Impact of dietary fat on the development of non-alcoholic fatty liver disease (NAFLD) in *Ldlr*$^{-/-}$ mice.

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ABSTRACT.

The prevalence of non-alcoholic fatty liver disease (**NAFLD**) has increased in parallel with central obesity and is now the most common chronic liver disease in developed countries. NAFLD is defined as excessive accumulation of lipid in the liver, i.e., hepatosteatosis. The severity of NAFLD ranges from simple fatty liver (steatosis) to non-alcoholic steatohepatitis (NASH). Simple steatosis is relatively benign until it progresses to NASH, which is characterized by hepatic injury, inflammation, oxidative stress and fibrosis. Hepatic fibrosis is a risk factor for cirrhosis and primary hepatocellular carcinoma (**HCC**). Our studies have focused on the impact of diet on the onset and progression of NASH. We developed a mouse model of NASH by feeding *Ldlr*$^{-/-}$ mice a "western diet" (**WD**); a diet moderately high in saturated and trans-fat, sucrose and cholesterol. The WD induced a NASH phenotype in *Ldlr*$^{-/-}$ mice that recapitulates many of the clinical features of human NASH. We also assessed the capacity of the dietary ω3-polyunsaturated fatty acids (ω3-**PUFA**), i.e., eicosapentaenoic acid (**EPA**, 20:5,ω3) and docosahexaenoic acid (**DHA**, 22:6,ω3), to prevent WD-induced NASH in *Ldlr*$^{-/-}$ mice. Histologic, transcriptomic, lipidomic and metabolomic analyses established that DHA was equal or superior to EPA at attenuating WD-induced dyslipidemia and hepatic injury, inflammation, oxidative stress and fibrosis. Dietary ω3-PUFA, however, had no significant effect on WD-induced changes in body weight, body fat or blood glucose. These studies provide a molecular and metabolic basis for understanding the strengths and weaknesses of using dietary ω3 PUFA to prevent NASH in humans.

Key words:

Non-alcoholic steatohepatitis, inflammation, oxidative stress, fibrosis, ω3-PUFA

Introduction

The Centers for Disease Control (**CDC**) estimates that nearly 80 million adults (1) and 13 million children (2) in the United States are obese. Obesity is a risk factor for chronic metabolic diseases, such as cardiovascular disease, metabolic syndrome (**MetS**), type 2 diabetes (**T2D**) and non-alcoholic fatty liver disease (**NAFLD**). Our studies have focused on NAFLD. The prevalence of NAFLD has increased in parallel with incidence of central obesity (3, 4), and is now the most common fatty liver disease in developed countries (5). NAFLD is defined as excessive lipid accumulation in the liver, i.e., hepatosteatosis (6, 7). NAFLD is the hepatic manifestation of MetS (8); MetS risk factors include obesity, elevated plasma triglycerides and LDL cholesterol, reduced HDL cholesterol, high blood pressure and fasting hyperglycemia (9). The prevalence of NAFLD in the general population is estimated to range from 6% to 30% depending on the method of analysis and population studied (10).

NAFLD ranges from benign hepatosteatosis to non-alcoholic steatohepatitis (**NASH**) (11), where NASH is defined as hepatosteatosis with inflammation and hepatic injury (12). Simple hepatosteatosis progresses to NASH in 30 to 40% of patients (13); representing ~3% to 5% of the general population (10). The T2D population has a higher prevalence ($\geq$60%) of NAFLD and NASH than the general population (14). NASH patients have higher mortality rates than NAFLD patients; and both are higher than the general population (15-17). NASH can progress to cirrhosis and hepatocellular carcinoma (**HCC**) (4, 13). Over a 10 year period, cirrhosis and liver related death occurs in 20% and 12% of NASH patients, respectively (18). Cirrhosis resulting from NASH is projected to be the leading cause of liver transplantation in the United States by 2020 (19). Given the increasing prevalence of NASH and its negative clinical outcomes, NASH is rapidly becoming a significant public health burden (20).

