DIET-INDUCED CHANGES IN MOUSE CELLS *IN VITRO* AND *IN VIVO* ZEBRAFISH MODELS OF ANGIOGENESIS

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE OF
BILKENT UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE
IN
MATERIAL SCIENCE AND NANOTECHNOLOGY

BY
SELVİN YILDIZ
January 2024

To all scientists that we lost due to the earthquake and to my beloved sister…

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By Selvin Yıldız

January 2024

I certify that I read this thesis and that, in my opinion, it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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ABSTRACT

DIET-INDUCED CHANGES *IN VITRO* MOUSE AND *IN VIVO* ZEBRAFISH MODELS OF ANGIOGENESIS AND REGENERATION

Selvin Yıldız

M.Sc. in Material science and Nanotechnology

Supervisor: Özlen Konu Karakayalı
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Cardiovascular disorders rank as the primary cause of global mortality. Being overweight or obese impacts the pathogenesis of cardiovascular disease, resulting in an imbalance in endothelial function, cell growth, and inflammatory activation. Disruption of these factors resulting from endothelial cell dysfunction serves as both an outcome and a catalyst for vascular disease processes. Endothelial cells (ECs) are a natural barrier between circulating blood and vessel components. They also play critical roles in multiple physiological and pathophysiological processes, such as angiogenesis, vascular permeability, and inflammation. Amelioration of endothelial dysfunction may be attained by weight loss; however, complementary *in vitro* and *in vivo* studies are needed to establish the effects of weight loss on endothelial function and angiogenesis. This study developed an *in vitro* model to understand better the diet-induced changes in angiogenesis for mouse endothelial cells. In addition, a novel *in vivo* model of diet-induced vascular changes and its potential reversal with a return to regular diet in a zebrafish model was also studied. *In vitro* studies showed that a serum from mice fed a high-fat diet (HFD) might lead to proliferation of endothelial cells, yet weight loss did not compensate for prior stress induced by HFD. *In vivo*, studies in adult zebrafish showed that egg yolk-based high-fat diet might affect cytological architecture in the adult fish liver. Switching to a normal diet could effectively reverse these changes. Moreover, a caudal fin inter-ray vascularization assay was developed and used to test whether vessel sprouting was affected by different diets. Overfeeding resulted in a higher number of vessels, yet future studies with higher sample sizes are needed. Similarly, the expressions of several angiogenesis-related genes, which were quantified using cDNAs from the whole larvae and adult caudal fin treated with different diets, showed significant changes in *vcam* in larvae and cdh5 in adult fin by diet. However, further experiments are needed due to high individual variability and low sample size. The findings herein show that *in vitro* mouse endothelial cells and zebrafish larvae and adults could be used as valuable models for studies involving reversal/weight loss of high fat or overfeeding dietary regimes. Furthermore, the caudal fin vascularization assay in Tg(*fli1*:eGFP) Casper fish could be a promising preclinical model for testing the effects of different diets on angiogenesis and endothelial dysfunction.

Keywords: angiogenesis, cardiovascular disease, dietary regimes, zebrafish

ÖZET

IN VITRO FARE VE *IN VIVO* ZEBRAFISH MODELLERİ İLE FARKLI DİYETLERİN ANJİYOGENEZE ETKİSİ

Selvin Yıldız
Malzeme Bilimi ve Nanoteknoloji Yüksek Lisans Programı

Danışman: Özlen Konu Karakayalı
Eş danışman: Sinem Karaman

Kardiyovasküler hastalıklar küresel ölümlerin başlıca nedeni olarak sıralanıyor. Aşırı kilolu veya obez olmak, kardiyovasküler hastalığın patogenezini etkileyerek endotel fonksiyonunda, hücre büyümesinde ve inflamatuar aktivasyonda dengesizliğe neden olur (Shrestha ve ark. 2020). Endotel hücre fonksiyon bozukluğundan kaynaklanan bu faktörlerin bozulması, vasküler hastalık süreçleri için hem sonuç hem de katalizör görevi görür. Endotel hücreleri (EC'ler), dolaşımdaki kan ve damar bileşenleri arasında doğal bir bariyer oluştururlar. Ayrıca anjiyogenez, vasküler geçirgenlik ve inflamasyon gibi birçok fizyolojik ve patofizyolojik süreçte de kritik roller oynarlar. Endotel disfonksiyonunun iyileştirilmesi kilo kaybıyla sağlanabilir; ancak kilo kaybının endotel fonksiyonu ve anjiyogenez üzerindeki etkilerini belirlemek için tamamlayıcı *in vitro* ve *in vivo* çalışmalara ihtiyaç vardır Bu çalışma ile, fare endotel hücrelerinde anjiyogenezde diyete bağlı değişiklikleri daha iyi anlamak için *bir in vitro* model geliştirdi. Ek olarak, bir zebra balığı modelinde diyete bağlı vasküler değişikliklerin yeni bir *in vivo* yüksek yağlı diet modeli ve bunun düzenli bir diyetle tersine çevrilme potansiyeli de incelenmiştir. *In vitro* çalışmalar, yüksek yağlı bir diyet (HFD) ile beslenen fare serumunun endotel hücreleri çoğalttığını ve kilo kaybının, HFD'nin neden olduğu önceki stresi telafi etmediğini gösterdi. Yetişkin balıklarda yapılan *in vivo* çalışmalar, tavuk yumurta sarısına dayalı yüksek yağlı diyetin, yetişkin balık karaciğerindeki sitolojik yapıyı etkileyebileceğini gösterdi. Normal bir diyete geçmenin bu değişiklikleri etkili bir şekilde tersine çevirebilecği gözlendi. Ayrıca, kuyruk yüzgeci uzantıları arası vaskülarizasyon tahlili geliştirildi ve damar filizlenmesinin farklı diyetlerden etkilenip etkilenmediğini test etmek için kullanıldı. Aşırı besleme, daha fazla sayıda damar filizlenmesi ile sonuçlanmakla birlikte, ancak daha yüksek denek sayısına sahip çalışmalara ihtiyaç olduğu görüldü. Benzer şekilde, farklı diyetlerle yetiştirilen tüm larvalardan ve yetişkin kuyruk yüzgecinden alınan cDNA'lar kullanılarak ölçülen anjiyogenezle ilişkili birkaç genin ekspresyonları çalışıldı ve, diyetle larvalarda vcam ve yetişkin yüzgeçlerinde cdh5'te önemli değişiklikler gözlendi. Ancak bireysel değişkenliğin yüksek olması ve örneklem büyüklüğünün düşük olması nedeniyle daha fazla deneye ihtiyaç vardır. Buradaki bulgular, *in vitro* fare endotel hücrelerinin ve zebra balığı larvalarının ve yetişkinlerinin, yüksek yağ veya aşırı beslenme diyet rejimlerinin tersine çevrilmesi/kilo kaybını içeren çalışmalar için değerli modeller olarak kullanılabileceğini göstermektedir. Ayrıca, Tg(*fli1*:EGFP) Casper balığındaki kaudal yüzgeç vaskülarizasyon deneyi, farklı diyetlerin anjiyogenez ve endotel disfonksiyonu üzerindeki etkilerini test etmek için umut verici bir klinik öncesi model olabilir.

Anahtar kelimeler: anjiyogenez, kardiyovasküler hastalıklar, diyet, zebra balığı

ACKNOWLEDGEMENTS

First, I would like to thank Dr. Özlen Konu Karakayalı and Dr. Sinem Karaman for their invaluable supervision during my study. Their guidance has enhanced my academic understanding and altered my journey, enabling me to grow and push my limits in ways I had not envisioned.

I express my deep gratitude to the jury members, Asst. Prof. Dr. Pınar Önal and Dr. Gülçin Çakan Akdoğan, for dedicating their valuable time to assess my thesis. I value their insightful perspectives and evaluations.

I would like to acknowledge Dr. Sinem Karaman and her lab member, Yelin Subashi, Madeleine Helena Lackman, and Satu Pauliina Paavonsalo, for accepting me as an intern and for their great friendship. I especially thank Vadim Le Joncour for teaching me everything about cell culture.

I am so thankful to Dr. Özlen Konu Karakayalı for accepting me as her student, and I am so happy to be a part of Konu Lab. All the members showed excellent friendship, support, and help. I want to thank our current lab members Rana Acar, Güneş Tok, Mert Demirdizen, Merve Vural Özdeniz, Moiz Aftab, Siber Güneş Kılınç, Çağdaş Yanyatan, Seda Aktürk, Zehra Fırat, Nil Gürgül, Algı Demirtaş, Betül Metin, Beril Çatalak Yılmaz, Emel Ahmad and Muazzez Çelebi Çınar for creating the best lab environment possible. I also thank our alumni, Rüya Tombuloğlu, for teaching me everything about the zebrafish lab. My special thanks to Rana Acar and Güneş Tok for their outstanding support and effort in my thesis study. In particular, I thank Dr. Seda Aktürk for performing histology of liver sections for the diet study in zebrafish adults.

Lastly, I would like to thank Bilkent University -UNAM for funding me and giving me such an opportunity to do my graduate studies. Also, thanks to the Erasmus+ grant and funding of Karaman-lab. The zebrafish study was conducted according to the requirements of ethical standards of Bilkent University under the Bilkent University Ethical Protocol No: 2023/3. The mouse experiments in this project are covered under the ethical license ESAVI/25789/2022 (license holder: Sinem Karaman), approved by the Committee for Animal Experiments of the District of Southern Finland.

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ABBREVIATIONS

bEnd.3: Brain tissue derived from a mouse with endothelioma

BMI: body mass index

cDNA: Complementary DNA

dpa: Days post amputation

dpf: Days post-fertilization

ECs: Endothelial cells

EGFP: Enhanced green fluorescent protein

EtOH: Ethanol

FBS: Fetal Bovine Serum

FITC: Fluorescein-5-isothiocyanate

Fli: Ets family of transcription factor

GFP: Green Fluorescent Protein

H&E: hematoxylin and eosin

HFD: High fat diet

hpf: Hours post fertilization

hpa: hours post amputation

kDa: Kilo Dalton

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

NFD: Normal-fat diet

PBS: Phosphate buffered saline

PCR: Polymerase Chain Reaction

PFA: paraformaldehyde

qPCR: Quantitative Polymerase Chain Reaction

RNA: Ribonucleic Acid

SL: Standard length

SREBP: sterol-responsive binding protein 1 and 2

VE-cadherin: Vascular endothelial-cadherin

VEGFA: Vascular endothelial growth factor A

VEGFR: Vascular endothelial growth receptor

1. INTRODUCTION

1.1. Being overweight and endothelial dysfunction

Being overweight and its associated health implications have become a pressing global concern in recent years (Shrestha et al. 2020). Endothelial dysfunction, a distinctive feature of vascular impairment, is a critical consequence of excess weight (Lee et al. 2013). The endothelium, a crucial cellular layer lining the blood vessels, is pivotal in maintaining vascular homeostasis, regulating blood flow, vascular tone, and angiogenesis. However, in obesity, this intricate balance becomes disrupted, leading to endothelial dysfunction, characterized by impaired endothelial function and integrity, eventually forming the groundwork for various cardiovascular complications (Simoneau, Houle, and Huot 2012). It is important to understand what mechanisms are involved in endothelial function disruption via *in vitro* and/or *in vivo* models. Moreover, molecular changes in RNA and protein levels of genes involved in obesity or angiogenesis and biomarkers of endothelial dysfunction can be studied using transcript-level analyses.

1.2. Angiogenesis and endothelial dysfunction

Vascular health serves as a crucial measure of general health, given that any impairment in its function is linked to both cardiovascular and overall well-being. The vascular system is made up of the vessels that carry blood and lymph through the body. As an indicator of a healthy vascular system, angiogenesis is the process of creating new blood vessels from existing ones. **Figure 1** shows the sprouting angiogenesis in a healthy blood vessel as a response to low oxygen levels in tissue. This cascade is triggered by the secretion of pro-angiogenic growth factors, with VEGF playing a crucial role. Then, nearby cells adjacent to blood vessels produce VEGF, forming a gradient ranging from high to low intensity. Next, the endothelial cell exposed to the strongest VEGF signals transforms into a tip cell. This process guides the extension of the developing vessel from the tip cell. Then, the tip cell induces notch signaling in neighboring cells, causing them to transition into stalk cells. This transformation occurs as the tip cell follows the VEGF gradient. Then, stalk cells undergo proliferation, propelling the outgrowth of the vessel (Tejerina-Miranda et al. 2024).

