Evaluation of the effects of the Colombian blueberry (*Vaccinium meridionale* Swartz) on markers of high-density lipoprotein (HDL) function, inflammation and oxidative stress in women with metabolic syndrome

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A dissertation submitted in requirement for the degree of Doctor in Food and Pharmaceutical Sciences

Program of Ophidism and Scorpionism
Area of Food and Therapeutic alternatives
Faculty of Pharmaceutical and Food Sciences
University of Antioquia
2019

Doctor in Food and Pharmaceutical Sciences dissertation

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2020

Acknowledgements

My sincere thanks to my family, especially to my mother and my husband for their unconditional support on this long road. For motivating me and encouraging me at all times.

To my tutor Jacqueline Barona for being an admirable professor who teaches and guides from love, affection and understanding.

To Dr. Christopher N. Blesso for receiving me so kindly in his laboratory and for his patience in my learning process.

To Dr. Maria Luz Fernandez, for being a source of admiration as a researcher, teacher and as a person. Thank you for your friendship, support, for listening to me and guiding me during my internship.

To Dr Vitelbina because she is an example as a researcher who is always aware of her students. Thank you for all the valuable suggestions, comments and corrections throughout my PhD studies.

To Yeisson A. Galvis for his unconditional support since the beginning of the project. For being an example of righteousness and discipline.

To Dr. Gelmy Ciro and Julian Quintero for contributing with all their knowledge as Food engineers in the development and characterization of the agraz beverage and in the development of the placebo.

To Claudia P. Franco for supporting in the screening and intervention process of the subjects, and for the design of the food consumption frequency format.

To Gregory H. Norris for his support in laboratory tests conducted during my internship at the University of Connecticut.

To Courtney L. Millar and Bruno Lemos for their kindness and willingness to support me in what I needed during my internship at the University of Connecticut.

To Belfran A. Carbonell of the Regeneration and Cancer research group of the University of Antioquia for their willingness to teach me the process of RNA extraction and nucleic acid electrophoresis.

To Jorge A. Lujan of the Immunovirology Research Group, for kindly facilitating the use of the spectrophotometric to measure the activity of antioxidant enzymes and the determination of serum antioxidant capacity.

To Esteban Marín of the Program of study and control of tropical diseases (PECET) for helping me with materials or supplies that I needed for my laboratory tests.

To Professor Ligia Luz Corrales for her generosity in providing me with supplies, equipment, and materials to standardize RNA integrity by electrophoresis.

To Julieta Vásquez for her support in the process of RNA electrophoresis standardization, and for her love and valuable friendship.

To Alejandro Serna for always being available to help in the laboratory, and always having a solution at hand to the problems with my computer, which facilitated the analysis.

To Ana Maria Naranjo for her support in the processing of the agraz nectar and for helping me with the doubts about the measurement of antioxidant capacity.

To Manuela Piedrahita for her great support in the measurements of antioxidant capacity and activity of antioxidant enzymes. For always being available to help, for her love and friendship.

To the Program of Ophidism and Scorpionism, area of food and pharmaceutical alternatives, belonging to the Faculty of Pharmaceutical and Food Sciences of the University of Antioquia, Medellín, Colombia.

To the Advanced Technology Laboratory (coordinated by Dr Christopher N. Blesso), Department of Nutritional Sciences, University of Connecticut, Storrs, CT, United States of America.

To the Ministerio de Ciencia, Tecnología e innovación (Minciencias) for the funding of this project through the call No. 657-2014; and for the doctoral scholarship granted to Catalina Marín Echeverri through the call for National Doctorates 727-2015.

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Abbreviations

8-OHdG	8-hydroxy 2 deoxyguanosine
ABCA1	ATP-binding cassette A1
ABCG1	ATP-binding cassette G1
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ACC	American College of Cardiology
AHA	American Heart Association
AOPP	Advanced oxidation protein products
AP-1	Activating protein 1
Apo	Apolipoprotein
ATF3	Activating Transcription Factor 3
ATPIII	Adult Panel Treatment III
BMI	Body mass index
CAT	Catalase
CETP	Cholesteryl ester transfer protein
CRP	C-reactive protein
CT	Total cholesterol
CVD	Cardiovascular diseases
CVR	Cardiovascular risk
CVRF	Cardiovascular risk factors
DPPH	2,2-Diphenyl-1-Picrylhydrazyl
EGIR	European Group for the Study of Insulin Resistance
eNOS	Endothelial nitric oxide synthase
Eq	Equivalent
ERK	Extracellular-regulated kinase
FF	Fresh fruit
FFA	Free fatty acids
FRAP	Ferric reducing ability of plasma
FW	Fresh weight

GAE	Gallic Acid Equivalents
GPx	Glutathione peroxidase
HAT	Hydrogen atom transfer
HDL-c	High density lipoprotein cholesterol
HIF	Hypoxia-inducible factor
HOCl	Hypochlorous acid
HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
hs-CRP	high sensitivity C-reactive protein
ICAM-1	Intercellular cell adhesion protein 1
IDF	International Diabetes Federation
IKK	IκB kinase
IL	Interleukin
IL-1R1	IL-1 receptor type 1
IRAK	IL-1R-associated kinase
IRF	Interferon regulatory factors
IκB	Inhibitor of κB
JAK	Janus tyrosine kinases
JNK	Jun N-terminal kinases
LCAT	Lecithin-cholesterol acyltransferase
LDL-c	Low density lipoprotein cholesterol
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MetS	Metabolic syndrome
MPO	Myeloperoxidase
mTORC1	Mammalian target of rapamycin complex 1
NADPH	Nicotinamide adenine dinucleotide phosphate
NCEP	National Cholesterol Education Program
NF-IL6	Nuclear factor for IL-6 expression
NF-κB	Nuclear factor kappa B
NLRP3	Nod-like receptor protein-3

NO	Nitric oxide
Nrf2	Nuclear factor erythroid 2-related factor 2
ORAC	Oxygen radical absorbance capacity
OX-LDL	Cxidized-LDL
OxS	Oxidative stress
p38MAPK	Mitogen activated protein kinase p38
PAF-AH	Platelet-activating factor-acetylhydrolase
PLTP	Phospholipid transfer protein
PBMC	Peripheral Blood Mononuclear Cell
PON1	Paraoxonase 1
PPAR-γ	Peroxisome proliferator-activated receptor gamma
PRR	Pattern recognition receptors
QUICKI	Quantitative Insulin Sensitivity Check Index
RCT	Reverse cholesterol transport
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SAA	Serum amyloid A
SET	Single-electron transfer
SOD	Superoxide dismutase
SR-BI	Scavenger receptor class B type I
STAT	Signal Transducer and Activator of Transcription
T2D	Type 2 diabetes
TAC	Total antioxidant capacity
TG	Triglycerides
TLR	Toll-like receptor
TNFSF	TNF superfamily
TNF-α	Tumor necrosis factor-α
VCAM-1	Vascular cell adhesion protein 1
WC	Waist circumference
WHO	World Heart Organization

Summary

Introduction: Metabolic syndrome (MetS) is defined by the National Cholesterol Education Program- Adult Panel Treatment III (NCEP-ATPIII) as the presence of three or more cardiovascular risk factors (CVRF). Around one quarter of the world population have this syndrome that increases twice the risk of developing cardiovascular diseases (CVD) in the next 5 to 10 years, the leading cause of mortality in the world. The consumption of fruit and vegetables have been associated with lower risk of MetS. This benefit has been attributed to several bioactive components such as phytochemicals with antioxidant properties and modulation of cellular signaling pathways. Fruits of the gender *Vaccinium* are rich in phytochemicals with predominance of polyphenols, especially anthocyanins, which exhibit a high antioxidant capacity. Human intervention studies with *Vaccinium* have demonstrated beneficial effects on reducing traditional CVRF, inflammation and oxidative stress markers and to improve antioxidant capacity and HDL function. In Colombia grows the specie *Vaccinium meridionale* Swartz, also called agraz, with demonstrated high antioxidant capacity. However, fruit consumption in Colombia is insufficient, there is not agroindustrial development of *Vaccinium* crops in Colombia and the information about the effects of chronic consumption of agraz on inflammation, oxidative stress and antioxidant markers in people at high risk of CVD is very limited.

General objective: To evaluate the effects of agraz (*V. meridionale* Swartz) on markers of high-density lipoprotein function, inflammation and oxidative stress in women with MetS.

Methodology: Forty women (25–60 years) from Medellín-Colombia with MetS according to NCEP-ATPIII criteria were included in this double-blind and crossover study. Volunteers were assigned to consume daily a dose of 200 mL of reconstituted freeze dried agraz (equivalent to the total phenols present in 200 g of fresh agraz fruits) or placebo (without polyphenols) beverage over 4 weeks. After a 4-week washout period, they consumed the alternate treatment for additional 4 weeks.

During the whole study, participants were asked to maintain their habitual physical activity and diet, except for the consumption of polyphenol-rich foods. Antropometrics [height, weight and waist circumference (WC)] and blood pressure were measured. Blood samples were obtained after a 12h overnight fasting to determine lipid profile and glucose at the beginning and end of each intervention period. In addition, the following markers were measured in samples obtained at the end of placebo and agraz periods: serum high-density lipoprotein (HDL) function markers (apolipoprotein-A1; paraoxonase 1 (PON1) activity; cholesterol efflux capacity), oxidative stress markers (serum myeloperoxidase -MPO) and advanced oxidation protein products -AOPP, and urinary F2-isoprostanes and 8-hydroxy 2 deoxyguanosine -8-OHDG), inflammatory markers (serum cytokines/chemokines and high sensitivity C-reactive protein (hs-CRP) levels, and the activation of nuclear factor-kB in peripheral blood mononuclear cells), endogenous antioxidant enzyme activity [superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx)], and serum total antioxidant capacity (TAC) determined by different methods [ABTS 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); FRAP (ferric reducing ability of plasma) and ORAC (Oxygen Radical Absorbance Capacity)]. For the statistical analysis, after evaluating the data distribution, repeated measures ANOVA or Friedman test were used in the analysis of variables with baseline measures. Paired samples *t*-test or Wilcoxon test were used to evaluate the association between the end of each intervention period. In addition, we analyzed the women separated into two groups [obese and overweight, according to body mass index (BMI)] to evaluate the differential response of agraz consumption. Furthermore, the results were analyzed according to blood antioxidant response after agraz intake. Mann- Whitney U test or Student's *t*-test were used to evaluate the associations between the independent groups. Finally, changes after agraz consumption (agraz minus placebo period) were calculated and the correlation between changes were determined with Pearson´s or Spearman´s correlation coefficient. All analyses were done using SPSS version 21 for Windows (SPSS, IBM Corporation, 2012). Differences with a value of $p<0.05$ were considered significant.

Results. Forty women (47.2 ± 9.4 years old) with MetS finished the study with an adherence above 90%. No differences in macronutrient intake and physical activity were observed during the whole study. We already published the effects of agraz on anthropometric, blood pressure and blood lipid profile and glucose, in this population. In the whole group, there were not significant differences in HDL function, inflammation, oxidative stress and antioxidant markers, after comparing the end of both intervention periods (placebo versus agraz) ($p > 0.05$). Regarding HDL function, interestingly, only after agraz period there were significant positive correlations between PON1 activities and cholesterol efflux. Additionally, there were significant inverse correlations between changes in inflammatory markers and HDL function markers and positive correlations with oxidative markers. When analyzing the effects of agraz consumption on inflammation in women according to BMI classification, we found significantly reductions in hs-CRP levels in overweight women compared to obese women (p=0.028). Further, regarding oxidative stress effects, there was a significant reduction in urinary 8-OHdG levels in obese women after agraz consumption compared to placebo (p=0.031). Women who increased SOD activity after agraz consumption, compared to placebo, significantly reduced oxidative stress markers like 8-OHdG (p=0.022) and F2-isoprostane (p=0.034). Likewise, those women who increased GPx activity after agraz intake, significantly reductions in total cholesterol (p=0.023) and low-density lipoprotein cholesterol (LDL-c) (p= 0.022) levels. Finally, the increase in serum TAC (determined by ABTS) after 4 weeks of agraz consumption, was significantly associated with reduction in waist circumference.

Conclusion: the chronic consumption of agraz over 4 weeks in a daily dose of 200mL (total phenols equivalent to 200g of fresh fruit) in 40 women with MetS did not significant improved HDL function, inflammation and antioxidant markers in the whole group. However, agraz consumption demonstrated to have a differential effect between obese and overweight women, with better anti-inflammatory effect in overweight women. Possibly, obese women have a more inflammatory state that requires a more drastic dietary intervention in this specific population. Interestingly, the effect on OxS markers was better in obese women. In addition, the group of

women who increase these antioxidant parameters significantly improved CVRF such as reduction in lipid and DNA oxidation, total cholesterol, LDL-c and waist circumference. We demonstrated differential responses to agraz consumption in this population, which could suggest there is an individual variability influencing the beneficial effects of this fruit.

CHAPTER 1

Introduction

1.1 Introduction

Several organizations have developed definitions of metabolic syndrome (MetS), such as the National Cholesterol Education program – Adult Treatment Panel III (ATPIII) based on the recommendations of the International Diabetes Federation (IDF), the one with the most suitable and widely used definition (1). This organization defines MetS as the presence of three or more cardiovascular risk factors (CVRF) in an individual, including central obesity, hypertension, high triglycerides (TG) and fasting glucose levels, and low high density lipoprotein cholesterol (HDL-c) concentration (2). MetS is a chronic condition that is accompanied by other alterations such as chronic low-grade inflammation, oxidative stress (OxS), dysregulated secretion of adipokines (3) and HDL dysfunction (4,5). In contrast, it is associated with decreased levels of anti-inflammatory cytokines and antioxidant factors (3,6). All these factors favor the development of endothelial dysfunction and insulin resistance (7,8).

Around one quarter of the population is affected by MetS worldwide (9). However, its prevalence varies according to the criteria used to define this syndrome (9). In Latin America, the average prevalence of MetS is 24.9% (range 18.8% to 43.3%), higher than in developed countries like United States, Spain, Portugal and Italy (10). In Colombia, there are not reports about MetS prevalence in the whole country, however, some studies have observed prevalences between 8.4 to 41% in different municipalities (11–17). Interestingly, a recent study in 285 health practitioners and employees (20-61 years) from the University of Antioquia-Medellin, found a prevalence of 17.5% in this population (18).

Although this syndrome affects both genders, the risk of incident cardiovascular events and death seems to be higher in women than in men (19), and the contribution of several metabolic components for the development of MetS is different in men compared to women, being abdominal obesity the dominant MetS feature in women, along with increased TG and low HDL-c (20).

This syndrome is linked to increased consumption of a high calorie diet, as well as sedentary lifestyle, and therefore, its prevalence grows as the obesity epidemic grows (9). In fact, obese people have a high prevalence of MetS, and it have been demonstrated that the cardiovascular risk increases as the body mass index (BMI) increases (21,22).

The high prevalence of MetS is a matter to be concerned, given that people with this syndrome have 5 times more probabilities of developing type 2 diabetes (T2D), and twice more risk of developing cardiovascular diseases (CVD) in the next 5 to 10 years, compared with individuals without the syndrome (2). Both diseases are an important public health problem globally, especially CVD as it is considered the first cause of mortality (23). In 2015 around 17.92 million people died from this cause (23), and 22 million deaths are projected for the year 2030 (24). In Colombia, ischemic heart disease is the leading cause of death with 38,618 cases in 2017, corresponding to 17% of total deaths (25,26). Additionally, CVD represents a high economic burden in the world, especially in low- and middle- income countries, with high cost for the health systems, households, productivity loss and number of years of healthy life loss (27). In Colombia, the health care expenditure for CVD had a total cost of $2.4 billion in 2015, occupying 7% of all health spending (28), which represents a problem for the financial sustainability of the health system.

Given the burden that MetS represents, several global strategies focused on prevention, treatment and monitoring of each risk factor, have been proposed to reduce incidence, morbidity and mortality from CVD and T2D (23). According to the Guidelines on the Primary Prevention of Cardiovascular Disease of the American College of Cardiology (ACC) and the American Heart Association (AHA), published in 2019, the most important recommendation for prevention is the promotion of lifestyle changes including tobacco abstinence, weight loss, exercise and a healthy diet (29). However, if the risk is very high, drug therapy is indicated to reduce each risk factor (29), for which, polypharmacy-related issues are common in people with MetS (30). This is due to the unavailability of a single drug for all metabolic risk factors. Fortunately, lifestyle modifications have demonstrated to be effective in

improving all alterations related with MetS (31,32), for which lifestyle changes have an important role in the prevention and treatment of CVRF.

In this sense, the consumption of fruit and vegetables have been associated with significant reduction in the risk of MetS (33), and a lower risk of mortality from all causes, including CVD and cancer. Specifically, eating five servings a day of fruits and vegetables reduces in 26% the risk of mortality from all causes (34). Even more, each serving of fruit per day is able to reduce in 5% the risk of mortality due to CVD (34). These benefits of fruits and vegetables have been attributed to several bioactive components including fiber (35), vitamins (36), minerals (37) and phytochemicals (38). Recently, phytochemicals -compounds produced by plants as secondary metabolites- have been an important focus of research given their beneficial effects on human health, which have been attributed largely to their antioxidant properties (39) and also to their capacity to modulate cellular signaling pathways (40,41). Although the health benefit of each single bioactive compounds have been demonstrated separately, evidence suggests that effects after consumption of whole food are due to the synergic action of these bioactive compounds and other nutrients (42).

Given the above, there is a growing interest in research related to the bioactive compounds present in different fruits in order to determine their effects on human health. A clear example are berries of the gender Vaccinium, which have an interesting phytochemical composition with predominance of polyphenols, especially anthocyanins, with a demonstrated high antioxidant capacity (43). Meta-analysis about numerous human intervention studies evaluating the effects of Vaccinium berries on health, have allowed to conclude several beneficial effects on traditional markers of cardiovascular risk such as: reductions of low density lipoprotein cholesterol (LDL-c), total cholesterol (TC), TG (44,45) and fasting glucose levels (45); increases in HDL-c levels; lowering of blood pressure (44,45), and body mass index (BMI) (45). Additionally, the effects of Vaccinium species on inflammation and oxidative stress markers are also highly studied for their strong association with MetS (6). In this sense, Vaccinium consumption have shown anti-inflammatory effect

through the reduction of inflammatory markers like tumor necrosis factor-α (TNF-α) (45,46) and C-reactive protein (CRP) (45). Vaccinium intake have shown also effects on the decrease of oxidation of lipids, proteins (47) and DNA (48); and improvement of antioxidant status (49).

Other interesting cardio-protective mechanism of Vaccinium consumption is the capacity to improve HDL function (50). This marker have been the focus of several studies in the recent years, given that it has been shown that regardless of HDL-c levels, the HDL particle has an atheroprotective role mediated by different functions such as the reverse transport of cholesterol, anti-oxidant and anti-inflammatory capacities, among others (51). Given the above, the HDL functionality has become a therapeutic target, and it have been demonstrated that lifestyle changes such as a healthy diet and physical activity can improve HDL function even in people with MetS, who have a dysfunctional HDL (52).

Studies have demonstrated bigger changes on traditional CVRF after Vaccinium consumption in young women (53), as well as in postmenopausal women who have an increased CVR given the loss of the protective role of estrogen (54). In addition, other polyphenol rich beverages have also shown better effects in overweight women compared with obese women, with reductions in body weight, systolic blood pressure and improvement in lipid profile (55). On the contrary, similar effects on oxidative stress and antioxidant status seem to occur in both healthy and unhealthy people (56). These results show the potential of these group of fruits on prevention and treatment especially in people with CVRF (i.e. with MetS and obesity).

Interestingly, Colombia is the second country that consumes most fruit juices in the world (0.80 portions a day) (57), which probably is due to the preference of Colombian people for fruit juices over whole fruits (58). In spite of that, fruit intake in the country is insufficient, with an estimated daily consumption of 88g per capita, which is below the World Heart Organization (WHO) recommendations (400g per capita) (59). Paradoxically, Colombia is one of the megadiverse countries (60), with a wide variety of native species of edible fruits, including a fruit of the gender

Vaccinium (61). Because of that, studies evaluating the protective properties of Colombian fruits could represent an opportunity for promote its consumption in the population, with potential beneficial effects for health.

Colombia has geographical characteristics that favor the production of the species *Vaccinium meridionale* Swartz, also called "agraz", "mortiño" or " andean blueberry" (62). However, in the country this fruit grows in the wild, there are not industrial mortiño crops and its merchandising is carried out by small merchants (62,63). This is possibly due to the lack of research around its social and economic benefits (62). Currently, it is consumed as fresh fruit or as part of processed food in several ways such as cakes, candies, yogurts, juices, wine and in the preparation of exotic dishes (63). Therefore, cultivating this fruit represents an opportunity to increase its national consumption and export.

Studies with agraz have reported a high concentration of anthocyanins, and other non-anthocyanin phenolic compounds such as chlorogenic acid and quercetin (64). Other studies have also shown a high antioxidant capacity of this fruit, indicating that its components have the ability to scavenge reactive species through two mechanism: hydrogen atom transfer (HAT) and single-electron transfer (SET) (64,65).

These characteristics make this fruit attractive from several perspectives. One of them is its potential for food industry, acting as an antioxidant ingredient, as well as its potential as a nutraceutical food (66–69). Likewise, this fruit is interesting for its potential on human health. In vitro studies in colon cancer cell lines, have shown that *V. meridionale* have antioxidant, cytotoxic, anti-proliferative and pro-apoptotic activities (70–72). In leukemic cell lines *V. meridionale* demonstrated to be a cytotoxic source (73), suggesting a potential effect against cancer. Moreover, in vivo studies in rats have also demonstrated beneficial effects of this fruit against ischemic injury, through mechanisms associated with the increase of endothelial nitric oxide synthase (eNOS) expression via Akt, the scavenging activity of reactive oxygen

species (ROS) and the increase of endogenous antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD) (74,75).

The previous findings have been encouraging to conduct studies in humans. Therefore, researchers from the University of Antioquia in collaboration with national and international researchers, have started carrying out studies evaluating the effects of *V. meridionale* consumption in human health, becoming pioneers in the field. Given the urgent need to find strategies to reduce CVRF, the focus of these studies have been evaluating the potential of this local fruit as a functional food in people with metabolic syndrome. Therefore, the aim of this research was to evaluate the effects of agraz on markers of high-density lipoprotein (HDL) function, inflammation and oxidative stress (OxS) in women with MetS. The results of this study could serve as a source to promote the exploitation of our natural resources for the benefit of people´s health and the economic development of our region.

1.2 Objective

1.2.1 General objective

To evaluate the effects of the agraz (*Vaccinium meridionale* Swartz) on markers of high-density lipoprotein function, inflammation and oxidative stress in women with metabolic syndrome.

1.2.2 Specific objetives

- To determine the effects of agraz, compared to placebo, on inflammation markers in women with MetS
- To evaluate the effects of agraz, compared to placebo, on oxidative stress markers in women with MetS.
- To evaluate the effects of agraz, compared to placebo, on HDL function in this group of women.
- To determine the association between the variables of interest with parameters of the MetS in this population.
- To compare the effects of agraz consumption according to the level of adiposity (overweight vs. obesity).

1.3 Research questions and hypotheses

Question 1:

Will chronic consumption of *V. meridionale* improve HDL function, inflammation and oxidative stress in women at high risk of CVD?

Hypotheses:

Compared with placebo, the consumption of *V. meridionale* beverage will improve alterations associated with MetS as follow: HDL function markers, pro-inflammatory markers, oxidation stress markers and the activity of endogenous antioxidant enzymes.

Likewise, we hypothesize that these beneficial effects will occur without significant changes in the diet and physical activity.

Question 2:

Since obesity is an important CVRF associated with inflammation and oxidative stress, the following question arose: How *V. meridionale* consumption influence MetS characteristics, inflammation, antioxidant status and oxidative stress in overweight versus obese women with metabolic syndrome?

Hypothesis

Chronic consumption of *V. meridionale*, compared to placebo, will reduce MetS parameters (hypertension, atherogenic dyslipidemia, hyperglycemia and abdominal obesity); pro-inflammatory markers (TNF-α, IL-6, IL-1β, IL-1 and MCP1); and

oxidative stress markers MPO and AOPP, in both overweight and obese women. However, a bigger change will be expected on obese women.

On the contrary, the antioxidant status (serum total antioxidant capacity [TAC] and endogenous antioxidant enzyme activity) will increase in both groups after *V. meridionale* intake, compared with placebo. Likewise, obese individuals would have more benefits.

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CHAPTER 2

Literature review

2.1 Metabolic syndrome (MetS) definition

The coexistence of metabolic abnormalities like hypertension, hyperglycemia, obesity, hyperuricemia and albuminuria, have been described since several years ago, especially since the 1920s, through clinical observations, which have allowed to determine their association with serious conditions such as diabetes, kidney disease, heart diseases and other circulatory diseases (1–4). Later, in 1947, android fat distribution was associated with diabetes, hypertension, atherosclerosis and gout (5) and obesity was associated with dyslipidemia and hyperglycemia (6). In the 1960s, the cluster of several metabolic alterations were named in different ways such as: “metabolic trisyndrome” in 1966, including people with gout, diabetes and hyperlipidemia (7); “plurimetabolic syndrome” in 1967 to define patients with hyperlipidemia, obesity and diabetes (8); “syndrome of affluence” in 1968, described as the association between unhealthy lifestyle and metabolic disturbances (9). Then, in 1981, in Germany, the term “Metabolic syndrome (MetS)” was used by Hanefeld and Leonhardt to describe the association between hyperlipidemia, hypertension, diabetes, obesity, gout and thrombophilia. This syndrome was also related to genetic predisposition and unhealthy lifestyle such as lack of physical exercise and overeating. All these alterations lead to the development of arteriosclerotic vascular disease and other complications (10). Later, in 1988, Gerald M Reaven introduced the term “Syndrome X” in which insulin resistance was described as the link with other metabolic disturbances such as impaired glucose tolerance, hypertension, high triglycerides levels and low high-density lipoprotein-cholesterol (HDL-c) levels, in people with and without type 2 diabetes (T2D), all increasing the risk of developing cardiovascular diseases (CVD) (11). Then, Norman Kaplan in 1989, highlighted the upper-body fat distribution or central obesity as an important factor associated with hypertension, diabetes, and hypertriglyceridemia. These alterations were called “The deadly quartet” (12). Later, in 1991 the term “insulin resistance syndrome” was described by Ralph A. DeFronzo and Eleuterio Ferrannini, as a clustering of metabolic disorders such as T2D, obesity, lipid abnormalities, hypertension and atherosclerosis (13).

Given the above, several internationals efforts have been carried out in order to define metabolic syndrome (MetS) and to establish diagnostic criteria (14). The first definition proposal came from the World Health Organization (WHO) in 1998, in which MetS was defined as the presence of insulin resistance (impaired fasting glucose or impaired glucose intolerance or T2D mellitus or insulin resistance [measured under hyperinsulinemic euglycemic conditions]) plus 2 additional risk factors such as hypertension, high triglycerides (TG) levels, low HDL-c levels, central obesity and microalbuminuria (15). Subsequently in 1999 the European Group for the Study of Insulin Resistance (EGIR), proposed a modification of the WHO criteria for the definition of the insulin resistance syndrome in nondiabetic individual, establishing also insulin resistance (or fasting hyperinsulinemia) as a central criterion, plus 2 additional risk factors such as fasting hyperglycemia, hypertension, dyslipidemia and central obesity. They did not include microalbuminuria as a criterion (16). Then, in 2001, the report of The National Cholesterol Education Program (NCEP) expert Panel on detection, evaluation and treatment of high Blood cholesterol in adults- Adult Treatment Panel III (ATP III) published the criteria for the clinical identification of MetS, without emphasis on a single risk factor. According to the NCEP-ATPIII panel, the presence of at least 3 of the following 5 components are enough to establish the diagnosis of MetS: abdominal obesity, hypertension, low HDL-c levels, high TG and elevated fasting glucose levels. In addition, they mention other risk factors related with MetS such as insulin resistance, proinflammatory state, and prothrombotic state; however, they are not routinely measured in the clinical setting (17). In 2003, the American Association of Clinical Endocrinologists (AACE) modified the NCEP-ATPIII criteria. Similar to the EGIR definition, they used the term “insulin resistance syndrome” and they also had a focus on insulin resistance for the diagnosis of non-diabetic individuals; however, they did not provide a specific definition (18). In 2005, the International Diabetes Federation (IDF) made some modifications to the NCEP-ATP III criteria as well. They recommended a definition centered in central obesity, plus 2 of the same criteria by the NCEP-ATPIII, with an update for the fasting glucose cut-off according to the American Diabetes Association updated definition of impaired fasting glucose, and with the waist

circumference cut-off according to specific gender and ethnic-groups (19). Finally, in 2009 as an attempt to harmonize the MetS definition, several international organizations (IDF, the National Heart, Lung, and Blood Institute (NHLB); American Heart Association (AHA); World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity) established a global consensus definition based on the IDF definition (or NCEP-ATPIII modified criteria), but without central obesity as an obligatory component (20). According to the AHA, this definition is currently the most suitable, since it is easy to use in the clinical setting and has the advantage of not emphazising any component. According to these criteria, diagnosis of MetS in adults is established when three or more of the following five factors are present: 1) abdominal obesity, measured by waist circumference with a cut-off depending of the population- and country-specific definitions; 2) TG ≥150 mg/dL or on drug treatment for elevated triglycerides; 3) HDL-c: men <40 mg/dL, women <50 mg/dL, or on drug treatment for reduced HDL-c; 4) blood pressure ≥130/≥85 mmHg, or on antihypertensive drug treatment; and 5) fasting plasma glucose ≥ 100 mg/dL, or on drug treatment for elevated glucose (20).

2.2 Inflammation and MetS

MetS is associated with chronic low-grade inflammation which is characterized by the increase of C-reactive protein (CRP) (21,22). In fact, CRP is considered as a stronger predictor of cardiovascular events (23). This protein was described by Tillet and Fracis in 1930; the name was attributed to the presence of this protein in response to acute inflammation caused by Streptococcus pneumonie polysaccharide C (24). CRP is an acute phase protein synthesized in the liver in response to pro-inflammatory cytokines such as IL-6, TNF-α and lL-1; however, small amounts of CRP can be produced locally, for example in atherosclerotic lesions by muscle smooth cells and monocytic cells (25,26).

CRP adds an independent prognostic value in terms of future cardiovascular risk in people with MetS, who have a risk of 3.4 per 1000 person-years of exposure for those with high-sensitive CRP (hs-CRP) values less than 3mg/L and a risk of 5.9 per 1000 person-years of exposure for those with CRP levels > 3mg/L. In addition, CRP levels increases as MetS components increases (27).