Multi-hit hypothesis for NASH development

The development of NASH has been proposed to follow a multi-hit model (21-23). The "1st Hit" involves excessive neutral lipid accumulation which sensitizes the liver to the "2nd Hit" (22) (**Fig. 1**). The "2nd Hit" is characterized by hepatic insulin resistance, inflammation, oxidative stress leading to in hepatic damage

that is associated with increased blood levels of hepatic enzymes/proteins, e.g., alanine aminotransferase [**ALT**] (3, 4, 24). The resulting hepatocellular death & necrosis promotes the "3rd Hit" which involves activation of resident stellate cells and subsequent deposition of extracellular (fibrotic) matrix. Fibrosis is a tissue repair mechanisms that results in scarring; it is mediated by hepatic stellate cell activation and myofibrillar cell infiltration of the liver. These cells produce extracellular matrix proteins, including collagen (collagen 1A1, **Col1A1**), elastin and smooth muscle α2 actin (25). Dietary (excess fat, cholesterol, glucose and fructose), metabolic (plasma and hepatic fatty acid profiles, hepatic ceramide, oxidized LDL, bile acid metabolites), endocrine (insulin, leptin & adiponectin), gut (endotoxin, microbial metabolites) and genetic (e.g., patatin-like phospholipase domain containing 3 [PNPLA3] polymorphisms) factors have been implicated as triggers for NASH progression (26-34).

Hepatosteatosis develops because of an imbalance of hepatic lipid metabolism leading to the accumulation of hepatic neutral lipids as triacylglycerols (**TAG**) and diacylglycerols (**DAG**) and cholesterol esters (**CE**). In humans with NAFLD, ~60% of the fat appearing in the liver is derived from circulating non-esterified fatty acids (**NEFA**) mobilized from adipose tissue; 26% are from *de novo* lipogenesis (**DNL**) and 15% are from the diet (35). Hepatic fatty acid oxidation (**FAO**) and very low density lipoprotein (**VLDL**) assembly and secretion represent pathways for removal of liver fat. Hepatosteatosis develops when lipid storage exceeds lipid export or FAO. Both hepatic and peripheral insulin resistance also contribute to the disruption of these metabolic pathways (36).

NASH patients consume a lower ratio of PUFA to saturated fatty acid (**SFA**) when compared to the general population (37, 38). Furthermore, consumption of a low ratio of dietary ω3 PUFA to ω6 PUFA is also associated with NAFLD development, while increased consumption of dietary long-chain ω3 PUFA decreases hepatic steatosis (39-41). Pachikian et al (42) recently reported that removal of all ω3 PUFA from a mouse diet promoted insulin resistance and hepatosteatosis in C57Bl/6J mice. While this diet lowered hepatic ω3 PUFA, including α-linolenic acid (**ALA**, 18:3,ω3), eicosapentaenoic acid (**EPA**, 20:5,ω3) and docosahexaenoic acid (**DHA**, 22:6,ω3), it did not affect hepatic ω6-PUFA content, i.e., linoleic acid (**LA**, 18:2,ω6) or arachidonic acid (**ARA**, 20:4,ω6). Several hepatic transcription factors are regulated by $C_{20\text{-}22}$ ω3 PUFA, including peroxisome proliferator activated receptor α (**PPARα**), sterol

regulatory element binding protein-1 (**SREBP1**), carbohydrate regulatory element binding protein (**ChREBP**) and Max-like factor X (**MLX**) (43). PPARα is a fatty acid-regulated nuclear receptor. Activation of PPARα increases expression of enzymes involved in FAO. SREBP1 and the ChREBP/MLX heterodimer regulate the expression of genes involved in DNL and triglyceride synthesis. Dietary ω3 PUFA suppress the nuclear abundance of SREBP1 and ChREBP/MLX leading to the attenuation of expression of genes involved in fatty acid and triglyceride synthesis. Lowering hepatic ω3 PUFA, as reported by Pachikian et al (42), promotes hepatosteatosis by suppressing hepatic FAO and stimulating fatty acid and triglyceride synthesis and storage. While trans-fatty acid (TFA) consumption is associated with insulin resistance and cardiovascular disease, the impact of TFA consumption on NAFLD in humans is less clear (44). In mice, however, TFA consumption is associated with hepatic steatosis and injury (45, 46).

High dietary cholesterol promotes hepatic inflammation (28, 47-49) and contributes to NASH development (50). In the *Ldlr*$^{-/-}$ mouse model, high fat-high cholesterol feeding results in a robust NASH phenotype (51). Kupffer cells, i.e., resident hepatic macrophage, become engorged with oxidized-LDL (**ox-LDL**), which induces inflammatory cytokine secretion. These locally secreted cytokines act on other hepatic cells and cause cellular injury. Kupffer cells also secrete chemokines (e.g., monocyte chemoattractant protein-1, **MCP1**) that recruit monocytes to the liver, further promoting an inflammatory environment in the liver. As such, reducing hepatic inflammation is an obvious target for NASH therapy.