There are several models of angiogenesis *in vitro* and *in vivo*. *In vitro* vascular models include cell lines that are obtained from primary cells or immortalized endothelial cell lines

(McCloskey et al. 2022). They are widely used to test the effects of drugs (Arioka et al. 2023) or different treatments (Rodrigo et al. 2023) on the development of new vessels (Zhang et al. 2023) or endothelial dysfunction, which is disruption of endothelial cell function or signaling. *In vivo* models, on the other hand, can mimic human endothelial dysfunction caused by genetics, or environmental factors, e.g., alcohol (Weeks et al. 2022) and high fat diet (Wang et al. 2022). Rodent and other vertebrate models, (e.g., zebrafish) have been widely used in understanding angiogenesis during development (Manikandan et al. 2022) as well as regeneration (Sojan et al. 2022). For example, caudal fin regeneration models of zebrafish have provided effective means to understand how new vessels originate and/ grow (Sipka et al. 2022).

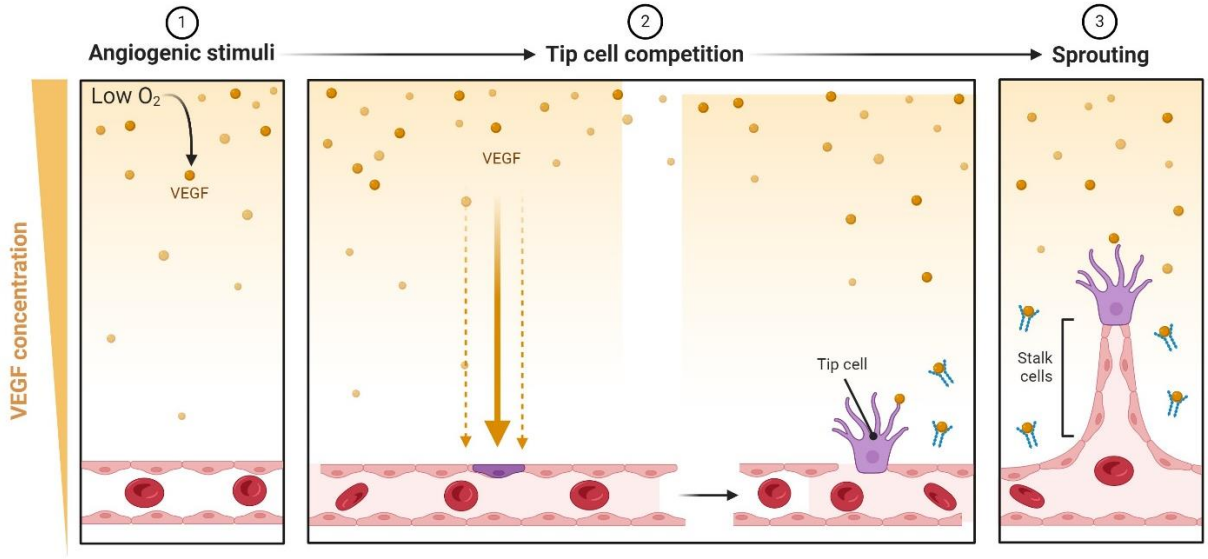

Figure 1: The process of sprouting angiogenesis in a healthy blood vessel. Created with BioRender.com

1.2.1. Endothelial growth factor (VEGF) promotes angiogenesis and regeneration

One key player in addressing endothelial dysfunction and its impact on vascular health is the vascular endothelial growth factor (VEGF) (Singh, Wu, and Dunn 2011). VEGF, a potent signaling protein, plays a pivotal role in regulating angiogenesis—forming new blood vessels from existing ones via acting through its cognate receptor VEGFR2. For instance, a recent

study shows the role of VEGF in the radial migration of endothelial cells (ECs) during retinal vascular development and angiogenesis (Rattner, Williams, and Nathans 2019). It has a significant role in this process, stimulating endothelial cell proliferation, migration, and the formation of new capillaries (Thapa et al. 2023). VEGF is also fundamental in normal physiological functions like embryonic development and regeneration (Tavakoli et al. 2023) and pathological conditions such as cardiovascular diseases and cancer, where abnormal angiogenesis contributes to disease progression (Thapa et al. 2023).

Angiogenesis, a complex biological process, involves the formation of new blood vessels from pre-existing ones. This mechanism is critical in various physiological processes, such as wound healing, organ development, and reproductive functions (Morland et al. 2017). However, when dysregulated, vascular overgrowth contributes to the progression of diseases like cancer, diabetic retinopathy, and inflammatory disorders (Cottrell et al. 2021). Regeneration is the body's remarkable capacity to repair or replace damaged cells, tissues, or organs, which heavily relies on angiogenesis (Cheng et al. 2018). Regeneration occurs when cells are wounded (Cheng et al. 2018), and newly formed blood vessels deliver oxygen, nutrients, and immune cells essential for tissue repair. For example, unlike mammals, zebrafish can regenerate their appendages, and studies have shown that new blood vessels originate from existing vasculature in fin regeneration and complete their development within 20 days (Chassot, Pury, and Jaźwińka 2016). VEGFA ortholog in zebrafish at the two paralogs, i.e., vegfaa/vegfa2 and vegfab, which are likely to be duplicated from a common ancestor (Bahary et al. 2007) and are among the signals that make possible regulation of vasculature development (Rauniyar, Bokharaie, and Jeltsch 2023).

1.3. *In vitro* mouse models for angiogenesis and regeneration

As opposed to zebrafish and other highly regenerative organisms, the mouse is more similar to humans and can regenerate only a limited number of tissues (McKellar et al. 2021). However, the mouse is one of the most widely used model organisms to understand human pathologies. *In vitro* mouse models are invaluable for studying angiogenesis and regeneration, offering controlled environments to dissect the molecular mechanisms underlying these processes, and have been used in the contexts of endothelial cells (Arnaoutova and Kleinman 2010) and stem cells (Schmohl et al. 2019). Endothelial cell culture assays involve culturing endothelial cells isolated from mouse or human tissues or cell lines under controlled laboratory conditions (Li et al. 2023). While the MTT assay is

widely used to assess cell viability and proliferation, it can also be used study the behavior of endothelial cells during angiogenesis (Wu et al. 2024). The wound healing or scratch assay is widely used *in vitro* to study angiogenesis and regeneration. This assay mimics the wound-healing process and allows researchers to observe and quantify the migration of cells, including endothelial cells, in response to various conditions or treatments (Shahzadi et al. 2022). It is a valuable assay for studying the movement capacity of cells and assessing the effects of various factors on wound closure. It is a versatile tool for testing different treatments on endothelial cell migration during angiogenesis and the regeneration process. For example, studies have used this assay to test different drugs, including bevacizumab (García-Romero et al. 2020) and lenalidomide (Li et al. 2022), and found that they inhibited angiogenesis by significantly reducing endothelial cell migration and proliferation in vitro.

Trans-well permeability assay can be employed to investigate a different aspect of endothelial cell behavior. It mimics the permeation process across biological barriers like endothelial cell layers or epithelial tissues (Lu et al. 2023). Accordingly, in the context of angiogenesis research, the trans-well assay can assess whether the endothelial cell barrier is intact and functional by mimicking the endothelial lining of blood vessels. For instance, it has been used to assess the impact of specific molecules on endothelial barrier function, and the authors found that certain compounds targeting VEGF signaling significantly reduced endothelial permeability (Wang et al. 2021).

Using these *in vitro* models in mice, researchers can a) dissect the cellular and molecular mechanisms involved in angiogenesis and regeneration, b) screen potential therapeutic compounds, and c) develop strategies to manipulate these processes for therapeutic purposes. One of the most exciting studies in this field has shown that brain remodeling and neurological recovery in mice is possible after focal cerebral anemia (Gregorius et al. 2021). The beauty of these models is their controlled experimental systems, which complement *in vivo* studies and contribute significantly to our understanding of vascular biology and tissue regeneration.

1.4. Zebrafish as a model system

Zebrafish is an excellent model system for studying human-related diseases due to its homologous structures with humans (Howe et al. 2013). Moreover, it is a tiny organism that is easy to handle and has a short breeding time with many offspring. Since it is transparent during the larval stage, it offers direct examination under the microscope. There are many

transgenic lines of zebrafish. For example, Tg(fli1:EGFP), where GFP is expressed under the fli1 promoter (Fallatah et al. 2019), this fluorescent tag enables the observation of specific tissues and organs, such as blood vessels (Pontes et al. 2017).

1.4.1. Zebrafish development

Zebrafish go through early and late larval stages followed by metamorphosis, leading to juvenile fish before adulthood (Parichy et al. 2009). During this process, various anatomical structures undergo significant changes, including the fins. Zebrafish fins develop from fin folds during embryonic and larval stages (Tzung et al. 2023). The development of the median fin fold is an essential aspect of embryonic development and is closely associated with the process of metamorphosis (Benard et al. 2023). Benard et al.'s study also showed convincing evidence that size was a good indicator of developmental progress while environmental parameters, e.g., temperature, and density of fish in a tank, affected the rate of development and higher temperatures accelerated the formation of milestones as exemplified based on morphological changes. Standard length (SL) is therefore a good predictor of different morphological developmental stages (i.e., early to late larvae, L1-L3; juvenile, J; and adult, A) and has been used to understand if a mutation could change the ontology (Singleman and Holtzman 2014). Accordingly, pigmentation patterns and fin ray visibility and development are two of the features that can be followed up for staging larval to juvenile and juvenile to adult transitions. Few studies have tested the effects of low to high levels of lipids on growth and metabolic parameters; one such study focused on Golden pompano, *Trachinotus ovatus*, a marine fish, and found that there was an optimum level of lipid content, characterized by the body weight gain rate (Xun et al. 2021).

1.4.1.1. Developmental rate and diet

Studies in literature also investigated the dependency of growth rate on dietary parameters and tested commercial diets and found that there were still significant differences among them (Fowler et al. 2019a; Gonzales and Law 2013). However, these were balanced diets and did not represent obesogenic diets, such as egg yolk. Interestingly, a recent study tested whether increasing levels of feed over the first month of development had effects on growth and found that feeding with 25% and 50% more feed than only 5% overfeeding had significant and disproportional effects on mass gain, specific growth rate, protein levels, and

lipid accumulation (Thompson et al. 2024). Guppies (*Poecilia reticulata*) have been studied with respect to weight gain under different dietary regimes, one of which was chicken egg yolk at increasing concentrations (Perera et al. 2023). As in the case of multiple diet types, egg-yolk also had an optimum level before and after, which caused the weight gain to be lower.

1.4.1.2. Zebrafish as a model organism to study angiogenesis and regeneration

In vivo models are needed to study blood vessel formation and function; this is also necessary to confirm the results obtained in vitro. To visualize zebrafish's vasculature well, we need unpigmented larvae that express endothelial GFP reporters. Tg(*fli1*:EGFP) represents a transgenic zebrafish strain where enhanced green fluorescent protein (EGFP) is produced under the regulation of the *fli1* promoter, a key element in vascular development (Liu et al. 2008).

Casper zebrafish has transparent appearance demonstrate a complete lack of all melanocytes and iridophores in both embryogenesis and adulthood (White et al. 2008). As mammalian and most vertebrate tissues are opaque, introducing the transparent zebrafish with GFP tagged vasculature Tg(fli1:EGFP) has transformed research in this field (Pontes et al. 2017). This transparency allows researchers to observe and track blood vessel development in real time, opening the door for *in vivo* studies of endothelial cells (Staal, Spaink, and Fibbe 2016).

Additionally, research suggests that the caudal fin regeneration model in the Tg(*fli1*:EGFP) transgenic line can be employed for screening antiangiogenic drugs, selectively inhibiting highly active, abnormal vessels while preserving quiescent vessels (Bayliss et al. 2006).

By using zebrafish as a model organism, scientists can uncover fundamental insights into the processes of angiogenesis and regeneration. These findings can have significant implications for developing new treatments or therapies for various human diseases and injuries related to impaired blood vessel formation or tissue regeneration

1.4.1.3. Vascular system in zebrafish

Zebrafish have a similar vascular anatomy to mammals, including humans (Isogai, Horiguchi, and Weinstein 2001). The zebrafish vascular system's similarities to that of

humans and its amenability to genetic manipulation and observation make it an excellent model for understanding vascular development, function, and disease. Developmental processes involved in vascular development, including angiogenesis, and vascular remodeling, are comparable between zebrafish and mammals (Gore et al. 2012). Different transgenic lines are available to visualize and analyze the vascular system in larval and adult zebrafish, such as Tg(fli1:EGFP) (Lawson and Weinstein 2002) and Tg(flt4:YFP) (Hogan et al. 2009) quantitatively.