The cardiovascular risk classification according to the concentration of hs-CRP was described for the first time in the survival analysis carried out by Paul M. et al., in 2002. They found that CRP levels <1 mg/L are indicative of low risk; from 1 to 3 mg/L, moderate risk; and levels >3 mg/L, are indicative of high risk of CVD (23).

Besides the above, MetS is associated with increased levels of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-6 (28,29). All these pro-inflammatory proteins have been strongly associated with insulin resistance, diabetes (30) and predict the risk of CVD and mortality (31). Other pro-inflammatory markers elevated in people with MetS and associated with an increased risk of developing CVD are IL-1β, IL-8 (32) and white blood cell (WBC) count (including monocyte, lymphocyte, neutrophil and eosinophil counts), compared with those without MetS (33,34).

Several pathophysiological mechanisms are involved in the development of systemic low-grade chronic inflammation, one of them is strongly linked with overnutrition. Dietary lipids for example induce the increase of intestinal permeability (35), which seems to be mediated by gut microbiota changes (36) such as reduction on Oscillospira (order Clostridiales), associated with a decrease of anti-inflammatory gene expression like IL-10, although the exact mechanism is not clear yet (37). The permeability alteration leads to metabolic endotoxemia caused by higher circulating levels of lipopolysaccharides (LPS) that are recognized by pattern recognition receptors (PRRs) like Toll-like receptor 4 (TLR4) in immune cells and other cells like adipocytes, muscle cells, hepatocytes, among others, triggering an inflammatory cascade with the activation of the nuclear factor kappa B (NF-κB) pathway, inducing cytokine expression and secretion (38). In addition, increased levels of plasmatic LPS are present in obese and diabetic people and is associated with insulin resistance (39).

In the same way, saturated fatty acids (a bacterial component) are also recognized by TLR2 and 4 (40), inducing an inflammatory response with increase of IL-6, TNF-α and MCP-1 mRNA expression, in a NF-κB dependent mechanism (41). Saturated fatty acids can also induce inflammasome-mediated IL-1β production after TLR2 activation (42).

Hypoxia is another chronic inflammation mechanism described in adipose tissue mediated by expansion of this tissue in obesity and the subsequent lowering of blood flow (43), affecting cell viability and function. In response to the hypoxia there is an increase of hypoxia-inducible factor 1α (HIF-1α), which induces a dysregulated expression and secretion of inflammation-related adipokines such as reduction of adiponectin levels and increase of IL-6 and leptin mRNA levels (44).

Another pro-inflammatory mechanism is the endoplasmic reticulum (ER) stress, caused by other mechanisms observed in people with MetS such as hypoxia (45), hyperglycemia (46) and high free fatty acid (FFA) levels (47). In response to ER stress, the activation of the unfolded protein response (UPR) occurs as an adaptive mechanism to restore the homeostasis, which is mediated by three ER membrane-associated proteins: protein kinase RNA (PKR)-like ER kinase (PERK), inositol requiring enzyme 1, (IRE1) and activating transcription factor-6 (ATF6). All these proteins induce inflammation through activation of NF-κB- IκB kinase (IKK) and mitogen-activated protein kinase (MAPK)- (c-Jun N-terminal kinases (JNK) pathways (48–51).

In adipose tissue, these inflammatory signals induce a change from an anti-inflammatory phenotype associated with a T-helper 2 (Th2) state, anti-inflammatory cytokines, M2- polarized macrophage (alternatively activated), and regulatory CD4+ T cells, to a pro-inflammatory phenotype with M1-polarized macrophages (classically activated), predominance of a Th1 state, inflammatory cytokines and Cd8+ T effector cells (52–57). This inflammatory process results on infiltration of lymphocytes T (58) and B (59), promoting the recruitment and activation of macrophages in this tissue mediated by MCP-1(57), some of which form distinct crown-like structures (CLS) around fat cells (60). The result is a local and systemic inflammation with increased inflammatory molecules and a deregulated secretion of adipokines (61). Other

tissues are also affected with pro-inflammatory changes in the endothelium with increases of cytokines levels (like IL-1β, TNF-α and MCP-1), monocytes adhesion and gene expression of the adhesion molecules vascular cell adhesion protein 1 (VCAM-1), intercellular cell adhesion protein 1 (ICAM-1) and IL-6 (62). Likewise, in endothelium, FFA and advanced glycation end-products (AGE) produced by chronic hyperglycemia and oxidative stress (OxS), can induce an inflammatory response associated with increased NF-κB activation (63,64). Finally, this pro-inflammatory state contributes with OxS (65) and insulin resistance associated to serine (Ser307) phosphorylation of the insulin receptor substrate–1 (IRS-1) (41), all of which promotes the atherosclerosis development and endothelial dysfunction.

The main transcription factor involved in pro-inflammatory protein expression is NF-Kb, a family of dimeric transcription factors composed by five proteins, including RelA (p65), RelB, c-Rel, NF-κB1 (p50), and NF-κB2 (p52), which share a Rel homology domain (RHD) responsible of the dimerization and binding of DNA and inhibitor of κB (IκB) proteins. In unstimulated cells, NF-κB is in its inactive form, sequestered in the cytoplasm by IκB proteins (including IκBα, IκBβ). In addition to IκB proteins, precursor's proteins of p52 and p50 like NF-κB1 (p105) and NF-κB2 (p100), respectively, contain a κB-like C-terminal portion that acts as IκB (also named IκBγ and IκBδ respectively) in the cytoplasm (66).

NF-κB signaling activation can be through canonical and noncanonical pathways (67). Pro-inflammatory signals such as the activation of TNF receptor (TNFR) family, cytokine receptors and TLRs, induce canonical NF-κB pathway, mediated by activation of TGFβ-activated kinase 1 (TAK1, also named mitogen-activated protein kinase kinase kinase [MAP3K]) which activates the IKK complex (IKKα, IKKβ and IKKγ [also known as NEMO]) (68). IKK phosphorylates IκBα and p105, inducing its ubiquitylation and proteasomal degradation (promoting total degradation of p105 or p50 generation). This allows the rapid nuclear translocation of heterodimers such as p50/RelA(p65), being this one the most abundant of Rel dimers in almost all cell types (69).

On the other hand, the noncanonical pathway is triggered in response signals of the TNFR superfamily members and TNF receptor-associated factor-3 (TRAF3)

proteasomal degradation, which promotes NF-κB-inducing kinase (NIK) synthesis, accumulation and activation (70), inducing phosphorylation of p100 in C-terminal serine residues (serines 866 and 870) and IKKα (71). Activated IKK also phosphorylates p100 in other serine residues (71). Then, phosphorylated sites are recognized by ubiquitin ligase complex and degraded in the proteasome, generating a mature NF-κB2 p52, and reducing its IκBδ function. Therefore, p52/RelB heterodimers are translocated to the cell nucleus. Unlike the canonical pathway, p52/RelB heterodimer is activated slowly (72).

In the cell nucleus, NF-κB dimers bind to specific DNA sites or κB sites in target genes' promoters, mediated by RHD. Likewise, dimers bind to its coactivators, mainly p300 and CBP (CREB-Binding Protein). Finally, modifications in NF-κB subunits by phosphorylation and acetylation have been reported as necessary to enhance transcriptional activity (73). These processes allow the transcription of NF-κB target genes involved in several biological processes included inflammation and immune response, such as pro-inflammatory cytokines, chemokines, cell adhesion molecules, acute phase proteins, regulators of proliferation and apoptosis.

Finally, NF-κB is negatively regulated by mechanisms such as re-synthesis of IκB proteins, p105 and p10 precursors, (NF-κB inducible genes), post-translational modifications of NF-κB subunits (74), ubiquitylation and proteosomal degradation of p65 (75).

2.2.1 Tumor necrosis factor-alpha (TNF-α)

TNF-α belongs to TNF superfamily (TNFSF) of 19 members, being TNF-α and TNF-β, the first identified (76–78). The gene encoding TNF-α is located on chromosome 6 (in the class III region of the major histocompatibility complex)(79), which is activated by several inducers such as ligands for receptors PRRs like TLRs (80) and antigen receptor ligands (81), and stimuli like pro-inflammatory cytokines (82), saturated fatty acids (83), LPS (84), viruses (85), among others. TNF-α transcription is mediated by different transcription factors including NF-κB (86), and LPS-induced

TNF-α factor (87), among others. TNF-α promotes the activation of NF-κB pathway with the target genes of inflammation and cell survival (88,89).

2.2.2 Interleukin-6 (IL-6)

IL-6 is mainly produced by cells of the immune system (T cells, monocytes/macrophages) (90,91), endothelial cells (92), adipocytes (93), fibroblasts (94), and hepatocytes (95). The gene encoding IL-6 is located on the chromosome 7 at position 15.3 (96). Its transcription is mediated by inducers like TNF-α, which regulate IL-6 production through activation of JAK/STAT (97). Likewise, LPS and fatty acids, activate IL-6 transcription by TLR4-induced NF-κB and MAPK signaling (93,98). This cytokine induces expression of pro-inflammatory genes including acute-phase proteins (99) and proliferative and survival genes (100).

2.2.3 Interleukin-1β (IL-1β)

IL-1β belongs to IL-1 family of 11 members. The gene encoding IL-1β is located on the chromosome 2 at position 14.1 (101). This interleukin is produced and secreted by a variety of cell types in response to pro-inflammatory stimuli in which, the transcription is mediated by the activation of several transcription factors including, NF-κB, IFN regulatory factors (IRFs) (102), and hypoxia-inducible factor-1 α (Hif-1α) (103). Once the mature interleukin is secreted, induces NF-κB activation and the subsequent inflammatory response (104).

2.2.4 Monocyte chemoattractant protein 1 (MCP-1)

MCP-1 is a pro-inflammatory cytokine, also known as chemokine (C-C motif) ligand 2 (CCL2), which belongs to the CC chemokine family. The gene encoding MCP-1 is

located in chromosome 17q11.2 (105), and it is expressed in several tissues and cells (106). MCP-1 transcription is regulated by classical NF-kB signaling (107) mediated by IL-1β (108), TNF-α, saturated fatty acids (109), LPS (110), among others. Once CCL2 is secreted, chemotaxis is stimulated through its interaction with CC chemokine receptor 2 (CCR2, also named MCP-1-R), a G-protein-coupled receptors (GPCRs), expressed in monocytes (111), T lymphocytes (112), dendritic cells (113), natural killer (NK) (114) and endothelial cells (115).

2.2.5 Interleukin 8 (IL-8)

IL-8 or C-X-C motif chemokine ligand 8 (CXCL), is a pro-inflammatory cytokine which belongs the CXC chemokine family. IL-8 is produced by different cell types like monocytes/macrophages (116), endothelial cells (117), epithelial cells (118) and smooth muscle cells (119).The gene encoding IL-8 is located on chromosome 4q, at position 13.3 (120). Its expression is regulated by several transcription factors including AP-1 (119), C/EBPβ, and NF-kB (118), cAMP response element-binding (CREB) and C/EBP Homologous Protein (CHOP) (121) in response to pro-inflammatory mediators like TNF-α and IL-1β. IL-8 mediates neutrophils (122), endothelial cell migration (123),cell proliferation and reduces cell apoptosis in tumorigenic processes (124).

2.3 High density lipoproteins (HDL) function

HDL is a heterogeneous lipoprotein, regarding to size, charge, density, composition and function. Concerning HDL density, studies with ultracentrifugation carried out by John Gofman et al. in 1949 and 1954 allowed to determine and separate HDL particles with a density between 1.063 and 1.21 g/mL. They were the first in identifying two major HDL subclasses, a less dense (1.063-1.125 g/mL) classified as HDL2, and other more dense (1.125-1.21 g/mL) classified as HDL3 (125,126).

Likewise, other methods like 2-dimensional agarose/polyacrylamide gel electrophoresis (2D-PAGE) classified HDL subclasses according to their size and net surface charge, that separate two HDL2 (HDL2a, 8.8.–9.7 nm; and HDL2b, 9.7–12.0 nm) and three HDL3 subclasses (HDL3c, 7.2–7.8 nm diameter; HDL3b, 7.8–8.2 nm; HDL3a, 8.2–8.8 nm) (127). Furthermore, nuclear magnetic resonance (NMR) spectroscopy allows the quantification of HDL particles and the separation of HDL into three subclasses: small (7.3–8.2 nm) medium (8.3–9.3 nm) and large (9.4–14.0 nm) (128).

HDL composition varies during the process of reverse cholesterol transport (RCT). HDL lipidome includes mainly phospholipids, cholesterol ester, sterol and triglycerides (129). In addition, in the analysis of HDL proteome more than 100 proteins have been identified, being apolipoprotein (Apo)A1 the most abundant, followed by ApoAII. Other HDL-associated proteins include ApoCs and ApoE; enzymes like lecithin-cholesterol acyltransferase (LCAT), cholesteryl ester transfer protein (CETP), phospholipid transfer protein (PLTP) and paraoxonase 1 (PON1); acute phase reactants like serum amyloid A (SAA); complement factors, among others (130).

Regarding HDL function, since HDL was identified, several prospective cohort studies have evaluated its relation with cardiovascular diseases. In 1966 in the Gofman's Livermore Cohort, low levels of HDL2 and HDL3 were associated with de novo ischemic heart (131). Then, in 1977 and 1988 The Framingham Study published the results of around 2800 individuals after 4 and a 12 years follow-up. They found that HDL-c levels were inversely associated with the incidence of coronary heart disease and mortality (132,133), for which HDL-c have been considered as a protective factor against CVD. In spite of that, other studies have demonstrated different results. For example, high HDL-c levels have been associated with major cardiac events risk factors (134), which seem to be associated with the presence of chronic inflammation (135). Contrary to the expected results with drug treatments, such as CETP inhibitors, to increase HDL-c levels, associations with increased CVD and mortality have been reported (136). Although it is well known that low HDL-c is associated with mortality; paradoxically, extremely

high levels of HDL-c have also been associated with all-cause mortality (133). Given these results, several studies have been carried out in order to understand the role of HDL particles, beyond HDL-c levels, and to determine its protective role and the association between HDL dysfunction with cardiovascular risk, CVD events and mortality (137).

HDL has an important role in the RCT, which is the cholesterol transport from peripheral tissues back to the liver to be eliminated (138). Once ApoA1 is synthetized in the liver or intestine and secreted as lipid free/lipid poor protein, it starts obtaining cholesterol and phospholipids mediated by the interaction with the cholesterol transporter ATP-binding cassette A1 (ABCA1), which is ubiquitously expressed on cell membranes (139). In this way, nascent HDL (preβ-HDL) is formed in which the amphipathic lipids are organized with the nonpolar region interacting with lipid components, and the polar domain exposed to the aqueous environment (140). Then, the cholesterol of nascent HDL is esterified by the activation of the LCAT enzyme, becoming a spherical lipoprotein, which has a surface monolayer of phospholipids and free cholesterol, with more than 70% of the surface covered by proteins, transporting cholesterol ester and triglycerides in the core (141). HDL also acquires cellular lipids by ABCG1 and scavenger receptor class B type I (SR-BI), that preferentially interact with mature HDL particles (142,143). In addition, RCT involves the exchange of lipids and proteins between HDL and ApoB-containing lipoproteins (144). In this way, HDL2 can be converted into HDL3 by lipid transfer mediated by CETP and PLTP to ApoB-containing lipoproteins, generating a TG-rich HDL (144,145). This TG-enriched particle can be then hydrolyzed by hepatic lipase and endothelial lipase, resulting in lipid poor HDL particles (146,147). Likewise, HDL lipids are catabolized via hepatic SR-BI and LDL apoB/E receptor that induce uptake of HDL cholesteryl ester. Finally, residual ApoA1 proteins are cleared by the kidneys (148).

In addition to RCT, HDL has other athero-protective roles including anti-inflammatory properties, mediated by several mechanisms such as: activation of Activating Transcription Factor 3 (ATF3), a transcriptional repressor of pro-inflammatory cytokines in macrophages, modification of the inflammatory response of TLR in

macrophages (149); binding of HDL/ApoM/S1P to sphingosine 1-phosphate receptor 1 (S1P1) in endothelial cells, that suppresses NF-κB activation by TNF-α, and the expression of adhesion molecules (150); suppression of inflammasome activation mediated by the binding of HDL to cholesterol crystals (151), among other mechanisms. However, during inflammatory conditions like diabetes and CVD, the anti-inflammatory properties of HDL are lost (152,153), becoming a dysfunctional lipoprotein, which is associated with changes on HDL composition with an increase of SAA and reduction in ApoA1, among other changes (154,155).

Another important function of HDL is its anti-oxidant activity mediated by proteins like Apo1, platelet-activating factor-acetylhydrolase (PAF-AH) (156), PON1 (157), LCAT (158), among others; which prevent the oxidative modification on LDL and HDL through mechanisms such as elimination of lipid hydroperoxides derived from oxidized LDL and the inhibition of ROS production (159,160). It has also been demonstrated that HDL3 protects LDL from oxidation better than HDL2 (161). However, in OxS and inflammatory conditions, the HDL anti-oxidant capacity is also affected, associated with increases in oxidized-LDL (ox-LDL) and myeloperoxidase (MPO) levels and reductions of ApoA1 and LCAT levels and PON1 activity (162,163).

2.3.1 Apolipoprotein A1 (ApoA1)

The gene encoding ApoA1 is located in the chromosome 11q23.3 (164). This protein in predominantly expressed in the liver and intestine. ApoA1 gene expression is controlled by some regulatory regions in the gene, which contains binding sites called hormone responsive elements (HREs), to which transcription factors bind, including various orphan receptors and ligand-dependent nuclear receptors. For example, transcription factors members of the steroid hormone receptor superfamily like homo- and heterodimers retinoic X receptor (RXR) bind to HRE in the apoAI promoter and it promotes its transcription in response to 9-cis-Retinoid acid (165–167).

ApoA-I is secreted as a mature protein (28 kDa, 243 amino acids) lipid-poor or lipid free that interacts with the cell-membrane transporter ABCA1 to promote the efflux of cholesterol and phospholipids (139), generating the nascent HDL particles or discoidal and representing major component of HDL (140). In this process, apoA-I-derived peptides interact and activate the enzyme LCAT, which induces the esterification of lipids (141), producing a spherical HDL. The interaction of ApoA1 with HDL is mediated by the polar C-terminal domain that interacts with phospholipid (PL)-stabilized emulsion particles (168). In this way, ApoA1 stabilizes the lipoprotein. For this, several arrangement of ApoA1 proteins on HDL particle have been described, including a double belt model and a trefoil model (169). In addition, ApoA1 can also be exchanged between HDL particles (170).

Its function is strongly affected by several conditions like the non-enzymatic glycation of ApoA1 as occurs in diabetes (171), oxidative modification by products of lipoperoxidation (172) or ApoA1 modifications mediated by MPO (173). In addition, low levels of ApoA1 have been associated with a diet high in carbohydrates (174), elevated risk of T2D (175), CVD (176), Parkinson's disease (177) and cancer (178). On the contrary, high ApoA1 levels have been associated with high physical activity, low BMI and non- smoking (174).

2.3.2 Paraoxonase 1 (PON1)

PON1 belongs to the paraoxonase family of three members, PON1, PON2 and PON3. This protein is predominantly synthesized and secreted by the liver (179). The encoding gene is located on the chromosome 7q21.3 (180), and its expression is regulated negatively by LPS and pro-inflammatory cytokines like TNF-α, IL-1β (181,182).

IL-6 promotes PON1 gene expression through PI-3K/AKT/NF-KB pathway, although it is no clear whether IL-6 play a protective role in this case (183). Likewise, high glucose levels have shown to upregulate PON1 expression mediated by the

activation of the transcription factor specificity protein 1 (Sp1) by PKC, which seems to be a compensatory mechanism in diabetes (184).

Furthermore, bioactive compounds present in plants, fruits and vegetables have also demonstrated to improve PON1 expression by several signaling pathways. Phytochemicals like quercetin for example, upregulates PON1 expression because it promotes translocation of the mature SREBP2 (185); polyphenols increase PON1 expression mediated by PPARγ -PKA-cAMP pathway (186); resveratrol, quercetin, catechin and other polyphenols have shown to induce PON1 gene expression by an aryl-hydrocarbon-receptor-(AhR-) dependent mechanism (187,188).

Regarding its function, PON1 was first identified in 1952 by Aldridge in animal studies as an esterase that hydrolyzes paraoxon, a metabolite of the organophosphate pesticide parathion (189). Then, human studies demonstrated that PON1 is an enzyme with paraoxonase and arylestesase activity in serum in a calcium-dependent manner (190,191). However, the native physiologic activity of PON1 seems to be the lactonase activity, due to its ability to hydrolyze lactone-based substrates, like homocysteine thiolactone (192,193).

Regarding the physiological role of PON1, in 1991 Mackness et al. found that paraoxonase is an HDL associated protein with an athero-protective role by preventing the accumulation of lipoperoxides in LDL (157). Other studies in this regard, have demonstrated that PON1 also protect HDL against lipid peroxidation because it hydrolyzes oxidized lipids (194). Human studies have shown that PON1 activity is inversely associated with CVD risk (195).

2.4 Oxidative stress (OxS)

In physiological conditions free radical species are produced as a result of the normal aerobic metabolism (196). Such species include reactive oxygen species (ROS) like superoxide anion (O2−.), singlet oxygen, hydroxyl radical (.OH), hydrogen peroxide (H2O2), hypochlorous acid (HOCl), and reactive nitrogen species (RNS) like nitric

oxide (NO) (196). Although these species are stronger oxidants and therefore dangerous, in a normal organism their production is balanced by antioxidant defense systems such as endogenous enzymatic and nonenzymatic antioxidants, like glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT), glutathione, uric acid, bilirubin and nicotinamide adenine dinucleotide phosphate (NADP). Also exogenous antioxidants, such as vitamins E (α-tocopherol) (197) and C (ascorbic acid) (198) help to balance free radical species. These antioxidant mechanisms allow to scavenge reactive species and keep their concentration at physiological levels, maintaining the cellular redox balance (196).

However, when there is an imbalance between prooxidant and antioxidant factors, in favor of the former, a state of OxS is developed. The accumulation of reactive species lead to cellular damage in macromolecules such as DNA, proteins, carbohydrates and lipids, leading to progressive development of different chronic diseases including CVD (199–201), cancer (202), major depressive disorder (203), among others. In addition, MetS is associated with increased ROS and OxS (204). In this sense, determination of oxidized products of DNA, lipids and proteins are the most common ways to evaluate OxS. Likewise, the reduction of antioxidant enzymes is associated with OxS that can be evaluated in this sense.

2.4.1 Advanced oxidation protein products (AOPP)

AOPP are highly oxidized proteins, generated in presence of chlorinated oxidants (HOCl or chloramines) and produced by MPO during neutrophils, monocytes and phagocytic cells respiratory burst (205). Fenton reaction has also been suggested as another mechanism of AOPP production (206). The interaction between HOCl with proteins, being albumin the most abundant, induces carbonyl proteins, protein cross linking and aggregation mediated by disulfide bonds or cross-link between two tyrosine residues (dityrosine) (207). Likewise, oxidized fibrinogen by HOCl seems to have an important role in AOPP generation (208), which affects the fibrinogen structure and function increasing its pro-coagulant activity (209).

AOPP have been shown to be pro-inflammatory mediators of monocyte activation, inducing TNF-α and ROS production mediated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation and p38 phosphorylation (210). AOPP also increase the expression of NF-κB, IL-6 and cyclooxygenase-2 (COX-2) in Human Embryonic Kidney Cells (206), and MCP-1 mRNA in vascular smooth muscle cells through p38 MAPK pathway (211). AOPP are accumulated in the liver and spleen, but they are mainly eliminated by the liver (212).

AOPP were first identified in 1996 in uremic plasma of end-stage renal disease patients on maintenance hemodialysis, and recognized as an oxidative stress marker (207). AOPP have been associated with a large number of diseases including type 1 and type 2 diabetes mellitus (213), which seems to be mediated by their capacity to induce β-cell destruction and dysfunction in a NADPH oxidase-dependent manner (214). Likewise, in these patients AOPP levels are correlated with the Framingham risk score, that could predict the risk of cardiovascular events (215). AOPP are also recognized as an independent risk factor for coronary artery disease (216), they area significantly increased in CVD patients, and in addition to AGE are considered biomarkers with predictive potential in the progression of this disease (217). Similarly, AOPP are significantly increased in alterations such as overweight/obesity, metabolic syndrome (218) (even more than lipid peroxidation) (219), diabetic nephropathy (220), acute coronary syndrome (221), atherosclerosis (222,223), cancer (224,225) and neurodegenerative diseases (226). AOPP are even increased in healthy young adults with CVRF, being also considered as a risk factor (227). The association between AOPP with atherosclerosis seems to be mediated by its capacity to increase ROS production in endothelial cells, mediated by NADPH oxidase activation (228,229); and by its capacity to block SR-BI, directly affecting the HDL metabolism (230). Likewise, AOPP have been shown to downregulate ABCA1, ABCG1 and liver X receptor alpha (LXRα) expression in apolipoprotein E knockout (apoE-KO) mice, contributing to atherosclerosis development (231).

Finally, AOPP is positively correlated with dityrosine (207), strongly related with AGE (213), 24-h proteinuria excretion and cystatin C (232). In contrast, they are negatively

associated with endogenous antioxidant enzymes SOD and GpX (220), and with adiponectin levels (233).

2.4.2 Mieloperoxidase (MPO)

MPO (EC 1.11.2.2) is a heme protein, member of the subfamily of peroxidases discovered in 1941 by Agner, K (234). MPO is mainly presents in immune system cells such as neutrophils, monocytes, macrophages (235) and lymphocytes (236). The gene encoding MPO is located on the chromosome 17q22 (237).

MPO was first described in 1965 with an antimicrobial function (238). In this sense, in the innate immune response against pathogens, phagocytic cells are activated, inducing respiratory burst, in which NADPH oxidase is activated generating superoxide anions within the phagosome, then, superoxide dismutation generates H2O2. MPO transfer two electrons to H2O2, forming the compound I. MPO in this state has high oxidizing ability and can induce peroxidation of substrates like Cl- and pseudohalides like thiocyanate, being HOCl the more abundant product (239). In this sense, MPO catalyzes the peroxidation of the chloride ion (Cl-) to produce HOCI, mediated by one-electron oxidations, forming compound II (240,241). HOCl is a potent microbicidal, and because of that, patients with MPO deficiency develop chronic infections (242).

MPO and its products can induce damage in pathogens but also host tissues through oxidation of biomolecules like proteins generating protein aggregation (243), oxidation in thiol groups on cysteines and glutathione (GSH) (244) and oxidation in other amino acids like methionine (245). In addition, MPO is associated with the production of other ROS such as hydroxyl radical (246) and RNS like nitrite (NO2–), the autoxidation product of NO (247). In this sense, MPO levels have been significantly increased in several chronic diseases like diabetes (248), CVD, atherosclerosis (249,250), among others, being a mediator of OxS. In addition, high MPO levels are associated with worse outcomes and risk of mortality in patients with acute coronary syndrome (251).

Atherosclerosis and CV risk have been associated with the MPO capacity to induce LDL oxidation though MPO-LDL interactions (252), amino acid oxidation in ApoB mediated by HOCl (253), aldehyde-modified LDL (254) and MPO-generated reactive nitrogen species, all of which promote foam cell formation (247). Likewise, MPO have also been shown to affect the ApoA1 function through oxidation, reducing cholesterol efflux and promoting a pro-inflammatory state and atherosclerosis development (255). Furthermore, MPO form a functional ternary complex in HDL with ApoA1 and PON1, with a reciprocal inhibition between MPO and PON1, in which, MPO affect PON1 activity by oxidation of tyrosine 71 (Tyr71), altering its interaction with HDL (256).

2.4.3 Superoxide dismutase (SOD)

Human SOD (SOD, EC 1.15.1.1) was first isolated in 1958 by Markowitz et al, in erythrocytes as a cuproprotein named erythrocuprein (257). In 1973, the SOD activity of this protein was determined, which catalyzes the dismutation of superoxide radicals to O2 and H2O. Its function is to protect against oxidative damage of superoxide free radicals (258). SOD is a ubiquitous metalloenzyme, of which have been described three isoforms, SOD1, SOD2 and SOD3.

Human SOD1 (Cu,ZnSOD) is encoded on the chromosome 21q22.11 (259). The protein is located in the cytosol and in the intermembrane space of mitochondria (260,261). The structure was described in 1992, which is highly conserved in eukaryotic cells (262). SOD1 have two subunits of 16kDa, each one forms a β-barrel (composed of eight antiparallel β-strands), a disulfide bond that provides structural stability and two metal ions, one copper and one zinc. The interaction with anion superoxide reduces Cu2+ to Cu1+ with reduced catalytical functions. In presence of other anion superoxide and 2H+, Cu1+ is oxidized to Cu2+, and H2O2 is generated (263).

In 1982 it was identified the human extracellular Cu,ZnSOD named SOD3, located in blood vessel walls. This enzyme is encoded on the chromosome 4p15.2 (264).

Structurally it has four equal noncovalently bound subunits that are similar to the human SOD1 dimers, each one with four copper atoms, with high affinity to heparin (265). Its catalytic function is identical to the SOD1 (266). This enzyme has been associated with improving the response to chemotherapy by stabilizing endothelial hypoxia-inducible factor-2α (HIF-2α) (267), reducing anti-inflammatory response in ischemic damages (268) and protecting against cardiac oxidative injury and hypertrophy (269).

Human SOD2 is manganese-dependent (MnSOD). The gene encoding this protein is located on the chromosome 6q25.3 (270). This enzyme is located in the mitochondria and is responsible for inducing the dismutation of the greatest amount of superoxide anion generated in the cell (271). SOD2 is a homotetrameric enzyme (dimer of dimers), in which each subunit contains a catalytic manganese site, two domains, alpha N-terminal and alpha/beta C-terminal domains, that stabilize the active sites, located between both domains (271). Interaction between a low ratio anion superoxide with MnSOD induces an oxidation and reduction of manganese, with production of H2O2, similar to the Cu,ZnSOD reaction. However, when anion superoxide exceeds enzyme levels, there is a depletion of the superoxide catalyzed with the formation of an inhibited complex between Mn2+SOD and O2•-, which is a possible mechanism why MnSOD prevents the accumulation of H2O2 in the cell (272).

The SOD activity has been shown to be significantly low in some diseases such as acute coronary syndrome (273), metabolic syndrome (274), pre-diabetes (275), inflammatory bowel disease (276), among others.

