TISSUE LYCOPENE ABSORPTION AND ACCUMULATION IN TRANSGENIC MICE LACKING ONE OR BOTH CAROTENOID CLEAVING ENZYMES

BY

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THESIS

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ABSTRACT

β-carotene oxygenase 1 (BCO1) and β-carotene oxygenase 2 (BCO2) are responsible for the cleavage of carotenoids in mammals. The goals of this study were to establish the relative contribution of each enzyme on lycopene accumulation in mice. We utilized male and female wild-type (WT), *Bco1*$^{-/-}$, *Bco2*$^{-/-}$, and *Bco1*$^{-/-}$ *Bco2*$^{-/-}$ double knockout (DKO) mice. We gavaged the mice with either 1 mg of lycopene resuspended in cottonseed oil or vehicle as a control group daily for a period of two weeks. In a second study, we evaluated the effect of dietary vitamin A on lycopene absorption and intestinal gene expression. We evaluated lycopene concentration isomer distribution by HPLC and evaluated gene expression by RT-PCR analyses. Hepatic mitochondria were isolated to determine lycopene content and isomer characterization. Of the 11 tissues measured, the liver accounted for 94-98% of the lycopene content across genotypes. We did not observe sex differences between genotypes, although *Bco1*$^{-/-}$ hepatic lycopene levels were approximately half in comparison to the other genotypes; *Bco1*$^{-/-}$ verses *Bco2*$^{-/-}$ ($p < 0.0001$), DKO mice ($p < 0.001$), WT (ns). Analyses of mitochondrial lycopene content revealed a 3-5 fold enrichment in comparison to total hepatic content ($p<0.05$) in all genotypes and sexes. In our second study, WT mice fed a vitamin A-deficient diet (VAD) accumulated greater amounts of lycopene in the liver than those fed a vitamin A sufficient diet (VAS) ($p<0.01$). These changes were accompanied by an upregulation of the vitamin A-responsive transcription factor intestine-specific homeobox (ISX) in mice fed VAD + lycopene and VAS + lycopene diets in comparison to VAD control-fed mice ($P<0.05$). Our data suggests that BCO2 is the primary lycopene cleavage enzyme in mice. Lycopene concentration was enriched in the mitochondria of hepatocytes independently of genotype, and lycopene stimulates vitamin A signaling in WT mice.

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CHAPTER 1

Literature Review

Introduction:

Lycopene is one of the most abundant carotenoids found in the human diet and tissues. It is a bioactive compound that gives fruits and vegetables such as tomatoes, watermelon, and guava their red colorings. Carotenoids are classified into two categories based on their chemical structure. Xanthophylls such as lutein and zeaxanthin contain oxygen in their structures, whereas carotenes such as lycopene and β-carotene are made up of only carbon and hydrogen atoms (1). Lycopene has 2 unconjugated double bonds and 11 double linear bonds making it extremely nonpolar, and susceptible to heat-mediated degradation (2). Following absorption by mammals, carotenoids are metabolized/cleaved by beta-carotene oxygenase 1 (BCO1) and/or beta-carotene oxygenase 2 (BCO2).

Lycopene is unable to be synthesized by the human body, and therefore must be supplied through the diet. Raw and processed tomatoes are the primary source of lycopene consumed in western countries and account for 80% of all lycopene consumed (3). In nature lycopene is found primarily in the *all-trans* form which is a linear hydrocarbon molecule (**Figure 1.1**). Lycopene accumulates in tomato fruit, for example in a, stacked, crystalline structure (4), whereas in mammalian tissues it is most often in non-linear, cis configurations (5). The isomerization from *all-trans* to *cis* can happen pre-consumption due to lycopene being susceptible to heat and light. It also undergoes extensive isomerization once consumed and metabolized by our bodies, with 9-*cis*, 13-*cis*, and 15-*cis* being common isomers amongst others (1). This isomerization was demonstrated by Moran et al. 2015 when human subjects were given a dose of 82% *all-trans* lycopene and subsequent the increasing appearance of serum *cis* isomers over time lead to a

shorter half-life of the *trans* compared to *cis* isomers (6). While lycopene does not have provitamin A activity, like some carotenoids, research and epidemiological studies have linked it to providing health benefits for a wide variety of diseases and illnesses as reviewed below.

Lycopene and Chronic Diseases:

Lycopene, among other carotenoids, has been reported to be highly effective at quenching reactive oxygen species like singlet oxygen that are generated by oxidative stress (7). This paired with its anti-inflammatory quality has been reported to be cardioprotective, scavenging potent oxidants associated with atherosclerosis (8), and some in studies demonstrating that lycopene has lipid lowering properties and reduces lipid peroxidation (9). Several studies have also provided evidence that it has positive effects in the treating of diabetes and improving cognitive function (10). Additionally, there is significant epidemiological support for a reduction of risk for development of prostate cancer (PCa). A systematic review conducted by Rowles et al included 42 studies on human consumption of lycopene and its association with prostate cancer. Their analysis found an inverse association with PCa risk and circulating and dietary levels of lycopene. There was a dose-response for dietary lycopene in which PCa risk went down 1% for every 2 mg of lycopene that was consumed. However, lycopene was not found to reduce the risk for advanced, or aggressive PCa (11).

A number of animal trials also suggest the effectiveness of lycopene at preventing prostate cancer (12, 13, 14). For example, one *in vivo* experiment utilized the transgenic adenocarcinoma mouse prostate (TRAMP) mouse model designed to study prostate cancer (12). Mice lacking the BCO2 enzyme were compared to control wild-type mice. Both groups were fed diets of either tomato powder, lycopene beadlets, or a control diet of powdered AIN93 G. All lycopene fed groups demonstrated a lower incidence of prostate cancer compared to the control-diet mice,

while the mice lacking the BCO2 enzyme showed a significant decrease in ability to prevent prostate cancer. This suggests that the metabolites of lycopene may play an important role in reducing PCa (12). Lycopene may not only be effective at preventing prostate cancer, but studies suggest its capability at abating ovarian cancer and general metastasis of cells (15,16).

Lycopene Digestion and Absorption:

For lycopene digestion, mechanical breakdown through peristalsis and mastication is imperative for releasing lycopene molecules from its food matrix. Once it reaches the stomach, acid and local enzymes also partially release carotenoids from the food matrix. Because lycopene is extremely nonpolar, it gets incorporated into lipid droplets before being released from the stomach into the small intestine. Once there, it is incorporated within lipid micelles for absorption through the enterocytes (1). The protein scavenger receptor type B, class 1 (SRB1) has been shown to be involved in the uptake of carotenoids in the gut, although it has not been established if the uptake of lycopene is also facilitated through this protein (17,18). SRB1 is a particularly important protein for the uptake of pro vitamin A carotenoids such as β-carotene (19). Therefore, proper regulation of SRB1 expression is needed to maintain ideal vitamin A levels in the body. It was discovered that the intestinal specific homeobox (ISX) transcription factor was a repressor of SRB1 (20). It was also demonstrated that ISX not only downregulates SRB1 expression, but also BCO1 expression, BCO1 being the enzyme responsible for the initial cleavage of pro vitamin A carotenoids. (21). The mechanisms behind how the expression of ISX itself is induced was not elucidated until recent years. Lobo et al. showed that retinoic acid, working through retinoic acid receptors (RARs), induced the upregulation of ISX, which then downregulated SRB1 and BCO1 expression (22) (**Figure 1.2**). Some studies have suggested that

lycopene's metabolites might also act upon these RAR receptors and could also influence this ISX/SRB1 axis, but there has not been enough evidence to prove this (23,24).

Both enzymes BCO1 and BCO2 are highly expressed in the intestine, and therefore lycopene may undergo partial cleavage in the intestinal epithelial cells (25). However, it is thought that most lycopene remains intact and is repackaged into chylomicrons which then travel through the lymphatic and are eventually taken up by the liver (26). Lycopene can be found in various lipoproteins, but it is primarily carried by low-density lipoproteins (27). The half-life of lycopene in the blood is around 6-8 days (6).

The liver is the main storage tissue of lycopene, but it is also distributed to tissues such as the adrenals, prostate, ovaries, kidneys, adipose tissue, and lungs. A study using Mongolian gerbils, a good model to study carotenoid absorption and tissue accumulation (28), found that when comparing the accumulation of the major carotenoids found in tomatoes (lycopene, phytofluene, phytoene), lycopene was the major carotenoid in the liver, comprising 55% of the carotenoids present (29). Additionally, Sy et al. also found that lycopene was the most abundant carotenoid in the liver of rats gavaged with pure carotenoids in oil. They suggested that this could be due to hydrophobic compounds tending to accumulate in the liver, with lycopene being the most nonpolar carotenoid (30).

The bioavailability of lycopene found in foods is closely linked to the matrix in which it is found (3). Lycopene's crystalline structure is a barrier to its bioavailability. However, studies have shown that the process of cooking releases lycopene from its matrix into the lipids found in the meal, increasing its bioavailability (31). Food processing techniques such as creating tomato paste or puree also results in increasing its bioavailability (32). Grainger et al demonstrated this through a study examining prostate levels of lycopene in males given either tomato soup, sauce,

or juice. The men receiving the soup and sauce had significantly higher levels of blood lycopene than the less processed tomato juice group (33). Additionally, because lycopene uptake into intestinal cells must be facilitated through incorporation into lipid micelles, to absorb lycopene a certain amount of fat must be included in the meal. A study by Brown et al. gave subjects green salads with tomatoes and a dressing containing 0,6, or 28g of fat from canola oil. They found that the subjects with the salad dressing lacking fat had negligible levels of lycopene in postprandial chylomicron fractions (34). Stahl et al demonstrated that lycopene uptake is improved when tomato juice is heated along with a lipid, in this case corn oil (5). One study has suggested that at least 5-10g of fat is the minimum for absorption for all carotenoids, although this number might vary based on the specific carotenoid (35).

Enzymatic Cleavage of Lycopene:

Previous research has identified two cleavage enzymes, BCO1 and BCO2, as the main cleavage enzymes of carotenoids, including lycopene (27, 36). BCO1 localizes in the cytosol and catalyzes the symmetric cleavage of carotenoids. Most research with BCO1 has been conducted regarding cleaving β-carotene at the 15, 15'double bond, as well as cleaving other pro-vitamin A carotenoids (37). BCO2 is located on the inner mitochondria membrane and cleaves carotenoids asymmetrically at the 9', 10' double bond. Additionally, BCO2 has a broader substrate specificity than BCO1 (38). Results from murine studies where animals were fed very high levels of the hydroxylated carotenoid, lutein, show that BCO2 may protect the mitochondria from toxicity caused by excess carotenoid accumulation in this organelle (38).

Based on data from the Human Protein Atlas (39), the tissues with the greatest protein levels of BCO1 in humans are the intestines, eyes, kidneys, liver, and reproductive organs. BCO2 protein expression is similar, with the liver the highest, followed by the intestines, eyes, kidneys, reproductive organs, and lungs (40). Also in humans, mRNA expression of both BCO1 and BCO2 has been reported in the testes, liver, kidney, adrenal glands, retina, and epithelial cells of the small intestines and stomach (41). BCO2, but not BCO1 expression, was seen in some additional tissues such as the cardiac skeletal muscle cells, prostate, endometrial connective tissue, and the endocrine pancreas. These additional expression sites suggest that BCO2 has a biochemical function beyond vitamin A synthesis, which is the main role of BCO1 (41).

Ferret, rat, and mouse models have been used to study carotenoid metabolism as they exhibit comparable expression of the carotenoid cleaving enzymes to humans, as well as similar absorption and tissue deposition patterns of the carotenoids (3). A study carried out in ferrets showed that BCO2 mRNA levels were highest in the liver, testes, kidney, heart, lung, spleen, prostate, and intestines (42). While no systemic tissue expression study has been conducted regarding BCO1 levels in rodents, Ford et al 2010 compared the expression of BCO1 and BCO2 in selected tissues and found that both enzymes were expressed in the liver, duodenum, and testes. Significantly, only BCO2 was found in the prostate, however, its expression in the testes was very low (43). The activity of BCO1 was measured in rats, with the highest levels of activity recorded in the intestine, liver, brain, lung, and kidneys (44).

Which of these enzymes is the primary cleaver of lycopene has been studied in the past, although without definitive results. Carlo dela Seña et al reported that lycopene can cleaved efficiently by BCO1 through studying its enzymatic activity on multiple carotenoids, and that BCO2 was inactive in the presence of *all-trans* lycopene (45,46). Previous work by our lab however showed

that lycopene failed to accumulate much in the livers of lycopene-fed, transgenic mice with BCO1 ablation (*Bco1* $^{-/-}$) compared to wild type (WT) mice, suggesting that BCO1 may not play a large role in the cleavage of lycopene (47). Additionally, we reported that *Bco2* $^{-/-}$ mice fed lycopene-containing tomato powder or lycopene-containing diets had enhanced tissue lycopene accumulation (48). These results suggest that lycopene may not be metabolized well by BCO1 *in vivo*, and that BCO2 might be the more active metabolizing enzyme for lycopene.

Lycopenoids, the Lycopene Metabolites:

The cleavage of lycopene results in metabolites called lycopenoids. These lycopenoids are apparently metabolized rapidly in the body and have only been found in barely detectable amounts (49,50). Due to these characteristics, it is extremely hard to study lycopenoids, and to determine if these metabolites are bioactive. While there have been many studies that have examined the formation of lycopenoids *in vitro*, it is not known if these cleavage products are relevant *in vivo* (51,52). However, because their structure and polarity are similar to that of retinoic acid, they may be able to interact with nuclear receptors such as RARs/RXRs/LXR/PXR/PPARs (49,50,53,54,). One of the previously studied lycopenoids was APO 10' lycopenoic acid (APO10) which would be the result of the cleavage of lycopene by BCO2 at the 9' 10' double bond (**Figure 1.3**). Gouranton et al. demonstrated that APO10 interacts with RARs to reduce pro-inflammatory cytokines interleukin-6 and interleukin-1β and thus attenuate inflammation in mice (55). Additional studies found that APO10 upregulated Sirtuin 1, a protein that regulates metabolic activity in response to cellular stress caused by inflammation and oxidative stress (56,57,58). In one of these studies, this upregulation contributed to a decrease in murine hepatic fat accumulation and protected the animals from hepatic steatosis while on a high-fat diet (56). There are barriers to studying lycopenoids due to it

being found in barely detectable levels in the body and a lack of commercially available standards (49,50,54), however continued research is needed due to the potential impact of their bioactivity in the body.

Factors Impacting Human Absorption and Metabolism of Lycopene:

While there are gaps in knowledge about how humans absorb and metabolize lycopene, and about how it exerts its bioactivity, there have been intrinsic and extrinsic factors identified that might affect these processes. Regarding lycopene absorption, it was found that in tomato products with greater ratios of *cis* isomers that there was greater lycopene absorption than high all-*trans* lycopene foods (59,60). This could be because both in *in vitro* and the ferret models it was demonstrated that *cis* lycopene isomers were more easily micellarized and absorbed than all-*trans* lycopene (61, 62). Some dietary compounds and nutrients might inhibit lycopene absorption. Plant sterol and stanol consumption has been shown to decrease plasma lycopene by 12-16%, and intake of minerals such as calcium, magnesium, and zinc might impede its bioaccessibility due to insoluble lipid-soap complex formation (63, 64). Other factors that may affect absorption include the form of lycopene in the food matrix, dietary lipid levels consumed with lycopene, and the ISX/SRB1 axis.

An intrinsic factor that could impact blood levels of lycopene are genetic variables such as single nucleotide polymorphisms (SNPs). These are single nucleotide variants in a base pair in a DNA sequence that cause a minor allele change (65). Most relevant to carotenoid levels in plasma are SNPs that have been identified for BCO1 and SRB1 (66,67). Studies have identified that SNPs of BCO1 and SRB1 have strong associations with plasma levels of carotenoids (66,67,68,69,70).

Some extrinsic factors that could affect lycopene bioactivity are medications that target blood cholesterol-lowering, as lycopene is circulated in lipoproteins. One study found that hypercholesterolemic men on statin treatments for 6 weeks had a reduction of circulating lycopene (71). Smoking was demonstrated to lead to autoxidation and degradation of carotenoids, including lycopene (72), and alcohol use has been linked to lower serum carotenoids (73).

Animal Models Used to Study Lycopene:

Animal models have been used for many decades to study carotenoids, with some of the most utilized models being the gerbil, ferret, and mouse. Animal models are beneficial to studying carotenoids as you have easy access to tissue for analysis, can use of radiolabeled compounds, and because human studies are more expensive and difficult to manage (28). As there is still much we don't know about carotenoid metabolism in humans, choosing the correct animal model can be difficult. Ideally, your animal model would be able to absorb carotenoids and display carotenoid distribution in tissues and serum similarly to humans (28). Another key factor to weigh in choosing an animal model is what animal would best model the disease/physiological state of interest. For instance, humans absorb high levels of carotenoids in their intact form, as do ferrets and gerbils, which make them optimal models to study carotenoid absorption (74). It is noteworthy however that mice and rats poorly absorb carotenoids and often require much higher dose levels to reach tissue amounts seen in humans. However, rodent models have frequently be used to study cancers, vitamin A deficiency, and atherosclerosis due to their low cost and the ability to manipulate their genetics (74).

Our lab and others have used gerbil, ferret, and mouse models to study lycopene tissue distribution (**Table 1.1**). Moran et al. found that in gerbils fed a diet of tomato powder, lycopene

accumulated in greatest amount in the liver, followed by the serum, adrenals, and spleen. (55% of all carotenoids) (29). A study with a ferret model also showed that the liver had the greatest stores of lycopene compared to the intestines, stomach, prostate, and plasma (75). There have been many studies that have utilized murine models to study lycopene's metabolism and relation to disease states (11,13,15). Lindshield et al. utilized this model to examine lycopene tissue biodistribution as well (47). Just as in the gerbil and ferret model, mice accumulated the most lycopene in the liver, with the spleen, adrenals, uterus, adipose, and kidneys following (47). Ford et al. also found using a mouse model that the liver accumulated the most lycopene (59 nmol/g), with the visceral adipose at 1 nmol/g and the gonadal adipose at 0.2 nmol/g (48).

