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Metabolic Response of Chardonnay Grape Marc in Hyperlipidemic, Overweight and Obese Adult Men and Women

By

FANNY LEE
DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

NUTRITIONAL BIOLOGY

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

Approved:

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2022

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Dissertation abstract

Atherosclerosis cardiovascular disease (ASCVD) is a complex, multifaceted condition that affects millions of adults; modifiable risk factors include weight, cholesterol, blood pressure and glucose control. Flavan-3-ols from cocoa and grapes and resveratrol from wine have been studied as strategies to improve vascular function, cholesterol, and glucose concentrations. Initiatives through the United States Department of Agriculture (USDA) have encouraged valorization of agricultural waste streams, such as wine production. The present study used Chardonnay grape marc (skins and seeds, also called pomace) blends, which builds upon the valorization efforts of the USDA yet also on the literature that phenolics provide a healthful addition to human diets. The purpose of this research was to determine whether results from previous animal trials that supplemented Chardonnay seed flour or Chardonnay seed extract, which identified improved cholesterol concentrations and glucose regulation, would translate to overweight, hyperlipidemic adult men and women. In addition, we were also interested in whether Chardonnay marc blends would impact gut hormones related to appetite control.

The present study was a 16-week, randomized, double blinded crossover trial. We collected data from a total of 27 participants, where 24 individuals completed the full trial, and 3 individuals withdrew from the study. Participants were recruited based on criteria of having elevated lipids but non-medicated. This study supplemented proprietary formulations of a high Chardonnay seed extract and Chardonnay marc blend (HE), high Chardonnay marc and Chardonnay seed extract blend (HM) and a non-grape comparator of microcrystalline cellulose (MCC) for 3-weeks per intervention.

Our first aim evaluated the impact of Chardonnay blends on ASCVD risk factors, specifically lipids and endothelial function. We concluded that there were no differences in fasting clinical lipid parameters of total cholesterol, low-density lipoprotein cholesterol (LDL-C), non-high density lipoprotein cholesterol (non-HDL-C) and triglycerides (TG). HDL-C was significantly lower following the HE intervention compared to both MCC and HM. Further NMR lipoprotein profiling revealed that the number of large HDL particles was significantly lower following HE compared to the MCC intervention. Postprandial triglyceride area under the curve was lower following the HM intervention compared to the HE intervention. There were no effects of the interventions on apolipoproteins AI, B, or CIII. Nor were there effects on oxidized LDL concentrations. Endothelial function, as measured by the finger plethysmography device EndoPat, was not affected by any of the interventions.

The next aim investigated whether the Chardonnay blends would have an impact on glucose regulation. The results indicated that following the HM intervention, fasting glucose, and insulin decreased, in addition to indexes of insulin resistance and sensitivity compared to the MCC intervention were improved. There were no changes following the interventions in postprandial glucose or insulin response. The HM intervention also resulted in decreased concentrations of acute phase inflammation marker, serum amyloid A (SAA), compared to both MCC and HE interventions. However, this decreased SAA was not associated with the improved glycemic outcomes.

The last aim explored the effects of the Chardonnay blends on gut hormones, ghrelin and peptide YY (PYY), as they related to appetite response. Here, we concluded

that the HE blend decreased subjective ratings of hunger compared to MCC, despite the lack of changes in ghrelin concentrations, which signals hunger. In addition, following the HM supplementation, the concentrations of the hormone PYY, which signals satiation, was significantly higher compared to HE. No other subjective ratings of appetite were significant.

This trial was the first to evaluate Chardonnay marc blends in numerous aspects of metabolism surrounding ASCVD. Phenolic compounds may not be the whole story when it comes to grape products and health potential. Our results indicate that the high marc blend has the potential to impact several risk factors and may be part of an overall strategy to reduce ASCVD risk. However, these are early results that should continue to be evaluated in future studies.

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Chapter 1

Chardonnay marc: A review of a wine byproduct's role in energy metabolism

1.1 Introduction

Chardonnay (*Vitis vinifera*) grapes are processed into two primary products: wine and marc (also called pomace) (*Figure 1.1*). Chardonnay wine is seen as the primary, desirable product. Chardonnay marc (CM) on the other hand, which are the remaining skins, seeds, and some stems not used in the winemaking process, is seen as a secondary, agricultural waste byproduct. In 2020, a total of 3.5 million tons of grapes were crushed in California where Chardonnay accounted for the largest proportion at 15.2% (Agriculture, 2020) Roughly 20-30% of crushed grapes is marc—meaning crushed Chardonnay grapes alone produced approximately 108,000 tons of marc.

Marc has been used to make grappa, as animal feed, composted, or discarded (Antonic et al., 2020). The winemaking process for white wines, including Chardonnay, involves pressing the grapes for the juice to ferment without the marc. In contrast to red winemaking, the grapes are crushed then the marc is macerated (i.e. fermented together) before the wine is pressed and separated from the marc. Grape seeds have been repurposed for grape seed oil in the culinary space and grape seed extract in the dietary supplement space, the skins are largely left unused. Whole marc is high in dietary fibers and phenolics— recent interest in valorization of this product has demonstrated effectiveness in using marc to fortify food products such as breads, dairy products and animal products as well as increasing the shelf-life of food items (Antonic et al., 2020). Due to the different winemaking processes, white wine grape marc retains both its extractable nutrients such as phenolics and non-extractable nutrients, while with

red wine grape marc much of its extractable nutrients are lost to the wine itself. Therefore, marc from white wine offers a rich source of both dietary fibers and phenolics that are often treated as agricultural waste.

With Chardonnay continuously being the single most produced wine variety in California, there is high potential for valorization of its marc. Utilizing the whole marc rather than isolating specific components allows for greater impact in diverting marc from waste streams. The purpose of this chapter is to review the compositional characteristics of CM and its potential effects on lipid, glucose metabolism and on gut hormones when ingested. In this chapter, we will predominantly consider the use of whole CM or CM components (e.g. seeds) and will consider grape seed extract (GSE) when studies with CM are not available.

1.2. Chardonnay marc compositional characterization

The "French paradox" brought attention to red wine. The prevailing theory was that the phenolic compounds in wine contributed to the low prevalence of coronary heart disease in the French population (Frankel et al., 1993; Kopp, 1998; Renaud & de Lorgeril, 1992). This likely spurred interest in studying the effects of grape seed extracts (GSE) on cardiovascular outcomes. However, grapes are highly complex and have more to offer than phenolics alone. This review is focused on Chardonnay specifically as it is a highly produced grape worldwide, including in California, thus making it an important agricultural product. Considering the winemaking process, upcycling its marc may have high potential for recapturing its nutrients that would otherwise be discarded.

The reported composition of CM is affected by a number of factors including but not limited to environmental growing conditions (e.g. soil, weather, drought, etc.),

growing regions and analysis techniques (e.g. extraction method) (Khanal et al., 2009; Pinasseau et al., 2017; Teixeira et al., 2013). CM is comprised of seeds, skins, pulp and some stems. Often, wine grape characterization papers focus on the seed component and phenolic compounds. Polyphenols are secondary plant metabolites that make up a vast group of diverse molecules. These molecules are commonly classified into broad groups of non-flavonoids (i.e. phenolic acids and stilbenes) and flavonoids (i.e. anthocyanins, flavones, flavanones, flavonols, flavanonol, isoflavones and flavan-3-ols). *Figure 1.2* shows the structures of select phenolics abundant in Chardonnay grapes. Chardonnay consistently is reported as having one of the highest amounts of total phenolics and flavan-3-ols compared to red wine grapes and other white wine grapes (de la Cerda-Carrasco et al., 2015; González-Centeno et al., 2013; Montealegre et al., 2006; Ricardo-Da-Silva et al., 1991; Yilmaz & Toledo, 2004).

Whole CM being a food ingredient goes beyond phenolics— dietary fibers, macro- and micronutrients must be considered as well. Due to the limited literature available on Chardonnay marc, compositional characterization was included for grape berries, skins alone, seeds alone, or whole marc in the common phenolics and macro- and micronutrients. *Table 1.1* summarizes the following sections' compositional phenolic descriptions.

1.2.1. Non-flavonoids

Phenolic acids

Phenolic acids are non-flavonoid phenolics that are categorized as either benzoic acid (e.g. vanillic acid, gallic acid) or cinnamic acid (e.g. caffeic acid, coumaric acid) derivatives. In Chardonnay grapes grown in Spain, the skin contained cis- and trans-

caftaric acid, cis- and trans-coutaric acid, and trans-fertaric acid, whereas the seeds only contained protocatechic acid (Montealegre et al., 2006). Gallic acid and vanillic acid have also been characterized from whole Chardonnay marc sourced from Sonoma, CA, Chile and New Zealand (de la Cerda-Carrasco et al., 2015; Lu & Yeap Foo, 1999; Sinrod et al., 2021). Seed fractions obtained from Italy characterized gallic acid and ellagic acid (Pasini et al., 2019). It is unclear whether the climate impacts the types of phenolic acids present in Chardonnay grapes and marc as these compounds are often not part of compositional characterizations.

Stilbenes

Stilbenes are a non-flavonoid polyphenolic compound and the mostly commonly known due to the "French paradox", is resveratrol. Stilbenes are primarily found in the skins of grapes and modestly in seeds. There is evidence that Chardonnay marc (whole and skins only components) contains resveratrol, and its seed fraction contains trans-resveratrol and trans-polydatin (Sinrod et al., 2021; Yilmaz & Toledo, 2004). However, currently there is limited characterization of stilbenes in Chardonnay grapes and marc to understand the quantity and other stilbene compounds that may be present.

1.2.2. Flavonoids

There are numerous classifications of flavonoids but 6 that are commonly found in the diet include: flavonols, flavones, flavanones, flavan-3-ols, anthocyanins and isoflavones (Ciumărnean et al., 2020; Manach et al., 2004). *Table 1.1* summarizes the phenolics discussed in this section. Isoflavones (e.g. daidzein, genistein) are predominantly found in soy rather than grapes, thus will not be further discussed here.

Flavonols

Flavonols are abundant in grape skins as production is stimulated by sunlight. Chardonnay grapes appear to increase production of flavonols with both water stress and increased sunlight (Teixeira et al., 2013). Chardonnay grown in a warm region of Spain was characterized to contain quercetin glucuronide, quercetin glucoside, kaempferol glucoside, and isorhamnetin glucoside in the skin (Montealegre et al., 2006). Whole Chardonnay marc from New Zealand characterized quercetin glucuronide, quercetin glucoside, kaempferol glucoside, and kaempferol galactoside (Lu & Yeap Foo, 1999). However, whole Chardonnay marc from Chile had no detectable levels of flavonols (de la Cerda-Carrasco et al., 2015). With limited available studies characterizing the flavonol profile of Chardonnay grapes and marc, it is unclear how much of an impact geography has on the types and quantity of flavonols present.

Flavones

Flavones (e.g. apigenin, luteolin) appear to be characterized in some red wines, and an array of both red and white Turkish grapes (Fang et al., 2007; Gambelli & Santaroni, 2004). However, it is unclear whether Chardonnay grapes and marc also contain flavones due to the lack of characterization.

Flavanones and flavanonols

Flavanones are widely found in citrus fruits and have been characterized in some red wines. It is thought that these compounds are created in the winemaking process (Mayr et al., 2018). It is unclear whether these compounds are present in Chardonnay grapes or marc, as these have not been characterized in the existing literature.

Flavanonols are not one of the commonly discussed classes of flavonoids, however are mentioned here as Chardonnay sourced from New Zealand were shown to contain astilbin and engeletin (Lu & Yeap Foo, 1999). These compounds have not been characterized in other studies, thus is it is unclear how prominent in Chardonnay grape and marc from different geographies.

Flavan-3-ols

Flavan-3-ols (or flavanols) exist as monomers and polymers. Its monomers exist as non-gallated (e.g. catechin, epicatechin) and gallated forms (e.g. gallocatechin, epigallocatechin). Its polymers are called *proanthocyanidins* (PAC) and the polymers which are comprised of only catechin and epicatechin monomer subunits are called *procyanidins* (PC). Flavan-3-ols are highly abundant on grape seed coating and are also found in the skins to a smaller degree. These compounds are the most widely discussed and characterized among Chardonnay grapes and marc. Teixeira et al. suggested that Chardonnay's levels of flavan-3-ols and PAC development are relatively consistent in different environments in the seeds, however in skins may be impacted by the environmental growing conditions (Teixeira et al., 2013).

Chardonnay marc has been characterized to contain the monomers: (+)-catechin, (-)-epicatechin, (-)-epicatechin gallate, (-)-gallocatechin, (-)-epigallocatechin, (-)-epigallocatechin gallate and (-)-catechin gallate (de la Cerda-Carrasco et al., 2015; Montealegre et al., 2006; Sinrod et al., 2021; Yilmaz & Toledo, 2004). Chardonnay marc has been characterized to contain the following polymers: procyanidins B1, B2, B3, B4, trimer 1 and 3 (de la Cerda-Carrasco et al., 2015; González-Centeno et al., 2013; Khanal et al., 2009; Ricardo-Da-Silva et al., 1991). In

terms of proanthocyanidins, it may be due to analytical challenges that there is currently only limited procyanidin characterization available in the literature.

Anthocyanins

Anthocyanins (e.g. malvidin, delphinidin) are responsible for the red, blue, purple color pigmentation. While these phenolics are abundant in red grapes, they are present in low amounts in white grapes (de la Cerda-Carrasco et al., 2015). For this reason, anthocyanins are not often quantified in white grapes therefore, will not be further discussed as a limitation in the literature.

1.2.3. Macronutrients

Carbohydrates

The carbohydrate fraction makes up a large portion of CM thus cannot be ignored. Whole CM is roughly 45% carbohydrates and roughly 24% dietary fiber, according to two reports (González-Centeno et al., 2010; Sinrod et al., 2021). Chardonnay marc contains the monosaccharides: mannose, arabinose, rhamnose, fucose, galactose, xylose, fructose, glucose, fructose; it also contains cellulose, hemicellulose and pectins.

Sinrod et al. characterized the oligosaccharide profile of whole Chardonnay marc and its components for the first time (Sinrod et al., 2021). They discovered a diverse array of unique oligosaccharides in the seedless and seed fractions. For example, the seedless fraction was abundant in Pent_4 HexA_1, whereas the seed fraction was abundant in Hex_3 Pent_1 HexA_1, among other oligosaccharides (Sinrod et al., 2021). Oligosaccharides are indigestible compounds that have documented prebiotic activity in

addition to other potential health promoting attributes such as glucose- and lipid-lowering effects (Muthukumaran et al., 2018).

Fat

The seed component contains the majority of the fat in the grape berry. Chardonnay seed flour and seed oil have been characterized and are abundant in monounsaturated fatty acids (MUFA) (~20%), and polyunsaturated fatty acids (PUFA) (~70%), particularly linolenic acid (18:2) (~70%) and oleic acid (18:1) (~20%) (Lutterodt et al., 2011; Parry et al., 2006; Wen et al., 2016).

Protein

The seed component contains much of the protein in the grape berry. Crude total protein levels of Chardonnay marc have been measured by Sinrod et. al where they reported whole marc contains roughly 16 mg/g dry weight and its seed portion contains 23.4mg/g dry weight (Sinrod et al., 2021). Amino acid characterization has not been conducted on Chardonnay grapes, however in Airén white wine grapes glutamic acid, glycine and glutamine were found to be the dominant amino acids in the seed fraction (Cejudo-Bastante et al., 2022). In other *Vitis Vinifera* varieties, amino acids of the respective marc's were rich in similar amino acids but also threonine as well (Chikwanha et al., 2018).

1.2.4. Micronutrients

Minerals

Minerals are present in the skin, flesh, and seeds of the grape berry and vary depending on the soil type that the grapes were grown. There are currently no published studies characterizing the mineral content of Chardonnay marc. However, in

Chardonnay grapes, there are at least 42 minerals that are found in the berry that are likely to be found in the marc as well (Bertoldi et al., 2011).

Vitamins

Vitamin content of Chardonnay grapes and marc are not often published. As part of a study evaluating Chardonnay seed oil and remaining seed flour for oxidative stability and antioxidant properties, carotenoids and tocopherols were measured (Lutterodt et al., 2011). Other *Vitis vinifera* varieties and table grapes contain riboflavin, thiamine, pyridoxine, ascorbic acid, pantothenic acid, tocopherol, and carotenoids (Aubert & Chalot, 2018; Juhász et al., 1987; Stranska et al., 2021). Chardonnay likely has a similar composition of vitamins; however, the current literature lacks this information. Notably though, drying techniques (e.g. freeze drying, infrared drying, drum drying) and milling techniques (e.g. hammer mill, impact mill, ball mill) vary on the intensity of heat and could affect nutrient loss.

1.3. Chardonnay marc component bioavailability

Currently, there are no studies that have evaluated the absorption, distribution, metabolism, and excretion (ADME) of CM in animal models or humans. Whole marc likely has a unique food matrix that could affect the bioavailability of its constituents. The limited literature on grape marc bioavailability in humans utilizes red grape marc extract and focuses on phenolics.

Two studies have evaluated red grape marc extract in humans, one was primarily focused on identifying the phenolic metabolites in the urine and the other on the pharmacokinetics of the phenolics in plasma and urine (Castello et al., 2018; Sasot et al., 2017). In the pharmacokinetics study, 10 men consumed 250mL of red grape marc

extract beverage and had plasma collected at fasting then every hour for 8h, then a fasting sample 24 hours later, while urine was collected over a 48h period (Castello et al., 2018). The red marc extract contained gallic acid, flavan-3-ols (catechins and procyanidins), flavonols and anthocyanins. Epicatechins and its derivatives peaked in the plasma ~2h post consumption and cleared in ~6h in the urine (Castello et al., 2018). Phenyl-γ-valerolactones (PVL) are the predominant colonic metabolites of flavan-3-ols and the data demonstrated that PVL's were the majority of the metabolites found in both plasma and urine; they peaked in the plasma around 4h and peaked around 6h in urine (Castello et al., 2018; Mena et al., 2019b). Hydroxybenzoic acid and other phenolic acids were abundant metabolites in the plasma (peaks at different times, 2, 4, 6h) and urine (peak at 3-6h), which may have been metabolites from anthocyanins (Castello et al., 2018). It is unclear what the metabolic fates of the other flavonoids were. Procyanidins and large proanthocyanidins are not as well absorbed, but are rather metabolized and fermented by colonic microbes (Mena et al., 2019b; Zhang et al., 2016).

The kinetics data corroborate well with a radiolabeled (-)-epicatechin bioavailability study that identified (-)-epicatechin and its derivatives as highly absorbed with much of the metabolites stemming from microbial metabolism (Ottaviani et al., 2016). The red marc study showed a plasma catechin level peak at around 2hs, while the pure (-)-epicatechin showed a plasma peak around 1h, which could indicate that a mix of different flavonoids and nutrients may affect the absorption.

Comparing the bioavailability of red marc extract (*Vitis vinifera* Tempranillo) and white marc extract (*Vitis vinifera* Verdejo) in Wistar rats demonstrated that there was a

dose dependence of plasma and urine metabolites in the white marc but not in the red (Gerardi et al., 2020). In addition, the phenolic metabolites peaked sooner in the plasma following the white marc compared to the red marc extract (2h vs 4h post intake, respectively) (Gerardi et al., 2020). While rat physiology is different than human physiology, this is the only direct comparison of red and white marc extracts and demonstrated a difference in bioavailability, and suggests that the components of white marc such as CM may have a different ADME than those of red marc. As discussed previously, whole CM also contains fatty acids, dietary fibers, minerals and more. While the focus is not to discuss each of the components individually, it is of note that they make up the whole food matrix, which further emphasizes the need to study the bioavailability of the nutrients that make up whole marc.

1.4. Cardiovascular health and Chardonnay marc

1.4.1. Atherosclerosis and cardiovascular disease risk factors

Atherosclerosis is the hardening and narrowing of the intima layer of arteries due to accumulation of fat, cholesterol, calcium and other material in plaques. Low density lipoprotein cholesterol (LDL-C) may accumulate and become oxidized, attracting monocytes and ultimately forming lipid-filled macrophages, also called foam cells (Kattoor et al., 2017). Plaque buildup limits the flow of blood and can affect any artery in the body's vascular system. Different vascular diseases may develop depending on the affected artery. Risk factors include: overweight or obesity; high levels of low density lipoprotein cholesterol (LDL-C); low levels of high density lipoprotein cholesterol (HDL-C); high levels of triglycerides (TG); elevated blood pressure; visceral adiposity; insulin resistance; inflammation; tobacco use; and low physical activity (Lechner et al., 2020;

Libby et al., 2019). This section will discuss the lipid-related factors. Glucose-related factors are addressed in a later section.

Chardonnay grape seed extract (ChSE) was the first material evaluated in a diet induced obese (DIO) hamster model. Like humans, hamsters have lipoprotein mediated reverse cholesterol transport mechanisms making them a preferred animal model for lipid metabolism. ChSE was supplemented via gavage to animals fed a high fat diet. ChSE supplemented hamsters gained less weight compared to the high fat fed controls (Decorde et al., 2009). Authors did not report lipoprotein cholesterol distribution, only measuring triglycerides which were not changed compared to controls (Decorde et al., 2009).

Years later, a different group of scientists utilized whole Chardonnay seed flour (ChSF) supplemented as 10% of a high fat diet first in a DIO hamster model to evaluate lipid metabolism, then in a DIO mouse model to explore lipid and glucose metabolism with the added use of global gene expression with microarrays. Following the ChSF feeding in DIO hamsters, there were significantly lower levels of very low density lipoprotein cholesterol (VLDL-C), LDL-C, total cholesterol (TC), no changes to HDL-C, decreased weight gain, hepatic lipid content and visceral fat compared to the high fat control group (Kim et al., 2014). In the DIO mice fed with ChSF, only LDL-C was lower while there was no change in the other lipoproteins. These mice also had less weight gain and lower hepatic lipid content compared to the control group (Seo et al., 2016). Impacts on liver and adipose suggested that ChSF may reduce CVD risk by decreasing inflammation and fatty acid synthesis while increasing bile acid synthesis, particularly from cholesterol, increasing cholesterol synthesis and increasing fatty acid oxidation

(Kim et al., 2014; Seo et al., 2016; Seo et al., 2015). Bile acids (BA) play an important role in lipid and glucose homeostasis through the farnesoid X receptor (FXR), a nuclear receptor highly expressed in hepatocytes and intestinal segments including the ileum and colon. GSE procyanidins may act as a co-ligand with the BA chenodeoxycholic acid (CDCA), to modulate TG lowering (Downing et al., 2017). However, with procyanidins having low bioavailability, it is questionable whether this is a viable mechanism of action on hepatocytes to lower TG.