Furthermore, the investigation of single-cell morphological dynamics in living larvae during vessel formation has been made possible by the integration of nuclear and cell membrane-specific fluorescent tags (Yu et al. 2015). It is known that *vegfa2* signaling is necessary for zebrafish islet vessel development (Toselli et al. 2019). Vascular endothelial growth factor kdr-like receptor (*kdrl*) is a well-known VEGF receptor that has been used in signaling studies in vasculature development of zebrafish (Koenig et al. 2016). Another well-known molecule for zebrafish angiogenesis is Angiopoietin-2 (*ang2*) which has role in vascular remodeling and maturation in zebrafish, is widely used for vasculature formation in brain development (Chen et al. 2024). The zebrafish cadherin 5 (*cdh5*) gene is orthologous to human *CDH5*, and it has been used in research studies together with vascular cell adhesion molecule (VCAM) as a marker for cell adhesion and cell-cell interactions in zebrafish (Larson et al. 2004; Li et al. 2018).

Consequently, the mechanisms regulating both angiogenic growth and inhibition can be explored *in vivo* within these experimental frameworks (Chávez et al. 2016).

1.4.1.3.1. Zebrafish caudal fin as a model for angiogenesis

Caudal fin development is well-established in zebrafish (Siomava et al. 2018). In zebrafish, each fin ray is equipped with an artery responsible for transporting blood to the tip of the fin, accompanied by two veins that return blood to the body. These blood vessels are observable under a microscope as shown in **Figure 2**, and can be studied throughout the fish's life cycle. During the typical fin development, the blood vessels undergo growth through a process known as vascular branching (Mancuso, Kuhnert, and Kuo 2008). This involves the emergence of sprouts that connect each artery to its adjacent veins, forming what is referred to as intra-ray vessels. Various mutations and pharmacological interventions can potentially influence the configuration of the fin blood vessels, impacting factors such as the density of intra-ray vessels.

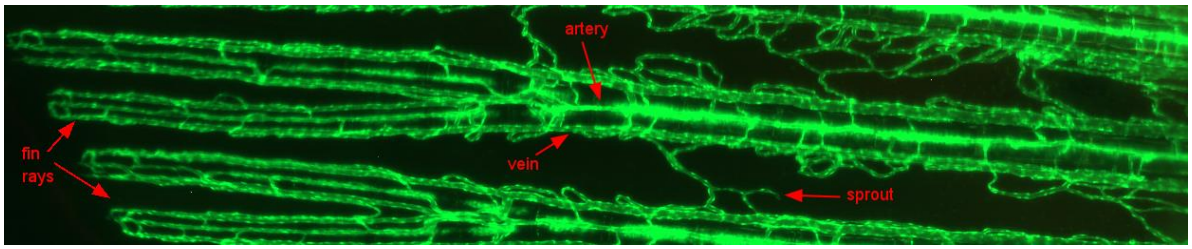

Figure 2: Blood vessels within the fin rays of zebrafish, labeled with green fluorescent protein. Created by ImageJ, photo belong to our lab (Konu-lab) taken by Invitrogen™ EVOS™ M5000 Imaging System.

Moreover, caudal fin has been used for regeneration studies (Dasgupta et al. 2023) and endothelial development (Bump et al. 2022). Scientists have shown by using inhibitors of VEGFR that endothelial development requires VEGFR signaling in the caudal fin of developing zebrafish larvae. The developmental timescale of endothelial cells and their remodeling follows three processes: 1) Endothelial sprouting; 2) Plexus elaboration; 3) Endothelial remodeling. The first step starts with sprouting, while the next two steps are required to add rays and their outgrowth and extension, which lasts from 10 to 16 rays. During this remodeling, the sensory system is also integrated into the vascular and skeletal systems.

Another angiogenesis model for the caudal fin is regeneration, which is used to observe and quantify the generation of new endothelial cells and measure their growth (Hlushchuk et al. 2016). In this study, different parameters have been quantified to analyze the newly generated vessel area and the antiangiogenic effects of inhibitor PTK787 could be studied. Caudal fin regeneration studies in zebrafish have yielded important information on how the vessels are organized nearby the rays. One such study showed that an artery was found in the center where two veins ran across the artery in between there were interlinking vessels from veins to artery and veins to veins (Huang et al. 2003). Upon cutting the fin 12 hours post amputation (hpa) the wound starts closing and between 36 hpa and 48 hpa anastomosis that leads to connection between the arteries and veins leading to restoration of the flow at the end of 48 hpa; and by 80 hpa new vessels start to develop from the rounded end of the regenerating caudal fin rays, resulting in the vascular plexus formation, which will go through remodeling by 96 hpa. Remodeling involves separation of arteries and veins where plexus is an intermediate stage and after 6 dpa vessel sprouting does not require presence of a plexus (Huang et al. 2003).

A stage called “intervessel pruning”, required to lessen the highly dense number of vessels found in the plexus was also observed in the study. Accordingly, the highest density

had been reached around 4 dpa and slowly decreased by the end of the process (30 dpa). Such caudal fin models are very useful in understanding genetic and environmental factors that can affect these processes that can be quantified, e.g., anastomosis, number of branches, and cell number. Live-imaging and characterization of bifurcating fin rays and vessel regeneration have also been used with other double transgenics to examine roles of null mutations, showing that veins lead to formation of new arteries at the tip of regenerating ends (Xu et al. 2014). In addition to the caudal fin, pectoral fin vasculature has already been studied in zebrafish and similarly an artery in the middle, surrounded by two paired-ray veins running across from which capillaries (interlinking vessels) extend is a characteristic feature at the fin rays (Paulissen et al. 2022).

1.4.2. Zebrafish as a model for human diseases and obesity

In addition to being a well-known model organism, zebrafish widely been used as model for human diseases. There are several examples of these, of which I will cite a few. One of the first studies showing the orthologous relationship between humans and zebrafish involved *sterol-responsive binding protein 1 and 2 (SREBP)* genes, whose upregulations are involved in lipid synthesis in mammalian and fish studies (Craig and Moon 2011). Another study showed that the lipid metabolism-related genes of zebrafish exhibit similarity in humans, indicating that researchers could also benefit from gene therapy for drug-induced diseases in zebrafish (X. Wang et al. 2018).

Zebrafish is also a promising model organism for understanding obesity and metabolic disorders due to its genetic similarity to humans. While zebrafish naturally develop obesity under specific conditions, researchers have also developed models to induce obesity in these fish. A recent study comparing zebrafish fed a high-fat diet (HFD) and a normal-fat diet (NFD) stated that both dietary regimens can induce a remarkable increase in body mass index (BMI) (Landgraf et al. 2017). These studies emphasize the suitability of zebrafish as a model for human complex diseases and its established role in developmental research.

1.4.3. Different dietary regimes in zebrafish

Zebrafish is a tropical freshwater species that in the lab feeds on dry (flake or pelleted) food and live prey such as artemia (brine shrimp) or rotifers (Sullins 2023). It has been used in the laboratory environment since the early 80s and has a well-defined feeding regime concerning the content of protein, fat, and fiber (Chen, Zheng, and Zhang 2018). Since it is a promising model for diet studies and has been commonly used in scientific research, various dietary regimes based on their developmental stages and research objectives have been tested (Fowler et al. 2019b; Printzi et al. 2023). The choice of dietary regime in zebrafish research often depends on the research objectives, availability of resources, and the specific parameters being studied, whether it is metabolism, development, behavior, or disease models. For example, high-fat diets have been introduced into zebrafish larvae and adults using different diets that range from egg yolk (W. Wang et al. 2019) to predefined commercially produced diets (Fowler et al. 2019c) or excess live feed,

e.g., artemia (Ghaddar et al. 2021). Zebrafish diets can be adjusted to mimic certain disease conditions or to study the impact of specific nutrients on disease progression (Landgraf et al. 2017a).

1.4.3.1.Egg yolk-based diets

Using an egg yolk diet in zebrafish adults as well as larvae presents a unique avenue to test effects of diet on different aspects of development and physiology. Egg yolk contains high levels of lipids but also is an excellent source of different proteins, minerals and vitamins(Réhault-Godbert, Guyot, and Nys 2019). Obesity in zebrafish can be induced and is characterized with a specific distribution and formation of adipose tissue in the body (has been reviewed in detail in Zang, Maddison, and Chen 2018). Chicken egg yolk is a lipid-rich meal due to its elevated levels of fats and cholesterol (lipids compose ~58% of chicken egg yolk, of which ~5% is cholesterol, 60% are triglycerides, and 35% are phospholipids). Chicken egg yolk provides more fat than typical commercial zebrafish micropellet foods (~15% lipids) (Otis and Farber 2016). Hence, the egg yolk diet-induced obesity paradigm in adult zebrafish has been used to induce weight gain and helped generate novel zebrafish obesity models. On the other hand, Tingaud-Sequeira et al. created one of the first models of diet-induced obesity in zebrafish larvae (Tingaud-Sequeira, Ouadah, and Babin 2011). 5 dpf zebrafish larva fed with hard-boiled chicken egg yolk as a high-fat diet (HFD) exhibited changes in adiposity levels at the end of treatment.

There are several protocols established for using egg yolk diets. One of these protocols involves feeding larvae with egg-yolk twice a day and cleaning the water every other day making sure daily and dead fish are collected (Balamurugan et al. 2022). Oil Red O (ORO) or Nile Red is used to measure the distribution and amount of lipids applied after fixation in addition to biochemical estimation.

As shown in **Table 1**, an egg yolk diet can be applied both for zebrafish larvae and adults. Den Broeder et al. demonstrated that zebrafish larvae, when fed a high-fat diet (HFD) from 6 to 15 dpf, exhibited a threefold increase in larvae with adipocytes compared to those on the regular feed diet (den Broeder et al. 2017). Nowadays, many scientists focus on drug therapies to limit to eliminate the effects of obesity. A recent study showed that an aqueous extract of *P. mauritianum* (0.25 g/L), which is a medicinal plant, lower the lipid accumulation in HFD-treated larvae

(Ghaddar et al. 2022).

Since adult zebrafish offers an excellent *in vivo* model for metabolic diseases like obesity, it has been used by many researchers—for example, Landgraf et al. introduce a method for inducing the metabolically explicit obesity phenotypes in adult zebrafish (Landgraf et al. 2017). Moreover, 8 weeks of egg yolk-based high-fat diet leads to a significant increase in body weight and adipose tissue mass accompanied by hyperglycemia and lipid accumulation in the liver of zebrafish (Landgraf et al. 2017b). A recent study demonstrated that adult zebrafish fed both high-fat diet and with egg yolk powder showed increase in body mass index (BMI), hepatic total cholesterol, and triglyceride (Li et al. 2023).

***Table 1**: List of different egg yolk diets on zebrafish larvae and adults.*

Step	Egg yolk dosage	Feeding period	Related article
Larva	0.4 % boiled egg yolk suspension	10 days	(den Broeder et al. 2017)
	0.1% egg yolk suspension	4 days	(Ghaddar et al. 2022)
	5% egg yolk suspension	1 day	(Cruz-Garcia and Schlegel 2014)
	0,5% egg yolk suspension	2 days	(Manuneedhi Cholan et al. 2022)
Adult	30 mg egg yolk powder	3 months	(Landgraf et al. 2017b)
	70 mg egg yolk powder	30 days	(Li et al. 2023a)
	5 mg egg yolk powder	16 days	(Picolo et al. 2021)

1.4.3.2.Weight loss models

In zebrafish, weight loss models primarily involve dietary and pharmacological interventions to induce a decrease in body weight or fat accumulation. Very little published research focuses on elucidating the role of nutrient restriction on zebrafish growth and obesity. However, a recent study shows that weight loss did not reverse obesity-triggered outcomes in muscle contractile function (Seebacher et al. 2017). These findings indicate that weight loss alone may not be a sufficient intervention (Seebacher et al. 2017). Another study revealed that zebrafish subjected to fasting experienced approximately a 10% reduction in their body mass throughout the 3-week experiment, accompanied by a corresponding decline in oxygen consumption (Craig and Moon 2011).

1.4.4. Tg(fli1:EGFP) zebrafish to test vascular diseases and diet interaction

The Tg(*fli1*:EGFP) zebrafish line has been used by many scientists as a model organism to study the relationship between diet and vasculature function. A recent study shows that berberine significantly decreases vascular dysfunction in hyperlipidemia models in zebrafish fed with high-cholesterol food (Zheng et al. 2023). Another research study concluded that the combination of a high-cholesterol diet and exposure to high glucose levels triggers a swift of vascular complications in zebrafish, mirroring the early stages of atherosclerotic vascular injuries observed in diabetic mammalian models; and hence this suggests the potential utility of zebrafish as a novel animal model for studying diabetic vasculopathy (Z.Wang et al. 2013). Fang et al. show that feeding Tg(*fli1*:EGFP) transgenic zebrafish a high-cholesterol diet triggers vascular processes known as atherogenesis, which is the process leading to coronary artery heart disease in humans (Fang, Liu, and Miller 2014). However there is not yet a Tg(*fli1*:EGFP) in the Casper background that tests the effects of the high-fat diet followed by a return to normal diet (aka. weight loss) regimen in zebrafish larvae or adults.

