The Zonation of Hepatic Lipid Metabolism:

Exploring the Evolution of the Current Model of Understanding

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Abstract

The purpose of this study was to conduct a literature review to explore the evolution of the current understanding of the zonation of hepatic lipid metabolism. Though much is known about zonation of other hepatic metabolic processes, there is limited research into lipid zonation. Towards this aim, a comprehensive literature review was conducted to investigate relevant studies dedicated to hepatic lipid zonation. The methods and findings of the studies were catalogued and compared in order to determine which results were consistent and which needed further study and exploration to be considered reliable. Ultimately, this review revealed that much of today's understanding of hepatic lipid zonation is predicated on the findings of several studies by Katz et al. in the early 1980s. Though there were a handful of other studies by other investigators, other data was deemed unreliable due to its reliance on digitonin perfusion, an experimental method that was proven to provide aberrant results due to comparisons drawn between incomparable study samples. Ultimately, while it appears that lipogenesis tends to occur in pericentral hepatocytes while beta oxidation occurs in periportal hepatocytes, further research is needed to elucidate the impact of various physiological factors (age, sex, breed of study subject, feeding state, etc.) on these zonation patterns. This work is crucial as the number of disorders and diseases that impact hepatic lipid zonation continues to rise. A greater understanding of the foundational principles of hepatic lipid zonation will allow for more targeted clinical interventions and improve health outcomes for affected individuals.

Dedication

Beau & Wells: the joy you spark defies all laws of the natural world. Thank you for being my constant inspiration to always do the very best that I can.

And to Trey, thanks for knowing I could do it even when I was sure I couldn't.

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An enormous thank you to my thesis director Dr. Sudha Biddinger. I am so grateful for the guidance, enthusiasm, and patience you've shown me over the last two years. Thank you also to my research advisor Dr. Jim Morris for the flexibility, encouragement and support through this process.

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Chapter I.

The Importance of the Zonation of Lipids

To appreciate the significance of the zonation of hepatic lipid metabolism, it is first critical to understand the physiology and organization of the liver itself. Sitting directly below the diaphragm and above the stomach, the liver is the largest organ in the human body. The liver acts as the metabolic gatekeeper for the body, as all blood exiting the intestines must first pass through the liver before entering systemic circulation. As such, the liver filters and metabolizes exogenous substances brought into the body through digestion. Among its many important functions, the liver is responsible for drug metabolism, production of bile, the conversion of excess glucose to glycogen (glycogenesis) and, conversely, the breakdown of glycogen to liberate glucose (glycogenolysis) (Liver: Anatomy and Functions, 2019).

The liver is comprised of a complex network of substructures that facilitate the enormous task of filtering the body's blood supply. The liver is physically separated into two main lobes, each of which is comprised of eight segments. Each of these eight segments is comprised of roughly one thousand lobules (Liver: Anatomy and Functions, 2019). In addition to this structural scaffolding, the liver is also functionally organized into regions known as "acini" – which are said to be the functional unit of the liver. Acini are zones of tissue defined by their proximity to the blood supply of the lobules. Each acinus features a portal triad (the hepatic artery, portal vein, and bile duct) at its center with a mass of parenchymal cells extending outwards towards the central veins. The

physical distance from different vasculature results in a distinct metabolite gradient that exposes the hepatocytes to vastly different environments. The hepatocytes closest to the portal triad are exposed to high concentrations of oxygen and nutrient rich blood. This region is known as the periportal region or zone one. As distance from the portal triad increases, the oxygen and nutrient concentrations decrease through zone two, before finally reaching the cells most proximate to the central vein. This region of hepatocytes is known as the pericentral region or zone three (Krishna et al., 2013). This organization can be visualized below:

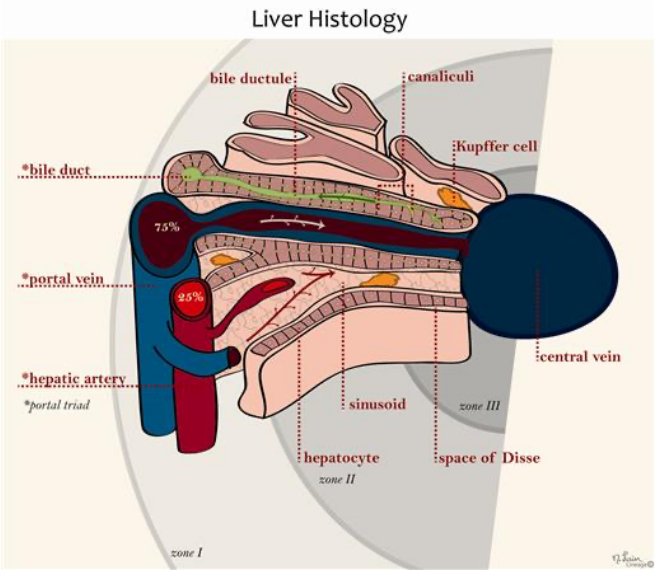

Figure 1: Identifying the Zones of the Liver.

The delineation of zones within the acini is based on proximity to vasculature. Zone 1 is known as the periportal zone for its proximity to the portal vein, while zone 3 is known as the pericentral zone due to its proximity to the central vein (Team, n.d.).

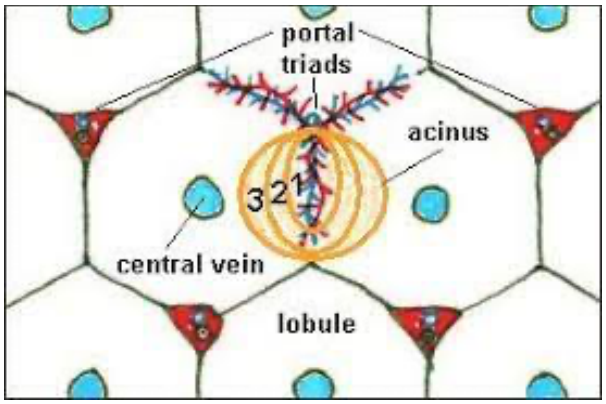

Figure 2: Organization of the Acini

Distinction between the hepatic lobule (green hexagons) and acinus (shown in yellow) demonstrates how the acini is organized around blood flow while the lobules are organized anchored by physical structures (The Hepatic Acinus Is the Functional Unit of the Liver, n.d.)

Hepatic Lipid Metabolism

The liver controls many homeostatic processes which facilitate the careful regulation of hepatic lipid concentrations. Lipids are a diverse class of macromolecules, serving a myriad of important roles in a range of metabolic processes from energy storage to intracellular signaling. There are a variety of ways that lipids can be processed in the liver: they can be generated from excess carbohydrates, taken up from the blood stream either to be broken down and utilized for energy or stored for later use, or exported from the liver for use at sites around the body (Alves-Bezerra & Cohen, 2017).

The *de novo* synthesis of lipids (*de novo* lipogenesis or simply lipogenesis) is an important process, particularly in times of carbohydrate excess. These carbohydrates can be converted to lipids and then these lipids can then be stored for later, providing the cell with an emergency fund of cellular energy. Lipogenesis occurs when amino acids and

glucose are converted into a class of lipids known as triacyl glycerides (TAGs) which can then be packaged into very-low-density-lipoproteins (VLDLs). VLDLs are then excreted from the hepatocytes for export to various sites around the body (Kim et al., 2023). Lipogenesis can be generally understood by the figure below:

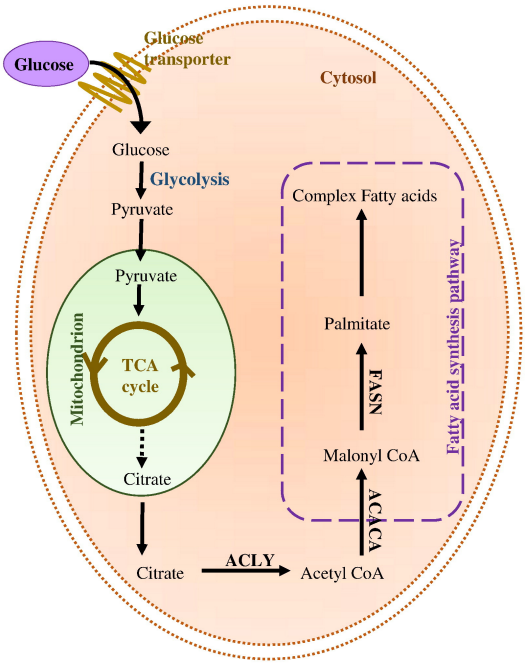

Figure 3: The Process of Lipogenesis

The general outline of lipogenesis is shown above with various processes taking place in different regions of the hepatocyte (Ameer et al., 2014).

Research suggests that under normal conditions lipogenesis takes place in the pericentral region of the lobule, but not in the periportal region (Hijmans et al., 2014). As such, one of the key takeaways from the figure above is the necessary presence of certain enzymes to facilitate fatty acid synthesis. More specifically, three enzymes are often

studies to understand the zonation of lipogenesis: ATP citrate lyase (ACLY), acetyl-coA carboxylase (ACC) and fatty acid synthase (FAS). ACLY catalyzes the first step of de-novo lipogenesis by converting citrate into acetyl-coA. Because of its involvement in lipogenesis, ACLY may serve as a potential therapeutic target for decreasing hepatic lipid production. Indeed, recent studies on murine models show that ACLY knockout mice exhibit reduced hepatic stores of malonyl-CoA and overall steatosis (Morrow et al., 2022). More research is needed to determine whether these results may be replicated in humans.

