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MASTER THESIS

Lyophilized Maqui (*Aristotelia chilensis*) improves carbohydrate metabolic markers and insulin sensitivity in high fat diet-induced obese mice but not when supplemented with a normal diet in healthy mice

Nutritional Cell Signalling Research Group

Department of Nutrition, Food Science and Gastronomy

Faculty of Pharmacy and Food Sciences, Universitat de Barcelona

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RESPONSIBLE TEACHER:

Dra. Joana Relat Pardo, PhD

jrelat@ub.edu

STUDENT:

Lic. Carmen Castillo Serrato

ccastillo1396@gmail.com

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ABBREVIATIONS

cDNA	*complementary Deoxyribonucleic Acid*
Chrebpα/β	*Carbohydrate response element (ChRE)-binding protein alpha/beta*
C3G	*Cyanidin 3-glucoside*
Glut4	*Glucose transporter type 4*
Gm-csf	*granulocyte-macrophage colony-stimulating factor*
HFD	*High Fat Diet*
H_2O_{mq}	Ultrapure water
IL-6	*Interleukin 6*
IR	*Insulin Resistance*
MCP-1	*Monocyte Chemoattractant Protein 1*
Mmp-3	*Matrix Metallopeptidase 3*
mRNA	*messenger Ribonucleic Acid*
NAFLD	*Non-alcoholic Fatty Liver Disease*
PI3K/Akt	*phosphatidylinositol 3-kinase/protein kinase B*
RT	*Retrotranscriptase*
RT-qPCR	*Real time quantitative Polimerase Chain Reaction*
scWAT	*subcutaneous White Adipose Tissue*
T2DM	*Type-2 Diabetes Mellitus*
TNF-α	*Tumor necrosis factor alpha*
WAT	*White Adipose Tissue*
WB	*Western Blot*

ABSTRACT

Introduction: Most of the non-comunicable chronic diseases are mainly caused by metabolic disruptions such as insulin resistance (IR) and obesity. Adipose tissue is one of the primary tissues where IR develops. Polyphenolic compounds found in food such as citrus fruits, grapes, onions, berries, cherries and more, have been documented as possible anti-diabetic agents [1]. An example of polyphenols are anthocyanidins found in berries such as Maqui berry (*Aristotelia chilensis*). A previous study has shown an improvement towards insulin sensitivity by ameliorating the insulin response, decreasing weight gain and activating thermogenesis in subcutaneous white adipose tissue (scWAT) [2]. The study lacked results for a normal diet on healthy mice and a normal diet on healthy mice with Maqui berry supplementation.

Materials and Methods: Healthy male C57BL/6J mice (n = 51) of 4 weeks of age in controlled housing conditions in terms of temperature and light cycles, were randomly divided into 4 experimental groups: control group (CTL) with normal diet and filtered water (n = 13), control group (CTLM) with normal diet supplemented with maqui (20 mg of freeze-dried maqui/mL in water) (n = 14), high-fat diet (HFD) group (n = 12), high-fat diet supplemented with maqui (HFDM) group (n = 12). The preparation of the maqui berry was of 1 g of lyophilized maqui added to 50 mL in filtered water.

Results: The high content of anthocyanins was of 45,052 mg/kg which translates to 4,5%. Maqui increases the expression of *Glut4* in the scWAT of mice fed in a HFD but not in CTLM. There was a significant increased gene expression for both *Chrebpα and β* in HFDM in scWAT when compared to the control HFD.

Conclusion: Maqui berry supplementation failed to demonstrate significant results in a normal diet on healthy mice. This evidence helps to prove that people with cardiometabolic risks are more likely to benefit from polyphenols when included in a balanced diet than healthy individuals.

