures which identifies an

appropriate senior staff member for the situation. At any time, you can request assistance from the on duty staff. You can also exit the calorimeter prior to the end of the measurement thereby choosing to end your participation in the study.

In the event of equipment failure, due to loss of power or other unforeseen circumstances, participants will be given the option to participate in a third diet period (1 week of a controlled diet) and a third calorimeter run with collections of blood, feces, and urine,.

If you agree to participate in this study you will be required to:

1) Consume a controlled diet provided by the USDA Nutrition Center for 2 weeks. On weekdays, you will come to the Center for breakfast and dinner, and lunch will be given to you for carry-out. On Friday evening, you will take the food we provide home with you to consume on the weekend. You must eat all of the food that we give to you and you cannot eat any food that we do not give to you.

2) Stay in one of the room calorimeters on 2 separate occasions. A calorimeter is a room (approximately 9'x12') that measures how much oxygen you consume and carbon dioxide you produce. This is done by determining the amount and the composition of air flowing into and out of the calorimeter. You will enter the calorimeter at 5:30 PM and exit the calorimeter at 5 PM the next day. Each room calorimeter has a bed, desk, television, computer, DVD player, radio, treadmill, air conditioning, toilet and personal hygiene facilities, and telephone. Prior to entering the calorimeter, you will be weighed. You will be provided two hot meals and the treatment test meal during the course of the day.

3) Have your blood taken while in the calorimeter. This requires that an intravenous (IV) line be placed in an arm vein. The line may be kept open by infusing a small amount of saline. A fasting blood sample (10 cc) will be taken when the IV is first placed. Blood samples (10 cc each) will then be collected from your IV line prior to and 30, 60, 90, 120, and 240 minutes following the start of breakfast. When blood samples are taken in the calorimeter, you will place your arm through a plastic sleeve that will prevent air in the calorimeter from escaping.

4) While in the calorimeter, collect all of your urine in containers we provide while in the calorimeter.

5) Collect all feces voided during two 3- to 5-day periods during each diet period.

6) Have your body composition measured using the BODPOD once during the study.

7) Be weighed daily on a bathroom type scale, prior to breakfast at the Center, during the controlled feeding diet periods.

8) Fill out a daily questionnaire about your general health and exercise during the controlled feeding diet period. It will take about 5 minutes per day.

Blood collection:
You will provide blood samples on 2 different study days (at the end of each diet period). The total amount of blood collected over the entire study will be 473 mL (25 mL for screening and 224 mL for each of two blood draw days). For comparison, the amount of blood taken by the Red Cross for a single donation is 473 mL or one pint, and a healthy person could safely donate this amount every 8 weeks. Blood will be analyzed for appearance of blackberry nutrients, as well as for plasma glucose, insulin, and triglycerides.

Urine and fecal collections. You will swallow a capsule of a nontoxic food coloring on day 4 of each diet period to mark the start of the fecal collection period. After swallowing the small capsule, you will start collecting your feces with your next bowel movement, and you will collect all of your feces and urine into plastic bags and containers that we give you. You will place your samples in a large Styrofoam cooler with dry ice that we give you. You will write the date and time of the void on the plastic bags. At your next visit to the Center you will drop off all samples collected. On day 6, you will swallow a second capsule of nontoxic food coloring and you will continue to collect your feces. We will tell you when to stop collecting your feces (usually 1 to 3 days later).

You also will collect all urine samples during each stay in the calorimeter.

Other study procedures:

Please tell the investigator about all medications including over the counter drugs or herbal supplement you are taking, even if you don't think they are important.

You may be required to withdraw from the study for reasons that you cannot control, including illness.

We would like to keep your name and address on file to help recruit for future studies. Please sign below if you agree to allow us to keep this information on file for this purpose.

Participant's Signature **Date of Signature**

The investigator may terminate your voluntary participation in this study if you do not follow the procedures listed above.

The treatments in this study that are considered experimental/investigational are the inclusion of blackberries in the diet to determine their effect on fuel management.