Over the last 30 years there has been a dramatic increase in obesity and NAFLD in the United States (3, 52-56). These changes in health status are associated with increased carbohydrate and total calorie consumption, but not total fat consumption. Elevated carbohydrate, and specifically fructose, consumption has been linked to the development of NAFLD and NASH progression (57-59). The liver expresses the fructose-specific transporter (**Glut5**) and is responsible for metabolizing up to 70% of dietary fructose (58, 59). Fructose metabolism is independent of insulin. When compared to glucose, fructose more readily enters the pathway for DNL and TAG synthesis. Fructose promotes all aspects of metabolic syndrome including hepatosteatosis, insulin resistance, dyslipidemia, hyperglycemia, obesity and hypertension (60). In contrast to fructose, hepatic glucose metabolism is well-regulated by insulin;

glucose is also converted to glycogen for storage. Excess glucose consumption does not promote hepatosteatosis as aggressively as excess fructose consumption. Fructose also affects several biochemical events that exacerbate NASH development, including formation of reactive oxygen species (**ROS**) and advanced glycation end-products (**AGEP**); (61-64).

Treatment Strategies for NAFLD.

General therapeutic strategies for NAFLD/NASH start with life style management (diet and exercise) and treating the co-morbidities associated with NAFLD/NASH, e.g., obesity, T2D, dyslipidemia. The best strategy for managing NASH, however, has not been established (65). Clinical approaches to manage NAFLD/NASH focus on: 1) a reduction in overall body weight by using dietary and exercise therapy; 2) control blood glucose and dyslipidemia (cholesterol and triglycerides) by using pharmaceutical and/or dietary supplements, such as metformin, fibrates, thiazolididiones, statins, and/or ω3 PUFA; 3) suppression of inflammation by using Toll-like receptor modulators or ω3 PUFA; and 4) suppression of oxidative stress by using vitamin E and other antioxidants (66-72). Therapeutic regulators of fibrosis, however, are less well-defined (73, 74).

Development of a mouse model of NASH.

We have used wild type C57BL/6J mice and mice with global ablation of the low density lipoprotein receptor (*Ldlr*$^{-/-}$, on the C57BL/6J background) to study dietary factors and molecular mechanisms involved in the onset and progression of diet-induced chronic fatty liver diseases (49, 75-80). We have assessed 3 diets for their capacity to promote a NASH phenotype that recapitulates human NASH: 1) the high fat diet [**HF**] (60% calories as fat, Research Diets [D12492] typically used to promote diet-induced obesity and T2D (76); 2) a high fat-high cholesterol diet (**HFHC,** Research Diets) used to induce fatty liver with elevated oxidative stress (49, 81); and 3) the western diet (**WD**)(Research Diets, D12079B) to induce NASH. The WD is moderately high in saturated and trans-fat (41% total calories), sucrose (30% total calories) and cholesterol (0.15 g%, w/w). Our studies established that the wild type mice develop hepatosteatosis and relatively mild hepatic inflammation and fibrosis when compared to WD-fed *Ldlr*$^{-/-}$

mice (**Table 1**). The combination of the WD and the *Ldlr*$^{-/-}$ mice yields a NASH- and MetS-like phenotype; a phenotype characterized by obesity, hyperglycemia, dyslipidemia, hepatosteatosis, hepatic inflammation, damage & fibrosis (77). Since humans (3, 4, 14) and *Ldlr*$^{-/-}$ mice (49, 75-80, 82) develop NAFLD and NASH in a context of obesity and insulin resistance, *Ldlr*$^{-/-}$ mice may be a useful preclinical model to investigate the development, progression and remission of NASH under defined laboratory conditions.

The WD is similar to a "fast-food" based diet (83) and human diets linked to obesity in the US (84, 85). Both the WD and "fast food" mouse models induced a NASH phenotype that recapitulates many of the phenotypic features of human NASH, including hepatic micro- and macro-steatosis, hepatocyte ballooning, hepatic injury including infiltration of leukocytes (inflammation), oxidative stress and branching fibrosis (77, 82). Moreover, NASH is associated with a major enrichment of both plasma and liver with saturated (SFA) and monounsaturated fatty acid (MUFA) and hepatic depletion of ω3 and ω6 PUFA (49, 77, 78), a phenomena that has been described in human NASH (86, 87).