1.5. Aim and rationale

Cardiovascular diseases are the pioneer cause of death worldwide. Being overweight or obese interferes with many physiological processes, notably affecting vascular health and leading to endothelial dysfunction. Understanding the intricate relationship between obesity and endothelial dysfunction holds significant implications for comprehending cardiovascular disease pathophysiology.

This thesis aimed to study the effect of different dietary regimes on vascularization and endothelial cell function. More specifically, I have tried to discover the relationship between obesity and vascular and endothelial cell dysfunctionality using imaging and mRNA expression studies. According to this aim, *in vitro* studies were performed using sera from mice fed with a regular, high-fat, and weight-loss diet. Similar dietary regimes were adapted for zebrafish, and *in vivo* studies were performed on both larval and adult zebrafish fed a normal, high-fat (or overfeeding), and weight-loss diet.

In more detail, I have the following original aims to discover:

1. Effects of sera from mice fed with a regular, high-fat, and weight-loss diet on bEnd3 cells.
   a) Do sera from mice fed with different diets affect the cell viability of bEnd3 cells?
   b) Does a high-fat diet affect the wound healing of bEnd3 cells?
   c) Can a permeability assay be developed for testing with bEnd3 cells with srea from a high-fat diet and a weight loss diet?
2. Effects of regular, high-fat or overfeeding, and weight-loss (high-fat/overfeeding followed by regular) diet on zebrafish.
   a) What are the effects of a high-fat diet (egg yolk) and weight loss diet on the the length and morphology of zebrafish larvae as well as selected angiogenesis marker gene expression?
   b) What are the effects of a high-fat diet and weight loss diet on the hepatic architecture of liver of adult zebrafish? Does the overfeeding diet have the same effect as a high-fat diet on adult zebrafish?
   c) How can we be sure that a high-fat/overfeeding diet induces obesity in adult zebrafish? Is body weight or length informative?

d) How do different dietary regimes affect the vasculature of adult zebrafish caudal fin and expression levels of angiogenesis markers?

2. MATERIALS AND METHODS

2.1.Materials

2.1.1. Chemicals, Reagents, and Kits

All chemicals, reagents, and kits were grouped and listed under the headings. Titles were determined according to the experimental procedure type and the study for which materials were used.

2.1.1.1. Cell Culture Solutions

The reagents and chemicals used for cell culture and maintenance are listed in **Table 1** with their companies and catalog numbers.

Table 2: List of materials used for mammalian cell culture and maintenance.

Product Name	Company & Country	Catalog Number
Lonza™ BioWhittaker™ Dulbecco's Modified Eagle's Medium with 1.0g L-Glucose per Liter, without L-Glutamine	Thermo Fisher Scientific, USA	BE12-707F
Fetal Bovine Serum (FBS), European Grade, Heat Inactivated	Biological Industries, USA	04-127-1A
Dulbecco's Phosphate Buffered Saline (PBS)	Biowest, France	L0615
Penicillin/Streptomycin, 100x	Thermo Fisher Scientific, USA	15140-122
Trypsin-EDTA	Biological Industries, USA	03-051-5B
Sodium Pyruvate 100mM	Thermo Fisher Scientific, USA	11360-039

2.1.1.2. Reagents and kits used in RNA isolation

Table 3: List of reagents and kits used for isolating RNA and measuring RNA levels.

RNA Isolation and Quantification Assays		
Product Name	**Company, Country**	**Catalogue Number**
AccuGENE™ Molecular Biology Water	Lonza, Switzerland	BE51200
QIAzol Lysis Reagent	Qiagen, Germany	79306
RNeasy Mini Kit	Qiagen, Germany	74104
Chloroform	Sigma-Aldrich, Germany	24216
2-Propanol	Sigma-Aldrich, Germany	24137
Ethanol	Sigma-Aldrich, Germany	32221
Sodium Acetate	Carlo Erba Reagents, Italy	366207
RevertAid First Strand cDNA Synthesis Kit	Thermo Fisher Scientific, USA	K1622
LightCycler® 480 SYBR Green I Master	Roche, Switzerland	4887352001

2.1.1.3. Reagents used in Histological Sectioning

Table 4: List of reagents and kits used in hematoxylin and eosin (H&E) staining.

H&E stain		
Product Name	**Company, Country**	**Catalogue Number**
4% paraformaldehyde (PFA)	Lonza, Switzerland	BE51200
Hematoxylin and Eosin	Qiagen, Germany	74104
Ethanol	Sigma-Aldrich, Germany	32221

2.1.2. Prepared Solutions and Recipes

The list of prepared solutions and buffers and their recipes were indicated in **Table 5;** solutions and buffers were categorized according to the experimental process they used.

Table 5: Solutions and buffers and their recipes used in MTT assay

MTT Cell Viability Assay	
Solution/Buffer	**Components**
MTT (12 mM)	5 mg of MTT dissolved into 1 ml of 1xPBS
SDS-HCl Solution (0.01 M)	1 g of SDS and 8 µl of HCl dissolved in 10 ml of ddH2O

2.1.3. qPCR Primers

The sequences of forward and reverse primers that used for qPCR experiments are listed in **Table 6**, with each pair's amplicon product size.

Table 6: Compendium of primers used in qPCR experiments. The eef1a (Elongation factor 1-alpha) gene was used as a reference gene for housekeeping gene normalization.

Gene ID	Primer Sequences
eef1a (Gene ID: 171361)	**F:** 5'- CCCTGGACACAGAGACTTCA -3' **R:** 5'- CAGCCTCAAACTCACCAACA -3'
vegfa2 (Gene ID: 7422)	**F:** 5'- GATGTGATTCCCTTCATGGATGTGT -3' **R:** 5'- GGATACTCCTGGATGATGTCTACCA -3'
vcam (Gene ID: 7412)	**F:** 5'- GGACCAAGAAGAGAGGCGAG -3' **R:** 5'- TGTCGCCACTCGCTAATTCA -3'
ang2 (Gene ID: 11601)	**F:** 5'- CCAATCTTCTAAGCCAATCAGCGGAA -3' **R:** 5'- CCACATCTGTCAGTTTGCGCGTGTTT -3'
cdh5 (Gene ID: 12562)	**F:** 5'- CCAAACAGAGTACACGTTTAGCGT -3' **R:** 5'- ACTATCTGGGTCTTTTGCTGAAACA -3'
kdrl (Gene ID: 796537)	**F:** 5'- GACCATAAAACAAGTGAGGCAGAAG -3' **R:** 5'- CTCCTGGTTTGACAGAGCGATA -3'

2.1.4. Laboratory Equipment

The laboratory equipment that was used to conduct experiments was listed in **Table 7;** the company of each equipment was specified.

Table 7: List of equipment used.

Instrument Name	Company, Country
NanoDrop ND-1000	Thermo Fisher Scientific, USA
PCR Thermal Cycler 2720	Applied Biosystems, USA
LightCycler® 96 Instrument	Roche, Switzerland
Synergy HT Microplate Reader	Biotek, USA
Amersham™ Imager 600	GE Healthcare Life Sciences, USA
DIC Microscope, DMi8	Leica, Germany
Biological Safety Cabinet	NuAire, USA
Air-Jacketed Automatic CO2 Incubator	NuAire, USA
Invitrogen™ EVOS™ M5000	UK.

2.2.Methods

2.2.1. In vitro mouse cell line-based studies

2.2.1.1.Wound assay

Regeneration of bEnd3 cells *in vitro* were measured using the wound healing assay. Briefly, bEnd3 cells were seeded into a 6-well plate with density of 20.000 cells per well and were incubated in a 5% CO2, 37 °C atmosphere. When reaching full cell culture confluence, a 200 µl pipette tip was used for scratching the bEnd3 cell monolayer. Then, each well was carefully washed with PBS twice to remove damaged scratched floating cells. Duplicate wells were then treated with the following conditions: negative control in serum-free medium, 1% serum from mice fed with a chow diet for 10 weeks, 1% serum from mice fed with a high-fat diet for 10 weeks, and 1% serum from mice fed with high-fat for 5 weeks then chow diets for 5 weeks. After incubation in 5% CO2 37 °C atmosphere from 24 to 48 h, wound healing was monitored using

bright field microscopy. The percentage of the scratch area was then quantified using ImageJ software.

2.2.1.2. MTT assay

To measure bEnd3 cell viability, we measured the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction into formazan by cellular NADPH (MTT assay). bEnd.3 cells (5000/well) were seeded in 96-well plates and treated with, 1% sera of mice fed either with chow, high-fat, or high-fat then chow diets (10 replicas each) and incubated in 37 °C, 5% CO2 atmosphere overnight. To quantify cell viability, 10 µL of MTT was added into each well and incubated in 37 °C, 5% CO2 atmosphere for two hours. Then, 100 µl of lysis buffer was added to each well to dissolve the formazan crystals and incubated at 37 °C overnight. Absorbance related to purple formazan metabolite production by cells, were measured at 570 nm using a FLUOstar Omega microplate reader.

2.2.2. In vivo zebrafish studies

2.2.2.1. Animal maintenance

Zebrafish (*Danio rerio*), housed in Bilkent University Zebrafish facility was used. AB strain and Tg(*fli*: GFP) and Casper zebrafish lines were used in this study. A cross between Casper and Tg(*fli1*:eGFP) fish was made to obtain the F1 progeny that contained heterozygous mutations in casper and Tg(*fli1*:eGFP). Offspring from the F1 x F1 cross was then used to select embryos that were with Casper; Tg(*fli1*:eGFP) phenotype. Zebrafish were kept at a temperature of 28.5°C. Euthanasia, performed by using a tricaine solution. (0.12% for adults and 0.08% for embryos and larvae). Sterile equipment was used to dissect adult fish and obtain various tissues.

2.2.2.1.1. Aquaria system and breeding

Experimental adult zebrafish of three months old or above were kept in glass aquaria filled with system water. Each aquarium had a standard filtering system and was equipped with 120-micron filter pad, 50-micron canister filter, biological filter and activated carbon absorption filter and 25-40% of the system water was filtered. The 14 h light/ 10 h dark period was kept constant.

Male and female casper Tg(*fli1*:eGFP) fish were paired and placed in breeding cages in the afternoon, with fertilized eggs collected the following morning.

2.2.2.1.2. Housing and of adult experimental fish

3-liter aquarium used for adult zebrafish in the control and treatment groups, up to 15 adults. Fresh water provided weekly. Adult fish fed twice a day with dry food flakes and *Artemia nauplia* until the start of the experiments.

2.2.2.1.3. Larval feeding

After larvae were selected according to Tg(*fli1*:eGFP);Casper phenotype, they were maintained at a density of 30-40 larvae per 3 L-tank and fed accordingly. Larvae were assigned to three dietary groups: one group was the control, fed a regular diet of 20 mg dry fry food per tank and 30 µl artemia per tank per day (L.ND) for three weeks. The second group was fed 20 mg of dry fry food per tank, 30 µl per tank per day, and 2.0 mL of egg yolk solution for 40 larvae (L.HFD) for three weeks. The egg yolk solution was prepared: 1 gr of egg yolk powder (59% fat, 32% proteins, 2% carbohydrates; Sigma; 100 mg) was added into a 15 ml E3 medium and shaken vigorously to ensure suspension. The fat percentage of egg yolk solution, after further dilution to 500 mL of E3 medium, was 0.02%. The third group was the weight loss group (L.WLD). The first two weeks the fish were fed the same with L.HFD conditions, and the last week were fed the same with L.ND. At the end of the three-week feeding experiment, larvae were anesthetized and imaged using an inverted light microscope.

2.2.2.1.4. Adult feeding

Both male and female zebrafish of the Tg(*fli1*:EGFP);Casper strain were used for feeding experiments. Tg(*fli1*:EGFP) Casper strain was chosen for two reasons: First, it is widely for research study and is frequently used for vascular fluorescence imaging because the *fli1* promoter express enhanced green fluorescent protein (GFP) in all blood vessels. Second, their transparency allows direct imaging of development vascularization and angiogenesis. 4 months post fertilization (mpf), zebrafish were assigned to six dietary groups: one group was the control, fed with regular diet, which is 3.9 mg dry food, and 143 ul artemia per fish per day (e.y. ND), the second group fed with 3.9 mg dry food, 143 ul artemia and 7.8 mg egg yolk powder (59% fat, 32% proteins, 2% carbohydrates; Sigma; 100 mg) per fish per day mimicking a high-fat-diet (HFD) in an isocaloric amount compared to (e.y.ND), the third group was weight loss group first 4 weeks fed same with HFD conditions, last 4 week fed same with e.y.ND group per fish per day (e.y.WLD), the fourth

group was another control group fed with regular diet (o.f.ND), the fifth group overfed with artemia and dry food to induce an obese state (15.6 mg dry food, 286 ul artemia per fish per day)(OFD), and the sixth group was another calorie restriction group that fed first 4 weeks with OFD conditions, last 4 weeks the same with o.f.ND group per fish per day (o.f.WLD). Zebrafish were maintained at 13 fish per 3 L-tank and fed accordingly. At week 8, zebrafish were fasted overnight and sacrificed. At the end (week 8) of the feeding experiment, each anesthetized zebrafish's body weight and length were recorded..