HOW LONG WILL I BE IN THE STUDY?

You will be in the study for 2 weeks (not including the break period). In the event of loss of data from equipment failure, you may choose to participate in a third week.

The investigator may decide to take you off this study if it is believed to be in your best interest, you fail to follow instructions, new information becomes known about the safety of the study, or for other reasons the investigator or sponsor believes are important.

You can stop participating at any time. However, if you decide to stop participating in the study, we encourage you to talk to the investigator and your regular doctor first so they can help you decide what other options may be best for your medical care once you are off study.

If you suddenly withdraw from the study, we may not be able to use any of the information gathered from your participation.

WHAT ARE THE RISKS AND SIDE EFFECTS OF THIS STUDY?

If you decide to participate in this study, you should know there may be risks. You should discuss these with the investigator and/or your regular doctor and you are encouraged to speak with your family and friends about any potential risks before making a decision. Potential risks and side effects related to this study include those listed below.

Risks and side effects ***that may occur*** include:

- Bruising from the placing of the catheter used to take blood from the arm

Risks and side effects ***that are less likely to occur*** include:

- Light-headedness during the blood draw
- Pain or discomfort during the blood draw

Risks and side effects ***that rarely occur*** include:

- Fainting during the blood draw

- Anxiety during the calorimeter stay

ARE THERE ANY BENEFITS TO TAKING PART IN THE STUDY?

You may or may not get any direct benefit from being in this study. We cannot promise that you will experience any benefits from participating in this study. We hope the information learned from this study will benefit others in the future.

WHAT OTHER OPTIONS ARE THERE?

Instead of being in this study, you have these options:

- You always have the option to not be in this study or to refuse any medical treatment.

WHAT ABOUT CONFIDENTIALITY?

Your personal health information (PHI) will be kept private to the extent allowed by law. You will not be identified by name in any publications resulting from this study. Data and samples from this study will be disposed of after all publications concerning the study are completed. As required by the U. S. Department of Agriculture, consent forms and medical screening data will be kept for 25 years, and then destroyed. All other data, records, and samples will be kept until manuscripts have been published, and then they will be destroyed. Samples collected at screening will be destroyed immediately after analysis. If you do not wish to sign this permission form you will not be allowed to participate in this study.

Information, that does not include personally identifiable information, concerning this clinical trial has been or may be submitted, at the appropriate and required time, to the government-operated clinical trial registry data bank, which contains registration, results, and other information about registered clinical trials. This data bank can be accessed by you and the general public at www.ClinicalTrials.gov. Federal law requires clinical trial information for certain clinical trials to be submitted to the data bank.

WILL I BE PAID FOR PARTICIPATING IN THIS STUDY?

You will be paid for being in this study. You will receive a total of $400 ($200 for the completion of each treatment period) for completion of the entire study, including all meals and all measurements. Payment will be issued upon completion of the entire study. If there is evidence that you have not complied with the study protocol, it is possible that we will remove you from the study with no monetary compensation. Because this is a short study, there will be no partial compensation for completing only part of the study. If there is loss of data from equipment failure and you choose to participate in a third treatment period, you will be compensated an additional $200 for completion of the

third treatment period. Compensation for research participants is considered taxable income. Amounts of $600.00 or more will be reported to the Internal Revenue Service (IRS).

WHAT ARE THE COSTS?

You do not have to pay anything to be in this study. However, if taking part in this study leads to procedures or care not included in the study, it may lead to added costs for you or your insurance company. You will not be charged for any tests or procedures that are part of this research study.

WHAT IF I'M INJURED OR BECOME ILL DURING THE STUDY?

We will make every effort to prevent injuries and illness from being in the study. In the case of an injury, illnesses, or other harm occurring during, or resulting from, the study, emergency medical treatment is available but will be given at the usual charge by an area hospital. You or your insurance company will be charged for any continuing medical care and/or hospitalization that are not a part of the study.