Rationale for using ω3 PUFA to prevent NASH.

Our studies have assessed the capacity of $C_{20\text{-}22}$ ω3 PUFA to prevent diet-induced NASH. $C_{20\text{-}22}$ ω3 PUFA are pleiotropic regulators of cell function affecting membrane structure and multiple cellular regulatory mechanisms (43). The impact of $C_{20\text{-}22}$ ω3 PUFA on lipid metabolism and inflammation is well documented making these dietary fats an attractive nutritional approach to combat NASH (43). Meta-analyses and other clinical studies suggest ω3-PUFA may lower liver fat in children and adults with NAFLD (71, 88-93). We identified 235 clinical trials (94) assessing NASH and NASH therapies. Twenty-three of these trials used ω3 PUFA as a treatment strategy where diets were supplemented with fish oil or a combination of EPA and DHA; few studies used EPA or DHA alone. Thus, dietary $C_{20\text{-}22}$ ω3 PUFA may have promise in reducing hepatic fat content in the NAFLD patient. These clinical studies, however, lack the capacity to assess the cellular, molecular and metabolic changes associated with NASH. As such, studies in mice

may provide insight into the molecular and metabolic processes associated with the onset, progression and remission of NASH and thus fill critical gaps in the field of chronic fatty liver disease.

ω3 PUFA attenuate WD-induced NASH in *Ldlr*$^{-/-}$ mice.

We assessed the capacity of EPA and DHA to prevent NASH in *Ldlr*$^{-/-}$ mice (77). The dietary level of EPA or DHA was at ~2% of total calories; olive oil was added to control diets to ensure all diets were isocaloric. The concentration of $C_{20\text{-}22}$ ω3 PUFA in the WD is comparable to the dose consumed by patients taking Lovaza™ (GSK) for treating dyslipidemia (95). Supplementing human diets with a DHA-enriched fish oil (6 g/day for 8 weeks) increased plasma DHA from 4 mol% to 8 mol% (96, 97). Humans consuming EPA + DHA ethyl esters (4 g/d for 12 weeks) increased plasma EPA + DHA from 5.5 mol% to 16.2 + 2.1 mol% (98). In our studies, mice consuming DHA at 2% total calories for 16 weeks increased plasma EPA, docosapentaenoic acid (DPA; 22:5,ω3) + DHA from 6.2 mol% to 15.2 mol%. As such, our protocol for $C_{20\text{-}22}$ ω3 PUFA supplementation yields a change in blood $C_{20\text{-}22}$ ω3 PUFA comparable to that seen in humans consuming $C_{20\text{-}22}$ ω3 PUFA at 4-6 g/d.

WD induces a robust NASH phenotype that recapitulates human NASH (**Fig. 2**) (77). Addition of EPA or DHA to the WD did not affect body weight, body fat or blood glucose, but the ω3 PUFA supplemented diets reduced WD-induced plasma lipids, hepatic lipids, inflammation, oxidative stress and fibrosis (77, 78). Moreover, these studies also established that DHA was equal or superior to EPA at attenuating all WD-induced NASH markers.

Feeding mice ω3 PUFA does not prevent WD-induced endotoxinemia.

Systemic inflammation is a major driver of NASH. Inflammatory signals contributing to NASH progression include: gut-derived microbial products (endotoxin, other bacterial toxins (**Fig.1**) (30, 99); ox-LDL (51, 74), adipokines (leptin/adiponectin) & cytokines (TNFα) (100) and products from hepatocellular death (23, 101). Feeding *Ldlr*$^{-/-}$ mice the WD leads to a 14-fold increase in plasma endotoxin. Including EPA or DHA in the WD did not prevent diet-induced endotoxinemia (78). The appearance of bacterial lipids (endotoxin,

a TLR-4 agonists) (102) in the plasma may represent a disturbance in gut physiology such as a change in microbial population, increased gut permeability (leaky gut), or simply co-transport of microbial lipids with chylomicron (30, 103, 104). A link between the gut microbiome and NAFLD has been established (30, 105, 106).