2.4.4 Catalase (CAT)

CAT (EC 1.11.1.6) is a heme enzyme described first by Loew in 1900 (277), that protects against the toxic effects of H2O2. The gene encoding CAT protein is located at chromosome 11p13, and it is expressed in almost all body tissues (278). Regarding the structure, human catalase or mammalian catalase was first described

in erythrocytes as a heme protein with 1997 amino acids, 393 water molecules in four identical subunits of 59.7 kDa (279). Each subunit forms a β-barrel, and has N- and C-terminal regions, wrapping loop, a heme group with the iron in ferric state and a tightly bound molecule NADPH (279,280).

Its catalytic activity was determined years later by Chance, who described the generation of three compounds (281–283). First, H2O2 interacts with the active site of CAT inducing oxidation of FeIII to FeIV, producing compound I (an oxoferryl porphyrin cation radical). Then, compound I reacts with other H2O2 reducing the FeIV to FeIII generating H2O and O2. When H2O2 is low, compound I can react with endogenous donors like phenols, NO and salicylic acid that donate one electron producing an inactive compound II (iron (IV) oxo-ligated porphyrin). This compound can return to its initial state through another one-electron reduction step (284). In addition, compound II also can interact with other H2O2, generating another inactive compound III (oxycatalase). However, NADPH acts as an electron donor preventing compound II formation, hence it keeps catalase active in vivo (285). A reduced CAT activity have been associated in OxS conditions like cancer (286,287) and CVD (288) with decreased ability to metabolize H2O2.

2.4.5 Glutathione peroxidase (GPx)

GPx (EC 1.11.1.9) is a family of antioxidant enzymes ubiquitously present in all cells. GPx was described first in erythrocytes, by Mills in 1957, as an enzyme with high ability to protect against hemoglobin oxidation (289). GPx catalyzes the reduction of H2O2 to $2H_20$ mediated by the conversion of reduced glutathione (GSH) to glutathione oxidized form or glutathione disulfide (GSSG) (289). Then, GSSG is reduced by a glutathione reductase in presence of NADPH2, generating 2GSH and NADP+. This enzyme has also been shown to reduce lipid hydroperoxides (290).

Eight isozymes of GPx have been described (GPx1-GPX8), which vary on structure, cellular location and substrate specificity. Structurally, GPx1-4 and GPx6 have a selenocysteine residue in the active site, while the rest only contain a Cys residue.

In addition, GPx4 is monomeric, GPx8 is dimeric and the rest are tetramers (291). The first three GPx enzymes have been more widely studied. GPx1 is the most abundant GPx isozyme, located in the cytoplasm and mitochondria and ubiquitously expressed, and its substrate is H_2O_2 (292). Its low activity has been associated with increased risk of cardiovascular events (293–295). GPx2 is another cytosolic enzyme, mainly expressed in the liver and gastrointestinal tract (296). This enzyme also reduces H_2O_2. GPx3 is abundant in plasma and as the previous enzymes, also catalyzes the H_2O_2 excess (297). It is mainly expressed in the kidney, (298–300). Down regulation of GPx3 is associated with insulin resistance (301), obesity (302) and contributes with OxS. In addition, this enzyme acts as a tumor suppressor (303). GPx4 is an intracellular enzyme located in the cytosol and membrane. It has a preference for lipid hydroperoxides instead of H_2O_2 (304).

2.5 Antioxidants and Total antioxidant capacity (TAC)

Antioxidants are defined as “substances that, when present at relatively low concentrations compared with those of the oxidizable substrates, significantly delay or inhibit oxidation of those substrates”. The antioxidants can act as preventive and chain-breaking. The first type of antioxidants acts to protect the system against initiation of oxidation via Fenton-type reaction, for example, chelating agents or some endogenous antioxidants enzymes (305). The second type or chain-breaking antioxidants, are also defined as peroxyl radical (LOO•) trapping antioxidants, and they represent the antioxidants able to stop the propagation process, transforming the LOO• in hydroperoxides (LOOH), while a reduced form of the antioxidant is oxidized to a radical (305). This antioxidant is not reactive enough to abstract a hydrogen atom from another lipid molecule (306). However, it can trap another peroxyl radical. This reaction finishes when the antioxidant is consumed (305).

There are several mechanisms antioxidants use to scavenging reactive species including: ROS/RNS scavenging activities; transfer either a hydrogen atom (H•) (also

called hydrogen atom transfer-HAT) and an electron (e−) (also called single electron transfer-SET) or both to inhibit the radical initiation or propagation steps; reducing power and metal chelation. Likewise, antioxidants inhibit the generation of free radicals or they may also act indirectly by upregulating endogenous antioxidant defenses (305).

Total antioxidant capacity (TAC) is the ability of a mixture of compounds to prevent oxidative reactions to another molecule, mediated by non-enzymatic antioxidants. This capacity depends on several factors such as the matrix, cumulative and synergistic interaction, nature of the oxidizing substrate, among others (307). There are several methods to determine TAC, which are considered under three major categories: HAT mechanisms, SET mechanisms or mixed-mode (both mechanisms). These methods measure the antioxidant capacity (AC) based on different strategies such as the use of colored and stable free radicals, the capacity of antioxidants to reduce cupric or ferric ions and the estimation of the antioxidant´s ability to protect a target molecule exposed to a free radical source (competitive methods) (305).

Some of the most common methods to determine AC are described below.

2.5.1 *Oxygen Radical Absorption Capacity (ORAC)*

This assay is one of the methodologies most frequently employed to evaluate the AC of foods (308). However, ORAC database previously available on the US Department of Agriculture website has been withdrawn based on lack of correlation between the ORAC index and in vivo situations (309).

ORAC is considered a competitive method given the oxidant reacts with a probe, while antioxidants compete with the probe and prevent its further oxidation. In other words, this method determines the capacity of a sample to delay the consumption of a target molecule (usually followed by UV-visible absorption or fluorescence spectroscopy) induced by peroxyl radicals (305).

ORAC is a HAT assay, which measures the capability of an antioxidant to quench free radicals by donating a hydrogen atom. This assay uses 2,2′ azobis (2-methylpropionamidine) dihydrochloride (AAPH) as a free radical generator, fluorescein as the target molecule and trolox (a hydro-soluble vitamin E analog) as the standard. The ORAC index is evaluated from the area under the curve (AUC) of the kinetic profiles of the consumption of the target molecule, and the results are expressed in trolox equivalents (TE) (310).

Given that ORAC method uses a biologically important free radical source (peroxyl radical) in human biology, this assay is relevant in in vivo conditions. In addition, this method has the advantage to be adaptable for numerous sample matrices including plasma, tissue and foods (305).

2.5.2 Ferric Reducing Antioxidant Power Assay (FRAP)

This is a SET assays, which detects the capacity of an antioxidant to transfer one electron to reduce metal ions, in this case, ferric ions. The FRAP assay is based on the reduction of ferric ions (Fe3+) to ferrous ions (Fe2+) by the electron donating antioxidants in the presence of tripyridyltriazine tridentate (TPTZ) ligand, forming a colored complex with Fe2+. Ferric salt (Fe3+-TPTZ) solution is of a yellow color but when it is reduced to the ferrous form, it changes to blue, and the absorbance at 593nm increases. This method has been widely used to assess the reducing power of an antioxidant in both food and biological fluid samples (311).

Antioxidants that react in the FRAP assay include ascorbic acid, α-tocopherol, uric acid, bilirubin, and polyphenolic compounds in plant-based foods (305). Therefore, this assay is used for a variety of purposes, including estimating of the antioxidant content of foods, to detect contamination of water and to evaluate the total antioxidant activity of biological fluids, including plasma, urine, saliva, follicular fluid, alveolar fluid, semen, tears, cerebrospinal fluid, and feces (305).

2.5.3 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation (ABTS)

This is a mixed-mode (HAT/SET) assay, which is used to determine the antioxidant activity since 1993, in the Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) equivalent antioxidant capacity (TEAC) assay (312). Currently it is used to evaluate the AC of pure compounds and foods (beverages, fruits, vegetables, and meats), and it has been adapted to be used in human biological samples (305).

This assay is based on ABTS•+ radical generation by several reagents being potassium persulfate one of the most used (313). This chemical generation is usually slower, in which 7 mM ABTS is mixed with 2.45 mM potassium persulfate in water and the solution is stored in the dark for 12–16 hours before use. Then, the concentrated solution is diluted with phosphate buffered saline (pH 7.4). Although this method was mainly developed in aqueous media to determine hydrophilic antioxidants, organic solvents could be used for lipophilic antioxidants (313).

The reaction is determined by decoloration or absorbance decrease, corresponding to the disappearance of the radical due to the antioxidant present in the sample. Quantification can be made by means of a calibration curve that relates the loss of the signal and the concentration of antioxidant present in the sample (313).

2.5.4 2,2-di(4-tert-octylphenyl) 1-picrylhydrazyl) radical scavenging colorimetric assay(s) (DPPH)

This method is also a mixed-mode (HAT/SET) assay through mechanisms such as: a) transference of an hydrogen atom, when the single occupied molecular orbital of the free radical is almost linearly oriented to the hydroxyl group of the phenol; b) proton-coupled electron transfer (PCET) (considered other version of HAT), in which an electron and a proton are transferred in a single step, but the electron and the proton can come from a different orbital or space; and c) sequential proton loss

electron transfer (SPLET) which involves the ionization of phenolic compounds and the electron transfer from the phenoxyl anion. Each mechanism depend on several variables like pH, medium and structural characteristics of phenols (305).

The DPPH• colorimetric assay was developed by Blois in 1958 (314) and modified by Brand-Williams et al. in 1995 (314). In addition, although this assay has been widely used in food and plants, Chrzczanowicz J et al. in 2008 adapted it to determine the AC in human serum (315). This method is based on the scavenging of a stable radical chromophore called 2,2-diphenyl-1-picrylhydrazyl (DPPH•) by antioxidants, by donating an electron or a hydrogen atom. The color´s change from purple of DPPH• radical to yellow of the reduced form of DPPH• allows the spectrophotometric determination of the AC (305).

2.6 *Vaccinium meridionale* Swartz

Vaccinium meridionale was described first by Peter Olof Swartz in 1788 (316). This fruit belongs to the Ericaceae family and *Vaccinium* gender, also called agraz, mortiño or Andean blueberry. This fruit is distributed geographically in Venezuela, Colombia, Perú and Ecuador, in places ranging from 2000 to 3800 meters above sea level. In Colombia, according to data reported by the scientific collections of the Institute of Natural Sciences (ICN) at the Universidad Nacional of Colombia, V meridionale has been mainly found in Antioquia, Cundinamarca, Magdalena and Boyacá (317), with two harvest per year, the first in April to May and the second in September to December (318).

This plant grows as shrubs of different size, between 0.3 to 5 meters, which produces terminal or axillary clusters with 8-15 fruits, with a variable diameter from 8-20mm (318). The fruits reach its physiological maturity between 60 to 80 days after flowering, being green in the immature state, and dark purple in the mature state. Usually, the pulp is light in color and contains a variable number of seeds (318). As the fruit ripens, there are increases of total soluble solids to 11 and 12 °Brix, associated with an increase of enzymes that degrade starch, producing more soluble

carbohydrates (319). In contrast, during maturation total titratable acidity and pH are reduced, which is due to the reduction of organic acids (319). Likewise, in the mature state, which is the state of consumption, *V. meridionale* has a moisture content of 77-83% and a pH between 2.2 to 2.7 (320).

Regarding the composition, the pulp of the mature *V. meridionale* fruit has an average energy of 66.1±5.4kcal/100g and it contains a concentration of total ash, fat, protein and carbohydrates of 0.3±0.08g/100g, 0.2±0.2g/100g, 1.2±1.4g/100g and 14.9±1.9g/100g, respectively (321). In addition, this fruit contains organic acids such as citric, malic and ascorbic acids in a concentration of around 1899, 1396 and 8 mg/100g fresh fruit, respectively (322). *V. meridionale* is an important polyphenol source with total phenol values that varies from 609±31mg to 758.6±62.3 Gallic Acid equivalents (Eq) (GAEq)/100g of fresh fruit (320,323). Anthocyanins are the main polyphenols of this fruit, which are located mainly in the epidermis and are responsible for the purple color in ripe fruit. This polyphenol is detectable in the mature fruit with a reported concentration of 200.6±10.2, 271.9 and 329±28mg Eq of cyanidine-3-glucoside/100g of fresh fruit (320,323,324). Five anthocyanins have been described in this fruit, including, Cyanidin 3-galactoside, Cyanidin 3-arabinoside, Cyanidin 3-glucoside, Delphinidin 3-hexoside and Delphinidin 3-pentoside. In addition, other phenolic compounds present in *V. meridionale* include quercetin glycosides, which represent 100% of the total flavonoids, and hydroxycinnamic acids with a concentration of 99.2±6.7 mg/100g fresh weight, including caffeoyl quinic acid isomers, caffeoyl methyl quinate and caffeic acid derivate isomers (323).

Several studies have reported a high antioxidant activity of this fruit measured by different methods such as: ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) whose reported values for total antioxidant capacity have been 8694±435µm Trolox Eq/100g of fresh fruit (320) and 45.5±2.3µmol Trolox Eq/g of fresh weight (323); FRAP (ferric reducing ability of plasma) method with values for total antioxidant capacity of 581±29mg ascorbic acid/100g of fresh fruit (320) and 87.0±17.8 lmol Trolox Eq/g of fresh weight; and DPPH (2,2-Diphenyl-1-

picrylhydrazyl) methodology with observed values of 2404±120µm Trolox Eq /100g of fresh fruit (323).

The physico-chemical characterization and antioxidant capacity of the freeze-dried V.meridionale used in this study, was previously reported by our laboratory, with a total phenol content of 1027.97 ± 41.99 mg GAE/g in 200mL, and a total cyanidin content of 4.66 mg cyanidin equivalents/g. In addition, antioxidant capacity of this beverage was determined by different methods obtaining the following results: 104.25 ± 15.14 µmol Trolox Eq /L measured with DPPH; 664.87 ± 73.47 µmol Trolox Eq /L with FRAP; 205.26 ± 9.99 µmol Trolox Eq /L with ABTS; and 4,702.31 ± 22.36 determined by ORAC (325).

Given these antioxidants properties, several studies have been carried out with V. meridionale to evaluate its effect in the prevention of some diseases. V. meridionale has been shown to have a cytotoxic and antiproliferative effect in colon adenocarcinoma cells (SW480) and derived metastatic cells (SW480) in in vitro studies (326). Studies in rats evaluating its effects in ischemia-reperfusion have demonstrated improvement on the systolic and diastolic function and antioxidant capacity, increasing SOD and catalase activity, reducing lipid peroxidation, and increasing endothelial nitric oxide synthase and Akt expression and proteins. These results indicate cardioprotective effects of V. meridionale against ischemia-reperfusion (327,328). In 2018, a study evaluating the bioaccessibility and intestinal permeability of a V. meridionale juice through in vitro gastrointestinal digestion and colonic fermentation was published. The researchers found that ascorbic acid, free phenolic compounds, and oligosaccharides were bioaccessible. Most of these compounds are fermented by the colon microbiota, exerting an antiproliferative effect (329). Recently, the first human interventions has been carried out led by researchers from the University of Antioquia. We conducted an intervention study in women with high CVRF, observing that the chronic consumption of V. meridionale increased serum antioxidant capacity and decreased a marker of DNA oxidative damage (330). In addition, Agudelo et al, in 2018 demonstrated in 19 healthy volunteers (18-60 years) that consuming a single dose of 250mL of Andean Berry juice during 14 days, significantly increased the antioxidant capacity measured by

the ABTS method, and reduced isoprostane levels. Likewise, this beverage reduced IL-6 levels in plasma. These results indicate an antioxidant and anti-inflammatory effect of V. meridionale (331). The authors also evaluated the effects of 21 days consuming 35 g/day of osmodehydrated Andean berry in overweight adults, finding improvements in systolic and diastolic blood pressure, BMI, weight and waist circumference, compared with the results obtained on day 1. However, glucose was significantly increased (332).

2.6.1 *Vaccinium* effects on inflammation

Studies in "healthy" people and with CVD risk have shown anti-inflammatory effects after chronic consumption of polyphenols derived from berries, decreasing serum pro-inflammatory proteins such as CRP, IL-6, IL-15, monokine induced by interferon gamma (333), IL-8, interferon-alpha and the regulated on activation, normal T cell expressed and secreted (RANTES) protein (334). In addition, a meta-analysis on randomized clinical trials, evaluating the impact of anthocyanins (from *Vaccinium* sp.), demonstrated that administration of higher doses of anthocyanins (> 300 mg/day) have an anti-inflammatory effect through reductions in CRP, IL-6, TNF-α, and VCAM-1 levels (335).

The anti-inflammatory effects of *Vaccinium* consumption over people with CVD risk have been shown to be better after a chronic consumption in comparison with an acute intervention (336). The potential mechanism by which *Vaccinium* exerts its anti-inflammatory effects is mediated by inhibition of NF-κB translocation by blocking IκB-α phosphorylation and degradation (337).

2.6.2 Vaccinium effects on oxidative stress

The chronic intake of *Vaccinium* fruits has shown significant increases in TAC determined by ABTS and ORAC methods, which is associated with increased levels

of anthocyanins in blood (338,339). In addition, the consumption of anthocyanins from *Vaccinium myrtillus*, directly removed ROS through their hydroxyl groups, or indirectly affecting the expression and activity of endogenous antioxidant enzyme systems such as SOD and CAT (340).

Studies in endothelial cells of human umbilical cord have shown that treatment with blueberry anthocyanins attenuate OxS by decreasing the level of ROS, xanthine oxidase-1 production (341) and oxidized glutathione (GSSG, or glutathione disulfide) (342). Additionally, these bioactive compounds increased the level of endogenous antioxidants such as SOD (341) and reduced glutathione (GSH) (342). A possible molecular mechanism by which these compounds induce increased expression of endogenous antioxidants is their demonstrated capacity to induce activation of the transcription factor nuclear factor (erythroid-derived 2) -like 2 (Nrf2), which regulates the expression of genes involved in the endogenous antioxidant defense (342).

Finally, the capacity of *Vaccinium* fruis to reduce oxidants agents like MPO has been demonstrated with V. macrocarpon in a model of acute pancreatitis (343). In addition, the chronic consumption on that same *Vaccinium* in people with high CVRF, had a positive effect in OxS through reduction in lipoperoxidation and protein oxidation levels assessed by AOPP (344).

2.6.3 Vaccinium effects on HDL function

Given that HDL particles have demonstrated a cardioprotective role, this lipoprotein has been proposed as a promising marker for cardiovascular risk. Investigations have focused on seeking treatment that would not only increase the plasma levels of HDL-C, but also to improve the function of this lipoprotein (345).

Different studies support the evidence of other protective effects of berries (*Vaccinium*) consumption against CVD, demonstrating that it increases serum concentration of HDL-C (346). In addition, anthocyanin consumption from *Vaccinium*

has demonstrated to improve HDL function by increasing cholesterol efflux capacity from macrophages (347) and PON-1 activity (348).

Studies in mice deficient in apolipoprotein E (ApoE -/-) (which are more prone to develop atherosclerosis) fed with anthocyanins, also showed elevated plasma concentrations of HDL-C, apoA-I, increased reverse cholesterol transport, increased activity of PON-1 and hepatic expression of Pon1 and Lcat (349). Finally, in vitro assays using macrophages have shown the effects of anthocyanin metabolites (protocatechuic acid, PCA) in increasing cholesterol efflux, associated with increased expression of ABCA1 and ABCG1, which encode proteins that facilitate the transport (efflux) of cholesterol from cells to the HDL particle (350).

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CHAPTER 3

Effect of agraz (*Vaccinium meridionale* Swartz) on high-density lipoprotein function and inflammation in women with Metabolic Syndrome

Effect of agraz (*Vaccinium meridionale* Swartz) on high-density lipoprotein function and inflammation in women with Metabolic Syndrome

3.1 Abstract

Metabolic syndrome (MetS) is associated with low-grade inflammation and high-density lipoprotein (HDL) dysfunction. Polyphenol-rich foods may improve these alterations. Agraz is a fruit rich in polyphenols (mainly anthocyanins); however, there is limited information about its effects on human health. We evaluated the effects of agraz consumption as compared to placebo on HDL function and inflammation in women with MetS. Forty volunteers (25–60 years) were included in this double-blind crossover study. Women consumed agraz or placebo over 4 weeks; separated by a 4-week washout period. HDL function (apoliprotein-A1; paraoxonase 1 (PON1) activity; cholesterol efflux capacity), oxidative stress (myeloperoxidase (MPO), advanced oxidation protein products) and inflammatory markers (serum cytokines/chemokines and peripheral blood mononuclear cell nuclear factor-kB) were measured after each period. Compared to placebo, agraz consumption did not significantly change any of the biomarkers measured. Interestingly, only after agraz period there were significant positive correlations between PON1 activities and cholesterol efflux. Additionally, there were significant inverse correlations between changes in inflammatory markers and HDL function markers and positive correlations with oxidative markers. Although polyphenol-rich foods have been shown to be beneficial for certain conditions; polyphenol-rich agraz fruit consumption did not impact inflammation and HDL function in the current study of women with MetS.

3.2 Keywords

Andean berry; cardiovascular risk factors; HDL dysfunction; inflammation; oxidative stress

3.3 Introduction

Metabolic syndrome (MetS) is a cluster of metabolic disorders shown to raise the risk of developing atherosclerotic cardiovascular diseases (CVDs) (1), which represent the leading cause of death worldwide (2). This syndrome is associated with low-grade chronic inflammation characterized by increased C-reactive protein (CRP) (3), cytokines such as interleukin (IL)-6, tumor necrosis factor-α (TNF-α) (4), monocyte chemoattractant protein-1 (MCP-1) (5)**,** and IL-8 (6), supporting the evidence that inflammation plays an important role in cardiovascular risk.

In addition, low high-density lipoprotein cholesterol (HDL-c) is another important component of MetS which is independently and inversely associated with cardiovascular risk (7). However, this association seems to be complex given that high/normal HDL-c levels have been observed in people with CVD (8,9). Disappointing results with drug therapies aiming to increase HDL-c have been reported, as some studies have not shown to prevent future cardiovascular events with increases in HDL-c (10,11). This information suggests that other aspects of HDL should be considered.

Independently of the HDL-c level, HDL particles have shown atheroprotective roles through different functions such as reverse cholesterol transport (12), as well as anti-inflammatory (13) and anti-oxidant (14) activities. In chronic inflammatory states like MetS, dysfunctional HDL has been observed (15), characterized by low cholesterol efflux (16) and depletion of HDL-associated atheroprotective proteins including apolipoprotein A-1 (ApoA-1) (17) and paraoxonase 1 (PON1), which are involved in cholesterol efflux (18,19), and antioxidant (20,21) and anti-inflammatory properties (22,23). Likewise, myeloperoxidase (MPO), a protein able to induce oxidative modification in HDL (24) and low-density lipoproteins (LDL) (25), has been found significantly elevated in people with MetS (26). Similarly, advanced oxidation protein products (AOPP), formed mainly from oxidation with hypochlorous acid (HOCl), generated by MPO (27), has been associated with MetS (28).

Improvements in HDL functionality are being considered and could be more effective approaches to ameliorate CVD risk. For example, several clinical studies have shown that consumption of flavonoids influence HDL functionality, through increases in HDL-c levels (29), PON1 activity (30,31), serum cholesterol efflux capacity (29,31), HDL antioxidant capacity, and reduction of HDL lipid hydroperoxides (31). These antioxidant compounds have also shown to inhibit the activation of nuclear factor kappa B (NF-κB)—an important transcription factor that regulates inflammatory responses (32,33).

Agraz *(Vaccinium meridionale* Swartz) is a fruit rich in flavonoids mainly anthocyanins which grows in Colombia as a wild berry. This fruit has shown high antioxidant activity (34) and ex vivo cardioprotective effect (35), which have aroused interest for its potential health benefits. However, currently there are limited published studies evaluating the effects of this berry in human health. We assessed the effects of agraz consumption, compared to placebo, on HDL function and inflammation markers in women with MetS. We hypothesized that agraz consumption, compared to placebo, would improve HDL function and decrease inflammation in this group of women.

3.4 Materials and Methods

3.4.1 Study Population

Forty women (*n* = 40; 25–60 years) with MetS, according to National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP-III) guidelines (36) were recruited from Medellin-Colombia. MetS was defined as the presence of three or more of the following risk factors: waist circumference ≥ 88 cm, triglycerides ≥ 150 mg/dL, HDL-c < 50 mg/dL, blood pressure ≥ 130/≥ 85 mmHg, and fasting plasma glucose ≥ 100 mg/dL (36). Those who had kidney disease, heart disease, diabetes, triglycerides ≥ 500 mg/dL, fasting plasma glucose ≥ 126 mg/dL, LDL cholesterol ≥ 190 mg/dL, blood pressure > 140/90 mm Hg, consumed anti-inflammatory, lipid-lowering, hypoglycemic, and/or anti-hypertensive medications, consumed more than 20 g alcohol per day, smoked, were pregnant or planning to become pregnant, were

high performance athletes, and/or consumed supplements or nutraceuticals, were excluded. This study was approved by the Human Bioethics Committee of the *Sede de Investigación Universitaria, Universidad de Antioquia* (Act No. 15-35-558-02). All participants signed the informed consent format.

3.4.2 Experimental Design

A double-blind study with a crossover design for 12 weeks was carried out. The volunteers were assigned to consume daily either agraz or placebo over 4 weeks, after which participants had a 4-week washout period, then they were allocated to the alternate treatment for additional 4 weeks (Figure 1). Freeze-dried agraz was reconstituted in 200 mL of water. The daily agraz dose was equivalent to the total phenols present in 200 g of fresh agraz (1027.97 ± 41.99 mg gallic acid equivalents (GAE)/L of agraz beverage). Placebo was designed to match the agraz beverage in terms of look, feel, taste, and macronutrients but without any polyphenols. The physico-chemical characterization, antioxidant capacity, total phenols and anthocyanin composition of agraz and placebo used in this study are presented in Table 1 and it has been previously described (37). During the whole study, including the washout period, volunteers were asked to abstain from consuming polyphenol-rich foods such as grapes, other berries, wine and tea or derived products. Participants registered daily consumption of the beverages, and a weekly questionnaire to assess adherence to the study to ensure they drank the beverages as indicated. When compliance was lower than 80%, participants were withdrawn from the study. Additionally, participants filled out a 7-day physical activity record and a food frequency questionnaire (38) at the beginning and end of each period to verify no changes in diet or exercise. Blood samples and data collection were obtained at the end of each consumption period.

3.4.3 Blood Collection and Peripheral Blood Mononuclear Cell (PBMC) Isolation

Blood samples were obtained after a 12-h overnight fast, from the antecubital vein using serum separator tube (yellow-topped tube) and tubes with EDTA (Vacutainer®, NJ, Franklin Lakes, USA). The blood collected with the yellow-topped tube was allowed to stand for 30 min, centrifuged at 2000 × g for 10 min, and the

serum frozen at −70 °C for further analysis. Whole blood collected with EDTA tubes was immediately used to isolate PBMC using Histopaque®-1077 (Sigma-Aldrich, St. Louis, MO, USA).

3.4.4 Anthropometric and Blood Pressure Measurements

Waist circumference was measured at the end of a normal expiration, at the superior border of the iliac crest using a nonflexible body tape (Lufkin W606PM, MD, Sparks, USA) with an accuracy of 0.1 cm. Systolic and diastolic blood pressure were measured with an automated monitor (Omron, Healthcare, Hoffman Estates, IL, USA) on the left arm, at the heart level after at least 5 min of resting in sitting position. Two measurements were made by at least 1 min of difference.

3.4.5 Biochemical Markers

Serum glucose and lipid profile concentrations were performed by colorimetric and enzymatic methods (Siemens®, Washington, DC, USA) using an automatic analyzer (Dimension RxL, Siemens, Washington, DC, USA). LDL cholesterol (LDL-c) concentration was calculated using the Friedewald formula (39).

3.4.6 PON1 Arylesterase Activity

This activity was measured in serum using phenyl acetate (SigmaAldrich, St. Louis, MO, USA) as a substrate, following the methodology described by Farrell et al. (40). Samples were diluted 400-fold in assay buffer (50 mM Tris, 1 mM $CaCl_2$, pH 8.0) and processed in duplicate in a UV-compatible half-area 96-well plate (Corning Inc., Corning, NY, USA). Then, substrate buffer (3 mM phenyl acetate, 50 mM Tris, 1 mM $CaCl_2$, pH 8.0) was added to each well and the reaction was finally measured at 270 nm (25 °C) every 20 seconds for 3 min using a microplate reader (Epoch Microplate UV/Vis Spec, VT, Winooski, USA). The results were expressed as kU/L using the molar extinction coefficient of phenol (0.00131 μM^{-1} cm^{-1}).

3.4.7 PON1 Lactonase Activity

PON1 lactonase activity was measured in serum diluted 200-fold in sample buffer (1 mM $CaCl_2$, 2.5 mM bicine, 200 mm NaCl, pH 8.3) using the method

described by Millar CL et al. (41). Each sample was added by duplicate in UV-compatible half-area 96-well plate (Corning Inc., Corning, NY, USA) and mixed with substrate buffer (0.2 mM m-cresol purple, 3 mM delta-valerolactone, pH 8.3). Finally, the reaction was measured at 577 nm (25 °C) every 20 seconds for 6 min using a microplate reader (Epoch Microplate UV/Vis Spec, VT, Winooski, USA). The results were obtained from a calibration curve with 0.1M HCl and substrate buffer, in a range of 0–115 µM.

3.4.8 Myeloperoxidase (MPO)

Serum MPO concentration was determined through an immunoenzymatic assay (MPO human ELISA (Enzyme-Linked ImmunoSorbent Assay) Kit, Cayman Chemical, MI, Ann Arbor, USA) following the manufacturer's instructions. Briefly, standard and samples were added to a coated plate with monoclonal antibodies specific for MPO and were incubated. Horseradish Peroxidase (HRP)-labeled MPO monoclonal antibody to detect the captured MPO was added. Finally, the chromogenic substrate TMB (3,3',5,5'-Tetramethylbenzidine) was added, incubated and the reaction was stopped with an acid solution. The yellow color formed was measured at 450 nm using a microplate reader (Multiskan Go, Thermo Scientific, MA, Waltham, USA). The intensity of the yellow color was directly proportional to the MPO concentration.

3.4.9 ApoB Precipitation

ApoB-depleted serum was obtained by precipitation of apoB-containing lipoproteins using polyethylene glycol (PEG, Pointe Scientific, INC. MI, Ann Arbor, USA), following the manufacturer's instructions. Briefly, serum was mixed with PEG in a 1:1 ratio. After mixing 10 times by inversion, samples were incubated on ice for 20 min and centrifuged at 10,000 × *g* for 10 min at 4 °C. Next, the supernatant (containing the HDL) was separated and used for the measurement of AOPP.