Human data on lycopene tissue accumulation is limited. Schmitz et al did an analysis of tissue carotenoid levels from a group of 20 deceased subjects. Just as in the animal models, the liver displayed the greatest levels of lycopene compared to the kidneys and lungs (76). However, in a study by Stahl with 9 subjects, the liver was third in accumulation behind the adrenals and testes (5). Other studies have examined lycopene tissue distribution, but only within one tissue (77,78). While these studies have been extremely important in advancing our understanding about lycopene biodistribution, the lack of more human studies shows just how important animal models have been in furthering our knowledge about lycopene absorption and biodistribution.

Conclusion:

Lycopene is a powerful antioxidant with epidemiological data linking it to having properties beneficial to our health, but there are still many gaps in knowledge about its metabolism and bioaccumulation in our body. This thesis is aimed at closing some of those gaps utilizing WT and transgenic mouse models. Our first objective was to elucidate which carotenoid cleaving enzyme, BCO1 or BCO2, was the main cleavage enzyme of lycopene. Based on previous

research, we hypothesized that BCO2 is the main enzyme responsible for lycopene cleavage in mammals. Additionally, we hypothesized that SRB1 facilitates the absorption of lycopene, and that lycopene, or its cleavage products, could mimic the action of retinoic acid in the regulation of RAR activity to influence the ISX/SRB1 axis.

FIGURES AND TABLES

Figure 1.1.

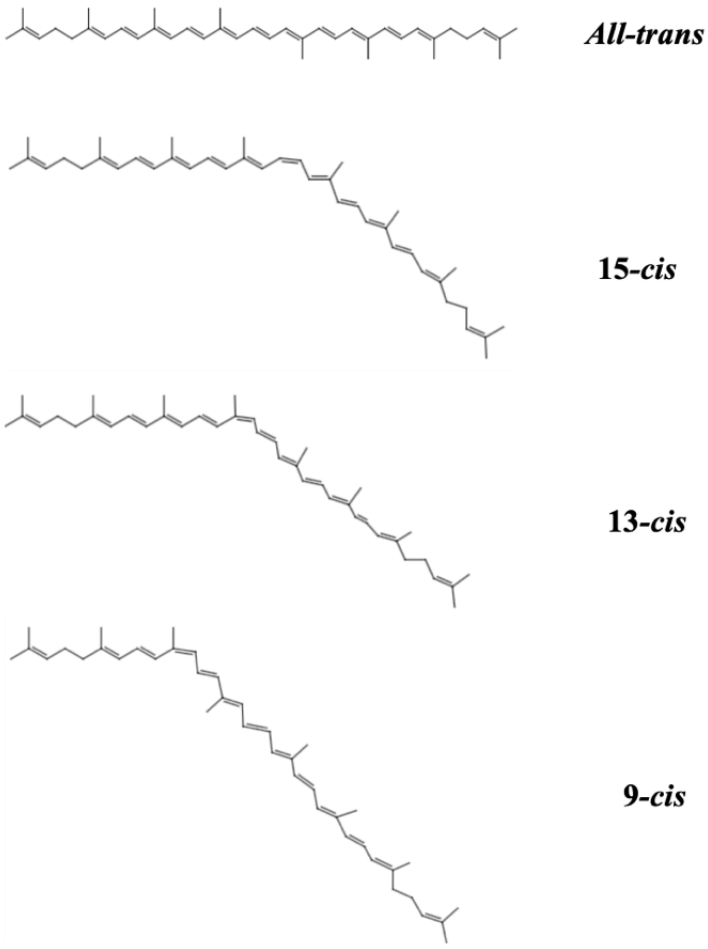


Figure 1.1: Lycopene's chemical structures for the *all-trans*, 15-*cis*, 13-*cis*, and 9-*cis* forms.

Figure 1.2.

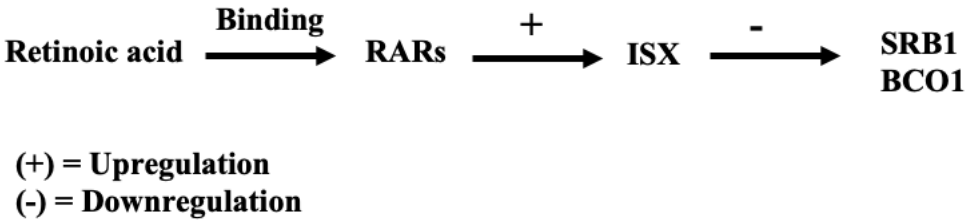


Figure 1.2: A schematic demonstrating how retinoic acid interacts with RAR receptors to influence the ISX/SRB1 axis.

Figure 1.3.

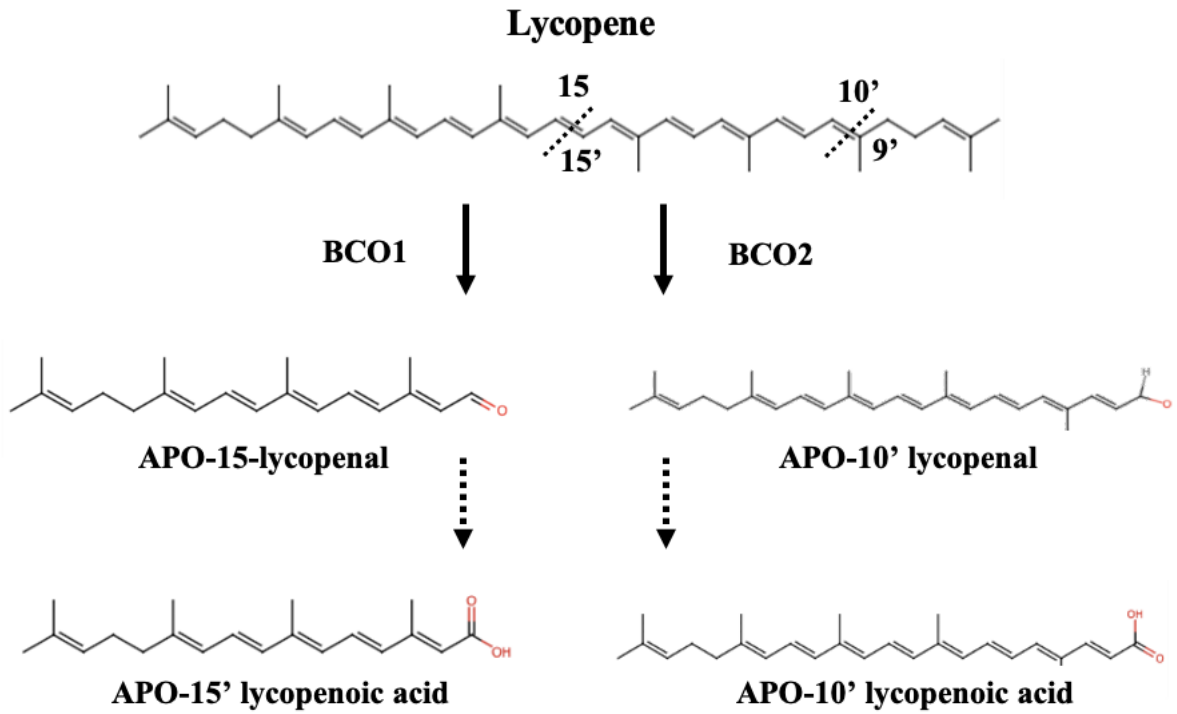


Figure 1.3: The metabolites of lycopene after BCO1 or BCO2 cleavage.

Table 1.1: An overview of lycopene tissue accumulation across a variety of animal models consuming lycopene from various sources

Study	Animal Model	Diet	Diet Duration	Lycopene Accumulation In Tissues
Moran et al., 2013	Mongolian Gerbils N=40 Males	AIN-93G Diet + Tomato Powder (10%)	26 Days	nmol/g Liver: 134 ± 30 Adrenals: 7.7±1.3 Spleen: 2.64±.30 Lung: 0.16±0.03 gWAT*: 0.07±0.02 Testes:0.40±0.08 Prostate/SV*: 0.12±0.02
Ferreira et al., 2000	Ferret N=6 Males	Tomato oleoresin Corn oil mix	9 Weeks	nmol/kg Liver: 933±155 Intestine: 73±29 Stomach: 9.3±1.5 Prostate: 12.7±8.2 Plasma: 11.2±7.5 (nmol/L)
Lindshield et al., 2008	Mouse (Wild Type) N=14 Males + Females	AIN-93G Diet + Lycopene Beadlets	60 Days	nmol/g Liver: 469±107 Spleen: 270±41 Adrenals: 48.8±15.3 Kidney: 2.60±0.21 Lungs: 1.94±0.38 Brain: 0.019± 0.005 Thymus: 4.71±1.03 Adipose: 3.06±0.79 Uterus: 4.43±0.81 Prostate: 0.81±0.08 Testes: 2.12±0.14 SV: 0.04±0.01
Ford et al., 2013	Mouse (Wild Type) N=7 Female	AIN-93G Diet + Tomato Powder (10%)	30 Days	nmol/g Liver: 59.5±16.4 gWAT: ~0.02 perigonadal AT*: 0.21±0.04 Serum: ~500 nmol/dl

Table 1.1: * gWat (gonadal adipose tissue), SV (seminal vesicles), AT (adipose tissue)

REFERENCES

1. Arballo J, Amengual J, Erdman JW Jr. Lycopene: A critical review of digestion, absorption, metabolism, and excretion. *Antioxidants* 2021;10(3):342.

2. Grabowska M., Wawrzyniak D., Rolle K., Chomczyński P., Oziewicz S., Jurga S., Barciszewski J. Let food be your medicine: Nutraceutical properties of lycopene. *Food Funct.* 2019;10:3090–3102.

3. Boileau TW-M, Boileau AC, Erdman JW Jr. Bioavailability of all-trans and cis–Isomers of Lycopene. *Experimental Biology and Medicine* 2002;227(10):914-919.

4. Schweiggert R, Mezger D, Schimpf F, Steingass C, Carle R. Influence of chromoplast morphology on carotenoid bioaccessibility of carrot, mango, papya, and tomato. *Food Chemistry* 2012;135(4):2736-2742.

5. Stahl W, Schwartz W, Sundquist A, Helmut S. cis-trans isomers of lycopene and β-Carotene in human serum and tissues. *Arch. Biochem. Biophys*. 1992;294(1):173-177.

6. Moran NE, Cichon MJ, Riedl KM, Grainger EM, Schwartz SJ, Novotny JA, et al. Compartmental and noncompartmental modeling of ^{13}C-lycopene absorption, isomerization, and distribution kinetics in healthy adults. *Am J Clin Nutr.* 2015;102(6):1436-49.

7. Przybylska S, Tokarczyk G. Lycopene in the prevention of cardiovascular diseases. *Int. J. Mol. Sci.* 2022;23(4):1957.

8. Arab L, Steck S. Lycopene and cardiovascular disease. *The American Journal of Clinical Nutritio*n 2000;71(6):1691-1695.

9. Sheriff S, Ibrahim S, Devaki T, Chakraborty S, Agarwal S, Perez-Sanchez. Lycopene prevents mitochondrial dysfunction during D-galactosamine/lipopolysaccharide-induced fulminant hepatic failure in albino rats. *J Proteome Res* 2017;6(9):3190-3199.

10. Valero MA, Vidal A, Burgos R, Calvo FL, Martinez C, Luengo LM, et al. Meta-analysis on the role of lycopene in type 2 diabetes mellitus. *Nutricion Hospitalaria* 2011;26(6):1236-1241.

11. Rowles J, Ranard K, Smith J, An R, Erdman JW Jr. Increased dietary and circulating lycopene are associated with reduced prostate cancer risk: a systemic review and meta-analysis. *Prostate Cancer and Prostatic Diseases* 2017;20(4):361-377.

12. Tan L, Thomas-Ahner J, Moran N, Cooperstone J, Erdman, JW Jr, Young G, et al. β-carotene 9′,10′ oxygenase modulates the anticancer activity of dietary tomato or lycopene on prostate carcinogenesis in the TRAMP model. *Cancer Prevention Research* 2017;10(2):161–169.

13. Smith J, Ford N, Thomas-Ahner J, Moran N, Bolton E, Wallig M, et al. Mice lacking β-carotene-15,15'-dioxygenase exhibit reduced serum testosterone, prostatic androgen receptor signaling, and prostatic cellular proliferation. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* 2016;311(6):1135-1148.

14. Wan L, Tan H, Thomas-Ahner J, Pearl D, Erdman JW Jr., Moran N, et al. Dietary tomato and lycopene impact androgen signaling- and carcinogenesis-related gene expression during early TRAMP prostate carcinogenesis. *Cancer Prevention Research* 2014;7(12):1228–1239.

15. Holzapfel N, Shokoohmand A, Wagner F, Landgraf M, Champ S, Holzapfel B, et al. Lycopene reduces ovarian tumor growth and intraperitoneal metastatic load. *American Journal of Cancer Research* 2017;7(6):1322–1336.

16. Shanbhag V. Lycopene in cancer therapy. *Journal of Pharmacy & Bioallied Sciences*, 2016;8(2):170–171.

17. Moussa M, Gouranton E, Gleize B, Yazidi C, Niot I, Besnard P, et al. CD36 is involved in lycopene and lutein uptake by adipocytes and adipose tissue cultures. *Molecular Nutrition & Food Research* 2011:55(4):578–584.

18. Shyam R, Vachali P, Gorusupudi A, Nelson K, Bernstein PS. All three human scavenger receptor class B proteins can bind and transport all three macular xanthophyll carotenoids. *Arch Biochem Biophys*. 2017;634:21-28.

19. Kiefer C, Sumser E, Wernet M, von Lintig J. A class B scavenger receptor mediates the uptake of carotenoids in *Drosopila. PNAS* 2002;99(16):10581-10586.

20. Choi M, Romer A, Hu M, Lepourcelet M, Mechoor A, Yesilaltay A, et al. A dynamic expression survey identifies transcription factors relevant in mouse digestive tract development. *Development (Cambridge, England)* 2006;133(20):4119–4129.

21. Seino, Y., Miki, T., Kiyonari, H., Abe, T., Fujimoto, W., Kimura, K., Takeuchi, A., Takahashi, Y., Oiso, Y., Iwanaga, T., and Seino, S. (2008) Isx participates in the maintenance of vitamin A metabolism by regulation of beta-carotene 15,15′-monooxygenase (Bcmo1) expression. *J. Biol. Chem.* **283**, 4905– 4911

22. Lobo GP, Hessel S, Eichinger A, Noy N, Moise AR, Wyss A, et al. ISX is a retinoic acid-sensitive gatekeeper that controls intestinal beta, beta-carotene absorption and vitamin A production. *FASEB J.* 2010;24(6):1656-66.

23. Bohn T, de Lera A, Landrier J-F, Carlsen H, Merk D, et al. State-of-the-art methodological investigation of carotenoid activity and metabolism-from organic synthesis via metabolism to biological activity-exemplified by a novel retinoid signalling pathway. Food Functional 2023;14(621).

24. Aydemir G, Kasiri Y, Birta E, Beke G, Garcia A, Bartok EM, et al. Lycopene-derived bioactive retinoic acid receptors/retinoid X receptors-activating metabolites may be relevant for lycopene's anti-cancer potential. *Molecular Nutrition and Food Research* 2013;57(5):739-747.

25. Raghuvanshi S, Reed V, Blaner W, Harrison E. Cellular localization of β-carotene-15′,15′-1 (BCO1) and β-carotene-9′,10′-oxygenase-2 (BCO2) in rat liver and intestine. *Archives of Biochemistry and Biophysics* 2015; 572:19-27.

26. Von Lintig J., Moon J., Lee J., Ramkumar S. Carotenoid metabolism at the intestinal barrier. *Biochim. Biophys. Acta Mol. Cell Biol. Lipids.* 2019:158580.

27. Wang X. Lycopene metabolism and its biological significance. *Am J Clin Nutr* 2012;96(5):1214-1222.

28. Lee C, Boileau A, Boileau T, Williams A, Swanson K, Heintz K, et al. Review of animal models in carotenoid research. *J Nutr*. 1999;129(12):2271-2277.

29. Moran N, Clinton S, Erdman JW Jr. Differential bioavailability, clearance, and tissue distribution of the acyclic tomato carotenoids lycopene and phytoene in Mongolian Gerbils. *J Nutr*. 2013;143(12):1920-1926.

30. Sy C, Gleize B, Dangles O, Landrier J-F, Veyrat C, Borel P. Effects of physicochemical properties of carotenoids on their bioaccessibility, intestinal cell uptake, and blood and tissue concentrations. *Mol. Nutr. Food Res.* 2012;56:1385-1397.

31. Williams AW, Boileau T'YM, Erdman JW Jr. Factors influencing the uptake and absorption of carotenoids. *Proc Exp Bio Med* 218:106-108, 1998.

32. Gartner C, Stahl W, Sies H. Lycopene is more bioavailable from tomato paste than from fresh tomatoes, *Am J Clin Nutr* 1997;66:116-122.

33. Grainger E, Hadley C, Moran N, Riedl K, Gong M, Pohar K, et al. A comparison of plasma and prostate lycopene in response to typical servings of tomato soup, sauce or juice in mean before prostatectomy. *Br J Nutr*. 2015;114(4):596-607.

34. Brown M, Ferruzzi M, Nguyen M, Cooper D, Eldridge A, Schwartz S, et al. Carotenoid bioavailability is higher from salads ingested with full-fat than with fat-reduced salad dressings as measured with electrochemical detection. *Am. J. Clin. Nutr.* 2004;80:396–403.

35. Roodenburg A, Leenen R, van het Hof KH, Westrate JA, Tijburg LBM. Amount of fat in the diet affects bioavailability of lutein esters but not of alpha-carotene, beta-carotene, and vitamin E in humans. *Am J Clin Nutr* 2000;71:1187-1193.

36. Våge DI, Boman IA. A nonsense mutation in the beta-carotene oxygenase 2 (BCO2) gene is tightly associated with accumulation of carotenoids in adipose tissue in sheep (Ovis aries). *BMC Genet* 2010;11(12).

37. Lindqvist A, Andersson S. Biochemical properties of purified recombinant human β-carotene 15,15′-monooxygenase. *JBC* 2002;277(26):23942-23948.