2.2.3. Determining angiogenesis by fluorescent imaging

In order to investigate the angiogenesis and complexity of the vascular network, the 2D structure of the adult caudal fin was introduced to vascular skeletonization related plugin of ImageJ. The Fiji (Fiji is Just ImageJ) version of ImageJ, which features the plugins Skeletonize (2D/3D) and Analyze Skeleton (2D/3D), Vascular Density Measurement were used to analyze the vessel network. An example of the vasculature and the obtained corresponding skeleton is presented in **Figure 3**.

First, the caudal fin for skeletonization has been reduced to the three dorsal fin rays in all groups. Secondly, the area of interest aligned according to the starting point of the fork of the fin ray (shown in a straight line). The inter-vessel area between two rays was chosen to be 500x40 pixels. Thirdly, the whole image is converted to 8-bit, and after threshold adjustment, the vascular density of the area of interest is measured using the Vascular Density Measurement plugin. Fourthly, the area of interest cropped. After making it binary it was skeletonized and finally the skeleton was analyzed using the Analyze Skeleton (2D/3D) plugin.

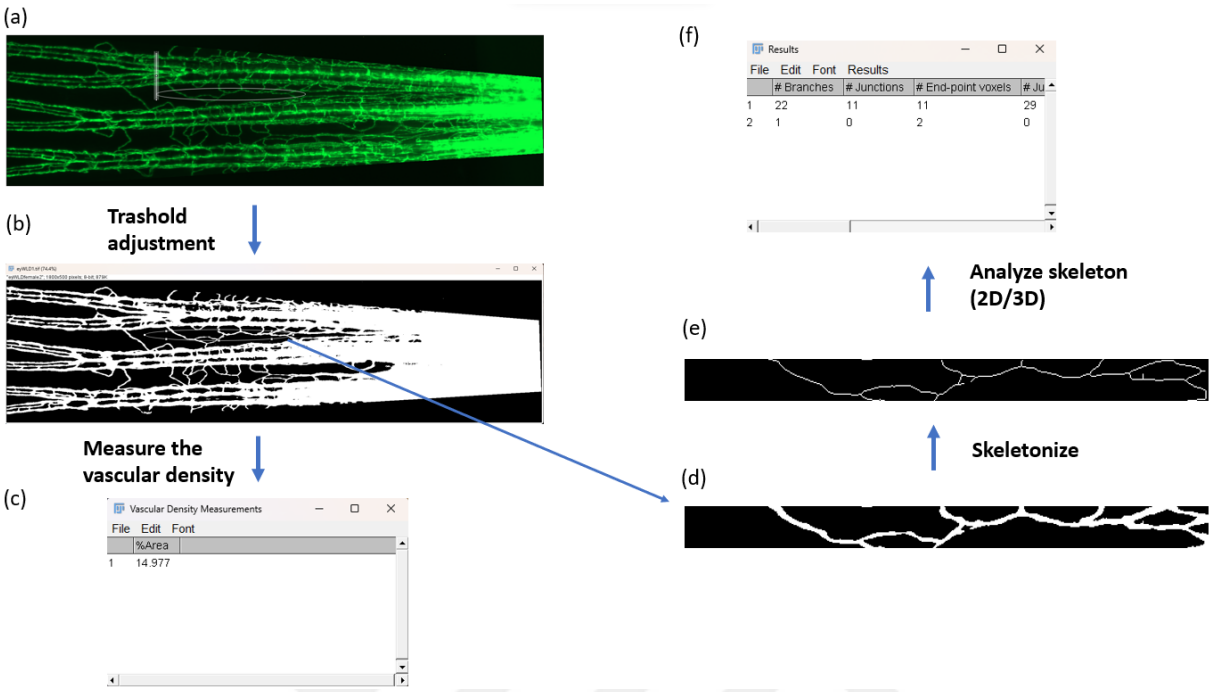

Figure 3: Representative workflow of image processing using ImageJ for morphometric analysis of angiogenesis. (a) The original colored image of the fin. (b) threshold adjustment of the colored image. (c) Results of vascular density measurement. (d) cropped version of are of the interest. (e) The skeletonized image of the binary image. (f) The number of the counted branches.

2.2.4. Histological Sectioning and Sample Preparation

Fish abdominal regions were exposed, and the liver was fixed in 4% paraformaldehyde (PFA) for one day after dehydration through a series of graded alcohol solutions, each sample was embedded in paraffin. Sections at five µm thickness were processed using a microtome. For histological analysis, liver samples were stained with hematoxylin and eosin (H&E). After mounting the slides, samples were imaged using an inverted light microscope (Zeiss, Axiovert 200 M).

2.2.5. Determination of gene expression

2.2.5.1. Total RNA isolation from adult zebrafish tissue

For RNA isolation, all materials and solutions underwent treatment with diethylpyrocarbonate (DEPC) to prevent RNase contamination during RNA isolations. The steps performed as follows;

1- Dissect fin tissues and wash with PBS and then snap-frozen with the help of liquid nitrogen;
2- Add 600 µl of Trizol (QIAGEN) to each sample;
3- Homogenize by up and down motion via 2ml syringes;
4- Add 130 µl of chloroform (Sigma Aldrich) each sample and mix well;
5- Incubate for 5 minutes at room temperature (RT) then centrifuge at 13,000 rpm for 20 minutes at +4 ºC;
6- Transfer the supernatant to a fresh tube;
7- Add 200 ul of isopropanol and gently mix;
8- Centrifugate at 13,000 rpm for 20 minutes;
9- Discarded supernatant the pellet and wash the pellet with 1 ml of 75% ethanol (EtOH);
10- Centrifugate at 13,000 rpm for 15 minutes;
11- Remove of the supernatant, wash with 100% EtOH;
12- Centrifugate at 13,000 rpm for 15 minutes;
13- Discard the supernatant, let the pellet to air-dry for 30 min;
14- Add 20 µl of RNAse-free water to the dried pellet.

Then concentration RNA was measured using NanoDrop (Thermo Fisher Scientific, USA) with the RNA option.

2.2.5.2. cDNA synthesis

The cDNA amplification steps were performed as follows and according to the OneScript® Hot cDNA Synthesis Kit:

1- 800 ng of RNA in a final volume of 13 µl of nuclease-free water, 4 µl 5X RT Buffer, 1 µl dNTP, 1 µl Oligo(dT), and 1 µl of OneScript® Hot Reverse Transcriptase were employed for each sample.
2- After incubation at 55°C for 15 minutes, followed by a 5-minute incubation at 85°C, in a PCR machine, cDNA samples were diluted at a ratio of 1:20 for qPCR synthesis.

2.2.5.3. Real-Time RT-PCR

qPCR reactions were done as follows:

1- Add 5 µl SYBR Green for each sample;
2- Add 1 µl of Forward, Reverse primer and 1 µl of RNAse free water;
3- Add 8 µl of master mix into 96-well plate for every reaction;
4- Add 2 µl of cDNA to each related well;
5- Negative control, was only consist 2ul water;
6- Centrifuge the plate at 1,500 rpm for one minute;
7- After setting the annealing temperature of each primer pair, the experiment was then started on Stratagene™ Mx3005P Instrument. The experimental steps, conditions, and the number of cycles of each step are described in **Table 8.** The plate was also tested across all wells in the machine using the same reference sample with a known dilution and wells with Ct values above 29 were eliminated from analysis.

Table 8: Reaction conditions of qPCR to detect gene expression levels.

Step	Hold Temperatures (°C)	Time	Number of Cycle
Pre-Incubation	95	5 min	1
Amplification	95	10 sec	45-50
	58-60	20 sec	
	72	20 sec	
Melting Curve	95	5 sec	1
	61	1 min	
	95	Acquisition per 5°C	
Cooling	40	30 sec	1

3. RESULTS

3.1. Scratch Wound Healing Assay and MTT

A scratch-wound healing assay was employed to determine the effects of sera from mice that were fed with different dietary conditions on angiogenesis and wound healing. As shown in Figures 4A and B, there is almost no healing or migration in the negative control, which does not contain FBS, i.e. FBS-. The healing rate of all groups were higher than the control FBS- however they did not exhibit differences between them. This showed that even 1% serum of mice fed with a regular diet (chow) was effective. The effect of the mice sera on viability of endothelial cells was evaluated by the MTT assay. As shown in Figure 4C, again all groups were similarly had higher survival and proliferation when compared with FBS- group. Sera regardless of the diet had increased wound healing and cell viability.

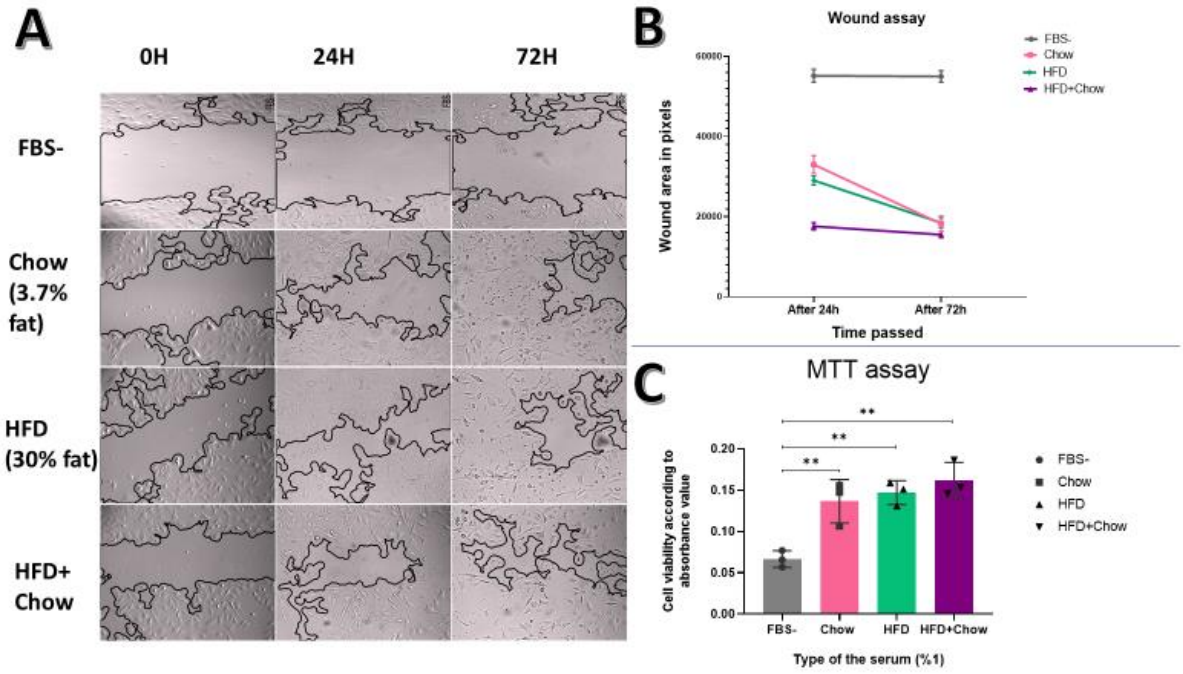

Figure 4: Effects of serums of mice fed with different dietary conditions on angiogenesis *in vitro*. A) bEnd.3 cells incubated without FBS, with %1 serum of mice fed with a chow diet (regular diet), %1 serum of mice fed with a high-fat diet, and %1 serum of mice fed first with high-fat diet then with chow diet (weight loss diet), (from top to bottom respectively) and the cell migration was photographed under Leica DMi1 Inverted Microscope at 24 h, and 72 h, respectively. The dimension of the bar on the images is 200 μm. B) Statistical migration/healing rates. C) Effect of serums of mice fed with different dietary conditions on bEnd.3 proliferation determined by the MTT assay. n = 3, according to Tukey's test ** $p < 0.01$.

3.2. A high-fat diet triggers developmental changes in larvae

To see the effect of egg yolk diet on zebrafish development, 5 dpf larvae were raised in different dietary conditions: control larvae normal diet (L.ND), larva fed high-fat diet (L.HFD), and larvae exposed to weight loss diet which involved switching from high-fat to normal diet (L.WLD). At the end of the three-week feeding experiment, at 26 dpf, larvae were anesthetized and imaged using an inverted light microscope (Leica DMi1 Inverted Microscope). As shown in Figure 4A, a high-fat diet sped up zebrafish development and triggered developmental changes in the caudal fin (Appendix 1). Although all the larvae were at the same stage, as seen in Figure 4A, individuals fed with L.HFD were more developed than those fed as control L.ND and L.WLD. Moreover, a weight loss diet involving switching to normal diet after going through a high fat diet showed a tendency to slow down the developmental process. It seemed that developmental speed could be modified in zebrafish embryos by different dietary regimes. Figure 4B displays the significant increase observed in the length of the L.HFD and L.WLD groups compared to the control group, L.ND. Although no significance was observed between L.HFD and L.WLD larvae there was an apparent tendency of a decrease in length in the L.WLD group.