Keywords: Polyphenols; Anthocyanins; Insulin Resistance; Obesity; Glut4; Chrebp; White Adipose Tissue; Maqui berry

I. INTRODUCTION

Most of the non-comunicable chronic diseases are mainly caused by metabolic disruptions such as insulin resistance (IR) and obesity, which can arise in the population due to unhealthy eating habits, an increase in a sedentary lifestyle, stress and other habits. The relationship between the development of IR in chronic diseases has shown an increase in mortality and morbidity, becoming a major concern worldwide [3]. IR has been related to type 2 diabetes (T2DM), tumors, cardiovascular and cerebrovascular diseases, non-alcoholic fatty liver disease (NAFLD) and many other diseases. The molecular mechanisms behind IR are behind the metabolic processes of the action of insulin, an anabolic hormone secreted by the pancreas. Abnormalities in receptor binding, intracelullar factors, and disruptions in the intestinal microecology can influence this pathological state [3]. Adipose tissue is one of the primary tissues where IR develops, therefore highlighting an important difference amongst insulin receptors in other tissues, that can later on develop a systematic IR [3].

Carbohydrates are the main source of energy for cells, therefore being an essential macronutrient that is widely recommended to be consumed daily. However, the excessive and unbalanced carbohydrate intake can possess harmful effects leading to health issues. At a molecular level, elements such as glucose transporter type 4 (*Glut4*) and carbohydrate response element binding protein (*Chrebp*) are necessary for certain metabolic pathways [4]. When the secretion of insulin is stimulated by the presence of glucose derived from food, *Glut4* on the cell membrane is in charge of the glucose uptake [5]. Metabolites from glucose can activate *Chrebp*, a transcription factor necessary for regulating glucose metabolism and lipogenesis [4]. A chronic excess in carbohydrate intake can cause IR and other metabolic alterations such as an increased adipose triglyceride accumulation [4]. Therefore, the activation of *Chrebp* elements and the role of *Glut4* are crucial to understand carbohydrate metabolism for the present work.

Different strategies have been developed in order to prevent the appearance of these metabolic alterations. The well-known health science of Nutrition is focused on the research of improving the population's eating habits to improve their health. Dietary

interventions such as precision nutrition, have shown important results when individual phenotypes are assessed for a more focused eating plan [6]. Exercise interventions had also shown great effectiveness when in comes to IR and T2DM [7]. Another innovative strategy is the identification of bioactive compounds with an significant effect on the development of IR. Polyphenolic compounds found in food such as citrus fruits, grapes, onions, berries, cherries and more, have been documented as possible anti-diabetic agents [1]. It is therefore crucial to understand the effects of these bioactive compounds at a molecular level.

Another example of polyphenols are anthocyanidins. Anthocyanidins have demostrated to improve glucose uptake, by stimulating *Glut4*, via upregulation through different pathways such as phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) signaling pathway [8]. Anthocyanidins found in berries such as Maqui berry *(Aristotelia chilensis)*, which have demonstrated positive effects on individuals in the treatment of metabolic disorders by improving glycemia and insulinemia [9]. More importantly, it has been previously published by the present research group crucial results on animal experimentation with lyophilized Maqui berry in High Fat Diet-induced (HFD) obese mice [2]. The previous study has shown an improvement towards insulin sensitivity by ameliorating the insulin response, decreasing weight gain and activating thermogenesis in subcutaneous white adipose tissue (scWAT) [2]. This study lacked a normal diet on healthy mice and a normal diet on healthy mice with Maqui berry supplementation.

Anthocyanins such as cyanidin 3-glucoside have also shown positive results regarding inflammation. Anti-inflammatory properties were detected when supplementation with cyanidin 3-glucoside (C3G) was used in HFD obese mice, lowering RNA levels of inflammatory cytokines such as tumor necrosis-*α* (TNF-*α*), interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) in white adipose tissue (WAT) [10]. An interest in studying possible anti-inflammatory effects in scWAT using maqui berry supplementation, with a high level of such anthocyanins, has been proposed for other investigations. In this work, an analysis using Real-time quantitative Polimerase Chain Reaction (qRT-PCR) was used to measure the gene expression of inflammatory cytokines and growth factors related to senescence-associated

secretory phenotype (SASP) genes. However, results for this ongoing experimentation are not described in this work.

Therefore, the current study aims to understand the molecular mechanisms involved when Maqui berry is supplemented in a chow diet (normal diet) in healthy mice and compare this approach with the effects of the same supplementation of Maqui berry in a HFD regarding insulin resistance and carbohydrate metabolism.

The results from this work concluded that supplementation of maqui berry in healthy mice with a normal diet, has no significant effect when compared to the effects in HFD obese mice. This statement helps to understand how cardiometabolic models, such as a diet-induced obesity, have a better use of this supplementation rich in anthocyanidins than healthier models.