Next, ACC catalyzes the conversion of acetyl coA to malonyl coA which begins the cascade of reactions culminating in the creation of various fatty acids. Within hepatocytes, ACC exists in two distinct isoforms: ACC1 which is present in the cytosol and ACC2 which is present in the mitochondria. The two isoforms give rise to two separate pools of malonyl co-A, each of which has implications for the fine-tuned regulation of hepatic fatty acid metabolism (Alves-Bezerra & Cohen, 2017). As the exploration of these enzymes shows, it is imperative to understand that the presence of an enzyme is not indicative of a process unless there is readily available substrate to drive the reaction forward.

Finally, once acetyl coA has been converted to malonyl coA, FAS converts malonyl coA into palmitate which can then act as a foundation upon which elongates (a class of enzymes tasked with lengthening the hydrocarbon tails of fatty acids) to generate longer chain fatty acids (Alves-Bezerra & Cohen, 2017). Once lipids are created, they can be stored as lipid droplets or transported elsewhere. Therefore, to understand more about

the zonation of lipogenesis, it may be helpful to assume that higher concentration of ACC, ACLY, or FAS likely indicate an increased rate of lipogenesis (Kim et al., 2023).

Because of their waxy nonpolar structures, lipids must be transported through the aqueous bloodstream via plasma membrane-associated proteins. These proteins serve as rafts and facilitate the transfer of lipids (packaged as triglycerides) in and out of hepatocytes. One important family of such enzymes are the apolipoproteins. These proteins provide a site for lipids to gather, forming lipoproteins which can then be transported from site to site (Mehta & Shapiro, 2021). Two apolipoproteins have provided interesting results in the context of hepatic lipid metabolism: ApoB and ApoE. ApoB is critical in the regulation of intrahepatic lipid concentration. When lipids are unavailable, it is not highly expressed; however, in times of lipid excess, ApoB production increases. Microsomal triglyceride transfer protein (MTP) transfers triglycerides to ApoB which ultimately creates very-low-density lipoproteins (VLDL) to be exported from the liver to provide triglycerides for catabolism to sites around the body (Meta & Shapiro). ApoE is also involved in the regulation of lipid concentration and regulates transfer and transport between tissues and cells. ApoE also appears to work to specifically promote lipid uptake via other carrier proteins such as lipoprotein receptor protein 1 (LRP1) which has a much higher binding affinity for ApoE than other lipoproteins. (Huang & Mahley, 2014). Given the current understanding regarding lipid metabolism, it may then be expected that hepatocytes with higher rates of beta-oxidation would express a higher concentration of ApoB and ApoE than those tasked with lipogenesis.

Once in the hepatocytes, triglycerides can be metabolized for energy via a process known as beta-oxidation (also referred to as fatty acid oxidation). This process involves the stepwise shortening of the carbohydrate tails of fatty acids with the liberation of chemical energy at each step. Because lipids must be returned to the liver for beta-oxidation to occur, many studies exploring the zonation of beta-oxidation focus on enzymes known to facilitate lipid uptake in hepatocytes. More specifically, there is a significant body of research exploring both carnitine palmitoyl transferase protein 1 (CPT-1) and liver fatty acid binding protein (L-FABP). Functioning as the key rate limiting enzyme for fatty acid oxidation, CPT-1 brings fatty acids into the intermembrane space of the mitochondria. CPT-2 then transports the fatty acids from the intermembrane space into the mitochondrial matrix. Once inside the mitochondria, the fatty acids are then available for beta-oxidation (Wang et al., 2021). Indeed, in instances of malfunctioning or absence CPT-1 proteins, affected individuals are unable to properly metabolize fats resulting in fatty acid buildup throughout the body.

Similarly, L-FABP is expressed in the cytoplasm of hepatocytes and through its two binding sites can carry free fatty acids for transport around the cell (Furuhashi & Hotamisligil, 2008). This is particularly important for uptake into the mitochondria for oxidation. Beta oxidation is generally associated with the periportal zone; therefore, higher activity and expression of CPT-1 and L-FABP are often studies as biomarkers.

If there is no cellular need for energy, lipids can be stored within the liver, typically in the form of triglycerides. Triglyceride synthesis is largely regulated by a few isoforms the GPAT enzymes. GPAT1 appears to play a critical role in allocating fatty acids for storage rather than breakdown via beta oxidation (Alves-Bezerra & Cohen,

2017). Another mechanism of storage is the generation of lipid droplets. These droplets serve as a transient reservoir for triglycerides within hepatocytes (Alves-Bezerra & Cohen, 2017). It has been observed that triglycerides seem to accumulate largely in the pericentral region as opposed to the periportal region (Hijmans et al., 2014). Free fatty acids stored in lipid droplets can be brought into the mitochondria for beta oxidation via the carnitine shuttle. This pathway is catalyzed by Carnitine palmitoyl transferase (CTP-1) and is therefore a critical enzyme in beta-oxidation as studies have shown that knockdown CTP-1 organisms display decreased ability for lipid mobilization (De Paula et al., 2023). Early studies of CTP-1 as a proxy for the zonation of beta-oxidation in hepatocytes demonstrate a largely periportal zonation pattern which is consistent with today's understanding of the zonation of beta-oxidation.

While there are many more enzymes and pathways involved in hepatic lipid metabolism, those described above represent a sample of frequently studied biomarkers as research seeks to elucidate the patterns of hepatic metabolic zonation of lipids.

Liver Zonation

Despite its homogenous appearance, the acini of the liver are distinguished by regions of unique metabolic functioning given their exposure to varying concentrations of metabolites. The difference in function throughout these regions is described as metabolic zonation (Hijmans et al., 2014). As previously mentioned, the acini can be divided into the three main zones: the periportal zone, those hepatocytes that are in direct proximity to the portal vein; the pericentral zone, those hepatocytes that are in direct proximity to the central vein; and the intermediate zones, those hepatocytes that fall somewhere between

the pericentral and periportal zones (Hijmans et al., 2014). Each region is characterized by a distinct gradient of metabolites. The periportal zone provides an oxygen and nutrient-rich environment while the pericentral zone is exposed to less oxygen and nutrients but more carbon dioxide (Hijmans et al., 2014). In addition to the different concentration of metabolites present in each zone, it has also been observed that certain processes appear to be localized within the different zones.

Many studies have explored the zonation of carbohydrate metabolism in the liver. It is generally accepted that gluconeogenesis is periportal while glycolysis is pericentral (Hijmans et al., 2014). These studies and findings have prompted further research into hepatic lipid zonation due to the interplay between carbohydrates and fat. Indeed, this observation was a driver for early research into hepatic zonation as researchers began to postulate that beta oxidation might provide the energy for gluconeogenesis (thus the periportal zonation); while glycolysis is able to provide a pool of acetyl coA for fatty acid synthesis via lipogenesis. Therefore, the metabolism of carbohydrates and lipids demonstrates a symbiotic and synergistic zonation pattern (Katz, 1983). Much of the work mapping out the zonation patterns of carbohydrate metabolism spanned the years of 1978-1982, with experiments into lipid zonation increasing in frequency around 1983.

The Current Understanding of Lipid Zonation

Exploring publications since the turn of the century reveals that much of the current understanding of lipid zonation relies on the results of experiments conducted nearly 40 years prior. In the seven papers published since 2000 devoted to lipid zonation, each has referenced the work of Katz in establishing the current landscape of knowledge.

As such, the current model is largely based on the work of a single researcher and an impactful but relatively limited pool of data. Interestingly, papers published since the early 2000s neglect to reference the work of Evans et al, who published a few key studies around the same time which provided conflicting data.

As such, the working hypothesis continues to generally assume that lipogenesis, by necessity, must take place in the pericentral region, while the opposing and antagonistic processes of beta oxidation takes place in the periportal region. Indeed, while the theory is tidy and logical, it fails to account for the confounding body of data that exists outside of the experiments of Katz et al. This exact conundrum has been taken on by two larger review studies devoted to understanding the conflicting data exploring lipid zonation (Schleicher, 2015) (Cunningham, 2021).

While papers published since 2000 are quick to agree with the model supported by the work of Katz, these recent review articles challenged the tidy and binary organization of relegating lipogenesis to the pericentral hepatocytes and oxidation to the pericentral. One review called for greater care in accounting for differences that may suggest a more flexible zonation pattern such as feeding state, sex, and circadian rhythm (Schleicher, 2015). This challenge carried through to a 2021 review which further expounded the limitations of technology an called for further research into zonation using mass spectroscopy imaging (Cunningham, 2021). As such, the current landscape of the zonation of hepatic lipid metabolism is one that plagued by uncertainty regarding the reliability of many of the foundational studies on the subject. Therefore, the primary aim of this paper is to explore the evolution of the current understanding of hepatic lipid

metabolism and evaluate what data can be regarded as reliable, and what further work needs to be done to fully understand the complex and dynamic balance of lipid zonation.

The Clinical Significance of Understanding Zonation

While understanding hepatic lipid zonation is an important endeavor unto itself, there are broader motivations to expand this body of knowledge. There are many diseases and disorders that arise when zonation patterns are disrupted in addition to those whose clinical presentation involve their own unique zonation patterns.