If you suffer an injury related to the study procedures, the reasonable costs of necessary medical treatment of the injury will not be reimbursed by the USDA to the extent these costs are not covered by your insurance or third party coverage. If you have an injury or illnesses occurring during, or resulting from the study, you, your medical insurance, a third-party payer, or a government program you've enrolled will be expected to provide coverage for your medical care. Federal government does not have any program to provide compensation to you if you experience injury or other bad effects that are not the fault of the investigators. If you are injured while participating in this research project as a result of the negligence of a United States Government employee who is involved in this research project, you may be able to be compensated for your injury in accordance with the requirements of the Federal Tort Claims Act. Compensation from individuals or organizations other than the United States might also be available to you. If you are a federal employee acting within the scope of your employment, you may be entitled to benefits in accordance with the Federal Employees Compensation Act.

No funds have been set aside, by the USDA, the MedStar Health Research Institute, MedStar Health, or its affiliated entities to repay you in case of injury, illness, or other harm occurring during, or resulting from the study and their current policies do not provide for payments for lost wages, cost of pain and suffering, or additional expenses. By agreeing to this you do not give up your rights to seek compensation in the courts.

WHAT ARE MY RIGHTS AS A PARTICIPANT?

- You have the right to be told about the nature and purpose of the study;
- You have the right to be given an explanation of the exactly what will be done in the study and given a description of potential risks, discomforts, or benefits that can reasonably be expected;

- You have the right to be informed of any appropriate alternatives to the study, including, if appropriate, any drugs or devices that might help you, along with their potential risks, discomforts and benefits;
- You have the right to ask any questions you may have about the study;
- You have the right to decide whether or not to be in the study without anyone misleading or deceiving you; and
- You have the right to receive a copy of this consent form.

By signing this form, you will not give up any legal rights you may have as a research participant. You may choose not to take part in or leave the study at any time. If you choose to not take part in or to leave the study, your regular care will not be affected and you will not lose any of the benefits you would have received normally. We will tell you about new information that may affect your health, welfare, or willingness to be in this study.

WHO DO I CALL IF I HAVE QUESTIONS OR PROBLEMS?

For questions about the study or a research-related injury, contact the investigator, Janet Novotny, PhD, at 301-504-8263. If you are having a medical emergency, you should call 911 or go directly to the nearest emergency room.

IRB number:	2013-037	**Clinical Site IC Version:**	Version 3
Project Title:	Blackberry Polyphenol Intake and Fuel Management		
Principal Investigator:	Janet A. Novotny	**Institution:**	USDA

For questions about your rights as a research participant, contact the MedStar Health Research Institute. Direct your questions to the Office of Research Integrity at:

Address: MedStar Health Research Institute
6525 Belcrest Rd.
Suite 700
Hyattsville, MD 20782

Telephone: (301) 560-2912
Toll Free: (800) 793-7175
Fax (301) 560-7336

IRB Approval Stamp
(ORA USE ONLY - DO NOT CHANGE ANY INFORMATION IN THIS SECTION)

Form Revision Date: 07/25/2010

IRB number:	2013-037	**Clinical Site IC Version:**	Version 3
Project Title:	Blackberry Polyphenol Intake and Fuel Management		
Principal Investigator:	Janet A. Novotny	**Institution:**	USDA

SIGNATURES

As a representative of this study, I have explained the purpose, the procedures, the possible benefits and risks that are involved in this research study. Any questions that have been raised have been answered to the individual's satisfaction.

Signature of Person Obtaining Consent _____ Date of Signature _____

Printed Name of Individual Obtaining Consent _____

I, the undersigned have been informed about this study's purpose, procedures, possible benefits and risks, and I have received a copy of this consent. I have been given the opportunity to ask questions before I sign, and I have been told that I can ask other questions at any time. I voluntarily agree to be in this study. I am free to stop being in the study at any time without need to justify my decision and if I stop being in the study I understand it will not in any way affect my future treatment or medical management. I agree to cooperate with Janet Novotny, PhD, and the research staff and to tell them immediately if I experience any unexpected or unusual symptoms.