Endothelial dysfunction is a CVD risk as it precedes atherosclerosis. One device that measures endothelial dysfunction is an EndoPat device, which measures endothelium-mediated changes from finger plethysmographic probes on each hand to determine peripheral arterial tone (PAT) (Axtell et al., 2010). It measures a reactive hypermia index (RHI), a blood pressure cuff is placed on the non-dominant arm and blood flow is occluded for 5 minutes (Axtell et al., 2010). The PAT measures blood flow before and after the occlusion period and calculates a ratio. Endothelial dysfunction as defined by an Endopat is considered when there is a RHI score of ≤ 2 (Corban et al., 2020).

A single reported human trial supplemented 4.8g of ChSF in the form of capsules, daily for 4 months. In this study, control supplements were low in phenolics but also included grape seed oil. Participants were recruited based on having endothelial dysfunction using an EndoPat RHI score of ≤ 2 and were otherwise healthy (Corban et al., 2020). Following the supplementation period, both the ChSF and control groups had a significant increase in RHI compared to baseline to normal endothelial function range, however the two interventions were not different from each other. There

were no significant changes in blood pressure or lipoproteins, however there was a significant decrease in TG seen in the control arm (Corban et al., 2020). ChSF did improve endothelial dysfunction, but the control with grape seed oil in it provided improvement as well. As this study did not have a non-grape control or "placebo", the possibility of a placebo effect remains unclear.

This result begs the question of how much of a factor do phenolics play a role. Compositionally speaking the ChSF had more phenolics and more dietary fiber, while the control had higher monounsaturated fatty acids. TG, while clinically normal at baseline for both groups, was decreased by the control and was significantly different from the ChSF group. The results are difficult to decipher as both arms had a grape product in them, however it may not be only the phenolics providing beneficial effects. More work needs to be done in humans to evaluate lipoproteins and other CVD risk factors to determine whether CM has effects that were seen in animal models. Oxidized LDL-C has not been measured in any Chardonnay studies to date, however it pays role in a pro-inflammatory state in the development of atherosclerosis. The evidence has demonstrated that Chardonnay reduces oxidative stress and has the downregulated genes related to inflammatory pathways, thus may have a potential to reduce the inflammatory state around LDL-C becoming oxidized, however there needs to be studies to evaluate this aspect as well.

1.4.2. Postprandial triglyceride response

Individuals commonly eat 3 meals and snacks throughout the day, thus spending upwards of 18 hours in the fed state— therefore, the non-fasting state may be more pertinent to examine than fasting. Non-fasting TG levels may be emerging as a

predictor of cardiovascular disease (CVD) (Enkhmaa et al., 2010). Data from the Women's Health Study found non-fasting TG were associated with cardiovascular events independent of other risk factors whereas fasting TG did not show an independent relationship (Bansal et al., 2007). Similarly, the Copenhagen Heart Study provided additional evidence of elevated non-fasting TG with cardiovascular events (Langsted et al., 2011; Nordestgaard et al., 2007).

To our knowledge, there are currently no published studies that have evaluated postprandial TG responses in animal models or in humans following CM consumption. However, briefly considering studies that used material with similar compositional characteristic elements may provide insight into postprandial TG response following CM consumption.

Gutiérrez-Salmeán et al supplemented pure (-)-epicatechin at 1 mg/kg in normal and overweight men and women prior to consuming an Ensure® beverage as an oral metabolic tolerance test meal (Gutierrez-Salmean et al., 2014). Blood samples were collected at 0, 2 and 4 h to assay for ppTG. Decreases in postprandial TG levels were seen in both normal and overweight individuals following the (-)-epicatechin consumption compared to the control (no supplementation), which was significantly different at 2 h; these results were corroborated with indirect calorimetry data that demonstrated a lower respiratory exchange ratio (RER) indicative of fat oxidation (Gutierrez-Salmean et al., 2014). These results were more pronounced in the overweight group than the normal weight group.

Two rodent studies utilizing tea catechin extracts, which primarily comprised of gallated catechins (i.e. epigallocatechin gallate) and some epicatechins, evaluated

postprandial TG response following lipid emulsions; they both observed decreases in postprandial TG response (Suzuki et al., 2005; Toyoda-Ono et al., 2007). Similarly in human trials using tea catechins supplemented at 200-800mg prior to consuming a high fat meal, there were decreases in postprandial TG at 2 h (de Morais Junior et al., 2020; Unno et al., 2005). Toyoda-Ono et al. and Suzuki et al. suggest that the tea catechins suppress the TG absorption via the lymphatic system.

Taken together, CM may have similar effects given the abundant catechin levels, however whole CM also includes both soluble and insoluble phenolics, in addition to dietary fibers. This dietary complexity is not seen in any of the studies described. However, consuming whole CM likely will not have the same high concentrated amounts of catechins as the previous studies.

1.4.3. Lipoprotein subfractions

Lipoprotein particles are complex assemblages of lipids and proteins that transport hydrophobic lipids (e.g. cholesterol and triglycerides). These particles are highly variable in size, density, and lipid content. The four major classes defined by density are the chylomicrons, VLDL, LDL and HDL. VLDL, LDL and HDL can be further sub-classified into large, medium, and small particles. Chylomicrons and VLDL are both TG rich particles, chylomicrons are the largest particles carrying dietary triglycerides from the intestine following a meal, while VLDL are large but smaller than chylomicrons and carry TG made by the liver. Intermediate density lipoprotein (IDL) and LDL are derived from VLDL, as the VLDL TGs are depleted by lipases in the periphery, that determines the size of the LDL. For instance, higher plasma TG levels are associated with more small-medium dense LDL particles (Borén, Chapman, et al., 2020). It is

thought that IDL and small dense LDL are pro-atherogenic and have been associated with higher CVD risk (Borén, Chapman, et al., 2020; Ivanova et al., 2017; Liou & Kaptoge, 2020; Pichler et al., 2018; Tribble et al., 1995; Williams et al., 2014). HDL is involved with reverse cholesterol efflux (i.e. transport of cholesterol from peripheral tissues to the liver) and low levels are associated with CVD risk. It is thought that large HDL particles are actually the particles responsible for the anti-atherogenic properties and there are studies that associate large HDL with lower CVD risk (Kontush, 2015; Li et al., 2016).

The ChSF animal trials evaluated lipoprotein (VLDL-C, LDL-C, HDL-C) fractions through size exclusion chromatography, however the authors did not report other lipoprotein subfractions (i.e. small, medium large particles). At present, a concord grape study and an oolong tea study are the most similar products that have evaluated lipoprotein particle size to some degree. The concord grapes have some flavan-3-ols and flavonols that are in common with CM, but predominantly have anthocyanins and oolong tea is rich in flavan-3-ols. In the concord grape power supplementation trial conducted in humans, observed a reduction in large LDL-C particles (Zunino et al., 2014). Following an oolong tea trial, LDL particles increased in size, while statistically significant, it is not clear how clinically relevant this finding is in terms of atherosclerosis risk (Shimada et al., 2004). More research is needed to gain a better understanding of this aspect of CVD risk and how CM could influence these factors. The existing literature on the major lipoprotein classes are promising but diving deeper into the subclasses is not clear for CM’s potential.

1.5. Chardonnay marc and glucose regulation

Roughly 10% (34.2 million) of people in the United States have been diagnosed with diabetes mellitus and 34.5% (88 million) have prediabetes (*National Diabetes Statistics Report, 2020*, 2020). In the case of prediabetes and Type 2 diabetes mellitus (T2DM), the dysregulation of glucose metabolism can be attributed to some form of insulin resistance (IR). Impaired fasting glucose (IFG) may be attributed to hepatic IR, whereas impaired glucose tolerance (IGT) may be attributed to muscular and adipose IR. Glucose homeostasis is highly complex and for the purpose of this review, we will limit the discussion to CM and/or GSE direct effects on glucose and insulin, factors that impact glucose homeostasis such as inflammation, adiponectin, and glucagon-like peptide-1 (GLP-1).

DIO hamsters given ChSE gavages lowered fasting glucose compared to the control but saw no impact on fasting insulin (Decorde et al., 2009). These hamsters had higher levels of adiponectin, less abdominal fat and gained less weight than control animals (Decorde et al., 2009; Li et al., 2020). DIO hamsters fed with 10% ChSF diets, on the other hand, did not see a change in fasting glucose, however they did have reduced hepatic lipid content (Kim et al., 2014). Similar to the ChSE supplemented hamsters, DIO mice fed with 10% ChSF diets had lower fasting glucose, and improved glucose and insulin tolerance (Seo et al., 2016; Seo et al., 2015). These mice gained less weight, had less visceral fat and less hepatic lipids (Seo et al., 2016; Seo et al., 2015). In addition, genes involved in inflammatory processes and ceramide de novo synthesis were downregulated (Seo et al., 2016; Seo et al., 2015). These data suggest that Chardonnay marc may affect glucose homeostasis in a multifaceted way. The

decreased hepatic steatosis and visceral fat suggest protection from hepatic IR that may have driven the lower fasting glucose. Since fat in the viscera is drained by the hepatic porta vein excess fat may expose the liver to excess free fatty acids, which could increase TG synthesis and disrupt insulin signaling in the liver (Neeland et al., 2019). Ceramides are integrally involved in intercellular insulin signaling (Galadari et al., 2013; Reali et al., 2017; Sokolowska & Blachnio-Zabielska, 2019). Excess ceramides are thought to negatively impact insulin signaling leading to insulin resistance, and their inhibition or degradation are associated with improved insulin sensitivity (Sokolowska & Blachnio-Zabielska, 2019).

While only the ChSE study measured adiponectin and the ChSF studies did not, this finding suggests that Chardonnay flavan-3-ols may increase plasma adiponectin levels which has insulin sensitizing effects, with proposed mechanism to reduce ectopic lipid deposition associated with hepatic and muscular IR, stimulate fatty acid oxidation and improve insulin signaling (Li et al., 2020). GLP-1 is an incretin hormone, secreted by intestinal L-cells, which enhances insulin secretion in response to glucose consumption (Röhrborn et al., 2015). Flavan-3-ol-rich GSE was shown to stimulate the release of GLP-1 in rat models, with procyanidins inhibiting the activity of dipeptidyl-peptidase 4 (DPP4), an enzyme which degrades and inactivates GLP1 (Röhrborn et al., 2015). In *in vitro* studies, flavan-3-ols also stimulated the differentiation of L-cells that secrete GLP-1 (Casanova-Martí et al., 2020; González-Abuín et al., 2012; González-Abuín et al., 2014). Intestinal FXR activation by BA can also stimulate the secretion of GLP-1, which is intriguing if procyanidins had an effect on intestinal FXR in this aspect on glucose homeostasis as they are more likely to reach the ileum and colon due to

their low bioavailability (Ahmad & Haeusler, 2019). There is also suggested mechanism of intestinal FXR modulating glucose homeostasis through lowering of ceramide production (Ahmad & Haeusler, 2019). While these data are in animal and *in vitro* models, they demonstrate the potential that Chardonnay may modulate glucose homeostasis.

In a human trial of normoglycemic individuals, ChSF supplemented in the form of capsules had no effect on fasting glucose (Corban et al., 2020). Future studies in hyperglycemic individuals are warranted. No studies have been conducted using whole CM to evaluate glucose homeostasis in humans, the animal trials give an indication that there may be a beneficial effect, but the existing human trial is not sufficient to evaluate its effect.

1.6. Chardonnay marc and satiety, food intake and hormones

Individuals with overweight or obesity are at a higher risk for numerous chronic metabolic conditions, particularly cardiovascular disease and Type 2 diabetes, as previously discussed. Weight loss is often recommended as a strategy to reduce the risk. Energy balance is achieved when energy intake equals energy expenditure, thus weight gain is positive energy balance and weight loss is a negative energy balance. However, regulation of energy balance as it relates to food intake is highly complex and involves both neural input as well as hormonal feedback. Appetite is regulated by adipokines (e.g. leptin and adiponectin), gastrointestinal hormones (e.g. ghrelin, peptide YY [PYY], insulin, glucagon, GLP-1), and neuroendocrine peptides (e.g. neuropeptide Y, agouti-related peptide). The satiating effects of fiber has been studied and debated,

however there is limited studies on the effects of flavonoids (Adam et al., 2016; Clark & Slavin, 2013; Warrilow et al., 2019).

The literature on CM has evaluated the adipokines leptin and adiponectin. However, these studies have been in the context of insulin resistance, inflammation, and fat oxidation rather than in the context of satiety. Plasma leptin was significantly lower following the consumption of ChSE and ChSF in DIO hamsters and mice, respectively (Decorde et al., 2009; Seo et al., 2016). Leptin receptors were upregulated following ChSF as well (Seo et al., 2016). Meanwhile, ChSF fed hamsters showed no reduction in expression of the leptin gene and plasma concentrations were not measured (Kim et al., 2014). These results were attributed to improving insulin sensitivity and reducing fat accumulation (Decorde et al., 2009; Seo et al., 2016). Interestingly, leptin is a hormone that reduces food intake, yet ChSF fed mice had significantly higher energy intake than the high fat diet controls but gained significantly less weight (Seo et al., 2016; Zanchi et al., 2017). However, with the hamsters given ChSE, their food intake was significantly less than high fat diet controls (Decorde et al., 2009). Phenolics, as secondary plant metabolites, have a bitter taste to protect the plant from predators (Soares et al., 2013). Flavan-3-ols in particular have been found to activate specific bitter taste receptors, thus it is unclear whether the decrease in food intake seen with the ChSE is due to food aversion or impacts on appetite or satiety (Soares et al., 2013).

Following the ChSE supplementation, plasma adiponectin levels were higher than the high fat diet control group; whereas following the ChSF diet in hamsters there was no difference in adiponectin gene expression and plasma adiponectin

concentrations were not measured (Decorde et al., 2009; Seo et al., 2016). This result was similarly attributed to increasing fatty acid oxidation and reducing insulin resistance (Decorde et al., 2009). Adiponectin acts to reduce food intake, however as mentioned previously it is unclear whether decrease in food intake seen with the ChSE supplementation is due to the bitter taste or a pharmacological effect of the high flavan-3-ols levels (Decorde et al., 2009; Kubota et al., 2007; Lee & Shao, 2014). Meanwhile, the mice on the high flavan-3-ol ChSF diet saw an increase in food intake; without measurements such as adiponectin or other satiety hormones, it is unclear whether the change in food intake was caused by a change in satiety hormones (Seo et al., 2016).

The effect of CM on gut hormones has not been evaluated, however there are limited data from other GSE studies. Ghrelin, GLP-1, PYY and cholecystokinin (CCK) are the gut hormones that have been evaluated thus far in the GSE literature. Ghrelin is a hormone that stimulates hunger, whereas GLP-1, PYY and CCK all increase satiety and inhibit gastric emptying (Zanchi et al., 2017). Serrano and colleagues published a series of studies conducted with Wister rats demonstrating GSE supplementation led to increased GLP-1, however also demonstrated that the food inhibiting behavior required a high enough dosage of GSE (González-Abuín et al., 2014; Serrano, Casanova-Martí, Blay, et al., 2016; Serrano, Casanova-Martí, Gil-Cardoso, et al., 2016). *In vitro* and *ex vivo* studies demonstrate the ability of GSEs to increase PYY, CCK and decrease ghrelin meanwhile rat studies utilizing specific higher doses of GSE translate these findings to effects on food intake (Ginés et al., 2019; Grau-Bové et al., 2020; Serrano et al., 2017; Serrano, Casanova-Martí, Blay, et al., 2016; Serrano, Casanova-Martí,

Depoortere, et al., 2016). Similar studies have not been conducted in humans to evaluate the translatability of these findings.

The only GSE supplementation study done on humans evaluating food intake and satiety utilized supervised ad libitum lunch and dinner meals, while satiety was measured through visual analog scales without hormone measurements (Vogels et al., 2004). Authors observed subjects who required <1800kcal/d decreased their food intake by 4% while other measures of satiety were unaffected (Vogels et al., 2004). Energy intake is difficult to measure especially in a controlled setting where study food fatigue may occur. Additional human trials are needed to evaluate this finding. While whole CM contains the flavan-3-ol monomers and oligomers that are found in the GSE used in these studies, if a high dosage of flavonoids is required to stimulate these actions, as seen in the animal studies, it is unlikely that CM will achieve those levels. However, CM has a complex composition that includes fiber thus it may have satiating effects through other mechanisms.

1.7. Conclusion

Chardonnay is the largest single varietal of grape produced in California and currently the marc is a large source of agricultural waste. Compositionally, Chardonnay marc is nutritionally complex and is particularly rich in flavonoids and fiber. The valorization of CM has high potential for not only diverting agricultural waste to other uses but also for human health. Currently, the literature is limited to CM components (e.g. seed flour and seed extract) however they demonstrate the multifaceted avenues that Chardonnay may improve glucose and lipid homeostasis. Future studies are

needed to evaluate whole CM to determine if it is as effective as its components and if those results are seen in humans.

Figures

Figure 1.1. Schematic of the two processing fates of Chardonnay grapes.

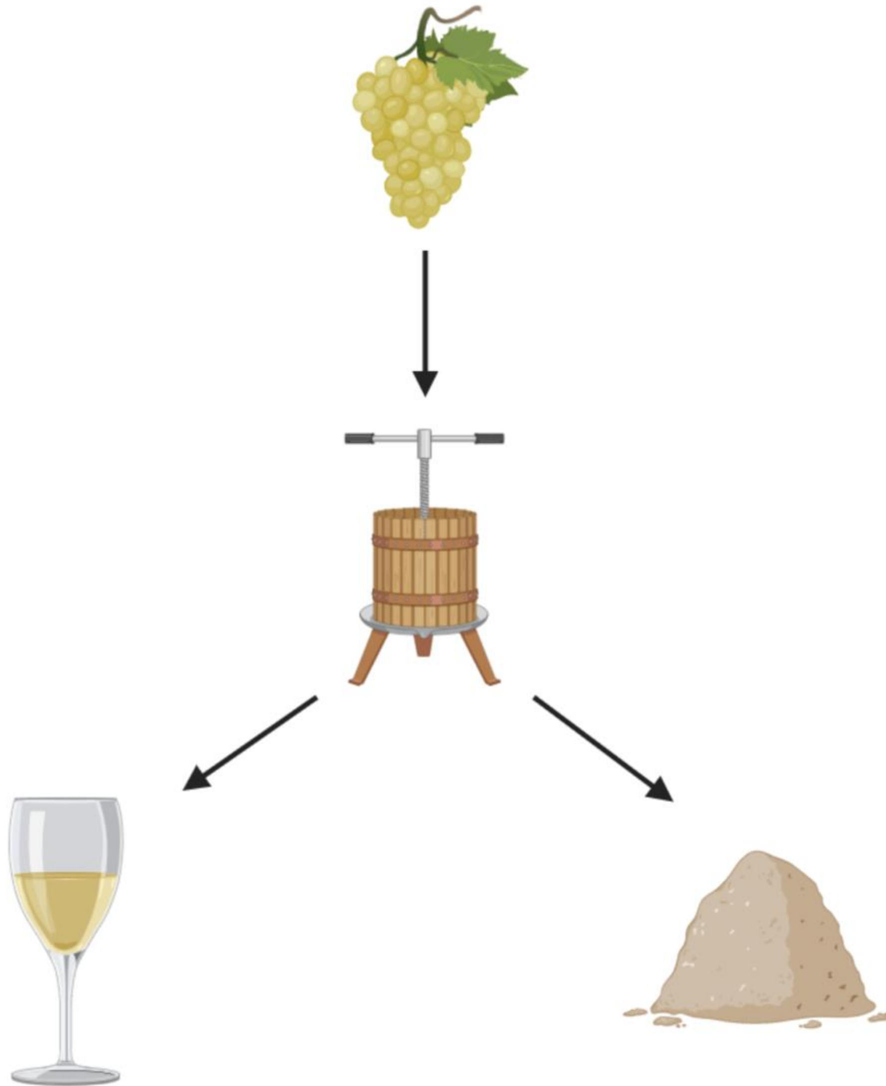

Figure 1.1. Chardonnay grapes have two processing fates where the pressed juice is fermented to become wine. The remaining skins, seeds and some stems in this case are dried and milled to create marc powder. Figure was created in Biorender.com

Figure 1.2. Select structures of abundant phenolics found in Chardonnay grapes

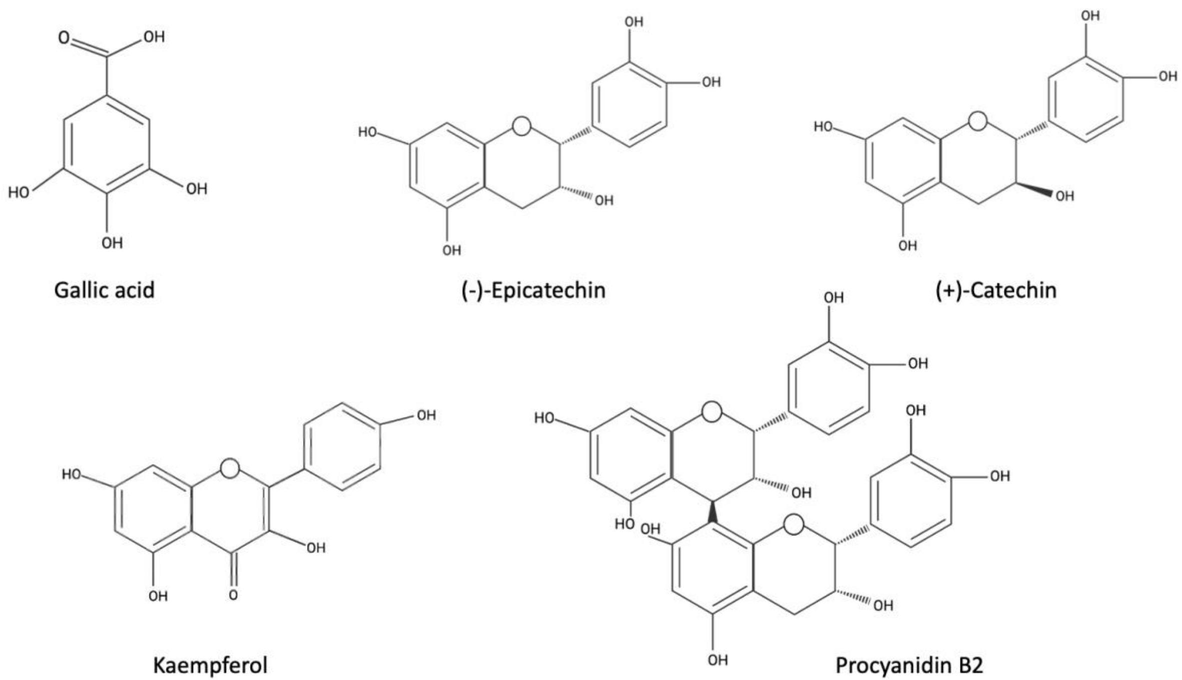

Figure 1.2. Select phenolics structures that are abundant in Chardonnay grapes are depicted. Figure was created in Biorender.com