ω3 PUFA attenuate hepatic inflammation

Analysis of the liver showed that including EPA or DHA in the WD attenuated WD-induced expression of multiple genes linked to inflammation including toll-like receptors (TLR-2, -4, -9) and TLR components (CD14; binds endotoxin), downstream targets of TLRs; like NFκB (p50 & P65 subunits) nuclear abundance, downstream targets of NFκB [chemokines (MCP1), inflammasome (NLRP3) and hepatic expression of cytokines, e.g., TNFα and IL1β (77, 78). As such, EPA and DHA attenuated WD-induced hepatic inflammation by down-regulating key cellular mediators of inflammation, including TLRs, CD14 (CD14 mRNA and protein), NFκB-p50 nuclear abundance.

ω3 PUFA have selective effects on hepatic oxidative stress

Hepatic oxidative stress is associated with NASH progression (107). Feeding mice the WD increased hepatic expression of transcripts linked to oxidative stress, e.g., NADPH oxidase (NOX) subunits [*Nox2*, *P22phox*, *P40phox* and *P67phox]*. The WD also induced the expression of Nrf2, a key transcription factor involved in the anti-oxidant response pathway (49, 77). Induction of Nrf2 was associated with increased expression of downstream targets of Nrf2 action, including Hmox1, Gst1α (78). Dietary ω3 PUFA had no effect on WD-mediated induction of hepatic Nrf2, Hmox1 or Gst1α. However, both EPA and DHA significantly attenuated WD-mediated induction of all NOX subunits (77). Thus, EPA and DHA do not attenuate the Nrf2-regulated anti-oxidant pathway, but target the NOX pathway to lower hepatic oxidative stress.

ω3 PUFA attenuate hepatic fibrosis

Hepatic fibrosis develops as a result of hepatocellular death brought on by inflammation and oxidative stress. Key regulators of fibrosis include TGFβ1, connective tissue growth factor (CTGF), platelet-derived growth factor (PDGF), oxidative stress (NOX), inflammatory mediators (endotoxin, TLR agonist), leptin and Notch signaling (34, 74, 108, 109). While EPA and DHA supplementation attenuated WD-mediated induction of hepatic inflammation and oxidative stress, only DHA attenuated hepatic fibrosis. The anti-fibrotic effect of DHA was assessed by quantifying the expression of key markers of hepatic fibrosis, including the expression of collagen 1A1 (Col1A1), tissue inhibitor of metalloprotease-1 (TIMP1), plasminogen activator inhibitor-1 (PAI1) and TGFβ1; as well as trichrome staining of liver for fibrosis (49, 77). These studies reveal an important difference in the capacity of EPA and DHA to attenuate NASH-associated hepatosteatosis, inflammation, oxidative stress and fibrosis.

The WD and ω3 PUFA affect all major hepatic metabolic pathways

To gain additional insight into NASH, we used a global non-targeted metabolomic approach to examine the impact of the WD and $C_{20\text{-}22}$ ω3 PUFA on hepatic metabolism. The analysis identified 320 known biochemicals (78). Both the WD and $C_{20\text{-}22}$ ω3 PUFA significantly affected the hepatic abundance of metabolites in all major metabolic pathways including amino acids & peptides, carbohydrate and energy, lipid, nucleotide and vitamins & cofactors. **Figure 3** illustrates the impact of diet on hepatic biochemicals associated with lipid, carbohydrate, amino acid and vitamin & cofactor metabolism. In each of the 4 pathways examined, at least 50% of the biochemicals were affected by the WD. The WD either increased or decreased the hepatic abundance of these metabolites. A closer examination of lipid metabolites shows that WD feeding increased 43 of 136 lipid metabolites, while inclusion of DHA in the WD attenuated the induction of 72% of the 43 metabolites. The WD also lowered hepatic levels of 31 lipids; DHA attenuated the WD effect on 87% of the 31 lipid metabolites. Similar effects were seen with carbohydrates, amino acids, vitamins and cofactors.

Overall, the metabolomic analysis expanded our understanding of the impact of the WD and DHA on hepatic metabolism. The onset of NASH is associated with major changes in overall hepatic metabolism and dietary DHA supplementation was able to reverse many of these WD-induced effects on hepatic metabolism. In addition to the pathways listed above, our analysis identified several key metabolites (oxidized lipids, advanced glycation end products, sphingolipids) that were regulated by WD and ω3 PUFA. Future studies will focus on evaluating the role these metabolites play in NASH progression and remission.