Participant's Signature _____ Date of Signature _____

Printed Name of Participant:

_____

As the Principal Investigator (or his designee) for this research study, I have reviewed this individual's eligibility for enrollment in the study and agree that the individual is eligible to be enrolled subject to results of screening.

_____ _____

Principal Investigator's Signature

IRB Approval Stamp

(ORA USE ONLY - DO NOT CHANGE ANY INFORMATION IN THIS SECTION)

Form Revision Date: 07/25/2010

Appendix 5: Two day menu rotation from blackberry controlled feeding study

DAY 1	Quantity (g)	Protein (g)	CHO (g)	Fat (g)	Energy (Kcal)	Energy from Fat (kcal)	% Energy from Fat
Breakfast		**33.84**	**85.83**	**29.92**	**703.84**	**268.21**	**38**
cereal, multigrain, Cheerios	35	2.91	28.49	1.43	129.5	12.88	10
milk, whole, 3.25%	179	5.64	8.56	5.85	109.19	52.68	48
eggs, raw	85	10.68	0.61	8.08	121.55	72.75	60
butter, unsalted	8	0.07	0	6.49	57.36	57.36	100
Jimmy Dean, Turkey Sausage Patties	60	10.99	0.85	6.76	92.96	60.85	65
blackberries, frozen, unsweetened	302	3.56	47.32	1.3	193.28	11.69	6
Lunch		**23.25**	**51.43**	**30.03**	**567.87**	**269.21**	**47**
Kaiser Roll- Ottenbergs	62	6	33	1.5	170	13.5	8
Turkey breast, sliced,Hormel	42	7.5	0.75	1.13	45	10.13	23
Provolone cheese- Sargento	27	7.11	0	7.11	99.47	63.95	64
lettuce, romaine, fresh, outer leaf	16	0.2	0.53	0.05	2.72	0.43	16
tomatoes, fresh	32	0.22	1.51	0.11	7.57	0.97	13
Heinz: Mayonnaise	13	0	0	9.75	86.67	86.67	100
mustard, yellow, prepared	5	0.19	0.29	0.17	3	1.5	50
potato chips, classic	29	2.05	15.34	10.23	153.44	92.06	60
Dinner		**26.29**	**75.16**	**29.36**	**703.12**	**264.27**	**38**
lettuce, iceberg, fresh, shredded	53	0.48	1.57	0.07	7.42	0.67	9
tomatoes, cherry, fresh, year round average	32	0.28	1.24	0.06	5.76	0.58	10
carrots, fresh, medium	26	0.24	2.49	0.06	10.66	0.56	5
cucumber, fresh, medium	32	0.32	0.97	0	4.85	0	0
cheese, cheddar, sharp, finely shredded	29	6.21	1.04	9.32	113.93	83.89	74
Chicken, deli breast seasoned whole- US Foods	50	8.93	--	1.34	44.64	12.05	27
Thousand Island Dressing- Kraft	29	0	4.14	12.43	134.64	111.86	83
Croutons, homestyle, plain	23	3.29	--	3.29	98.57	29.57	30
Dinner Roll - Sysco	35	2.98	16.38	1.49	89.36	13.4	15
blackberries, frozen, unsweetened	302	3.56	47.32	1.3	193.28	11.69	6
Evening Snack		**6.23**	**57.72**	**17.25**	**423.89**	**155.24**	**37**
graham cracker, honey, Honey Maid	40	2.58	30.97	3.87	180.65	34.84	19
Nutella hazelnut chocolate spread	45	3.65	26.76	13.38	243.24	120.41	50
Daily Total		**89.61**	**270.15**	**106.56**	**2398.71**	**956.93**	**40**