38. Amengual J, Lobo GP, Golczak M, Li HN, Klimova T, Hoppel CL, et al. A mitochondrial enzyme degrades carotenoids and protects against oxidative stress. *FASEB J.* 2011;25(3):948-59.

39. Proteinatlas.org [Internet] The Human Protein Atlas; [cited 2023 Jan 30] Available from: https://www.proteinatlas.org/ENSG00000135697-BCO1

40. Uhlen M, Fagerberg L, Hallstrom B, Lindskog C, Oksvold P, Mardinoglu A. Proteomics. Tissue-based map of the human proteome. *Science* 2015;347(6220):1260419.

41. Lindqvist A, He YG, Andersson S. Cell type-specific expression of β-carotene-9′,10′ monooxygenase in human tissues. *Journal of Histochemistry & Cytochemistry* 2005;53:11.

42. Hu KQ, Liu C, Ernst H, Krinsky NI, Russell RM, Wang XD. The biochemical characterization of ferret carotene-9',10'-monooxygenase catalyzing cleavage of carotenoids in vitro and in vivo. *J Biol Chem*. 2006;14;281(28):19327-38.

43. Ford N, Clinton S, von Lintig J, Wyss A, Erdman JW Jr. Loss of carotene-9',10'-monooxygenase expression increases serum and tissue lycopene concentrations in lycopene-fed mice. *J Nutr*. 2010;140(12):2134-2138.

44. During A, Nagao A, Hoshino C, Terao J. Assay of β-carotene-15′,15′-dioxygenase activity by reverse-phase high-pressure liquid chromatography. *Analytical Biochemistry* 1996;241(2):199-205.

45. dela Seña C, Narayanasamy S, Riedl KM, Curley RW Jr, Schwartz SJ, Harrison EH. Substrate specificity of purified recombinant human β-carotene 15,15'-oxygenase (BCO1). *J Biol Chem*. 2013;288(52):37094-103.

46. dela Seña C, Sun J, Narayanasamy S, Riedl K,Yuan Y, Curley Jr. R, et al. Substrate specificity of purified recombinant chicken β-carotene-9′, 10′-oxygenase (BCO2)*. *Metabolism* 2016;291(28):14609-14619.

47. Lindshield B, King J, Wyss A, Goralczyk R, Lu C-H, Ford N, et al. Lycopene biodistribution is altered in 15,15'-carotenoid monooxygenase knockout mice. *J Nutr.* 2008; 138:2367-2371.

48. Ford N, Elsen A, Erdman JW Jr. Genetic ablation of carotene oxygenases and consumption of lycopene or tomato powder diets modulate carotenoid and lipid metabolism in mice. *Nutrition Research* 2013;33(9):733-742.

49. Harrison E. Carotenoids, β-apocarotenoids, and retinoids: The long and the short of it. *Nutrients* 2022;14(7):1411.

50. Kopec R, Riedl K, Harrison E, Curley R Jr., Hruszkewycz, Clinton S, et al. Identification and quantification of apo-lycopenals in fruits, vegetables, and human plasma. *J Agric. Food Chem.* 2010;58(6):3290-3296.

51. Zhang H, Kotake-Nara E, Ono H, Nagao A. A novel cleavage product formed by autoxidation of lycopene induces apoptosis in HL-60 cells. *Free Radical Biology and Medicine* 2003;35(12):1653-1663.

52. don Anjos Ferreira A, Yeum KJ, Russell R, Krinsky N, Tang G. Enzymatic and oxidative metabolites of lycopene. *J Nutr Biochem*. 2004;15(8):493-502.

53. Lindshield B, Canene-Adams K, Erdman JW Jr. Lycopenoids: Are lycopene metabolites bioactive? *Arch Biochem. Biophys*. 2007;458(2):136-140.

54. Cichon MJ, Moran NE, Riedl KM, Schwartz SJ, Clinton SK. Identification of an epoxide metabolite of lycopene in human plasma using 13C-labeling and QTOF-MS. *Metabolites* 2018; 8(1):24.

55. Gouranton E, Aydemir G, Reynaud E, Marcotorchino J, Malezet C, Caris-Veyrat C, et al. Apo-10′-lycopenoic acid impacts adipose tissue biology via the retinoic acid receptors. *Biochim. Biophys. Acta Mol. Cell Biol. Lipids.* 2011;1811:1105–1114.

56. Chung J, Koo K, Lian F, Hu KQ, Ernst H, Wang XD. Apo-10'-lycopenoic acid, a lycopene metabolite, increases Sirtuin 1 mRNA and protein levels and decreases hepatic fat accumulation in ob/ob Mice. *J Nutr.* 2012;142:405–410.

57. Ip B, Liu C, Lichtenstein AH., von Lintig J, Wang XD. Lycopene and Apo-10′-lycopenoic acid have differential mechanisms of protection against hepatic steatosis in β-Carotene-9′,10′-oxygenase knockout male mice. *J Nutr.* 2014;145:268–276.

58. Hadar A, Gozes I, Gurwitz D. RGS2 and SIRT1 link renin angiotensin aldosterone system to alzheimer's disease. *Academic Press* 2017;12.

59. Cooperstone J, Ralston R, Riedl K, Haufe T, Schweiggert R, King S, et al. Enhanced bioavailability of lycopene when consumed as cis-isomers from tangerine compared to red tomato juice, a randomized, cross-over clinical trial. *Mol Nutr Food Res* 2015;59(4):658-669.

60. Unlu N, Bohn T, Francis D, Clinton S, Schwartz S. Carotenoid absorption in humans consuming tomato sauces obtained from tangerine or high β-carotene varieties of tomatoes. *J. Agric. Food Chem.* 2007;55(4):1597-1603.

61. Boileau A, Merchen N, Wasson K, Atkinson A, Erdman JW Jr. *Cis*-lycopene is more bioavailable than *trans*-lycopene *in vitro* and *in vivo* in lymph-cannulated ferrets. *J Nutr.* 1999;129(6):1176–1181.

62. Failla M, Chitchumroonchokchai C, Ishida B. In vitro micellularization and intestinal cell uptake of *cis* isomers of lycopene exceed those of all-*trans* lycopene. *J Nutr.* 2008;138(3):482-486.

63. Baumgartner S, Ras R, Trautwein E, Mensink R, Plat J. Plasma fat-soluble vitamin and carotenoid concentrations after plant sterol and plant stanol consumption: a meta-analysis of randomized controlled trials. *Eur J Nutr* 2017;56(3):909-923.

64. Corte-Real J, Iddir M, Soukoulis C, Richling E, Hoffmann L, Bohn T. Effect of divalent minerals on bioaccessibility of pure carotenoids and physical properties of gastro-intestinal fluids. *Food Chemistry* 2016;197:546-553.

65. Frazer K, Murray S, Schork N, Topol E. Human genetic variations and its contribution to complex traits. *Nature Reviews. Genetics* 2009;10(4).

66. Mckay G, Loane E, Nolan J, Patterson C, Meyers K, Mares J. Investigation of genetic variation in scavenger receptor calss B, member 1 (SCARB1) and association with serum carotenoids. *Ophthalmology* 2013;120(8):1632-1640.

67. Grabmann S, Pivovarova-Ramich O, Henze A, Raila J, Amoako Y, Nyamekye R. SNP rs6564851 in the BCO1 gene is associated with varying provitamin A plasma concentrations but not with retinol concentrations among adolescents from rural Ghana. *Nutrients* 2020;12(6):1786.

68. Amengual J, Coronel J, Marques C, Aradillas-Garcia, Vargas Morales JM, et al. β-carotene oxygenase 1 activity modulates circulating cholesterol concentrations in mice and humans. *J Nutr*. 2020;150(8):2023-2030.

69. Borel P, Moussa M, Reboul E, Lyan B, Defoort C, Vincent-Baudry S. Human plasma levels of vitamin E and carotenoids are associated with genetic polymorphisms in genes involved in lipid metabolism. *J Nutr*. 2007;137(12):2653-2659.

70. Hendrickson S, Hazra A, Chen C, Eliassen A, Kraft P, Rosner B. β-Carotene-15,15′-monooxygenase 1 single nucleotide polymorphisms in relation to plasma carotenoid and retinol concentrations in women of European descent. *Am J Clin Nutr* 2012;96(6):1379-1389.

71. Ryden M, Leanderson P, Kastbom KO, Jonasson L. Effects of simvastin on carotenoid status in plasma. *Nutr Metab Cardiovasc Dis*. 2012;22(1):66-71.

72. Hurst J, Contreras J, Siems W, Van Kujik F. Oxidation of carotenoids by heat and tobacco smoke. *BioFactors* 2004;20(1):23-35.

73. Kitamura Y, Tanaka K, Kiyohara C, Hirohata T, Tomita Y, Ishibashi M. Relationship of alcohol use, physical activity, and dietary habits with serum carotenoids, retinol and alpha-

tocopherol among male Japanese smokers. *International Journal of Epidemiology* 1997;26(2):307-314.

74. Kim J, Kim Y. Animal models in carotenoids research and lung cancer prevention. *Translational Oncology* 2011;4(5):271-281.

75. Ferreira A, Yeum KJ, Smith D, Krinsky N, Wang XD, Russell R. Tissue distribution of lycopene in ferrets and rats after lycopene supplementation. *J Nutr*. 2000;130(5):1256-1260.

76. Schmitz H, Poor Christopher, Wellman RB, Erdman JW Jr. Concentrations of selected carotenoids and vitamin A in human liver, kidney, and lung tissue. *J Nutr*. 1991;121(10):1613-1621.

77. Parker RS. Carotenoid and tocopherol composition of human adipose tissue. *Am J Clin Nutr* 1988;47(1):33-36.

78. Tanumihardjo SA, Furr HC, Amedee-Manesme O, Olson JA. Retinyl ester (Vitamin A ester) and carotenoid composition in human liver. *International Journal for Vitamin and Nutrition Research* 1990;60(4):307-313.

CHAPTER 2

Tissue lycopene absorption and accumulation in transgenic mice lacking one or both carotenoid cleaving enzymes

Introduction

Lycopene is one of the most abundant carotenoids found in the human diet and tissues. Over the past decades, the antioxidant properties of lycopene have led researchers to explore its potential in the treatment and prevention of chronic diseases such as cardiovascular disease, non-alcoholic fatty liver disease, oxidative stress, and prostate cancer (1). However, the mechanisms that regulate lycopene cleavage and accumulation in tissues remain relatively unstudied, as most research has been focused on its biological and physiochemical properties (1). While the metabolism of lycopene is not fully elucidated, metabolites called lycopenoids have been identified in foods and tissues, but are difficult to study as they are only found in very low concentrations and are likely metabolized rapidly in tissues (2,3,4,5,6).

It is well established that two enzymes, β-carotene oxygenase 1 (BCO1) and β-carotene oxygenase 2 (BCO2), are responsible for mammalian cleavage of most carotenoids, including lycopene (7,8,9). BCO1 is a cytosolic enzyme that symmetrically cleaves carotenoids such as β-carotene at the 15, 15'double bond to form vitamin A (10). BCO2 cleaves carotenoids asymmetrically between the carbons in position 9',10', is localized in the mitochondria, and has a broader substrate specificity than BCO1 (11,12).

Carlo dela Seña et al reported that lycopene could be cleaved *in vitro* using recombinant human BCO1 (13), but not by chicken BCO2 (14), suggesting BCO1 is the main lycopene cleaving enzyme. In contrast, we showed substantially lower hepatic lycopene accumulation in *Bco1* $^{-/-}$

mice compared to wild type (WT) mice, suggesting that *in vitro* enzymatic activities might differ from animal studies (15).

The goal of this study was to probe which enzyme cleaves lycopene *in vivo*. For this work, we cross-bred C57BL/6J WT mice with *Bco1*$^{-/-}$ and *Bco2*$^{-/-}$ mice for 10 generations to assure similar genetic backgrounds of all genotypes. Mice lacking both BCOs (double knockout, DKO) were also derived following backcrossing. We dosed these mice with lycopene and evaluated accumulation of lycopene in tissues, plasma, and isolated hepatic mitochondria. In a separate study, we also evaluated the impact of lycopene on the expression of intestine specific homeobox (ISX), a vitamin A-sensitive gene implicated in the uptake of dietary lipids including cycled carotenoids (16).

Materials and Methods

Mice breeding:

The University of Illinois Institutional Animal Care and Use Committee reviewed and approved the experimental procedures used in these studies. Breeding colonies of C57BL/6J WT, *Bco1*$^{-/-}$, *Bco2*$^{-/-}$, and DKO mice were maintained at the Animal Care Facility. Mouse colonies were backcrossed for 10 generations with C57BL/6J mice in order to ensure mice were in the same genetic background, as done in the past (17). Genotyping was routinely performed using DNA isolated from the tail snips at 10 days of age. DNA samples were isolated using the Extract-N-Amp™ Tissue PCR kits (Sigma-Aldrich, St. Louis, Missouri).

Lycopene oil dilution and resuspension:

The lycopene oil used to gavage the mice was Redivivo® lycopene oil 10% fluid suspension and was a gift from DSM, Basel, Switzerland. We prepared a working solution containing 1 mg lycopene/0.2 mL of oil using cottonseed oil. We aliquoted the working solution into 2 mL Eppendorf tubes, flushed with argon and immediately sealed with parafilm. Working solutions were stored at 4 °C for a maximum of 2 weeks until used for gavage.

Animal trials:

For the first study, we utilized male (n = 4-7/group) and female (n = 4-6/group) WT, *Bco1*$^{-/-}$, *Bco2*$^{-/-}$, and DKO mice (Study 1 design found in **Appendix A Figure 1**). After weaning, mice continued on a chow diet (Teklad Global 18% protein rodent diet 2918; Envigo, Indianapolis, Indiana) for one week before being placed on a semi-purified powder AIN-93G diet containing cottonseed oil as a source of fat to minimize carotenoid contamination in their feed. (Dietary composition found in **Appendix A Table 1)**. Mice remained on this diet for the entire duration of the study. We provided at least five grams of diet/mouse/day to ensure mice had constant access to food. After two weeks, mice were gavaged daily with 1 mg lycopene suspended in 0.2 mL of cottonseed oil. A subset of WT mice were gavaged with 0.2 mL of cottonseed oil alone to serve as a control. We measured body weight gain once mice started on the AIN-93G diet. After two weeks of daily gavage, mice were fasted overnight and sacrificed for tissue and blood collection.

In a second study, we utilized WT male and female mice (n=3-6 per sex, n=6-8 per group) **(**Study 2 design found in **Appendix A Figure 2)**. After weaning, mice continued on a chow diet (Teklad Global 18% protein rodent diet 2918; Envigo) for one week, and then were switched to a vitamin A deficient (VAD) AIN-93G diet (Dietary composition found in **Appendix A Table 2**)

supplemented with either 100 mg of lycopene/kg diet or the same diet without lycopene. Lycopene was added to the diets in the form of 10% lycopene beadlets (Redivivo® DSM). The VAD diet without lycopene contained the same amount of control beadlets (Redivivo® DSM). A third subset of mice received an AIN-93G vitamin A sufficient diet (VAS) (4 IU/kg) supplemented with 100 mg of lycopene/kg diet in the form of beadlets. After four weeks on these diets, mice were fasted overnight and sacrificed for tissue and blood collection.

Necropsy:

Mice were injected intraperitoneally with a dose of a mixture containing ketamine/xylazine (87mg/kg ketamine, 13mg/kg xylazine) to anesthetize. Once mice were unresponsive, the blood was collected via cardiac puncture followed by cervical dislocation. Serum was separated from blood by centrifuging (2400 x *g* for 15 minutes, 4 °C) and stored at -80 °C until analysis. The liver, duodenum, gonadal white adipose tissue (gWAT), kidneys, spleen, heart, adrenals, prostate, seminal vesicles, testes, and ovaries were harvested. For study two, the jejunum, duodenum, and the ileum were harvested.

For mRNA expression analyses, a portion of the gWAT, duodenum, jejunum, ileum, the dorsal lobe of the prostate, and the caudal lobe of the liver were preserved in RNAlater ® (Thermo Fisher Scientific, Waltham, MA), following manufacturer's instructions.

Carotenoid extraction and high-performance liquid chromatography (HPLC) analysis:

Tissue lycopene was extracted, and concentrations and isomers were analyzed by HPLC using a well-established method from our lab (18,19) utilizing approximately 0.05-0.1 g of tissue or 200 µl of serum. For the adrenals, prostate, and ovaries, tissues were pooled for n=1 per

sex/genotype. Lycopene was identified using commercially available standards and comparing their spectra and elution times to the sample. Isomers were characterized by relative retention times and spectral characteristics.

All HPLC reagents utilized for lycopene analyses were HPLC grade. Ethanol was obtained from Decon Laboratories (King of Prussia, PA). Butylated hydroxytoluene was purchased from Sigma-Aldrich. All other chemicals utilized are American Chemical Society grade and were purchased from Thermo Fischer Scientific.

Lipid analyses:

Hepatic lipids were extracted using the Folch method, with small modifications (20). Total cholesterol was measured using the total cholesteryl and cholesteryl ester colorimetric/fluorometric assay kit (BioVision, Milpitas, CA), following the manufacturer's instructions.

RNA isolation:

We isolated mRNA from approximately 50 mg of tissue using the RNA isolation kit (Thermo Fisher Scientific). The tissues were homogenized in 1mL of TRIzol ™ Reagent (Thermo Fisher Scientific) with a sonicator and the isolation was carried out based on the manufacturer's instructions. The concentration and purity of the RNA was measured with a Nano-drop 2000 spectrophotometer (Thermo Fisher Scientific).

Real time PCR Analyses:

Real time PCRs were conducted using either TaqMan Fast Advanced Reaction Mix (Applied Biosystems, Foster City, CA) or PowerUp SYBR Green Master Mix (Applied Biosystems). Primers were purchased from Integrated DNA Technologies (Coralville, IA): intestine-specific homeobox (ISX; 5'-ATCTGGGCTTGTCCTTCTCC-3' and 5'-TTTTCTCTTCTTGGGGCTGA-3'), Scavenger receptor type B, class 1 (SRB1; 5'-CTCATCAAGCAGCAGGTGCTCA-3' and 5'-GAGGATTCGGGTGTCATGAA-3'), BCO1 (Bco1; 5'-CGGAAGTATGTGGCGGTAAA-3'), BCO2 (Bco2; 5'-GCACATCCTCATTACGACCC3' and 5'-CCCCGGGCTCTTTCTTTTT-3'), mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 5'-TTGGCATTGTGGAAGGGCTCAT-3' and 5'-GATGACCTTGCCCACAGCCTT-3') mouse α-tubulin (5'-CAGGGCTTCTTGGTTTTCC-3' and 5'-GGTGGTGTGGGTGGTGAG-3'). Probes were purchased from Thermo Fisher Scientific: (SRB1; Mm00450234). Gene expression analysis was conducted with the StepOnePlus Real-Time PCR System, and the calculations were made utilizing either GAPDH (study 1) or α-tubulin as a housekeeping gene (study 2). RNA expression levels were determined using the Pfaffl method as done in the past (21).