Tables

Table 1.1. Summary of phenolic compounds in Chardonnay components

Non-Flavonoids			Flavonoids		
	Skin	Seeds		Skin	Seeds
Phenolic acids			**Flavonols**		
cis- and trans-caftaric acid	+	?	quercetin glucuronide	+	
cis- and trans-coutaric acid	+	?	quercetin glucoside	+	
trans-fertaric acid	+	?	kaempferol glucoside	+	
protocatechic acid	-	+	isorhamnetin glucoside	+	
ellagic acid	?	+	**Flavones**		
gallic acid	+	++	apigenin	?	?
vanillic acid	+	++	luteolin	?	?
Stilbenes			**Flavanones**	?	?
trans-resveratrol	+	+	**Flavanonols**		
trans-polydatin	-	+	astilbin	?	?
			engeletin	?	?
			Flavan-3-ols		
			(+)-catechin	+	++
			(-)-epicatechin	-	++
			(-)-epicatechin gallate	+	+
			(-)-gallocatechin	+	-
			(-)-gallocatechin gallate	-	+
			(-)-epigallocatechin	-	++
			(-)-epigallocatechin gallate	-	++
			(-)-catechin gallate	-	++
			Procyanidin B1		++
			Procyanidin B2		++
			Procyanidin B3		++
			Procyanidin B4		++
			Procyanidin trimer 1		++
			Procyanidin trimer 3		++
			Anthocyanins		
			malvidin	-	
			delphinidin	-	

++ highly abundant; + present; - not detected; ? unknown

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Chapter 2

Effects of Chardonnay marc on atherosclerosis cardiovascular disease risk factors

2.1. Introduction

Atherosclerosis is a slow progressing condition that can virtually affect any blood vessel in the body and has complex ties to many chronic conditions (e.g. cardiovascular disease, diabetes, high blood pressure, kidney disease etc.). It is characterized by the hardening and thickening of blood vessels from the accumulation of fat, cholesterol, and other substances in arterial walls. Atherosclerosis cardiovascular disease (ASCVD) specifically afflicts the cardiovascular system and may lead to decreased blood flow or even a cardiovascular event. Endothelial dysfunction (i.e. abnormal vascular reactivity) is one of the earliest indications of the ASCVD (Gimbrone & García-Cardeña, 2016). Risk factors include: overweight or obesity, high concentrations of low density lipoprotein cholesterol (LDL-C), high triglycerides (TG), elevated blood pressure, low concentrations of high density lipoprotein cholesterol (HDL-C), insulin resistance and low levels of physical activity (Lechner et al., 2020; Libby et al., 2019).

The management of ASCVD is complex and should be multifaceted to reflect the numerous treatable risk factors. Currently the most common pharmaceutical target has been LDL-C using statin drugs—however, residual risk of cardiovascular events remains high (Matsuura et al., 2019; Wong et al., 2017). Non-fasting TG is an emerging independent risk factor for CVD and cardiovascular events, thus should also be

considered in disease management (Bansal et al., 2007; Keirns et al., 2021; Kolovou et al., 2019; Nordestgaard et al., 2007).

The “French Paradox” sparked interest in red wine phenolic compounds (particularly resveratrol) and its relationship to cardiovascular protection (Renaud & de Lorgeril, 1992). In a recent meta-analysis evaluating grape polyphenols, predominantly from red grapes, authors point to positive benefits of grape phenolics on CVD risk factors, but specifically point to whole grapes having more benefit than extracts (Lupoli et al., 2020). While much attention is on red wine and red grapes, white wines and their respective grapes have had less attention. When wine grapes are processed, the juice is used for winemaking and the marc (also called pomace, which is the remaining skins, seeds, and stems) is treated as agricultural waste. In red winemaking, the marc is macerated and in white winemaking the marc is discarded, therefore white wine grape marc retains both its extractable nutrients such as phenolics and non-extractable nutrients. However, with red wine grape marc much of its extractable nutrients are lost to the wine itself. Roughly 20-30% of crushed grapes is marc and with Chardonnay being the single most produced variety of wine grape in California, there is high potential for diverting agricultural waste and recapturing the nutrient rich material for valorization. Beyond having a rich source of phenolics, such as (-)-epicatechin, Chardonnay marc is high in dietary fiber and interestingly has a diverse oligosaccharide profile (de la Cerda-Carrasco et al., 2015; Sinrod et al., 2021).

Chardonnay seed flour (ChSF) supplementation in diet induced obese hamster and mice models resulted in lower very-low density lipoprotein cholesterol (VLDL-C), LDL-C, total cholesterol (TC), overall weight gain, and hepatic fat accumulation

compared to the non-grape comparator fed animals (Kim et al., 2014; Seo et al., 2016). These data suggest that ChSF may potentially improve ASCVD risk factors. Currently, the only human trial conducted supplementing ChSF saw improvements in endothelial dysfunction but not in other ASCVD risk factors (Corban et al., 2020). In this study, participants were specifically recruited with endothelial dysfunction and had otherwise clinically normal lipid concentrations. Whether whole Chardonnay marc or its components has similar effects on cholesterol as seen in animal trials is unclear.

The first aim of this study was to evaluate and compare the effectiveness in improving ASCVD risk factors, specifically plasma cholesterol and triglyceride concentrations, following supplementation with a Chardonnay marc rich blend and a Chardonnay seed extract rich blend in adult men and women. This study was the first to test these blends, so the following hypotheses are based on evidence in the literature and knowledge of action of blend component compounds. As the Chardonnay marc-rich blend and ChSF both contain fiber and phenolics, with marc blend phenolics being much lower, the hypothesis is based on previous ChSF studies with the addition of fiber effects on ASCVD risk. I hypothesized that total cholesterol and LDL-C concentrations would be improved in response to the Chardonnay marc rich blend, whereas there would be no effect on HDL-C, triglycerides, or endothelial function. With the Chardonnay seed extract rich blend, I based my hypothesis on previous animal studies utilizing Chardonnay seed flour rich in flavan-3-ols in addition to flavan-3-ol effects on ASCVD risk factors. Therefore, I hypothesized that the Chardonnay seed extract rich blend would lead to improved total cholesterol, LDL-C, triglycerides but have no effect on HDL-C or endothelial function.

2.2. Methods

2.2.1. Study participants

Men and women between the ages of 35-65 years were included in this study to increase the generalizability of the results. Other eligibility criteria included body mass index (BMI) ≥25 but <40 kg/m^2 and at least one marker of dyslipidemia as defined as, total cholesterol (TC) >190 mg/dL but <300 mg/dL, low density lipoprotein cholesterol (LDL-C) >130 mg/dL but <180 mg/dL, high density lipoprotein cholesterol (HDL-C) <40 mg/dL (men)/<50 mg/dL (women) and fasting triglycerides (TG) >150 mg/dL but <400 mg/dL. These specific ranges were chosen as they are the upper end of normal plasma lipid concentrations. The broad lipid inclusion factor was a decision to increase the likelihood of recruiting participants on a constrained budget.

Participants were excluded from the study if they self-reported metabolic diseases, cardiovascular disease, gastrointestinal disorders, or other chronic conditions; history of cardiovascular events, presence of atrial fibrillation, pacemaker or other internal electrical device controlling the rhythm or pacing of the heart; pregnancy or lactation; use of lipid-lowering, glucose-lowering, anti-hypertensive or weight-loss medication; use of antibiotics in the past 3 months; food sensitivities, allergies or aversions to foods or food components provided in the standard meals or capsules; use of herbal/plant-based supplements, omega-3 fatty acids and fish oils in the past 6 months or any individuals who fall into the vulnerable categories.

Recruitment and screening

Participants were recruited between August 2017 and March 2019 from the Davis and greater Sacramento area through posting flyers, newspaper advertising,

Craigslist advertisement, Western Nutrition Research Center (WHNRC) website and referrals. The first step in the screening process was an initial telephone assessment of medical history, self-reported height, weight, age, and interest in the study. Next, for those who met the general criteria, they were scheduled an in-person screening appointment.

Participants were asked to fast for 12-hrs leading up to their appointments. During the appointment, height was measured to the nearest tenth of a centimeter using a wall-mounted stadiometer (Ayrton Corporation, model S100) and weight was measured to the nearest tenth of a kilogram using an electronic scale (Tanita BWB-627A Class III electronic scale; Toledo Scale). Systolic and diastolic blood pressure, pulse and temperature were measured after participants rested undisturbed for 5-minutes using a blood pressure monitor (GE CARESCAPE™ V100). Fasting blood was then drawn from an antecubital vein by a licensed phlebotomist using VacuTainers (Becton Dickinson, Rutherford, New Jersey) for a clinical comprehensive metabolic panel (CMP), lipid panel and complete blood count (CBC). Blood was sent to the UC Davis Medical Center clinical pathology laboratory for analysis. Participants also completed an additional detailed medical history questionnaire and the Stanford Brief Physical Activity questionnaire before concluding the visit. Participant screening characteristics are shown in *Table 2.1*.

The CONSORT (Consolidated Standards of Reporting Trials) diagram (*Figure 2.1*) depicts the total number of people who responded to advertisements, who were screened for eligibility and who ultimately completed the study. A total of 31 participants (9 men, 22 women) enrolled into the study, data were collected from 27 participants (8

men, 19 women) and 24 participants (8 men, 16 women) completed the study in its entirety.

Study approvals

The study was approved by the University of California, Davis Institutional Review Board and registered on clinicaltrials.gov (NCT03203915). Study volunteers provided written informed consent to be screened and to participate in the full study if they qualified.

2.2.2. Study design and timeline

The study was a 16-week double-blinded, randomized control crossover design. Each intervention arm was 3-weeks followed by a 3-week washout period before beginning the next intervention arm (*Figure 2.2*). If eligibility requirements were met, participants were oriented to study protocol and expectations. Participants were asked to maintain their usual dietary patterns, current physical activity habits and to maintain their current weight during the study. During active study periods, participants came to the WHNRC on a weekly basis to pick up capsules. On the third week of capsule pick up, participants also picked up a standardized dinner to consume the evening before testing. Testing occurred at the end of the 3-week supplementation period. Participants were not required to come to the WHNRC during the washout period, but contact was maintained until the next active study period.

Randomization and blinding

A statistician randomized the sequence of the intervention by creating 6 blocks of the 6 permutations of the intervention sequence and randomly shuffled the order within each block. The generated list was given to the study coordinator to assign intervention

sequence to participants once they completed the study orientation. Sonomaceuticals, LLC provided the study capsules, which were coded by the manufacturer and then recoded with colored stickers by the Metabolic Kitchen and Human Feeding Laboratory. Investigators were unblinded following the analyses of the outcome measures.

Study capsules and test meals

Participants consumed the study material, Chardonnay grape marc powder, in the form of capsules every day for 20 days at home and on the 21st day they consumed their last set of capsules on their test day. Participants consumed 3 capsules (or 1500mg) of study material, daily. They were instructed to consume all 3 capsules with their first meal of the day. There were 3 different study capsules used in this study, two capsules had proprietary blends of Chardonnay grape seed extract and whole Chardonnay marc of varying ratios and one capsule of only microcrystalline cellulose (MCC), which served as the non-grape control. The study capsules were designated: high Chardonnay grape seed extract blend (HE), high Chardonnay marc blend (HM) and MCC (*Table 2.2*). Randal Optimal Nutrients, LLC manufactured study capsules (Santa Rosa, CA) and Eurofins (Petaluma, CA) analyzed the nutrient composition (*Table 2.3*). To monitor compliance, participants were asked to complete a daily capsule checklist to note how many capsules were consumed and at what time. They were asked to come to the WHNRC on a weekly basis to pick up each week's worth of capsules and to return the previous weeks packaging along with any unconsumed capsules.

On the capsule pick up during the week leading up to testing, participants also received a standardized meal to consume the evening prior of the test day. Harris

Benedict equation using information collected from the in-person screening visit was used to estimate caloric needs for study meals. The pre-test dinner was chicken angel hair pasta with cream sauce, nutrient composition information available in *Table 2.4*. The calorie levels of the meals distributed were either 500kcal or 800kcal, based on 30% of 1700kcal and 30% of 2700kcal, respectively. Participants were instructed to consume only this meal for dinner and to begin their 12-hour fast after consuming the meal. The test day breakfast meal was a breakfast casserole with egg, rice, potatoes, turkey sausage, cheddar cheese and mango-orange juice. Participants also consumed the last set of capsules with breakfast. Test day meals were scaled to meet 30% of daily caloric needs based on the Harris-Benedict equation estimation.

2.2.3. Protocols

Metabolic testing

Participants arrived at the WHNRC in the morning, following a 12-hour fast for a 6-hour metabolic test day. Height was measured to the nearest tenth of a centimeter using a wall-mounted stadiometer, weight was measured using an electronic scale and these were used to calculate BMI. Participants rested silently before having their blood pressure measured. A licensed phlebotomist drew fasting blood through the antecubital vein in vacutainers. Blood was subsequently drawn at 1, 2 and 3 hours after consuming the high fat challenge meal (previously described). The literature suggests a range between 3 - 6 h for a postprandial TG peak, highlighting the wide variability in human response (Emerson et al., 2018; Kolovou et al., 2019; Langsted et al., 2011; Lim et al., 2021; Mazidi et al., 2021; Shokry et al., 2019) – however we ultimately decided on 3 h due to financial limitations and a history of previous studies

conducted at the WHNRC (unpublished data) demonstrating that TG peak occurred on average roughly around 3 h.

Once blood was drawn, serum vacutainers were held at room temperature for 30 minutes to coagulate, meanwhile, plasma vacutainers were immediately chilled on ice. All vacutainers were centrifuged in a refrigerated Centra CL3R (International Equipment Co.) for 10 minutes at 100 x g at 4°C, then aliquoted to cryotubes with a transfer pipet before storing at -80°C until ready for analyses. All 3 test days followed the same protocol.

EndoPAT

To evaluate endothelial dysfunction, peripheral endothelial function was measured non-invasively using the EndoPAT 2000 (Itamar Medical, Israel). Participants were instructed to lay supine, limit movement, refrain from engaging in conversation with the investigator and to stay conscious throughout the measurement. Finger probes were placed onto the participants two index fingers and a blood pressure cuff on their upper non-dominant arm. The dominant arm without the blood pressure cuff was used as the control arm. The EndoPAT measurement included 7 minutes of baseline, 5 minutes of occlusion period and 5 minutes of post-occlusion. During the baseline measurement, the EndoPAT was measuring normal, non-occluded blood flow through the finger probes. During the occlusion period, the blood pressure cuff was inflated to 120psi to occlude blood flow to the non-dominant arm for 5 minutes. Lastly, after the occlusion period, the blood pressure cuff was immediately released and measured for an additional 5 minutes. The Reactive Hyperemic Index (RHI), which is a measure of endothelial dysfunction, was calculated as the post-to-pre occlusion signal ratio of the

non-dominant, occluded arm to the same post-to-pre occlusion signal ratio of the control arm. Normal vascular tone is considered >1.67. The Augmentation index (AI), which is a measure of arterial stiffness, was calculated based on the pulse wave of the EndoPAT signal in the occluded arm. Because the AI is related to heart rate, AI is corrected to a standard heart rate of 75 beats per minute. Lower AI values indicate less arterial stiffness and is intended only for research purposes. Analyses were completed with the 24 completed participants and 3 participants who withdrew.

Lipoproteins

To evaluate clinical lipid parameters related to ASCVD risk factors, fasting serum TC, LDL-C, HDL-C values were determined using an enzyme-linked colorimetric assay on a clinical chemistry analyzer (Cobas Integra 400+; Roche Diagnostics). All reagents were purchased from Roche Diagnostics and used based on the manufacturer's instructions. Analyses were completed with samples from 24 completed participants and available samples from 3 participants who withdrew.

Triglycerides

To evaluate ASCVD risk related to TG and non-fasting TG, serum TG was measured at fasting, 1, 2, and 3 hours postprandially. TG values were determined by an enzyme-linked colorimetric assay on the same analyzer as described. Reagents were purchased from Roche Diagnostics and assays were conducted based on the manufacturer's instructions. Analyses were completed with samples from 24 completed participants and available samples from 3 participants who withdrew. Area under the curve (AUC) and incremental area under the curve (iAUC) were calculated for the postprandial response using the trapezoid rule. The iAUC calculation allowed values to

be negative if they went below baseline. AUC was derived from the entire acute timeframe including fasting values and iAUC accounts for the acute response while not including the fasting area.

NMR lipid profiling

Lipoproteins are highly variable in size. Certain particles and particle sizes are thought to be more atherogenic than others, thus, to determine the distribution of lipoproteins and their respective sizes, plasma samples at fasting and 3 hours postprandial were sent out to LabCorp for NMR LipoProfile® analysis (LabCorp; Morrisville, NC). The lipid profiling included VLDL, chylomicron, intermediate density lipoprotein (IDL), HDL, LDL and TG particle concentration and sizing (small, medium, and large). This secondary analysis was completed with samples from 24 completed participants only.

Apolipoproteins

Apolipoproteins (apo) are structural proteins of lipoproteins, where apo-AI is the primary structural protein of HDL-C, Apo-B is the primary constituent of atherogenic lipoproteins (i.e. VLDL-C, LDL-C, IDL-C), and Apo-CIII has been associated with higher CVD risk and plays an important role in TG metabolism (Kampoli et al., 2009; Morita, 2016; Stock, 2019). Therefore, to evaluate atherosclerosis risk from a structural protein point of view, serum Apo-AI, B and CIII were measured at fasting, 2 and 3 hours postprandially. In addition, Apo-B:Apo-AI ratio was calculated to evaluate atherogenic risk (Walldius & Jungner, 2006). Apo-AI, B, and CIII concentrations were determined by an enzyme-linked colorimetric assay on the same analyzer as described, with reagents purchased from Kamiya Biomedical (Seattle, Washington) and analyses were

conducted based on the manufacturer's instructions. This secondary analysis was completed with samples from 24 completed participants only. AUC and iAUC were calculated for the postprandial response.

Oxidized LDL

LDL-C may penetrate the endothelium and become susceptible to oxidation, which increases its atherogenicity (Khatana et al., 2020). To evaluate this aspect of atherosclerosis risk, plasma oxidized LDL (oxLDL) was measured at fasting, 1, 2, and 3 hours postprandially using an enzyme-linked immunosorbent assay (Mercodia, Uppsala, Sweden) according to the manufacturer's instructions. This secondary analysis was completed with samples from 24 completed participants and 3 participants who withdrew. AUC and iAUC were calculated for the postprandial response.

2.2.4. Statistical analysis

Data from all volunteers who began the 16-week protocol with testing results were included in the primary intent-to-treat analyses, which included clinical lipoproteins and triglyceride response. Secondary analyses only included the full set of completing participants, unless otherwise stated—these analyses included Endopat, NMR lipid profiling, apolipoproteins, and oxLDL. All data were evaluated for normality using Shapiro-Wilks, were transformed if necessary and evaluated for outliers. AUC and iAUC were calculated for all outcomes with postprandial measures using R. Linear mixed-model ANOVAs were used to analyze variables on JMP Pro 16 (SAS Institute).

For single variables, mixed models were constructed with the test day (within-subject measure) and interventions (between-subject measure) as main effects and test day by intervention interaction term represented the crossover effect. For time response

variables, mixed models were constructed with the main effects: test day, sampling times (both as within-subject measures) and intervention (between-subject measure) as the main effects and included respective interaction terms. Tukey's test was used for multiple comparisons with statistically significant findings. Effect size, or the standard difference, was calculated for each intervention relative to each other for each outcome (*Supplemental Table 2.1*).

To determine the sample size needed for testing the effect of consuming the 2 Chardonnay marc blends along with a non-grape comparator on plasma TG levels is a 6-sequence, 3-period, 3-treatment crossover design that is balanced with respect to first-order carryover effects. For power calculation and using 177 mg/dl TG ± 40mg/dl SD as an average hypertriglyceride level, testing 24 subjects using this design gives a power of 0.9 with an alpha of 0.05 to detect a 10-20% effect of grape marc blends on plasma TG.

2.3. Results

2.3.1. Participant characteristics

Participants were asked to maintain their weight and not to change their physical activity behaviors. There were no significant changes in weight (p=0.37) and BMI (p=0.32), data are found in *Table 2.5*.

2.3.2. Blood pressure and EndoPAT

There was a significant main effect of intervention in systolic blood pressure (SBP) (p=0.047) but not in diastolic blood pressure (DBP) (p=0.34; *Table 2.5*). Pairwise comparisons revealed a lower SBP following the HE supplementation compared to HM (p=0.038). There was no difference between HM and MCC (p=0.56) or between HE and

MCC (p=0.29). There was no significant main effect of intervention with RHI and AI@75 following the supplementation (p=0.96 and p=0.29, respectively; *Table 2.5*).

2.3.3. Lipoproteins

Following the supplementation period, there was no significant main effect of intervention in fasting levels of TC, LCL-C, TC:HDL and non-HDL-C. Following the HM and MCC supplementation, there was a significant main effect of intervention where there was higher HDL-C compared to HE (p=0.007 and p=0.02, respectively). However, there was no difference between HM and MCC (p=0.87). Data are found in *Table 2.5*.

2.3.4. Triglycerides

Both fasting and postprandial triglyceride concentrations were evaluated. There were no significant differences between the fasting TG levels following the supplementation period (p=0.16). There was a trend towards significance in the main effect of intervention in the postprandial TG response (p=0.06). There was a significant main effect of intervention (p=0.05) in TG AUC, where HM had a significantly lower AUC than HE (p=0.041) and no difference between MCC and HE (p=0.30) or MCC and HM (p=0.57). In TG iAUC, there was a trend towards significant main effect of intervention (p=0.07). Data are depicted in *Figure 2.3*.