3.2.1. Egg yolk diet modulates angiogenesis related *vcam* in zebrafish larvae

In order to investigate the angiogenetic effects of the high-fat and weight-loss diets on the larvae, the mRNA levels of angiogenesis-related genes were determined by RNA extraction followed by qPCR. *vegfa2* is a well-known indicator of physiological and pathophysiological angiogenesis (Tang et al. 2010). It showed an increasing trend both in L.HFD and L.WLD groups yet were not significant. According to the ZFIN database (http://zfin.org/ZDB-GENE-070209–238), one of the molecules involved in the control of vascular adhesion associated with inflammation is Vascular Cell Adhesion Molecule-1 (*vcam1*), which showed a significant increase both in L.HFD and L.WLD groups. *Cdh5* also known as VE-cadherin is an endothelial specific, transmembrane protein, that promotes cell-cell adhesion (Anderson et al. 2015). *Kdrl* is one of the VEGF-Receptor-related gene in the zebrafish (Tang et al. 2010). It also can be used as an angiogenesis marker, although it did not show statistically significant increase there was a trend of an upregulation both in L.HFD and L.WLD groups.

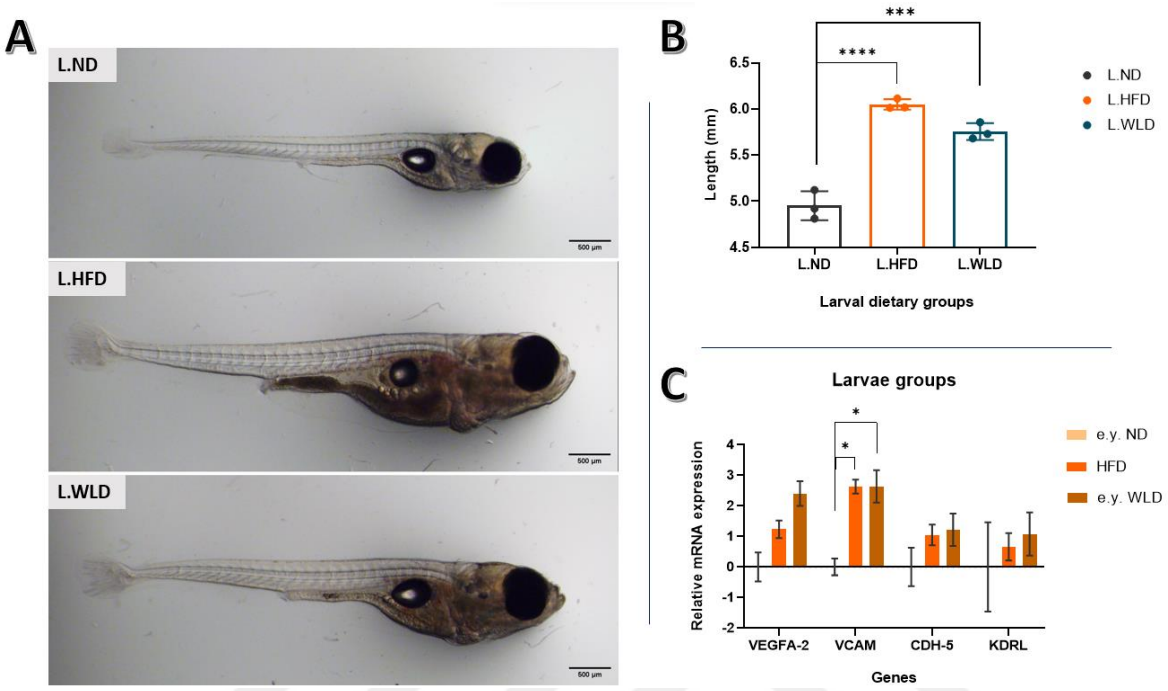

Figure 5: A) Bright-field images of 26 dpf larvae fed with normal diet (L.ND), with high-fat diet (L.HFD), and with weight loss diet (L.WLD), respectively. The dimension of the bar on the images is 300 µm. B) Length of the 26 dpf larvae fed with normal diet (L.ND), with high-fat diet (L.HFD), and with weight loss diet (L.WLD), respectively. N = 3, *** $p < 0.001$, **** $p < 0.0001$. C) Each dietary group's expression of angiogenesis-related genes in the whole larvae. The analysis of gene expression was done by using cDNA prepared from pools of larvae in each group at the end of the feeding experiment. (n = 10, according to Tukey's test * $p < 0.05$).

3.3. Skeletonization of images yields information about angiogenesis.

During normal fin development, the blood vessels grow by vascular branching morphogenesis, which gives an idea about angiogenesis. The goal was to develop a methodology to quantify and compare the sprouting angiogenesis rate in zebrafish. For that two inter-vessel areas were chosen then the number of branches and vascular density of two such areas from an individual were measured and summed.

3.3.1. Egg- yolk based diet trigger angiogenesis

Figure 5A has exemplified the chosen and skeletonized areas, i.e./ two inter-vessel area chosen for each biological replica, and four areas in total for each group. In **Figure 6B**, HFD group had a trend for higher number of branches yet was not significantly different from the normal diet due

to high variation when compared to the other groups. In **Figure 6C**, both the HFD and e.y.WLD showed increasing tendency in terms of vascular density yet were not significantly different from the control group upon Tukey's multiple test corrections.

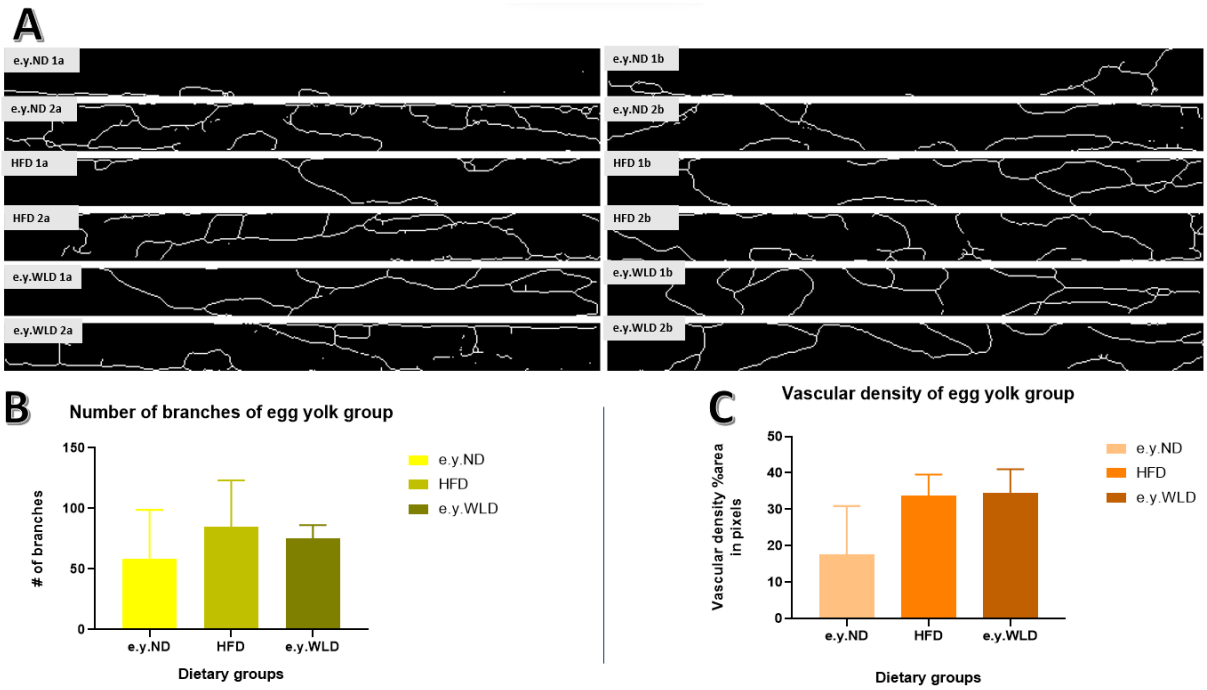

Figure 6: A) skeletonized areas of fish fed with normal diet (e.y.ND1,2), with high-fat diet (HFD1,2), with weight loss diet (e.y.WLD1,2,) respectively. The dimension of the bar on the images is 100 µm. B) number of the branches of each group n = 2. C) vascular density of each group, n = 2.

3.3.2. Overfeeding diet significantly increases the vascularization

Figure 7A shows the chosen and skeletonized areas, the two inter-vessel areas were chosen for each biological replica, amounting to four areas in total for each group. In **Figure 7B**, OFD group had significantly higher branch number compared to the control group. In **Figure 7C**, both the OFD and o.f.WLD showed an increasing trend in terms of vascular density that were not significant upon Tukey's multiple test correction.

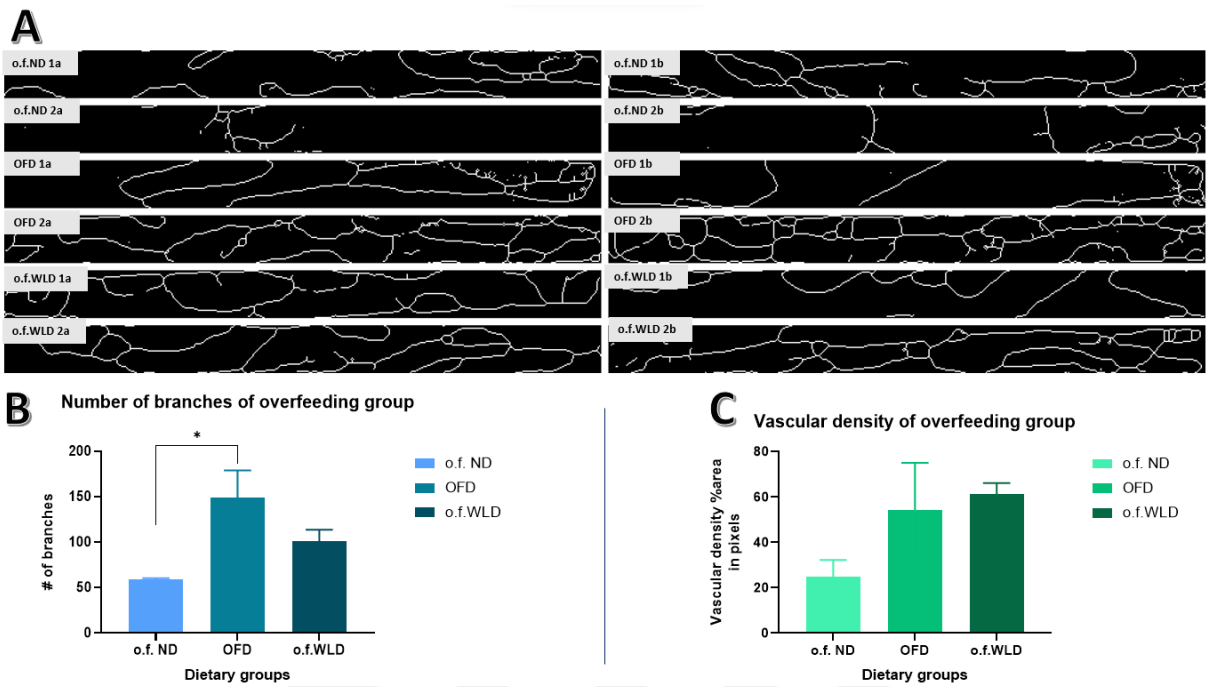

Figure 7: A) skeletonized areas of fish fed with normal diet (o.f.ND1,2), with overfeeding diet (OFD1,2), with weight loss diet (o.f.WLD1,2,) respectively. The dimension of the bar on the images is 100 µm. B). number of the branches of each group n = 2, according to Tukey's test * $p < 0.05$. C) vascular density of each group n = 2.

3.4. High-fat diet, but not overfeeding diet, induces hepatic changes in adult zebrafish liver architecture

To validate the reliability of the egg yolk induced high fat diet, i.e. e.y.HFD model, liver samples of each group were H&E stained for histological analysis, and each individual's body weight was measured at the end of the eight-week feeding experiment. As shown in **Figure 8A**, the HFD group had a significantly higher vacuoles suggesting formation of increased number of lipid droplets when compared to the control diet (e.y.ND) while the egg yolk weight loss diet, i.e. e.y.WLD group showed a distinct decrease in big vacuoles caused by e.y.HFD. As seen, there was no apparent differences between the over feeding groups, i.e. o.f.ND, OFD, and o.f.WLD, in the manner of vacuoles likely representing accumulation of lipid droplets. It can be seen in **Figure 8B** that high-fat diet feeding (HFD) and over-feeding diet (OFD) for eight weeks led to a substantial increase in body weight. In addition, there was a drastic decrease in body weight e.y.WLD and o.f.WLD after four weeks of a weight-loss diet. **Figure 8C** showed no indicative change between the length of any of the groups.