3.4.10 Advanced Oxidation Protein Products (AOPP)

ApoB-depleted serum was diluted in 1X Phosphate Buffered Saline-PBS in a 1:5 ratio. Then, 200 µL of each diluted sample were mixed in duplicate with 10 µL of

1.16 M potassium iodide and 20 µL of glacial acetic acid. The mixture was centrifuged at 4000 RPM for 10 min at 4 °C and the supernatant was read in duplicate at 340 nm in a UV-compatible half-area 96-well plate (Corning Inc., Corning, NY, USA). Finally, the results were compared with a calibration curve with chloramine T in a range of 0 to 100 µM. The results were expressed in units of chloramine T.

3.4.11 Cholesterol Efflux

J774 macrophages (ATCC, Manassas, VA, USA) were used to measure the cholesterol efflux capacity in apoB-depleted serum obtained after both intervention periods (agraz versus placebo). Cholesterol efflux was measured using the protocol described by Millar et al. (41) Cells were cultured on 24-well plates (0.7×10^5 cells/well), with RPMI (Roswell Park Memorial Institute) media (Sigma-Aldrich, MO, St. Louis, USA), 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and penicillin/streptomycin (ThermoFisher Scientific, Waltham, MA, USA) and incubated at 37 °C with 5% CO_2. After 24 h, cells were treated with RPMI media and 1% FBS, and loaded with [1,2-3H(N)]-cholesterol (Perkin Elmer, MA, Waltham, USA) and CI 976 4 mg/mL (2,2-dimethyl-N-(2,4,6-trimethoxyphenyl) dodecanamide) (Sigma-Aldrich, MO, St. Louis, USA) for 24 h in the same incubation conditions. Then, cells were treated with Cpt-cAMP 25 mg/mL (8-(4-Chlorophenylthio) adenosine 3′,5′-cyclic monophosphate) (Sigma-Aldrich, MO, St. Louis, USA) in RPMI media and 0.2% bovine serum albumin (BSA) and incubated for 16 h at 37 °C with 5% CO_2, to promote the activation of ATP-binding cassette transporter A1 (ABCA1). After washing the cells, 2.8% of apoB-depleted serum in RPMI media with 0.2% BSA was added to the cells (in triplicate). Efflux was performed for 4 h at 37 °C with 5% CO_2 followed by collection of cell media and cell lysates. Cell lysates were obtained by washing cells with 0.1 N NaOH and collection of the supernatant. Then, cell media and cell lysates were diluted into liquid scintillation cocktail and counted on the Beckman LS 6500 Scintillation Counter. Percent of cholesterol efflux was calculated as follows:

$$\left[\frac{3H - cholesterol\ radioactivity\ in\ media}{(3H - cholesterol\ radioactivity\ in\ media + 3H - cholesterol\ radioactivity\ in\ cell\ lysate)}\right] * 100$$

3.4.12 Inflammatory Markers

Serum concentration of TNF-α, MCP-1, IL-6, IL-8, and IL-1β were measured using the Human cytokine magnetic panel kit (catalog number HCYTOMAG-60K-05, Millipore Corporation, MA, Burlington, USA), using Luminex xMAP® technology (Millipore Corporation, MA, Burlington, USA) and following the manufacturer's instructions. For the NF-κB measurement, the nuclear component from the PBMC was extracted first, through a nuclear extraction kit (Abcam ab113474) according to the manufacturer´s protocol. Then, the NF-κB transcription factor was measured using the ELISA NFkB p65 transcription factor assay kit (Abcam ab133112) according to manufacturer´s instructions. Absorbances were obtained at 450 nm.

3.4.13 Statistical Analysis

Sample size was estimated using Epidat (version 3), based on data of a previous study following a similar protocol as this study, in which blood pressure was reduced significantly after grape supplementation (a fruit rich in polyphenols), compared to placebo (42). After using Grubbs' test, outlier data for some variables were eliminated for the analysis.

Results are described based on summary measures such as mean and standard deviation (SD). The data distribution was analyze using the normality test of Shapiro Wilk. Data without normal distribution were log-transformed. Paired samples -tests were conducted to analyze differences between the agraz and placebo periods. Pearson and Spearman correlations coefficient were used. All analyses were done using SPSS version 21 for Windows (SPSS, IBM Corporation, IL, Chicago, USA). Differences were considered significant at the levels *p < 0.05, **p < 0.01, ***p < 0.001.

3.5 Results

3.5.1 Participant Characteristics and MetS Criteria

Forty women (47.2 ± 9.4 years old) with MetS finished the study with an adherence of 95%. In addition, there were no differences in macronutrient intake and physical activity during the whole study.

Baseline MetS characteristics are shown in Table 1. The HDL-c mean for these women was 42.2 ± 6.4 mg/dL at the beginning of the study (Table 2).

3.5.2 HDL Function and Related Oxidative Markers

There were no differences in apoA-1 concentrations, PON1 activities, cholesterol efflux capacity, MPO concentration, MPO/PON1 ratio and AOPP, after comparing the end of both intervention periods (placebo versus agraz) ($p > 0.05$, Table 3).

After agraz consumption, but not after placebo consumption, there were moderate positives correlations between cholesterol efflux capacity with PON1 arylesterase activity ($r = 0.516$, $p = 0.006$) and lactonase activity ($r = 0.597$, $p = 0.001$) (Figure 2). Likewise, there was a negative correlation between the changes in HDL-c and changes in AOPP ($r = –0.400$; $p = 0.031$) (Figure 3), a marker of oxidative stress associated with HDL.

3.5.3 Inflammatory Markers

There were no significant differences in inflammatory markers in this group of women after agraz consumption compared placebo (Table 4).

However, changes in inflammatory markers were inversely correlated with changes in PON1 activity and cholesterol efflux capacity. Moreover, changes in MPO and MPO/PON1 ratio were positively correlated with inflammatory markers (Table 5). Likewise, NF-κB levels were significantly and negatively correlated with PON1 arylesterase activity ($r = −0.431$, $p = 0.009$), only after placebo consumption. This correlation was not observed after agraz period (Figure 4).

3.6 Discussion

The MetS is strongly linked to low-grade chronic inflammation, oxidative stress and HDL dysfunction. In this study, we evaluated the effects of consuming a fruit (agraz) rich in polyphenols (mainly anthocyanins) on these factors, which increase cardiovascular risk.

PON1 activity was measured as a HDL function marker for its atheroprotective role. Improvements in PON1 activity have been reported following the consumption of polyphenol-rich beverages like pomegranate. An increase in PON1 activity was observed following the daily consumption of a pomegranate beverage (total phenol content of 2600 mg GAE/L of juice) for 4 weeks in 30 patients with type 2 diabetes (30). In our study, we did not detect any significant effect on PON1 activity measures. This could be related to the lower dose provided (total phenols: 1027.97 ± 41.99 mg GAE/L of agraz beverage) compared to the above studies.

Cholesterol efflux capacity is another important marker that reflects the role of HDL in atheroprotection. Several studies have evaluated the effects of dietary interventions on this HDL-dependent process. For example, anthocyanin supplementation (80 mg of anthocyanins twice per day, Medox®, Sandnes, Norway) over 24 weeks showed an increase in cholesterol efflux in macrophages cultured with the serum of hypercholesterolemic subjects (55.3 ± 5.0 years) (31). In our study, after agraz consumption, cholesterol efflux did not change compared to placebo. Similarly, one-week intervention with 250 mL of polyphenol-rich beverages (6800 ± 100 mg GAE/L of juice) in 6 healthy male subjects (25–30 years), did not significantly improve cholesterol efflux rate from cells to serum, compared to serum baseline. However, cholesterol accumulation decreased significantly in macrophages cultured in the post-treatment serum. The authors of this last study concluded these results are probably due to inhibition of cholesterol-rich lipoprotein uptake by the cells, mediated by serum-associated polyphenols (43).

PON1 has been shown to stimulate HDL binding and HDL-mediated macrophage cholesterol efflux via the ABCA1 transporter (18). We found a positive correlation

between PON1 activity and cholesterol efflux after agraz consumption, but not after placebo consumption, suggesting a stronger link between these HDL markers with agraz consumption. A study with anthocyanin supplementation in subjects with hypercholesterolemia demonstrated an increased in HDL-associated PON1 activity and an improvement in cholesterol efflux capacity. This suggests that modulation of PON1 activity is a potential mechanism by which anthocyanins regulate cholesterol efflux capacity (31). This increase in PON1 activity by polyphenols could be also associated with their capacity to modulate the expression level of the PON1 gene, acting as ligands for the aryl hydrocarbon receptor -AhR (44); or for the peroxisome proliferator-activated receptor gamma (PPAR-γ) pathway (45).

MPO is able to induce HDL dysfunction through oxidative modifications, while PON1 exerts antioxidant activities on HDL (20,21,24). Therefore, an increase in the MPO/PON1 ratio is considered as a potential marker of HDL dysfunction (46). We did not observe significant changes in these parameters. However, a study in 55 healthy males evaluated the consumption of a polyphenol rich-food (cocoa extract) during 4 weeks observed that MPO was significantly reduced compared to baseline; however, no changes were found compared with the placebo group (47). In contrast to this healthy population, our volunteers had several cardiovascular risk factors, which mediate a higher state of HDL dysfunction and oxidative stress.

Additionally, MPO is the main generator of AOPP (27)–a marker of protein oxidation which contributes to atherosclerotic plaque development- (48). AOPP was not statistically different between treatment periods. A study with 31 healthy subjects consuming a single dose of polyphenol-rich beverage with higher total phenol content (total polyphenols 4000 mg GAE/L of beverage), reported that AOPP was reduced by 39% during the first 60 min and tended to return to baseline within 1 and 4 hours after consumption (49). From this acute study, it is evident the reduction in this marker is of short duration. In our study, blood samples were taken after 12 hours of overnight fasting. Therefore, at this time of blood sampling, any acute effect on AOPP may not have been detected. However, chronic interventions have shown positive effects in this variable. For example, after 60 days of intervention with 200

mL of cranberry juice in 56 people with MetS, AOPP decreased in relationship to the baseline values (50). Our intervention lasted 30 days, thus, longer intervention may be necessary to obtain significant changes in this variable.

AOPP is mainly generated by MPO through HOCl (27). MPO oxidizes important proteins transported by HDL, like apoA-1, producing an oxidized apoA-1 (a type of AOPP) and affecting its capacity to remove cholesterol from cells (reverse cholesterol transport) (51). Thus, the cholesterol-enrichment of HDL is impaired and consequently the HDL-c levels are decreased. We observed a significant negative correlation between changes in AOPP and HDL-c after agraz consumption, compared to placebo. This could be associated with the inhibitory effect of some polyphenols on MPO, through direct binding of the polyphenol molecule with the active site of this enzyme (52,53), with the corresponding decrease in AOPP formation.

Inflammation is another important component of MetS (3–6). Various studies in both healthy populations and individuals with elevated CVD risk have found anti-inflammatory effects after chronic consumption of polyphenol-rich bilberries (54,55). In our study, there was no differences between agraz and placebo period. Interestingly, after agraz consumption, PON1 activity (HDL function marker) had a negative correlation with inflammatory markers as compared to placebo. PON1 activity is significantly reduced in pro-inflammatory conditions such as MetS (56), possibly due to the capacity of pro-inflammatory cytokines to inhibit PON1 expression (57). A study with polyphenols have demonstrated anti-inflammatory effects through inhibition of NF-κB activation and consequent inflammatory cascade (33). Another study showed the capacity of polyphenols to increase PON1 expression (44), as a mechanism to improve HDL function.

Similarly, we observed significant negative correlations between cholesterol efflux capacity and pro-inflammatory markers. Although this was not a study to explore mechanisms, another (in vitro) study demonstrated that anthocyanins increased ABCA1 mRNA—a cholesterol efflux regulatory protein- and consequently

increased cholesterol efflux via PPARα and LXRα, as well as inhibited the nuclear translocation of NF-κB with a reduction in pro-inflammatory protein expression (58).

Given that there are no previously published studies evaluating agraz consumption in people with MetS, there were no antecedents about the dose or time of intervention to be assessed with this fruit. Therefore, the daily dose used in this study was aimed to be comparable to a habitual juice consumption. The experimental design followed in this study has been employed by others evaluating polyphenol-rich fruits in people with MetS (41,42). In conclusion, the dose of agraz used in this study during 4 weeks did not impact the HDL function, inflammatory and oxidative stress markers measured in this study, compared to placebo, in this group of women with MetS.

3.7 References

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3.8 Tables and figures

Table 1. Physicochemical and nutritious properties of the nectar of *Vaccinium meridionale* and placebo

Physicochemical analysis	Freeze-dried *V. meridionale* reconstituted in 200 mL (nectar)	Placebo (200 mL)
Calories per dose (Kcal)	26.00 ± 0.18 a	21.86 ± 0.21 a
Carbohydrates (glucides) (%)	2.08 ±0.19 a	2.50±0.19 a
Protein (%)	0.05±0.01 a	0.21 ±0.05 b
Ashes (%)	0.06±0.01 a	0.18 ±0.03 b
pH	2.56±0.23a	2.42±0.29a
Humidity (%)	97.00 ±0.23 a	97.47 ±0.21a
Density (g/mL)	0.999 ± 0.010a	0.998 ± 0.01a
Total phenols (mg GAE/g or L)	1027.97 ± 41.99a	31.91 ± 3.15b
DPPH (µM TE/L)	104.25 ± 15.14a	0.43 ± 0.03b
FRAP (µM TE/L)	664.87 ± 73.47a	58.37 ± 3.78b
ABTS (µM TE/L)	205.26 ± 9.99a	83.39 ± 2.34b
ORAC (µM TE/g)	4,702.31 ± 22.36 a	0.44 ± 0.024b

Different superscript letters for nectar and placebo indicate significant differences ($p<0.05$) according to Tukey's test. GAE, Gallic Acid Equivalents; TE, Trolox Equivalents.

Table 2. Baseline metabolic syndrome characteristics of the studied women (*n* = 40).

Variables	Mean ± SD
Age (years)	47.2 ± 9.4
Waist circumference (cm)	102 ± 9.2
Systolic blood pressure (mm Hg)	118.1 ± 12.5
Diastolic blood pressure (mm Hg)	76.1 ± 9.3
Fasting glucose (mg/dL)	94.2 ± 7.3
HDL-c (mg/dL)	42.2 ± 6.4
Triglycerides (mg/dL)	220.6 ± 88.9

SD, standard deviation; HDL-c, high-density lipoprotein cholesterol.

Table 3. High-density lipoprotein (HDL) function markers after 4 weeks of agraz consumption, compared to placebo, in women with metabolic syndrome.

Variables	Placebo		Agraz		Δ Change (agraz-placebo)	*p*
	n	Mean ± SD	*n*	Mean ± SD	Mean ± SD	
HDL function markers						
Apo A1 (mg/dL)	34	127.6 ± 43.1	29	132 ± 49	3.1 ± 40.8	0.597
PON1 Arylesterase Activity (kU/L)	38	77.1 ± 17.5	38	76.5 ± 17.5	−0.7 ± 8.8	0.643
PON1 Lactonase Activity (kU/L)	38	12.6 ± 2.7	38	12.6 ± 2.8	0.2 ± 1.6	0.862
Cholesterol efflux (%)	27	8.2 ± 3.6	27	8.7 ± 3.8	0.5 ± 2.9	0.324
HDL-related oxidative markers						
MPO (ng/mL)	34	177.8 ± 74.6	34	175 ± 72.7	−11.1 ± 72	0.795
MPO/PON1 arylesterase ratio	34	2.7 ± 1.6	34	2.6 ± 1.3	−0.1 ± 1.2	0.770
MPO/PON1 lactonase ratio	34	15.5 ± 7.4	34	14.9 ± 6.9	−0.7 ± 6.7	0.515
AOPP (μM)	29	99.5 ± 20.9	29	97.5 ± 17	−2.0 ± 19.8	0.703

SD, standard deviation; Apo, apolipoprotein; PON1, paraoxonase 1; MPO, myeloperoxidase; AOPP, advanced oxidation protein products. Paired *t*-test was used for the analysis. Significance $p < 0.05$.

Table 4. Inflammation markers after 4 weeks' placebo and agraz consumption.

Variables	Placebo		Agraz		Δ Change (agraz-placebo)	*p*
	n	Mean ± SD	*n*	Mean ± SD	Mean ± SD	
IL-1β (pg/mL)	37	0.8 ± 0.4	37	0.8 ± 0.4	0.0 ± 0.2	0.748
IL-6 (pg/mL)	37	2.6 ± 2.1	37	2.1 ± 1.2	−0.5 ± 1.5	0.271
IL-8 (pg/mL)	37	12.6 ± 5.6	37	12.1 ± 5.5	−0.3 ± 2.6	0.322
MCP-1 (pg/mL)	38	251 ± 103	38	248.3 ± 106.6	−2.6 ± 47.3	0.479
TNF-α (pg/mL)	38	4.7 ± 1.8	38	4.6 ± 1.5	−0.1 ± 0.8	0.257
NF-κB (abs)	38	0.1 ± 0.02	38	0.1 ± 0.02	0.0 ± 0.02	0.290

SD, standard deviation; TNF-α, tumor necrosis factor-alpha; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; NF-κB, nuclear factor kappa B. Paired *t*-test was used for the analysis. Significance $p < 0.05$.

Table 5. Correlations[1] between changes in high-density lipoprotein (HDL) function and inflammation markers after agraz consumption, compared to placebo, in women with metabolic syndrome.

Changes in variables	IL-1β (pg/mL)	IL-6 (pg/mL)	IL-8 (pg/mL)	MCP-1 (pg/mL)	TNF-α (pg/mL)
Apo A1 (mg/dL)	0.151	0,022	0.056	−0.087	0.030
PON1 Arylesterase Activity (kU/L)	0.215	−0.273	−0.106	−0.060	−0.012
PON1 Lactonase Activity (kU/L)	0.060	−0.390*	−0.169	0.145	−0.213
MPO (ng/mL)	0.102	0.707***	0.338	0.413*	0.196
MPO/PON1 arylesterase ratio	0.097	0.682***	0.349	0.393*	0.229
MPO/PON1 lactonase ratio	0.099	0.701***	0.323	0.295	0.202
AOPP (μM)	0.098	0.080	−0.228	0.170	−0.087
Cholesterol efflux (%)	−0.594***	−0.283	−0.128	−0.148	−0.496**

[1] Pearson and Spearman correlation coefficients. Significance at *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Apo, apolipoprotein; PON1, paraoxonase 1; MPO, myeloperoxidase; AOPP, advanced oxidation protein products; TNF-α, tumor necrosis factor-alpha; IL, interleukin; MCP-1, monocyte chemoattractant protein-1.

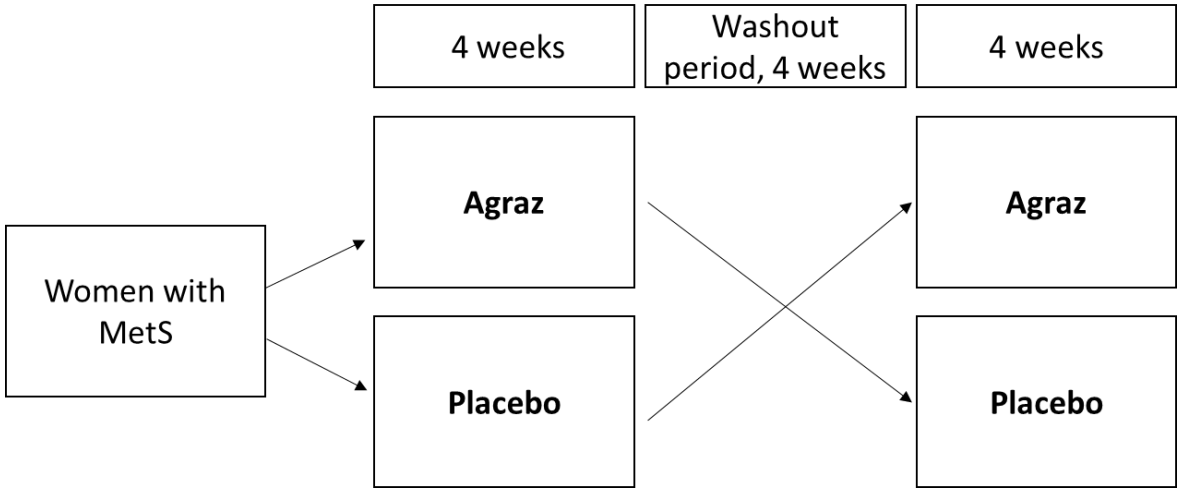

Figure 1. Study protocol. Women consumed agraz and placebo in a crossover design during 12 weeks (including a washout period). MetS: metabolic syndrome

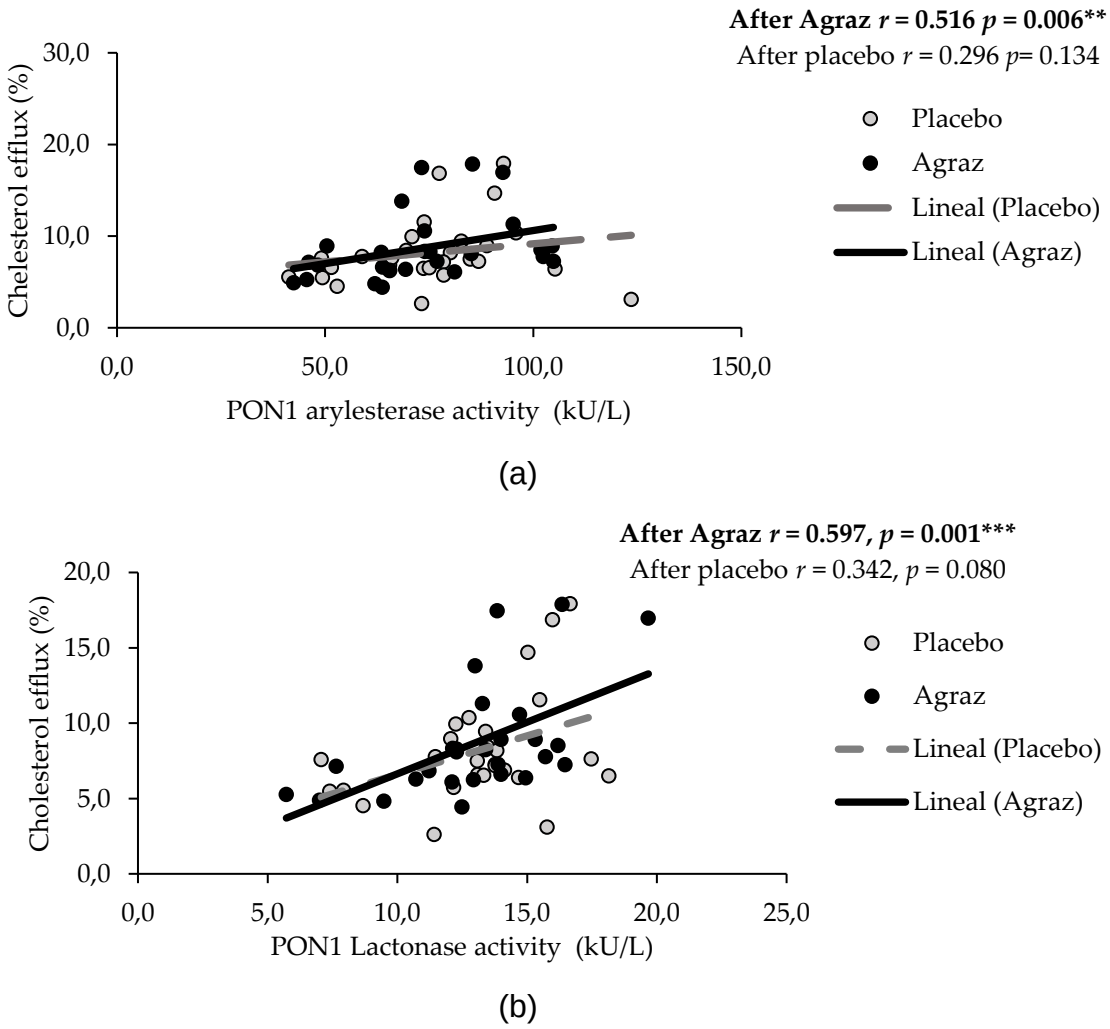

Figure 2. Spearman correlations between cholesterol efflux with (**a**) PON1 (paraoxonase 1) arylesterase and (**b**) lactonase activity after agraz consumption, compared to placebo. Significance *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

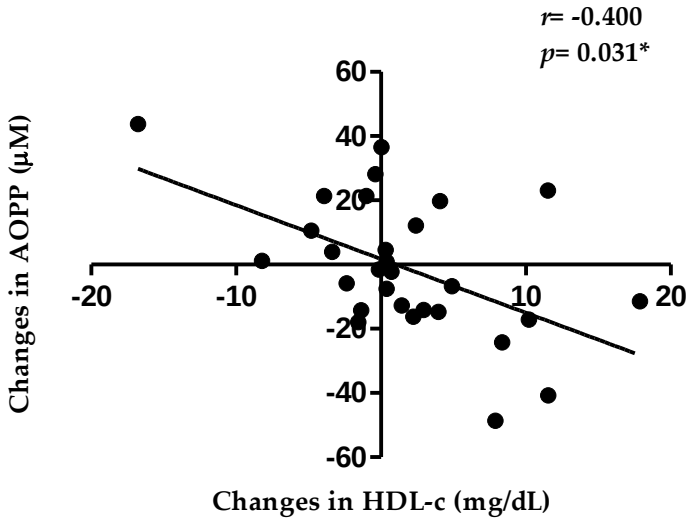

Figure 3. Pearson correlation between changes in high-density lipoprotein cholesterol (HDL-c) and advanced oxidation protein products (AOPP) after agraz consumption, compared to placebo. Significance *$p < 0.05$.

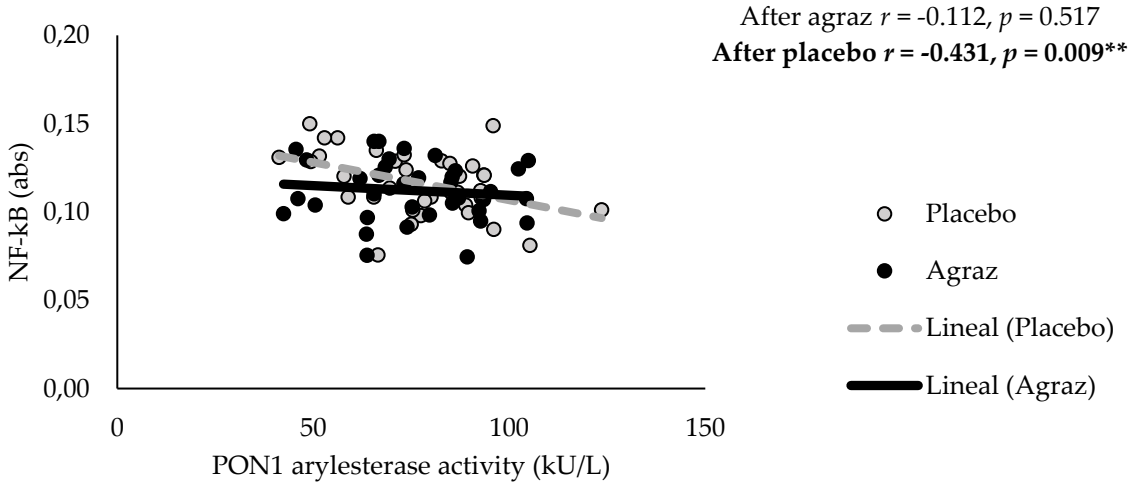

Figure 4. Pearson correlation between nuclear factor kappa B (NF-κB) and paraoxonase 1 (PON1) after agraz consumption, compared to placebo. Significance **$p < 0.01$.

CHAPTER 4

Improvements in antioxidant status after agraz consumption was associated to reductions in cardiovascular risk factors in women with metabolic syndrome

4.1 Abstract

Scope. In this study, we aimed to evaluate the effects of the chronic consumption of agraz (*Vaccinium meridionale*) on antioxidant status and oxidative stress markers in women with metabolic syndrome (MetS).

Methods: 40 women with MetS (47.2±9.4 years) participated in a double blind, crossover design study, in which participants consumed daily agraz or placebo during 4 weeks, separated by a 4-wk washout period. At the end of each intervention period, endogenous antioxidant enzymes activity (superoxide dismutase, catalase and glutathione peroxidase), serum total antioxidant capacity (TAC) (ferric reducing ability of plasma [FRAP]; Oxygen Radical Absorbance Capacity [ORAC] and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) [ABTS]), and oxidative stress markers (Oxo-2'-deoxyguanosine (8-OHdG) and F2-isoprostane) were determined.

Results: Women who increased endogenous antioxidant enzymes activity and serum TAC after agraz consumption, compared to placebo, significantly reduced oxidative stress markers, total cholesterol, LDL-cholesterol (LDL-c) levels, and waist circumference. However, when analyzing the group as a whole, there were no significant differences in endogenous antioxidants enzymes and serum TAC after 4 weeks consuming agraz, compared to placebo.

Conclusion: the chronic consumption of agraz demonstrated beneficial effects in the group of women in whom antioxidant parameters increased after agraz consumption, evidencing an individual variability in response to the beverage evaluated.

4.2 Keywords

Andean berry, antioxidant enzymes, oxidative stress, total antioxidant capacity,

Vaccinium meridionale

4.3 Introduction

People with metabolic syndrome (MetS) have an increased risk to develop cardiovascular diseases (CVD), which represent the leading cause of mortality in the world (1). This syndrome is defined by the National Cholesterol Education Program, Adult Treatment Panel III (NCEP-ATPIII) as the presence of three or more cardiovascular risk factors (CVRF) including high waist circumference, high blood pressure, elevated levels of fasting blood triglycerides (TG) and glucose levels, and low high density lipoprotein cholesterol (HDL-c) levels (2).

In addition to these CVRF, people with MetS also presents oxidative stress (3), which has been defined as an unbalance between the generation of oxidative species and the antioxidant status, in favor of the oxidative species (4). In this sense, compared with people without MetS, people with MetS have higher lipoperoxidation and oxidative DNA damage (3), and a reduced endogenous antioxidant enzymes activity such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) (5). In addition, total antioxidant activity (TAC) -defined as the “global antioxidant efficiency mirroring the multiple aspects of redox interactions” (6) -, is significantly reduced as the components of the syndrome increase (7). All these alterations on the antioxidant status are significantly related to CVRF (8).

Contrarily, it has been demonstrated that an antioxidant-rich diet like fruit and vegetables consumption has beneficial effects on the reduction of CVD risk (9), associated with increases of TAC in blood (10), endogenous antioxidant enzyme activity (11) and reduction of lipids, insulin resistance, inflammation (9), hypertension (12), among other CVRF. These beneficial effects have been related to bioactive compounds such as polyphenols, a group of secondary metabolites of plants, and common antioxidants in a great variety of foods (13). In this sense, dietary polyphenol intake has shown to reduce the prevalence of MetS components (14).