Non-alcoholic Fatty Liver Disease (NAFLD) occurs through the accumulation of fat in the liver, a process known as steatosis. Statistics estimate that up to 25% of adults in the United States have NAFLD (Rivera, 2024) and NAFLD is estimated to become the leading cause for liver transplant by 2030 (Byrne et al., 2015). These statistics and predictions underscore the importance of understanding lipid zonation given the current, and climbing, number of affected individuals. This disease is particularly concerning, as it significantly increases the risk of Type 2 Diabetes, cardiovascular disease and chronic kidney disease (Byrne et al,, 2015). Curiously, NAFLD has been shown to increase lipid accumulation in the pericentral region, later progressing to the periportal region as the disease progresses (Chalasani et al., 2008). This is noteworthy as early studies on lipid metabolism suggest that lipogenesis occurs mainly in the pericentral region (Hijmans et al., 2014). Therefore, it will be key to understand what changes allow steatosis to progress to the periportal region to try to combat its spread. To date, there have been a small number of studies dedicated to metabolic zonation in NAFLD; thus, it would be

beneficial aggregate data to provide a comprehensive zonation resource towards understanding the metabolic zonation of lipids in NAFLD.

While NAFLD itself appears to present with a zonated pattern of lipid accumulation, there are also data that demonstrate that further disruption can arise with exposure to other substances. For example, in pediatric NAFLD patients, an increased consumption of dietary fructose (abundantly available in many processed foods) resulted in lipid accumulation in both the periportal and pericentral regions, entirely abolishing the zone-specific progression of disease (Nobili et al., 2018).

Beyond NAFLD, there are a myriad of other conditions that would benefit from greater understanding of hepatic lipid zonation. Cancer cachexia causes noteworthy disruptions in zonation patterns between controls and study groups, where a reverse in zonation of L-FABP from periportal to pericentral and enhanced activity of CPT-II in the pericentral region (Kazantzis & Seelaender, 2005). Cancer cachexia impacts an estimated 80% of cancer patients, with 20% of those ultimately dying due to side effects from cachexia (Mariean et al., 2023). Therefore, more research into this area has the potential to improve the outcomes for an enormous number of people across the globe

Similarly, chronic ethanol feeding was shown to eliminate the zonation patterns of lipogenesis observed in control animals in addition to shifting fatty acid oxidation and activity of CPT-1 to the pericentral region. With roughly 10% of Americans being diagnosed with Alcohol Use Disorder (AUD), greater understanding into the impact of ethanol on zonation patterns could provide important therapeutic targets in mitigating and reversing the damages done by prolonged ethanol exposure. As such a stronger

understanding of what factors and conditions impact and influence hepatic zonation is critical in creating therapeutics for these conditions.

Definition of Terms

"Acinus": the functional unit of the liver. Defined as a region of hepatocytes surrounding the hepatic triad and extending out towards the central venules.

"The central vein": carries oxygen and nutrient-depleted blood away from the liver.

"*de novo* Lipogenesis": the process by which excess carbohydrates are converted to fatty acids within the liver.

"Fatty acids": A molecule composed of a hydrocarbon chain (tail) and a terminal carboxyl group (head). These molecules are the building blocks of triglycerides.

"Fatty acid oxidation/Beta oxidation": the process by which the energy stored in fatty acids is liberated.

"Hepatic Triad": a vascular bundle containing the portal vein, hepatic artery, and bile duct.

"Hepatocytes": specialized parenchymal cells that make up 80-90% of liver cells.

"Fatty acid uptake": the process by which fatty acids are brought into cells from the blood stream.

"Fatty acid export": the process by which fatty acids are returned to the bloodstream from cells.

"Lipid Droplets (LDs)": a transient form of lipid storage in the liver.

"Lipogenesis": the generation of lipids from fatty acid and triglyceride synthesis.

“Mass spectrometry imaging (MSI)”: an analytic technique that provides a spatial map of metabolites within tissue samples.

“Metabolic zonation”: the process by which certain metabolic functions (i.e. lipogenesis, beta oxidation, etc.) are spatially anchored to a particular region of the liver.

“Non-alcoholic fatty liver disease (NAFLD)”: a condition characterized by the accumulation of triglycerides in the liver.

“Pericentral region”: hepatocytes that surround the central vein within the acini.

“Periportal region”: hepatocytes that surround the portal vein/hepatic triad within the acini.

“Steatosis”: an abnormal accumulation of fat within cells.

“The portal vein”: carries oxygen and nutrient rich blood to the liver.

“Triglycerides (TGs)”: a lipid composed of three fatty acids and a glycerol.

Chapter II.

Methods

The purpose of this study was to conduct a literature review to explore the evolution of the current understanding of the zonation of hepatic lipid metabolism. Though there is a large body of evidence supporting the zonation of carbohydrates and amino acids, lipid zonation remains largely unexplored, and the current model is limited in its applications. As such, this study set out to review relevant data pertaining to lipid zonation and understand the evolution of the current model.

Towards this aim, the study used PubMed to research publications relating to lipid zonation in the liver. This search was conducted using a query of "(pericentral OR perivenous OR periportal OR zonation) AND (liver OR hepato*) AND (fatty acid OR triglyceride OR lipoprotein OR cholesterol). The search yielded 693 results. From there, each abstract was reviewed for relevance. Studies were determined to be relevant and subsequently included if they included references to zonation patterns or specific processes within lipid metabolism. Relevant articles were reviewed, and data was collected in a spreadsheet to allow for comparison across studies. The references of these articles were also examined for further studies not yielded by the initial PubMed search. For each relevant article, the following data was noted: year of publication, relationship to lipid zonation, the reported zonation pattern, the experimental method and any other relevant notes.

Once this selection of data was collected, data relating to the same process and/or enzyme was analyzed in greater detail. In addition to the information above, the articles were further examined for differences between study subjects, age of subjects, sex of subjects, and rigor of experiment to allow for more context with which to interpret both supporting and discordant results.

Ultimately, this data was used to construct a series of tables demonstrating the evolution of the data over time, the challenges in interpreting the data given conflicting results, and organizing the results by process. The data was also used to generate a timeline figure to provide a visual aid to understand the pivotal moments in establishing the current understanding of lipid zonation.

Ultimately, this study was limited by an availability of data. While there were many studies in the 1980s and 1990s examining zonation patterns of specific enzymes within lipid metabolism, the focus of studies shifted towards broader application of zonation in the context of disease and disfunction. Rather than isolating a single enzyme, many studies began exploring other hepatic disorders such as NAFLD and their clinical presentation often included references to zonation without centering the study around it.

Chapter III.

Results

A selection of the data from the literature review is grouped by enzyme to highlight the discordant results among studies exploring the same enzyme. It is these conflicting results that provided the motivation for the comprehensive literature review.

Table 1 CPT-1 Zonation Data

Role in Lipid Metabolism	Enzyme/Protein/Process	Which Zone is higher?	Research Method	Reference	Year of Publication
Beta Oxidation	CPT-1	PP	Digitonin collagenase perfusion	Glucagon regulation of gluconeogenesis and ketogenesis in periportal and perivenous rat hepatocytes. Heterogeneity of hormone action and of the mitochondrial redox state	1988
Beta Oxidation	CPT-1	PC	Digitonin collagenase perfusion	Properties of the mitochondrial membrane and carnitine palmitoyl transferase in the periportal and the perivenous zone of the liver. Effects of chronic ethanol feeding	1991
Beta Oxidation	CPT1	*Slightly* PP	Digitonin collagenase perfusion	Flexibility of zonation of fatty acid oxidation in rat liver	1995
Beta Oxidation	CPT1	No zonation	Immunohistochemistry	Cancer cachexia modifies the zonal distribution of lipid metabolism-related proteins in rat liver	2005
Beta Oxidation	CPT1 activity	PP	Digitonin-collagenase perfusion and micro assays	Zonation of fatty acid metabolism in rat liver	1989
Beta Oxidation	CPT1 activity	PP	Assays from mitochondria-isolated and permeabilized (dual digitonin pulse perfusion) hepatocytes	Zonal heterogeneity of the effects of chronic ethanol feeding on hepatic fatty acid metabolism	1990

Role in Lipid Metabolism	Enzyme/Protein/Process	Which Zone is higher?	Research Method	Reference	Year of Publication
Beta Oxidation	CPT2	PC	Immunohistochemistry	Cancer cachexia modifies the zonal distribution of lipid metabolism-related proteins in rat liver	2005

CPT-1 brings fatty acids into the intermembrane space of the mitochondria and is often used as a biomarker for the presence of beta-oxidation. Data reporting the presence of the enzyme is conflicted ranging from reports of no zonation, to pericentral and, most frequently, periportal.

Table 2 L-FABP Zonation Data

Role in Lipid Metabolism	Enzyme/Protein/Process	Which Zone is higher?	Research Method	Reference	Year of Publication
Beta Oxidation	L-FABP	PP	Immunostaining	Immunohistochemical studies on the distribution and frequency of fatty-acid-binding protein positive cells in human fetal, newborn and adult liver tissues	1987
Beta Oxidation	L-FABP	PP	Immunocytochemistry	Acinar Heterogeneity of Fatty Acid Binding Protein Expression in the Livers of Male, Female and Clofibrate Treated Rats	1989
Beta Oxidation	L-FABP	PP	Digitonin perfusion	Acinar Heterogeneity of Fatty Acid Binding Protein Expression in the Livers of Male, Female and Clofibrate Treated Rats	1989
Beta Oxidation	L-FABP	PP	Immunohistochemistry	Cancer cachexia modifies the zonal distribution of lipid metabolism-related proteins in rat liver	2005

L-FABP is responsible for the uptake of fatty acids into the liver and is therefore also used as a proxy for beta oxidation activity. Across a variety of different experimental methods, activity of L-FABP was unanimously reported to be periportal.