Summary

NAFLD and its progression to NASH is a major public health concern. To help better understand the molecular and metabolic basis for the disease process, we developed a mouse model of NASH. The WD induces a robust NASH phenotype in *Ldlr*$^{-/-}$ mice that recapitulates human NASH. Addition of DHA to the WD attenuates NASH development without promoting weight loss or a reduction in body fat. While EPA and DHA did not attenuate WD-induced markers of systemic inflammation (endotoxin), dietary ω3 PUFA attenuated WD-induced hepatic inflammation by targeting key mediators of hepatic inflammation; specifically a key transcriptional mediator of inflammation (NFκB-p50) and several downstream NFκB targets, e.g., TLR receptors (TLR-2, -4, -9) and co-factors (CD14) and inflammasome components (NLRP3). The WD induced several oxidative stress pathways (Nrf2, Nrf2-regulated pathways and NOX-subtype). DHA attenuated the NOX-pathway while preserving the Nrf2-regulated anti-oxidant pathway. Finally, dietary DHA, but not EPA, attenuated WD-induced hepatic fibrosis. Together, these findings suggest that DHA may have potential for use as a therapeutic agent to treat human NASH.

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Conflicts:

No conflicts of interest.

Authorship:

All authors contributed to the writing and editing of the manuscript.

Table 1: Comparison of mouse models of NASH*.

	Wild Type			*Ldlr*$^{-/-}$	
Diet	**RD**	**HF**	**HFHC**	**RD**	**WD**
Body weight (g)	28	45	43	31	42
Plasma parameters:					
Glucose (mg/dl)	6	12	8	8	11
Triglyceride (mg/dl)	120	90	66	86	229
Cholesterol (mg/dl)	52	108	138	232	1018
ALT (U/L)	4	19	20	5	44
Hepatic parameters:					
% Body Weight	4	3	5	4	5
Triglyceride (mg/g protein)	51	157	141	77	328
Cholesterol (mg/g)	7	6	8	12	34
Gene Expression *(Fold Change)*					
Scd1 mRNA	1	2	8	1	7
Mcp1 mRNA	1	7	8	1	32
Col1A1 mRNA	1	7	15	1	18

*****The wild type mice are C57BL/6J and the *Ldlr*$^{-/-}$ mice are on the C57BL/6J background.
RD: reference diet (chow); HF: high fat diet; HFHC: high fat high cholesterol
WD: western diet; ALT: alanine aminotransferase; Scd1: stearoyl CoA desaturase-1;
Mcp1: monocyte chemoattractant protein-1; Col1A1: collagen 1A1.

Figure Legends:

Figure 1: **Factors contributing to the onset and progression of NASH.**

Figure 2: **Effects of the western diet and $C_{20\text{-}22}$ ω3 PUFA on the prevention of NASH *Ldlr*$^{-/-}$ mice.** The effect of diet on NASH parameters was assessed (77). The comparison is between mice fed the reference diet (chow) versus the western diet supplemented with olive oil, EPA or DHA. The effects are graded from minimal effect (+) to maximum effect (++++) of diet on specific parameters.

Figure 3: Effects of the western diet and $C_{20\text{-}22}$ ω3 PUFA on hepatic metabolites. A non-targeted metabolomic analysis was carried out as described (78). The pie plots represent the effects of diet on the total number of identified lipids (136 biochemicals), carbohydrates (34 biochemicals), amino acids (78 biochemicals) and vitamins & cofactors (16 biochemicals). Hepatic levels of some biochemicals were not affected by diet (No Change, gray); some were increased by the WD (red) and some were decreased by the WD (green). The top number in the fraction represents the total number of biochemicals increased or decreased by the WD. The bottom number is the percent of the WD affected biochemicals that were attenuated by including DHA in the WD.