Vaccinium meridionale also called agraz, is an important source of polyphenols with a total phenol content between 609±31mg to 758.6±62.3 Gallic Acid equivalents/100 g fresh fruit, specially anthocyanins, with a high TAC (15,16). In 2018, a study

conducted with rats evaluating effects of this fruit in ischemia-reperfusion, demonstrated that agraz significantly improved systolic and diastolic function and antioxidant capacity, increased SOD and catalase activity and reduced lipid peroxidation (17). Given these characteristics, agraz has generated great interest for its potential effects on human health. Currently, there is very limited information in humans about the effects of agraz on endogenous antioxidant enzymes activity, serum TAC and its relationship with CVRF. Therefore, we aimed to evaluate the effects of the chronic consumption of agraz on antioxidant and oxidative stress markers in women with CVRF, i.e. with MetS.

4.4 Methods

4.4.1 Study population

In this study, 40 women from Medellín- Colombia, between 25 to 60 years old and with MetS, according to the NCEP ATP-III, were included (2). MetS was defined as having three or more of the following risk factors: waist circumference ≥ 88 cm; triglycerides ≥ 150 mg/dL; HDL-c < 50 mg/dL; blood pressure ≥ 130/≥85 mmHg, and fasting plasma glucose ≥ 100 mg/dL. Exclusion criteria included the presence of kidney disease, heart disease, diabetes; having TG ≥ 500 mg/dL, fasting plasma glucose ≥ 126 mg/dL, low density lipoprotein cholesterol (LDL-c) ≥ 190 mg/dL, blood pressure ≥140/90 mm Hg; smoking; taking anti-inflammatory, lipid-lowering, hypoglycemic, and anti-hypertensive medications; being pregnant or planning to become pregnant; consuming supplements or nutraceuticals and the intake of more than 20 g alcohol per day. This study was conducted according to the Declaration of Helsinki and it was approved by the Human Bioethics Committee of the Sede de Investigación Universitaria, University of Antioquia (Act No. 15-35-558-02). Written informed consent was obtained from all subjects.

4.4.2 Dosage information

Agraz fruits were bought in the east of Antioquia (Colombia), and processed in a food laboratory from the University of Antioquia to produce a lyophilized product to preserve its bioactive compounds. The daily dose of the agraz beverage was prepared with 7.38g of lyophilized product dissolved in 200 mL of pure water, which was equivalent to the total phenols present in 200 g of fresh agraz fruit (1,027.9 mg Gallic Acid equivalents in 200mL). The placebo was designed by food engineers from the University of Antioquia using food-grade ingredients to simulate organoleptic and physicochemical characteristics of the agraz beverage, but lacking polyphenols. The daily dose during placebo period was also 200mL. Microbiologic and physicochemical characterization of the agraz and placebo beverages have been previously described (18).

4.4.3 Study design

This was a double blind, placebo-controlled, crossover design study. The method used for the beverage assignation was the alternating quasi-randomization allocation method, in which, one subject received the placebo, and the next received the agraz beverage, and so on. The total duration of the study was 12 weeks: four weeks of agraz or placebo consumption, 4 weeks of washout, and finally 4 weeks of the alternate treatment (Figure 1). During the study, women were asked to avoid consumption of polyphenol-rich foods, such as grapes, wine, green tea, other berries, among others. To verify the daily consumption of the beverage in each intervention period and the abstinence of polyphenol-rich foods, a questionnaire was used (19). Adherence less than 70% was considered a criterium to withdraw a volunteer from the study. Likewise, participants were asked to keep their usual physical activity and diet throughout the study. To verify compliance with this criteria, physical activity was monitored thought a seven-day physical activity record at the baseline and end of each intervention period. In addition, diet was also evaluated using a modified food frequency questionnaire (FFQ) designed and validated previously in the University of Antioquia (20). This FFQ included 9 different food groups and 143 foods, allowing to determine the kilocalories and macronutrients

consumed in the last month; it was completed at baseline and end of each intervention period.

4.4.4 Antropometrics, Blood Pressure and Blood Collection

Waist circumference and blood pressure were determined as previously reported (21). Venous blood was obtained after 12h overnight fasting using serum separator (yellow-topped) tubes. Then, samples were centrifuged at 2000× g for 10 min, the serum was separated and stored at -70°C.

4.4.5 Endogenous antioxidant enzymes activity

The endogenous antioxidant enzymes activity was determined at the end of each intervention period. The activity of SOD was determined with the commercial OxiSelect™ kit Superoxide Dismutase Activity Assay (Cell Biolabs, Inc, CA, San Diego, USA), following manufacturer's instructions. Results were expressed in U/mL. Likewise, CAT activity was measured using the OxiSelect™ Catalase Activity Assay Kit (Cell Biolabs, Inc, CA, San Diego, USA) following manufacturer's instructions. Results were also expressed in U/mL. Finally, GPx activity was determined with the Glutathione Peroxidase Assay Kit (Cayman Chemical, MI, USA), following manufacturer's instructions. Results were expressed in nmol/min/mL.

4.4.6 Serum total antioxidant capacity (TAC)

Serum TAC was determined at the end of each intervention periods, using different methods:

ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] was determined using a modified method described by Re, *et al* (1999) (22). Briefly, ABTS cation radical (ABTS•+) was generated with ABTS 7mM and potassium persulfate 2,45mM, incubating by 12-16 hours at room temperature. Then, ABTS was dissolved in PBS 5mM pH 7.4 (to obtain an absorbance of 0.70 (±0.02) at 734nm at room temperature). This method was processed in 96-well microplate, in which 250µL of

ABTS solution was mixed with 2.5 µL of serum or Trolox standards (concentration 0–2000 µM) by triplicate. After 5 minutes´ incubation at room temperature, the absorbance was measured at 734nm using a microplate reader (Multiskan Go, Thermo Scientific, MA, Walthamcity, USA). Concentrations were determined using the standard curve and results were expressed in terms of µmol Trolox equivalents (TE)/mL.

The ferric reducing ability of plasma (FRAP) was measured using a modified method of Benzie and Strain (1996) (23), in which FRAP reagent was prepared with 25mL of 0.3M acetate buffer-pH 3.6, 2.5mL of TPTZ (2,4,6-tri-pyridyl-s-triazine) 10mM and 2.5mL of ferric chloride hexahydrate ($FeCl_3.6H_2O$) 20mM. In a 96-well microplate 10µl of serum diluted 1:3 with H_2O, Trolox standards (concentration 0–400 µM) or reagent blank was mixed with 300µL of freshly prepared FRAP warmed at 37°C. The plate was incubated at 37°C by 8 minutes and absorbances were measured at 593nm using a microplate reader (Multiskan Go, Thermo Scientific, MA, Walthamcity, USA). All reactions were processed in triplicate. Results were expressed in terms of µmol TE/mL.

ORAC (Oxygen Radical Absorbance Capacity) was determined using a modified method of Du *et al*. (2001) (24), in which radical AAPH (2,2'-Azobis(2-amidinopropane) dihydrochloride) generates a peroxyl radical, which oxidizes fluorescein, losing its fluorescence. In presence of serum antioxidants, the loss of fluorescence decreases. Serum samples were diluted 1:400 using phosphate buffer (pH 7.3) and Trolox was used as a standard (concentration 0-100 µM). The kinetics of oxidative degradation of fluorescein was determined using the differences in areas under the curves (AUC) at the excitation and emission wavelength of 485 and 520 nm, respectively. Results were expressed in µmol TE/mL., and were obtained using the following formula

$$\frac{\mu\text{mol TE}}{\text{mL sample}} = \frac{\mu\text{mol}}{L}(of\ the\ curve) * \text{DF} * \frac{1L}{1000mL}$$

DF: Dilution factor

4.4.7 Oxidative stress markers

Oxidative stress markers were determined at the end of each intervention period. 8-Oxo-2'-deoxyguanosine (8-OHdG) and F2-isoprostane levels were determined in 24-hour urine, using the commercial kit 8-hydroxy 2 deoxyguanosine ELISA Kit (Abcam, Cambridge, MA, USA) and OxiSelect™ 8-iso-prostaglandin F2 ELISA kit (Cell Biolabs, Inc., San Diego, CA, USA), respectively, following manufacturer's instructions. Results were expressed in terms of ng/mg creatinine.

4.4.8 Statistical analysis

To evaluate data distribution, Shapiro Wilk test was used. Variables with non-normal distribution were log-transformed and verified again with Shapiro Wilk test. Data are presented as mean and standard deviation (SD). To determine the differences between the results obtained at the end of each intervention period, a paired T-test was implemented. In addition, changes after agraz consumption compared to placebo were calculated subtracting the results obtained after agraz consumption minus those obtained after placebo consumption. Thus, it was possible to determine increases or reduction in each variable, and to determine the association of the changes among variables through a one-way ANCOVA, with adherence as a covariate. All analyses were done using SPSS version 21 for Windows (SPSS, IBM Corporation, 2012). Differences with a value of $p<0.05$ were considered significant.

4.5 Results

Forty women with MetS (47.2 ± 9.4 years old) were included in this study. Adherence during the study was around 95%, indicating an adequate intake of each beverage (placebo and agraz) and abstinence from polyphenol-rich foods. In addition, no statistical differences were found in diet (kilocalories and macronutrients intake) and physical activity during the whole study ($p\geq0.05$).

There were no statistical differences in endogenous antioxidants enzymes and serum TAC measured by ABTS, FRAP and ORAC, after 4 weeks consuming agraz compared to placebo ($p< 0.05$) (Table 1).

Regarding oxidative stress markers, we previously had reported a significant reduction in 8-OHdG of −0.27±0.72 ng/mg creatinine after agraz consumption compared to placebo ($p= 0.041$). However, no changes were found in F2-isoprostanes in this group of women (25).

Interestingly, women who increased SOD activity after agraz consumption (n=21), compared to placebo, significantly reduced oxidative stress markers like 8-OHdG ($p=0.022$) and F2-isoprostane ($p=0.034$) (Figure 2). Likewise, in those women in whom GPx activity increased after agraz consumption (n=18), compared to placebo, significant reductions in total cholesterol ($p=0.023$) and LDL-c ($p= 0.022$) levels were found (Figure 3). Finally, the increase in serum TAC (determined by ABTS) after 4 weeks of agraz consumption, was significantly associated with waist circumference reduction ($p= 0.017$) (Figure 4).

4.6 Discussion

To the best of our knowledge, this study is the first in evaluating the effect of *V. meridionale* consumption on endogenous antioxidant enzymes activity and to determine TAC by the methods described, in people at high risk of CVD. We demonstrated that chronic consumption (4 weeks) of agraz, with the daily dose provided in this study, did not significantly modify antioxidant enzymes activity when compared to placebo, in this group of women. Similarly, Kardum *et al.*, did not observe significant changes in endogenous antioxidant enzyme activity in people with CVRF, in a crossover design study, evaluating the effects after 6 weeks of consuming a blueberry drink (26). Contrarily, studies evaluating the acute and chronic effects of polyphenols rich berries in healthy people, demonstrated beneficial effects with significant increases in the activity of enzymes such as SOD, CAT and GPx, influenced by polyphenols (27–29). These beneficial effects could be explained

by the lower levels of oxidative stress (OxS) observed in healthy people versus the higher levels in people with metabolic syndrome (3,5); given that inactivation of enzymes activity mediated by products generated during oxidation has been reported (30.) In fact, we reported a better anti-inflammatory effect after 4 weeks of agraz consumption in women having 3 MetS parameters in comparison with those with 4 MetS components (25). Therefore, people included in this study might need strong lifestyle changes for achieving better results.

Interestingly, there was a group of women with increases in the activity of endogenous antioxidant enzymes after agraz consumption, in whom a protective effect was evidenced. Studies with anthocyanins, the main polyphenol present in berries, have shown reductions in OxS levels through significant increases in endogenous antioxidant enzymes activity of CAT, SOD and GPx, and reducing reactive oxygen species (31,32). Although a clear mechanism has not been established, some authors have indicated that polyphenols and its metabolites, via phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) induce the activation of nuclear factor erythroid 2-related factor 2 (Nrf2), an important transcription factor in the antioxidant response (33,34).

In this study, women who increased SOD activity after agraz consumption, compared to placebo, significantly reduced markers of oxidative damage of lipids (F2-isoprostane) and DNA (8-OHdG), indicating a protective effect against oxidative damage of superoxide free radical in this group of people. Likewise, Lee *et al*, have demonstrated a negative correlation between SOD and F2-isoprostanes (35) and 8-OHdG (36). The protective effect of SOD could be explained by its biological role catalyzing the dismutation of superoxide radicals to O_2 and H_2O,(37) given that superoxide radical (O_2^-) participates in the hydroxyl radical production, a powerful oxidant involved in the oxidative damage of macromolecules such as lipids (38) and DNA (39).

In addition, it is well known that GPx is an enzyme responsible for catalyzing hydrogen peroxide (H_2O_2) reduction to $2H_2O$ mediated by the conversion of reduced glutathione (GSH) to the glutathione oxidized form or glutathione disulfide (GSSG), thus, removing H_2O_2 from the tissues (40). Due to its important role in OxS, the

reduced activity of this enzyme has been associated with a higher risk of CVD (41). Interestingly, we also found that women with increases in GPx after agraz consumption, compared to placebo, have a significant reduction of CVRF such as total cholesterol and LDL-c. These results are in accordance with a report by Blankenberg *et al.*, who demonstrated that people with higher GPx activity have significantly less risk of future cardiovascular events, compared with people with lower GPx activity (41).

In this study, we determined TAC after agraz and placebo consumption, as a measure to evaluate the antioxidant capacity mediated by both exogenous (dietary) and endogenous antioxidants. It has been reported that anthocyanins (the main polyphenol in *V.meridionale*) have a low bioavailability (42). However, a maximal concentration of its phenolic metabolites in blood between 2 and 30 hours, and a half-life between 0.5 and 96 hours, have been demonstrated after its consumption (43). In fact, studies evaluating blood TAC after an acute consumption (during 4 hours) of *Vaccinium* beverages, have shown significant increases in TAC determined by ABTS and ORAC methods, which is associated with increased levels of anthocyanins in blood (44,45). We did not find significant improvement in TAC after 4 weeks of agraz intake, compared to placebo, in this group of women. Similarly, Karsen *et al.*, did not observe increases in TAC determined by the same methods (FRAP, ORAC and ABTS) in a group of 31 subjects with at least one CVRF, after consuming bilberry juice during 4 weeks (46). Contrarily, Basu, *et al*., demonstrated that an 8-week intervention with cranberry juice in 31 women with MetS was enough to significantly increase TAC determined by the ABTS method, when compared with the baseline (47). The intervention period of this last study was the double of the time of our study and with a different fruit. Another study demonstrated increases in TAC, measured by the ABTS method, after 2 weeks of consuming an anthocyanin-rich juice, however these results were obtained in healthy individuals (28). Our study included women with several CVRF with high levels of OxS, as others have reported (5). Although in this study blood samples were collected after 12 hours of overnight fasting, a period where there could be phenolic metabolites in circulation, it is possible the given dose was not sufficient to

neutralize the high levels of OxS in this group of women. Therefore, further studies exploring higher dosage or an extended duration of the supplementation are warranted.

Interestingly, we found that women who increased TAC determined by ABTS after 4 weeks of agraz consumption (n=21), compared to placebo, had reduced significantly their waist circumference. TAC has been inversely associated with central obesity (48), and recently a significant reduction in waist circumference (4.1%) after 21 days consuming 35g/day of osmodehydrated agraz in 25 overweight adults was reported. (49). Although there is not a clear mechanism, anthocyanins like cyanidin 3–glucoside have been shown to ameliorate obesity by upregulating brown adipose tissue mitochondrial function and inducing beige adipocyte phenotypes or "browning" of white fat, increasing so the energy expenditure and improving insulin sensitivity (50,51). These findings indicate a cardioprotective effect of agraz in those women in whom the level or activity of antioxidant system increased after its consumption, positively impacting central obesity and OxS, two important risk factors for the development of a cardiovascular event.

This study presented some limitations. Although the randomization would be ideal, the method used for the alternating beverage assignation was used in order to equilibrate the number of participants to begin each period (agraz versus placebo), to reduce bias due to behavioral issues (collective holidays, climate conditions along the year). In addition, the sample size was small, which reduces the possibility to detect association in the studied variables. In spite of that, the crossover design used in this study have some advantages: a smaller sample size is required in comparison to parallel studies, and patients serve as their own controls, which reduces the influence of confounders.

In conclusion, the chronic consumption (4 weeks) of a daily dose of agraz equivalent to 200g of fresh fruit, in 40 women with MetS, did not significantly increase serum TAC and endogenous antioxidant enzymes activity in the group as a whole. However, women in whom these antioxidant parameters increased after agraz consumption, several CVRF were improved, such as reductions in lipid and DNA oxidation, total cholesterol, LDL-c and waist circumference, demonstrating a

beneficial effect of agraz in this sub-group of women. These results evidence an individual variability that influences the response to agraz consumption, which could be mediated by factors such as intestinal microbioma, food matrices, absorption, genetic variation, among others (52). More studies will be necessary in this regard.

4.7 References

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4.8 Tables and figures

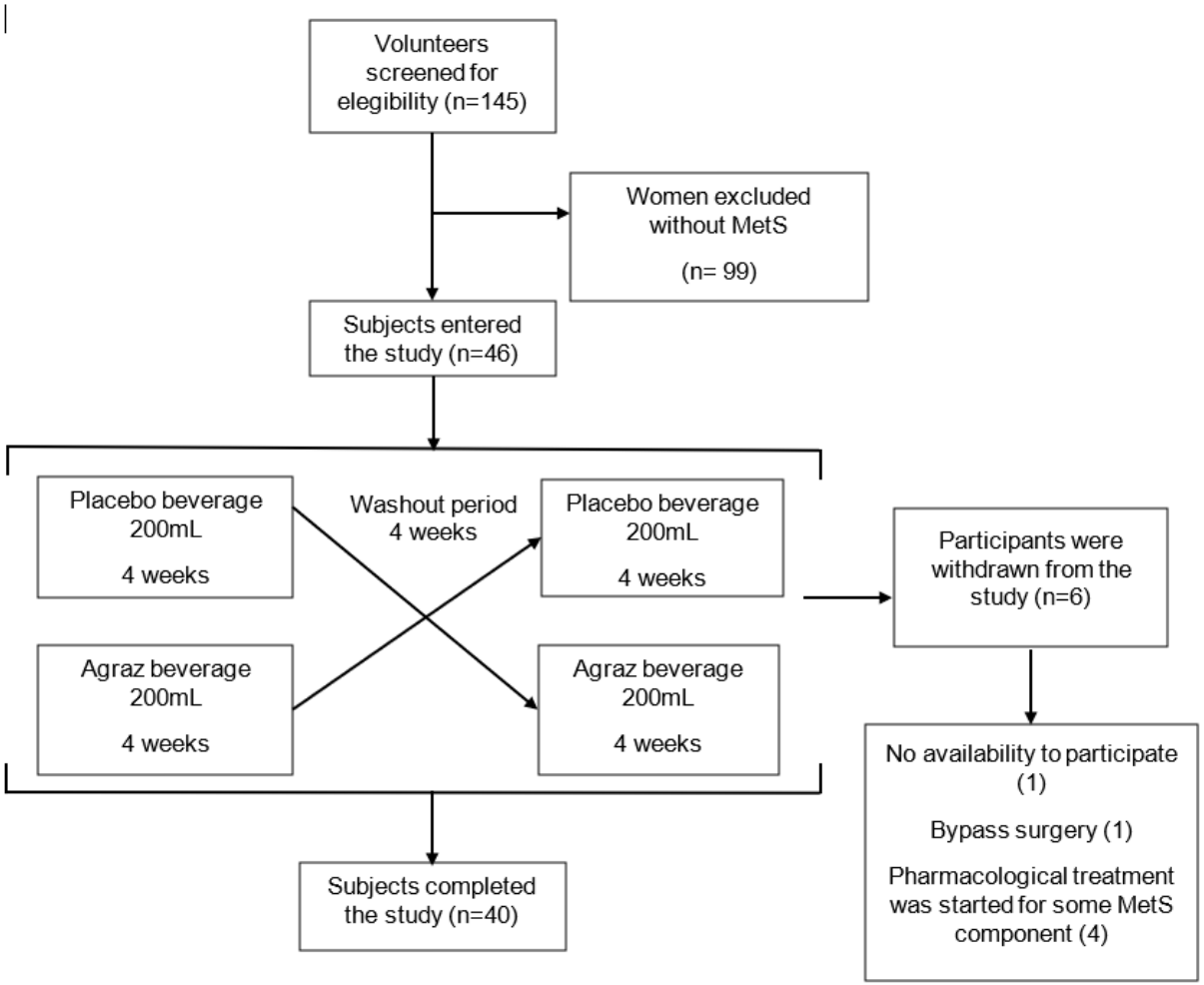

Figure 1. Study protocol

Table 1. Antioxidant enzymes activity and serum total antioxidant capacity after 4 weeks of agraz consumption, compared to placebo, in women with metabolic syndrome

Variables	Placebo		Agraz		Δ Change (Agraz-placebo)	p
	n	Mean ±SD or median (p25-p75)	n	Mean ±SD or median (p25-p75)	Mean ±SD	
SOD (U/mL) a	37	228.6 (185.4-266.7)	34	231.4 (196.4-291.7)	14.7 ± 68.4	0.402
CAT (U/mL) a	37	300.5 (250.6-357.7)	37	296.7 (264-330.6)	7.1 ± 78.2	0.667
GPx (nmol/min/ml) a	40	84.7 (65.7-148.5)	40	83.5 (59.6-121.2)	-2 (-23.4, 31)	0.670
ABTS (µM Trolox Eq/mL) a	40	2.2 (2.2-2.4)	40	2.3 (2.2-2.3)	0.0 ± 0.1	0.771
FRAP (µM Trolox Eq/mL) b	40	814.6 ± 162.5	40	810.2 ± 176.9	-4.5 ± 111.3	0.996
ORAC (µM Trolox Eq/mL) b	40	17.3 ± 4.8	40	17.4 ± 4.9	0.1 ± 3.4	0.848

a. T paired or b. Wilcoxon test; * significance p<0.05

Abbreviations: SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; ABTS, 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); FRAP, Ferric reducing ability of plasma; ORAC, Oxygen radical absorbance capacity

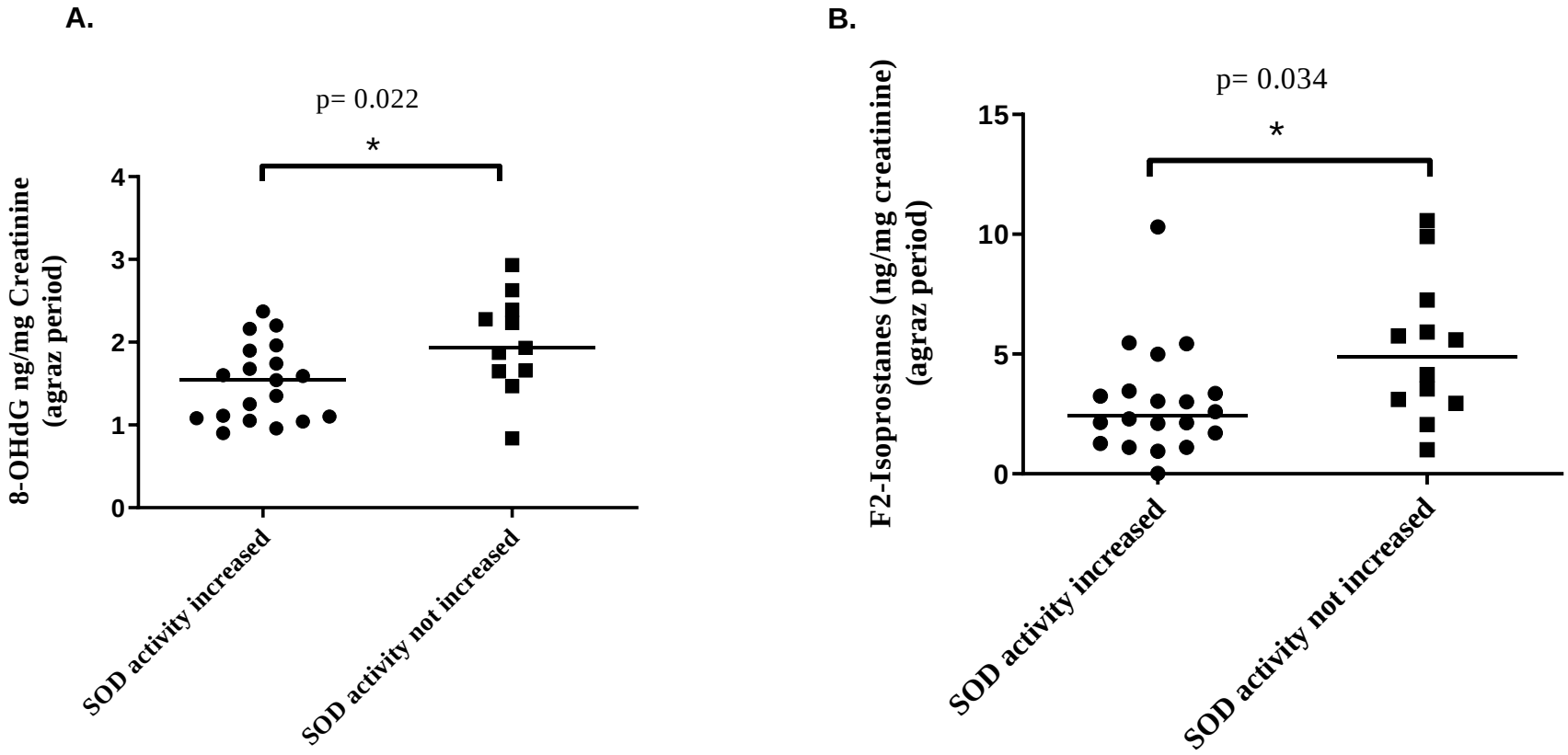

Figure 2. Changes in superoxide dismutase (SOD) activity and effects on oxidative stress markers after 4 weeks of agraz consumption, compared to placebo, in women with metabolic syndrome. A) Changes in SOD and effects on urinary 8-OHdG. B) Changes in SOD and effects on 8-isoprostane levels. ANOVA adjusted by adherence. * Significance $p<0.05$

Abbreviations: SOD, superoxide dismutase; 8-OHdG, 8-hydroxydeoxyguanosine.

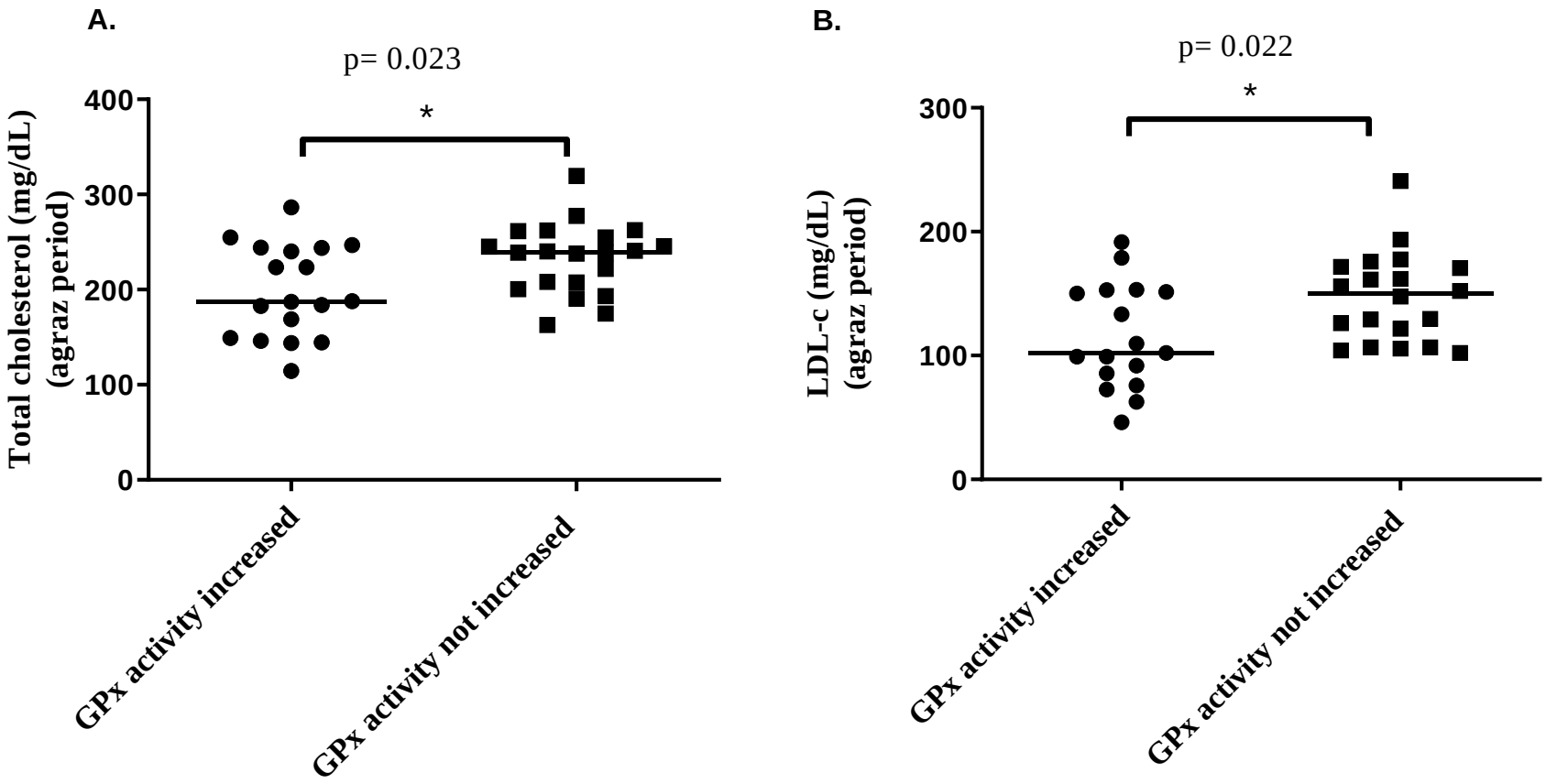

Figure 3. Changes in glutathione peroxidase (GPx) activity and effects on cardio-metabolic risk factors, after 4 weeks of agraz consumption, compared to placebo, in women with metabolic syndrome. Changes in GPx activity and effects on: (A) total cholesterol; (B) low-density lipoprotein cholesterol levels. ANOVA adjusted by adherence. * Significance $p<0.05$

Abbreviations: GPx, glutathione peroxidase; LDL-c, low-density lipoprotein cholesterol.