Table 3 ACC Zonation Data

Role in Lipid Metabolism	Enzyme/Protein/Process	Which Zone is higher?	Research Method	Reference	Year of Publication
Fatty Acid Synthesis	ACC	PC	Microdissection & micro assays	Distribution of enzymes of fatty acid and ketone body metabolism in periportal and perivenous rat-liver tissue	1983
Fatty Acid Synthesis	ACC	PP	Dual-digitonin-pulse perfusion & immunoblotting	Zonation of hepatic lipogenic enzymes identified by dual-digitonin-pulse perfusion	1989
Fatty Acid Synthesis	ACC	PP	Dual-digitonin-pulse-perfusion	Periportal zonation of the cytosolic acetyl-CoA synthetase of male rat liver	1992
Fatty Acid Synthesis	ACC	PC	Microdissection	Hepatocyte heterogeneity in the metabolism of fatty acids: discrepancies on zonation of acetyl-CoA carboxylase	1992
Fatty Acid Synthesis	ACC activity	PP	Dual-digitonin-pulse perfusion	Hepatic zonation of acetyl-CoA carboxylase activity	1990
Fatty Acid Synthesis	ACC mass	PP	Dual-digitonin-pulse perfusion	Hepatic zonation of acetyl-CoA carboxylase activity	1990
Fatty Acid Synthesis	ACC specific activity	PP	Dual digitonin pulse perfusion	Hepatocyte heterogeneity in the metabolism of fatty acids: discrepancies on zonation of acetyl-CoA carboxylase	1992
Fatty Acid Synthesis	ACC specific activity	PP (fasted 48h)	Dual-digitonin-pulse perfusion	Hepatic 5′-AMP-activated protein kinase: zonal distribution and relationship to acetyl-CoA carboxylase activity in varying nutritional states	1994
Fatty Acid Synthesis	acetyl-CoA synthetase	PP	Dual digitonin pulse perfusion	Periportal zonation of the cytosolic acetyl-CoA synthetase of male rat liver	1992
Fatty Acid Synthesis	acetyl-CoA synthetase	PP	Dual-digitonin-pulse-perfusion	Periportal zonation of the cytosolic acetyl-CoA synthetase of male rat liver	1992
Fatty Acid Synthesis	activity of ACC	PC	Digitonin-collagenase perfusion and micro assays	Zonation of fatty acid metabolism in rat liver	1989
Fatty Acid Synthesis	Activity of ACC	PC	Digitonin-collagenase perfusion and micro assays	Zonal heterogeneity of the effects of chronic ethanol feeding on hepatic fatty acid metabolism	1990
Fatty Acid Synthesis	Activity of ACC	no zonation	Digitonin-collagenase perfusion and micro assays	Flexibility of zonation of fatty acid oxidation in rat liver	1995
Fatty Acid Synthesis	Activity of FAS	no zonation	Digitonin-collagenase perfusion and micro assays	Flexibility of zonation of fatty acid oxidation in rat liver	1995

As the most frequently researched biomarker for fatty acid synthesis, data on ACC is widely variable. With very little variation in technique, the disparate results are particularly noteworthy; however, it is worth noting that these studies report a variety of metrics ranging from the presence of ACC, to the activity, to the specific activity.

Table 4 ACLY Zonation Data

Role in Lipid Metabolism	Enzyme/Protein/Process	Which Zone is higher?	Research Method (mRNA, protein, or enzyme activity)	Reference	Year of Publication
Fatty Acid Synthesis	ATP citrate lyase	PC	radiochemical micro test	Heterogeneous distribution of ATP citrate lyase in rat-liver parenchyma. Micro radiochemical determination in micro dissected periportal and perivenous liver tissue	1983
Fatty Acid Synthesis	ATP citrate lyase	PC	microdissection	Hepatocyte heterogeneity in the metabolism of fatty acids: discrepancies on zonation of acetyl-CoA carboxylase	1992
Fatty Acid Synthesis	ATP citrate lyase	PP	Digitonin-collagenase perfusion and micro assays	Differential gene expression in periportal and perivenous mouse hepatocytes	2006
Fatty Acid Synthesis	ATP citrate lyase	PC	microdissections & micro assays	Heterogeneous Distribution of ATP Citrate Lyase in Rat-Liver Parenchyma	1983
Fatty Acid Synthesis	ATP citrate-lyase	PP	dual-digitonin-pulse perfusion & immunoblotting	Zonation of hepatic lipogenic enzymes identified by dual-digitonin-pulse perfusion	1989

As a hallmark of lipogenesis, the presence of ACLY is typically regarded as a sign of lipogenesis. Data into zonation of ACLY reports highly variable results across a wide range of experimental methods.

Table 5 FAS Zonation Data

Role in Lipid Metabolism	Enzyme/Protein/Process	Which Zone is higher?	Research Method	Reference	Year of Publication
Fatty Acid Synthesis	fatty acid synthase	PC (females only)	radiochemical assay	Zonal distribution of fatty acid synthase in liver parenchyma of male and female rats	1989
Fatty Acid Synthesis	fatty acid synthase	PP	dual-digitonin-pulse perfusion & immunoblotting	Zonation of hepatic lipogenic enzymes identified by dual-digitonin-pulse perfusion	1989
Fatty Acid Synthesis	fatty acid synthase	PP	run on assay	Positional and temporal regulation of lipogenic gene expression in mouse liver	1993
Fatty Acid Synthesis	fatty acid synthesis	no zonation	digitonin perfusion	Periportal and perivenous hepatocytes retain their zonal characteristics in primary culture	1986

Studies exploring the zonation of FAS have yielded variable and incongruent results. Across a variety of experimental methods and different experimenters, there is little agreement on the pattern of zonation of this enzyme involved in lipogenesis.

Table 6 Lipid Transport Zonation Data

Role in Lipid Metabolism	Enzyme/Protein/Process	Which Zone is higher?	Research Method	Reference	Year of Publication
Lipid Transfer	ApoE	PP in male, no zonation in female (rats)	in situ hybridization	Differential expression of apolipoprotein E messenger RNA within the rat liver lobule determined by in situ hybridization	1999
Lipid Transfer	ApoB	PP	collagenase/digitonin perfusion & microarray analysis	Endotoxin promotes preferential periportal upregulation of VLDL secretion in the rat liver	2005
Lipid Transfer	microsomal triglyceride transfer protein mRNA	PP	collagenase/digitonin perfusion & microarray analysis	Endotoxin promotes preferential periportal upregulation of VLDL secretion in the rat liver	2005
Lipid Transfer	LRP1	PC	nanostring	Hepatic LRP-1 plays an important role in amyloidosis in Alzheimer's disease mice: Potential role in chronic heavy alcohol feeding	2024

Studies exploring the zonation of the transport of lipids have found variable patterns of zonation. Often regarded as a proxy for beta oxidation, studies exploring the zonation of apolipoproteins support a periportal pattern of zonation, while a study into LRP-1 (which has many important roles but notably serves as the apolipoprotein-E receptor) demonstrated a pericentral zonation pattern.

Chapter IV.

Discussion

Part One: The Evolution of Our Understanding.

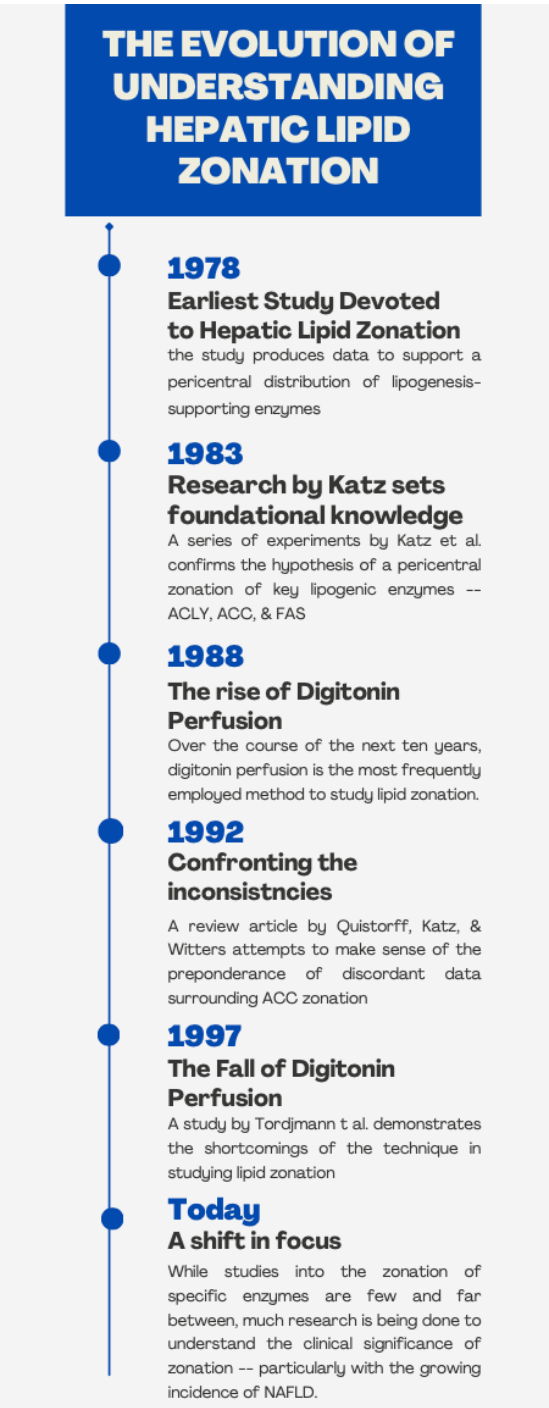

Figure 4: The Evolution of the Current Understanding of Hepatic Lipid Zonation

A timeline demonstrating the key moments in the evolution of the current model of understanding for the zonation of hepatic lipid metabolism. The timeline includes the publication of key findings and the rise and fall of the promising yet problematic method of digitonin perfusion.