Mitochondrial purification:

Hepatic mitochondria were isolated using established protocols, with small modifications (22). Briefly, approximately 400 mg of liver was placed in a Dounce homogenizer with 3 mL of the homogenization buffer (75mM sucrose, 225mM mannitol, 5mM Tris-HCL pH 7.4, phenylmethylsulfonyl fluoride (PMSF), 1x protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO) and ground over ice exactly 80 times. We kept an aliquot as total liver homogenate, while we employed the remaining homogenate (~2.25 mL) for mitochondrial purification. First, the homogenate was centrifuged at 2,800 x *g* for 5 minutes at 4° C twice to pellet undigested tissues

and cells. The supernatant was centrifuged at 10,500 x *g* for 5 minutes at 4° C, and the pellet was then resuspended in 0.3 mL of the homogenization buffer and centrifuged at 10,500 x *g* for 10 minutes at 4° C. The supernatant was discarded, and the pellet left comprised the mitochondrial fraction. This fraction was resuspended in 0.2 mL homogenization buffer and stored at -80°C. The purity of the isolation was confirmed through a western blot analysis. Mitochondrial purity was estimated using western blot for translocase of outer mitochondria membrane 20 (TOM20) and GAPDH as mitochondrial and cytosolic markers, respectively (22).

Western blot:

The western blot methods including protein quantification, electrophoresis, and detection method were the same as previously detailed in Coronel et al (23). The total protein quantification was performed using the bicinchoninic acid assay, following the manufacturer's protocol (Thermo Fisher Scientific). Between 20-40 μg of protein was loaded onto an SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA). After blocking with fat free milk powder (5% w/v) the membrane was washed with Tris-buffered saline (15 mM NaCl and mM Tris/HCL, pH 7.5) and 1% of triton-100 (TBS-T). After washing, the membranes were incubated overnight with the antibody of interest. The primary antibodies and their dilutions are as follows: GAPDH, 1:1000 (Invitrogen #PA1-988) and TOM20, 1:1000 (Cell Signaling #42406). Secondary antibodies that were either infrared fluorescent-labeled (Li-CorBioscience, Lincoln, NE) or HRP-conjugated were prepared in TBS-T and 5% fat-free milk powder and incubated at room temperature for 1 hour. These immunoblots were developed with the ECL system (Thermo Fisher Scientific), and the band intensities were visualized through ImageQuant LAS 4000 (GE Healthcare) (23).

Statistical Analysis:

GraphPad Prism software (GraphPad Software, Inc.) was utilized to perform statistical analysis. We performed a 4 x 2 multifactorial two-way analysis of variance (ANOVA) to explore interactions between genotypes and sex. No sex differences within genotypes were found for any tissue, so we continued our analyses comparing the effect of genotype on lycopene levels by one-way ANOVA, utilizing Tukey's post-hoc test. When assumptions of ANOVA were violated, the data either underwent a transformation or a non-parametric test was conducted. For analyzing mouse weight gain, repeated measurement analysis was used. During study 2 gene analysis, a two-way ANOVA was utilized for comparison of treatment groups. For all analyses, a P value <0.05 was considered statistically significant.

Results

Study 1

Mice body and tissue weights:

There was a sex effect in mouse weight across all genotypes with males having higher final weights than females ($p=0.0012$, $p=0.033$, $p=0.0024$, $p<0.0001$, $p<0.0001$) (**Figure 2.1**). Additionally, when examining the genotype effect among each sex, the WT (vehicle) females weighed significantly less than the female WT, *Bco2*$^{-/-}$, and DKO mice ($p=0.024$, $p=0.0001$, $p=0.039$). When analyzed for genotype effect regardless of sex, Bco*2*$^{-/-}$ mice had a significantly higher weight than the *Bco1*$^{-/-}$ mice ($p=0.001$). Regarding tissue weights, the *Bco2*$^{-/-}$ mice had a greater gWAT tissue weight than the *Bco1*$^{-/-}$ mice ($p< 0.01$). In the *Bco2*$^{-/-}$ and DKO groups, the males had a significantly higher gWAT tissue weight than females ($p< 0.001$). However, in the

rest of the tissues measured, there were no further sex effects, and there were only genotype effects seen when males and females were analyzed separately (**Appendix A Tables 3, 4**). These results did not differ when the tissue weights were analyzed as a percent of the total body weight (**Appendix A Tables 5, 6**).

Impact of genotype on tissue lycopene accumulation:

In the liver, which accounted for 94-98% of total measured lycopene, the *Bco1*$^{-/-}$ mice exhibited less lycopene accumulation than the *Bco2*$^{-/-}$ ($p < 0.0001$) or the DKO group ($p < 0.001$) (**Figure 2.2A**). No differences were observed between WT and *Bco1*$^{-/-}$ mice. *Bco2*$^{-/-}$ mice also accumulated more splenic lycopene than the *Bco1*$^{-/-}$ mice ($p < 0.001$).

In the serum and the rest of extrahepatic tissues measured, *Bco1*$^{-/-}$ mice tended to have a higher accumulation of lycopene than *Bco2*$^{-/-}$ mice. In the serum, the *Bco2*$^{-/-}$ and WT groups accumulated less lycopene than the DKO group ($p<0.0001$) ($p<0.01$) (**Figure 2.2B**).

The *Bco2*$^{-/-}$ group accumulated less lycopene than the DKO group in the heart ($p <0.0001$), kidneys ($p <0.0001$), duodenum ($p <0.01$), gonadal adipose ($p <0.01$), and testes ($p <0.001$) (Table 1). Compared to the *Bco1*$^{-/-}$ mice, the *Bco2*$^{-/-}$ group accumulated significantly less lycopene in the kidneys ($p <0.001$). The pooled tissues also exhibited a similar extrahepatic genotype accumulation trend, with DKO mice accumulating the most lycopene followed by the *Bco1*$^{-/-}$ group and then the *Bco2*$^{-/-}$ group (**Table 2.1**). The WT mice accumulated less lycopene than the *Bco2*$^{-/-}$ mice in the spleen ($p<0.001$) and heart ($p<0.001$), and less than the DKO mice in the kidneys ($p<0.01$) and heart ($p<0.01$). Additionally, the WT mice accumulated less than the *Bco1*$^{-/-}$ mice in the heart ($p<0.001$).

Lycopene accumulates in the mitochondria:

BCO2 is localized in the inner membrane of the mitochondria, and previous studies show that cycled carotenoids can accumulate in this organelle (11,24). To examine whether lycopene can also accumulate in the mitochondria, we isolated hepatic mitochondria from WT, *Bco1*$^{-/-}$, *Bco2*$^{-/-}$ and DKO mice (**Figure 2.3A**). Lycopene accumulation was obvious when comparing the color of isolated mitochondria samples from WT mice dosed with vehicle and lycopene (**Figure 2.3B**). HPLC analysis showed mitochondria are enriched with lycopene in comparison to liver homogenates, independently of the genotype (**Figure 2.3C**).

We also evaluated whole liver and mitochondrial lycopene isomeric profile. First, however, to gain better context, we examined the isomer configuration in the gavage dose of lycopene, the serum, and the liver. The dose of lycopene that was given to the mice via gavage contained 84% *all-trans*, 10% 5 *cis*, and 6% other *cis* isomers. Tissues contained substantially lower percentage of *trans* isomers than the dose. For a WT mouse dosed with lycopene, as an example, the serum profile was 13% *all-trans*, 55% 5 *cis*, and 32% other *cis* while the liver contained 29% *all-trans*, 41% 5 *cis*, and 30% other *cis* (**Figure 2.4 A-C)**. For the whole liver and mitochondrial evaluation, the ratio of *all-trans*, 5-*cis*, and other *cis* lycopene isomers between the homogenate and mitochondria remained largely unaltered across all genotypes (**Figure 2.4D and E).** However, *Bco1*$^{-/-}$ mice had a reduction in *all-trans* isomers in comparison to the other genotypes. (**Figure 2.4D and E**). This reduction resulted in an increase in *cis* isomers across the whole liver and mitochondria, including 5 *cis*. Isomer configurations for all harvested tissues were also analyzed (**Appendix A Figure 3**).

Total liver lipids and cholesterol:

Previous research has shown the ablation of BCO1 or BCO2 can lead to alterations in hepatic lipid content (11,25,26,27). Both groups of WT mice displayed greater total lipid level content than mice lacking one or both BCOs ($p<0.01$) (**Figure 2.5A**). Additionally, the DKO mice had a higher percentage of lipids than the *Bco1*$^{-/-}$ mice ($p<0.05$).

Hepatic total cholesterol levels mirrored the results described in our total lipid analysis, showing that the WT vehicle mice had greater cholesterol levels than the knockout mice ($p<0.05$), and the WT mice dosed with lycopene had greater levels than the single knockout mice ($p<0.01$) (**Figure 2.5B**).

RNA expression levels:

Portions of the liver, duodenum, gWAT, and prostate were used for RNA isolation and RT-PCRs for relevant genes related to lycopene metabolism (gWAT and prostate data not shown) (**Appendix A Figure 4)**. Lycopene dosing in the control group did not enhance the expression of any tested genes in the liver. The absence of either BCO1 or BCO2 did not result in an upregulation in the expression of the alternate cleavage enzyme, and in the *Bco1*$^{-/-}$ mice hepatic BCO2 expression was significantly downregulated compared to the WT groups ($P<0.01$) (**Figure 2.6A**).

In the duodenum, the WT mice dosed with lycopene exhibited a significant downregulation of SRB1 expression compared to the WT vehicle. Additionally, ISX expression in DKO mice was downregulated compared the WT groups ($P<0.01$) (**Figure 2.6B**).

Study 2

Lycopene accumulation in VAS and VAD mice

To evaluate the effect of vitamin A status on lycopene accumulation, we measured lycopene in the liver and serum in WT mice fed either VAS or VAD diets for four weeks. Hepatic lycopene was higher in VAD mice in comparison to VAS mice (**Figure 2.7A**, $p < 0.05$), while we did not observe significant differences in plasma lycopene levels between these groups (**Figure 2.7B**).

Gene expression

Next, we evaluated gene expression between groups at the level of the duodenum, jejunum, and ileum. In comparison to mice fed a VAD diet without lycopene, mice fed either VAD or VAS diets supplemented with lycopene showed an upregulation of the transcription factor ISX ($p=0.02$) and ($p<0.0001$), respectively (**Figure 2.7C**). We also evaluated the expression of two target genes of ISX: SRB1 and BCO1. Only those mice fed VAS presented lower SRB1 and BCO1 expression ($p=0.02$), and ($p<0.0001$), in comparison to WT mice fed a VAD vehicle diet (**Figure 2.7D and E**).

Discussion

We have had long-time interest in lycopene metabolism and tissue deposition using rodent, ferret, and gerbil models (1,6,18,28,29,30). With the availability of transgenic mouse models with an ablated expression of single or both carotenoid cleavage enzymes, it is possible to investigate which enzyme might be the main contributor to lycopene cleavage. Our previous publications with *Bco1*$^{-/-}$ and *Bco2*$^{-/-}$ mice strongly suggested that the primary enzyme

responsible for the initial cleavage of lycopene was BCO2 (15,29,31). The genetic background of the WT and transgenic BCO/DKO mice were not identical for those studies, however. We overcame this limitation in our current study, allowing us to compare lycopene distribution more accurately in mice.

In humans, BCO1 and BCO2 are expressed in the liver, kidney, adrenal glands, testes, retina, and epithelial cells of the small intestines and stomach (32). Based on data from the Human Protein Atlas (proteinatlas.org) (33), the tissues with the greatest levels of BCO1 and BCO2 are the liver, intestines, and eyes (34). Additionally, our study found that the in the liver accounted for 94-98% of the total lycopene measured across genotypes. Because of the high lycopene liver stores and high hepatic expression of BCOs, we inferred that the data derived from this organ would inform us about which enzyme was most important for the cleavage of lycopene. The results reported in this manuscript support our notion that BCO2 is the most important cleavage enzyme for lycopene based on the substantial differences in lycopene accumulation in the liver. *Bco1*$^{-/-}$ mice had significantly lower hepatic and total body lycopene concentrations than all other genotypes. While serum and many extrahepatic tissues did not follow this trend, these tissues were minor accumulation sites for lycopene under our experimental conditions.

It was also found that lycopene preferentially accumulated in the mitochondria, as observed for other carotenoids in the past (11,24). Previously, mice fed high levels of lutein resulted in impaired mitochondrial function and accumulation of uncharacterized lutein isomers in this organelle, probably due to the capacity of carotenoids to act as electron scavengers in mice (11). While we did not explore whether lycopene accumulation resulted in the impairment of

mitochondrial function, the presence of a comparable isomeric profile between mitochondria and total liver homogenate suggests that this carotenoid did not affect the respiratory chain.

SRB1 is a protein that has been shown to be involved in the uptake of carotenoids (35,36), and additionally, the transcriptional factor ISX has been shown to be involved in the regulation of SRB1 expression. Lobo et al found that retinoic acid induced ISX expression via retinoic acid receptors (RARs), which in turn downregulated SRB1 and BCO1 (16). Just as BCO1 cleaves β-carotene to form retinoids, we have hypothesized that lycopene is cleaved into derivatives known as lycopenoids (6). It is believed that BCO1 derived lycopenoids could bind and serve as agonists/antagonists for RARs and retinoid x receptors (RXRs) (13,37). Results from study 1 demonstrated that in the duodenum, where this ISX/SRB1 axis exists, the WT group dosed with lycopene had a significant decrease in SRB1 expression compared to the WT control group. Thus, lycopene, like dietary beta carotene (16) , might suppress intestinal SRB1. Therefore, study 2 utilized VAS and VAD diets to further explore this axis and to determine if SRB1 is involved in the uptake of lycopene.

In study 2 VAD mice supplemented with lycopene had significantly greater lycopene accumulation in the liver than VAS, lycopene supplemented mice. This suggests that SRB1 plays a role in facilitating the uptake of lycopene. Gene expression results in the three intestinal sections demonstrated that lycopene fed VAD and VAS mice expressed significantly higher levels of ISX than the VAD vehicle mice. The opposite effect was seen in SRB1 and BCO1 expression where VAS mice expressed significantly less levels of both genes than the VAD vehicle mice. These results suggest that lycopene, or its metabolites, might act similarly to retinoic acid on RAR receptors and influence the ISX/SRB1 axis as some studies have

speculated (37,38). Lycopenoids may act as agonists or antagonists for other nuclear receptors but there is still much we do not know about the effect of lycopenoids in the body. These metabolites are difficult to study due to their short half-life, lack of commercially available standards, and because they are only found in very low concentrations in tissues (2,4,5).

Previous studies conducted by our lab explored the bioavailability/accumulation of *all-trans* and *cis* isomers of lycopene. Common findings from these studies and the current results showed that while the dose of lycopene that was provided was largely in *all-trans* form (84% in the current study), tissue and serum lycopene were mostly in *cis* forms. It has been estimated that 90% of the lycopene humans receive from foods is in the *all-trans* form (5). However, a study from our lab (39) where humans were provided with a single dose of 82% *all-trans* 13-C lycopene revealed that *cis* isomers of lycopene increase over time in body tissues, leading to a shorter half-life of the *all-trans* isomer (5.3 versus 8.8 h) and a shorter time to reach maximal plasma concentrations (28 versus 48 h) (39). Results from a ferret study by Hu et al. suggested that ferret BCO2 does not cleave *all-trans* lycopene efficiently or at all, and that based on the K_m value when examining the BCO2 interaction with the 5-*cis* lycopene and *all-trans* substrates, *cis* lycopene was preferentially cleaved by BCO2 (40). However, our results revealed that the isomer composition across all tissues, except for the duodenum, showed a lower percentage of *all-trans* isomers than either 5 *cis* or other *cis*. Additionally, in the liver, isolated hepatic mitochondria, kidneys, spleen, adipose, and testes, the *Bco1*$^{-/-}$ mice had significantly less percentages of *all-trans* isomers than the other groups, suggesting that BCO2 prefers an *all-trans* substrate. The differences in results between these two studies could be due to interspecific differences between ferret and mice.

Another observation we had was that our gene expression results for BCO1 and BCO2 in the liver revealed that the ablation of one enzyme did not enhance the expression of the other

enzyme, but rather resulted in a significant downregulation of the other enzyme. Coronel et al. found that BCO1 over-expression in adipocytes of *Bco1*$^{-/-}$ mice also failed to alter BCO2 expression in the adipose tissue (23).

Additionally, our results of male mice having significantly higher weights than the females in all genotypes is a trend that has seen in other murine studies (41). Additionally, the weights of the female *Bco2*$^{-/-}$ mice in our study being significantly greater than the rest of the female genotype groups is in agreement with the work of Lim et al who showed that *Bco2*$^{-/-}$ mice had a greater food intake than WT mice (25). They also found that the cholesterol levels in the hypothalamus were lower in the *Bco2*$^{-/-}$ mice compared to the WT group. Similarly, both our male and female *Bco2*$^{-/-}$ mice had lower cholesterol in the liver compared to the WT vehicle group. Wu et al also suggested that *Bco2*$^{-/-}$ mice had an increased food intake compared to WT mice was due to *Bco2*$^{-/-}$ mice having mitochondrial dysfunction and altered energy metabolism (27, 42), although in one study they found that despite an increased food intake, BCO2 KO mice had lower weights than the WT mice (43). This lower weight was stated to be caused by mitochondrial dysfunction, and as lycopene has been shown to improve/protect mitochondrial function (44, 45), this could be why our *Bco2*$^{-/-}$ mice did not show a reduction in weight compared to other genotypes.