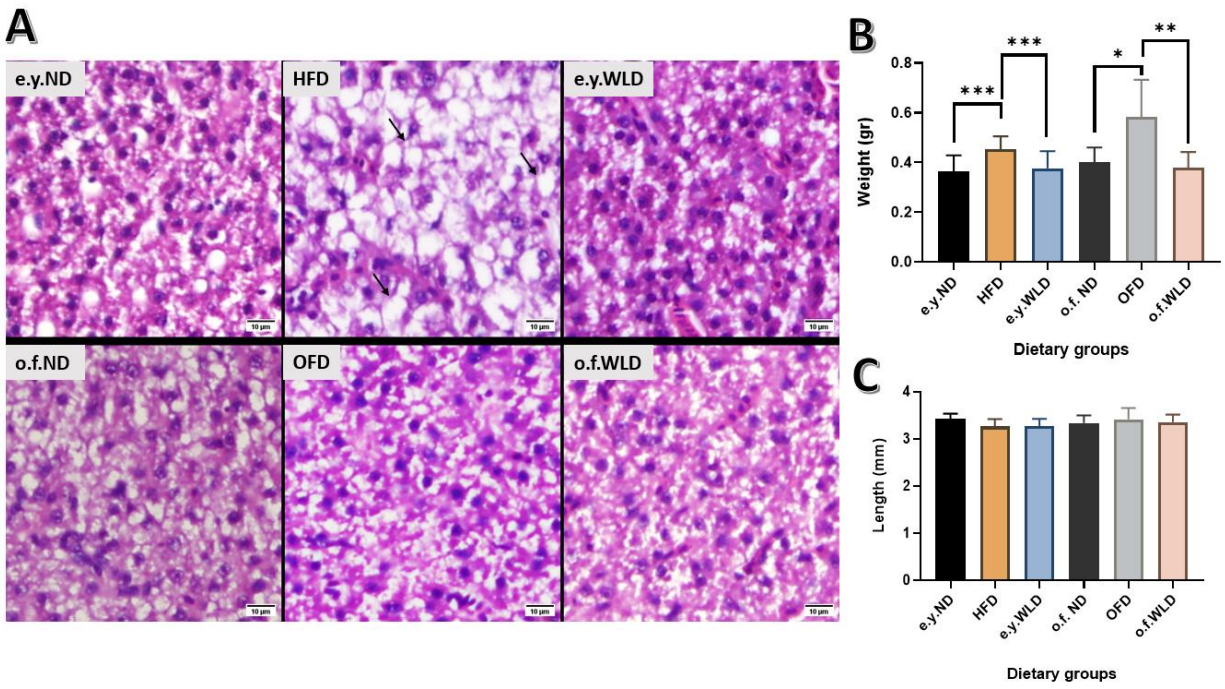

Figure 8: A) Hematoxylin and eosin (H&E) staining of liver sections from zebrafish that fed in different lipid contents and quantity of feeding. Black arrows indicate vacuole lipid droplets. The dimension of the bar on the images is 10 μm. B) Body weight results of each group of zebrafish's. n = 13, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. C) Body length results of each group of zebrafish's. n = 13, according to Tukey's test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.5. High fat and overfeeding diets modulate angiogenesis related gene expression in adult fish

After determining the vascularization changes on the caudal fins via image analysis, qPCR was performed to check the levels of angiogenesis-related genes. For that RNA was extracted from caudal fins of adult zebrafish that fed with different dietary regimes for 8 weeks. Moreover, cDNA synthesis followed by qPCR was performed. Same *vegfa2*, *cdh5* and *kdlr* primers were used for adult as well. As shown in **Figure 9A, B**, *vegfa2* showed an increasing tred in e.y.WLD, OFD and o.f.WLD groups but none were significant. Ang2 (Angiopoietin 2) plays an important role in angiogenesis such as vascular remodeling and maturation in zebrafish (He et al. 2023). Its expression showed an increasing trend in both HFD and e.y.WLD that could not reach significant levels. *Cdh5* showed a significant increase in OFD group while *kdrl* expression showed an increasing trend in OFD and o.f.WLD groups yet these were not significant.

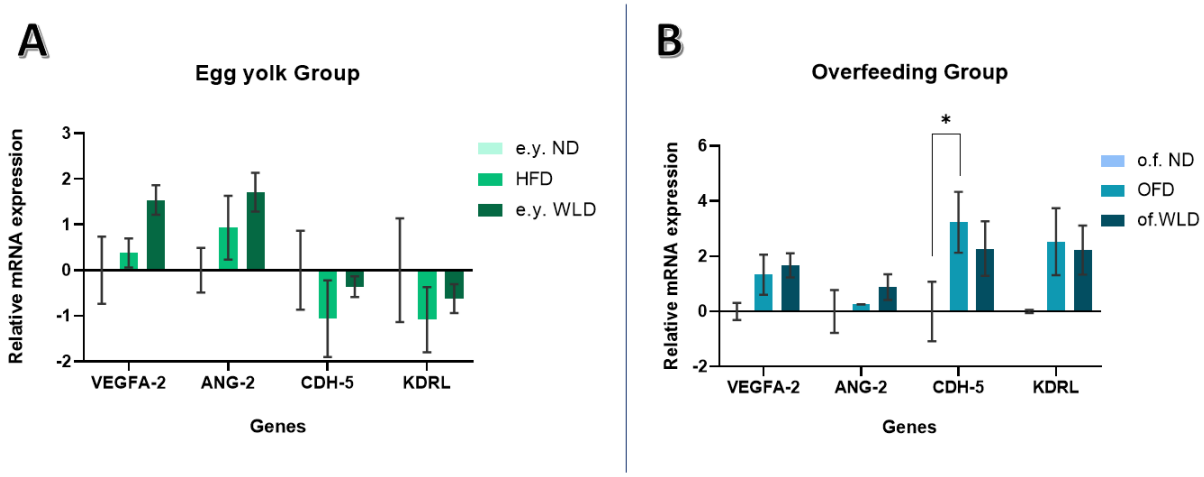

Figure 9: Each dietary group's expression of angiogenesis-related genes in the caudal fin. Gene expression was analyzed using cDNA prepared from pools of adults in each group after eight weeks of the feeding experiment. (n = 2) according to Tukey's test,* $p < 0.05$.

4. CONCLUSION, DISCUSSION AND FUTURE PERSPECTIVES

4.1. Understanding the effects of different dietary regimes on endothelial cells

To investigate the effect of different dietary regimes on endothelial cell dysfunctionality, *in vitro* assays were performed by using 1% serum from mice fed with a chow diet, 1% serum from mice fed with a high-fat diet, and 1% serum from mice fed with high-fat then chow diets on bEnd3 cells.

According to the findings of the MTT assay, all mice sera resulted in an increase in absorbance values. As seen in Figure 2C, metabolic activity of endothelial cells could increase for sera collected from mice fed with HFD, even in the weight loss setup. This might suggest that weight loss may not compensate for prior stress induced by HFD. However, these experiments need to be repeated with higher number of individual replicates and higher amounts of sera to test whether the amount of sera from different dietary regimes would differ from each other.

The results of the scratch-wound healing assay showed that all mice serum-treated samples displayed a higher healing rate compared to negative control, which was FBS-. These results were consistent with MTT assay results regarding the proliferative effect of mice sera on bEnd3 cells. The experiment need to be replicated to obtain significance levels on these increases. However, these two tests, MTT and wound healing, support each other. This thesis provided a preclinical and effective *in vitro* model to test dietary effects of mouse sera on endothelial cells.

4.2. Diet-induced changes in zebrafish larvae

To see the effect of egg yolk diet on zebrafish development, 5 dpf larvae were raised in different dietary conditions: control, high-fat, and high-fat + control diet (weight loss). According to the results, individuals fed a high-fat diet were more advance in their development than those fed a normal diet and to a lesser degree, than the weight loss diet. A high-fat diet seemed to speed up zebrafish development significantly and potentially triggering early metamorphosis start (Appendix), and this can be seen in morphological changes shown in **Figure 5A** and length measurements in **Figure 5B**. This outcome could be a result of the lipid and protein richness of

chicken egg yolk (59% fat, lipids make ~58% of chicken egg yolk, of which ~5% is cholesterol, 60% are triglycerides, and 35% are phospholipids); 32% proteins, 2% carbohydrates) when compared to typical commercial zebrafish micropellet foods (~15% lipids and 25% protein).

In order to investigate the angiogenesis at the RNA level, qPCR was performed. The mRNA levels of angiogenesis-related genes were determined by RNA extraction followed by qPCR. According to the ZFIN database (http://zfin.org/ZDB-GENE-070209–238), zebrafish vascular cell adhesion molecule-1 (vcam1) is a protein that regulate inflammation. In this thesis, there was a significant increase in *vcam1* expression both in L.HFD and L.WLD groups. Since inflammation was known to be one of the significant consequences of obesity, it can be said that this result supported the effectivity of diet-induced obesity in zebrafish larvae (Russo et al. 2023). Other markers did not show a statistically significant increase, probably because of high standard deviation. Since whole larvae are used for RNA extraction, the mRNA from all the tissues and organs could be misleading and result in high standard deviation. Using more specific tissues or organs would result in more reliable outcomes. This study should be repeated with different zebrafish lines such as AB wild-type lines, to enhance the reliability and to observe other developmental markers, such as specific pigmentation patterns. Moreover, a larger number of individual replicates are needed for expression studies.

4.3. Adult zebrafish fin reveals information about angiogenesis

During the development of obesity, angiogenesis is known to be accelerated by hypoxia, inflammation, and structural remodeling of blood vessels (Herold and Kalucka 2021). Hypoxia-induced pathological angiogenesis has been shown in zebrafish (Zhao et al. 2016). Based on these findings, I have hypothesized that obesity induces inflammation and could trigger angiogenesis in zebrafish tissue, and the caudal fin serves as a great environment to study vasculature. During normal fin development, the blood vessels grow by vascular branching morphogenesis, which can give an idea about the degree of angiogenesis. In order to develop a methodology to quantify and compare the sprouting angiogenesis rate in zebrafish, ImageJ has been used as a tool. Two inter-vessel areas were chosen to analyze the angiogenesis, and the number of branches and vascular density of each area were measured. There are multiple studies in the literature that used similar methodologies for vascular analysis (Rling et al. 2023; Caceres et al. 2019; Ma et al. 2021). In this

study, I have used ImageJ's skeletonize and vascular density measurement plugins.

Many scientific studies and research have indicated that high-fat diet (HFD) elevates the concentrations of growth factors that activate transcription factors, leading to the upregulation of numerous genes associated with promoting inflammation, angiogenesis, and cellular proliferation (H. Park et al. 2012; Miura et al. 2019). In this study, the fins of zebrafish fed with the egg yolk (e.y) high-fat diet (HFD) displayed a tendency of an increase of angiogenic sprouts and branches when compared to the control group however variation was high and hence the results were not significant. This was due to high variation in between samples that belong to the same group suggesting that a higher sample size was needed to increase statistical power. Hence future studies should include more than two samples per group.

My approach was relatively novel since I have used skeletonize function rather than simple counting or pixel intensity. Another reason for not finding a significant change could be due to the measurement method; I have used an equal sized but relatively small ecliptic area to measure the branches and density in the inter-vessel region. Additional analyses should test whether spanning a larger area in between fin rays could yield different results.

OFD group on the other hand had a significantly higher branching number when compared to the control group. Moreover, the emerging angiogenic sprouts were noticeable originating from matured blood vessels, closely resembling the conditions observed in human patients (Schaafhausen et al. 2013). The measurement of vessel density showed a tendency of an increase in the vascular network of OFD and o.f.WLD groups as paralleled in egg yolk group's results. Interestingly, weight loss did not result in a significant change in the HFD fed adults fish.

This study was limited into one zebrafish line, Tg(*fli1*:EGFP) in the Casper background, other zebrafish lines should be included as well as the sample number should be increased for further studies.