Up until the late 1970s, hepatic lipid metabolism rarely served as the central subject of research papers. In the early 80s, a series of experiments by Jungermann et al. established that several metabolic processes (such as glycolysis and gluconeogenesis) took place in distinct zones across the liver. These studies were among the first to establish a gradient of functions across what had previously appeared to be a homogenous organ. As this body of knowledge expanded, so too did the questions around what other processes might exhibit similar zonation patterns.

From here, research began to be published exploring the zonation of lipids. The earliest paper with lipid zonation at its center (rather than a secondary element of the conclusion), explored the zonation of NADPH returning enzymes which served as proxies for fatty acid synthesis. Lipogenesis relies on a continuous supply of NADPH to supply the energy needed to lengthen the growing fatty acid chain (Rieder, 1978). The study demonstrated a pericentral pattern of expression of these enzymes, thus providing some of the earliest data supporting the hypothesis of a lipogenic region among pericentral hepatocytes (Rieder 1978 & 1981). These early studies relied on the polyvinyl alcohol (PVA) method of histochemistry to generate visual data confirming the presence of a particular enzyme in a specific region of a tissue sample. The results were uniform among the four enzymes studied and marked the beginning of the effort to understand lipid zonation.

From here, more studies began to explore the zonation of enzymes – particularly those three key enzymes involved in lipogenesis: ACC, ACLY, and FAS. Relying largely on microdissection and subsequent micro assays, the early data was robust and in agreement that the three key enzymes of lipogenesis were indeed pericentrally zonated, thus suggesting that the process of lipogenesis was likely pericentrally zonated as well (Katz, 1983).

Shortly thereafter, a study produced a result that did not fit with the hypothesis. A 1983 study exploring the zonation of beta oxidation by way of 3-hydroxyacyl-coA dehydrogenase reported that this enzyme exhibited no zonation by way of microdissection and micro assay (Katz, 1983). Though researchers had anticipated a higher expression pattern in the periportal hepatocytes, they reasoned that their pericentral results made sense given the liver's important role in glucose homeostasis. Therefore, they argued, it was reasonable that oxidation must be able to occur anywhere in the liver and should not be limited to one zone given its paramount importance in maintain homeostasis (Katz, 1983). As such, the finding was largely disregarded, though the hypothesis of a periportal zonation of beta-oxidation continued to dominate the introductions and discussions of subsequent publications.

Indeed, per Table 7, all published data on the zonation of beta oxidation from 1987 through 1989 continued to unanimously report and perpetuate the theory of a periportal pattern of zonation for beta-oxidation. No subsequent studies referred to the glucostatic function of the liver as a factor that may interrupt or preclude zonation, and all continued to report that their findings confirmed the logical hypothesis of beta oxidation occurring in the periportal region. This data came from different studies exploring five

different markers of beta-oxidation: rate of fatty acid oxidation, the presence of L-FABP, the presence and activity of CPT-1, and the presence of liver lipase.

Table 7: Data Published on the Zonation of Beta Oxidation from 1987 - 1989

Role in Lipid Metabolism	Enzyme/Protein/Process	Which Zone is higher?	Research Method	Reference	Year of Publication
Beta Oxidation	L-FABP	PP	immunostaining	Immunohistochemical studies on the distribution and frequency of fatty-acid-binding protein positive cells in human fetal, newborn and adult liver tissues	1987
Beta Oxidation	CPT-1	PP	digitonin collagenase perfusion	Glucagon regulation of gluconeogenesis and ketogenesis in periportal and perivenous rat hepatocytes. Heterogeneity of hormone action and of the mitochondrial redox state	1988
Beta Oxidation	CPT1 activity	PP	digitonin collagenase perfusion	Zonation of fatty acid metabolism in rat liver	1989
Beta Oxidation	L-FABP	PP	immunocytochemistry	Acinar Heterogeneity of Fatty Acid Binding Protein Expression in the Livers of Male, Female and Clofibrate Treated Rats	1989
Beta Oxidation	L-FABP	PP	digitonin perfusion	Acinar Heterogeneity of Fatty Acid Binding Protein Expression in the Livers of Male, Female and Clofibrate Treated Rats	1989
Beta Oxidation	liver lipase	PP	isopycnic centrifugation & digitonin/collagenase perfusion	Secretion of liver lipase activity by periportal and perivenous hepatocytes	1989
Beta Oxidation	rate of FA oxidation	PP	measured by conversion of palmitate into oxidation products	Zonation of fatty acid metabolism in rat liver	1989

Around this time, a new experimental method began to appear and the dual-digitonin pulse perfusion method of Katz and Chen (1988) took hold as the dominant experimental method for its apparent precision in isolating periportal and pericentral hepatocytes from liver samples. The method caught on quickly, and in 1989 alone, four

papers were published and all four employed digitonin perfusion to obtain their zone-specific data. Here is where the conflicting data emerged.

Table 8 Conflicting Data Surrounding Fatty Acid Synthesis Zonation

Role in Lipid Metabolism	Enzyme/Protein/Process	Which Zone is higher?	Research Method	Reference	Year of Publication
Fatty Acid Synthesis	ACC	PP	dual-digitonin-pulse perfusion & immunoblotting	Zonation of hepatic lipogenic enzymes identified by dual-digitonin-pulse perfusion	1989
Fatty Acid Synthesis	activity of ACC	PC	digitonin-collagenase perfusion and microassays	Zonation of fatty acid metabolism in rat liver	1989
Fatty Acid Synthesis	ATP citrate-lyase	PP	dual-digitonin-pulse perfusion & immunoblotting	Zonation of hepatic lipogenic enzymes identified by dual-digitonin-pulse perfusion	1989
Fatty Acid Synthesis	fatty acid synthase	PC (females only)	radiochemical assay	Zonal distribution of fatty acid synthase in liver parenchyma of male and female rats	1989
Fatty Acid Synthesis	fatty acid synthase	PP	dual-digitonin-pulse perfusion & immunoblotting	Zonation of hepatic lipogenic enzymes identified by dual-digitonin-pulse perfusion	1989
Fatty Acid Synthesis	rate of FA synthesis	PC	digitonin-collagenase perfusion and microassays	Zonation of fatty acid metabolism in rat liver.	1989

Data from this year demonstrated a conflicting narrative not only on the zonation of specific enzymes, for example fatty acid synthase, but also within the broader process of lipogenesis as ACC appeared in the periportal zone, while ACLY appeared in the pericentral zone. The one thing these studies had in common was their experimental method: they all relied on digitonin perfusion for the isolation of hepatocytes. It is worth stating that while the digitonin perfusion method accounted for all the discordant data, it also produced data that affirmed the periportal/pericentral zonation of fatty acid oxidation and synthesis. Thus, it may be possible that this method might be appropriate for studying the periportal zone, while some factor rendered it unreliable for studying the pericentral.

Further research is required to elucidate the inconsistencies of these results as only the assumed pericentral enzymes were impacted.

Researchers confronted these inconsistent results in a variety of ways. Rather than grappling with the unexpected results, one group of researchers declared that their data supported a periportal zonation of key lipogenic enzymes in the livers of normally feeding male rats (Evans, 1989). The researchers explained that the presence of a given enzyme in a given place did not necessarily imply anything about the ability or inability of a particular set of cells to carry out a particular function given the lack of data exploring the availability of substrate and cofactors (Evans, 1989). These comments undercut the entire purpose of their study as the presumed pretense of the experiment was exploring the zonation of these enzymes to understand more about the zonation of the processes they catalyze. It is also worth noting that in studies for which the results supported the dominant zonation hypothesis, no such qualifying comments were provided, i.e. although our results support a pericentral distribution of ACC, this does not necessarily mean that lipogenesis occurs in the pericentral region.

This relatively unsatisfactory lack of engagement with experimental data discrepancies was quickly reinforced in the early 1990s when a review article exploring L-FABP expression similarly shared that even with a periportal expression of the enzyme, it was impossible to make any subsequent deductions around the lipogenic or oxidative capabilities of that zone of cells (Bass, 1990). It is important to note that the combination of digitonin perfusion and many of the micro assays such as e.l.i.s.a tests and immunoblotting were designed to quantify the presence of an enzyme rather than its specific activity. As such, much of the body of research up until this point can be

considered only suggestive of zonation patterns, as very few of the experiments explored rate of reactions or enzyme activity. A more thorough explanation of the results for enzyme activity and rates of synthesis will be explored later in this section.

As time went on, conflicting results continued to be published. Studies generally reached one of two possible conclusions: that the zonation of their chosen enzymes affirmed zonation of the related process; or those for which the unexpected zonation of enzymes could be explained away with a reminder that enzyme expression does not always correlate with enzyme activity and that any experiment into enzyme expression is unreliable without further study into the availability of substrates and cofactors (Bass, 1990) (Evans, 1989).