2.3.5. NMR lipid profiling

Among the VLDL and chylomicron particle species, the total number, large, medium, or small particles were not affected by intervention (p=0.11, p=0.42, p=0.12 and p=0.97, respectively). Among the LDL particles, there was no significant main effect of intervention in the total number, large or small particles (p=0.51, p=0.33 and p=0.21, respectively). There was no significant main effect of intervention on the total number of

IDL particles (p=0.98). Among the HDL particles, there was no significant main effect of intervention on total number, medium or small particles (p=0.52, p=0.95, p=0.83, respectively). There was a significant main effect of intervention on large HDL particles (p=0.03; *Figure 2.4*). Paired comparison tests reveal following the HE supplementation there are lower numbers of large HDL particles with 7.05 ± 0.7 μmol/L compared to MCC with 7.7 ± 0.7 μmol/L (p=0.03) but no differences between HE and HM (HM with 7.6 ± 0.7 μmol/L of large HDL particles) (p=0.78) or between MCC and HM (p=0.12). Finally, there was no effect of intervention on the mean particle sizes of VLDL, LDL or HDL (p=0.66, p=0.95 and p=0.10, respectively). All data are found in *Supplemental Table 2.2.*, unless otherwise stated.

2.3.6. Apolipoproteins

At fasting, there were no significant main effects of intervention on Apo-AI, Apo-B and Apo-CIII (p=0.15, p=0.35, p=0.2, respectively; *Supplemental Table 2.3*). In the postprandial response there were no significant main effects of intervention in Apo-AI, Apo-B and Apo-CIII (p=0.10, p=0.58 and p=0.73, respectively). There were no significant main effects of intervention seen in Apo-AI AUC, Apo-B AUC, Apo-CIII AUC (p=0.096, p=0.37, p=0.48, respectively). Following the supplementation period, there were no significant effects in iAUC in Apo-AI or B (p=0.95, p=0.86, respectively); there was a trend towards significance in Apo-CIII iAUC (p=0.06). Data are depicted in *Figures 2.5-2.7*. There were no differences in Apo-B:Apo-AI ratio (p=0.11, *Table 2.5*).

2.3.7. Oxidized LDL

There was no significant main effect of intervention on fasting oxLDL levels (p=0.28). In the postprandial oxLDL response, there was no significant main effect of

intervention (p=0.88). There was no significant main effect of intervention in oxLDL AUC (p=0.52) or iAUC (p=0.52). Data are depicted in *Supplemental Figure 2.1.*

2.4. Discussion

This was the first study to utilize whole Chardonnay marc in humans in the form of capsules, and to evaluate various cardiovascular end points that may contribute to atherosclerosis risk. Clinical parameters involving fasting lipoproteins such as high LDL-C, low HDL-C and high TG are implicated in ASCVD risk. ChSF supplementation in high fat diet induced obese hamsters resulted in lower fasting TC, LDL-C and VLDL-C (Kim et al., 2014). However, these results were not seen in the present trial in either of the Chardonnay interventions, therefore not supporting my hypothesis regarding fasting concentrations of TC, LDL-C, and TG. In the human trial that supplemented ChSF at 4.8g per day, there were no changes in lipoprotein levels either, albeit the participants did not have evident dyslipidemia entering the study (Corban et al., 2020).

HDL-C is involved with reverse cholesterol transport (RCT), in other words, it transports cholesterol from peripheral tissues to the liver for removal, positioning it as an important factor in reducing ASCVD risk (Marques et al., 2018). Following the high extract blend (HE) supplementation in the present trial, there was a significant decrease in HDL-C plasma concentrations compared to both microcrystalline cellulose (MCC) and high marc blend (HM) supplementation. These study results did not support my hypothesis that HDL-C would be unaffected by either Chardonnay intervention. In the ChSF hamster study, there was a trend towards lowered HDL-C following the ChSF supplementation, thus the results from the present study support that observation.

In addition, NMR lipid profiling showed a decreased number in large HDL particles following HE compared to MCC supplementation. Large HDL particles are thought to be the primary HDL species responsible for RCT, but this concept is still debated as some groups have found positive associations with reduced ASCVD while others have found no association (Duparc et al., 2020; Kontush, 2015; Li et al., 2016; Paavola et al., 2017). Apo-AI is associated with all HDL particles, and it has been argued that Apo-AI has more relevance in ASCVD mortality (Duparc et al., 2020; Wu et al., 2021). The present study did not see any changes in Apo-AI across interventions.

Fasting triglycerides have long been part of the atherosclerosis conversation, while non-fasting triglyceride levels are emerging as a practical and relevant measure of CVD risk (Keirns et al., 2021). Epidemiological studies have pointed to the relationship between high non-fasting TG levels and CVD compared to fasting levels of TG (Bansal et al., 2007; Kolovou et al., 2019; Langsted et al., 2011; Nordestgaard et al., 2007). While it was only a statistical trend, there was a pattern of a lower postprandial TG response following the HM intervention. More importantly, a summary measure of postprandial triglycerides, the TG AUC following the HM supplementation was significantly decreased compared to HE. Studies that utilized grape seed extract or pure (-)-epicatechin, demonstrated a reduced TG response, therefore my hypothesis was partly supported based on these findings where the HE intervention would have similar results on postprandial TG response (Gutierrez-Salmean et al., 2014; Suzuki et al., 2005; Toyoda-Ono et al., 2007). However, the HE intervention resulted in the highest postprandial TG response which does not align with previous study results that suggested high flavan-3-ol content decreased TG response and therefore not

supporting my hypothesis. The HM intervention resulting in a trend towards lower TG response, and a lower TG AUC may suggest that high flavan-3-ol content alone may not be the complete story behind TG response improvement. The present study did not include postprandial substrate oxidation measurements—in the ChSF studies, gene expression analyses showed an upregulation in genes related to β-oxidation (Kim et al., 2014; Seo et al., 2016). It is conceivable that upregulation of these genes could be translated to increased fat combustion following a high fat meal and therefore a lower TG response.

While this study was not designed to evaluate mechanisms of action, Apo-CIII was measured to gain insight whether this could be one aspect of how CM could be affecting TG concentrations as Apo-CIII inhibits TG hydrolysis and may contribute to the development of ASCVD (Borén, Packard, et al., 2020). The results did not show statistical differences in Apo-CIII response across the interventions, but the pattern of the response was similar to the postprandial TG response, where the HM supplementation resulted in lower responses. In addition, Apo-CIII iAUC had a trend towards significance similar to the postprandial TG response. This result could be an indication of how CM may be affecting TG concentrations and should be further explored. In future studies, it may be interesting to also measure Apo-CII levels as it acts as a cofactor for lipoprotein lipase in TG hydrolysis (Olivecrona & Beisiegel, 1997).

Endothelial dysfunction is an early indication of atherosclerosis cardiovascular disease (ASCVD)—the current study at hand did not demonstrate an improvement in EndoPAT measurements with either Chardonnay formulations as seen following the ChSF supplementation in the previous study conducted by Corban et al. (Corban et al.,

2020). Participants of the present study were relatively healthy and did not have endothelial dysfunction (defined as RHI <1.67), therefore this result was not completely unexpected, thus aligned with my initial hypothesis. Blood pressure is another early indication of ASCVD (Karmali et al., 2015). SBP was significantly lower following HE compared to HM. Flavan-3-ols have been cited to improve blood pressure through increasing nitric oxide levels to increase vasodilation (Ottaviani et al., 2020; Schroeter et al., 2006). The compositional analysis of the study material included a gross total phenolic content and did not include specifically flavan-3-ols. However, from the Yokoyama group, it is clear that Chardonnay is high in flavan-3-ols and that the HE arm had higher total phenolics than the HM arm (Kim et al., 2014), so it is possible that the lower SBP may be attributed to the higher phenolics, but additional studies will need to validate this finding. Notably, the ChSF supplementation levels were substantially higher with the hamster model used in the Yokoyama study whereas in the present study whole CM was used with Chardonnay seed extract blended to increase phenolic content.

Oxidized LDL (oxLDL) plays a critical role in the pathophysiology of atherosclerotic progression (Poznyak et al., 2020; Zmysłowski & Szterk, 2017). The current literature on Chardonnay marc or seeds in ASCVD risk factors has not evaluated effects on oxLDL directly. However, animals supplemented with ChSF has demonstrated to have downregulate genes related to oxidative stress and reduce inflammatory markers, which have roles in the oxidation of LDL particles (Kim et al., 2014; Seo et al., 2016; Zmysłowski & Szterk, 2017). *In vitro* studies and animal models treated with epicatechins and grape powder, respectively, reduced LDL oxidation and

cellular uptake of oxLDL (Chen et al., 2017; Fuhrman et al., 2005). Previous studies supplementing humans with red grape marc extract or red grape seed extract have resulted in decreased oxLDL levels from baseline, which suggests that phenolics from Chardonnay marc could potentially have similar results due to similar phenolic profiles (Annunziata et al., 2021; Sano et al., 2007). However, the present study saw no differences in oxLDL. These results do not support the red grape data but are similar to the non-significant oxLDL result following a pure (-)-epicatechin supplementation in humans (Kirch et al., 2018).

Limitations

Both men and women were recruited on broad inclusion criteria where they only needed to meet one of the lipid qualifications. While this was a conscious decision for logistical recruitment reasons, it nonetheless introduced variability and heterogeneity into the sample population. On average, many of the lipid criteria at enrollment were on the low end of normal and could have had too much variability to detect appreciable differences. We sought to evaluate a more general population by including both men and women, however we did not take menstrual cycle or menopause into account when recruiting women, which could have affected the results as well. While we had testing in 6-week increments, we are still uncertain whether we were consistently testing in the same phase of the menstrual cycle in premenopausal women. To reduce participant burden we did not have baseline testing at the beginning of each intervention arm and as a result, we were not able to determine changes in outcomes from beginning to end of each intervention and could only compare relative to the other interventions.

The testing was a total of 4 hours where only 3 hours of postprandial data were collected, thus it is unclear whether the TG peak was captured, and we do not have information regarding TG clearance. Future studies should consider narrowing the studied population further to continue exploring the effectiveness of CM in improving postprandial TG. A longer postprandial period should be considered to evaluate the rise and fall of circulating triglycerides. In addition, the 3-hr postprandial timeframe likely captured the effects from the more bioavailable monomeric flavan-3-ols (e.g. [-]-epicatechin and [+]-catechin), but likely was not long enough timeframe to capture the potential impact of the colonic fermentation products (e.g. valerolactones) (Ottaviani et al., 2016) may also have on the outcomes, thus should be considered in future applications.

2.5. Conclusion

In conclusion, high phenolics alone may not offer cardio-protection and the HM supplementation with more nutritional complexity has potential to be part of a regimen to reduce ASCVD risk. Changes in SBP, HDL-C and non-fasting TG were observed in the present trial. All these outcomes have a role in ASCVD risk, however more research is needed to build upon these early results.

Figures

Figure 2.1. Consort Diagram

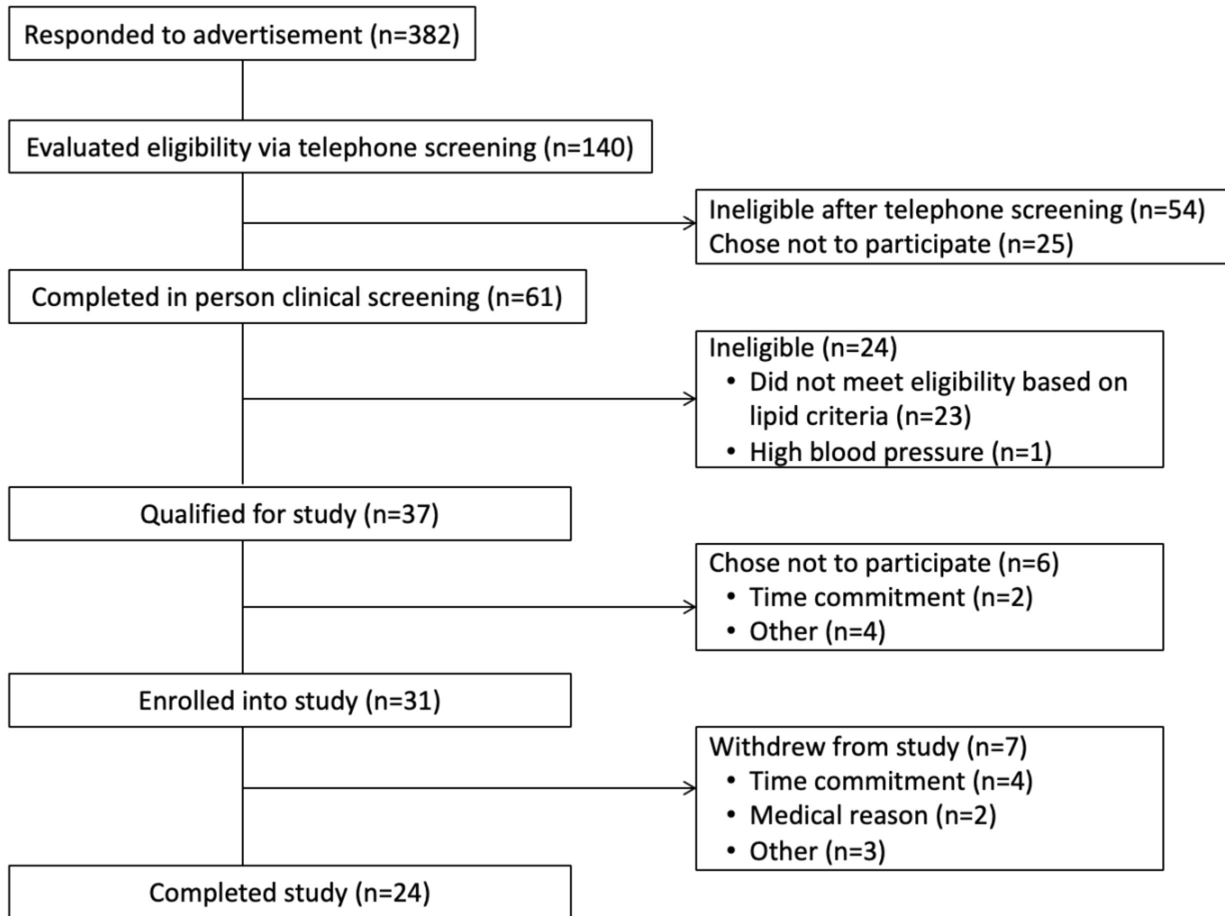


Figure 2.1. Consort diagram illustrates the final recruitment of the study.

Figure 2.2. Study Design

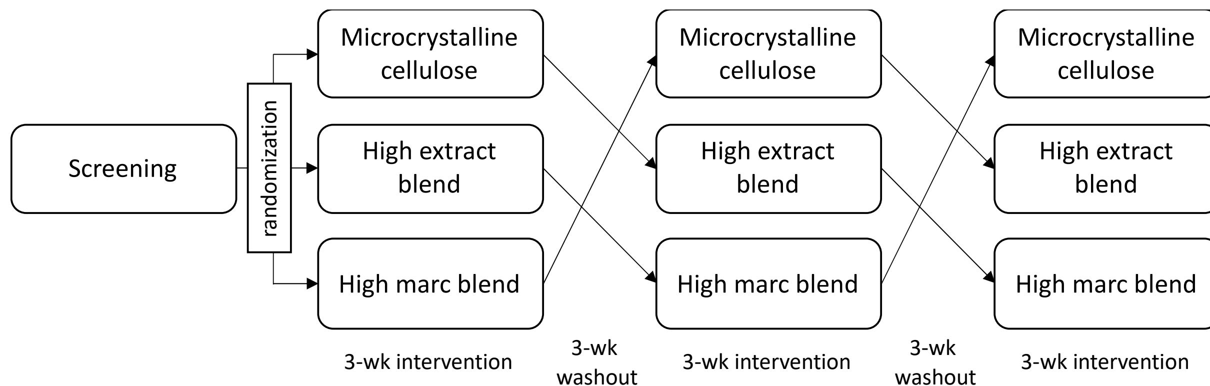


Figure 2.2. Study design that depicts an example of a randomization scheme of the interventions.

Figure 2.3. Postprandial triglyceride response

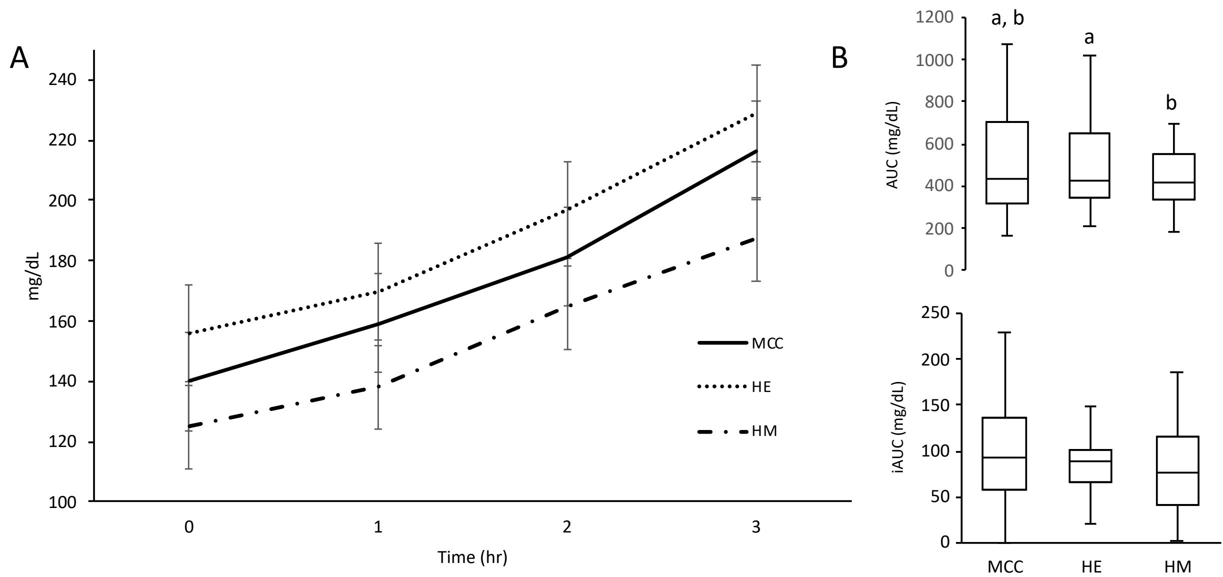


Figure 2.3. Panel A: Postprandial triglyceride response following high fat meal challenge. Data are shown as mean ± SEM. There is a trend towards significance in the main effect of intervention (p=0.06). Panel B: Area under the curve (AUC) and incremental area under the curve are depicted. There is a significant main effect of intervention with AUC between HE and HM interventions. MCC – microcrystalline cellulose, HE – high Chardonnay extract blend, HM – high Chardonnay marc blend.

Figure 2.4. Large HDL particles

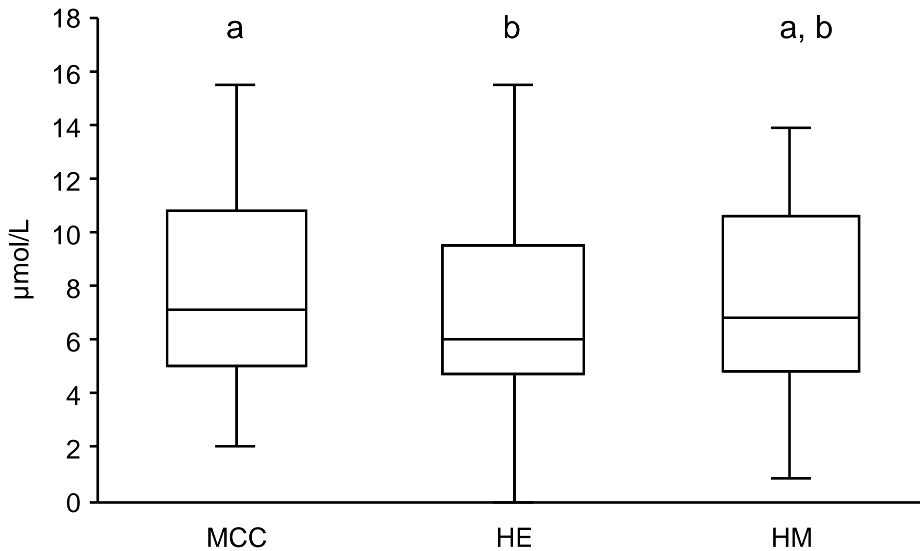


Figure 2.4. Large HDL particle data are shown as box and whisker plots by intervention. Different letters indicate significant differences.

Figure 2.5. Apo-AI response

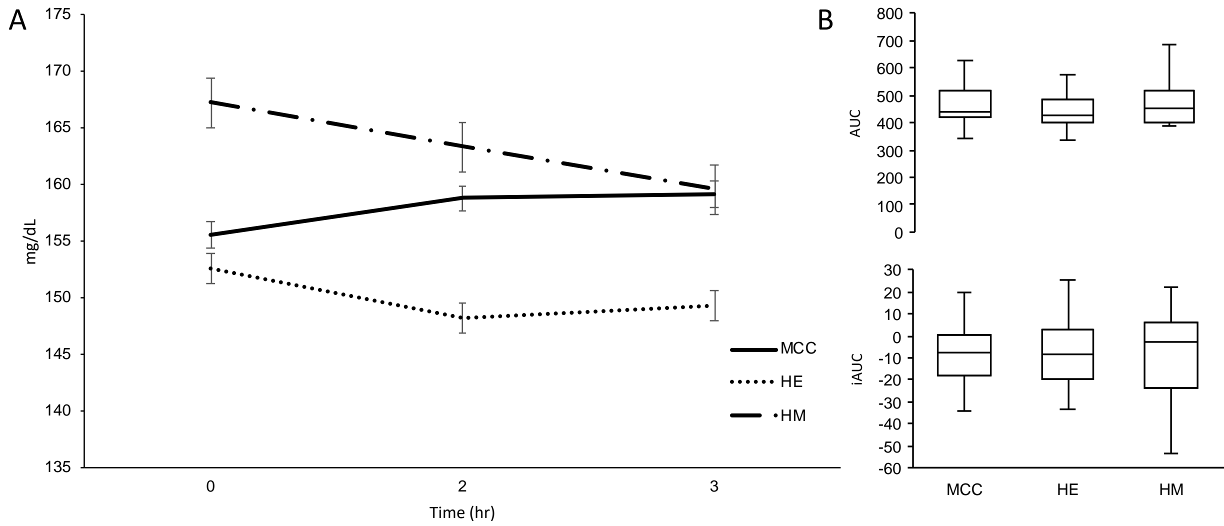


Figure 2.5. Panel A: Postprandial Apo-AI response following a high fat meal challenge. Data are shown as mean ± SEM. Panel B: Area under the curve and incremental area under the curve are shown. MCC – microcrystalline cellulose, HE – high Chardonnay extract blend, HM – high Chardonnay marc blend.