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Figure 1

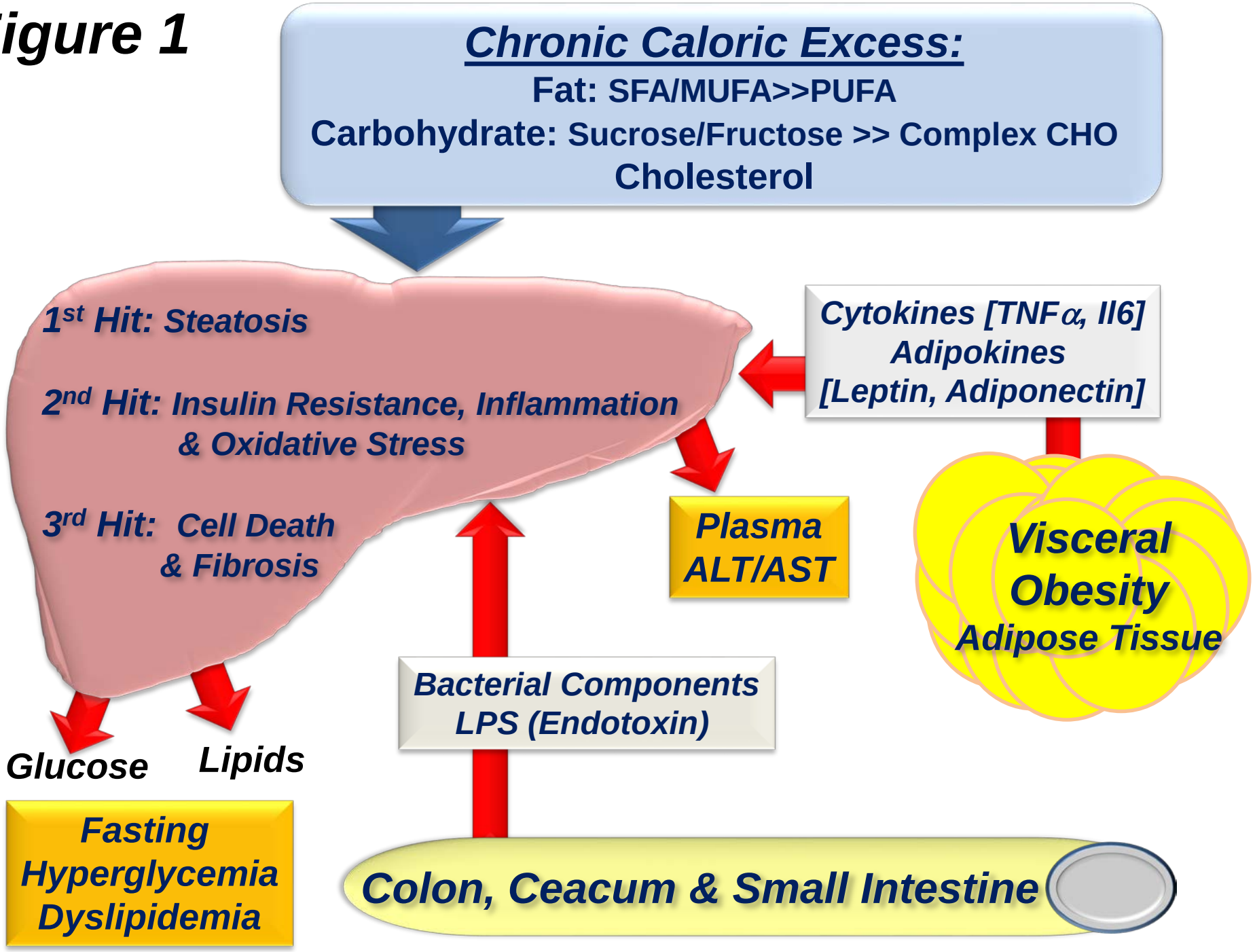
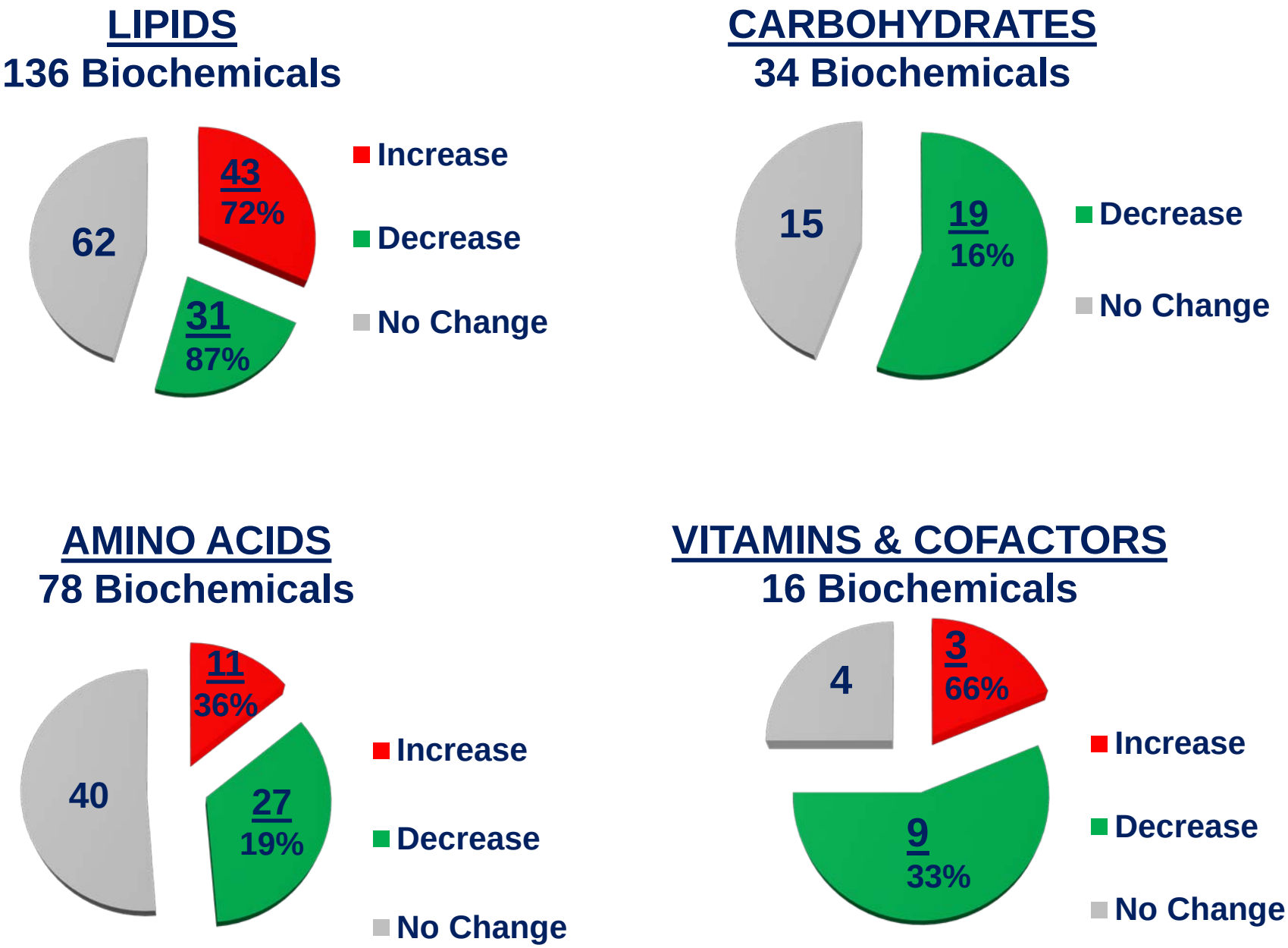