TABLES AND FIGURES

Table 2.1.

		(Lycopene µg/g; µg/ml)				
Tissues	Genotype P-value	WT (vehicle)	WT	*Bco1*$^{-/-}$	*Bco2*$^{-/-}$	DKO
Kidney	.0001	n.d.	.23±.033^{ab}	.32±.034^{ac}	.23±.014^{b}	.46±.037^{c}
Heart	.0001	n.d.	.15±.023^{a}	.38±.031^{bc}	.32±.012^{b}	.58±.047^{c}
Duodenum	.0101	n.d.	5.2±.871^{ab}	4.5±1.41^{a}	2.9±.341^{a}	7.0±1.40^{b}
Spleen	.0001	n.d.	.89±.264^{a}	.90±.078^{ab}	2.0±.268^{b}	1.8±.220^{b}
Adipose	.0088	n.d.	.37±.064^{ab}	.66±.105^{a}	.36±.048^{b}	.82±.149^{a}
Testes	.0001	n.d.	1.2±.223^{a}	2.1±.273^{a}	3.8±.150^{a}	12.0±.323^{b}
Seminal Vesicles	.3327	n.d.	.14±.077	.11±.013	.14±.011	.22±.061
Adrenals	NA	n.d.	3.6	8.9	3.8	10.7
Prostate	NA	n.d.	.34	.53	.30	.80
Ovaries	NA	n.d.	2.3	3.1	.95	9.7

Table 2.1: Lycopene accumulation in tissues. One-way ANOVA was used to analyze for statistical significance. N=9-12 per group (N=4-7 for testes and seminal vesicles). Adrenals were pooled (n=2) as well as prostate and ovaries (n=1). Tissues not sharing the same letter in a row are significantly different ($p<0.05$). NA = not applicable. n.d.= not detected.

Figure 2.1.

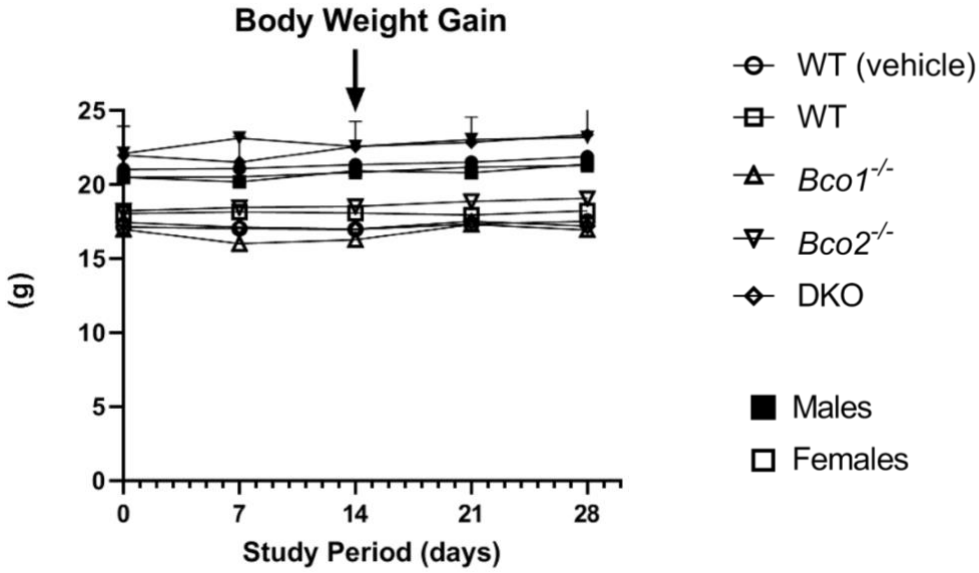


Figure 2.1: Body weight of mice (WT (vehicle), WT, *Bco1*$^{-/-}$, *Bco2*$^{-/-}$, DKO) over the treatment period of 28 days. The weights were tracked starting when mice were placed on the AIN-93G diet at 4 weeks of age (day zero in figure). Days 0-14 they were fed powdered diet, and from days 14-28 they also received a daily oral gavage (start of gavage indicated by arrow). Males weighed significantly more than females on the final treatment day in all genotypes (WT (vehicle), WT, *Bco1*$^{-/-}$, *Bco2*$^{-/-}$, DKO) ($p=0.0012$, $p=0.03$, $p=0.0024$, $p<0.0001$, $p<0.0001$). Female WT vehicle mice weighed significantly less than female WT, *Bco2*$^{-/-}$, and DKO mice ($p=0.02$, $p=0.0001$, $p=0.04$). The *Bco2*$^{-/-}$ mice had a significantly higher weight than the *Bco1*$^{-/-}$ mice ($p=0.001$) when sexes were combined. There was no genotype effect among male mice. One-way and two-way ANOVAs were used to measure significance between sexes and genotypes. N=4-7 per group. Error bars represent SEM.

Figure 2.2.

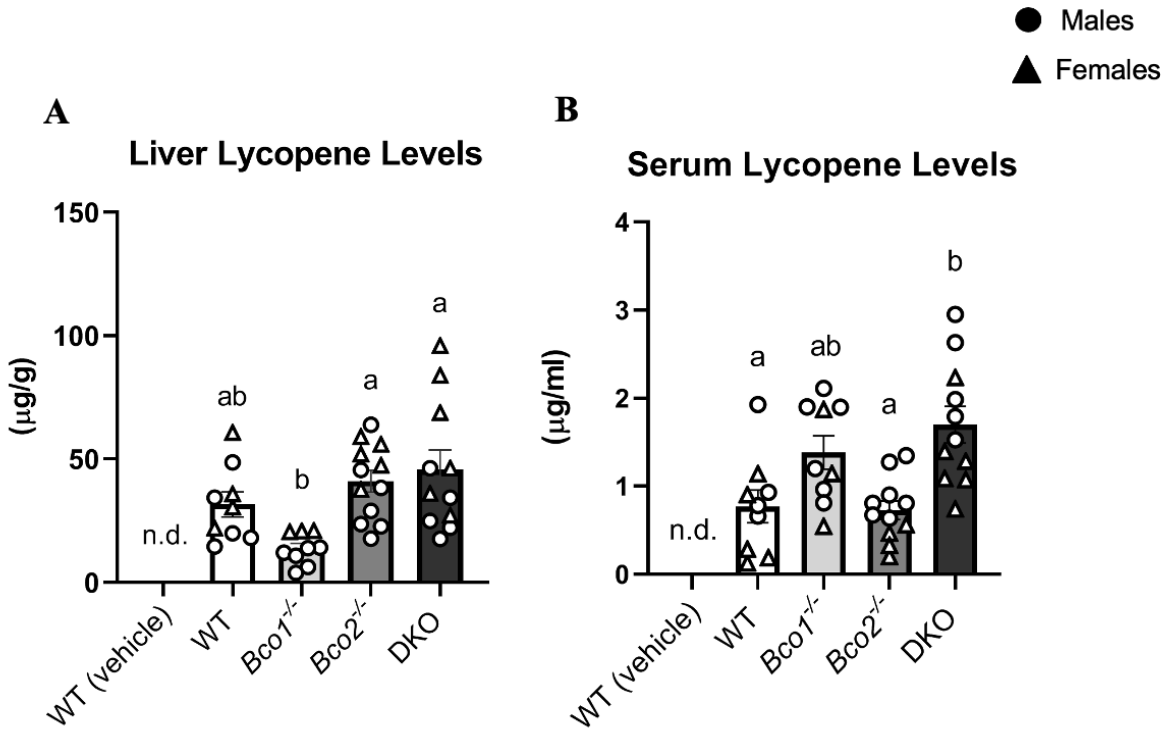


Figure 2.2: Lycopene accumulation in mouse liver and serum. (A) Hepatic lycopene accumulation in mice (WT (vehicle), WT, *Bco1*$^{-/-}$, *Bco2*$^{-/-}$, DKO). The *Bco2*$^{-/-}$ and DKO mice accumulated significantly more lycopene than the *Bco1*$^{-/-}$ mice. **(B)** Serum lycopene accumulation in mice. The DKO mice accumulated significantly more lycopene than the *Bco1*$^{-/-}$ and WT mice. There was no significant sex effect. One-way ANOVA was used to analyze for statistical significance. N=9-12 per group. For each gene, bars not sharing the same letter are significantly different ($p<0.05$). Circles represent males, triangles females. n.d. = not detected. Error bars represent SEM.

Figure 2.3.

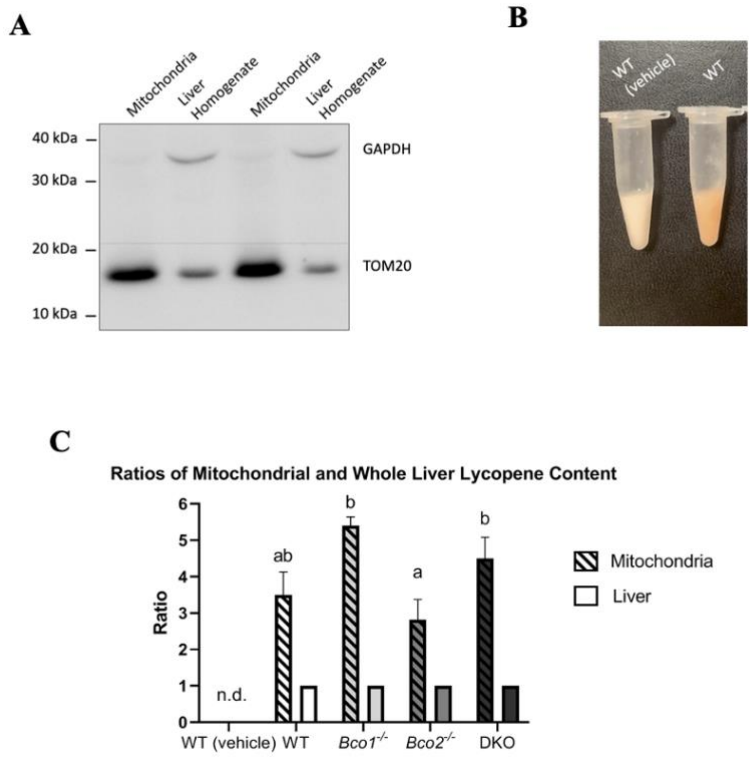


Figure 2.3: Mitochondrial and whole liver lycopene accumulation. (A) Western blot of liver homogenate and purified mitochondrial fractions. The cytosolic housekeeping gene GAPDH was detected in the liver homogenate only, and the mitochondrial protein TOM20 showed stronger bands in the purified mitochondrial samples. **(B)** Lycopene accumulation in the mitochondria of a WT mouse dosed with lycopene. **(C)** Ratios generated from the accumulation of lycopene (µg/g protein) in mitochondrial fractions compared to whole liver homogenate. In all genotypes, the ratio of mitochondria levels of lycopene compared to liver levels was enhanced. The *Bco2*$^{-/-}$ mice had a smaller ratio difference than the *Bco2*$^{-/-}$ mice ($p<0.004$) and the DKO mice ($p<0.04$). One-way ANOVAs was utilized, n= 9-12 per group. Bars not sharing the same letter are significantly different ($p<0.05$). Error bars represent SEM.

Figure 2.4.

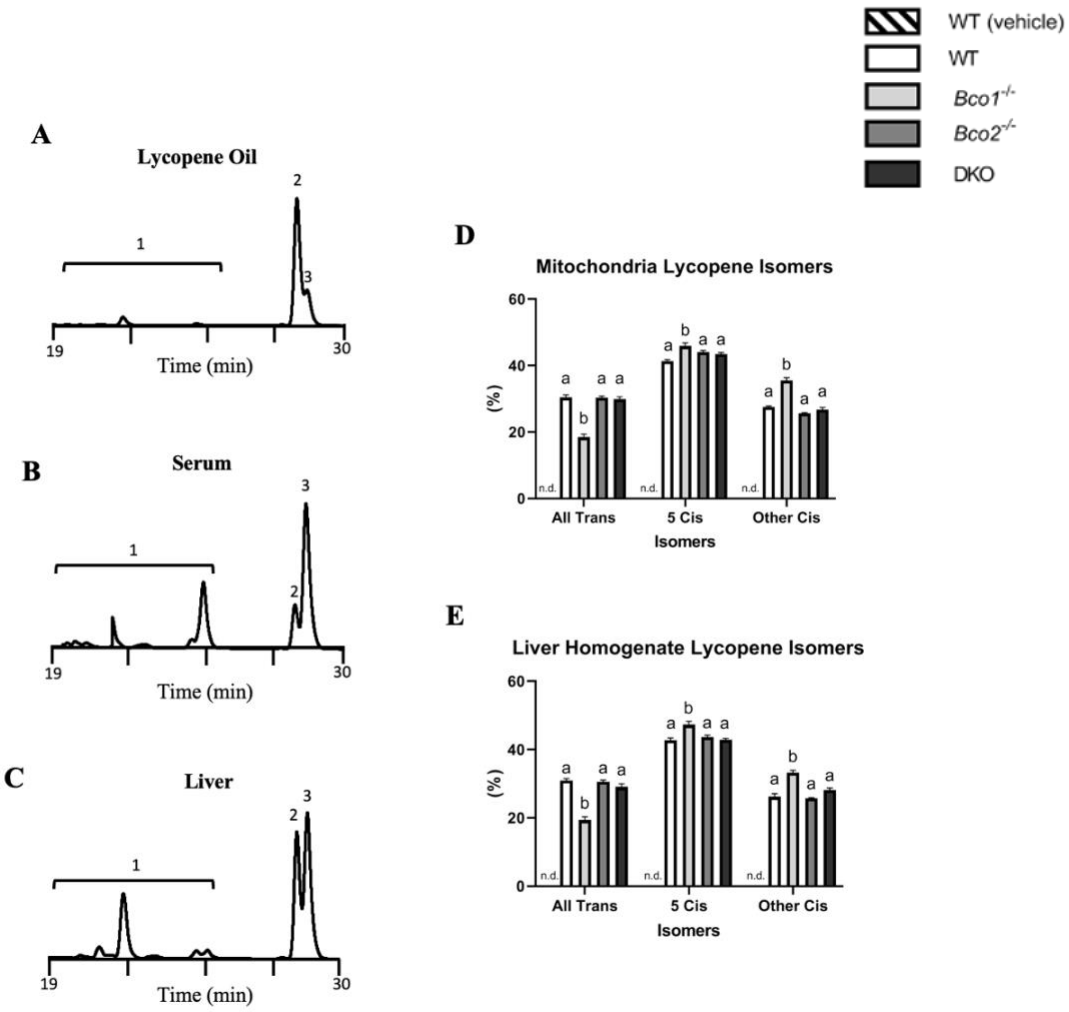


Figure 2.4: HPLC chromatograms of the dose of lycopene given, and a liver and serum sample, and the mitochondria and liver homogenate isomers. Retention times for lycopene isomers HPLC chromatograms (measured at 470 nm) beginning at 19 and ending at 30 minutes. The number (1) indicates other *cis* peaks, while (2) and (3) are *all-trans* and 5 *cis*, respectively. **(A)** The isomeric profile of the dose gavaged to the mice was 84% *all-trans*, 10% 5 *cis*, and 6% other *cis*. **(B)(C)** In the serum of a WT mouse dosed with lycopene, there was 13% *all-trans*, 55% 5 *cis*, and 32% other *cis* while the liver contained 29% *all-trans*, 41% 5 *cis*, and 30% other *cis*. (**D)(E)** When comparing the isomers in the mitochondria enriched samples to the liver homogenate samples, there was no difference seen for any genotypes. However, both the mitochondria and liver homogenate samples from *Bco1*$^{-/-}$ mice had significantly lower levels of *all-trans*, and higher levels of 5 *cis* and other *cis* ($p<0.05$). One-way ANOVAs was utilized, n= 9-12 per group. Bars not sharing the same letter are significantly different ($p<0.05$). Error bars represent SEM.

Figure 2.5.

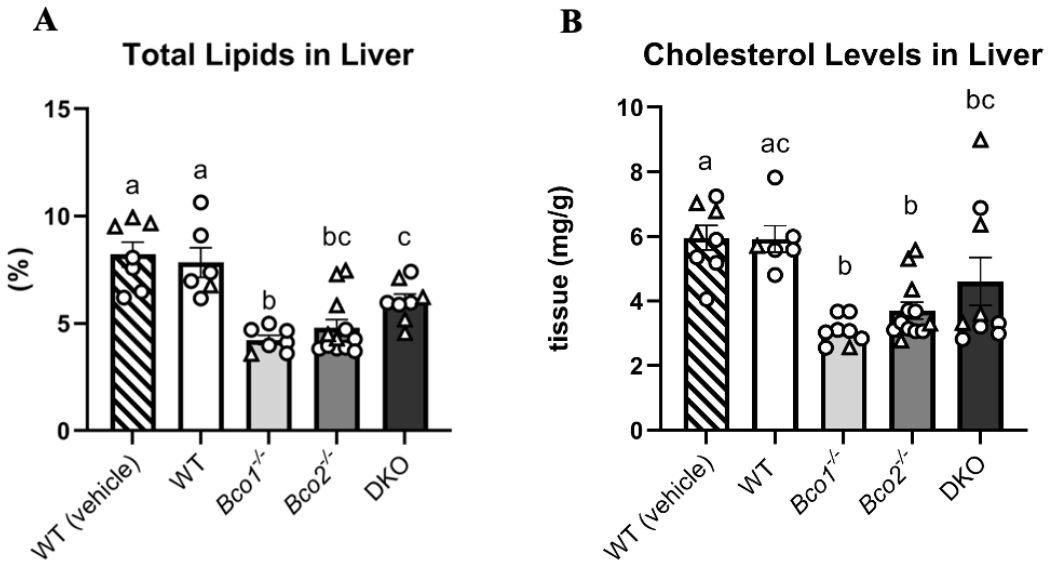


Figure 2.5: Total hepatic lipid and cholesterol levels. (A) Percentage of lipids in livers of mice (WT (vehicle), WT, *Bco1*$^{-/-}$, *Bco2*$^{-/-}$, DKO). The WT vehicle group had significantly higher lipid levels than the knockout groups ($p<0.0001$, $p<0.0001$, $p=0.01$), as did the WT group ($p<0.0001$, $p=0.0002$, $p=0.05$). Additionally, the DKO mice had a higher percentage of lipids than the *Bco1*$^{-/-}$ mice ($p=0.046$). **(B)** Cholesterol levels in the livers of mice (WT (vehicle), WT, *Bco1*$^{-/-}$, *Bco2*$^{-/-}$, DKO) showed that the WT vehicle group had significantly higher levels than the knockout groups ($p<0.0001$, $p=0.002$, $p=0.05$), and the WT group had higher levels than the single KO groups ($p=0.0001$, $p=0.005$) Results were calculated through two-way ANOVA. N=10-12 for both **A** and **B**. No sex effect was seen. For each gene, bars not sharing the same letter are significantly different ($p<0.05$). Circles represent males, triangles females. Error bars represent SEM.