Figure 2.6. Apo-B response

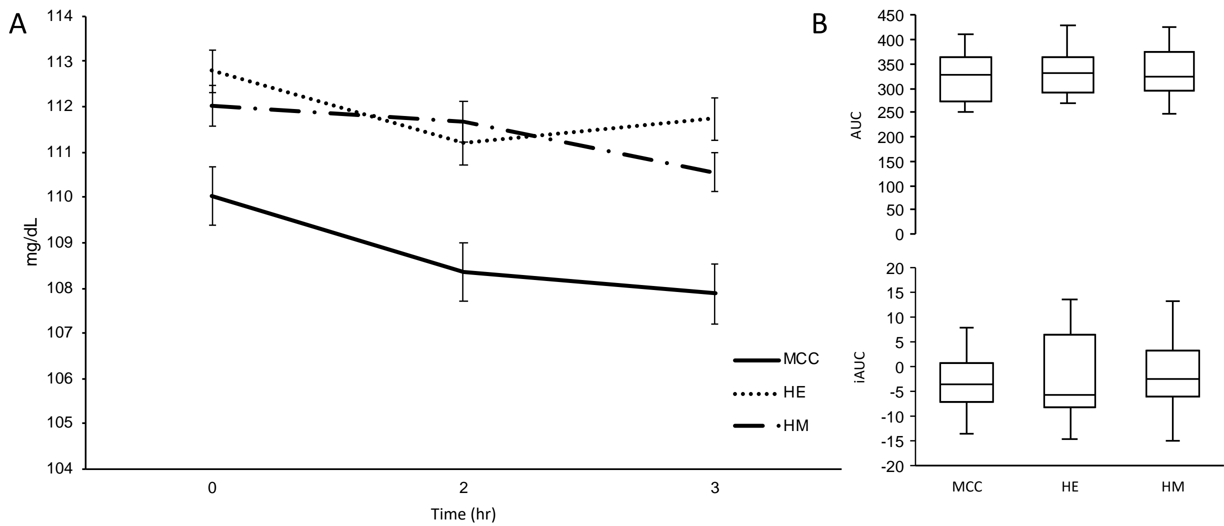


Figure 2.6. Panel A: Postprandial Apo-B response following a high fat meal challenge. Data are shown as mean ± SEM. Panel B: Area under the curve and incremental area under the curve are shown. MCC – microcrystalline cellulose, HE – high Chardonnay extract blend, HM – high Chardonnay marc blend.

Figure 2.7. Apo-CIII response

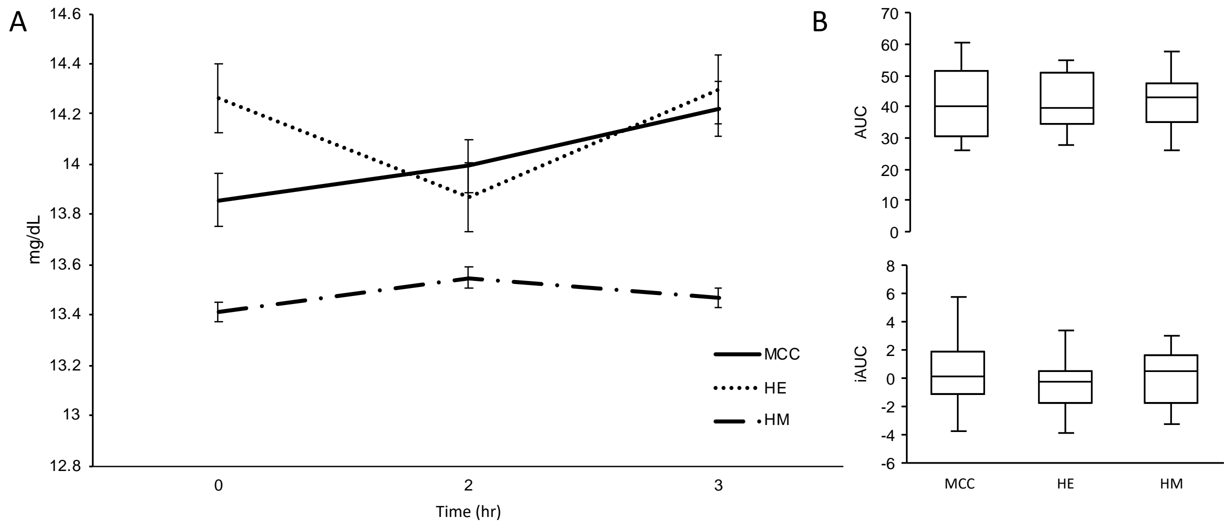


Figure 2.7. Panel A: Postprandial Apo-CIII response following a high fat meal challenge. Data are shown as mean ± SEM. Panel B: Area under the curve and incremental area under the curve are shown. There is a trend towards significance in the incremental area under the curve (p=0.06). MCC – microcrystalline cellulose, HE – high Chardonnay extract blend, HM – high Chardonnay marc blend.

Tables

Table 2.1. Screening characteristics

Age (years)	53.9 ± 1.7
BMI (kg/m^2)	28.1 ± 0.5
Systolic BP[1] (mmHg)	117.9 ± 2.2
Diastolic BP (mmHg)	71.2 ± 1.5
Total cholesterol (mg/dL)	230.5 ± 6.8
LDL-C[2] (mg/dL)	144.6 ± 4.8
HDL-C[3] (mg/dL)	54.9 ± 2.3
TC:HDL[4] (mg/dL)	4.4 ± 0.3
Non-HDL-C (mg/dL)	175.6 ± 7.3
Triglyceride (mg/dL)	132.1 ± 16.7

Data are presented in mean ± SEM, n=27

[1]BP – blood pressure, [2]LDL-C – low density lipoprotein cholesterol, [3]HDL-C – high density lipoprotein cholesterol, [4]TC:HDL – total cholesterol to HDL-C ratio

Table 2.2. Relative composition of study capsules

	Chardonnay seed extract	Whole Chardonnay marc	Microcrystalline cellulose
Microcrystalline cellulose capsule	N/A	N/A	++
High extract capsule	↑↑	↑	↑
High marc capsule	*	**	N/A

Interventions were a proprietary blend, where each blend was created independent of the other interventions. N/A – not applicable, or not present; ++ - higher amounts; ↑↑ - higher amounts, ↑ - lower amounts; ** - higher amounts; * - lower amounts

Table 2.3. Nutrient composition of Chardonnay interventions per 1500mg

	High extract blend	High marc blend
Energy (kcal)	0.2	5.6
Total carbohydrates (mg)	37.0	997.67
Total protein (mg)	5.4	144.5
Total fat (mg)	4	108
Total dietary fiber (mg)	26.7	720.9
Total polyphenol (mg)	119.7	75.0

Table 2.4. Study foods nutritional composition

	Pre-test dinner (500kcal)	Pre-test dinner (800kcal)	Test day meal (700kcal)
Total energy (kcal)	516	826	699
Total carbohydrate (g)	57.3	91.7	79.1
Total protein (g)	19.0	30.5	25.6
Total fat (g)	23.5	37.6	31.5
Total dietary fiber (g)	3.8	6.1	4.1
Total saturated fatty acids (g)	13.6	21.7	15.9
% calories from carbohydrate	45.0	45.1	45.3
% calories from protein	14.9	14.9	14.5
% calories from fat	40.0	40.0	40.1

700kcal representative is shown for the test day meal composition.

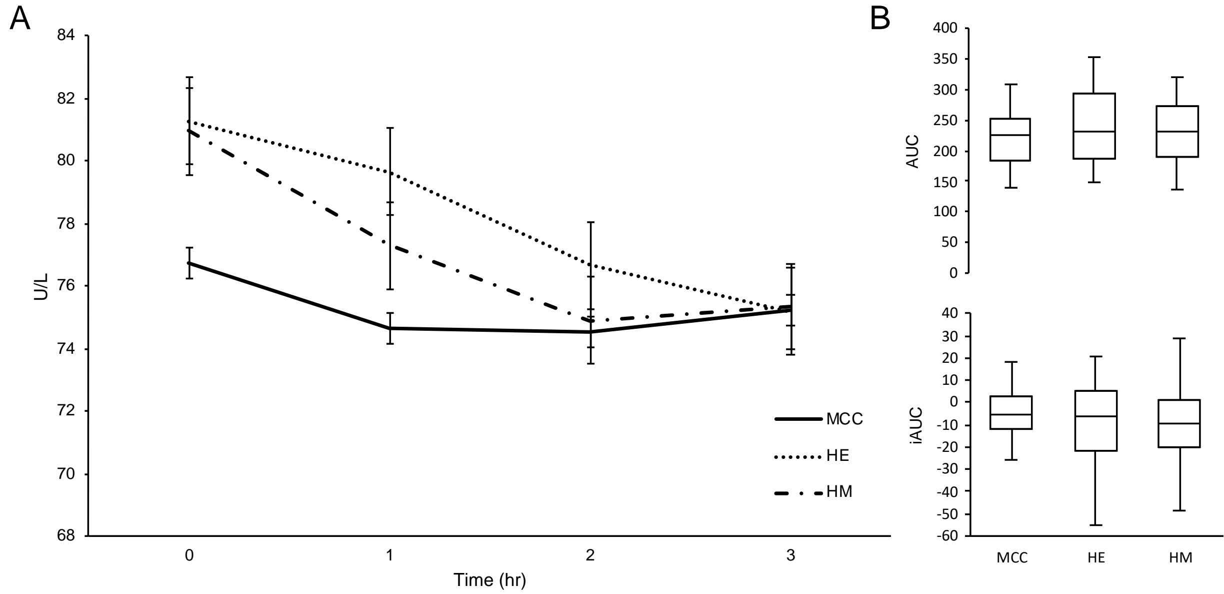
Table 2.5. Clinical parameters following supplementation

	Microcrystalline cellulose (n=25)	High extract blend (n=25)	High marc blend (n=26)
Weight (kg)	78.1 ± 2.3	78.7 ± 2.3	77.3 ± 2.2
BMI (kg/m^2)	28.2 ± 0.56	28.3 ± 0.58	28.1 ± 0.56
TC[1] (mg/dL)	221.2 ± 9.1	221.8 ± 7.9	221.3 ± 6.5
LDL-C[2] (mg/dL)	142.6 ± 6.3	142.8 ± 6.1	146.5 ± 6.2
HDL-C[3] (mg/dL)	55.7 ± 2.5[b]	52.4 ± 2.5[a]	56.6 ± 2.6[b]
TC:HDL[4] (mg/dL)	4.1 ± 0.3	4.5 ± 0.3	4.1 ± 0.2
Non-HDL-C (mg/dL)	165.5 ± 9.0	169.4 ± 8.2	164.7 ± 6.1
Triglyceride (mg/dL)	140.1 ± 17.5	156.0 ± 21.2	125.0 ± 12.0
Systolic BP[5] (mmHg)	123.8 ± 3.7[b]	118.8 ± 2.8[a]	125.6 ± 2.5[b]
Diastolic BP (mmHg)	72.4 ± 1.7	69.9 ± 1.8	72.0 ± 1.8
RHI[6]	2.3 ± 0.1	2.3 ± 0.1	2.3 ± 0.1
AI@75[7]	6.3 ± 3.0	8.4 ± 3.4	12.5 ± 4.3
Apo[8]-B:Apo-AI	0.72 ± 0.03	0.76 ± 0.03	0.70 ± 0.03

Data are presented in mean ± SEM, n=27. Different letters indicate significance.
[1]TC – total cholesterol, [2]LDL-C – low density lipoprotein cholesterol, [3]HDL-C – high density lipoprotein cholesterol, [4]TC:HDL – total cholesterol to HDL-C ratio, [5]BP – blood pressure, [6]RHI – reactive hyperemic index, [7]AI@75 – augmentation index standardized to 75 beats per minute, [8]Apo – apolipoprotein

Supplemental material

Supplemental Figure 2.1. Oxidized LDL-C response



Supplemental Figure 2.1. Panel A: Oxidized low density lipoprotein cholesterol (LDL-C) response following a high fat meal challenge over 3 hours is depicted. Data are shown as mean ± SEM. Panel B: Area under the curve and incremental area under the curve are shown. MCC – microcrystalline cellulose, HE – high Chardonnay extract blend, HM – high Chardonnay marc blend.

Supplemental Table 2.1. Effect size of outcome variables

	MCC - HE	**MCC - HM**	**HE – HM**
TC (mg/dL)	0.02	<0.001	0.01
LDL-C (mg/dL)	0.01	0.12	0.12
HDL-C (mg/dL)	0.26	0.07	0.33
TC:HDL (mg/dL)	0.25	0.06	0.32
Non-HDL-C (mg/dL)	0.10	0.02	0.12
Fasting TG (mg/dL)	0.18	0.17	0.36
TG response (mg/dL)	0.14	0.21	0.34
TG AUC	0.14	0.21	0.35
TG iAUC	0.14	0.29	0.15
Systolic BP (mmHg)	0.33	0.11	0.44

Diastolic BP (mmHg)	0.28	0.04	0.24
Endopat RHI	0.06	0.03	0.09
Endopat AI@75	0.12	0.34	0.22
Fasting oxLDL (U/L)	0.23	0.21	0.02
oxLDL response	0.15	0.09	0.06

Cohen's *d* was used to calculate effect sizes. Small effect with *d* = 0.2; medium effect with *d* = 0.5; large effect with *d* = 0.8. TC – total cholesterol; LDL-C – low density lipoprotein cholesterol; HDL-C – high density lipoprotein cholesterol; TC:HDL – total cholesterol to HDL-C ratio; TG – triglyceride; BP – blood pressure; RHI – reactive hyperemic index; AI – augmentation index; oxLDL – oxidized LDL; MCC – microcrystalline cellulose, HE – high Chardonnay extract blend, HM – high Chardonnay marc blend.

Supplemental Table 2.2. Summary of NMR lipid profile data

	MCC[5]			HE[6]			HM[7]		
Total VLDL[1] & chylomicron particles (nmol/L)	60.99	±	6.44	67.16	±	6.54	57.39	±	5.64
Large VLDL & chylomicron particles (nmol/L)	6.18	±	1.17	7.55	±	1.47	5.88	±	0.90
Medium VLDL particles (nmol/L)	18.99	±	3.40	24.19	±	3.75	16.65	±	2.08
Small VLDL particles (nmol/L)	35.84	±	5.04	35.40	±	3.97	34.85	±	4.27
Total LDL[2] particles (nmol/L)	1283.29	±	64.40	1326.71	±	60.57	1300.92	±	60.46
IDL[3] particles (nmol/L)	236.67	±	34.70	238.42	±	28.90	222.58	±	19.58
Large LDL particles (nmol/L)	419.54	±	50.12	414.13	±	51.34	460.29	±	52.39
Total small LDL particles (nmol/L)	627.21	±	68.91	674.21	±	73.88	617.83	±	68.56
Large HDL[4] particles (μmol/L)	7.74	±	0.73	7.05	±	0.69	7.58	±	0.67
Medium HDL particles (μmol/L)	10.72	±	1.14	10.94	±	1.27	11.07	±	0.74

Small HDL particles (μmol/L)	15.37	±	1.58	15.85	±	1.28	15.87	±	1.17
VLDL size (nm)	50.92	±	1.65	51.49	±	2.03	50.13	±	1.69
LDL size (nm)	20.83	±	0.16	20.85	±	0.17	20.85	±	0.15
HDL size (nm)	9.38	±	0.12	9.26	±	0.11	9.30	±	0.10

Data are shown as mean ± SEM. [1]VLDL – very low density lipoprotein, [2]LDL – low density lipoprotein, [3]IDL – intermediate density lipoprotein, [4]HDL – high density lipoprotein, [5]MCC – microcrystalline cellulose, [6]HE – high Chardonnay extract blend, [7]HM – high Chardonnay marc blend

Supplemental Table 2.3. Fasting apolipoprotein concentrations

	MCC[2]			HE[3]			HM[4]		
Apo[1]-AI (mg/dL)	154.93	±	4.98	151.80	±	4.80	165.62	±	9.50
Apo-B (mg/dL)	109.11	±	3.55	112.11	±	3.24	111.20	±	3.22
Apo-CIII (mg/dL)	14.06	±	0.77	14.75	±	0.88	13.65	±	0.55

Data are shown as mean ± SEM. [1]Apo – apolipoprotein, [2]MCC – microcrystalline cellulose, [3]HE – high Chardonnay extract blend, [4]HM – high Chardonnay marc blend

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Chapter 3

Effects of Chardonnay marc on glucose regulation

3.1. Introduction

Glucose dysregulation may present as various phenotypes (e.g. impaired fasting glucose, glucose intolerance, etc.) and manifest long before type 2 diabetes mellitus (T2DM) is diagnosed (Galicia-Garcia et al., 2020; Skyler et al., 2017; Wagner et al., 2021). Insulin resistance (IR) is central to the pathophysiology of type 2 diabetes mellitus (Galicia-Garcia et al., 2020). The progression to IR is largely multifaceted and complex. Inflammation has been long discussed as an important factor in the development of IR. Proposed mechanisms of action include disruptions in insulin secretion in pancreatic β-cells, blocking of insulin receptor activation and action, thus reducing systemic inflammation could be one strategy in combating insulin resistance (Rehman & Akash, 2016).

Beyond inflammation, bile acids appear to play a role in regulating glucose homeostasis. Bile acids act as signaling molecules in various aspects of metabolism, including glucose metabolism (Ahmad & Haeusler, 2019; Alberto González-Regueiro et al., 2017; Staels & Fonseca, 2009). Bile acids are the primary ligands for the nuclear receptor, farnesoid X receptor (FXR) and activates membrane protein Takeda-G-protein-receptor-5 (TGR5) (Makishima et al., 1999; Maruyama et al., 2002). FXR is involved in numerous areas of metabolism—it plays a role in regulating glucose metabolism through improving insulin sensitivity and suppressing gluconeogenesis (Ahmad & Haeusler, 2019). Bile acid activation of TGR5 is thought to stimulate the secretion of glucagon-like peptide-1 (GLP-1), which promotes insulin release and has

been used in diabetes management (Ahmad & Haeusler, 2019; Katsuma et al., 2005). Kim et al., has suggested that FXR and TGR5 are interrelated in the secretion of GLP-1 (Kim & Fang, 2018).

Grape products, specifically marc, provides a source of flavonoids and fiber that may benefit glucose homeostasis (Moodi et al., 2021). Recently, there has been a shift from studying grape seed extracts to now also including whole marc or marc extracts. Particularly red wine grape marc has been studied in high fat fed animal models where marc supplemented animals were protected from IR (Daniel et al., 2021; Khanal et al., 2011; Rodriguez Lanzi et al., 2016; Rodriguez Lanzi et al., 2020). The current body of literature lacks trials that utilize whole grape marc in humans. Several that have evaluated the effect of whole grape marc on glucose metabolism in humans have used different red wine grape varieties (e.g. Cabernet Sauvignon). Red wine undergoes a maceration process, where nutrients such as phenolics and oligosaccharides are extracted from the marc into the wine; whereas white wine (e.g. Chardonnay) do not undergo maceration and its corresponding marc retain much more of these nutrients in comparison (Guaita & Bosso, 2019; Kim et al., 2014; Sinrod et al., 2021). Previous trials that used Chardonnay grapes as an intervention has been conducted using diet induced obese animals where Chardonnay seed flour or seed extract was supplemented and resulted in lower fasting glucose and insulin compared to controls (Decorde et al., 2009; Seo et al., 2016; Seo et al., 2015).

The second aim of this study was to explore the interrelationships between glucose regulation in terms of inflammatory markers and circulating plasma bile acids following the supplementation of two different whole Chardonnay marc blends. Previous

animal studies have evaluated glucose and insulin concentrations and gene expression to consider mechanisms of action; however, the present study is the first to evaluate these interrelationships of glucose regulation in humans using whole Chardonnay marc blends.

3.2. Methods

Details of the study design and participants were fully described and can be found in Chapter 2.2. Participants were recruited specifically for the previously described lipid criteria. Glycemic outcomes were secondary objectives, specific glycemic criteria were not included aside from excluding participants who reported having type 2 diabetes mellitus. Based on self-reported history and initial screening glucose values measured (92.9 ± 1.9 mg/dL), we recruited a mixed population who were normoglycemic (n=23) and with impaired fasting glucose (≥100 mg/dL; n=8). Methods that were not previously described are detailed in the following section.

3.2.1. Protocols

Metabolic testing

Participants arrived at the WHNRC in the morning, following a 12-hour fast for a 6-hour metabolic test day. They consumed a high fat breakfast challenge meal, which comprised of a breakfast casserole with egg, rice, potatoes, turkey sausage, cheddar cheese and mango-orange juice. Participants also consumed the last set of capsules with breakfast. A licensed phlebotomist drew fasting blood through the antecubital vein in ethylenediaminetetraacetic acid (EDTA) and serum vacutainers (Becton Dickinson, Rutherford, New Jersey). Blood was subsequently drawn at 1, 2 and 3 hours after consuming the high fat challenge meal. Once blood was drawn, plasma

EDTA vacutainers were immediately chilled on ice, while the serum vacutainers were kept at room temperature for 30 minutes to clot. All vacutainers were centrifuged in a refrigerated Centra CL3R (International Equipment Co.) for 10 minutes at 100 x *g* at 4°C, then aliquoted before storing at -80°C until ready for analyses. All 3 test days followed the same protocol.

Glucose and insulin

To evaluate glycemic regulation, plasma glucose and serum insulin were measured at fasting, 1, 2, and 3 hours postprandially. Plasma glucose concentrations were analyzed using an enzyme-linked colorimetric assay on a clinical chemistry analyzer (Cobas Integra 400+; Roche Diagnostics, Indianapolis, IN). Serum insulin concentrations were determined by an electrochemiluminescence immunoassay on an immunoanalyzer (Cobas e 411; Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions.