This pattern continued until 1990 when two studies were published that explored ACC activity. These studies had the potential to be quite reliable, as they both explored enzyme activity – not just expression. Although both studies used digitonin pulse perfusion, they ultimately produced contradictory results. In the study exploring the impact of ethanol feeding on zonation patterns, ACC activity was determined to be pericentral. These results were not reviewed in the discussion as they supported the largely accepted pattern of zonation, and they were the findings of the control rats. Conversely, the other study determined ACC activity to be periportal. Boldly, this article embraced these unexpected results by declaring their data to be more reliable than those previous studies into ACC zonation given that they explored specific activity rather than expression. They reasoned that since specific activity accounted for allosteric regulation and covalent modification in ways that a simple test for enzyme expression could not, their results must have been correct. As such, the paper concluded that ACC activity must

be periportal and called for more work into enzyme specific activity for a comprehensive understanding of lipid zonation (Evans, 1990).

Although it appeared that studies might continue to be published in an endless cycle of discordant results, celebrating affirming findings and minimizing discrepancies, a 1992 review by Quistorff et al. finally took on the contradictory body of work exploring ACC zonation. This was a pivotal moment in the understanding of hepatic lipid zonation, as up until this point, data and studies had been produced and discussed in siloes, with very little reference to other works and little attempt to synthesize results towards a comprehensive picture of lipid zonation.

This important study marked the first written acknowledgment of the contradictory body of data and the first attempt to make sense of the discrepancies. Ultimately, the study explored the inconsistencies in the lipogenesis results and pointed to the variations in experimental methods as critical in understanding the incongruent results. While a greater analysis of variations in experimental methods will be provided later, it is worth noting the significance of this review as a key moment in confronting the variations in experimental results.

And still, the next five years saw continued use of this inconsistent and controversial technique. Indeed, from 1992 through 1997, another four studies were published using dual digitonin pulse perfusion to isolate zone-specific hepatocytes. The studies continued to report confounding results, showing acetyl coA synthetase as periportal (Knudsen, 1992), ACC specific activity as periportal (Quistorff, 1992), and peroxisomal [14-C] palmitate oxidation as pericentral (Guzman, 1995). Even more

confusing still was a study that reported that ACC and FAS activity demonstrated no zonation patterns (Guzman, 1995), though this result was not addressed in the discussion.

While a reliable and reproducible experimentally determined understanding of lipid zonation continued to evade researchers, another critical moment came with a 1997 study which exposed the limitations imposed by various experimental methods. Although not directly related to lipid zonation, Tordjmann et al. conducted an experiment demonstrating the capricious nature of digitonin pulse perfusion. To illustrate their point, researchers designed an experiment to explore the zonation of angiotensin II sensitivity. A variety of experimental methods were employed, including digitonin perfusion. Only use of digitonin perfusion produced results that demonstrated a distinct zonation pattern for the enzyme. The paper intentionally chose an angiotensin II, an enzyme for which there was no established zonation pattern, to demonstrate the flaws of the method. Following the publication of this study, the use of digitonin perfusion went from ubiquitous to sporadic, as illustrated below:

Table 9 Examining the Experimental Methods Post-1977

Role in Lipid Metabolism	Enzyme/Protein/Process	Which Zone is higher?	Research Method	Reference	Year of Publication
Lipid Transfer	ApoE	PP in male, no zonation in female (rats)	in situ hybridization	Differential expression of apolipoprotein E messenger RNA within the rat liver lobule determined by in situ hybridization	1999
Fatty Acid Synthesis	Enrichment of lipogenic acetyl co-A	PP	mass isotopomer analysis	Zonation of Labeling of Lipogenic Acetyl-CoA across the Liver: IMPLICATIONS FOR STUDIES OF LIPOGENESIS BY MASS ISOTOPOMER ANALYSIS	2004
Beta Oxidation	CPT1	no zonation	Immunohistochemistry	Cancer cachexia modifies the zonal distribution of lipid metabolism-related proteins in rat liver	2005
Beta Oxidation	CPT2	PC	Immunohistochemistry	Cancer cachexia modifies the zonal distribution of lipid metabolism-related proteins in rat liver	2005

Role in Lipid Metabolism	Enzyme/Protein/Process	Which Zone is higher?	Research Method	Reference	Year of Publication
Beta Oxidation	L-FABP	PP	Immunohistochemistry	Cancer cachexia modifies the zonal distribution of lipid metabolism-related proteins in rat liver	2005
Lipid Transfer	ApoB	PP	collagenase/digitonin perfusion & microarray analysis	Endotoxin promotes preferential periportal upregulation of VLDL secretion in the rat liver	2005
Lipid Transfer	microsomal triglyceride transfer protein mRNA	PP	collagenase/digitonin perfusion & microarray analysis	Endotoxin promotes preferential periportal upregulation of VLDL secretion in the rat liver	2005
Beta Oxidation	fatty acid oxidation	PP	collagenase/digitonin perfusion & microarray analysis	Differential gene expression in periportal and perivenous mouse hepatocytes	2006
Fatty Acid Synthesis	ATP citrate lyase	PP	collagenase/digitonin perfusion & microarray analysis	Differential gene expression in periportal and perivenous mouse hepatocytes	2006
Beta Oxidation	fatty acid oxidation	PP	intravital microscopy, spatial proteomics, and functional assessment	A spatial map of hepatic mitochondria uncovers functional heterogeneity shaped by nutrient-sensing signaling	2024
Fatty Acid Synthesis	lipogenesis	PC	intravital microscopy, spatial proteomics, and functional assessment	A spatial map of hepatic mitochondria uncovers functional heterogeneity shaped by nutrient-sensing signaling	2024
Lipid Uptake	LRP1	PC	nanostring	Hepatic LRP-1 plays an important role in amyloidosis in Alzheimer's disease mice: Potential role in chronic heavy alcohol feeding	2024

Table 9 clearly illustrates the once dominant technique fall out of favor as advances in experimental methods gave rise to more reliable and sophisticated methods of determining zonation. Indeed, as the experimental methods increased in sophistication, so too did the experimental questions. Rather than focusing on the zonation patterns of singular enzymes, studies set out to explore lipid zonation in the context of different disorders such as Huntington's (Bragg, 2023), cancer (Kazantis, 2005), and, of course, NAFLD.

As recently as 2023, researchers are now conducting experiments exploring the spatiotemporal zonation of different processes, allowing them to look not just at how

different metabolic processes happen in different places and under varying physiological conditions, but also how those patterns change over time (Martini, 2023). These recent advancements in experimental methods and techniques are prompting greater research into a wide variety of topics, and, indeed, a PubMed search for "steatosis AND liver AND (periportal OR pericentral OR perivenous OR zone 1 OR zone 2 OR zone 3)" yields a noteworthy 553 results since 2000.

Part Two: A Synthesis of the Data

While Part One explored the chronological evolution of the current understanding of hepatic lipid zonation, part two will aim to determine what results are the most reliable and convincing and should therefore be used to scaffold a modern and reliable understanding of lipid zonation.

As previously noted, the key difference between studies was the chosen experimental method. As such, the pros and cons of the three most popular methods (immunostaining/in situ hybridization, microdissection, and digitonin perfusion) will be discussed with further discussion on the relevant data. It is important to note that each method does indeed have its pros and cons, and while one method may be less reliable in one experiment, it may be perfectly suited to another. For example, while immunofluorescence can illustrate where a particular enzyme is located, it cannot provide information on how the expression of that enzyme changes under different conditions. Digitonin perfusion and microdissection allow for investigation into specific activity and enzyme flux; however, they require the destruction of the tissue sample thus limiting their

ability to reflect *in vivo* conditions. As such, the goal of this examination will be to determine which method provided the most reliable results for hepatic lipid zonation.

Immunostaining/In Situ Hybridization is often regarded as one of the most precise and reliable methods for determining the location of a particular protein. The technique employs a complementary nucleotide sequence which binds to a DNA or RNA segment of interest in the target protein. The presence of that probe and the DNA/RNA sequence is typically visualized by a marker tag. The drawbacks of this method lie in identifying a probe sequence that has low DNA/RNA copies (Jensen, 2014). Even still, once an appropriate probe is identified, the method is an accurate and efficient method for visualizing the presence of a particular nucleotide sequence. Given that the method provides visual data, it is less quantifiable than other identification methods; however, different variations of the technique can be more quantifiable than others and the technique continues to be improved (Jensen, 2014). Immunostaining (or immunohistochemistry) follows a similar principle; however, rather than using a complementary nucleotide sequence to tag a target compound, it relies on the use of antibodies. While it is useful for gene/protein localization, it is limited in elucidating expression and activity.

Lipid zonation data obtained via immunostaining and in-situ hybridization tends to follow the predicted patterns of zonation for lipogenesis, beta oxidation, and lipid transport. Indeed, of the ten studies that used these methods only one put forth data inconsistent with the current understanding lipid zonation. In this study, CPT-1 was found to exhibit no zonation pattern while CPT-2 appeared in an unexpected periportal

pattern (Kazantis, 2005). While the study did not comment on this unexpected zonation, it is possible that further research could elucidate the reason for these results.

Microdissection is another reliable method for isolating populations of cells to detect the presence of a target protein. Microdissection involves the use of a microscope to accurately dissect a tissue or object from a sample for further study and analysis. Microdissection is cost-effective and reliable, making it one of the most popular methods for clinical practices (Walsh & Halushka, 2022). Although the method is known for precision, it is also extremely time consuming and requires significant training and expertise, therefore tissue samples are not standardized across experiments and can be subject to the expertise of the clinician. Given this element of subjectivity, it ranks at slightly less reliable than in-situ hybridization and immunostaining. Furthermore, microdissection requires the removal of a sample from the study subject, thus it is a static image, providing no information about dynamic shifts in expression over time.