Figure 2.6.

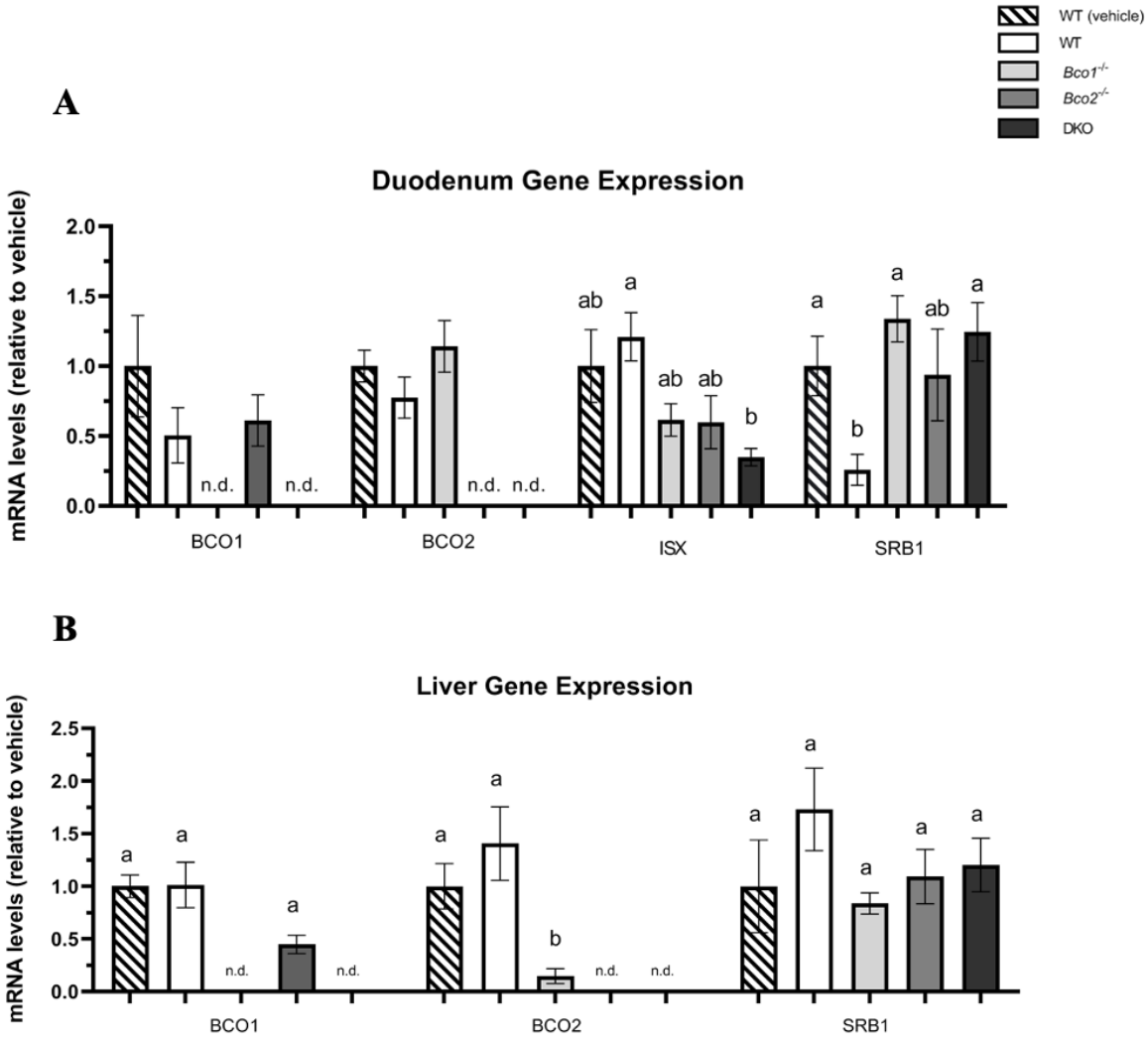


Figure 2.6: Gene expression of Bco1, Bco2, ISX, and SRB1 in the liver and duodenum of different mouse genotypes. (A) In the liver, BCO1 expression in the *Bco2*$^{-/-}$ group was significantly downregulated compared to the WT groups. Similarly, the BCO2 expression in the *Bco1*$^{-/-}$ group was also downregulated compared to the WT groups. **(B)** In the duodenum, the WT group dosed with lycopene exhibited a significant downregulation of SRB1 compared to the WT control. DKO mice had downregulated ISX expression compared to WT groups. n=9-12 per group, one-way ANOVA was used to analyze statistical significance. For each gene, bars not sharing the same letter are significantly different ($p<0.05$). Error bars represent SEM.

Figure 2.7.

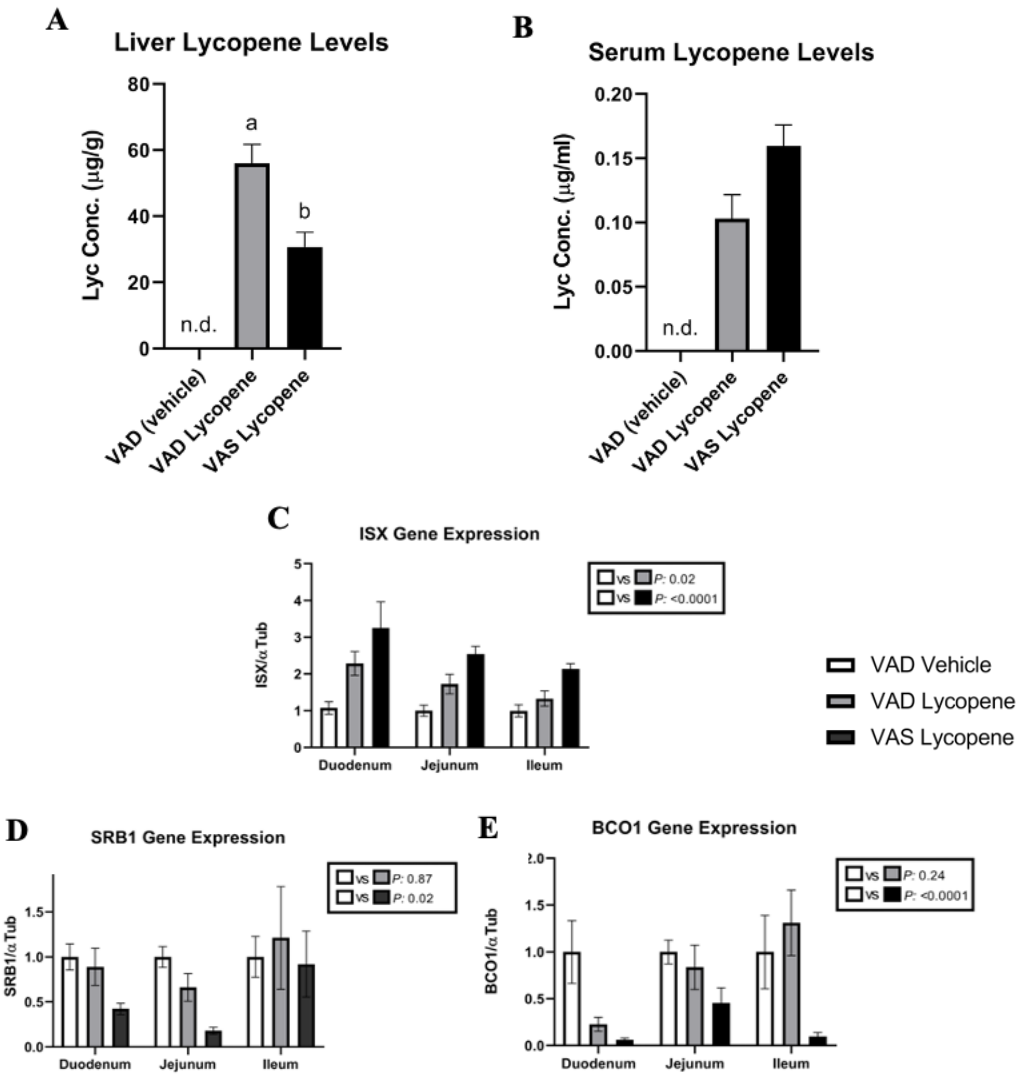


Figure 2.7: Lycopene concentration levels in the liver and serum of mice supplemented with lycopene on VAS and VAD diets and gene expression levels of the genes ISX, SRB1, and BCO1 in VAS, VAD, and VAD (vehicle) mice. (A) In the liver, the VAD mice had significantly greater levels of lycopene than the VAS mice ($p<0.05$). **(B)** In the serum, no significant difference was seen between the treatment groups. **(C)** Both the VAD and VAS mice had higher ISX expression than the VAD vehicle mice. **(D)(E)** VAS mice had lower SRB1 and BCO1 expression than the VAD vehicle mice. n=7-9 per group, two-way ANOVA was used to analyze statistical significance. For each tissue, bars not sharing the same letter are significantly different ($p<0.05$). For each gene expression comparison, a value of $p<0.05$ is significant. Error bars represent SEM.

REFERENCES

1. Arballo J, Amengual J, Erdman JW Jr. Lycopene: A critical review of digestion, absorption, metabolism, and excretion. *Antioxidants* 2021;10(3):342.

2. Cichon MJ, Moran NE, Riedl KM, Schwartz SJ, Clinton SK. Identification of an epoxide metabolite of lycopene in human plasma using 13C-labeling and QTOF-MS. *Metabolites* 2018; 8(1):24.

3. Erdman JW Jr., Ford NA, Lindshield BL. Are the health attributes of lycopene related to its antioxidant function? *Arch. Biochem. Biophys.* 2009:483:229-35.

4. Harrison E. Carotenoids, β-apocarotenoids, and retinoids: The long and the short of it. *Nutrients* 2022;14(7):1411.

5. Kopec R, Riedl K, Harrison E, Curley R Jr., Hruszkewycz, Clinton S, et al. Identification and quantification of apo-lycopenals in fruits, vegetables, and human plasma. *J Agric. Food Chem.* 2010;58(6):3290-3296.

6. Lindshield B, Canene-Adams K, Erdman JW Jr. Lycopenoids: Are lycopene metabolites bioactive? *Arch. Biochem. Biophys.* 2007;458(2):136-140.

7. Wang XD. Lycopene metabolism and its biological significance. *The American journal of clinical nutrition* 2012; 96(5).

8. Moran N, Mohn E, Hason N, Erdman JW Jr., Johnson E. Intrinsic and extrinsic factors impacting absorption, metabolism, and health effects of dietary carotenoids. *Adv. Nutr.* 2018;9.

9. Våge DI, Boman IA. A nonsense mutation in the beta-carotene oxygenase 2 (BCO2) gene is tightly associated with accumulation of carotenoids in adipose tissue in sheep (Ovis aries). *BMC Genet* 2010:11(12).

10. von Lintig J, Vogt K. Filling the gap in vitamin A research. Molecular identification of an enzyme cleaving beta-carotene to retinal. *J Biol Chem* 2000;275(16):11915-20.

11. Amengual J, Lobo GP, Golczak M, Li HN, Klimova T, Hoppel CL, et al. A mitochondrial enzyme degrades carotenoids and protects against oxidative stress. *FASEB J.* 2011;25(3):948-59.

12. Kiefer C, Hessel S, Lampert J, Vogt K, Lederer M, Breithaupt D, et al. Identification and characterization of a mammalian enzyme catalyzing the asymmetric oxidative cleavage of provitamin A. *J Biol Chem*. 2001;276(17):14110-6.

13. dela Seña C, Narayanasamy S, Riedl KM, Curley RW Jr, Schwartz SJ, Harrison EH. Substrate specificity of purified recombinant human β-carotene 15,15'-oxygenase (BCO1). *J Biol Chem*. 2013;288(52):37094-103.

14. dela Seña C, Sun J, Narayanasamy S, Riedl K,Yuan Y, Curley Jr. R, et al. Substrate specificity of purified recombinant chicken β-carotene-9′, 10′-oxygenase (BCO2)*. *Metabolism* 2016;291(28):14609-14619.

15. Ford N, Elsen A, Erdman JW Jr. Genetic ablation of carotene oxygenases and consumption of lycopene or tomato powder diets modulate carotenoid and lipid metabolism in mice. *Nutrition Research* 2013;33(9):733-742.

16. Lobo GP, Hessel S, Eichinger A, Noy N, Moise AR, Wyss A, et al. ISX is a retinoic acid-sensitive gatekeeper that controls intestinal beta,beta-carotene absorption and vitamin A production. *FASEB J.* 2010;24(6):1656-66.

17. Amengual J, Coronel J, Marques C, Aradillas-Garcia C, Vargas Morales JM, Andrade F, et al. β-carotene oxygenase 1 activity modulates circulating cholesterol concentrations in mice and humans. *J Nutr*. 2020;150(8):2023-2030.

18. Moran N, Clinton S, Erdman JW Jr. Differential bioavailability, clearance, and tissue distribution of the acyclic tomato carotenoids lycopene and phytoene in Mongolian Gerbils. *J. Nutr.* 2013;143(12):1920-1926.

19. Zuniga KE, Erdman JW Jr. Combined consumption of soy germ and tomato powders results in altered isoflavone and carotenoid bioavailability in rats. *J Agric Food Chem*. 2011;59(10):5335-41.

20. Folch J, Lees M, Stanley G H S. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem.* 1957:226(1):497-509.

21. Amengual J, Petrov P, Bonet M, Ribot J, Palou A. Induction of carnitine palmitoyl transferase 1 and fatty acid oxidation by retinoic acid in HepG2 cells. *Int. J. Biochem. Cell Biol.* 2012;44(11):2019-2027.

22. Solesio ME, Xie L, McIntyre B, Ellenberger M, Mitaishvili E, Bhadra-Lobo S, et al. Depletion of mitochondrial inorganic polyphosphate (polyP) in mammalian cells causes metabolic shift from oxidative phosphorylation to glycolysis. *Biochem J*. 2021;478(8):1631-1646.

23. Coronel J, Yu J, Pilli N, Kane M, Amengual J. The conversion of β-carotene to vitamin A in adipocytes drives the anti-obesogenic effects of β-carotene in mice. *Molecular Metabolism* 2022;66.

24. Palczewski G, Amengual J, Hoppel C, von Lintig J. Evidence for compartmentalization of mammalian carotenoid metabolism. *FASEB* 2014;28(10):4457-4469.

25. Hessel S, Eichinger A, Isken A, Amengual J, Hunzelmann S, Hoeller U, et al. CMO1 deficiency abolishes vitamin A production from beta-carotene and alters lipid metabolism in mice. *J Biol Chem*. 2007;282(46):33553-33561.

26. Lim J, Liu C, Hu K, Smith D, Wang XD. Ablation of the carotenoid cleavage enzymes (BCO1 and BCO2) induced hepatic steatosis by altering the farsenoid X receptor/miR-34a/sirtuin 1 pathway. *Arch. Biochem. Biophys* 2018;654:1-9.

27. Wu L, Guo X, Wong S, Lu P, Hartson S, Medeiros D, et al. Deficiency of β-carotene oxygenase 2 induces mitochondrial fragmentation and activates the STING-IRF3 pathway in the mouse hypothalamus. *JNB* 2021:88:108542.

28. Boileau A, Merchen N, Wasson K, Atkinson A, Erdman JW Jr. *Cis*-lycopene is more bioavailable than *trans*-lycopene *in vitro* and *in vivo* in lymph-cannulated ferrets. *J. Nutr.* 1999;129(6):1176–1181.

29. Ford N, Clinton S, von Lintig J, Wyss A, Erdman JW Jr. Loss of carotene-9',10'-monooxygenase expression increases serum and tissue lycopene concentrations in lycopene-fed mice. *J Nutr*. 2010;140(12):2134-2138.

30. Boileau TW-M, Boileau AC, Erdman JW Jr. Bioavailability of all-trans and cis–Isomers of Lycopene. *Experimental Biology and Medicine* 2002;227(10):914-919.

31. Lindshield B, King J, Wyss A, Goralczyk R, Lu C-H, Ford N, et al. Lycopene biodistribution is altered in 15,15'-carotenoid monooxygenase knockout mice. *J. Nutr.* 2008; 138:2367-2371.

32. Lindqvist A, He YG, Andersson S. Cell type-specific expression of β-carotene-9′,10′ monooxygenase in human tissues. *Journal of Histochemistry & Cytochemistry* 2005;53:11.

33. Proteinatlas.org [Internet] The Human Protein Atlas; [cited 2023 Jan 30] Available from: https://www.proteinatlas.org/ENSG00000135697-BCO1

34. Uhlen M, Fagerberg L, Hallstrom B, Lindskog C, Oksvold P, Mardinoglu A. Proteomics. Tissue-based map of the human proteome. *Science* 2015;347(6220):1260419.

35. Kiefer C, Sumser E, Wernet M, von Lintig J. A class B scavenger receptor mediates the uptake of carotenoids in *Drosopila. PNAS* 2002;99(16):10581-10586.

36. Shyam R, Vachali P, Gorusupudi A, Nelson K, Bernstein PS. All three human scavenger receptor class B proteins can bind and transport all three macular xanthophyll carotenoids. *Arch Biochem Biophys*. 2017;634:21-28.

37. Bohn T, de Lera A, Landrier J-F, Carlsen H, Merk D, et al. State-of-the-art methodological investigation of carotenoid activity and metabolism-from organic synthesis via metabolism to biological activity-exemplified by a novel retinoid signalling pathway. *Food Functional* 2023;14(621).