II. HYPOTHESIS AND OBJECTIVES

2.1 Hypothesis and Objectives

The hypothesis for this study is: if maqui berry has a positive effect on carbohydrate metabolism and insulin sensitivity in high fat diet-induced obese mice, it may also have a positive effect on mice on a normal diet. Therefore the following research objectives are:

- Determine if maqui has a similar effect regarding carbohydrate metabolism and insulin sensitivity on a normal diet as on a high fat diet in mice.
- Identify the differences between groups regarding the gene expression of *Glut4* and *Chrebpα* and *β* by RT-PCR.
- Compare the results obtained with current scientific evidence.

III. MATERIALS AND METHODS

3.1 Lyophilized maqui and animal processing

This part was already finished when this TFM started but as it is considered crucial to understand the results presented, it has been briefly described in this section.

Healthy male C57BL/6J mice (n = 51) of 4 weeks of age in controlled housing conditions in terms of temperature and light cycles, were randomly divided into 4 experimental groups: control group (CTL) with normal diet and filtered water (n = 13), control group (CTLM) with normal diet supplemented with maqui (20 mg of freeze-dried maqui/mL in water) (n = 14), high-fat diet (HFD) group (n = 12), high-fat diet supplemented with maqui (HFDM) group (n = 12). The HFD and HFDM groups had free access to the high-fat diet. In the case of the HFDM group, they also had free access to filtered water with maqui. The high-fat diet consisted of 45% of calories from fat. Regarding the preparation of the maqui, 1 g of lyophilized maqui was added to 50 mL in water [2]. The intervention lasted 16 weeks and they were subsequently euthanized.

Body weight, food and drink intake were recorded every other day twice a week during the intervention, as previously described [2]. Samples of different tissues are then extracted, including subcutaneous white adipose tissue (scWAT), which was used for this study, and subsequently stored at a temperature of - 80^{o}C.

3.2 RNA Extraction and RT (Retrotranscriptase)

The start of this work occurs with the receipt of scWAT tissue samples. The process began with the extraction of RNA from these samples using TRI Reagent^{TM} Solution (AM9738, Thermo Fisher Scientific, Waltham, USA). The RNA from scWAT was extracted from frozen scWAT using 1000 µL of TRI reagent solution added to each sample in a new eppendorf. The tissue was then crushed and centrifuged for 10 minutes at 12000xg at 4^{o} C. The supernatant is removed and placed in a new eppendorf where 200 µL of chloroform is added to each sample to separate the phases. After a mixing and centrifugation process of 12 minutes at 12000xg at 4^{o} C,

the supernatant is transferred to a new eppendorf. Then, 500 µL of isopropanol are added and centrifuged again for 8 minutes at 12000xg at 14º C. A total of 1 mL of 70% ethanol is added to the new supernatant and centrifuged but this time at 7500xg for 5 minutes at 14º C. The content is resuspended using 30 µL ultrapure water (H_2O_{mq}) and finally placed in a water bath for 5 minutes at 65º C. The final product is frozen at - 80º C until it is used for the next process.

Once the RNA is obtained, a total quantification of the samples is done to know the concentrations of each sample. The concentrations ranged from 100 ng/µL to 800 ng/ µL. Later, the *High-Capacity cDNA Reverse Transcription* (4368814, Thermo Fisher Scientific, Waltham, USA) kit was used for the synthesis of cDNA from 1µg of total RNA. The dosage used of each reagent were: 3 µL of *10xRT Buffer*, 1,2 µL of 25X *dNTP Mix* (100 mM), 3,0 µL of 10x *RT Random Primers*, 1,0 uL of *MultiScribe Reverse Transcriptase*, 6,8 µL of H_2O_{mq} for each sample. Controls were performed, an enzymatic control (without RT) and a DNA control (only with H_2O_{mq}). After completing this step, the plate was placed on the thermal cycler at different ranges of temperature, up to 85º C, with the reaction volume set to 20 µL for optimization.