DAY 2	Quantity (g)	Protein (g)	CHO (g)	Fat (g)	Energy (Kcal)	Energy from Fat (kcal)	% Energy from Fat
Breakfast		**28.21**	**114.44**	**19.59**	**709.86**	**176.3**	**25**
cereal, Wheaties	54	4.48	44.98	1.24	190.62	11.18	6
milk, whole, 3.25%	177	5.58	8.46	5.79	107.97	52.09	48
eggs, raw	94	11.81	0.68	8.94	134.42	80.45	60
bread, cinnamon swirl	26	2.79	13	2.32	83.57	20.89	25
blackberries, frozen, unsweetened	302	3.56	47.32	1.3	193.28	11.69	6
Lunch		**22.87**	**50.61**	**34.99**	**609**	**313.91**	**52**
Tortilla- Chi-Chi's	53	3.72	26.96	3.25	151.97	29.29	20
Chicken Breast, Fajita Flavor	60	11.79	2.14	2.68	79.84	24.1	30
lettuce, romaine, fresh, head	26	0.32	0.86	0.08	5.44	0.7	16
Parmesan cheese, grated, Kraft	6	2.4	0	1.8	25.8	16.2	68
salad dressing, Caesar	29	1.04	1.04	19.68	185.44	176.07	100
cracker, cheese, gold fish	33	3.61	19.61	7.5	160.38	67.54	42
Dinner		**20.67**	**122.39**	**43.76**	**966**	**394**	**41**
Pot Pie, Marie Calllender's	210	12.73	40.91	28.18	468.18	253.64	54
vegetables, California-style blend, frozen	80	0.94	4.71	0	22.6	0	0
whipped topping, frozen, non dairy	18	0	4	3	43	27	63
cake, pound, home baked style	52	3.44	25.45	11.28	217.08	101.56	47
blackberries, frozen, unsweetened	302	3.56	47.32	1.3	215.22	11.69	5
Evening Snack		**0.36**	**7.41**	**11.27**	**133**	**96.24**	**77**
carrots, baby, fresh	56	0.36	4.61	0.07	20.51	0.66	3
salad dressing, Ranch	28	0	2.8	11.2	112	95.59	90
Daily Total		**72.11**	**294.85**	**109.61**	**2417.86**	**980.45**	**41**

Appendix 6: 3T3-L1 Media compositions from ZenBio, inc.

3T3-L1 MEDIA COMPOSTIONS

3T3-L1 Adipocyte Medium (cat # AM-1-L1)	3T3-L1 Preadipocyte Medium (cat # PM-1-L1)
DMEM / Ham's F-12 medium (1:1, v/v)	DMEM, high glucose
HEPES pH 7.4	HEPES pH 7.4
Fetal Bovine Serum (FBS)	Bovine Calf Serum (BCS)
Biotin	Penicillin
Pantothenate	Streptomycin
Human insulin	Amphotericin B
Dexamethasone	
Penicillin	
Streptomycin	
Amphotericin B	

3T3-L1 Differentiation Medium (cat # DM-2-L1)	3T3-L1 Basal Medium (cat # BM-1-L1)
DMEM / Ham's F-12 medium (1:1, v/v)	DMEM/Ham's F-12 medium (1:1, v/v)
HEPES pH 7.4	HEPES pH 7.4
Fetal Bovine Serum (FBS)	Biotin
Biotin	Pantothenate
Pantothenate	
Human insulin	
Dexamethasone	
Penicillin	
Streptomycin	
Amphotericin B	
3-Isobutyl-1-methylxanthine (IBMX)	
PPARγ agonist	

NOTE:

All media except cat# PM-1-L1 contain 3.15g/L D-glucose.

PM-1-L1 contains 4.5g/L D-glucose.

All media are also available without serum and/or phenol red free.

Please inquire for custom media requests.

MEDIA EXPIRATION DATES:

If placed at 4°C upon arrival, the media is stable until the expiration date on the bottle label. If stored at -20°C upon arrival, it is stable for 6 months. Add fresh antibiotics when you are ready to use.

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