Chronic Caloric Excess:
Fat: SFA/MUFA>>PUFA
Carbohydrate: Sucrose/Fructose >> Complex CHO
Cholesterol
1st Hit: Steatosis
2nd Hit: Insulin Resistance, Inflammation & Oxidative Stress
3rd Hit: Cell Death & Fibrosis
Cytokines [TNFα, Il6]
Adipokines
[Leptin, Adiponectin]
Plasma ALT/AST
Visceral Obesity
Adipose Tissue
Bacterial Components
LPS (Endotoxin)
Glucose
Lipids
Fasting Hyperglycemia Dyslipidemia
Colon, Ceacum & Small Intestine

Figure 2

	Western Diet		
	+Olive	+EPA	+DHA
Body Weight & Fat Mass	++++	++++	++++
Fasting Plasma Cholesterol	++++	+++	++
Fasting Plasma Triglycerides	++++	+++	++
Hepatic Damage (ALT/AST)	++++	+++	++
Plasma Endotoxin	++++	++++	++++
Hepatosteatosis (Triglycerides & Cholesterol)	++++	+++	++
Oxidative Stress (NOX2, P67Phox)	++++	++	++
Inflammation (MCP1, TLR, CD68)	++++	++	++
Fibrosis (ProCol1A, Trichrome Stain)	++++	++++	+

Figure 3


LIPIDS
136 Biochemicals
43
72%
62
31
87%
Increase
Decrease
No Change
CARBOHYDRATES
34 Biochemicals
15
19
16%
Decrease
No Change
AMINO ACIDS
78 Biochemicals
11
36%
40
27
19%
Increase
Decrease
No Change
VITAMINS & COFACTORS
16 Biochemicals
4
3
66%
9
33%
Increase
Decrease
No Change