4.4. Does high fat diet trigger obesity which can be reversed by weight loss in adult zebrafish?

To assess the effects of different dietary regimes on the morphology of adult zebrafish, six dietary groups were assigned: two groups were the control (e.y. ND) and (o.f. ND), one group fed

with high-fat-diet (HFD), another group overfed with artemia and dry food (OFD), two of them were weight loss group, respectively for the egg yolk and overfeeding diets, i.e. (e.y. WLD) and (o.f. WLD). Results showed that high-fat feeding (HFD) and over-feeding diet (OFD) for eight weeks substantially increased the body weight. In addition, there was a drastic decrease in body weight, e.y.WLD and o.f.WLD after four weeks of a weight-loss diet. Since the changes in lengths were insignificant this could indicate obesity in both dietary regimes.

H&E stained liver sections showed that the HFD group had a higher occurrence of large vacuoles suggestive of lipid droplets when compared to the control (e.y. ND). Where the e.y.WLD group showed a distinct reversal in these big vacuoles. There was no visible change between o.f.ND, OFD, and o.f.WLD in the manner of lipid droplets. It has been known that obesity induces hepatic steatosis both in the liver of zebrafish and humans (Oka et al. 2010; K. H. Park et al. 2019; Li et al. 2023b). Therefore, based on these results, my results suggested that egg yolk diet more likely induced obesity in zebrafish, whereas there was no significant change for overfeeding group. This unexpected outcome could be a result of the low lipid composition of typical commercial zebrafish micropellet foods (~15% lipids) or artemia compared to chicken egg yolk (59% fat, (lipids compose ~58% of chicken egg yolk, of which ~5% is cholesterol, 60% are triglycerides, and 35% are phospholipids). However, Turola et al. showed that overfeeding induced hepatic steatosis in zebrafish by using oil red O (ORO) staining (Turola et al. 2015). For further studies and reliable outcomes ORO staining should be performed in these samples; this will help identify and quantify the large and smaller sized oil droplets.

4.5. Studying changes in angiogenesis based on expression level markers

After determining the vascularization on the caudal fins via image analysis, qPCR was performed to check the levels of angiogenesis-related genes. *Vegfa2* showed a tendency of an increase in all high fat or overfeeding groups although non-significant. It is well known that *vegfa2* mRNA levels were upregulated during caudal fin regeneration (Rathinasamy, Paneerselvan, and Ragunathan 2014; Vivek and Malathi 2017). Future studies could use more samples to increase statistical power hence the effects of high fat or overfeeding diet on *vegfa2* expression could be better distinguished.

Ang2 (Angiopoietin 2) plays a vital role in angiogenesis, such as vascular remodeling and

maturation in zebrafish (He et al. 2023). As expected its expression showed tendency of an increase in HFD, yet was not significant. Studies show that ang2 has a specific role in endothelial repair and vascular development (Francis, Claflin, and Kushner 2021). Such a tendency to increase in egg yolk diet but not in overfeeding diet could signal there were differences in diets with respect to endothelial effects that need to be studied further.

According to the literature, reducing CDH5/VE-cadherin expression caused vascular fragility and increased permeability (Dejana, Orsenigo, and Lampugnani 2008). The significant increase in the OFD group could indicate abnormal vascularization upon overfeeding but this was not observed in the egg yolk diet. Because according to the literature, upregulation of CDH5 expression was shown in tumor cells and cancer tissues (Mao et al. 2013; Y. Wang et al. 2021). This significant finding should be further studied with higher sample sizes and differing dietary regimens whether *cdh5* expression in zebrafish is modulated by increased metabolism that is due to higher amounts of nutrients (proteins, lipids and carbohydrates) and not primarily lipids.

One study showed that *kdrl* expression was upregulated after an injury in zebrafish, leading to injury recovery through angiogenesis (Cai et al. 2023). The observed tendency of increased expression in kdlr could correspond to a potential response to endothelial changes occurring in OFD and o.f.WLD, in association with the observed higher rate of vascular branching and density in OFD and o.f.WLD groups. However, these experiments should be repeated with a bigger sample pool. Moreover, angiogenesis and branching number may be modulated by changes at the post-transcriptional (Chang et al. 2013) as well as post-translational levels (Lu et al. 2023) and future studies should study these as well.

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Appendix 1

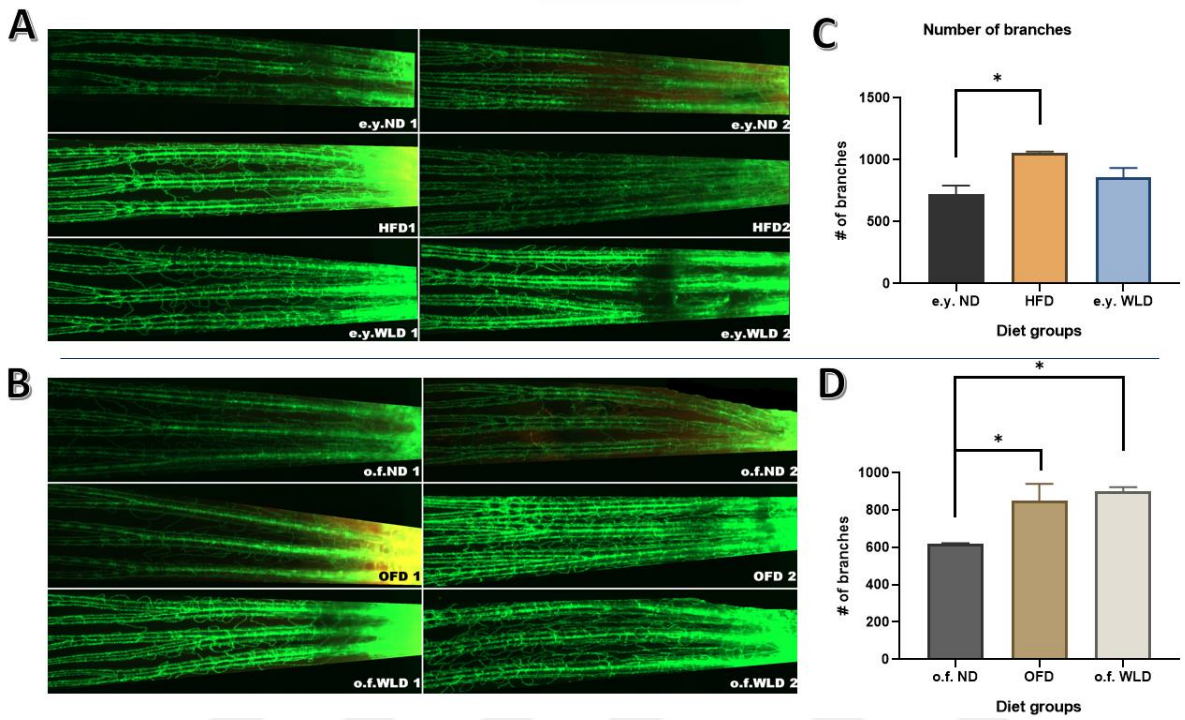

Figure appendix 1: *Fluorescent images of caudal fins. The vessels appear green (eGFP in fli1a positive arterial endothelial cells).* ***A)*** *central three fin ray of fish fed with normal diet (e.y.ND1,2), with high-fat diet (HFD1,2), with weight loss diet (e.y.WLD1,2,) respectively. The dimension of the bar on the images is 100 µm.* ***B)*** *central three fin ray of fish fed with normal diet (o.f.ND1,2), with overfeeding diet (OFD1,2), with weight loss diet (o.f.WLD1,2,) respectively. The dimension of the bar on the images is 500 µm.* ***C, D)*** *number of the branches in all three rays of each group according to methods that we established in this study (branch number analyzed by ImageJ program) n = 2, * p < 0.05.*

Appendix 2

Figure appendix 2.1: 26 dpf larvae fed with normal diet.

Figure appendix 2.2.: 26 dpf larvae fed with high fat diet from 5 dpf.

Figure appendix 2.3.: 26 dpf larvae fed with high fat diet for 2 weeks and normal diet for 1 week.

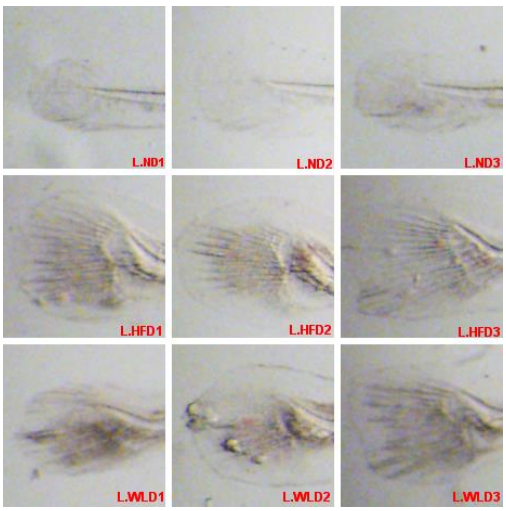

Figure appendix 2.4.: caudal fin of the larvae

Appendix 3 Permeability Assay using *in vitro* mouse cells

Method: bEnd.3 cells were cultured onto 24 mm Transwell inserts (pore size 0.4 µm, Corning Inc., Corning, NY, USA) at 1 x 105 cells/cm2 density. After cells formed a confluent monolayer, media was carefully replaced with either serum free, 5% FBS, 1% sera of mice fed either with chow, high-fat, or high-fat then chow diets (2 replicas each). FITC-dextran (50 µM, Sigma-Aldrich, St. Louis, MO, USA) was added to the medium in the apical chamber. After tracer addition to the upper chamber, media samples from both apical and basolateral sides were collected after 5 min., 30 min., and 120 min. The fluorescent absorbance of FITC-dextran was measured using a fluorescence plate reader (Molecular Devices, LLC, San Jose, CA, USA) at an excitation/emission wavelength of 490 nm/520 nm, respectively. Cell monolayer permeability value was determined with the formula:

permeability = (dQ / (dt *A*C0))

With dQ (transported amount, basolateral side fluorescence), dT (time, in s), A (surface of the barrier, in cm) and C° (initial concentration, apical side fluorescence).

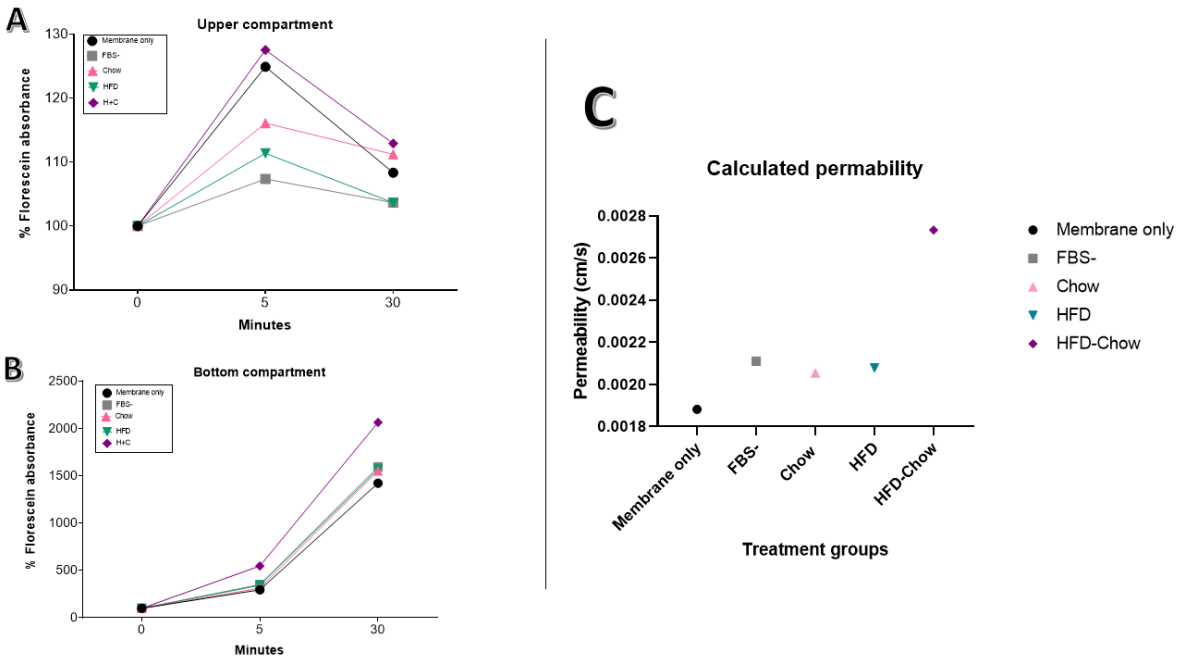

Figure appendix 3: Results of permeability assay. The vertical axis indicates the fluorescence intensity of FITC-conjugated dextran. The horizontal axis shows the time after treatment of bEnd.3

cells with %5 FBS, %1 serum of mice fed with a chow diet, %1 serum of mice fed with a high-fat diet, and %1 serum of mice fed with both a chow and high-fat diet. The measurement of the leakage of FITC-conjugated dextran fluorescence intensity from the upper to the lower chambers of Trans-well membranes was conducted in A) upper and B) lower chambers at specific time points.