3.3 RNA dilution and qPCR

After the RT process, a 1:50 dilution of all samples is carried out and they are then quantitatively measured by PCR (qPCR) using *SYBR™ Select Master Mix for CFX* (4472942, Thermo Fisher Scientific, Waltham, USA). Two house keepers were used, *36b4* and *beta-actins*. The genes used during this experimental period were: *Glut4, Chrebpα* and *Chrebpβ*. The sequences for these primers to perform the following qPCR are found in the following table **(appendix A).**

3.4 Statistical Analysis

The qPCR results were analyzed by the ΔCt method. The efficiency of the primers was calculated by performing a standard curve. Using the qPCR values for the two housekeepers, *36B4* and *beta-actins*, the *bestkeeper* value of Cts was obtained and used to analyze the changed in gene expression of the studied genes. Statistics was done by applying a two-tailed Student T-test. The statistical significance was determined with a *p*-value of < 0.05 in all cases. The data were expressed as the

mean ± SEM. The statistical analyses were performed using GraphPad Prism version 8.02 (GraphPad, San Diego, CA, USA).

IV. RESULTS

4.1 Anthocyanin content in Maqui berry

The content of anthocyanins in the lyophilized maqui berry used has been previously described [2]. The high content of anthocyanins was of 45,052 mg/kg which translates to 4,5%, a very high percentage compared to other products such as dried blueberries and raspberries. For a more comprehensive understanding of the matter, the table from the previous study has been included **(table 1)**. There is an 80% of the following anthocyanins: delphinidin-3-O-sambubioside-5-O-glucoside and delphinidin-3-O-sambubioside in the total composition **(table 1)**.

Table 1. Anthocyanidin composition of the lyophilized maqui berry. Anthocyanins content was previously determined by UPLC-DAD. The table shows the concentration in mg/g and mmol/kg of the different anthocyanidins detected. The following data was obtained from a previous publication. [2]

Compound	Conc. (mg/g)	Conc. (mmol/kg)	Retention Time (min)	LOD (mg/L)	LOQ (mg/L)
Delphinidin-3-O-sambubioside-5-O-glucoside	19.645 ± 0.788	24.71 ± 0.99	3.5	1.81	6.02
Delphinidin-3-O-sambubioside	17.770 ± 1.178	28.07 ± 1.86	5.	0.30	1.00
Cyanidin-3-O-sambubioside-5-O-glucoside	2.447 ± 0.063	3.14 ± 0.08	5.5	0.75	2.50
Not identified (quantified as cyd-3-0-glu)	0.402 ± 0.050	0.83 ± 0.10	6.5	-	-
Cyanidin-3-O-glucoside	2.148 ± 0.158	4.43 ± 0.33	7	0.11	0.35
Cyanidin-3-O-sambubioside	2.642 ± 0.201	4.28 ± 0.33	7.3	0.17	0.56
TOTAL	45.052	65.46			

4.2 Maqui induces gene expression of Glut4 on high fat diet in scWAT but not in non-obese mice

Changes in *Glut4* have been highly linked with oxidative stress and insulin resistance, therefore, affecting insulin stimulation and glucose uptake on individuals [11]. In this case, the data shows that maqui increases the expression of *Glut4* in the scWAT of mice fed in a HFD **(figure 1)**. This result coincides with the previously published work [2]. However, when maqui is added to non-obese mice (CTLM), there

was no significant differences in the expression of *Glut4* compared to the control group (CTL).

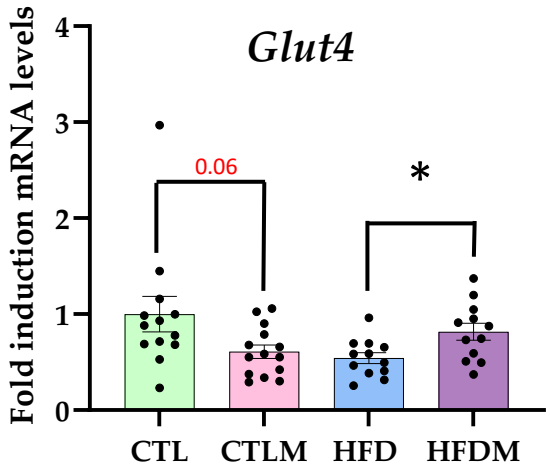

Figure 1. The relative mRNA levels of *Glut4* were measured by qPCR in scWAT of CTL, CTLM, HFD, HFDM mice. Maqui supplementation shows no statistical differences between CTL and CTLM. Maqui induces the expression of *Glut4* in scWAT in HFDM. Bars represent the fold induction in the mRNA levels versus the CTL to which is assigned with an arbitrary value of 1. Data is represented as the mean ± SEM. *$p < 0.05$.