Despite its shortcomings, data obtained via microdissection was largely consistent and produced only one aberrant result reporting 3-hydroxyacyl-CoA dehydrogenase as exhibiting no zonation when it would be expected to follow a periportal pattern for its role in beta oxidation (Katz, 1983). All other results followed the expected pattern of zonation.

Finally, one of the most common and least consistent experimental methods among this data is digitonin perfusion. In this process, developed by Quistorff et al. in 1987 (Quistorff & Grunnet, 1987), digitonin is perfused through the central vein to isolate pericentral hepatocytes and then through the portal vein to isolate periportal hepatocytes. Digitonin acts as a mild detergent, associating with cholesterol in the plasma

membrane of the hepatocytes, generating holes in the membrane. These holes can vary in size depending on the concentration of digitonin employed. Ultimately, these holes allow for internal contents of the hepatocytes to be released into the elution which can then be collected and analyzed (Geelan, 2005). In another variation, digitonin perfusion is followed by a perfusion of collagenase which is known to breakdown collagen, thus further permeating the sample cells.

One major drawback of this study is that, prior to 1997, it required the destruction of one zone of cells to obtain the other zone, therefore samples had to be drawn from separate animals. Comparing cell samples from separate animals may have caused unreliable results and greater disparity between the pericentral and periportal zones as most results determined a periportal vs pericentral zonation pattern based on the ratio of enzyme expression. Therefore, if a liver with naturally higher levels of both lipogenic and oxidative enzymes was compared with a liver with naturally lower levels of enzymes, then a zonation pattern may be reported when in fact the data is simply reflective of a different baseline among different individuals. As previously discussed, Tordjmann et al. demonstrated the inconsistency of this method by exploring levels of angiotensin. They obtained compelling results and significant data demonstrated zonation in angiotensin II sensitivity by way of this method with no zonation occurring via other methods (Tordjmann et al., 1997). In their study, they also pioneered a new method of digitonin perfusion which allowed for hepatocytes to be isolated from the same animal, thus eliminating the inconsistencies posed by use of separate animals. As such, results obtained via digitonin perfusion prior to the update in technique from 1997 would have required samples from separate livers of separate animals, leading to data that could

diminish or amplify the presence or absence of a particular enzyme. Indeed, data obtained via digitonin perfusion is variable and inconsistent, as demonstrated in Table 10.

Table 10 Inconsistencies Among Data Obtained via Digitonin Perfusion

Role in Lipid Metabolism	Enzyme/Protein/Process	Which Zone is higher?	Research Method	Reference	Year of Publication
Beta Oxidation	CPT-1	PC	digitonin collagenase perfusion	Properties of the mitochondrial membrane and carnitine palmitoyl transferase in the periportal and the perivenous zone of the liver. Effects of chronic ethanol feeding	1991
Beta Oxidation	CPT-1	PP	digitonin collagenase perfusion	Glucagon regulation of gluconeogenesis and ketogenesis in periportal and perivenous rat hepatocytes. Heterogeneity of hormone action and of the mitochondrial redox state	1988
Beta Oxidation	CPT1	Slightly PP	digitonin collagenase perfusion	Flexibility of zonation of fatty acid oxidation in rat liver	1995
Beta Oxidation	CPT1 activity	PP	collagenase/digitonin perfusion & microarray analysis	Zonal heterogeneity of the effects of chronic ethanol feeding on hepatic fatty acid metabolism	1990
Beta Oxidation	CPT1 activity	PP	digitonin collagenase perfusion	Zonation of fatty acid metabolism in rat liver	1989
Beta Oxidation	fatty acid oxidation	PP	collagenase/digitonin perfusion & microarray analysis	Differential gene expression in periportal and perivenous mouse hepatocytes	2006
Beta Oxidation	L-FABP	PP	digitonin perfusion	Acinar Heterogeneity of Fatty Acid Binding Protein Expression in the Livers of Male, Female and ClofibrateTreated Rats	1989
Beta Oxidation	liver lipase	PP	isopycnic centrifugation & digitonin/collagenase perfusion	Secretion of liver lipase activity by periportal and perivenous hepatocytes	1989
Beta Oxidation	peroxisomal [14C]palmitate oxidation	PC	digitonin collagenase perfusion	Flexibility of zonation of fatty acid oxidation in rat liver	1995
Fatty Acid Synthesis	ACC	PP	dual-digitonin-pulse perfusion & immunoblotting	Zonation of hepatic lipogenic enzymes identified by dual-digitonin-pulse perfusion	1989
Fatty Acid Synthesis	ACC	PP	dual-digitonin-pulse-perfusion	Periportal zonation of the cytosolic acetyl-CoA synthetase of male rat liver	1992
Fatty Acid Synthesis	ACC activity	PP	dual-digitonin-pulse perfusion	Hepatic zonation of acetyl-CoA carboxylase activity	1990
Fatty Acid Synthesis	ACC mass	PP	dual-digitonin-pulse perfusion	Hepatic zonation of acetyl-CoA carboxylase activity	1990
Fatty Acid Synthesis	ACC specific activity	PP	dual digitonin pulse perfusion	Hepatocyte heterogeneity in the metabolism of fatty acids: discrepancies on zonation of acetyl-CoA carboxylase	1992

Role in Lipid Metabolism	Enzyme/Protein/Process	Which Zone is higher?	Research Method	Reference	Year of Publication
Fatty Acid Synthesis	acetyl-CoA synthetase	PP	dual digitonin pulse perfusion	Periportal zonation of the cytosolic acetyl-CoA synthetase of male rat liver	1992
Fatty Acid Synthesis	acetyl-CoA synthetase	PP	dual-digitonin-pulse-perfusion	Periportal zonation of the cytosolic acetyl-CoA synthetase of male rat liver	1992
Fatty Acid Synthesis	Activity of ACC	no zonation	digitonin-collagenase perfusion and microassays	Flexibility of zonation of fatty acid oxidation in rat liver	1995
Fatty Acid Synthesis	activity of ACC	PC	digitonin-collagenase perfusion and microassays	Zonation of fatty acid metabolism in rat liver	1989
Fatty Acid Synthesis	Activity of ACC	PC	digitonin-collagenase perfusion and microassays	Zonal heterogeneity of the effects of chronic ethanol feeding on hepatic fatty acid metabolism	1990
Fatty Acid Synthesis	Activity of FAS	no zonation	digitonin-collagenase perfusion and microassays	Flexibility of zonation of fatty acid oxidation in rat liver	1995
Fatty Acid Synthesis	ATP citrate-lyase	PP	dual-digitonin-pulse perfusion & immunoblotting	Zonation of hepatic lipogenic enzymes identified by dual-digitonin-pulse perfusion	1989
Fatty Acid Synthesis	fatty acid synthase	PP	dual-digitonin-pulse perfusion & immunoblotting	Zonation of hepatic lipogenic enzymes identified by dual-digitonin-pulse perfusion	1989
Fatty Acid Synthesis	fatty acid synthesis	no zonation	digitonin perfusion	Periportal and perivenous hepatocytes retain their zonal characteristics in primary culture	1986
Fatty Acid Synthesis	lipogenesis	PC	digitonin-collagenase perfusion and microassays	Zonal heterogeneity of the effects of chronic ethanol feeding on hepatic fatty acid metabolism	1990
Fatty Acid Synthesis	rate of FA synthesis	PC	digitonin-collagenase perfusion and microassays	Zonation of fatty acid metabolism in rat liver.	1989
Lipid Transfer	ApoB	PP	collagenase/digitonin perfusion & microarray analysis	Endotoxin promotes preferential periportal upregulation of VLDL secretion in the rat liver	2005
Lipid Transfer	microsomal triglyceride transfer protein mRNA	PP	collagenase/digitonin perfusion & microarray analysis	Endotoxin promotes preferential periportal upregulation of VLDL secretion in the rat liver	2005

In addition to the experimental method, it is also important to note what enzymes and processes were studied, as some are more reliable than others: flux and specific activity are highly indicative of a particular process occurring in a particular place; enzyme presence is strongly suggestive though not absolute; and the expression of mRNA may or may not indicate the activity of an enzyme in that region and should

therefore be considered the least reliable metric. The vast majority of studies on lipid zonation investigated enzyme presence, though a few studied specific activity.

Finally, it is also important to note the potential impact of the inconsistencies between the breed of study subjects. Each of the studies that reported a periportal zonation of ACC (counter to the current understanding of lipogenesis) used Wistar rats. A 2015 study explored differences in patterns of steatosis and lipid metabolism between Wistar rats and Fischer rats, found that Fischer rats followed a periportal lipid accumulation more similar to that observed in humans, while Wistar rats showed a mid-zone pattern of accumulation. This study illustrated the discrepancies between different breeds of mice, therefore more work is needed to establish in what ways these model organisms differ from one another and what results can be extrapolated and used to target disease in human beings (Bhapole, 2015).

Finally, it should also be noted that while some studies included tests on both male and female rats, many used only male rats. The studies which included female rats ultimately reported a periportal and pericentral zonation of ACC respectively; however, no significant zonation was observed in females (Katz, 1989) (Katz, 1983). As such, it is important to recognize that zonation patterns appear to vary between sexes and therefore we may need a more flexible model of ACC zonation that accounts for breed and sex.