Area under the curve (AUC) and incremental AUC (iAUC) were calculated for the postprandial response using the trapezoid rule. The iAUC calculation allowed values to be negative if vales fell below baseline. AUC was derived from the entire acute timeframe including fasting values and iAUC accounts for the acute response while not including the fasting area.

To estimate insulin resistance, Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) and the McAuley index were calculated using fasting glucose and insulin concentrations (McAuley et al., 2001; Wallace et al., 2004). To estimate insulin sensitivity, quantitative insulin-sensitivity check index (QUICKI) was calculated

using fasting glucose and insulin concentrations (Chen et al., 2005). Samples from 27 participants were used in these analyses.

Inflammatory markers

To evaluate inflammation levels, inflammation markers were measured at fasting with an immunoassay, MSD® Multi-spot assay (Meso Scale Diagnostics, Rockville, MD). Acute phase proteins, C-reactive protein (CRP) and serum amyloid A (SAA) were measured on this assay according to the manufacturer's instructions. Samples from 27 participants were used in these analyses.

Bile acids

Bile acids have been emerging as a regulator of glucose metabolism through mechanisms such as promoting incretin hormone secretion, therefore plasma bile acids were measured at fasting, 1, 2, and 3 hours postprandially (Ahmad & Haeusler, 2019). Samples were prepared and analyzed using the method as reported by Pederson et al (Pedersen et al., 2021). Briefly, plasma samples were first prepared by precipitating out proteins before being enriched with deuterated bile acids in 5 µL methanol, butylated hydroxytoluene/EDTA in 5 µL 1:1 methanol/water and 1 cyclohexyluredio, 3-dodecanoic acid and 1-phenylureido, 3-hexanoic acid (PUHA) in 5 µL methanol. Then, samples were diluted with 200 µL methanol:acetonitrile (1:1) and, vortexed for 3 min. Most of the protein precipitated and the debris were removed by centrifugation for 10 min at 4,500 RCF. The supernatant was filtered by centrifugation through 0.2 µm PVDF membranes in a 96 well format (Agilent Technologies, Santa Clara, CA, USA) at 4,500 RCF for 3 min. When samples were ready for the experiment, they were processed with two method blanks, two plasma laboratory reference materials (UTAK Plasma; UTAK

Laboratories Inc, Valencia, CA) and two plasma standard reference materials (NIST-1950; Sigma-Aldrich, St. Louis, MO). Bile acids were then quantified using internal standard methodology against authentic calibration standards detected by negative mode electrospray ionization and scheduled multiple reaction monitoring on an API 6500 QTrap (AB Sciex, Framingham, MA, USA). Data was processed with AB Sciex MultiQuant v 3.0.1 with integrations reviewed. A total of 24 completed participants were used in this secondary analysis, 2 samples were excluded due to technical issues in the analysis process.

A total of 19 bile acids were separated and identified from the analysis. Of these bile acids, 9 were primary bile acids where 3 were taurine conjugates and 2 were glycine conjugates, 10 were secondary bile acids where 3 were taurine conjugates and 4 were glycine conjugates (*Supplemental Table 1*).

<u>3.2.2. Statistical analysis</u>

All data were included from 27 participants in analyses when available unless otherwise stated. All data were checked for normality using the Shapiro-Wilks test, transformed if necessary and evaluated for outliers. AUC and iAUC were calculated for postprandial measures using R (R statistical software). Linear mixed-model ANOVAs were used to analyze outcome variables on JMP Pro 16 (SAS institute). Spearman's correlation were used to evaluate relationships between inflammatory markers and glycemic outcomes.

Bile acid data were range-scaled by centering the data to individual subjects' mean value across the 3 test days and all time points, then scaled to the maximum range of each subjects' data (Krishnan et al., 2012; van den Berg et al., 2006).

Multivariate cluster analysis by intervention was done using the range-scaled bile acids data to identify clusters of bile acids that were associated with glycemic outcomes. Spearman's correlations were used to identify relationships between 1) fasting concentrations of plasma bile acids and glycemic outcomes and 2) clustered postprandial bile acid responses and glycemic response among respective interventions.

3.3. Results

3.3.1. Glucose and insulin

There was a statistically significant difference in intervention in fasting glucose (p=0.03) following the supplementation period, where there was a modestly lower concentration of 99.1 $\pm$ 1.6 mg/dL following the HM intervention compared to MCC with 101.8 $\pm$ 1.6 mg/dL (p=0.03). No differences between MCC and HE (concentration of 99.7 $\pm$ 1.9 mg/dL; p=0.11) or HM and HE (p=0.84) were observed. Fasting insulin mirrored these results (p=0.04) where there was a lower concentration of 62.7 $\pm$ 10 pmol/L following the HM supplementation compared to MCC of 75.4 $\pm$ 12 pmol/L (p=0.03), but no differences between MCC and HE (concentration of 66.5 $\pm$ 10.4 pmol/L; p=0.30) or between HM and HE (p=0.56). These data are depicted in *Figures 3.1* and *3.2*.

Although, when evaluating the postprandial glucose response following the high fat meal, there were no differences between interventions (p=0.99), which was also reflected in the AUC and iAUC (p=0.55 and p=0.14, respectively). Similarly, insulin response following the challenge meal did not result in differences (p=0.51). Insulin

AUC and iAUC corroborated the response data (p=0.61 and p=0.10, respectively). *Figures 3.3* and *3.4* illustrate these data.

To estimate insulin resistance (i.e., HOMA-IR and McAuley) and sensitivity (i.e., QUICKI) indexes, fasting concentrations of glucose and insulin were used. The McAuley index also includes fasting triglyceride levels to estimate insulin resistance. The HM supplementation resulted in a lower HOMA-IR compared to MCC (p=0.04), whereas there were no differences between MCC and HE (p=0.22) or HM and HE (p=0.69). There was no significant difference in the McAuley index (p=0.15). Lastly, QUICKI was significantly higher following the HM supplementation compared to MCC (p=0.03), but no difference between MCC and HE (p=0.16) or HM and HE (p=0.74). Data are found in *Table 3.1*.

3.3.2. Inflammatory markers

Among the acute phase inflammatory markers, there was a significantly lower concentration of SAA following the HM intervention compared to MCC (concentrations of 6888.5 ± 1218.5 versus 9204.6 ± 1646 pg/mL, respectively; p=0.007) and compared to HE (concentration of 8567.3 ± 1292 pg/mL; p=0.05), but no difference between MCC and HE (p=0.75). There was a trend towards significance in intervention in CRP (concentrations of 6382.3 ± 1024.4 in MCC, 6401.3 ± 1067.3 in HE and 4769.3 ± 789.5 pg/mL; p=0.07). Data are found in *Figure 3.5*.

To identify whether the acute phase inflammatory markers had a relationship with the glycemic outcomes, Spearman’s correlations were conducted by intervention. While there were significant differences in CRP and SAA, there were no significant

correlations between fasting glucose, insulin, insulin resistance or sensitivity indexes. Data are found in *Tables 3.2-3.4*.

3.3.3. Bile acids

Fasting bile acids were used in Spearman's correlation with glycemic outcomes by intervention period. Significant and trends towards significance in correlations by intervention are shown in *Tables 3.5-3.7*. In the MCC intervention 1 primary bile acid (cholic acid [CA]) and 1 secondary bile acid (ursodeoxycholic acid [UDCA]) were the two bile acids that had notable correlations to insulin and insulin resistance indexes. In the HE intervention, there were 2 primary bile acid (tauro-α-muricholic acid [T- α-MCA], and chenodeoxycholic acid [CDCA]) and 5 secondary bile acids (glycochenodeoxycholic acid [GCDCA], taurodeoxycholic acid [TDCA], UDCA, glycocholic acid [GCA], and deoxycholic acid [DCA]) with significant correlations with fasting glucose, insulin, and insulin resistance indexes. Lastly, in the HM intervention, there was 1 primary bile acid (α-muricholic acid [α-MCA]) and 1 secondary bile acid (glycoursodeoxycholic acid [GUDCA]) with significant correlations with fasting glucose, insulin, and insulin resistance indexes.

Postprandial bile acid responses for all 19 observed bile acids were clustered by intervention. The MCC and HM interventions resulted in 4 clusters, while the HE intervention resulted in 5 clusters. Cluster members for each intervention are shown in *Table 3.8-3.10* alongside the representative variable for each cluster. Clusters are created and ranked based on the amount of the total variance that is explained. The composition of cluster 1 was identical in HE and HM interventions, while completely different bile acids made up cluster 1 in MCC. Cluster 2 and 3 had no similarities among

the three interventions. In cluster 4, two bile acids were the same in the HE and HM interventions. The representative variable in cluster 1, GCDCA, and in cluster 4, β-muricholic acid (β-MCA), were the same in the HE and HM interventions. MCC clusters and representative variables did not share commonalities as the two grape interventions shared. This suggests that HE and HM have similar bile acids that describe most of the variance, however the MCC clusters are much different and have a different impact on bile acid metabolism.

Respective whole cluster components were used in Spearman's correlations to identify relationships between the postprandial bile acid response and the glycemic postprandial responses for each respective intervention. *Table 3.11* lists the significant and trend toward significant cluster correlations following each of the interventions. In the MCC intervention, there was a trend towards significance ($p=0.06$, $\rho=0.2$) between cluster 4 and insulin response. Following the HE intervention, there was a significant correlation between cluster 1 ($p=0.002$, $\rho=0.31$) and 3 ($p=0.003$, $\rho=0.3$) with insulin response, while there was a trend towards significance between cluster 5 and glucose response ($p=0.06$, $\rho=0.2$). There was a significant correlation between cluster 2 and insulin response ($p=0.04$, $\rho=0.21$) following the HM intervention.

3.4. Discussion

The present study supplemented two formulations of Chardonnay marc blended products in humans. Fasting glucose concentrations were modestly lower following the high marc blend (HM) intervention compared to microcrystalline cellulose (MCC), but no differences with the high extract blend (HE). Similar results in fasting glucose were seen in diet induced obese (DIO) mice supplemented with Chardonnay seed flour (ChSF)

and DIO hamsters given Chardonnay seed extract (ChSE) gavages. This lowered fasting glucose result was not seen in the red grape marc (RGM) trials in humans (Costabile et al., 2019; Martinez-Maqueda et al., 2018; Urquiaga et al., 2015). While statistically significant, our results may not be clinically significant considering the variability in response. There was a 2.7 mg/dL difference in fasting glucose concentrations between HM and MCC, whereas oral glucose lowering medications has been documented to decrease fasting glucose by 30% (Hundal et al., 2000). Neither of the Chardonnay products resulted in changes in the postprandial glucose response, whereas the DIO mice supplemented with ChSF resulted in a lower oral glucose tolerance response compared to control (Seo et al., 2016; Seo et al., 2015). The glucose peak may have been missed due to blood sampling after an hour rather than 30 minutes postprandially— the RGM trials similarly did not report changes in postprandial glucose response (Costabile et al., 2019; Martinez-Maqueda et al., 2018).

The aforementioned animal and human trials reported lower fasting insulin (Costabile et al., 2019; Martinez-Maqueda et al., 2018; Seo et al., 2016; Seo et al., 2015). Our data demonstrated that there was a significantly lower fasting insulin concentration following the HM supplementation compared to MCC in this population. Unlike the previous studies, our trial did not demonstrate differences in insulin response. Indexes of insulin resistance (HOMA-IR and McAuley) and sensitivity (QUICKI) were calculated following the supplementation periods. Following the HM supplementation, there was a significant decrease in HOMA-IR compared to MCC but that difference disappeared when triglycerides were included to calculate the McAuley index, suggesting the impact is focused on carbohydrate metabolism. HOMA-IR is a commonly

used index to identify insulin resistance, yet a clear cut off to determine normal versus insulin resistant is complicated as it varies with sex, ethnicity, body mass index and more (Gayoso-Diz et al., 2011; Kim et al., 2018; Qu et al., 2011; Singh et al., 2013). For this study, we chose a cut off of ≤2 based on the work of Gayoso-Diz et al. that also considered cardio-metabolic risk factors (Gayoso-Diz et al., 2013). The participants who exhibited insulin resistance, consistently had improved HOMA-IR following HM supplementation compared to MCC. There was also an increase in QUICKI following the HM intervention compared to MCC, indicating an increase in insulin sensitivity. Future studies should evaluate this finding in a larger and more specific population.

A proposed mechanism for Chardonnay flavanols to improve insulin resistance (IR) includes reducing inflammation (Seo et al., 2016; Seo et al., 2015). The ChSF trials measured gene expression and found genes related to inflammation (e.g. genes in the family of tumor necrosis factors, interleukins, proliferator-activated receptors) were significantly downregulated and attributed this as one potential mechanism of improved insulin resistance (Kim et al., 2014; Seo et al., 2016; Seo et al., 2015). None of the ChSF studies measured specific inflammatory marker concentrations. Gene expression data, while important, are not necessarily indicative of actual protein expression levels.

A meta-analysis of grape products concluded that long supplementation periods (≥12 weeks) and >500mg/day of grape polyphenols was needed to decrease C-reactive protein (CRP) concentrations (Sarkhosh-Khorasani & Hosseinzadeh, 2021). The present study supplemented for 3 weeks with roughly 75 and 120mg of total polyphenols in the HM and HE interventions, respectively. Despite the present study's lower supplementation levels, there was a trend towards significance in CRP, and upon

visual inspection following the HM intervention there was lower CRP than the other two interventions. Serum amyloid A (SAA), another acute phase inflammatory marker, was significantly reduced following HM intervention compared to the other interventions. CRP and SAA are both correlated with insulin resistance (Filippin-Monteiro et al., 2012; Marzi et al., 2013; Scheja et al., 2008; Tan et al., 2004). The HM intervention appears to impact both of these markers, however the correlation analysis did not reveal a relationship between the inflammatory markers and the glycemic outcomes. Our data does not indicate that the lowered inflammatory markers are correlated to the lowered fasting insulin and HOMA-IR.

The present study appears to be the first to evaluate the effect grape phenolics on bile acids in the context of glucose metabolism in humans. Procyanidins, oligomers comprised of the flavan-3-ols catechin and epicatechin, act as a co-agonist with bile acids for FXR as well as alter the gut microbiota as shown in animal and *in vitro* experiments (Downing et al., 2017; Tveter et al., 2020). Procyanidins are large molecules that are not highly bioavailable to humans but are metabolized by the gut microbiome (Mena et al., 2019a; Ou & Gu, 2014; Zhang et al., 2016). It is unclear whether the limited data available regarding procyanidin's effect on bile acid metabolism are translatable to humans.

Previous ChSF studies measured gene expression related to bile acid synthesis from cholesterol but did not consider potential relationships to glucose regulation (Kim et al., 2014; Seo et al., 2016; Seo et al., 2015). The HM intervention resulted in improved glycemic markers—interestingly α-muricholic acid (α-MCA), a primary bile acid, was negatively correlated to fasting insulin and HOMA-IR while positively

correlated to QUICKI and the McAuley index. While α-MCA is predominantly found in murine animal models, they are also found in small amounts in humans (Goto et al., 1992; Ikawa et al., 1987). In mice, this compound has been shown to be associated with improved glucose metabolism (Bonde et al., 2016). The HE intervention, predominantly had secondary bile acids that were significantly correlated with the fasting glycemic outcomes. Secondary bile acids are formed through gut microbial modification of primary bile acids (Ridlon et al., 2014). While the postprandial measurements were not different following the different interventions, the significant cluster correlations were represented mostly by secondary bile acids. The discussion of the microbiome is beyond the scope of this dissertation, but it is worth noting the interrelationship as many larger phenolic compounds are metabolized by the microbiome as well. Tveter et al. suggested that grape polyphenols led to decreased secondary bile acids, which ultimately inhibited FXR to improve glucose regulation (Tveter et al., 2020). As of this writing, we are still awaiting GLP-1 to make further sense of the relationship between the bile acids and glycemic outcomes.

3.5. Conclusion

Following the HM intervention, fasting glucose, insulin and indexes of insulin resistance and sensitivity improved compared to the non-grape comparator, microcrystalline cellulose. Acute phase inflammatory markers were similarly improved by HM, which may contribute to the improved glycemic outcomes. Future studies should evaluate the clinical significance of these findings.

Figures

Figure 3.1. Fasting glucose

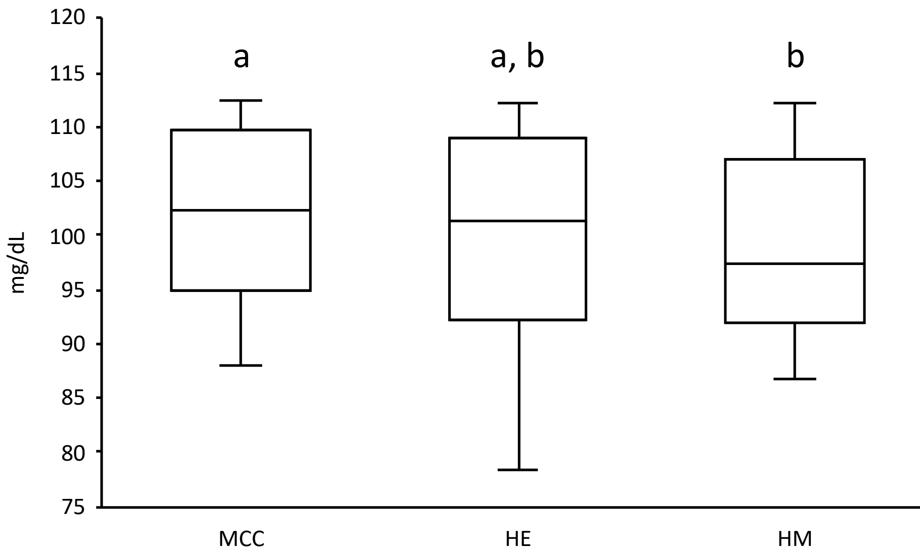

Figure 3.1. Fasting glucose data are depicted as box and whisker plots (n=27). Fasting glucose was lower following the HM intervention compared to MCC (p=0.03), but not between MCC and HE (p=0.11) or HM and HE (p=0.84). MCC – microcrystalline cellulose, HE – high Chardonnay extract blend, HM – high Chardonnay marc blend

Figure 3.2. Fasting insulin

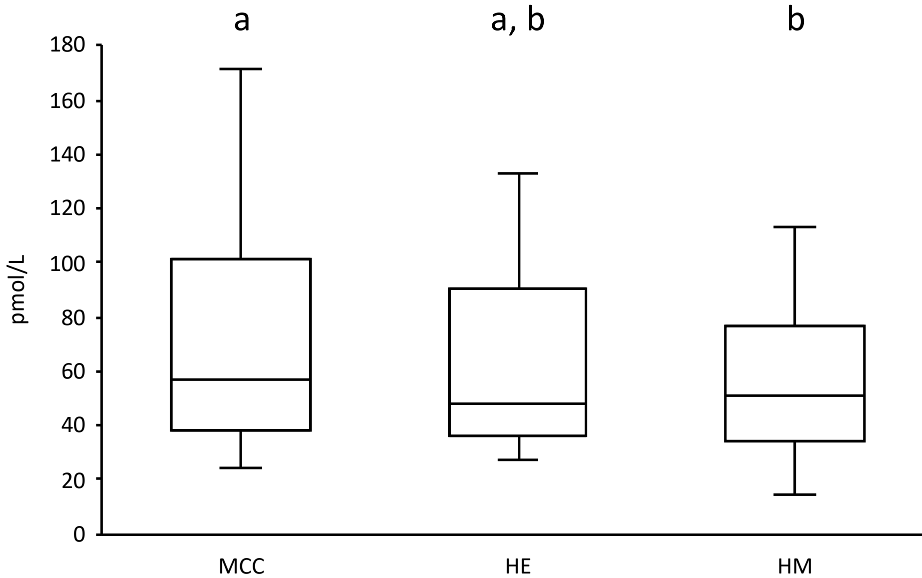

Figure 3.2. Fasting insulin data are depicted as box and whisker plots (n=27). Fasting glucose was lower following the HM intervention compared to MCC (p=0.03), but not between MCC and HE (p=0.3) or HM and HE (p=0.56). MCC – microcrystalline cellulose, HE – high Chardonnay extract blend, HM – high Chardonnay marc blend

Figure 3.3. Glucose response

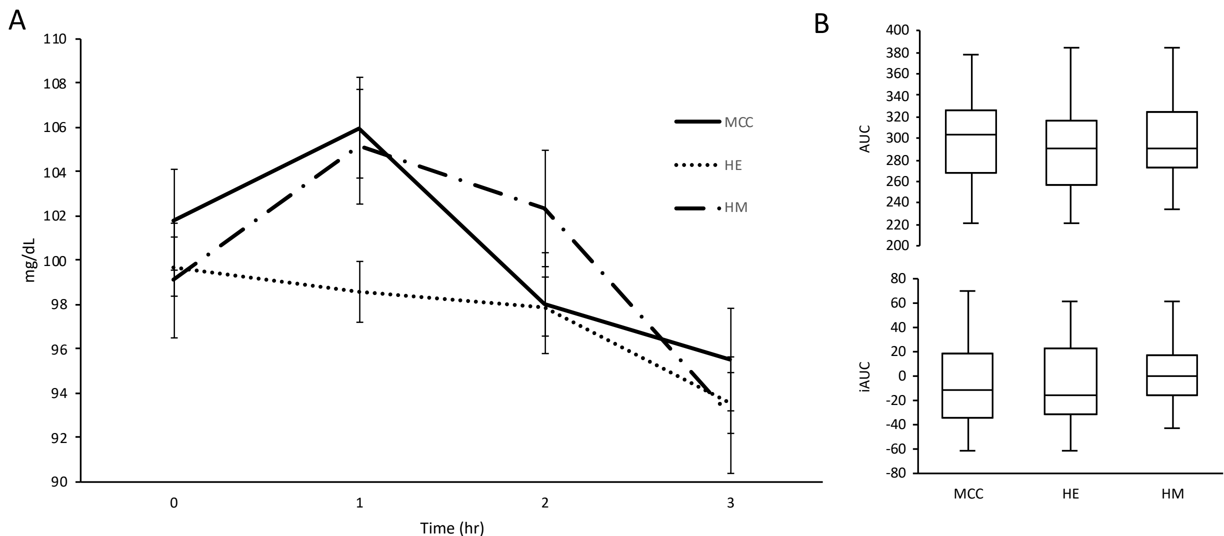

Figure 3.3. Panel A: Glucose response is shown from fasting through 3 hours postprandially (n=27). Data are presented as mean ± SEM. Panel B: Area under the curve and incremental area under the curve are shown as box and whisker plots. MCC – microcrystalline cellulose, HE – high Chardonnay extract blend, HM – high Chardonnay marc blend