38. Aydemir G, Kasiri Y, Birta E, Beke G, Garcia A, Bartok EM, et al. Lycopene-derived bioactive retinoic acid receptors/retinoid X receptors-activating metabolites may be relevant for lycopene's anti-cancer potential. *Molecular Nutrition and Food Research* 2013;57(5):739-747.

39. Moran NE, Cichon MJ, Riedl KM, Grainger EM, Schwartz SJ, Novotny JA, et al. Compartmental and noncompartmental modeling of ^{13}C-lycopene absorption, isomerization, and distribution kinetics in healthy adults. *Am J Clin Nutr.* 2015;102(6):1436-49.

40. Hu KQ, Liu C, Ernst H, Krinsky NI, Russell RM, Wang XD. The biochemical characterization of ferret carotene-9',10'-monooxygenase catalyzing cleavage of carotenoids *in vitro* and *in vivo*. *J Biol Chem*. 2006;14;281(28):19327-38.

41. Wang C, He Y, Xu P, Yang Y, Saito K, Xia Y, et al. TAp63 contributes to sexual dimorphism in POMC neuron functions and energy homeostasis. *Nature* 2018;9(1544).

42. Wu L, Guo X, Hartson S, Davis M, He H, Medeiros D, et al. Lack of β, β-carotene-9′, 10′-oxygenase 2 leads to hepatic mitochondrial dysfunction and cellular oxidative stress in mice. *Mol. Nutr. Food Res* 2017;61(5).

43. Wu L, Lyu Y, Clarke S, Lucas E, Smith B, Hildebrand D, et al. Targeted metabolomics reveals abnormal hepatic energy metabolism by depletion of β-carotene oxygenase 2 in mic. *Scientific Reports* 2017;7(14624).

44. Qu M, Jiang Z, Liao Y, Song Z, Nan X. Lycopene prevents amyloid (Beta)-induced mitochondrial oxidative stress and dysfunctions in cultured rat cortical neurons. *Neurochem. Res.* 2016;41(6):1354-64.

45. Sheriff S, Ibrahim S, Devaki T, Chakraborty S, Agarwal S, Perez-Sanchez. Lycopene prevents mitochondrial dysfunction during D-galactosamine/lipopolysaccharide-induced fulminant hepatic failure in albino rats. *J Proteome Res* 2017;6(9):3190-3199.

CHAPTER 3

Future Studies and Conclusion

In the completed studies, we explored the bioaccumulation of lycopene in tissues in WT and knockout mice. We found that with ablation of BCO2, there was a substantial increase in hepatic lycopene accumulation compared to *Bco1*$^{-/-}$ mice. In order to further probe lycopene tissue metabolism, a new study was designed to evaluate lycopene turnover. Male and female WT and DKO mice will be fed a VAD AIN-93G diet for two weeks prior to lycopene consumption. Then the mice will be on a AIN-93G diet supplemented with 10% lycopene beadlets (put in mg/kg diet here) for two weeks. After the two weeks, groups of WT and DKO mice will be terminated for baseline data. Remaining mice will be placed back on a lycopene-free VAD AIN-93G diet either for one day or three days before takedown. HPLC will be utilized to compare the lycopene levels in tissues between mice from baseline and the two time points. This will provide insight into tissue specific lycopene degradation and turnover rate. This will provide novel data as no studies to our knowledge have directly evaluated lycopene turnover in preclinical animal models.

Other results from the current studies suggested that lycopene's metabolites could act upon RAR receptors to influence the ISX/SRB1 axis. This is a novel finding to our knowledge and stimulates a lot of questions. One obvious question is what specific lycopene metabolites interact with these RAR/RXR receptors? While this is a relevant and important question, we do not yet have the means to study lycopene metabolites effectively. A second question that this research raised was if there was a way to provide further evidence that lycopene metabolites were acting specifically through RAR receptors. This question led us to propose a second new study. We will utilize an RAR antagonist that blocks all RAR activity. This design will allow us to test the question of whether lycopene influences the ISX/SRB1 axis via stimulation of RAR signaling, in

a manner similar to retinoic acid. Briefly, four groups of WT mice will be on the VAD AIN-93G diet to prep for carotenoid uptake. Two groups will continue on this diet for the remaining 10 days, with one group receiving a gavage dose of retinoic acid as a positive control 24 hours before sacrifice. The other two groups will receive the VAD AIN-93G diet supplemented with lycopene beadlets (10%) for the 10 days, with one of these groups receiving the RAR antagonist suspended in cottonseed oil via gavage. Based on previous studies (1,2), the RAR antagonist will be given at the dose of 5mg/kg body weight, and the mouse will receive the doses 30 and 6 hours before sacrifice.

In conclusion, these results support the hypothesis that BCO2 is the most important cleavage enzyme of lycopene in the liver, the tissue where the majority of the lycopene accumulated across mouse genotypes (94-98% of lycopene from 11 tissues). Lycopene concentration was enriched in the hepatic mitochondria (where BCO2 resides) independently of the expression of BCO2. Additionally, the similar lycopene isomer profile of whole liver and isolated mitochondria suggests that there is not preferential cleavage of *cis* forms of lycopene compared to *all-trans* lycopene. Finally, SRB1 appears to play a role in facilitating the uptake of lycopene in the intestines, and lycopene's metabolites may act on RAR receptors to influence the ISX/SRB1 axis.

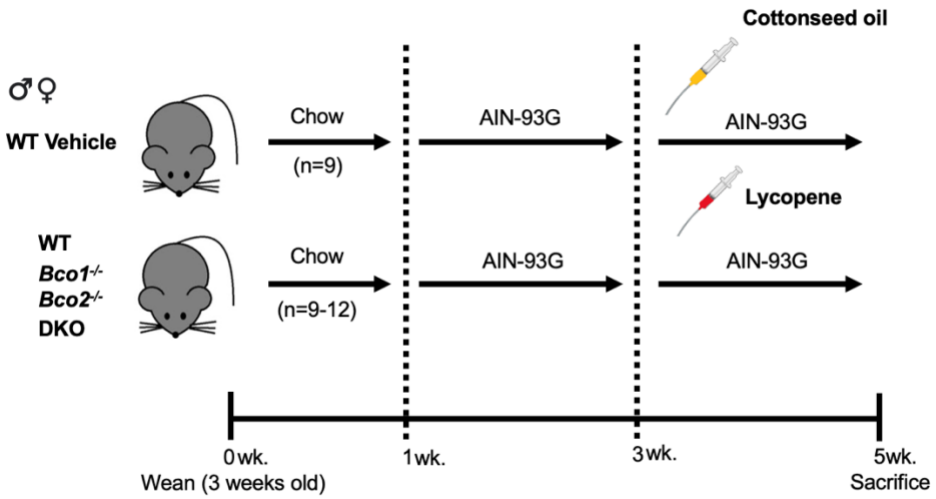
REFERENCES

1.Ramkumar S, Moon J, Golczak, von Lintig. LRAT coordinates the negative-feedback regulation of intestinal retinoid biosynthesis from β-carotene. *J. Lipid Res*. 2021;62:100055.

2. Chung S, Wang X, Roberts S, Griffey S, Reczek P, Wolgemuth D. Oral administration of a retinoic acid receptor antagonist reversibly inhibits dermatogenesis in mice. *Endocrinology* 2011;152(6):2492-2502.

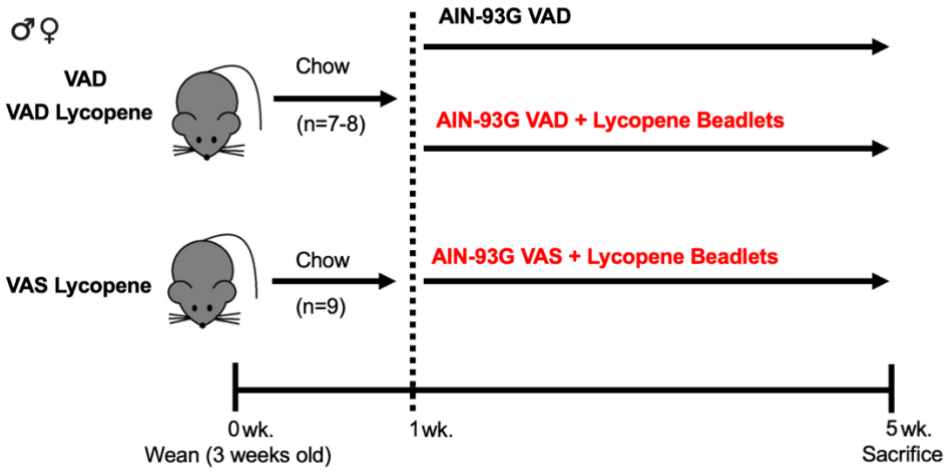
APPENDIX A

Appendix Figure A.1.



Appendix Figure A.1: Animal model study design for study 1.

Appendix Figure A.2.



Appendix Figure A.2: Animal model study design for study 2.

Appendix Table A.1.

AIN-93G	
Ingredients	**Amount(g)**
Corn Starch	397.5
Casein	200
Maltodextrin	132
Sucrose	100
Alphacel (cellulose)	50
Mineral Mix (AIN93M-MX)	35
Vitamin Mix (AIN93-Vx)	10
L-Cysteine	3
Choline Bitartrate	2.5
Cottonseed Oil	70
Total	1000

Appendix Table A.1: Composition of modified (cottonseed oil replaced soybean oil) AIN-93G diet.

Appendix Table A.2.

AIN-93G Vit A Free	
Ingredients	**Amount(g)**
Corn Starch	397.5
Casein	200
Maltodextrin	132
Sucrose	100
Alphacel (cellulose)	50
Mineral Mix (AIN93M-MX)	35
Vitamin Mix (AIN93-Vx) Vit A Free	10
L-Cysteine	3
Choline Bitartrate	2.5
Cottonseed Oil	70
Lycopene Beadlets (redivivo® DSM)	0.1
Total	1000.1

Appendix Table 2. Composition of Vitamin A free AIN-93G diet with lycopene beadlets

Appendix Table A.3.

		Male	(Weight of tissues/g)		
Tissues	Genotype P-value	*WT*	*Bco1*$^{-/-}$	*Bco2*$^{-/-}$	*DKO*
Liver	.0057	.80±.035^{a}	1.3±.113^{b}	1.1±.030^{ab}	.89±.088^{ab}
Kidney	.0471	.27±.010^{a}	.31±.009^{ab}	.33±.006^{b}	.31±.022^{ab}
Heart	.1047	.10±.006	.12±.006	.12±.003	.12±.012
Duodenum	.4672	.10±.014	.11±.004	.12±.006	.11±.005
Spleen	.1099	.06±.004	.07±.002	.06±.002	.06±.003
Adipose	.0440	.38±.020	.25±.014	.52±.056	.50±.131
Adrenals	.2376	.01±.001	.01±.001	.01±.007	.01±.005
Testes	.3058	.16±.009	.17±.003	.18±.008	.17±.014
Seminal Vesicles	.3026	.13±.006	.13±.005	.16±.007	.14±.022
Total Prostate	.0021	.03±.002^{a}	.04±.001^{ab}	.08±.022^{b}	.04±.006^{ab}

Appendix Table A.3: Weight of tissues in male mice. One-way ANOVA was used to analyze for statistical significance. N=4-7 per group. Adrenals were pooled (n=2) as well as prostate (n=1). Tissues not sharing the same letter in a row are significantly different ($p<0.05$).

Appendix Table A.4.

		Female (Weight of tissues/g)			
Tissues	Genotype P-value	*WT*	*Bco1*$^{-/-}$	*Bco2*$^{-/-}$	*DKO*
Liver	0.1684	.73±.063	.81±.010	.85±.039	.75±.020
Kidney	0.4316	.24±.020	.24±.006	.25±.006	.24±.008
Heart	0.3707	.11±.002	.11±.001	.11±.004	.10±.002
Duodenum	0.1456	.11±.005	.12±.020	.13±.010	.10±.006
Spleen	0.1987	.08±.010	.07±.005	.05±.013	.06±.003
Adipose	0.0343	.24±.040	.13±.004	.24±.017	.17±.019
Adrenals	0.0001	.01±.000^{a}	.01±.000^{a}	.07±.015^{b}	.01±.000^{a}
Ovaries	0.0622	.01±.000	.01±.002	.04±.021	.01±.001

Appendix Table A.4: Weight of tissues in female mice. One-way ANOVA was used to analyze for statistical significance. N=4-8 per group. Adrenals were pooled (n=2) as well as ovaries (n=1). Tissues not sharing the same letter in a row are significantly different ($p<0.05$).

Appendix Table A.5.

		Male (Weight as % body weight)			
Tissues	Genotype P-value	*WT*	*Bco1*$^{-/-}$	*Bco1*$^{-/-}$	*DKO*
Liver	.0009	3.7±.095^{a}	5.9±.452^{b}	4.5±.142^{ab}	3.78±.150^{a}
Kidney	.0028	1.2±.019^{a}	1.5±.028^{b}	1.4±.024^{b}	1.3±.021^{ab}
Heart	.1155	.48±.021	.55±.022	.52±.010	.52±.024
Duodenum	.9257	.47±.055	.50±.026	.50±.024	.47±.029
Spleen	.0001	.29±.014^{ab}	.32±.006^{b}	.25±.006^{a}	.26±.011^{a}
Adipose	.0063	1.8±.135^{ab}	1.2±.052^{a}	2.2±.198^{b}	2.0±.109^{ab}
Adrenals	.4270	.03±.003	.03±.003	.06±.031	.02±.003
Testes	.0724	.74±.025	.79±.018	.79±.034	.71±.012
Seminal Vesicles	.2680	1.2±.054	1.2±.050	1.4±.045	1.2±.121
Total Prostate	.0030	.12±.008^{a}	.18±.007^{ab}	.35±.102^{b}	.17±.013^{ab}

Appendix Table A.5: Weight of tissues as percent body weight in male mice. One-way ANOVA was used to analyze for statistical significance. N=4-7 per group. Adrenals were pooled (n=2) as well as prostate (n=1). Tissues not sharing the same letter in a row are significantly different ($p<0.05$).

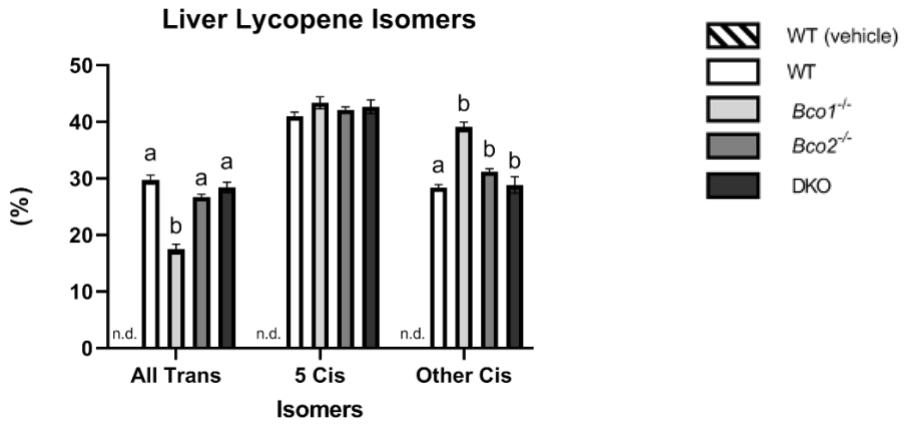
Appendix Table A.6.

		Female (Weight as % body weight)			
Tissues	Genotype P-value	*WT*	*Bco1*$^{-/-}$	*Bco2*$^{-/-}$	*DKO*
Liver	0.2089	4.0±.337	4.8±.044	4.5±.216	4.4±.238
Kidney	0.4378	1.3±.067	1.4±.021	1.4±.036	1.4±.035
Heart	0.0913	.58±.015	.63±.013	.56±.020	.60±.021
Duodenum	0.3362	.62±.023	.68±.120	.68±.052	.57±.030
Spleen	0.1695	.42±.055	.43±.024	.28±.068	.33±.025
Adipose	0.1680	1.3±.275	.74±.035	1.3±.097	1.0±.135
Adrenals	0.0001	.04±.002^{a}	.04±.002^{a}	.35±.078^{b}	.04±.002^{a}
Ovaries	0.0633	.04±.002	.05±.011	.23±.109	.05±.006

Appendix Table A.6: Weight of tissues as percent body weight in female mice. One-way ANOVA was used to analyze for statistical significance. N=4-8 per group. Adrenals were pooled (n=2) as well as ovaries (n=1). Tissues not sharing the same letter in a row are significantly different ($p<0.05$).

Appendix Figures A.3A-L.