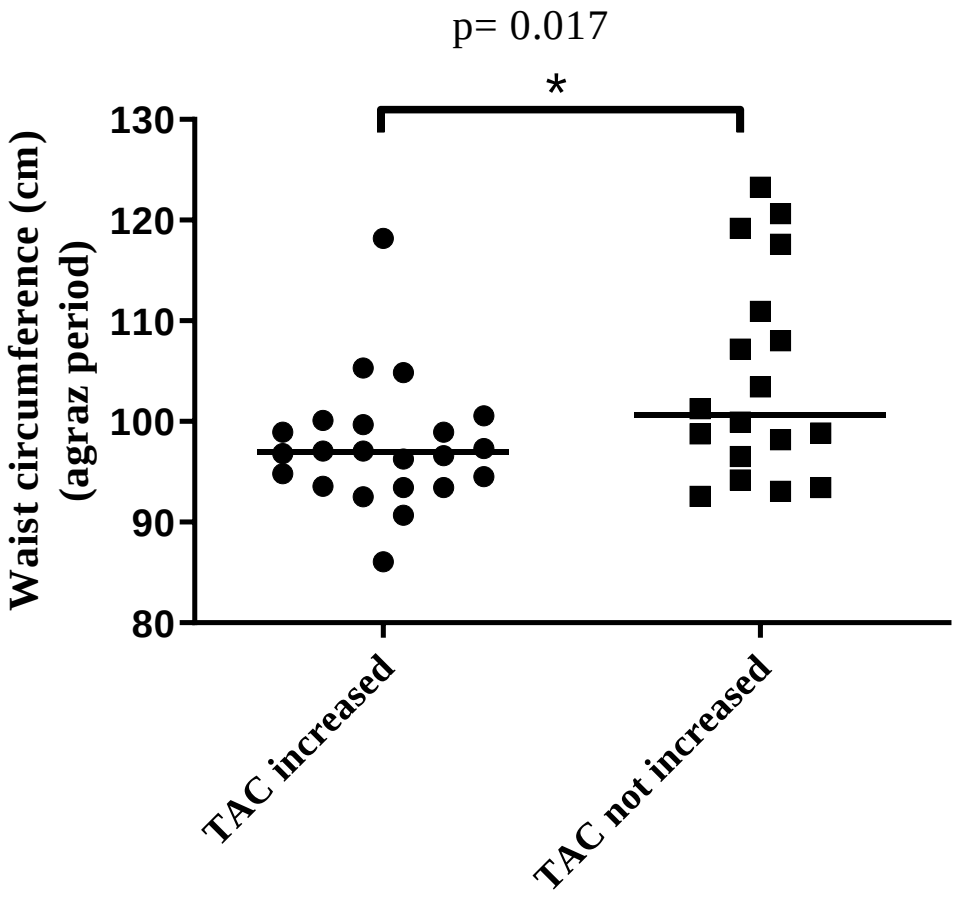

Figure 4. Effects of changes in serum total antioxidant capacity (TAC; measured by ABTS) on waist circumference, after 4 weeks of agraz consumption, compared to placebo, in women with metabolic syndrome. ANOVA adjusted by adherence. *Significance p<0.05

Abbreviations: TAC, total antioxidant capacity.

CHAPTER 5

Differential effects of agraz (*Vaccinium meridionale* Swartz) consumption in overweight and obese women with metabolic syndrome

Differential effects of agraz (*Vaccinium meridionale* Swartz) consumption in overweight and obese women with metabolic syndrome

5.1 Abstract

Background: Obesity implies higher cardiovascular risk (CVR) than overweight. Polyphenol-rich fruits have shown to ameliorate CVR factors (CVRF). It is not clear whether differential effects could be observed between obese and overweight people consuming these fruits.

Objective: To evaluate the effects of agraz on CVRF in overweight and obese women with metabolic syndrome (MetS).

Methods: Overweight (n=22) and obese (n=18) women (25-60 years) with MetS, were included in this crossover, double-blind and placebo-controlled study. They consumed agraz or placebo over 4-weeks separated by a 4-wk washout period. At the end of each period, the following parameters were measured: anthropometrics, blood pressure, serum lipid profile, glucose, insulin, adipokines, apolipoprotein (apo)-A1, high sensitivity C-reactive protein (hs-CRP), serum total antioxidant capacity (TAC), endogenous antioxidant enzymes and oxidative stress (OxS) markers.

Results: Compared to placebo, agraz consumption significantly ($p<0.05$) reduced hs-CRP and urinary 8-hydroxy 2 deoxyguanosine (8-OHdG) levels in overweight and obese women, respectively. In both groups, changes in antioxidant markers were significant ($p<0.05$) and negatively correlated with changes in CVR factors and OxS markers, respectively. Positive correlations were observed with cardioprotective markers.

Conclusions: Agraz consumption had differential effects in overweight and obese women, with better effects on inflammation and OxS markers, respectively. Further studies should consider these differential responses when analyzing the results of an intervention, and eventually adjust to get better outcomes.

5.2 Keywords

Andean berry, obesity, overweight, oxidative stress, total antioxidant capacity, antioxidant enzymes, inflammation, cardiovascular risk factors.

5.3 Introduction

Obesity is a global epidemic and represents an important public health problem (1). Obese people have higher risk of coronary heart disease than overweight people (61% and 22% respectively) (2). Compared with overweight subjects, obese people have higher prevalence of metabolic syndrome (MetS) (3), a complex of cardiometabolic factors that doubles the risk for developing cardiovascular diseases (CVD) (4), which represent the first cause of mortality worldwide (5).

Besides the cardiometabolic alterations in obese people, endogenous antioxidant enzymes are not enough to neutralize the overproduction of reactive oxidative species (6), resulting in oxidative stress (OxS) and high levels of oxidation markers (7) (8). These alterations lead to dysregulated adipocytokine production, chronic inflammation, insulin resistance (9,10), and atherosclerotic lesion development (11). Lifestyle changes have shown to contribute importantly to the treatment and prevention of cardiovascular risk factors (CVRF) in overweight and obese people. For example, studies in people with high BMI (≥25 kg/m^2) have shown that chronic consumption of polyphenol-rich foods results in reductions of lipid peroxidation (12,13) and protein oxidation markers (14); and increases in antioxidant markers like blood total antioxidant capacity [TAC] (15) and antioxidant enzymes activity (16,17). Likewise, polyphenol consumption has shown to improve insulin resistance (18), adiponectin levels (14), BMI, waist circumference (WC), LDL-cholesterol (LDL-c) (19), systolic and diastolic blood pressure (13) and inflammation (e.g., by reducing high sensitivity C-reactive protein [hs-CRP] levels) (20). In contrast, some studies did not find beneficial effects of polyphenol consumption in people with high BMI (21,22). One reason for these contradictory results might be a differential response to polyphenol consumption between obese and overweight people. Recently, Herranz-López *et al.*, found that consumption of polyphenol-rich extracts

(Metabolaid®, 500 mg/day) had a better effect in reducing BMI, WC, body fat percentage and blood pressure in overweight, compared to obese participants (23), suggesting that it is important to evaluate differences in the response between overweight and obese participants in intervention studies.

Lately, there has been a great interest in studying the effects of *Vaccinium meridionale* Swartz (also called agraz or Andean berry) in human health, due to its high antioxidant capacity and phenol concentration (mainly anthocyanins) (24). In 2018, Torres *et al.*, showed that 21 days of osmodehydrated Andean berry consumption in overweight subjects caused reductions of some CVRF like blood pressure, BMI and WC (25). Previously, we reported beneficial effects after 4-weeks of agraz consumption in the same group of women with MetS, with increases in TAC (26) and reduction in DNA oxidative damage (27). However, there are limited studies evaluating the effect of consumption of a polyphenol-rich beverage of agraz on OxS markers and antioxidant capacity comparing people with different BMI classification. Therefore, we aimed to evaluate the effects of agraz, compared to placebo, in cardiometabolic variables, OxS markers, endogenous antioxidant enzyme activity and serum TAC in overweight and obese women with MetS.

5.4 Materials and methods

5.4.1 Study design

Forty women (25-60 years) with MetS from Medellín- Colombia, were included in this double-blind, crossover design and placebo-controlled study. MetS was defined according to the NCEP ATP-III guidelines (4), as previously described (27). Women were analyzed according to their BMI as overweight (n=22) and obese (n=18) (28). Exclusion criteria included kidney disease, heart disease, diabetes; having TG ≥ 500 mg/dL, fasting plasma glucose ≥ 126 mg/dL, LDL-c ≥ 190 mg/dL, blood pressure >140/90 mmHg. In addition, consumption of anti-inflammatory, lipid-lowering, hypoglycemic, and anti-hypertensive medications; smoking, consuming more than

20 g alcohol per day, being pregnant or planning to become pregnant, being a professional athlete, and consumption of supplements or nutraceuticals.

Women were assigned through a the alternating quasi-randomization allocation method, to consume over 4 weeks either a freeze-dried agraz powder dissolved in 200 mL of water (containing 1027.97 ± 41.99 mg gallic acid equivalents /L of total phenols) or 200 mL of placebo (with sensorial and physicochemical characteristics of the agraz beverage, but devoid of any polyphenols). Both beverages were prepared and characterized by food engineers of the University of Antioquia as previously described (26). There was a 4-week washout between both periods, in which neither agraz nor placebo were consumed.

During the whole study, women were asked to continue with their habitual diet and exercise, as well as to avoid the consumption of polyphenol-rich foods. To verify compliance, a 7-day physical activity record and a food frequency questionnaire were used at the beginning and end of each period. This last questionnaire was designed and adjusted specifically for the academic community of the University of Antioquia (29), where the participants were recruited. The frequency of consumption of some foods and the energy and macronutrients consumed during each period were obtained. In addition, the adherence to the study was evaluated on a weekly basis using a questionnaire to verify the daily beverage consumption and abstinence from consuming polyphenol-rich foods. An adherence below 80% was a criterion for withdrawing study participants.

This study was performed according to the Helsinki declaration and was approved by the Human Bioethics Committee of the *Sede de Investigación Universitaria*, *University of Antioquia* (Act No. 15-35-558-02). Before the intervention, all participants signed an informed consent.

5.4.2 Blood collection

Blood samples were collected after 12 hours of overnight fasting at baseline and at the end of each intervention period, using serum separator tube (Vacutainer ®,

Franklin Lakes, NJ, USA). Samples were centrifuged at 2000 x *g* for 10 minutes and kept frozen at -70°C until analysis.

5.4.3 Anthropometric, metabolic and inflammatory markers

Body weight was measured using a calibrated digital scale (Seca 813, Seca, Chino, CA, USA) and height was determined with a portable stadiometer (Seca 213, Seca, Chino, CA, USA). BMI was calculated by dividing body weight (kg) by height squared (m^2). WC was measured using a non-stretching body measuring tape (Lufkin W606PM, Sparks, MD, USA) according to the Anthropometry Procedures Manual of the CDC (2017) (30). Systolic and diastolic blood pressure were measured using an automated monitor (Omron, Healthcare, Hoffman Estates, IL, USA) and following the protocol described in the Health Tech/ Blood Pressure Procedures Manual of the CDC (2007) (31). Serum glucose and lipid profile concentrations were measured with colorimetric and enzymatic kits (Siemens, Washington, DC, USA). The levels of hs-CRP were measured with a turbidimetric immunoassay (Siemens, Washington, DC, USA); values above 10mg/L were excluded for the analysis. All measurements were made using an automatic analyzer (Dimension RxL®, Siemens, Washington, DC, USA). Insulin levels were determined with direct chemiluminescence technology following manufacturer's instructions (Siemens, Washington, DC, USA) and using ADVIA Centaur® CP immunoassay System (Siemens, Washington, DC, USA). Adiponectin and resistin levels were determined with the human adipocyte magnetic panel kit following manufacturer's instructions and using Luminex® technology (Millipore Sigma, Burlington, MA, USA). Apo-A1 was determined with the apo-A1 (human) ELISA kit based on a competitive assay (Cayman Chemical, MI, USA) in a microplate reader (Multiskan Go®, Thermo Scientific, MA, USA). The Friedewald formula was used to calculate LDL-c (32). HOMA-IR index was calculated with the formula described by Matthews *et al* (33), and insulin sensitivity was calculated using Quantitative Insulin Sensitivity Check Index (QUICKI index) developed by Katz, *et al* (34).

5.4.4 Serum total antioxidant capacity (TAC)

Serum TAC was measured through several methods: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was determined via a modified method described by Re, *et al* (1999) (35); ferric reducing ability of plasma (FRAP) was measured using the modified method of Benzie and Strain (1996) (36); oxygen radical absorbance capacity (ORAC) and 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) were measured following the methodology reported by Quintero *et al* (26).

5.4.5 Endogenous antioxidant enzymes

Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) enzyme activities were determined in serum using the following kits, respectively: OxiSelect™ kit Superoxide Dismutase Activity Assay (Cell Biolabs, Inc, CA, San Diego, USA), OxiSelect™ Catalase Activity Assay Kit (Cell Biolabs, Inc, CA, San Diego, USA), and Glutathione Peroxidase Assay Kit (Cayman Chemical, MI, USA), following manufacturer's instructions. The results were expressed in U/mL for SOD and CAT enzyme activities and in nmol/min/mL for GPx activity.

Paraoxonase 1 (PON1) lactonase activity was measured according to the methodology described by Millar *et al* (37). The concentration was determined using a standard curve generated with 0.1M HCl (0-115 µM) and results were expressed in kU/L.

5.4.6 Oxidative stress (OxS) markers

Advanced oxidation protein products (AOPP) levels were measured in apo-B depleted serum as previously described (38). The results were expressed in µM. Myeloperoxidase was determined using the Myeloperoxidase human ELISA Kit, (Cayman Chemical, MI, USA) following manufacturer's instructions. The results were expressed in ng/mL.

8-isoprostane levels and 8-hydroxy 2 deoxyguanosine (8-OHdG) were measured in 24-hours' urine using the following kits, respectively: OxiSelect™ 8-iso-prostaglandin F2 ELISA kit (Cell Biolabs, Inc., San Diego, CA, USA) and 8-OHdG ELISA Kit (Abcam, Cambridge, MA, USA), following manufacturer's instructions. The concentrations were calculated with standard curves. Finally, results were normalized to urinary creatinine levels and expressed in ng/mg creatinine.

5.4.7 Statistical analysis

Shapiro Wilk test was used to evaluate data distribution. Accordingly, data are presented as mean ± SD or median and 25 (p25) and 75 (p75) percentiles. Repeated measures ANOVA was used to determine the effect of time, intervention and the interaction between time*intervention in variables measured at baseline and end of intervention (anthropometric measures, blood pressure, lipid profile and fasting glucose). For variables measured only at the end of intervention, paired samples t-test or Wilcoxon test were used to compare the results between agraz and placebo periods, according to data distribution. Mann- Whitney U test or Student's t-test were used to evaluate the differences between obese and overweight women. Finally, changes after agraz consumption (agraz minus placebo period) were calculated and the correlations between changes were determined with Pearson´s or Spearman´s correlation coefficients. All analyses were done using SPSS version 21 for Windows (SPSS, IBM Corporation, 2012). Differences with a p value <0.05 were considered significant.

5.5 Results

5.5.1 Study population characteristics

Forty women, 22 overweight (49±9 years old) and 18 obese (45±9 years old) with MetS finished the study (**Table 1**). Adherence was 94.8%, indicating a suitable

consumption of both beverages. No changes in diet and physical activity were found during the whole intervention (agraz versus placebo) as previously reported (27). Importantly, there were not differences in age, energy, fat, carbohydrate, and protein intakes, nor physical activity between obese and overweight women (**Table 1** and **Table 2**).

5.5.2 Anthropometric, metabolic and inflammatory markers.

As expected, at baseline, women with obesity had significantly higher body weight, BMI, WC, systolic and diastolic blood pressure ($p<0.05$), compared with overweight women. However, there were no significant differences in fasting glucose and lipid profile (**Table 1**).

After agraz consumption, differences between obese and overweight women, in other cardiovascular variables after agraz intake were not observed (**Table 3**). However, changes in hs-CRP levels were significantly different between obese and overweight women ($p=0.028$) (**Table 4**), with a significant reduction of hs-CRP levels in overweight women ($p=0.011$) after agraz consumption, compared to placebo (**Figure 1**).

In obese women, there were significant correlations between changes in different cardiometabolic factors after agraz consumption compared to placebo (**Figure 2**). Changes in HDL-cholesterol (HDL-c) levels had a positive correlation with changes in QUICKI index ($r= 0.57$) (Figure 2A) and a negative correlation with changes in fasting glucose levels ($r= -0.59$) (Figure 2B). Likewise, changes in apo-A1 concentration had negative significant correlations with changes in LDL-c, Non-HDL-c and TC levels ($r= -0.608$; $r= -0.585$; $r= -0.615$, respectively) (Figure 2C-E).

5.5.3 Antioxidant and oxidative stress (OxS) markers

There was a significant reduction in urinary 8-OHdG levels in obese women after agraz consumption compared to placebo (p=0.031) (**Figure 1)**. No other significant changes were observed **(Table 5).**

The correlation analyses showed significant correlations among antioxidant, metabolic and inflammatory markers in obese women (**Figure 3**). Changes in PON1 lactonase activity were negatively correlated with changes in WC and hs-CRP levels (r= -0.52; r= -0.57, respectively) (Figure 3A and B). Likewise, changes in CAT activity correlated negatively with changes in HOMA-IR (r= -0.60) (Figure 3D) and 8-isoprostanes (r= -0.55) (Figure 3E). In addition, changes in TAC measured by DPPH had a positive correlation with changes in HDL-c levels (r= 0.51) (Figure 3C).

Important correlations were also found in overweight women (**Figure 4**). Changes in ORAC were negatively correlated with BMI (Figure 4A), and changes in SOD activity were also negatively correlated with changes in 8-OHdG levels (Figure 4C). On the contrary, positive correlations were found between changes in DPPH and changes in adiponectin concentration (Figure 4B). Changes in FRAP were also positively correlated with changes in PON1 lactonase activity (Figure 4D).

5.6 Discussion

In this study, we evaluated the effects of agraz consumption on anthropometric, metabolic, inflammatory, antioxidant and OxS markers in women with MetS according to their BMI classification (overweight and obesity). We found that after agraz intake, the effects on inflammatory and oxidative markers were different in obese and overweight women.

It is well known that BMI is highly associated with CVRF (39). As expected, obese women included in this study had a greater risk profile with higher WC and blood pressure than those with overweight, as others have reported (40).

A meta-analysis including 1251 subjects demonstrated that berry consumption has improved CVRF like obesity, hypertension, hyperglycemia, and dyslipidemia, not only in normal weight subjects, but also in overweight and obese people (41).

Interestingly, these beneficial effects were observed only after interventions longer than 8 weeks (41). The time of intervention of our study was 4 weeks, which seemed to be insufficient to modify traditional cardiovascular markers in both groups of women. In addition, the beneficial effects reported in the meta-analysis, were observed only in studies with a parallel design, but not in those with a crossover design (41). Although our study did not show effects in these CVRF, a crossover design study, as the one we used, has some advantages over studies with a parallel design, such as higher statistical power, as it requires a smaller sample size and it reduces the inter-individual variation because each person is her/his own control (42). Interestingly, Torres *et al*. demonstrated that *V. meridionale* consumption over 21 days, significantly improved blood pressure, WC and BMI in overweight adults (25). However, they used a pretest/post-test design with a single group.

We observed that positive changes in atheroprotective markers (HDL-c and apo-A1) were negatively and significantly associated with CVRF. Currently, identifying potential therapies targeting improvements in HDL functionality is of great interest, given the relationship of HDL with CVD reduction (43–45). A study in adults with prediabetes consuming anthocyanins during 12 weeks showed increases in apo-A1 levels (46) - the major component of HDL with atheroprotective roles (47). Although we did not measure the expression of the *ApoA1* gene in this women to explain the improvement in apo-A1 levels, a study in mice demonstrated that anthocyanin consumption significantly increased *ApoA1* gene expression and reduced atherosclerosis progression (48). The authors concluded these effects could be attributed to the anthocyanin content in the black elderberry extract consumed by the mice.

In obesity, adipose tissue expansion is an important source of cytokines triggering a systemic inflammation (49), which is associated with high levels of CRP, an independent predictor of cardiac risk (50). It has been demonstrated that the levels of pro-inflammatory markers are significantly different between overweight and obese individuals, being higher in the latter (51). As expected, in our study, hs-CRP

levels were higher in obese than overweight women, however, in both groups the levels of this pro-inflammatory marker were above 3 mg/L, indicating a high risk of CVD (50). Interestingly, the levels of hs-CRP significantly decreased by more than 1 mg/L after consuming agraz during 4 weeks, only in overweight women; indicating a better effect in this group of women. This result is similar to the reported by Karlsen *et al.* who observed a reduction in hs-CRP, among other cytokines, in overweight subjects consuming bilberry juice (*V. myrtillus*) over 4 weeks (52). These anti-inflammatory effects of polyphenols could be associated to their capacity of reducing pro-inflammatory protein expression through the inhibition of NF-kB translocation (52) and activation of liver X receptor alpha (LXRα) pathway (which induces a trans-repression of NF-kB) (53). The lack of *Vaccinium* anti-inflammatory effects in obese women have also been reported by others, even after 6 (18) and 8 weeks of intervention (13). It is possible that obese subjects, with a more inflammatory environment, need to make more drastic changes in their lifestyle or receive longer treatments to achieve more consistent results.

Interestingly, we found that after agraz consumption, obese women with lower levels of hs-CRP correlated with higher activity of the antioxidant enzyme PON1. Other mechanistic studies with polyphenols have reported increases in PON1 expression (54) and the reduction of inflammation mediated by NF-kB (52). However, molecular studies with *V. meridionale* will be necessary to corroborate this mechanism.

In addition to inflammation, people with MetS have low levels of TAC which is inversely associated with some CVRF (55). In this study, agraz consumption showed positive effects on serum TAC in the participants. In obese women, there was a tendency to increase serum TAC measured by DPPH scavenging capacity by 18.2% after agraz consumption, compared to placebo. Recently, we reported an increase in serum antioxidant status by 19% with this method after 30 days of agraz consumption, in the whole group of women with MetS (26). This could indicate there was a greater neutralization of DPPH radicals in serum through donation of an electron and/or a hydrogen by antioxidant molecules (i.e. in agraz). This could be

hypothesized as a protective effect of agraz intake given that it has been demonstrated that TAC is negatively associated with OxS (56).

Likewise, changes in serum TAC correlated positively with changes in HDL-c levels in obese women, and with changes in PON1 activity in overweight women, after agraz consumption, compared to placebo. OxS affects the antioxidant activity on the HDL particle (57), which also alters its ability to accept cholesterol, thereby decreasing cholesterol transported by HDL (58). Therefore, when antioxidant capacity is improved, there is also an increase in the cholesterol efflux capacity by the HDL particle, increasing HDL-c levels. This was demonstrated in a study where anthocyanin consumption improved HDL-associated PON1 activity and cholesterol efflux capacity (59). Thus, anthocyanins present in the agraz could have the same effect in these women.

Increased levels of adiponectin- secreted by adipose tissue with insulin sensitizing effects (60)- have been reported in people with high TAC and vice versa (61). Although, we did not observe significant changes in this hormone, we found a positive correlation between serum TAC and adiponectin levels in overweight women after consuming agraz, compared to placebo. This could be associated to the antioxidants present in agraz which showed to increase serum TAC (26).

We also evaluated the effects of agraz consumption in endogenous antioxidant enzymes (CAT, SOD and GPx) which have an important role in the prevention of oxidative damage in the cells. A study reported that obese women had significant lower levels of these endogenous enzymes than overweight women (6); these low enzyme levels were strongly associated with abdominal obesity (6). In our study, there were not significant changes in antioxidant enzyme activities in overweight and obese women after agraz intake, compared to placebo. Nevertheless, changes in CAT activity had negative and significant correlations with a lipid peroxidation marker in obese women; and changes in SOD activity were negatively correlated with a DNA oxidative marker in overweight women. Our results are similar to the reported by Lee

et al. in subjects consuming a chokeberry supplement (56). They found a negative association between SOD activity and 8-isoprostane levels, and concluded these results indicate the prevention of lipid peroxidation mediated by the endogenous antioxidant system (56). In addition, a recent study in rats evaluating the effects of *Vaccinium meridionale,* demonstrated the treatment with this extract significantly increased the activity of CAT and SOD associated with an increase of AKT expression (62), which seems to be modulated by polyphenols (63), as a defense mechanism against free radical damage.

Catalase has an important role as an antioxidant enzyme through the decomposition of the reactive species of hydrogen peroxide (H_2O_2) in O_2 and water, which indirectly could diminish insulin resistance (64). We found that changes in CAT activity were negatively correlated with changes in HOMA-IR- an insulin resistance marker- after agraz consumption, compared to placebo, in obese women. This could be associated with reductions in H_2O_2 levels mediated by this endogenous enzyme.

Similarly, agraz consumption had positive effects over OxS markers in both groups of women. The effects were more evident in obese women, in which there was a significant reduction in urinary 8-OHdG levels. This biomarker of DNA oxidative damage is associated with CVD (65). The reduction of this marker was also reported in a study with a pretest-post-test design (without a control group) evaluating healthy people consuming a strawberry beverage for one month (66). Different from our study, they also found decreases in other OxS markers such as malondialdehyde and isoprostane levels (66). Other studies with parallel designs in obese people with MetS have reported significant reductions in OxS markers, including oxidized LDL, malondialdehyde, hydroxynonenal and AOPP, after 8 weeks of consuming berry beverages (13–15). It is important to note, these studies had different designs (pretest-post-test without a control group, parallel arm) than the one used in our study (crossover placebo-controlled design). In addition, most of them had double the time of supplementation than our study (8 versus 4 weeks). It seems that a longer

intervention is required to observe significant changes in OxS, especially in people with high OxS, as the women included in this study.

In conclusion, in this study we evaluated anthropometric, metabolic, inflammatory, antioxidant and OxS markers, to determine the differential effects of agraz consumption between overweight and obese women with MetS. Agraz consumption had a better anti-inflammatory effect in overweight women. Interestingly, the effect on OxS markers was better in obese women. Regarding the anti-inflammatory effects of *Vaccinium* supplementation, it seems to be difficult to observe these effects in obese cohorts, possibly associated with a more inflammatory state that requires a more drastic dietary intervention in this specific population. Although there were positive effects on OxS markers in obese individuals in this study, evaluation of agraz intervention using different dosages and a longer duration of supplementation should be explored to obtain better responses in this population. Finally, the associations observed after agraz intake on antioxidant markers in both groups of women, suggest a potential antioxidant role of the bioactive compounds present in this fruit.

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5.8 Tables and figures

Table 1. Baseline characteristics of overweight and obese women with metabolic syndrome included in the study[1]

Variables	Overweight (n= 22)	Obesity (n=18)	p value
	Mean ±SD or median (p25-p75)	Mean ±SD or median (p25-p75)	
Age (years)[a]	49.0 ± 9.0	45.0 ± 9.0	0.210
Weight (Kg)[a]	69.8 ± 7.4	84.5 ± 10.4	**0.000***
BMI (kg/cm^2)[b]	28.1 (27.3 - 28.9)	32 (30.9 - 33.7)	**0.000***
Waist Circumference (cm)[a]	97.7 ± 3.5	107.2 ± 11.2	**0.001***
Systolic blood pressure (mm Hg)[a]	112.8 ± 11.2	124.6 ± 11.0	**0.002***
Diastolic blood pressure (mm Hg)[a]	72.5 ± 9.9	80.5 ± 6.6	**0.006***
Fasting glucose (mg/dL)[a]	94.7 ± 7.4	93.5 ± 7.2	0.609
TC (mg/dL)[b]	229.8 (209.8 - 255.9)	225 (192.6 - 239.9)	0.447
HDL-c (mg/dL)[a]	43.5 ± 7.1	40.6 ± 5.2	0.164
Triglycerides (mg/dL)[b]	204.2 (175.8 - 281)	194.8 (149.4 - 274.9)	0.254
TG/HDL ratio [a]	4.6 (3.8 - 6.8)	4.5 (3.7 - 6.9)	0.775
LDL-c (mg/dL)[a]	136.2 ± 37.5	131.8 ± 31.7	0.692
Non HDL-c (mg/dL)[a]	182.8 ± 36.6	172.9 ± 39.9	0.418

[a] t student test; [b] Mann-Whitney U test; * significance $p<0.05$

[1]Women were classified according to BMI (28) and metabolic syndrome definition by ATP-III (4). Abbreviations: HDL-c, HDL-cholesterol; LDL-c, LDL-cholesterol; p25, percentile 25; p75, percentile 75; TC, total cholesterol.