4.3 Maqui induces gene expression of Chrebpα/β on high fat diet in scWAT but not in non-obese mice

Chrebp has an important role in the homeostasis of glucose. An impairment in these proteins can lead to altered insulin sensitivity and metabolic alterations [12]. The results from this study show a significant increased gene expression for both *Chrebpα and β* in HFDM in scWAT when compared to the control HFD **(figures 2a and 2c)**. In the case of *Chrebpα* for the CTLM group, there were no significant differences detected between CTL and CTLM **(figure 2a)**. For *Chrebpβ*, there were significant differences amongst groups **(figure 2b)**. Although, when comparing CTL with CTLM, there were no significant differences **(figure 2b)**.

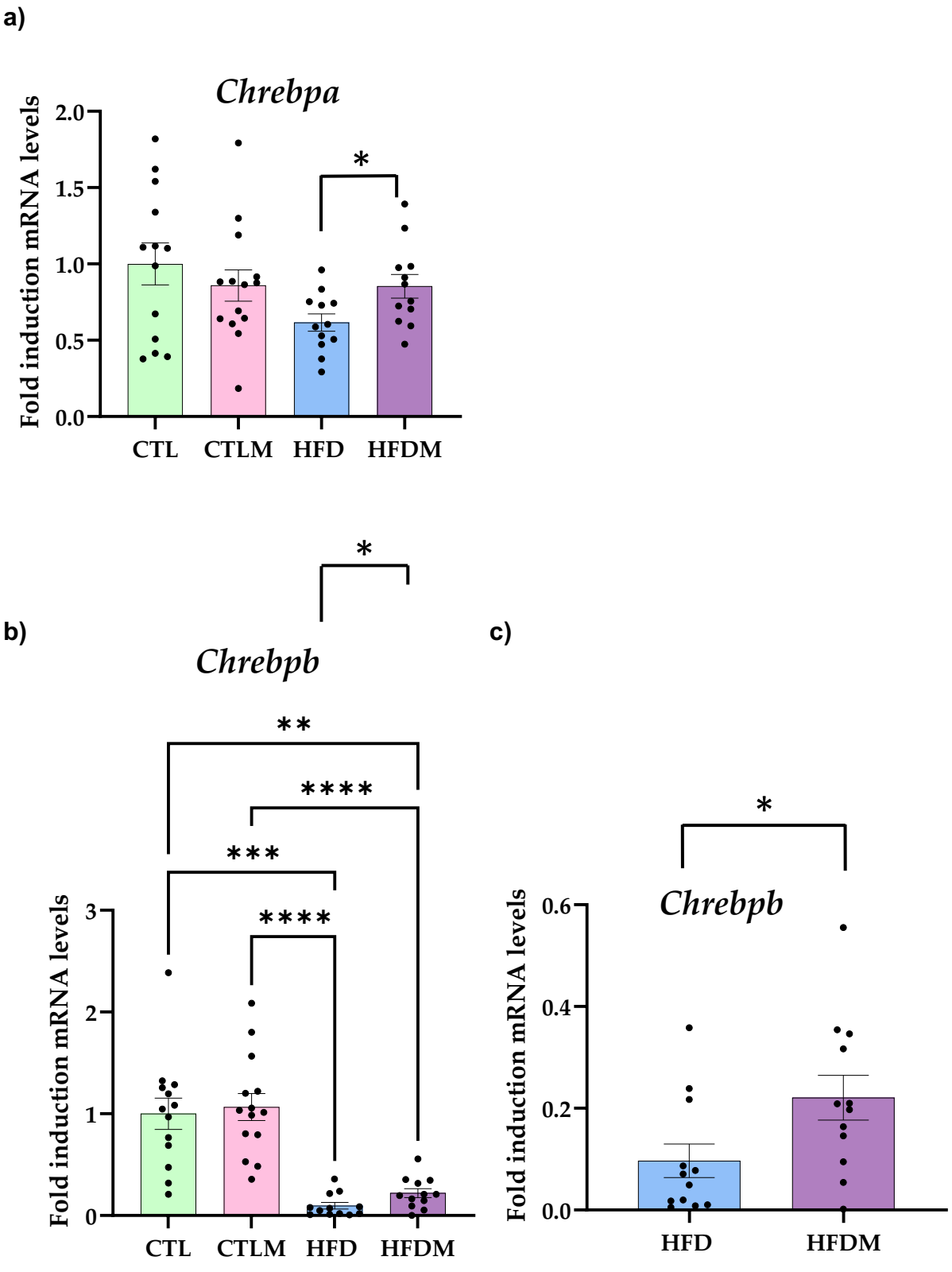

Figure 2. The relative mRNA levels of *Chrebpα* (a) *and Chrebpβ* (b,c) were measured by qRT-PCR in scWAT of CTL, CTLM, HFD, HFDM mice. **a)** Maqui induces the expression of *Chrebpα* in HFDM but no statistical differences between CTL and CTLM were observed. **b)** Significant differences between CTL vs HFDM, CTL vs HFD, CTLM vs HFD, CTLM vs HFDM

were detected**. c)** Maqui induces the expression of *Chrebpβ* in scWAT in HFDM vs HFD. Bars represent the fold induction in the mRNA levels versus the CTL to which is assigned with an arbitrary value of 1. Data is represented as the mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; ****$p < 0.0001$.

4.4 Inflammation markers using RT-PCR were inconclusive in all groups

At the beginning of this study, we aimed to determine mRNA levels for genes related to inflammation through RT-PCR as well. The genes selected for this approach were: interleukin-6 (*IL-6),* tumor necrosis factor alpha *(Tnf-α),* granulocyte-macrophage colony-stimulating factor *(Gm-csf)* and matrix metallopeptidase-3 *(Mmp-3)*. Nonetheless, the results for this approach were inconclusive using this method on scWAT due to the lower expression of these genes. Therefore, the results are not shown for this study.

V. DISCUSSION