Figure A.3A



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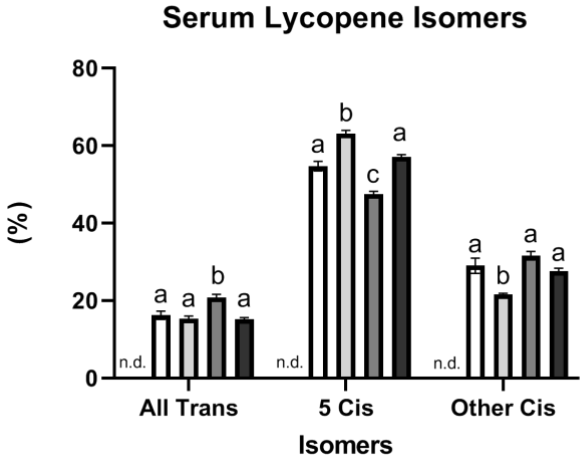

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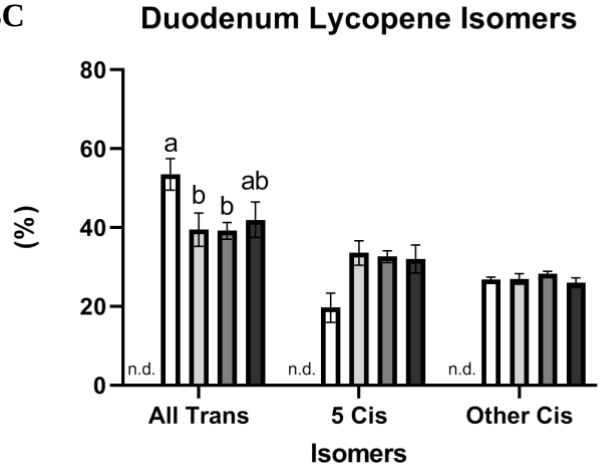

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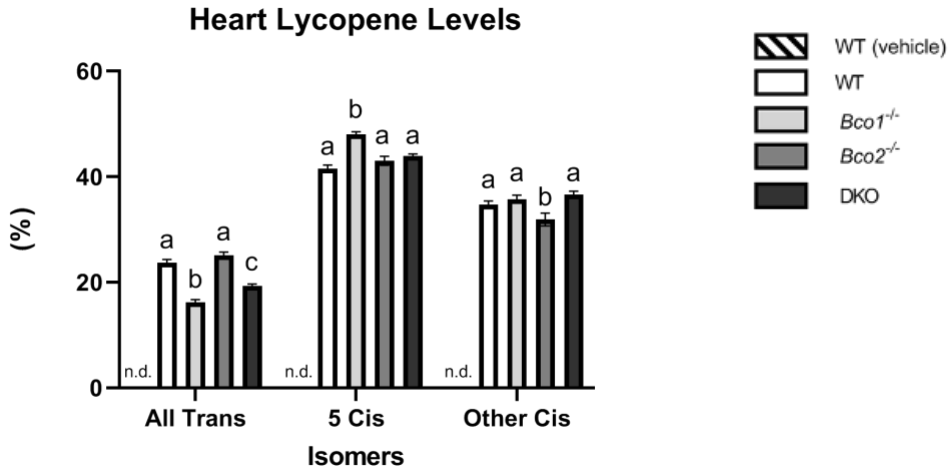

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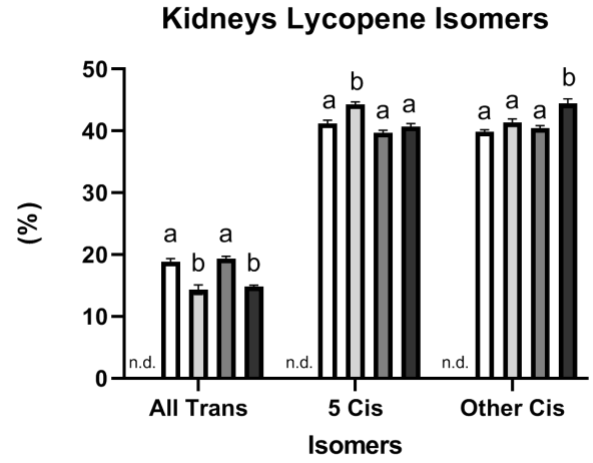

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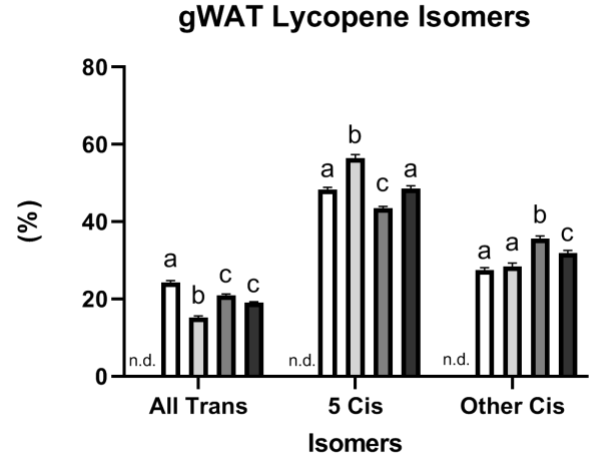

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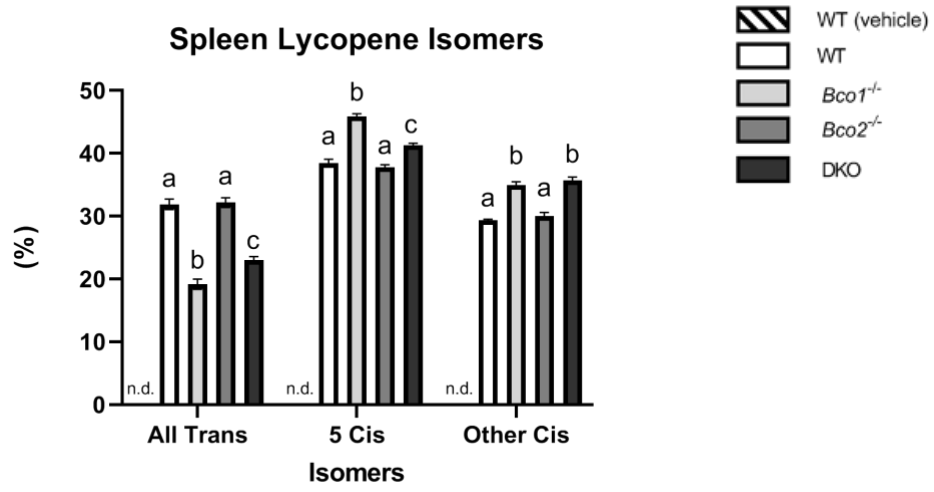

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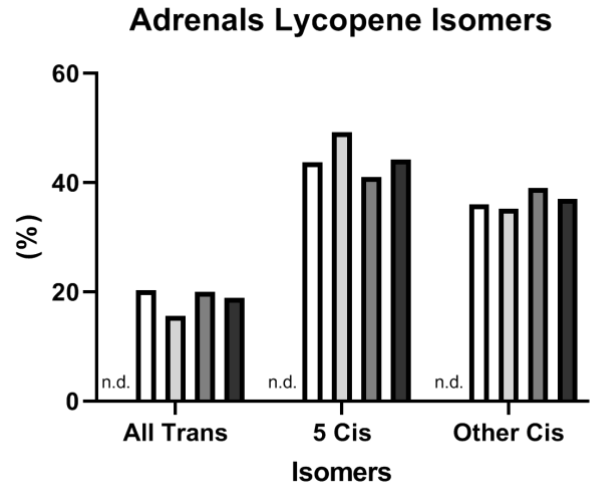

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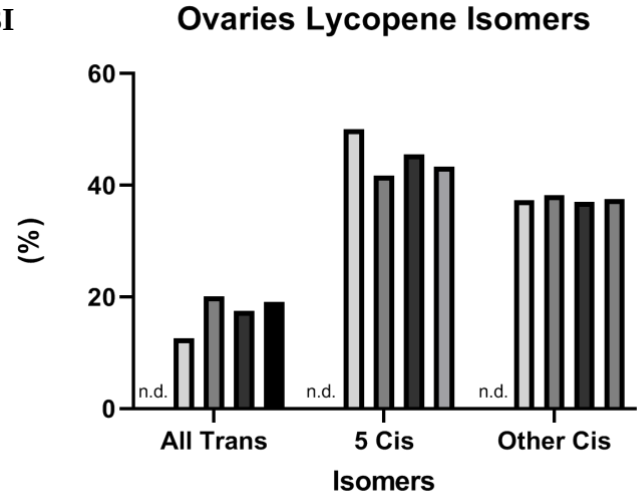

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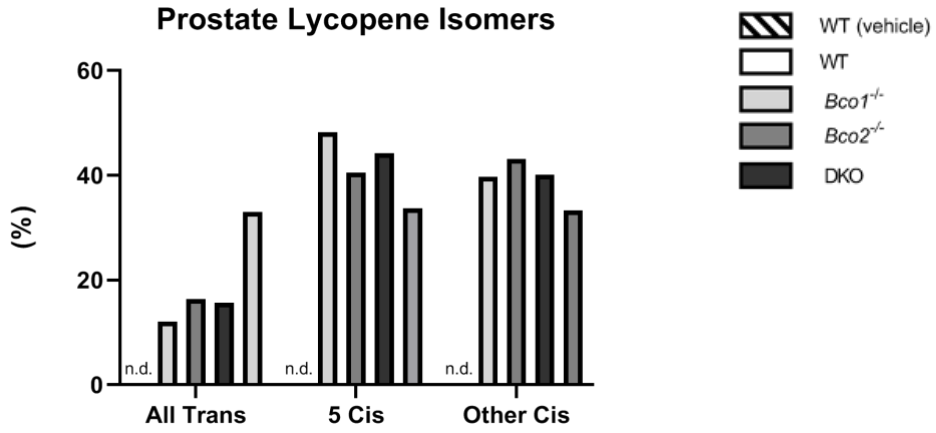

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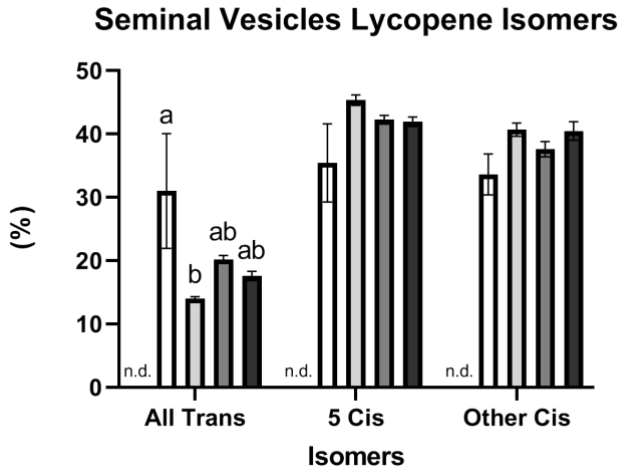

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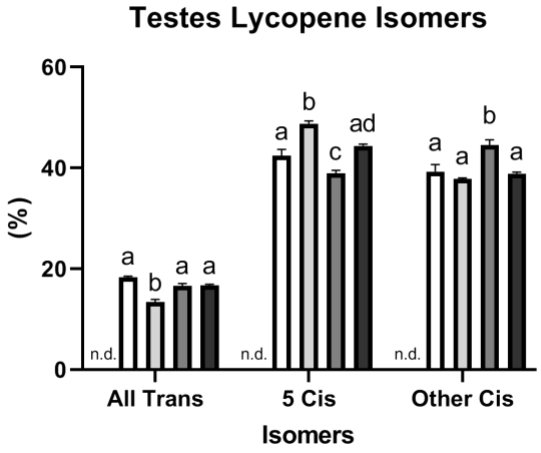

Appendix Figures A.3A-L: Lycopene isomer configurations in various tissues. One-way ANOVAs was utilized, n= 9-12 per group. Bars not sharing the same letter are significantly different ($p<0.05$). Error bars represent SEM.

Appendix Figures A.4A-D.

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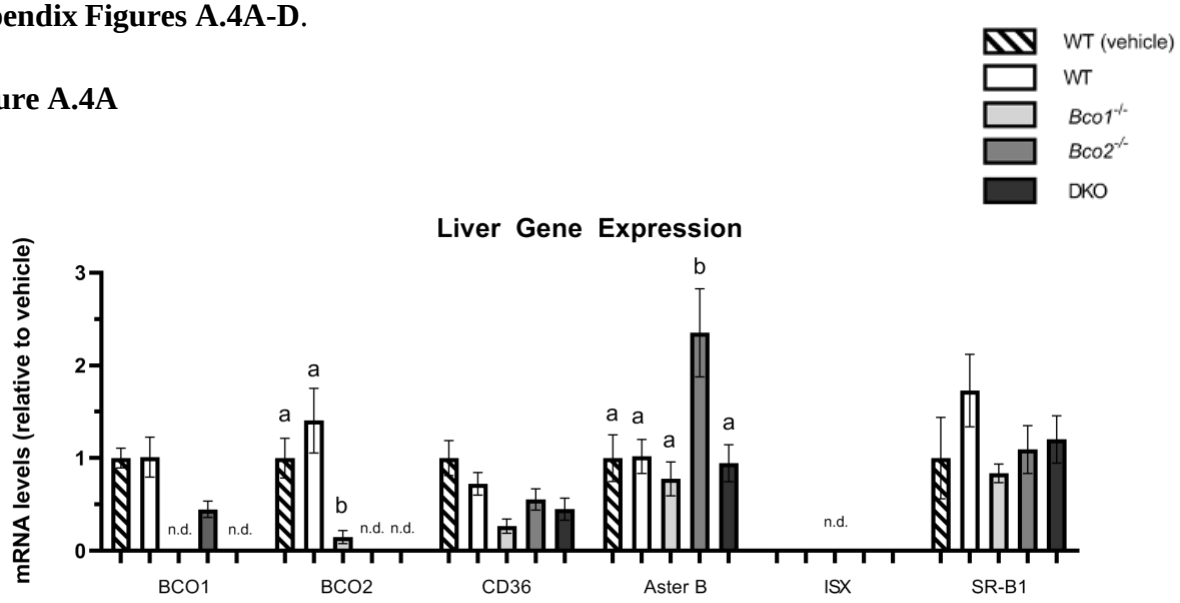

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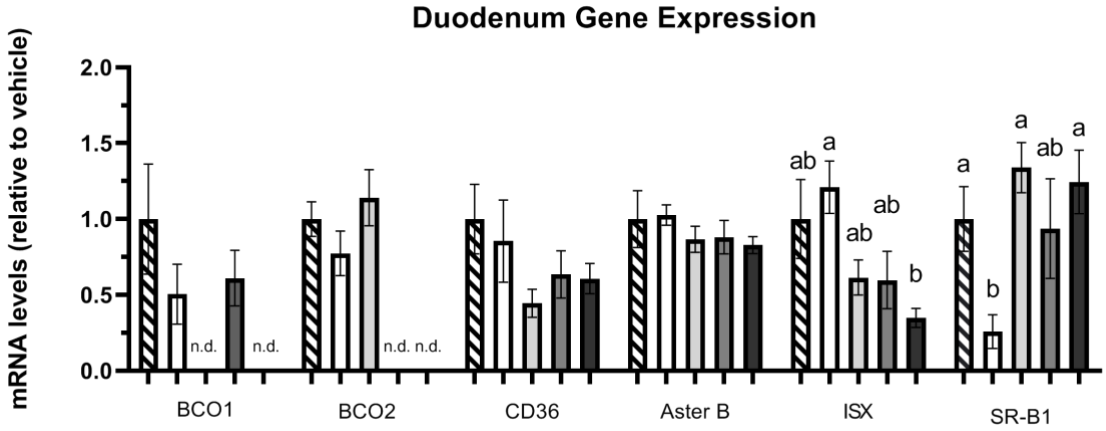

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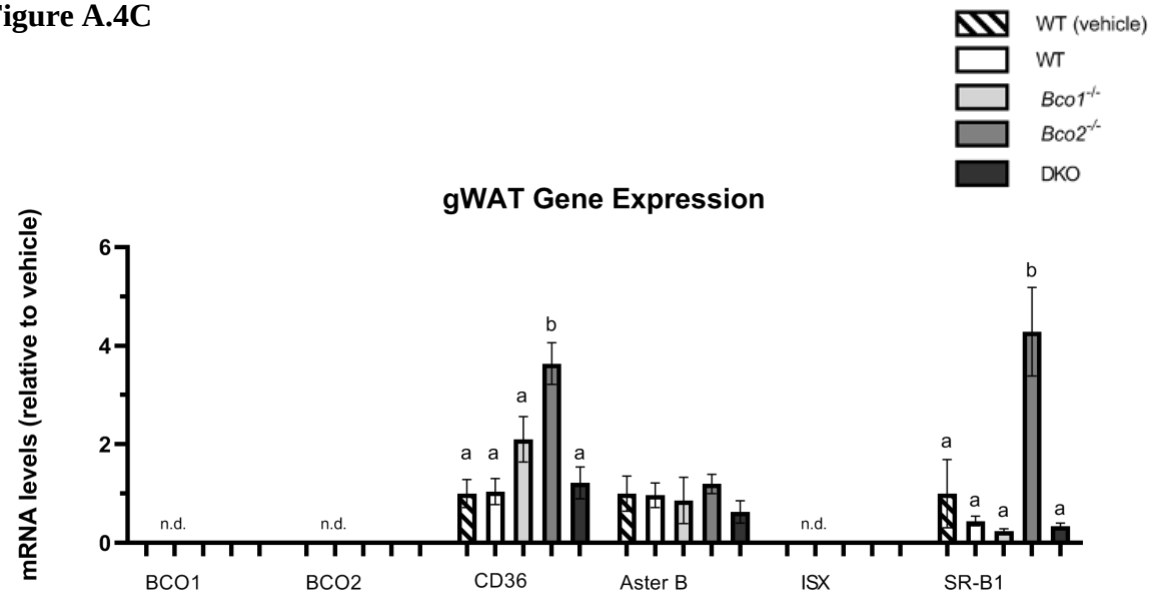

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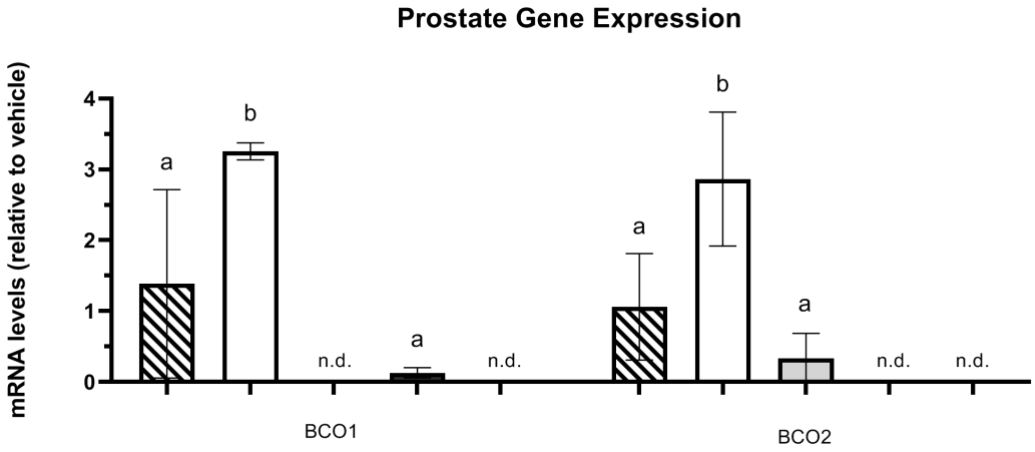

Appendix Figures A.4A-D: Study 1 gene expression results for liver, duodenum, gWAT, and prostate. One-way ANOVAs was utilized, n= 9-12 per group. Bars not sharing the same letter are significantly different ($p<0.05$). Error bars represent SEM.