Figure 3.4. Insulin response

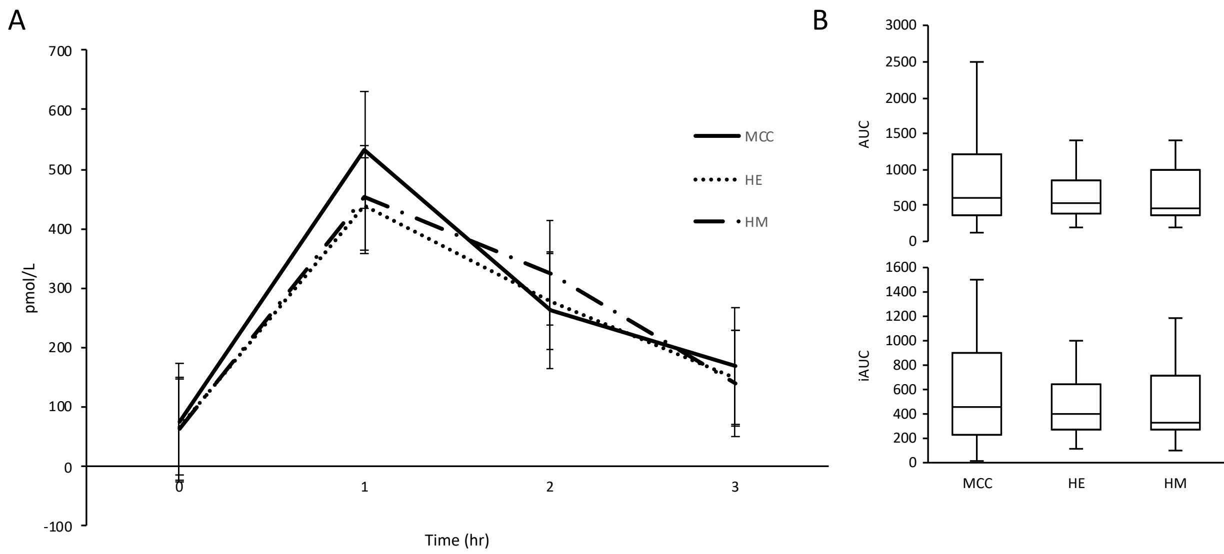

Figure 3.4. Panel A: Insulin response is shown from fasting through 3 hours postprandially (n=27). Data are presented as mean ± SEM. Panel B: Area under the curve and incremental area under the curve are shown as box and whisker plots. MCC – microcrystalline cellulose, HE – high Chardonnay extract blend, HM – high Chardonnay marc blend

Figure 3.5. Acute phase inflammatory markers

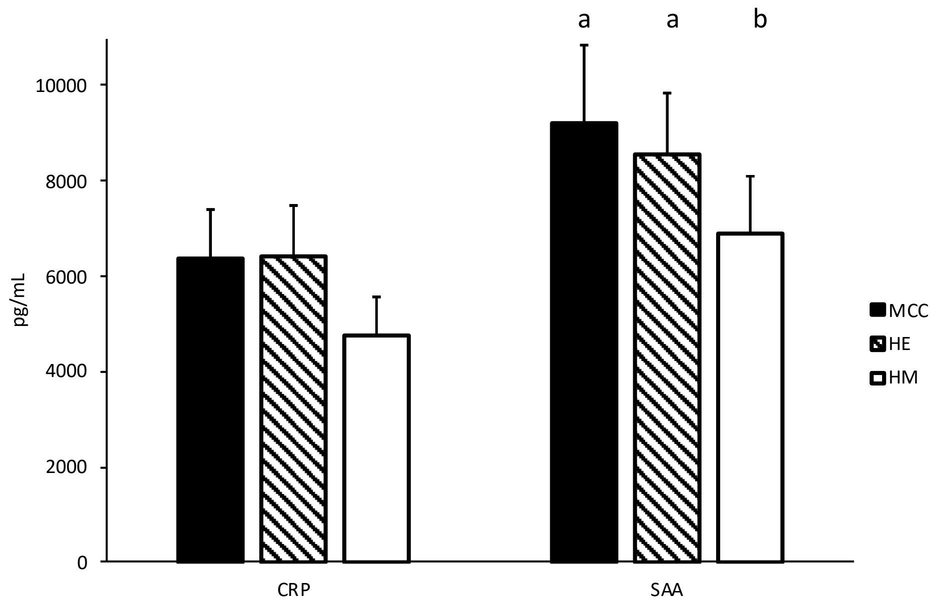

Figure 3.5. Acute phase inflammatory markers are shown following respective interventions (n=27). No significant effects in CRP (p=0.07). SAA was significantly lower following the HM intervention compared to both MCC (p=0.007) and HE (p=0.05) interventions. Data are presented as mean ± SEM. MCC – microcrystalline cellulose, HE – high Chardonnay extract blend, HM – high Chardonnay marc blend; CRP – C-reactive protein, SAA – serum amyloid A

Tables

Table 3.1. Insulin indexes

	Microcrystalline cellulose (n=25)	High extract blend (n=25)	High marc blend (n=26)
HOMA-IR[1]	2.76 ± 0.45[a]	2.43 ± 0.42[a,b]	2.26 ± 0.39[b]
QUICKI[2]	0.34 ± 0.01[a]	0.35 ± 0.01[a,b]	0.35 ± 0.01[b]
McAuley index	7.11 ± 0.39	7.14 ± 0.39	7.59 ± 0.39

Values are mean ± SEM. Different letters indicate significance.

[1]HOMA-IR - Homeostatic Model Assessment for Insulin Resistance, [2]QUICKI - quantitative insulin-sensitivity check index

Table 3.2. Inflammatory marker and glycemic outcome correlation following microcrystalline cellulose intervention

Intervention	**Inflammatory marker variable**	**Glucose/Insulin Variable**	**Spearman ρ**	**P value**
MCC[1]	CRP[2]	Fasting glucose	-0.04	0.83
MCC	CRP	Fasting insulin	0.16	0.46
MCC	CRP	HOMA-IR[4]	0.17	0.41
MCC	CRP	QUICKI[5]	-0.17	0.41
MCC	CRP	McAuley	-0.05	0.80
MCC	SAA[3]	Fasting glucose	-0.08	0.71
MCC	SAA	Fasting insulin	0.29	0.16
MCC	SAA	HOMA-IR	0.27	0.19
MCC	SAA	QUICKI	-0.27	0.19
MCC	SAA	McAuley	-0.31	0.13

[1]Microcrystalline cellulose; [2]C-reactive protein; [3]serum amyloid A; [4]Homeostatic Model Assessment for Insulin Resistance, [5]quantitative insulin-sensitivity check index

Table 3.3. Inflammatory marker and glycemic outcome correlation following high extract blend intervention

Intervention	Inflammatory variable	Glucose/Insulin Variable	Spearman ρ	P value
HE[1]	CRP[2]	Fasting glucose	-0.03	0.88
HE	CRP	Fasting insulin	0.26	0.21
HE	CRP	HOMA-IR[4]	0.25	0.23
HE	CRP	QUICKI[5]	-0.25	0.23
HE	CRP	McAuley	-0.18	0.39
HE	SAA[3]	Fasting glucose	-0.09	0.67
HE	SAA	Fasting insulin	0.28	0.18
HE	SAA	HOMA-IR	0.26	0.22
HE	SAA	QUICKI	-0.26	0.22
HE	SAA	McAuley	-0.35	0.08

[1]High extract blend; [2]C-reactive protein; [3]serum amyloid A; [4]Homeostatic Model Assessment for Insulin Resistance, [5]quantitative insulin-sensitivity check index

Table 3.4. Inflammatory marker and glycemic outcome correlation following high marc blend intervention

Intervention	Inflammatory variable	Glucose/Insulin Variable	Spearman ρ	P value
HM[1]	CRP[2]	Fasting glucose	0.26	0.19
HM	CRP	Fasting insulin	0.16	0.45
HM	CRP	HOMA-IR[4]	0.21	0.30
HM	CRP	QUICKI[5]	-0.21	0.30
HM	CRP	McAuley	-0.23	0.25
HM	SAA[3]	Fasting glucose	-0.01	0.95
HM	SAA	Fasting insulin	0.17	0.40
HM	SAA	HOMA-IR	0.19	0.35
HM	SAA	QUICKI	-0.19	0.35
HM	SAA	McAuley	-0.09	0.66

[1]High marc blend; [2]C-reactive protein; [3]serum amyloid A; [4]Homeostatic Model Assessment for Insulin Resistance, [5]quantitative insulin-sensitivity check index

Table 3.5. Bile acid and glycemic outcome correlation following microcrystalline cellulose intervention

Intervention	Bile acid variable	Glucose/Insulin variable	Spearman ρ	P value
MCC[1]	CA[2]	HOMA-IR[4]	0.47	0.02
MCC	CA	QUICKI[5]	-0.46	0.02
MCC	CA	Fasting insulin	0.46	0.02
MCC	UDCA[3]	Fasting insulin	0.41	0.04
MCC	UDCA	HOMA-IR	0.40	0.05
MCC	UDCA	QUICKI	-0.40	0.06

[1]Microcrystalline cellulose, [2]cholic acid, [3]ursodeoxycholic acid, [4]HOMA-IR - Homeostatic Model Assessment for Insulin Resistance, [5]QUICKI - quantitative insulin-sensitivity check index

Table 3.6. Bile acid and glycemic outcome correlation following high extract blend intervention

Intervention	Bile acid variable	Glucose/Insulin variable	Spearman ρ	P value
HE[1]	GDCA[2]	Fasting glucose	0.52	0.01
HE	T-α-MCA[3]	Fasting glucose	0.46	0.02
HE	TDCA[4]	Fasting glucose	0.44	0.03
HE	CDCA[5]	Fasting insulin	-0.44	0.03
HE	UDCA[6]	HOMA-IR[9]	-0.43	0.03
HE	UDCA	Fasting insulin	-0.43	0.04
HE	UDCA	QUICKI[10]	0.43	0.04
HE	CDCA	HOMA-IR	-0.42	0.04
HE	CDCA	QUICKI	0.41	0.05
HE	GCA[7]	Fasting glucose	0.40	0.06
HE	DCA[8]	Fasting insulin	-0.39	0.06

[1]High extract blend, [2]glycochenodeoxycholic acid, [3]tauro-α-muricholic acid, [4]taurodeoxycholic acid, [5]chenodeoxycholic acid, [6]ursodeoxycholic acid, [7]glycocholic acid, [8]deoxycholic acid, [9]HOMA-IR - Homeostatic Model Assessment for Insulin Resistance, [10]QUICKI - quantitative insulin-sensitivity check index

Table 3.7. Bile acid and glycemic outcome correlation following high marc blend intervention

Intervention	Bile acid variable	Glucose/Insulin variable	Spearman ρ	P value
HM[1]	α-MCA[2]	McAuley	0.50	0.01
HM	α-MCA	Fasting insulin	-0.45	0.03
HM	GUDCA[3]	Fasting glucose	-0.45	0.03
HM	α-MCA	HOMA-IR[4]	-0.41	0.05
HM	α-MCA	QUICKI[5]	0.40	0.05

[1]High marc blend, [2]α-muricholic acid, [3]glycoursodeoxycholic acid, [4]HOMA-IR - Homeostatic Model Assessment for Insulin Resistance, [5]QUICKI - quantitative insulin-sensitivity check index

Table 3.8. Bile acid clusters - Microcrystalline cellulose

Cluster	Members	Representative variable
1	TLCA[1] GHDCA[2] T-α-MCA[3] α-MCA[4] TUDCA[5] LCA[6] TCA[7] UDCA[8]	TLCA
2	GCDCA[9] GCA[10] GUDCA[11] TCDCA[12] β-MCA[13]	GCDCA
3	CA[14] CDCA[15]	CA
4	GDCA[16] TDCA[17] GLCA[18] DCA[19]	GDCA

[1]Glycochenodeoxycholic acid, [2]glycocholic acid, [3]taurochenodesoxycholic acid, [4]taurocholic acid, [5]glycoursodeoxycholic acid, [6]glycohyodeoxycholic acid, [7]tauroursodeoxycholic acid, [8]deoxycholic acid, [9]ursodeoxycholic acid, [10]α-muricholic acid, [11]taurodeoxycholic acid, [12]glycodeoxycholic acid, [13]taurolithocholate, [14]tauro-α-Muricholic acid, [15]glycolithocholate, [16]β-muricholic acid, [17]lithocholic acid, [18]cholic acid, [19]chenodeoxycholic acid

Table 3.9. Bile acid clusters - High extract blend

Cluster	Members	Representative variable
1	GCDCA[1] GCA[2] TCDCA[3] TCA[4] GUDCA[5] GHDCA[6] TUDCA[7]	GCDCA
2	DCA[8] UDCA[9] α-MCA[10]	DCA
3	TDCA[11] GDCA[12] TLCA[13] T-α-MCA[14] GLCA[15]	TDCA
4	β-MCA[16] LCA[17]	β-MCA
5	CA[18] CDCA[19]	CA

[1]Taurolithocholate, [2]glycohyodeoxycholic acid, [3]tauro-α-Muricholic acid, [4]α-muricholic acid, [5]taurochenodesoxycholic acid, [6]lithocholic acid, [7]taurocholic acid, [8]ursodeoxycholic acid, [9]glycochenodeoxycholic acid, [10]glycocholic acid, [11]glycoursodeoxycholic acid, [12]tauroursodeoxycholic acid, [13]β-muricholic acid, [14]cholic acid, [15]chenodeoxycholic acid, [16]glycodeoxycholic acid, [17]taurodeoxycholic acid, [18]glycolithocholate, [19]deoxycholic acid

Table 3.10. Bile acid clusters – High marc blend

Cluster	Members	Representative variable
1	GCDCA[1] GCA[2] TCDCA[3] TCA[4] GUDCA[5] TUDCA[6] GHDCA[7]	GCDCA
2	TDCA[8] TLCA[9] GDCA[10] T-α-MCA[11] GLCA[12]	TDCA
3	DCA[13] CDCA[14] α-MCA[15] UDCA[16]	DCA
4	β-MCA[17] LCA[18] CA[19]	β-MCA

[1]Glycochenodeoxycholic acid, [2]glycocholic acid, [3]taurochenodesoxycholic acid, [4]taurocholic acid, [5]glycoursodeoxycholic acid, [6]tauroursodeoxycholic acid, [7]glycohyodeoxycholic acid, [8]taurodeoxycholic acid, [9]taurolithocholate, [10]glycodeoxycholic acid, [11]tauro-α-Muricholic acid, [12]glycolithocholate, [13]deoxycholic acid, [14]chenodeoxycholic acid, [15]α-muricholic acid, [16]ursodeoxycholic acid, [17]β-muricholic acid, [18]lithocholic acid, [19]cholic acid

Table 3.11. Bile acid response cluster correlation analysis

Intervention	Bile acid cluster	Glucose/insulin variable	Spearman ρ	P value
MCC[1]	MCC cluster 4 components	Insulin response	0.20	0.06
HE[2]	HE cluster 1 components	Insulin response	0.31	0.002
HE	HE cluster 3 components	Insulin response	0.30	0.003
HE	HE cluster 5 components	Glucose response	0.20	0.06
HM[3]	HM cluster 2 components	Insulin response	0.21	0.04

[1]Microcrystalline cellulose, [2]high extract blend, [3]high marc blend

Supplemental Tables

Supplemental Table 1. Observed bile acids

Bile acid name	Abbreviation	Chemical class
Cholic acid	CA	Primary bile acid
Chenodeoxycholic acid	CDCA	Primary bile acid
α-Muricholic acid	α-MCA	Primary bile acid
β-Muricholic acid	β-MCA	Primary bile acid
Taurocholic acid	TCA	Primary bile acid – taurine conjugate
Taurochenodesoxycholic acid	TCDCA	Primary bile acid – taurine conjugate
Tauro-α-Muricholic acid	T-α-MCA	Primary bile acid – taurine conjugate
Glycocholic acid	GCA	Primary bile acid – glycine conjugate
Glycochenodeoxycholic acid	GCDCA	Primary bile acid – glycine conjugate
Ursodeoxycholic acid	UDCA	Secondary bile acid
Deoxycholic acid	DCA	Secondary bile acid
Lithocholic acid	LCA	Secondary bile acid
Tauroursodeoxycholic acid	TUDCA	Secondary bile acid – taurine conjugate
Taurodeoxycholic acid	TDCA	Secondary bile acid – taurine conjugate
Taurolithocholate	TLCA	Secondary bile acid – taurine conjugate
Glycoursodeoxycholic acid	GUDCA	Secondary bile acid – glycine conjugate
Glycodeoxycholic acid	GDCA	Secondary bile acid – glycine conjugate
Glycohyodeoxycholic acid	GHDCA	Secondary bile acid – glycine conjugate
Glycolithocholate	GLCA	Secondary bile acid – glycine conjugate

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Chapter 4

Effects of Chardonnay marc on appetite regulation

4. 1. Introduction

Overweight and obesity are risk factors for numerous chronic conditions such as type 2 diabetes mellitus, cardiovascular disease, high blood pressure and more. Modest weight loss is recommended as a method to combat these issues (Haase et al., 2021). Regulation of energy balance in terms of appetite and satiety is a highly complex process that involves neural input and hormonal feedback that impacts eating behaviors such as desiring food, inducing fullness or meal termination (Heisler & Lam, 2017; Murphy & Bloom, 2006). Flavan-3-ols such as proanthocyanidins and catechins have been demonstrated to increase fat oxidation in humans, thus may be a potential method of weight control, however there are limited studies evaluating the role of polyphenols on appetite regulation as another method of weight control (Gutierrez-Salmean et al., 2014; Kim et al., 2014).

High doses of grape seed extract (GSE) in rat models were effective in lowering food intake (Serrano, Casanova-Martí, Gil-Cardoso, et al., 2016). The authors suggested that the accompanying effects on gastrointestinal (GI) hormones were one aspect contributing to the decreased food intake as ghrelin, a hormone that stimulates hunger, was inhibited and peptide YY (PYY), a hormone that increases satiety and delays gastric emptying, was increased (Casanova-Martí et al., 2020; Serrano, Casanova-Martí, Depoortere, et al., 2016; Serrano, Casanova-Martí, Gil-Cardoso, et al., 2016). Interestingly, Chardonnay grape seed flour (ChSF) supplementation resulted in an increased food intake in animals, without adverse effects on weight or other

metabolic outcomes (Kim et al., 2014; Seo et al., 2016; Seo et al., 2015). ChSF is rich in polyphenols, particularly flavan-3-ols, yet had increased intake. These trials did not measure any GI hormones related to appetite regulation. Currently, there is one study that has fed humans ChSF roughly 4.8g per day for 4 months that did not see changes in body weight and did not measure food intake or appetite related GI hormones.

Chardonnay marc is composed of primarily skins and seeds remaining from winemaking, thus is nutritionally complex with polyphenols, dietary fibers and other micro- and macronutrients. ChSF supplementation led to an increase in food intake in animals, however it is unclear if Chardonnay marc has effects on appetite regulation. The secondary aim of the study was to evaluate subjective and hormonal measures of acute satiety following supplementation with a Chardonnay marc rich blend and a Chardonnay seed extract rich blend in adult men and women. We hypothesized that the Chardonnay seed extract rich blend will increase both subjective and hormonal measures of satiety, while the Chardonnay marc blend would not.

4.2. Methods

A complete description of the study design and participant inclusion/exclusion criteria were described in section 2.2. Analyses and protocol not previously described are included here.

4.2.1. Protocols

Metabolic testing

Participants arrived at the WHNRC in the morning, following a 12-hour fast for a 6-hour metabolic test day. They consumed a high fat breakfast challenge meal, which comprised of a breakfast casserole with egg, rice, potatoes, turkey

sausage, cheddar cheese and mango-orange juice. Participants also consumed the last set of capsules with breakfast. Before breakfast, a licensed phlebotomist drew fasting blood through the antecubital vein in ethylenediaminetetraacetic acid (EDTA) vacutainers (Becton Dickinson, Rutherford, New Jersey). Blood was subsequently drawn at 1, 2 and 3 hours after consuming the high fat challenge meal. All plasma samples had dipeptidyl-peptidase IV (DPP-IV) and aprotinin inhibitors added to the vacutainers prior to collection. Once blood was drawn, plasma vacutainers were immediately chilled on ice. All vacutainers were centrifuged in a refrigerated Centra CL3R (International Equipment Co.) for 10 minutes at 100 x *g* at 4°C, then aliquoted before storing at -80°C until ready for analyses. All 3 test days followed the same protocol.

Visual analog scales

To evaluate subjective measures of satiety, 100mm visual analog scales (VAS) were administered on a palm pilot (PalmOne Zire 80) at intervals of approximately 20-40 minutes over the 6-hour test day, for a total of 9 measurements. Two fasting measurements were averaged before proceeding with analyses. Four variables were included: hunger, fullness, desire to eat and prospective consumption, nausea was used to determine whether scores were valid to use. Three responses were excluded from the analysis due to consistent ratings of nausea throughout the test day. Participants were instructed to rate their current perception of each parameter on the scale to questions such as “How hungry do you feel right now?” with either end being anchored by opposing descriptions (e.g. “not at all hungry” and “extremely hungry”). All questions are presented in *Table 1*.

Satiety hormones

To evaluate satiety objectively, the gastrointestinal hormones ghrelin and peptide YY (PYY) were measured in plasma at fasting, 1, 2 and 3 hours postprandially. Total active ghrelin was measured with a metabolic plate immunoassay utilizing a biotinylated capture antibody on the plate surface and all samples were acidified with hydrochloric acid prior to analysis (Meso Scale Diagnostics, Rockville, MD). PYY_{3-36} was measured with a radioactive immunoassay (Millipore, Billerica, MA).