With these considerations in mind, the most reliable data can be aggregated into the following table:

Table 11 Experimental Results Excluding Use of Digitonin Perfusion

Role in Lipid Metabolism	Enzyme/Protein/Process	Which Zone is higher?	Research Method	Reference	Year of Publication
Beta Oxidation	3-hydroxyacyl-CoA dehydrogenase	No Zonation	microdissection & microassays	Distribution of enzymes of fatty acid and ketone body metabolism in periportal and perivenous rat-liver tissue	1983
Beta Oxidation	CPT1	No Zonation	Immunohistochemistry	Cancer cachexia modifies the zonal distribution of lipid metabolism-related proteins in rat liver	2005
Beta Oxidation	CPT2	PC	Immunohistochemistry	Cancer cachexia modifies the zonal distribution of lipid metabolism-related proteins in rat liver	2005
Beta Oxidation	fatty acid oxidation	PP	radioactive counting	Zonal heterogeneity of the effects of chronic ethanol feeding on hepatic fatty acid metabolism	1990
Beta Oxidation	fatty acid oxidation	PP	intravital microscopy, spatial proteomics, and functional assessment	A spatial map of hepatic mitochondria uncovers functional heterogeneity shaped by nutrient-sensing signaling	2024
Beta Oxidation	hepatic peroxisome activity	PC (in older animals)	morphometrical analysis	The impact of aging on enzyme proteins of rat liver peroxisomes: quantitative analysis by immunoblotting and immunoelectron microscopy	1993
Beta Oxidation	L-FABP	PP	immunocytochemistry	Acinar Heterogeneity of Fatty Acid Binding Protein Expression in the Livers of Male, Female and ClofibrateTreated Rats	1989
Beta Oxidation	L-FABP	PP	Immunohistochemistry	Cancer cachexia modifies the zonal distribution of lipid metabolism-related proteins in rat liver	2005
Beta Oxidation	L-FABP	PP	immunostaining	Immunohistochemical studies on the distribution and frequency of fatty-acid-binding protein positive cells in human fetal, newborn and adult liver tissues	1987
Beta Oxidation	peroxisomal beta-oxidation	PP	Immunohistochemistry	Effects of n-3 and n-6 polyunsaturated fatty acid-enriched diets on lipid metabolism in periportal and pericentral compartments of female rat liver lobules and the consequences for cell proliferation after partial hepatectomy	1995
Beta Oxidation	rate of FA oxidation	PP	measured by conversion of palmitate into oxidation products	Zonation of fatty acid metabolism in rat liver	1989

Role in Lipid Metabolism	Enzyme/Protein/Process	Which Zone is higher?	Research Method	Reference	Year of Publication
Fatty Acid Synthesis	6-phosphogluconate dehydrogenase	PC	PVA method histochemistry	NADP-dependent dehydrogenases in rat liver parenchyma. I. Methodological studies on the qualitative histochemistry of G6PDH, 6PGDH, malic enzyme and ICDH	1978
Fatty Acid Synthesis	6-phosphogluconate dehydrogenase	PC	PVA method histochemistry	NADP-dependent dehydrogenases in rat liver parenchyma. III. The description of a liponeogenic area on the basis of histochemically demonstrated enzyme activities and the neutral fat content during fasting and refeeding	1981
Fatty Acid Synthesis	ACC	PC	microdissection & microassays	Distribution of enzymes of fatty acid and ketone body metabolism in periportal and perivenous rat-liver tissue	1983
Fatty Acid Synthesis	ACC	PC	microdissection	Hepatocyte heterogeneity in the metabolism of fatty acids: discrepancies on zonation of acetyl-CoA carboxylase	1992
Fatty Acid Synthesis	ATP citrate lyase	PC	microdissection	Hepatocyte heterogeneity in the metabolism of fatty acids: discrepancies on zonation of acetyl-CoA carboxylase	1992
Fatty Acid Synthesis	ATP citrate lyase	PC	radiochemical microtest	Distribution of enzymes of fatty acid and ketone body metabolism in periportal and perivenous rat-liver tissue	1983
Fatty Acid Synthesis	ATP citrate lyase	PC	microdissections & microassays	Heterogeneous Distribution of ATP Citrate Lyase in Rat-Liver Parenchyma	1983
Fatty Acid Synthesis	Distribution of ACC isoforms	No Zonation	immunoprecipitation and visualized with autoradiography	Flexibility of zonation of fatty acid oxidation in rat liver	1995
Fatty Acid Synthesis	fatty acid synthase	PC (females only)	radiochemical assay	Zonal distribution of fatty acid synthase in liver parenchyma of male and female rats	1989
Fatty Acid Synthesis	fatty acid synthase	PP	mRNA in situ hybridization	Positional and temporal regulation of lipogenic gene expression in mouse liver	1993
Fatty Acid Synthesis	glucose-6-phosphate dehydrogenase	PC	PVA method histochemistry	NADP-dependent dehydrogenases in rat liver parenchyma. I. Methodological studies on the qualitative histochemistry of G6PDH, 6PGDH, malic enzyme and ICDH	1978

Role in Lipid Metabolism	Enzyme/Protein/Process	Which Zone is higher?	Research Method	Reference	Year of Publication
Fatty Acid Synthesis	glucose-6-phosphate dehydrogenase	PC in female rats (no zonation in males	PVA method histochemistry	NADP-dependent dehydrogenases in rat liver parenchyma. III. The description of a liponeogenic area on the basis of histochemically demonstrated enzyme activities and the neutral fat content during fasting and refeeding	1981
Fatty Acid Synthesis	isocitrate dehydrogenase	PC	PVA method histochemistry	NADP-dependent dehydrogenases in rat liver parenchyma. I. Methodological studies on the qualitative histochemistry of G6PDH, 6PGDH, malic enzyme and ICDH	1978
Fatty Acid Synthesis	lipogenesis	PC	intravital microscopy, spatial proteomics, and functional assessment	A spatial map of hepatic mitochondria uncovers functional heterogeneity shaped by nutrient-sensing signaling	2024
Fatty Acid Synthesis	malic enzyme	PC	PVA method histochemistry	NADP-dependent dehydrogenases in rat liver parenchyma. I. Methodological studies on the qualitative histochemistry of G6PDH, 6PGDH, malic enzyme and ICDH	1978
Fatty Acid Synthesis	malic enzyme	PC	PVA method histochemistry	NADP-dependent dehydrogenases in rat liver parenchyma. III. The description of a liponeogenic area on the basis of histochemically demonstrated enzyme activities and the neutral fat content during fasting and refeeding	1981
Fatty Acid Synthesis	malic enzyme	PP	mRNA in situ hybridization	Positional and temporal regulation of lipogenic gene expression in mouse liver	1983
Fatty Acid Synthesis	peroxisomal [14+B63A62:G72C]palmitate oxidation	PP	mass isotopomer analysis	Zonation of Labeling of Lipogenic Acetyl-CoA across the Liver: IMPLICATIONS FOR STUDIES OF LIPOGENESIS BY MASS ISOTOPOMER ANALYSIS	2004
Fatty Acid Synthesis	Rate of FA synthesis	no zonation	DCP analysis of [malonyl coA]	Flexibility of zonation of fatty acid oxidation in rat liver	1995
Fatty Acid Synthesis	S14 gene & ACC activity	PC	Immunohistochemistry	Thyroid hormone and dietary carbohydrate induce different hepatic zonation of both "spot 14" and acetyl-coenzyme-A carboxylase: a novel mechanism of coregulation	1993

Role in Lipid Metabolism	Enzyme/Protein/Process	Which Zone is higher?	Research Method	Reference	Year of Publication
Lipid Transfer	ApoE	PP in male, no zonation in female (rats)	in situ hybridization	Differential expression of apolipoprotein E messenger RNA within the rat liver lobule determined by in situ hybridization	1999
Lipid Uptake	LRP1	PC	nanostring	Hepatic LRP-1 plays an important role in amyloidosis in Alzheimer's disease mice: Potential role in chronic heavy alcohol feeding	2024

This data entirely excludes results obtained via digitonin perfusion. Of the studies enumerated, there are a few that standout for their robust and reliable reporting.

The first two studies exploring lipogenesis were thorough, consistent, and reproducible. While the first study explored the impact of different techniques for determining the presence of their target enzymes, researchers then conducted a follow up study in which they used the most reliable technique and tested the impact of different fed states on the presence of those enzymes. The controls in the subsequent study supported the initial findings from the first, thus demonstrating the reliability and reproducibility of the methods and results (Rieder 1978 & 1981). These data demonstrated a pericentral zonation of the presence of several lipogenesis-supporting enzymes, and therefore provide reliable support of this pattern of zonation.

The studies that investigated the specific activities of enzymes should also be considered highly reliable. This would include the results that demonstrated a periportal zonation of fatty acid oxidation (Guzman, 1989), pericentral zonation of lipogenesis (Kang, 2024), and a pericentral zonation of ACC activity (Kinlaw, 1993). While these results do not address the intricacies and flexibility of lipid zonation, they provide a reliable and fundamental starting point from which to explore the impact of disruptions to

lipid zonation and how these patterns can best be utilized in the mitigation and treatment of a myriad of diseases and disorders.

Conclusion

Using this data, it is reasonable to conclude that in normally feeding male rats, lipogenesis tends to be pericentral, while beta oxidation tends to be periportal. It is important to note that these processes remain under constant flux and are easily influenced by other physiological conditions such as temperature, sex hormones, other endogenous influences (such as insulin and glucagon) and certainly exogenous substances (such as ethanol). To better understand the zonation of lipids, more research is needed into the baseline patterns across individuals and how those patterns shift in response to physiological stressors such as those named above. This data will provide a better understanding of how to target the therapeutic reversal and mitigation of conditions such as NAFLD.

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