Table 2. Baseline kilocalories, macronutrients intake and physical activity in obese and overweight women with metabolic syndrome[1]

Variables	Overweight (n =22)	Obese (n = 18)	p value
	Mean ±SD or median (p25-p75)	Mean ±SD or median (p25-p75)	
Kilocalories (Kcal)[a]	1800.7 (1519.6 - 2419.1)	1643 (1227.3 - 2486.8)	0.610
Protein (g)[a]	65.9 (54.1 - 81.2)	63 (47.7 - 81.5)	0.770
Total fat (g)[a]	58.8 (52.1 - 84.5)	63.1 (49.5 - 88)	0.830
Saturated fatty acids (g)[a]	21.4 (19 - 31.2)	23.6 (17.5 - 33.1)	0.550
Monounsaturated fatty acids (g)[a]	23.1 (19.8 - 33.9)	22.7 (17.5 - 34.3)	0.980
Polyunsaturated fatty acids (g)[a]	11.8 (8.3 - 15.9)	12 (8 - 17)	0.790
Cholesterol (mg)[b]	313.4 ± 151.3	310.5 ± 131.7	0.950
Total carbohydrates (g)[a]	255.8 (203.1 - 358.8)	206.2 (174.1 - 329.5)	0.230
Dietary Fiber (mg)[a]	20.3 (13.6 - 25.1)	14.9 (9.8 - 18.5)	0.100
Physical activity (min) [a]	210(110-330)	220 (70-300)	0.730

[a] Mann-Whitney U test; [b] t student test; * significance $p<0.05$

[1]Women were classified according to BMI (28)

Table 3. Effects of 4 weeks' intervention with agraz or placebo on anthropometric and biochemical characteristics in obese and overweight women with metabolic syndrome[1]

	Placebo		Agraz		Repeated Measures ANOVA p-value		
	Before	After	Before	After			
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Time	Intervention	Time * Intervention
Overweight							
Weight (Kg)	69.6 ± 7.2	70.3 ± 7.5	69.8 ± 7.3	69.9 ± 7.6	0.346	0.054	0.231
BMI (kg/cm^2)	27.9 ± 1.2	28.2 ± 1.5	27.9 ± 1.2	28.0 ± 1.3	0.276	0.062	0.272
Waist Circumference (cm)	96.8 ± 3.5	97.3 ± 4.1	96.5 ± 3.9	96.7 ± 4.2	0.307	0.414	0.69
SBP (mm Hg)	113 ± 13	114 ± 12	114 ± 13	112 ± 12	0.541	0.979	0.284
DBP (mm Hg)	73 ± 10	74 ± 9	73 ± 8	72 ± 10	0.275	0.952	0.422
Fasting glucose (mg/dL)	95.0 ± 6.1	95.0 ± 7.8	96.6 ± 7.9	95.2 ± 7.6	0.364	0.543	0.268
TC (mg/dL)	232.9 ± 38.1	226.9 ± 41.9	226.8 ± 44.6	223.8 ± 46.8	0.182	0.374	0.83
HDL-c (mg/dL)	44.2 ± 7.9	43.0 ± 6.6	43.1 ± 6.3	43.5 ± 6.9	0.659	0.613	0.339
Triglycerides (mg/dL)	224.7 ± 79.3	227.9 ± 92.7	198.1 ± 73.3	201.7 ± 74.0	0.009	0.752	0.987
TG/HDL ratio ‡	5.2 ± 2.0	5.5 ± 2.7	4.7 ± 2.2	4.8 ± 2.0	0.050	0.846	0.983
LDL-c (mg/dL)	143.7 ± 32.0	138.4 ± 39.1	144.4 ± 41.2	139.9 ± 44.0	0.781	0.391	0.991
Non HDL-c (mg/dL)	188.7 ± 34.5	184.0 ± 39.2	183.8 ± 43.6	180.3 ± 45.2	0.186	0.386	0.927
Obese							
Weight (Kg)	84.5 ± 10.3	84.8 ± 10.6	85.0 ± 10.3	84.7 ± 10.4	0.269	0.975	0.104
BMI (kg/cm^2) ‡	33.0 ± 2.9	33.1 ± 3.0	33.1 ± 2.9	33.0 ± 3.0		0.772 †	
Waist Circumference (cm)	106.8 ± 10.5	105.5 ± 10.9	106.3 ± 10.7	105.3 ± 10.7	0.479	0.077	0.87
SBP (mm Hg)	124 ± 12	122 ± 12	118 ± 11	120 ± 10	0.012	0.778	0.138
DBP (mm Hg)	80 ± 8	79 ± 9	77 ± 8	78 ± 9	0.034	0.763	0.157

Fasting glucose (mg/dL) ‡	93.8 ± 6.9	96.3 ± 8.3	97.1 ± 8.6	96.8 ± 8.6	0.120	0.309	0.040
TC (mg/dL)	210.1 ± 37.8	213.6 ± 40.7	210.8 ± 41.2	206.8 ± 42.2	0.230	0.952	0.283
HDL-c (mg/dL)	40.4 ± 5.3	40.2 ± 4.9	39.9 ± 6.1	39.6 ± 6.2	0.334	0.678	0.926
Triglycerides (mg/dL)	204.2 ± 80.8	222.3 ± 115.2	200.2 ± 80.3	208.7 ± 97.6	0.706	0.232	0.676
TG/HDL ratio ‡	5.2 ± 2.3	5.8 ± 3.3	5.2 ± 2.6	5.6 ± 3.0	0.515	0.739	0.999
LDL-c (mg/dL)	128.9 ± 33.8	129.4 ± 29.8	129.9 ± 35.0	121.2 ± 35.8	0.109	0.302	0.241
Non HDL-c (mg/dL)	169.7 ± 36.9	173.5 ± 40.9	170.9 ± 40.1	167.3 ± 42.1	0.311	0.981	0.280

[1]Women were classified according to BMI (28) and ATP-III (4).
‡ Log-transformed variable; † p-value from Friedman test; significance $p<0.05$

Abbreviations: p25, percentile 25; p75, percentile 75; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL-c, HDL-cholesterol; LDL-c, LDL-cholesterol; TC, total cholesterol; TG, triglycerides.

Table 4. Changes in anthropometric, metabolic and inflammatory markers between agraz and placebo periods for participants classified with overweight *versus* those with obesity[1]

Change between agraz and Placebo	Overwerweigh	Obesity	p
	Mean ±SD or median (p25-p75)	Mean ±SD or median (p25-p75)	
Weight (Kg)[a]	-0.1 (-0.5, 0.5)	-0.4 (-1.4, 0.4)	0.360
BMI (kg/cm^2)[a]	0.0 (-0.2, 0.2)	-0.2 (-0.5, 0.1)	0.310
Waist Circumference (cm)[a]	-0.1 (-1.4, 0.8)	0.4 (-2.3, 3.5)	0.438
SBP (mm Hg) [b]	-2.6 ± 11.3	4.8 ± 13.0	0.061
DBP (mm Hg) [b]	-1.4 ± 8.2	2.6 ± 7.4	0.116
Fasting glucose (mg/dL) [b]	-1.4 ± 5.9	-2.8 ± 5.4	0.450
TC (mg/dL)[b]	2.9 ± 26.9	-7.5 ± 28.6	0.258
HDL-c (mg/dL) [b]	1.6 ± 7.8	-0.1 ± 4.5	0.411
Non HDL-c (mg/dL) [b]	2.0 ± 27.6	-7.4 ± 28.0	0.307
TG (mg/dL) [b]	0.5 ± 142.8	-3.6 ± 81.5	0.914
LDL-c (mg/dL) [b]	1.3 ± 25.6	-6.5 ± 20.7	0.351
TG/HDL-c ratio [b]	-0.3 ± 4.3	0.0 ± 2.3	0.846
Apo A1 (mg/dL) [b]	-4.8 ± 37.0	13.5 ± 44.5	0.229
Insulin (µUI/mL) [a]	0.7 (-2.6, 2.3)	0.1 (-2.2, 1.9)	0.887
HOMA-IR Index [a]	0.2 (-0.6, 0.4)	0.1 (-0.7, 0.7)	0.910
QUICKI Index [b]	0.0 ± 0.0	0.0 ± 0.0	1.000
Adiponectin (µg/mL) [a]	0.9 (-1.5, 3.0)	0.9 (-2.3, 2.4)	0.665
hs-CRP (mg/L) [a]	-1.0 (-2.5, -0.5)	0.4 (-0.5, 1.2)	**0.028***

[1]Women were classified according to BMI (28); [a] Mann-Whitney U test; [b] Student t test; * significance p<0.05

Abbreviations: p25, percentile 25; p75, percentile 75; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL-c, HDL-cholesterol; LDL-c, LDL-cholesterol; TC, total cholesterol; TG, triglycerides; Apo, apolipoprotein; HOMA, Homeostatic Model Assessment for Insulin Resistance; QUICKI Index, Quantitative Insulin Sensitivity Check Index; hs-CRP, high sensitivity C-reactive protein

Table 5. Changes in antioxidant and oxidative stress markers between agraz and placebo periods for participants classified with overweight *versus* those with obesity[1]

Change between agraz and Placebo	Overweight		Obesity		p
	n	Mean ±SD	n	Mean ±SD)	
Antioxidants markers					
Total phenols (mgGA/L)	22	7.9 ± 65.0	18	16.2 ± 73.7	0.159
DPPH (% Scavenging effect)	21	1.2 ± 5.2	18	1.9 ± 3.9	0.642
ORAC (µM Trolox Eq/mL)	22	-0.4 ± 3.4	18	0.7 ± 3.3	0.337
ABTS (µM Trolox Eq/mL)	22	0.0 ± 0.1	18	0.0 ± 0.1	0.500
FRAP (µM Trolox Eq/mL)	22	-20.1 ± 115.5	18	14.7 ± 106.0	0.331
SOD activity (U/mL)	20	32.5 ± 80.0	17	-6.1 ± 45.6	0.088
CAT activity (U/mL)	21	15.9 ± 61.7	18	-3.2 ± 94.7	0.454
GPx activity (nmol/min/mL)	22	4.0 ± 58.7	17	-8.5 ± 48.2	0.482
PON1 Lactonase Activity (kU/L)	22	-1.1 ± 8.3	16	-0.1 ± 9.7	0.736
Oxidative stress markers					
AOPP (µM)	16	-3.2 ± 22.4	13	-0.5 ± 16.8	0.727
8-Isoprostane (pg/mL)	19	0.5 ± 2.2	15	-0.3 ± 2.2	0.321
Urinary 8-OHdG (mg/g creatinine)	19	-0.1 ± 0.7	16	-0.4 ± 0.7	0.186
MPO (ng/mL)	19	-3.5 ± 82.4	17	-19.7 ± 59.5	0.197

[1]Women were classified according to BMI (28). Student t test; * significance $p<0.05$

Abbreviations: p25, percentile 25; p75, percentile 75; DPPH, 2,2-Diphenyl-1-Picrylhydrazyl; ORAC, Oxygen radical absorbance capacity; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); FRAP, ferric reducing ability of plasma; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; PON1, paraoxonase1; AOPP, advanced oxidation protein products; 8-OHdG, 8-hydroxy 2 deoxyguanosine; MPO, myeloperoxidase.

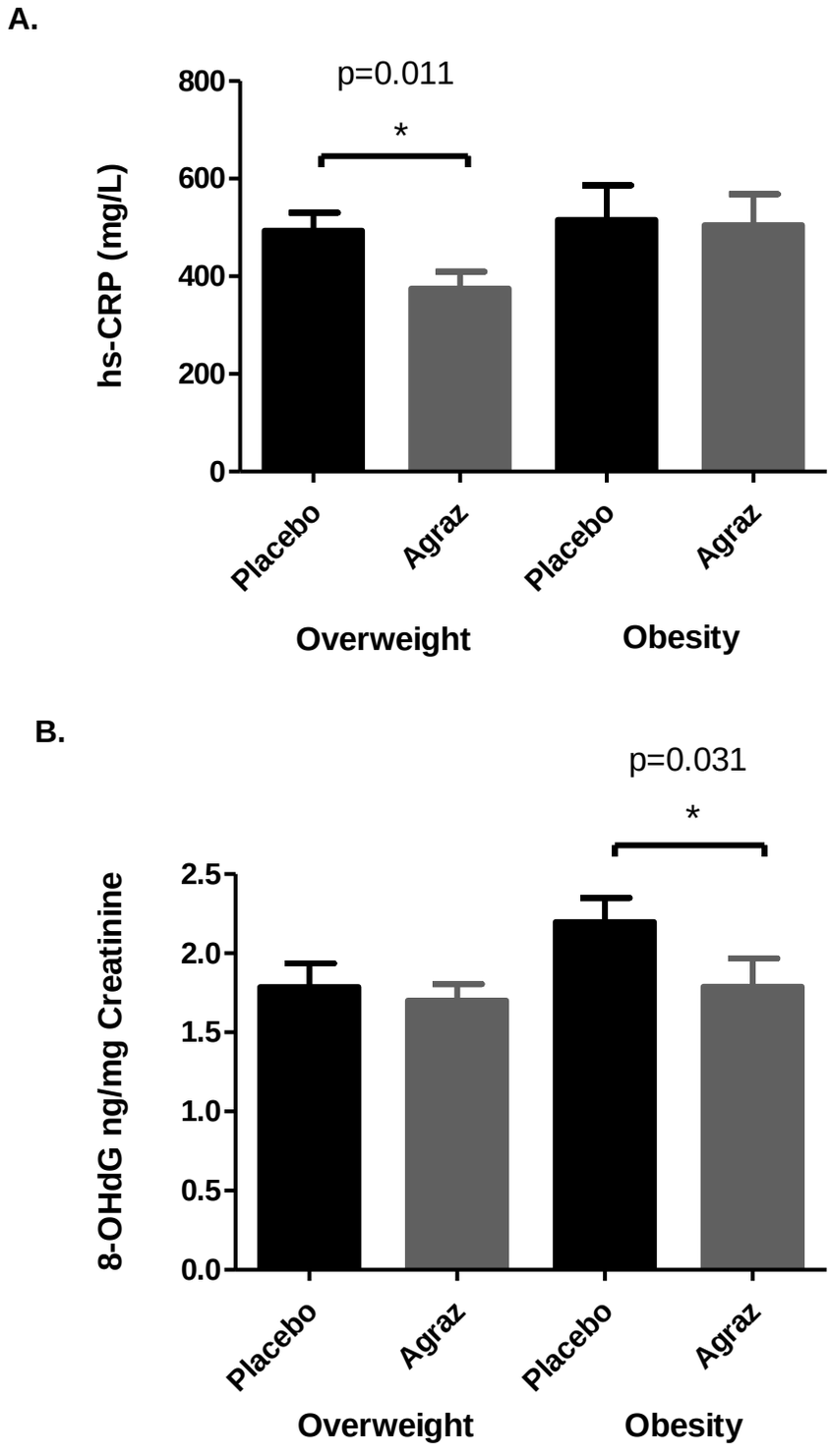

Figure 1. Differential effects after 4 weeks of agraz consumption, compared to placebo on (A) inflammation and (B) oxidative stress in overweight and obese women.

Abbreviations: hs-CRP, high sensitivity C-reactive protein; 8-OHdG, 8-hydroxy 2 deoxyguanosine.

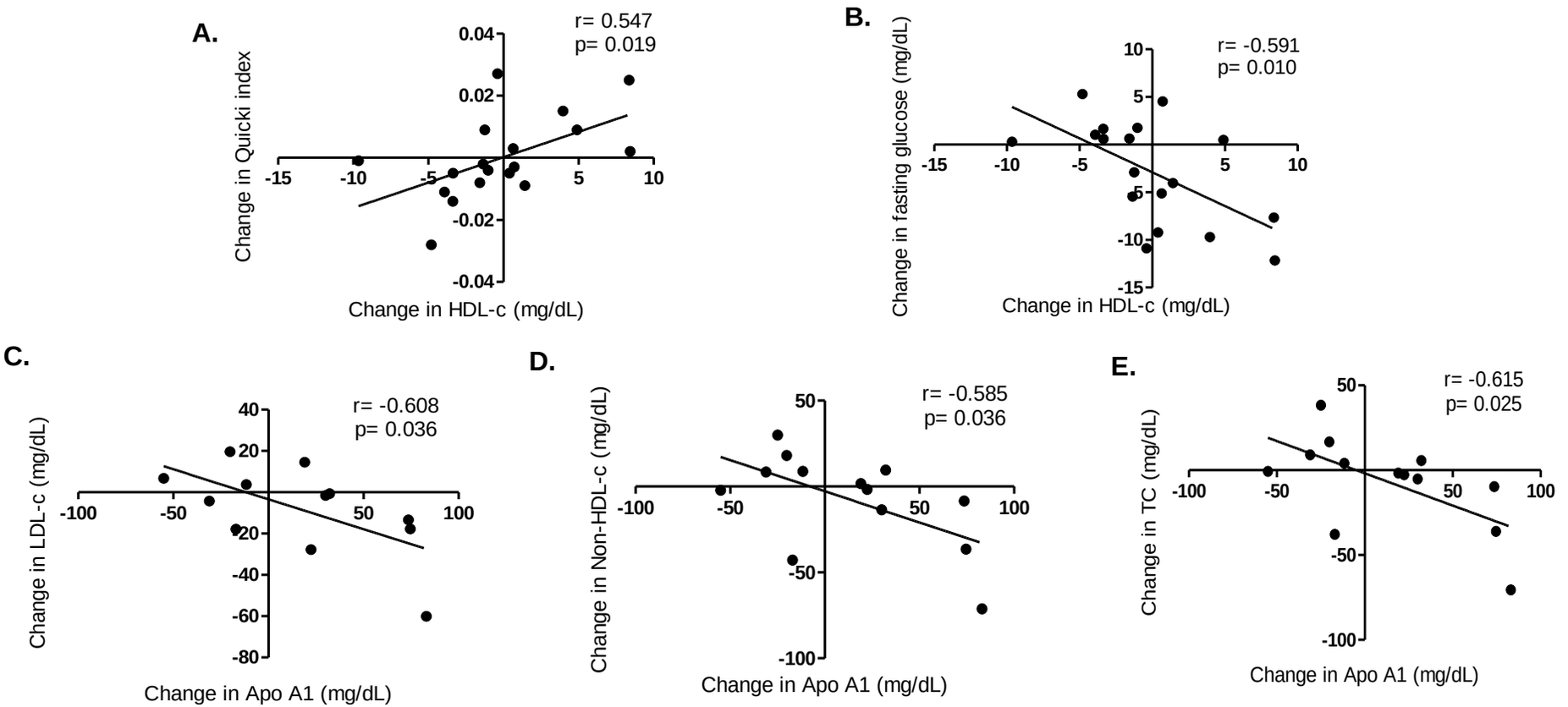

Figure 2. Pearson correlations between changes in cardiometabolic factors after agraz consumption, in obese women with MetS. Significance $p<0.05$. Abbreviations. HDL-c, high-density lipoprotein-cholesterol; LDL-c, low-density lipoprotein-colesterol; Apo, apolipoprotein; TC, total cholesterol.

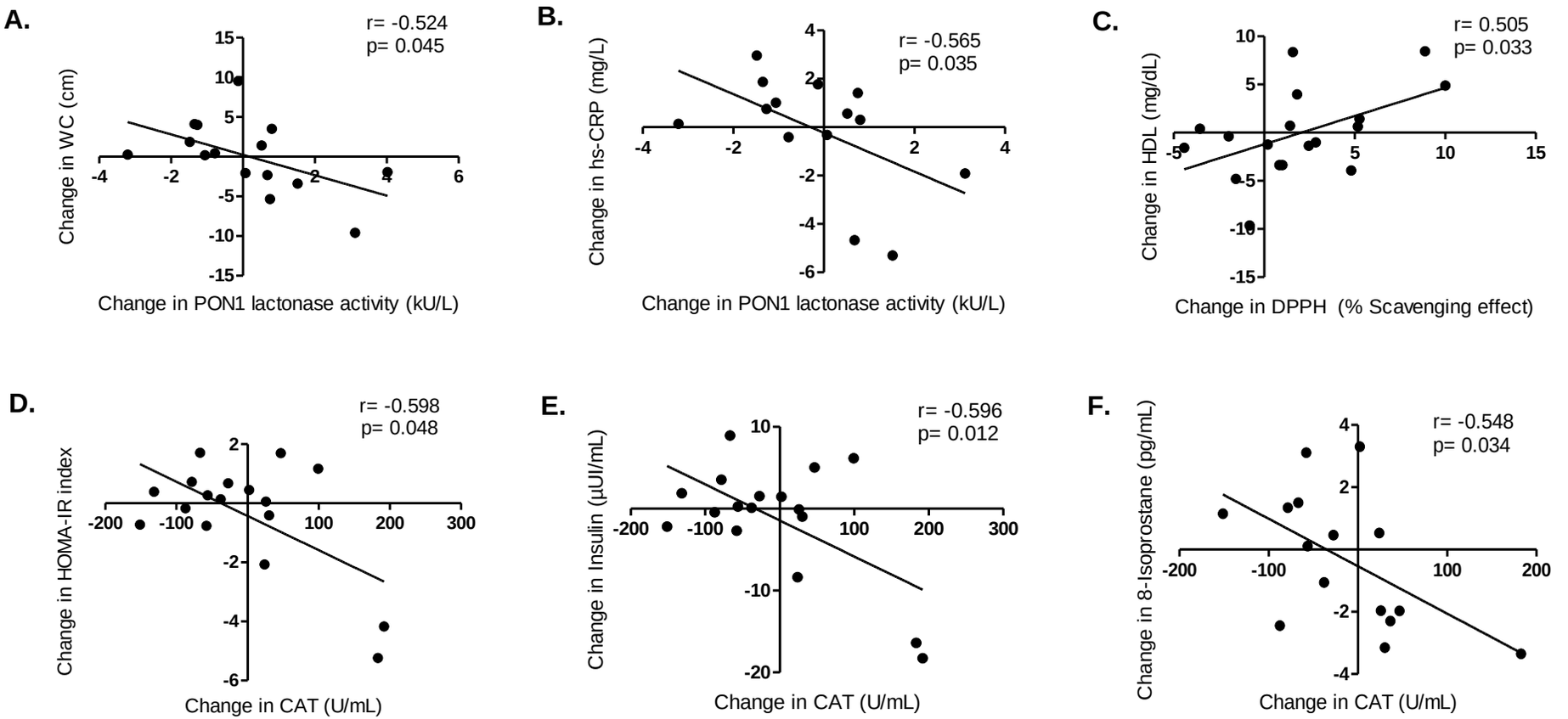

Figure 3. Correlations between changes in antioxidant markers and changes in cardiometabolic factors after agraz consumption, in obese women with MetS. Significance <0.05. A, C and F, Pearson correlations; B, D and E, Spearman correlations

Abbreviations. WC, waist cercumference; PON1, paraoxonase 1; hs-CRP, high sensitivity- C reactive protein; HDL-c, high-density lipoprotein-cholesterol; HOMA-IR, Homeostatic Model Assessment for Insulin Resistance index; DPPH, 2,2-Diphenyl-1-Picrylhydrazyl; CAT, catalase.

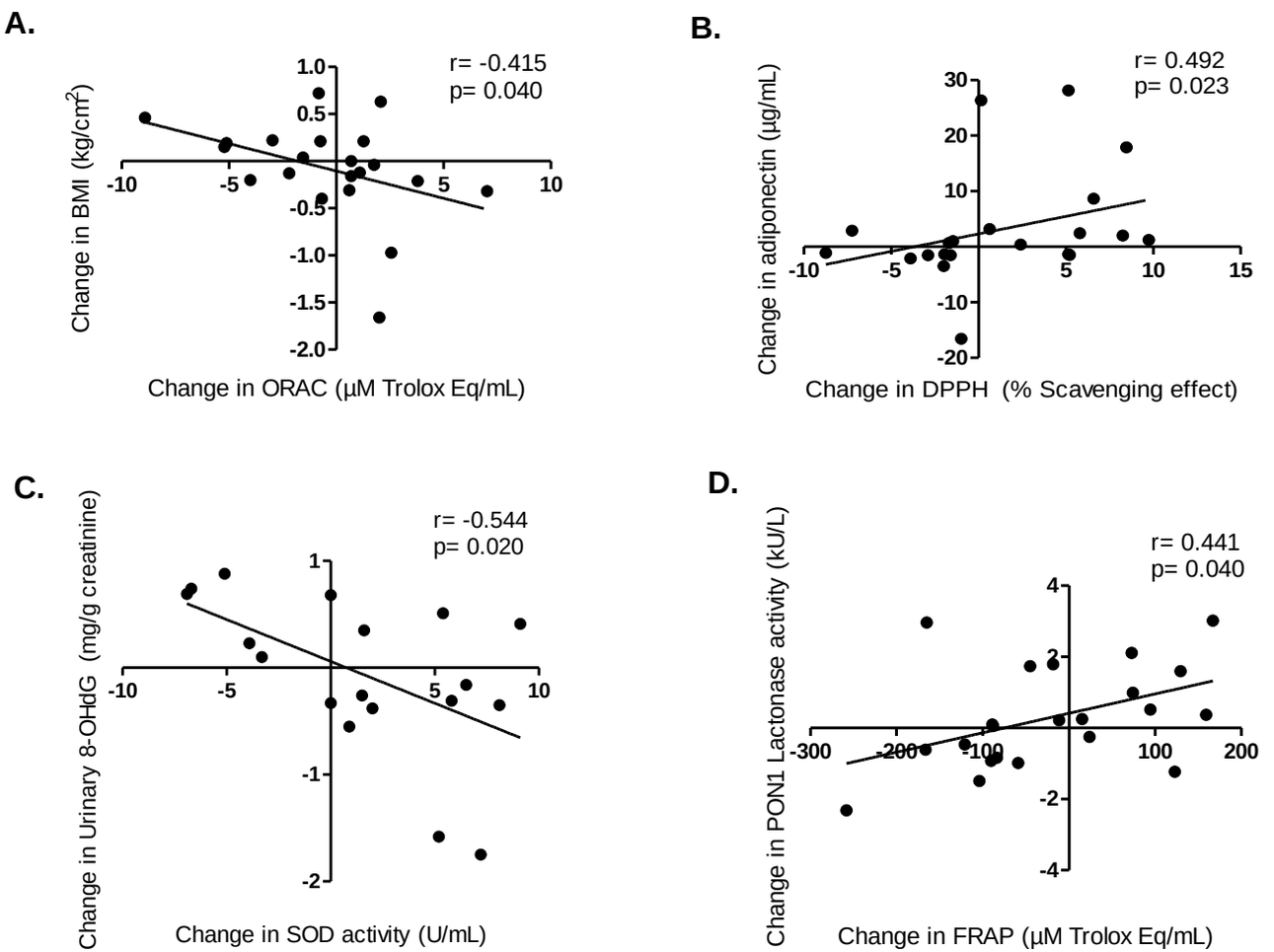

Figure 4. Correlations between changes in antioxidant markers, cardiometabolic factors and oxidative stress markers, after agraz consumption, in overweight women with MetS. Significance $p<0.05$; A and B Spearman correlations; C and D Pearson correlations.

Abbreviations. BMI, body mass index; ORAC, Oxygen radical absorbance capacity; DPPH, 2,2-Diphenyl-1-Picrylhydrazyl; PON1, paraoxonase 1; SOD, superoxide dismutase; 8-OHdG, 8-hydroxy 2 deoxyguanosine; FRAP, ferric reducing ability of plasma.

CHAPTER 6

Conclusions and perspectives

To the best of our knowledge, this intervention is the first evaluating the effects of the chronic consumption (4 weeks) of *V. meridionale* in women with metabolic syndrome (MetS). Therefore, there were no antecedents about the dose or time of intervention to be assessed with this fruit, for which, the daily dose used in this study had the purpose of simulate the effects of a habitual dose of juice consumption. The results obtained in this study were not influenced by changes in macronutrient intake or exercise. In addition, there was a high adherence to the study, indicating the daily consumption of the beverage by the participants.

We evaluated the effects of *V. meridionale* on high-density lipoprotein (HDL) function markers, oxidative stress (OxS) and low-grade chronic inflammation, given that MetS is strongly linked with those alterations.

We observed that after 4 weeks of *V. meridionale* consumption in the whole group of women there was a significant positive relationship between HDL function markers [i.e. paraoxonase 1 (PON1) activity and cholesterol efflux capacity], and a negative correlation between HDL function markers and inflammatory markers. These correlations could be mediated by the capacity of some polyphenols to modulate some signaling pathways involved in: a) the increase of PON1 expression and activity, which is implicated in the regulation of cholesterol efflux capacity; and b) inhibition of the nuclear translocation of the nuclear factor κB (NF-κB), with a reduction in pro-inflammatory protein expression and levels. However, mechanistic studies will be necessary to demonstrate the molecular effect of *V. meridionale* in this regard.

In this study we demonstrated the response to *V. meridionale* consumption was different in some individuals, which indicates that individual variability should be taken into consideration when analyzing the beneficial effects of this fruit and other human interventions. In these regard, overweight women had better anti-inflammatory effect with reduction in C-reactive protein levels. It is possible that obese subjects, with a more inflammatory environment, need to make more drastic changes in their lifestyle or receive longer treatments to achieve more consistent results in these markers. We also showed that women who increased their endogenous antioxidant activity, improved several cardiovascular risk factors, such as reductions in lipid and DNA oxidation, total cholesterol and low-density lipoprotein cholesterol levels, and waist

circumference. These differential responses could be mediated by factors such as intestinal microbioma, food matrices, absorption, genetic variation, among others, however more studies will be necessary in this regard.

In the sub-groups analysis, although both overweight and obese women improved their antioxidant status after *V. meridionale* consumption, there were different responses. While in overweight women there was a tendency to improve serum total antioxidant capacity (TAC), in obese women there was a reduction in 8-hydroxy-2' -Deoxyguanosine (8-OHdG) levels, a biomarker of DNA oxidative damage associated with cardiovascular diseases. These effects could indicate a greater neutralization of radicals in serum through donation of an electron and/or a hydrogen by antioxidant molecules present in the fruit, avoiding oxidation or damage of molecules of biological importance, even in condition of high oxidative stress (OxS).

In spite of the positive results of this intervention, in the whole group, there were not significant differences in HDL function, inflammation, oxidative stress and antioxidant markers, after comparing the end of both periods (placebo versus *V. meridionale*). It is important to know the women included in this study were at high risk for cardiovascular diseases, with HDL dysfunction, oxidative stress and low-grade systemic and chronic inflammation. The dose used in this study was provided as a regular dose of juice consumption in our country. Therefore, it could be necessary to explore higher doses and/or longer interventions to promote significant changes in this population.

This study presented some limitations. The method used for the beverage assignation was the alternating quasi-randomization allocation method, in which, one subject received the placebo, and the next received the *V. meridionale* beverage, and so on. We selected this method to reduce bias due to behavioral issues (collective holidays, climate conditions along the year). In addition, the sample size was small, which reduces the possibility to detect associations in some of the studied variables.

In spite of the limitations, this study had several strengths such as: a) the crossover design used, which requires a smaller sample size compared with a parallel design, and it also reduces the inter-individual variation because each women was her own control, reducing the influence of confounders; b) the double blind study design, which diminishes bias in the analysis due to participants or investigator's expectations; c) the design of a placebo with similar organoleptic and physicochemical characteristics to the agraz beverage, but lacking polyphenols. The use of a true placebo is important to differentiate the real effects of the agraz consumption from the possible effects associated with the belief in the beverage´s ability to cure or improve health; d) the use of a food frequency questionnaire designed and validated previously in the University of Antioquia (the place where the study was carried out), which allowed to evaluate the kilocalories and macronutrients consumed locally before and during the study, in order to determine that diet did not influence the results of the study; e) monitoring physical activity using a seven-day physical activity record at baseline and end of each intervention period, allowing to determine not changes during the study; and f) the use of a weekly adherence questionnaire, which allowed to verify the high adherence of the participants to the study, indicating the daily consumption of the beverage in each intervention period and the abstinence of other polyphenol-rich foods.

In addition, this is one of the first human interventions with *V. meridionale* in women at high cardiovascular risk, providing the basis for future studies.

CHAPTER 7

Appendices

Escuela de Microbiología - Escuela de Nutrición y Dietética –

Línea alternativas terapéuticas y alimentarias, Grupo de Ofidismo

Anexo 1. Consentimiento informado para participar en un estudio

Título del estudio: "Evaluación de los efectos del agraz colombiano *Vaccinium meridionale* Swartz sobre dislipidemias, hipertensión, inflamación y estrés oxidativo en mujeres con Síndrome metabólico".