Polyphenols have been widely known for their different metabolic properties in obese individuals. In a study where a Mediterranean diet was supplemented with additional plant-based sources of polyphenols, researchers observed a notable reduction in visceral adiposity [13]. In another randomized controlled trial (RCT) with 25 overweight and obese humans, the abdominal subcutaneous adipose tissue was collected and analyzed [14]. Most et al., found a significant downregulation of pathways related to adipogenesis, energy metabolism, oxidative stress and inflammation when supplemented with a combination of epigallocatechin-gallate and resveratrol [14]. In a diabetic rat model study, delphinidin had a major improvement in postprandial blood glucose by the inhibition of a Na+ dependent glucose transporter [15]. It has been reported that delphinidin constitutes 80% of the total anthocyanidins compounds found in Maqui berry as registered in table 1. These compounds have also been discovered in other foods, such as sweet potatoes, where they are linked to anti-diabetic mechanisms [16]. Hence, this information serves as crucial support for utilizing Maqui berry due to its significant anthocyanidin content.

A direct association between IR and adipose tissue dysfunction has been studied for years leading to the identification of key molecules such as *Glut4*, which is affected

in obese subjects [17]. In this study, an evident increased gene expression of *Glut4* in HFDM has been recorded **(figure 1)**. In tissues such as muscles as well as adipose tissue, *Glut4* is in charge of the glucose uptake by the action of insulin, therefore being one key regulator for glucose homeostasis [18]. When compared to human clinical trials with cardiometabolic risks, supplementation of polyphenols exerts a positive effect in glucose homeostasis [19]. Similar to this results, Cheng et al, demonstrated an improvement in metabolic syndrome parameters including glucose metabolism after Rutgers Scarlet Lettuce extract supplementation in diet-induced obese mice [20]. However, the remaining question of whether they have the same effects on healthy individuals remains briefly researched. Interestingly, in the present study, when comparing *Glut4* mRNA levels with the control group following a normal diet, Maqui berry fails to demonstrate a significant effect.