4.2.2. Statistical analysis

Data from participants who completed the entire 16-wk trial were included (n=24) in this secondary analysis. Area under the curve (AUC) and incremental area under the curve (iAUC) were calculated for VAS and satiety hormone response data using R (R statistical software) using the trapezoid rule. The iAUC calculation allowed values to be negative if they went below baseline. AUC is derived from the entire acute timeframe including fasting values and iAUC accounts for the acute response while not including the fasting area. Linear mixed models were used to compare the effects of the intervention and Spearman's correlation were used to evaluate the relationship between the subjective and objective data for each intervention. All analyses were performed using JMP Pro 16 (SAS Institute). Data were checked for normality using the Shapiro-Wilk test and non-normal data were logarithmically transformed.

4.3. Results

4.3.1. Visual analog scales

Following the supplementation period there was a significant main effect of intervention in hunger AUC (p=0.039; *Figure 4.1.*). Multiple comparisons revealed a

significantly lower AUC following HE compared to MCC (p=0.049), but no difference between HE and HM (p =0.1) or between HM and MCC (p=0.9). There was no significant main effect of intervention in ratings of fullness AUC (p=0.8; *Supplemental Figure 4.1.*), a weak trend for rating of desire to eat AUC (p=0.1; *Supplemental Figure 4.2.*) and no significant difference in ratings of prospective food consumption AUC (p=0.2; *Supplemental Figure 4.3.*).

There were no significant main effects of intervention seen in hunger iAUC (p=0.8; *Figure 4.1.),* fullness iAUC (p=0.4; *Supplemental Figure 4.1.*) or desire to eat iAUC (p=0.4; *Supplemental Figure 4.2.).* However, there was weak evidence of an effect in prospective food consumption iAUC (p=0.06; *Supplemental Figure 4.3.*).

4.3.2. Satiety hormones

Fasting

At fasting, there were no significant main effects of intervention in levels of ghrelin (p=0.6) following the supplementation period. Similarly, there were no significant main effects of intervention in fasting levels of PYY_{3-36} (p=0.4).

Postprandial response

There were no significant main effects of intervention in postprandial ghrelin response (p=0.9; *Figure 4.2.*). Interestingly, there was a significant main effect of intervention in postprandial PYY_{3-36} (p=0.04). Multiple comparisons revealed a lower PYY_{3-36} response following the HE supplementation compared to HM (p=0.05), but no differences between MCC and HE (p=0.9) or MCC and HM (p=0.09; *Figure 4.3.*).

AUC and iAUC

There were no significant main effects of intervention in ghrelin AUC (p=0.9; *Figure 4.2.*). There was strong evidence of an effect of intervention on PYY_{3-36} AUC (p=0.007; *Figure 4.3.*). Multiple comparisons revealed a lower PYY_{3-36} AUC following the HE supplementation compared to HM (p=0.015), lower PYY_{3-36} AUC following the MCC supplementation compared to HM (p=0.019) but no difference between MCC and HE (p=1; *Figure 4.3.*).

Ghrelin iAUC did not have significant differences (p=0.6; *Figure 4.2.*). However, there was evidence of a main effect of intervention on PYY_{3-36} iAUC (p=0.020; *Figure 4.3.*), which corroborated the postprandial PYY response finding. Multiple comparisons showed there was a significantly lower PYY_{3-36} iAUC following the MCC supplementation compared to HM (p=0.019) but not between MCC and HE (p=0.1) or HE and HM (p=0.7; *Figure 4.3.*).

4.4. Discussion

To my knowledge, this is the first study to evaluate both subjective and hormonal measures of satiety in humans using Chardonnay marc, as there is currently limited existing literature on flavonoids and satiety. Previous animal trials using Chardonnay seed flour (ChSF), which is high in flavan-3-ols, particularly (-)-epicatechins, noted an increased food intake, but did not measure gastrointestinal hormones (Kim et al., 2014; Seo et al., 2016). Grape seed extract (GSE) rich in proanthocyanidins, which are also abundant in Chardonnay, have been demonstrated to decrease ghrelin and increase peptide YY (PYY) in *in vitro* and *ex vivo* studies (Ginés et al., 2019; Grau-Bové et al., 2020; Serrano, Casanova-Martí, Depoortere, et al., 2016). The current study

supplemented relatively low doses of total polyphenols in humans compared to the animal *in vivo* and *ex vivo* trials. None of the interventions had an impact on ghrelin at fasting or following the challenge meal. However, in the subjective visual analog scale (VAS) ratings there was an overall lower total hunger response following the high extract blend (HE) supplementation compared to microcrystalline cellulose (MCC) without the corresponding hormonal difference.

The HE intervention with higher levels of polyphenols, resulted in lower PYY_{3-36} levels, compared to the high marc blend (HM) intervention with lower polyphenols but higher fiber that elevated PYY_{3-36} levels. These hormonal data did not support previous GSE studies conducted *in vivo* and *ex vivo*. One function of PYY is to increase satiety, and PYY is thought to act after non-digestible components of a meal enter the large intestine, stimulating PYY release. The higher fiber content of the HM could have led to the higher PYY_{3-36} that was measured. However, the protocol used in this study only collected data for three hours following meal ingestion, and it is likely that this timeframe was not sufficiently long to result in significant subjective ratings of fullness or decreased desire to eat. None of the interventions resulted in a significant subjective assessment of increased satiety. These results are similar to the VAS results seen in humans supplemented with GSE (Vogels et al., 2004).

Satiety and subsequent eating behavior are highly complex in humans. Changes in circulating hormone concentrations may not necessarily reflect those in the brain that would lead to change in behavior or changes in feelings of satiety. Food intake was not assessed, however, VAS rating for prospective food intake was measured. While there was increased PYY, there was no corresponding difference in prospective food intake

or desire to eat VAS ratings. High doses of GSE inhibited food intake in rats, while ChSF increased food intake as noted previously (Kim et al., 2014; Seo et al., 2016; Serrano et al., 2017; Serrano, Casanova-Martí, Blay, et al., 2016; Serrano, Casanova-Martí, Gil-Cardoso, et al., 2016). Polyphenols have a bitter taste and has been shown to activate specific bitter taste receptors however, Serrano et al. conducted their studies by directly injecting the GSE intragastrically to bypass issues with taste (Soares et al., 2013). This method may imply that the compounds in GSE directly may inhibit food intake however translating these results to humans may be problematic as injecting supplements directly into the stomach is an uncommon mode of delivery. The current study does not measure food intake but should be considered in future studies.

The limited measurements that were done in this study were exploratory in nature and not designed to thoroughly evaluate other aspects of satiety. It would be interesting to consider whether the difference in PYY response is connected to the microbiome or to insulin regulation as there were no resulting subjective changes in satiety. Future directions should consider including additional hormones (e.g. adiponectin, leptin), evaluating food intake and varying supplementation amounts.

4.6. Conclusion

In summary, participants felt overall less hungry following the high extract blend supplementation compared to the microcrystalline cellulose, despite a lack of differences in ghrelin concentrations between interventions. Peptide YY was significantly higher following high marc blend compared to high extract blend supplementation, but this did not result in a change in subjective feelings of fullness or

satiety. Additional studies are needed to further understand how Chardonnay marc may affect appetite and satiety.

Figures

Figure 4.1. Visual analog scale – Hunger

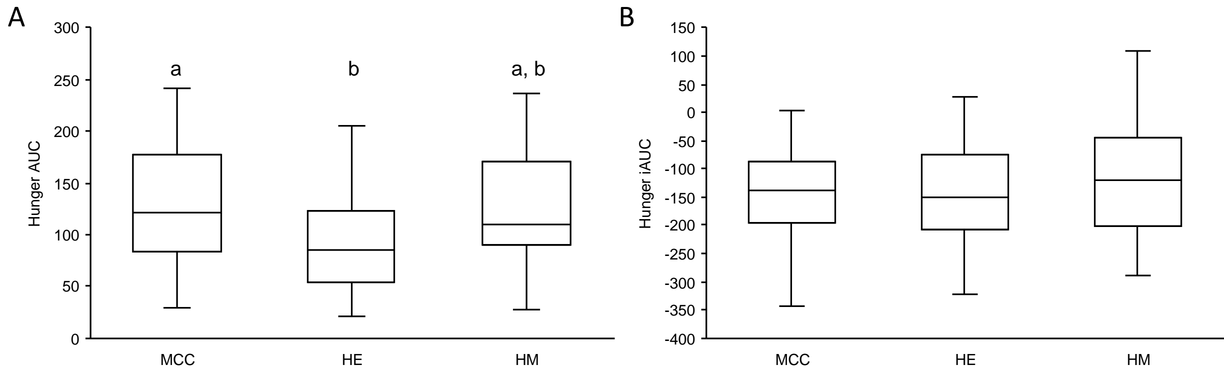


Figure 4.1. Panel A: Visual anaolg scale hunger area under the curve (AUC) is depicted in a box and whisker plot. Different letters indicate significant differences between interventions. Panel B: Visual analog scale hunger incremental area under the curve (iAUC) is depicted in a box and whisker plot.

Figure 4.2. Postprandial ghrelin response

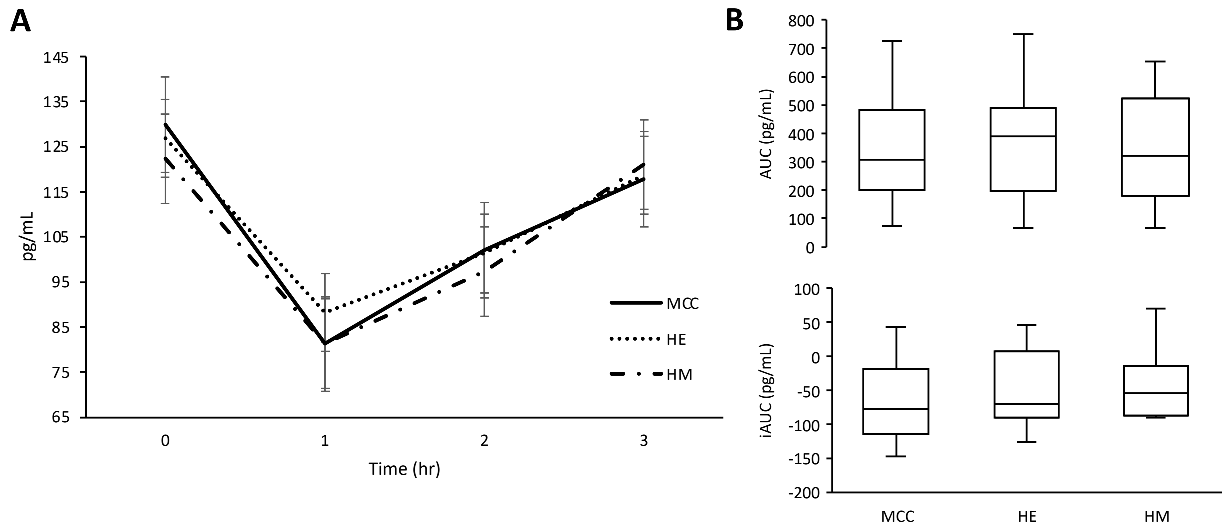


Figure 4.2. Panel A: Ghrelin response is shown from fasting through 3 hours postprandially. Data are presented as mean ± SEM. Panel B: Area under the curve and incremental area under the curve are shown as box and whisker plots. MCC – microcrystalline cellulose, HE – high Chardonnay extract blend, HM – high Chardonnay marc blend

Figure 4.3. Postprandial peptide YY response

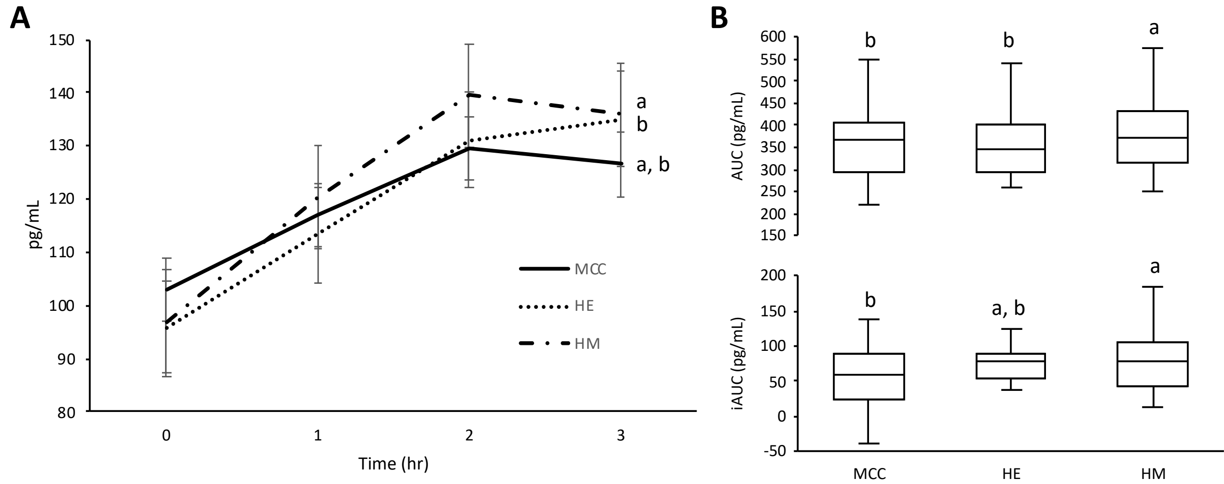


Figure 4.3. Panel A: Peptide YY response is shown from fasting through 3 hours postprandially. Significant differences in intervention are shown with different letters. Data are presented as mean ± SEM. Panel B: Area under the curve and incremental area under the curve are shown as box and whisker plots. MCC – microcrystalline cellulose, HE – high Chardonnay extract blend, HM – high Chardonnay marc blend

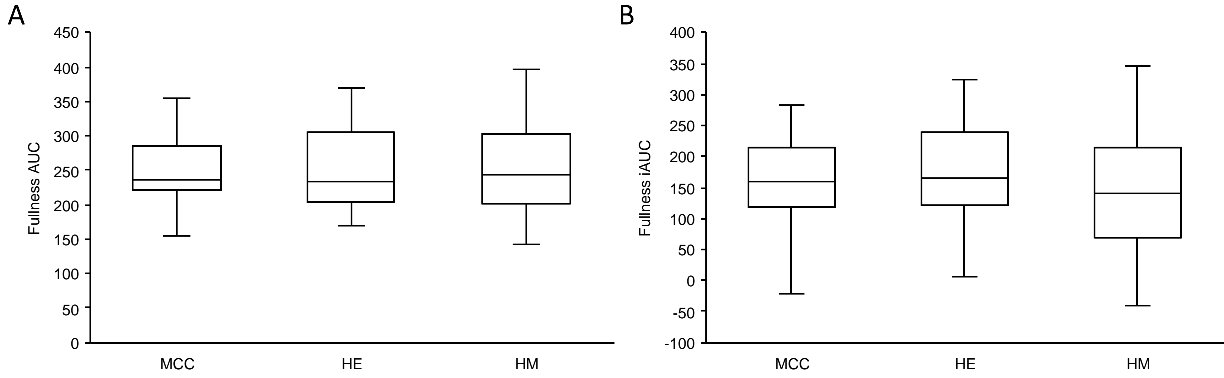
Tables

Table 4.1. Visual analog scale questions

Question	Negative anchor	Positive anchor
1. How hungry do you feel right now?	Not at all hungry	Extremely hungry
2. How full is your stomach right now?	Not at all full	Extremely full
3. How strong is your desire to eat right now?	Not strong at all	Extremely strong desire
4. How much food could you eat right now?	No food at all	Extreme amount
5. How nauseous do you feel right now?	Not at all nauseous	Extremely nauseous

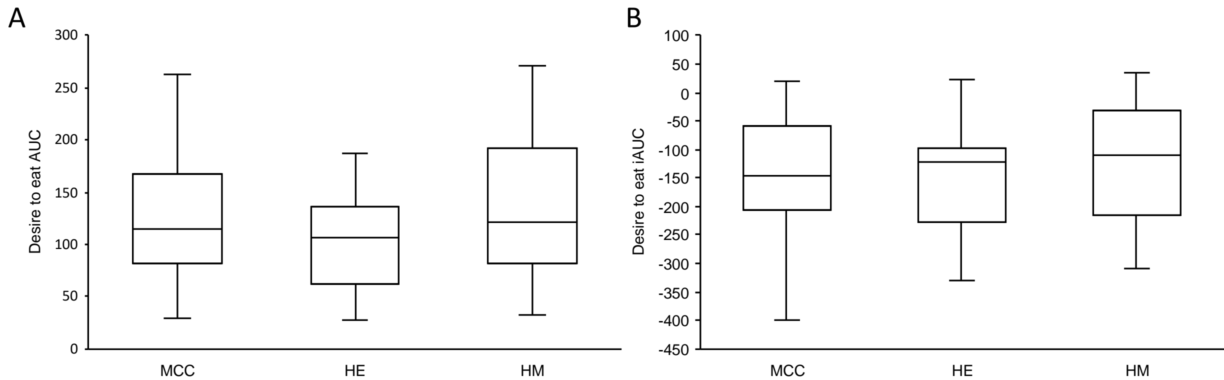
Supplemental Material

Supplemental Figure 4.1. Visual analog scale – Fullness



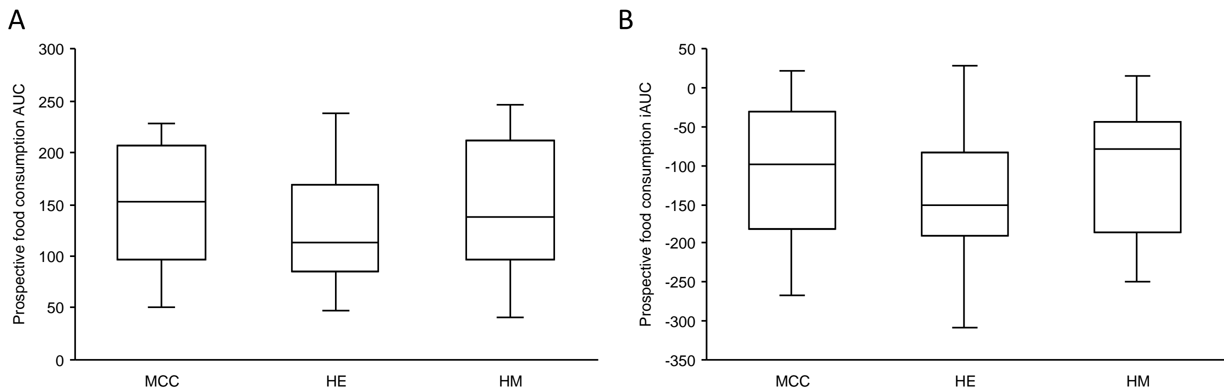
Supplemental Figure 4.1. Panel A: Visual anaolg scale fullness area under the curve (AUC) is depicted in a box and whisker plot. Panel B: Visual analog scale fullness incremental area under the curve (iAUC) is depicted in a box and whisker plot.

Supplemental Figure 4.2. Visual anaolg scale – Desire to eat



Supplemental Figure 4.2. Panel A: Visual anaolg scale desire to eat area under the curve (AUC) is depicted in a box and whisker plot. Panel B: Visual analog scale desire to eat incremental area under the curve (iAUC) is depicted in a box and whisker plot.

Supplemental Figure 4.3. Visual analog scale – Prospective food intake



Supplemental Figure 4.3. Panel A: Visual anaolg scale prospective food consumption area under the curve (AUC) is depicted in a box and whisker plot. Panel B: Visual analog scale prospective food consumption incremental area under the curve (iAUC) is depicted in a box and whisker plot.

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Chapter 5

Final remarks

The effects of Chardonnay marc blends were discussed separately between lipid, glucose, and appetite regulation, despite these three areas having interconnected relationships. This last section will summarize key findings, connections between outcomes and ultimately atherosclerosis cardiovascular disease (ASCVD) risk factors.

Following supplementation of HE, high density lipoprotein cholesterol (HDL-C) concentrations were significantly lower compared to both the microcrystalline cellulose and high Chardonnay marc blend (HM) interventions. The NMR lipoprotein profiling revealed that there were less large HDL particles following the HE supplementation compared to MCC. On the other hand, HE supplementation resulted in decreased systolic blood pressured compared to HM. Additional studies are needed to evaluate the validity of these findings and to determine how it may ultimately affect ASCVD risk.

There was weak evidence that postprandial triglyceride response was affected by the interventions (p=0.06). Postprandial TG area under the curve (AUC) was significantly lower following the HM intervention compared to HE. While results were weakly significant, the apolipoprotein (APO)-CIII response and incremental area under the curve (iAUC) (p=0.06) were similarly lower after HM supplementation. Apo-CIII acts to inhibit TG hydrolysis thus decreasing Apo-CIII concentrations could be one mechanism by which HM is acting to lower TG (Borén et al., 2020).

Apo-CIII expression is regulated by insulin, whereby its expression is increased with insulin resistance (Altomonte et al., 2004; Chen et al., 1994). We found that HM supplementation decreased fasting insulin and improved indexes of insulin resistance

and sensitivity compared to MCC. However, while notable for the physiological relationship between insulin and Apo-CIII, our data are insufficient to confidently point to a relationship connecting these outcomes. Independently, the trend towards lower Apo-CIII and improved glucose regulation suggest improvement in ASCVD risk factors.

We found that among the acute phase inflammatory markers, HM had a weak effect in decreasing C-reactive protein concentrations (p=0.07) and a strong effect in decreasing serum amyloid A concentrations (p=0.006) compared to MCC. There was no indication that this change in inflammation was related to the improved glycemic outcomes—whether these data are related to decreasing ASCVD risk is unclear.

Finally, we demonstrated that postprandial PYY_{3-36} concentrations were higher following the HM intervention compared to HE; PYY_{3-36} AUC was higher in HM compared to both HE and MCC and PYY_{3-36} iAUC was higher in HM compared to HE. PYY acts to increase satiation following a meal— this study was not designed to determine whether food intake behavior was altered following supplementing with the interventions, nor were there changes to ratings of satiety and fullness. Appetite regulation is tied to weight management but in our study was a first exploration into whether grape products had effects on gut hormones and appetite. Future studies should further consider, PYY's connection to insulin action in addition to its digestive roles (Boey et al., 2006; Shi et al., 2015; van den Hoek et al., 2004; Viardot et al., 2008).

To our surprise, the high Chardonnay marc blend (HM) supplementation with less phenolics compared to the high Chardonnay seed extract blend (HE) resulted in

more improved ASCVD risk factors than HE. Future studies with more refined methodologies and populations are needed to confirm our early findings.

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