Investigador principal: Jacqueline Barona A., Bacterióloga, MSc., PhD. Ciencias Nutricionales

Co-investigadores: Juan Carlos Aristizábal R., Nutricionista, MSc., PhD. Vitelbina Núñez R., Bacterióloga, MSc., PhD. Gelmy Ciro, Ingeniera de alimentos, PhD.

Financia: Colciencias Convocatoria 657-2014, Universidad de Antioquia.

Introducción

Usted está invitado para participar en un estudio que investigará los efectos de un jugo de agraz (mortiño) o un placebo en su colesterol, triglicéridos, presión sanguínea, y en los efectos protectores del agraz comparado con un placebo para disminuir su riesgo de diabetes (azúcar aumentada en la sangre) y enfermedad cardiovascular.

Este consentimiento informado le dará la información necesaria para entender por qué este estudio se está realizando y por qué usted está invitado a participar. También describirá qué necesita usted para participar y cualquier riesgo conocido, incomodidades o inconveniencias que pueda tener mientras esté participando en este estudio. Usted puede hacer preguntas respecto al estudio en cualquier momento. Si usted decide participar, le pediremos que firme este formato de consentimiento informado, el cual será un registro de que usted está de acuerdo en participar. Se le dará el original de este formato.

¿Por qué se está realizando este estudio?

El síndrome metabólico afecta aproximadamente entre un 20-30% de la población adulta en cada país. En Colombia hay muy pocos estudios sobre la prevalencia (frecuencia) del síndrome metabólico, y en Medellín se encontró un alto número de

personas con este síndrome, incluso en personal del área de la salud. Este síndrome está relacionado con obesidad, desórdenes de los lípidos sanguíneos, acumulación de placas de grasa en las arterias (aterosclerosis), enfermedades del corazón y predisposición a diabetes tipo 2. Adicionalmente, a medida que las personas envejecen, ellas incrementan la producción de radicales libres (sustancias oxidantes) y la integridad de sus vasos sanguíneos puede alterarse, los cuales son factores importantes en el desarrollo de aterosclerosis, presión alta y falla cardíaca. El propósito de este estudio es evaluar los efectos de un jugo de agraz en una población clasificada con síndrome metabólico, quienes están en alto riesgo de sufrir diabetes tipo 2 y enfermedad coronaria.

¿Cuáles son los procedimientos del estudio? ¿Qué me pedirán que haga?

Visita informativa: el objetivo de esta visita es brindarle toda la información necesaria para que usted tome la decisión o no de participar en este estudio. Se le explicarán todos los procedimientos experimentales y el compromiso de tiempo para usted. Esta visita durará aproximadamente 20 minutos durante los cuales usted leerá detenidamente este documento de consentimiento. Estaremos atentos para solucionar cualquier duda que tenga antes de tomar la decisión de participar.

El estudio consta de dos partes:

Visita de tamizaje e ingreso al estudio: Esta visita se realizará en el edificio de la Sede de Investigación Universitaria (SIU) de la Universidad de Antioquia y durará aproximadamente 25 minutos. Una vez usted haya leído y firmado este formato de consentimiento le solicitaremos inicialmente que nos permita obtener una muestra de sangre en ayunas. Le pediremos que llene un formato de su historia médica y le mediremos su presión arterial, perímetro de cintura, altura y peso corporal para determinar si usted cumple con los criterios de inclusión, es decir si tiene síndrome metabólico y puede participar.

Criterios de Inclusión

-Edad: 25 a 60 años.

-Sexo femenino

-Tener síndrome metabólico si usted tiene 3 de las siguientes características:

Perímetro de cintura ≥88 cm para mujeres

Triglicéridos ≥150 mg/dL

HDL colesterol <50 mg/dL en mujeres

Presión arterial ≥130/85 mm Hg

Glucosa plasmática (azúcar sanguínea) de ayuno ≥100 mg/dL.

Criterios de Exclusión

Usted será excluída y no podrá ingresar al estudio, si usted tiene cualquiera de lo descrito a continuación:

Si usted tiene enfermedad renal, diabetes, o enfermedad cardiaca.

Si sus triglicéridos son □500 mg/dL, su glucosa (azúcar) es □126 mg/dL o ha sido diagnosticado con diabetes, su colesterol LDL es □190 mg/dL o sus valores de presión arterial son >140/90 mmHg (en más dos visitas). En estos casos, usted requiere tratamiento médico y/ó farmacológico.

Si usted consume medicamentos para disminuir los lípidos sanguíneos (ej. Lovastatina, gemfibrozil), para disminuir el azúcar en sangre (ej. Metformina), o para bajar la presión sanguínea (ej. Hidroclorotiazida).

Si usted fuma.

Si usted consume licor en exceso (más de dos tragos/copas diarias).

Si usted está tomando aspirina, warfarina u otro medicamento para anticoagular la sangre tales como motrin, ibuprofeno, plavix, naproxeno, dipiridamol, y cualquier otro medicamento anti-inflamatorio.

Si está embarazada o planea embarazarse. Si está tomando terapia de reemplazo hormonal.

Si usted es deportista de alto rendimiento.

Si usted consume suplementos o nutracéuticos.

Si usted cumple con los criterios de inclusión, le pediremos que registre su dieta o lo que consume habitualmente y la actividad física que realiza usualmente. Le entregaremos instrucciones y le mostraremos cómo llenar un registro de toda la comida y bebida que consume (dieta) durante 3 días; y de su actividad física por una semana. Se le pedirá que complete estos registros de consumo y actividad física 3 veces más durante el estudio.

Período experimental: este estudio durará 12 semanas, durante las cuales usted recibirá dos tratamientos que consisten en un jugo natural de la fruta llamada mortiño y de un refresco similar a éste. Usted será asignado ciegamente y al azar para consumir el placebo o el jugo de agraz durante 30 días. Después de este tiempo, habrá un período de “lavado” (sin consumir ningún tratamiento) de 4 semanas y usted será cambiado al tratamiento alternativo. Tanto el placebo como el jugo de agraz estarán marcados como A ó B, y usted no sabrá cuál es el que está consumiendo ni en qué orden, pero usted recibirá ambos tratamientos en diferentes períodos. Durante los períodos de tratamiento, usted completará tres (3) registros de su dieta durante 3

días (un registro al final de cada período y después del “lavado”) y tres registros de actividad física durante 7 días (uno en cada período y luego del lavado). Igualmente, anotará diariamente en formatos que le entregaremos, en qué momento consumió el placebo o el jugo de agraz y cualquier evento de salud que pueda tener, por ejemplo: gripe, vómito, diarrea, fractura, etc., y cualquier medicamento que haya tenido que consumir, ya que es necesario valorar su participación en el estudio.

Usted deberá abstenerse de consumir cualquier tipo de agraz, arándanos, uvas, fresas, cerezas, té, y vinos durante todo el estudio, incluyendo el período de lavado. Usted deberá continuar con el resto de su dieta (lo que consume habitualmente) y nivel de actividad física usual.

Usted necesitará venir al edificio de la SIU o a la IPS Universitaria una vez cada dos semanas para medir su peso, y acordaremos con usted el mejor horario para consumir diariamente su tratamiento (sea el placebo o jugo de agraz), excepto durante las 4 semanas de lavado.

Durante esta fase experimental del estudio, necesitaremos extraerle sangre venosa durante 3 veces, una muestra de sangre después de las primeras 4 semanas, una muestra de sangre después del período de lavado y una muestra de sangre al final del estudio (semana 12). El total de sangre extraída durante todo el estudio será aproximadamente de 50mL (equivalente a 7 cucharadas). El compromiso de tiempo durante cada extracción de sangre será de alrededor 15 minutos. Las extracciones de sangre se realizarán en el laboratorio de la IPS Universitaria-Sede Prado. Igualmente, le pediremos que colecte su orina durante 24h en dos ocasiones, al final de cada periodo de consumo (jugo y placebo).

<u>¿Qué otras opciones existen?</u>

Este estudio de intervención no tiene la intención de reemplazar ningún tratamiento clínico. Pero, se ha demostrado que pequeñas modificaciones en la dieta, como el aumento en la ingesta de frutas y verduras, representan una alternativa eficaz y viable para personas que pueden estar en riesgo de padecer diabetes o enfermedades del corazón. Dado que no siempre es posible consumir gran cantidad de frutas, la dosis de jugo de agraz que se utilizará en este estudio representa una opción más práctica.

<u>¿Cuáles son los riesgos o inconvenientes del estudio?</u>

Se le pedirá que done sangre venosa 5 veces durante el estudio (incuyendo el tamizaje) después de un ayuno de 12 horas. La flebotomía o extracción de sangre será programada entre 7 a 9 de la mañana. Un profesional del laboratorio clínico será quien le haga la extracción de sangre, por lo que se espera un número mínimo de

moretones. Si usted se siente débil o mareado durante la extracción de sangre, tenemos la experiencia y una camilla para ayudar a que usted se sienta mejor.

Se le pedirá que colecte en dos ocasiones, una muestra de orina durante 24h continuas. Para facilitar la recolección de orina es preferible que se encuentre en su casa, lo cual podría ser inconveniente.

Todos los participantes, independiente del grupo al cual sean asignados, no deben consumir cualquier tipo de agraz, arándano, uvas, moras, fresas, cerezas y deben evitar ingerir cualquier clase de vino y té durante todo el estudio (12 semanas).

¿Cuáles son los beneficios del estudio?

Al participar en este estudio, usted conocerá sus niveles de colesterol, triglicéridos y otros factores de riesgo para diabetes tipo 2 y enfermedad cardiovascular y sabrá cómo cambian durante el tratamiento con el jugo de agraz. Así mismo, esperamos que su participación en este estudio pueda ayudar a determinar cómo el agraz afecta el colesterol en la sangre y puede reducir la formación de radicales libres (sustancias oxidantes) y mejorar la funcionalidad de los vasos sanguíneos en esta población específica de hombres de 25-60 años.

¿Voy a recibir algún pago por mi participación? ¿Existen costos por participar?

Usted no recibirá ninguna retribución económica, pero se le entregará y explicará de forma gratuita todos los resultados de los exámenes de laboratorio que se le realicen. No existe ningún costo por participar.

¿Cómo se protegerán mi información personal?

Todos los datos recogidos serán confidenciales y nunca se identificará su nombre cuando se divulguen los resultados. Además, no se compartirán los resultados con ninguna persona externa a la investigación sin su consentimiento. Todos los registros y los resultados de este estudio se mantendrán guardados en un lugar seguro en el edificio de la SIU (piso 6 Lab 631) hasta por 10 años. Sólo los investigadores tendrán acceso al código utilizado para identificarlo. Además, las muestras de sangre sobrantes se conservarán durante un período hasta de 3 años. En dichas muestras se pueden realizar otras mediciones, para lo cual los investigadores de este estudio solicitan su autorización, firmando este consentimiento, para emplearlas en estudios posteriores. En la conclusión de este estudio, los investigadores pueden publicar sus hallazgos. La información se presentará en formato resumido y usted no será identificado en publicaciones o presentaciones.

El Comité de Bioética de la SIU puede inspeccionar los registros del estudio como parte de su programa de auditoría, pero estos comentarios sólo se centrarán en los investigadores y no en los participantes del estudio. El Comité de Bioética es un grupo de expertos que supervisa los estudios de investigación para proteger los derechos y el bienestar de los participantes de la investigación.

¿Qué sucede si me lesiono o me enfermo durante la participación en el estudio?

No se espera que usted se enferme por participar en este estudio, dado que el tratamiento consiste en ingerir un jugo natural de fruta o un refresco similar y donará 5 muestras de sangre durante los 3 meses que dura el estudio. Sin embargo, en el caso de que usted se lesione o enferme en el transcurso de la investigación le solicitamos que lo reporte en el diario de consumo del producto. En caso de que necesite asistencia médica, debe buscar inmediatamente atención clínica con la EPS a la cual usted esté afiliado. Seguidamente, debe notificar al investigador principal o a un miembro del equipo de investigación, dado que el tratamiento que eventualmente le prescriban puede afectar su participación en el estudio.

¿Puedo dejar de participar en el estudio y cuáles son mis derechos?

Usted no tiene que participar en este estudio, si no quiere. Si usted acepta participar en el estudio, pero después cambia de opinión, se puede retirar en cualquier momento. No hay consecuencias de ningún tipo ni sanciones si decide que no quiere participar más. Se le pedirá que abandone el estudio si su cumplimiento con la ingesta del jugo o placebo es inferior al 80%. Se le notificará de cualquier hallazgo importante durante el curso del estudio que pueda afectar su participación en el mismo.

¿A quién contacto si tengo preguntas sobre el estudio?

Tómese el tiempo que necesite antes de tomar una decisión. Estamos atentos para responder cualquier pregunta que usted tenga acerca de este estudio. Si tiene más preguntas sobre este estudio o si usted tiene un problema relacionado con el mismo, puede comunicarse con el investigador principal: Jacqueline Barona, tel. 219 5493, o los co-investigadores (Juan Aristizábal, tel. 219 9224; Vitelbina Núñez, tel. 219 6536; Gelmy Ciro, tel. 19 6536). Si usted tiene alguna pregunta acerca de sus derechos como voluntario de la investigación, puede comunicarse con el Comité de Bioética de la SIU, Universidad de Antioquia, tel. 219 66 47.

Documentación del consentimiento:

He leído libremente este formulario y he decidido participar en el estudio descrito anteriormente. El propósito general, los detalles de la participación y los posibles riesgos e inconvenientes han sido explicados a mi satisfacción. Entiendo que puedo

retirarme en cualquier momento. Recibiré el original de este documento y la copia será conservada por los investigadores.

Nombre completo Participante	Firma del Participante	Fecha

Teléfonos: Oficina_____Casa_____Celular _____

Dirección:_____

Nombre completo - Testigo 1	Firma Testigo 1	Fecha

Parentesco:_Teléfonos:_____

Dirección:_____

Nombre completo - Testigo 1	Firma Testigo 1	Fecha

Parentesco:_Teléfonos:_____

Dirección:_____

Nombre completo del investigador que obtiene el consentimiento	Firma del investigador	Fecha

Nombre abreviado del estudio: “Evaluación de los efectos del consumo del agraz (mortiño) colombiano *Vaccinium meridionale* Swartz en hombres y mujeres con Síndrome metabólico”.

Anexo 2. Visita de tamizaje

Fecha:_____Número (código) asignado: _____

Nombre completo del participante: _____

Números telefónicos: trabajo _____Casa: _____Celular: _____

Correo electrónico: _____

Fecha de nacimiento:_____Edad: _____

Presión arterial (mm Hg):_____Pulso: _____

Talla (metros):_____ Peso (Kg):_____

IMC:_____(kg/m^2)

Perímetro abdominal (cm):_____Promedio P. abdominal: _____

Datos de laboratorio:

Analito	**Resultados (mg/dL)**	**Intervalo biológico de referencia (IBR)**
Colesterol total		< 200 mg/dL
LDL-C		< 100 mg/dL
TG		< 150 mg/dL
HDL-C		> 40 mg/dL (hombres)
Glucosa		70-99 mg/dL

Escuela de Microbiología - Escuela de Nutrición y Dietética - Línea de alternativas terapéuticas y alimentarias, Grupo de Ofidismo

Nombre abreviado del estudio: "Evaluación de los efectos del consumo del agraz (mortiño) colombiano *Vaccinium meridionale* Swartz en hombres y mujeres con Síndrome metabólico".

Anexo 3. Cuestionario de Historia Médica

Instrucciones: Por favor llene este formulario con la información más precisa posible.

Nombre:_____Fecha:_____

Dirección:_____

Números telefónicos
Casa:_____trabajo:_____Celular:_____

Correo electrónico:
_____

Edad:_____Fecha de nacimiento (dd/mm/año)_____

Estatura:_____ Peso _____

Medicamentos:

¿Cuáles medicamentos mandados por el médico toma usted? (si es mujer incluya el uso de anticonceptivos).

_____

_____

_____

_____

¿Cuáles medicamentos **no** mandados por el médico toma usted?

_____

Alergias:

¿Sufre usted alergias? Por favor, indique si tiene alergias a medicamentos, alimentos o a factores ambientales.

_____

_____

_____

¿Cuáles son sus valores de colesterol y triglicéridos (si se los han realizado recientemente)?

Colesterol_____(estimación) Triglicéridos_____ (estimación)

¿Es usted vegetariano? Sí_____ No_____

Sí es vegetariano, por favor diga que alimentos evita

_____

_____

_____

_____

¿Es usted fumador? Sí No_____

_____

Sí fuma, ¿cuántos paquetes por día?_____1 o menos de uno _____2 o más por día?

Sí usted era fumador,

¿Cuantos años fumó antes de parar de fumar?_____años Cuando dejo de fumar? _

¿Consume usted licor? Sí_____Cantidad:_____Frecuencia:_____

Tipo de licor:_____No consume _____

¿Ha sufrido de alguna de estas enfermedades? Señale todas las que haya sufrido

Enfermedad renal ( )	Diabetes ( )
Enfermedad cardíaca ( )	Dolor de pecho ( )
Colesterol elevado ( )	Enfermedad tiroidea ( )
Fiebre reumática ( )	Murmuros cardiacos ( )
Hipertensión ( )	Látidos cardiacos anormales ( )
Enfermedad hepática( )	Falta de respiración ( )
Aritmias cardiacas ( )	Calambres en las piernas sin hacer ejercicio ( )
Asma ( )	Falta de respiración sin hacer ejercicio ( )
Huesos dislocados o quebrados ( )	Tos con silbido ( )
Bronquitis crónica ( )	Tos con sangre ( )
Neumonia a repetición ( )	Pérdida de conciencia ( )
Tos crónica ( )	Mareos ( )
Anemia ( )	Enfermedad aguda febril ( )
Desmayo durante el ejercicio ( )	Epilepsia ( )
Golpe de calor durante ejercicio ( )	Cancer ( )

Si marco alguna de las enfermedades anteriores, diga de forma general, como fue su enfermedad:

_____

_____

_____

_____

_____

¿Ha sufrido alguna otra enfermedad o condición médica la cuál nosotros debamos saber?

Si____No_____explique:

_____

Suplementos Dietarios:

¿Ha consumido usted suplementos dietarios (multivitaminas, calcio, hierbas medicinales, productos con efedrina, creatina, etc.). Por favor, diga cuándo, cuales suplementos y cantidad.

Por favor, liste los suplementos que consume regularmente, incluya marca y cantidad.

Ejercicio

¿Practica usted algún deporte?_____Sí_____No

Si su respuesta fue "Sí", por favor describa su rutina actual de ejercicio, incluya el tipo, la frecuencia y cantidad. Además indique si realiza ejercicio el fin de semana o también durante la semana.

Historia Familiar

Sus parientes sanguíneos han sufrido alguna de las siguientes enfermedades? (señale todas las que hayan sufrido)

	Padres	Abuelos	Hemanos (as)
Diabetes	( )	( )	( )
Enfermedad Cardíaca	( )	( )	( )
Colesterol elevado	( )	( )	( )

Enfermedad renal	( )	( )	( )
Epilepsia	( )	( )	( )
Paro cardiaco	( )	( )	( )
Hipertensión	( )	( )	( )
Enfisema	( )	( )	( )
Cancer	( )	( )	( )
Obesidad	( )	( )	( )

Existe alguna otra condición médica en la familia que nosotros debamos conocer?

Si_____No_____Explique:

_____

Yo, certifico que la información aquí reportada es completa, veraz y precisa, además que da cuenta de mi historia médica de acuerdo a mis conocimientos.

Nombre completo:

_____

Firma: _____

Fecha: _____

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Anexo 4. Alimentos que no deben ser consumidos durante las doce semanas que dura el estudio

			
Otros arándanos	**Uvas**	**Moras**	**Fresas**
			
Ciruelas	**Uvas pasas**	**Cerezas**	**Frambuesas**
			
Nueces	**Linaza**	**Alcaparras**	**Mermeladas y salsas de estos alimentos**
			
Vino	**Té negro**	**Té verde**	**Otras preparaciones donde se incluyan estos alimentos**

Anexo 5. Cuestionario de frecuencia de consumo

Fecha: _____ Nombre: _____COD: _____Periodo: _____

Por favor revise cada uno de los alimentos de la lista; registre el número de porciones consumidas según sea el caso; es decir si lo consumió en el último mes D (diario), S (semanal) ó M (mensual), si el alimento no lo consumió en el último mes, coloque una "X" debajo de la letra "N". Para orientarse acerca del tamaño de las porciones puede orientarse con el albúm de imágenes, el número de porciones consumidas se pueden registrar en números enteros o fracciones.

	Alimento	Frecuencia de C.			
		N	D	S	M
1. LÁCTEOS	Leche de vaca entera (incluye deslactosada) / 1 vaso pequeño				
	Leche de vaca semidescremada (incluye deslactosada)/1 vaso pequeño				
	Leche de vaca descremada (incluye deslactosada) / 1 vaso pequeño				
	Leche en polvo entera (incluye deslactosada) / 1 cuch. dulcera colmada				
	Leche en polvo descremada (incluye deslactosada) / 1 cuch. dulcera colmada				
	Yogurt entero / 1 vaso o bolsita estándar				
	Yogurt light / 1 vaso o bolsita estándar				
	Cuajada / 1 tajada delgada				
	Queso crema / 1 cucharada tintera alta				
	Queso mozarrella / 1 tajada delgada				
	Queso duro / 1 tajada delgada				
	Queso blando o fresco / 1 tajada delgada				
	Queso lonchita / 1 unidad				
2. HUEVOS, CARNES, PESCADOS	Huevo de gallina / 1 unidad				
	Pollo CON piel / 1 pieza o ración pequeña				
	Pollo SIN piel / 1 pieza o ración pequeña				
	Carne de res / 1 ración pequeña, 1/8 libra				
	Carne de cerdo / 1 ración pequeña, 1/8 libra				
	Chicharrón / 1 ración pequeña				
	Hígado (res, cerdo, pollo) / 1 ración pequeña, 1/8 libra				
	Otras vísceras (sesos, corazón, mollegas) , 1/8 libra				
	Chorizo / 1 unidad mediana				
	Salchichón / 1 tajada mediana				
	Morcilla / 1 unidad				
	Mortadela / 1 unidad				
	Jamón tajado / 1 unidad				
	Salchicha / 1 unidad pequeña				
	Carne de hamburguesa (1 unidad), albóndigas (3 unidades pequeñas)				
	Bagre / 1 filete				
	Trucha / 1 unidad pequeña				
	Tilapia / 1 filete				

	Alimento	Frecuencia de C.			
		N	D	S	M
	Salmón / 1 filete				
	Ostras, ostiones, almejas, mejillones y similares / 6 unidades				
	Calamares, pulpo / 1 ración				
	Crustáceos: camarones, langostinos / 4-5 piezas				
	Pescados enlatados en agua: sardinas, atún / 1 lata pequeña				
	Pescados enlatados en aceite: sardinas, atún / 1 lata pequeña				
3. LEGUMINOSAS Y HARINAS	Lentejas / 1 cucharón mediano				
	Fríjoles / 1 cucharón mediano				
	Garbanzos / 1 cucharón mediano				
	Soya / 1 cucharón mediano				
	Tostadas / 1 unidad				
	Pan blanco / 1 tajada				
	Pan integral / 1 tajada				
	Almohabana, pandebono, pandequeso / 1 unidad mediana				
	Buñuelo / 1 unidad pequeña				
	Empanada / 1 unidad				
	Pastel de pollo / 1 unidad				
	Papa rellena / 1 unidad				
	Panzerotti / 1 unidad				
	Palo de queso / 1 unidad				
	Pastel de queso, jamón, hawaiano/ 1 unidad				
	Galletas de sal: dux, club social / 1 paquete				
	Cereales para el desayuno: zucaritas, muesli, copos de avena, all-bran, granola / 1 pocillo				
	Arepa tela / 1 unidad delgada				
	Choclo 1 unidad pequeña o tierno enlatado medio pocillo				
	Arroz blanco / 1 pocillo chocolatero				
	Pasta:fideos, macarrones, espaguetis, otras / ½ pocillo				
	Pizza /1 ración				
	Plátano verde o maduro / ½ unidad mediana				
	Papa / 1 unidad mediana o 3 criollas pequeñas				
	Yuca / 1 trozo mediano				
	Arracacha /1 trozo mediano				
4. FRUTAS	Naranja / 1 unidad pequeña				
	Mandarina / 1 unidad mediana				
	Banano / 1 unidad pequeña				
	Manzana o Pera con cáscara / 1 unidad				
	Fresas ó frambuesas / 8 unidades				
	Sandía, melón, piña / 1 rebanada				
	Papaya, papayuela / 1 rebanada				
	Uvas rojas o verdes / 10 unidades				

	Alimento	Frecuencia de C.			
		N	D	S	M
	Mango / 1 unidad mediana				
	Guayaba / 1 unidad mediana				
	Guanábana / 2 cucharadas soperas				
	Tomate de árbol / 1 unidad pequeña				
	Mora / 8 unidades				
	Arándanos				
	Cerezas				
	Ciruelas / 2 unidades pequeñas o común 12 unidades				
	Granadilla / 1 unidad				
	Maracuyá / 1 unidad				
	Limón / 1 unidad pequeña				
5. VERDURAS Y HORTALIZAS	Espinaca / 1 pocillo				
	Col, coliflor, brócoli / ½ pocillo				
	Lechuga / 1 pocillo				
	Repollo / ½ pocillo				
	Tomate rojo / 1 unidad mediana o medio pocillo				
	Tomate verde / 4 rodajas				
	Zanahoria / ¼ pocillo				
	Remolacha / ¼ pocillo				
	Pepino / ½ pocillo				
	Cebolla / ½ unidad mediana o 3 cucharadas soperas				
	Setas, champiñones / ½ pocillo				
	Verduras enlatadas / 1 lata				
6. ACEITES Y GRASAS	Frutos secos: maní, almendra, nueces / ½ pocillo tintero				
	Aceite vegetal: oliva, maíz, girasol, soja, canola, mezclas / 1 cucharada sopera				
	Aguacate / 1/8 unidad				
	Margarina / 1 cucharadita				
	Mantequilla / 1 cucharadita				
	Manteca vegetal / 1 cucharada sopera				
	Manteca de cerdo / 1 cucharada sopera				
7. DULCES Y POSTRES	Arequipe, mermelada / 1 cucharada sopera				
	Panelita de leche / 1 unidad				
	Bocadillo / 2 cubos				
	Chocolatina / 1 unidad pequeña				
	Galletas dulces / 1 paquete				
	Pasteles dulces guayaba, arequipe / 1 unidad				
	Torta dulce / 1 porción				
	Donas industrializadas / 1 unidad				
	Churros azucarados, churros rellenos / 1 ración				
	Brownie / 1 unidad				
	Helado / 1 bola pequeña				

	Alimento	Frecuencia de C.			
		N	D	S	M
	Caramelos, confites / 2 unidades				
	Salsas dulces o mermeladas de frutos rojos / 2 cucharadas soperas				
	Ciruelas pasas / 8 unidades medianas				
	Fruta en almíbar durazno, breva, cereza / 1 porción				
	Leche condensada / 2 cucharadas soperas				
8. MISCELÁNEA	Sopas y cremas de sobre / 1 plato				
	Mostaza / 1 cucharadita				
	Mayonesa comercial / 1 cucharada sopera				
	Salsa de tomate / 1 cucharadita				
	Sal (1 pizca)				
	Azúcar morena o blanca / 1 cucharada tintera o 1 sobre				
	Azúcar light / 1 cuchara tintera				
	Edulcorantes artificiales				
	Mecato tipo papitas, platanitos / 1 paquete				
	Rosquitas / 1 paquete				
9. BEBIDAS	Gaseosa con azúcar o malta / 1 botella				
	Gaseosa baja en calorías / 1 botella				
	Agua de fruta o sabor / 1 vaso				
	Agua panela / 1 taza				
	Jugos naturales de fruta y/o verdura / 1 vaso				
	Jugos de frutas en botella o enlatados / 1 vaso				
	Café / 1 pocillo				
	Chocolate / 1 pocillo				
	Bebidas energizantes / 1 lata				
	Bebidas hidratantes / 1 unidad				
	Vino tinto / 1 copa				
	Vino blanco / 1 copa				
	Cerveza / 1 botella				
	Licores: amaranto, café / 1 copa				
	Destilados: whisky, vodka, ginebra, tequila, aguardiente, ron / 1 copa				
	Té en infusión / 1 sobre				
	Alimento o suplemento	**Frecuencia de C.**			
		N	**D**	**S**	**M**

Ajustado: J. M.ª Monsalve Álvarez y L. I. González Zapata. Nutr Hosp. 2011;26(6):1333-134

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Anexo 6. Formato Registro Actividad física durante 7-días

Instrucciones para completar el formato

Es importante mantener un registro adecuado de su actividad física usual para participar en este estudio. Por favor anote toda la actividad física o ejercicio que realice durante 7 días consecutivos durante la última semana de consumo de su tratamiento. Es muy importante que mantenga el mismo nivel y las mismas actividades físicas que venía realizando antes de ingresar al estudio.

Nombre completo: _____Código (#)**:** _____

DIA / FECHA	**Tipo de actividad física** (Ejemplo: caminar, correr, jardinería, etc.)	**Cantidad de tiempo** (en minutos)
LUNES Fecha: _____		
MARTES Fecha: _____		
MIÉRCOLES Fecha: _____		
JUEVES Fecha: _____		
VIERNES Fecha: _____		
SÁBADO Fecha: _____		
DOMINGO Fecha: _____		

Para ser completado por el investigador

Cantidad total de tiempo/semana invertida en actividad física:_____ (minutos)

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Anexo 8. Registro de consumo del producto

Instrucciones para completar el formato (para llenar todos los días)

Es importante que usted registre el consumo del jugo (A ó B) que le estamos entregando. Para ayudarle a recordar y para asegurar que usted está consumiendo el producto, le pedimos que por favor anote a qué hora lo consumió cada día.

Nombre: _____ código: ____ Semana: ____ Período: 1 ___ 2 ___

FECHA (dd/mm/año)	**¿Consumió su jugo hoy?** (encierre en un círculo)	**¿Qué hora era cuando lo consumió?**	**Observaciones***
Lunes (dd/mm/año)	**Sí / No**		
Martes (dd/mm/año)	**Sí / No**		
Miércoles (dd/mm/año)	**Sí / No**		
Jueves (dd/mm/año)	**Sí / No**		
Viernes (dd/mm/año)	**Sí / No**		
Sábado (dd/mm/año)	**Sí / No**		
Domingo (dd/mm/año)	**Sí / No**		

*__Observaciones__: Por favor reporte cualquier inconveniente de salud que tenga (por ejemplo: gripe, fiebre, diarrea, dolor de cabeza, etc.) o cualquier medicamento que haya tenido que consumir (ejemplo: dolex gripa, ibuprofeno, omeprazol, etc.)