The expression of *Chrebp* is abundant in white adipose tissue as well as liver and brown adipose tissue, having a key role in carbohydrate metabolism [21]. Studies have shown that *Glut4* in adipose tissue has important regulatory roles in metabolic processes such as glycolysis and fatty acid synthesis [22]. It responds to the presence of glucose, especially following a high-carbohydrate intake, by metabolizing it for storage as glycogen in muscle and fat in adipose tissue [21]. In this study, supplementation with Maqui berry shows a significant increase of the gene expression on both transcription factors, Chrebp*α* and Chrebp*β,* in HFDM mice **(figure 2a, figure 2b)**, in accordance with the previously published results from Sandoval et al [2]. There is a high concordance between the action of Maqui berry's anthocyanidins upregulating *Glut4* and *Chrebpα/β* and ameliorating insulin resistance in a HFD on scWAT. Although, when comparing mice on the CTL group with CTLM, there are no statistical differences shown. However, for *Chrebpβ*, maqui supplementation in HFDM shows high statistical significance.

The reasons as to why Maqui berry lacks to demonstrate an upregulation in a normocaloric diet but its effectiveness on a high fat diet is evident, remain unknown. However, studies have shown the benefits of polyphenols in a variety of functional foods, including the improvemente of insulin resistance and other cardiometabolic risks [23]. Perhaps the supplementation of certain dietary compounds in a healthy individual offers no additional benefits compared to those already experiencing

metabolic alterations present. Nonetheless, this approach remains neccessary for gaining a comprehensive overview when it comes to supplementation with Maqui berry in different populations such as the experimental groups involved.

The lack of significant results when comparing obese to healthy models regarding cardiometabolic parameters has been reported in several studies. When comparing antioxidant effects in obese mice and non-obese mice using cranberry extract-enriched diets, Boušová et al, found out this diet modulates the antioxidant activity in obese mice, ameliorating consequences in obesity better than non-obese mice [24]. Anthocyanins in blackcurrant have also reported positive effects regarding glucose metabolism, showing no effect in non-obese mice in a low-fat diet but an effect on obese mice models with blackcurrant supplementation [25]. This gives rise to more evidence as to how certain food or supplementation works better in pathological states. Consequently, it demonstrates how a balanced diet containing all essential nutrients can be sufficient for healthy individuals, eliminating the need to take multiple supplements simultaneously.

Lastly, another approach firstly proposed was the analysis of inflammatory markers such as IL-6, Tnf-α, granulocyte-macrophage colony-stimulating factor (Gm-csf) and matrix metallopeptidase-3 (Mmp-3) in scWAT. However, qPCR seemed to not be the best way to evaluate these biomarkers due to their low expression in scWAT. At the end of this work a new experimetanl approach was being performed by using western blot (WB) and ELISA techniques to analyze changes in protein levels of these inflammatory factors, as they provide evidence for the presence of specific proteins [26].

VI. CONCLUSIONS

According to the results of this approach, Maqui berry supplementation remains an excellent dietary intervention in a HFD, showing significant upregulated gene expression of *Glut4* and *Chrebpα/β*, by improving glucose homeostasis and insulin sensitivity. However, when compared to mice in the normal diet, no significant change was detected, therefore concluding that bioactive compounds such as anthocyanidins found in Maqui berry have a greater impact in carbohydrate metabolism in obese

models rather than a healthy population. This evidence helps to prove that people with cardiometabolic risks are more likely to benefit from these polyphenols when included in a balanced diet and a constant routine of physical activity to improve their health status.

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VIII. CONFLICT OF INTEREST

The author declares there was no conflict of interest.

IX. APPENDIXES

APPENDIX A

Table A. Primers for real-time PCR

Genes names		Primes / Sequences (5' – 3')
mβ-actin	Forward	GCTCTGGCTCCTAGCACCAT
	Reverse	GCCACCGATCCACACAGAGT
mChrebpα	Forward	CGACACTCACCCACCTCTTC
	Reverse	TTGTTCAGCCGGATCTTGTC
mChrebpβ	Forward	TCTGCAGATCGGTGGAG
	Reverse	CTTGTCCCGGCATAGCAAC
mGlut4	Forward	ACTCATTCTTGGACGGTTCCTC
	Reverse	CACCCCGAAGATGAGTGGG
m36b4	Forward	AGATGCAGCAGATCCGCAT
	Reverse	GTTCTTGCCCATCAGCACC

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