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Quantifying the effect of high fructose feeding on the intestinal permeability of endotoxins

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica do Professor Doutor John Jones (Universidade de Coimbra) e do Professor Doutor Carlos Palmeira (Universidade de Coimbra)

Cristiano de Almeida Ferreira Caramelo dos Santos

2013

“Science! true daughter of Old Time thou art!

Who alterest all things with thy peering eyes.

Why preyest thou thus upon the poet's heart,

Vulture, whose wings are dull realities?

How should he love thee? or how deem thee wise?

Who wouldst not leave him in his wandering

To seek for treasure in the jewelled skies”

“Sonnet: To Science”

Edgar Allan Poe

Acknowledgments

Even the smallest step in the scientific field requires different parties to come together, with that said, I couldn't have made it this far without help of the following people:

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Table of contents

Acknowledgments III

Index of images V

Abstract VII

Resumo VIII

1. Introduction 2

1.1 Fructose in diet & Consumption 2

1.2. NAFLD: an important outcome of high fructose consumption 2

1.3 Fructose uptake & metabolism 4

1.5 Intestinal microflora 8

1.5 Intestinal permeability 11

1.6 Endotoxin and inflammation mechanisms 13

1.3 Nuclear Receptors: a link between inflammatory factors and metabolism 15

1.3.1 Peroxisome Proliferator Activated Receptors 16

1.8 Objectives / Scope of this work 19

2. Methods 22

2.1 Mice keeping and diet 22

2.2 Blood collection and Intestinal Permeability 22

2.3 Tissue Collection 23

2.4 Western Blots 23

2.4.1 Cell lysate preparation 24

2.4.2 Protein Quantification 24

2.4.3 SDS-PAGE 25

2.4.4 Electric Transfer to PVDF Membrane 26

2.4.5 Antibody blocking and detection 26

2.5.4 Results analysis 27

3. Results 29

3.1 Weight Gain 29

3.2 Intestinal Permeability 30

3.3 Western Blots 31

PPAR α 31

PPARβ 32

Tumor necrosis factor α (TNF-α) 33

Glucose transporter 2 (GLUT2) 34

4. Discussion.. 36
5. Conclusions 40
Bibliography 43

Index of images

Figure 1: Overview of the reactions that fructose can partake in the cell. Adapted from Tran et al, 2009 [55]. 6
Figure 2 Schematic representation of how the muscle, liver and adipocytes are dependent on the intestinal metabolic reactions. SCFA stands for short chain fatty acids, and FIAF stands for fasting induced adipose factors Adapted from Cani et al, 2011, [49.] 8
Figure 3 Schematic representation of the Crabtree effect in microorganisms. "1" represents glycolysis pathway. "2" represents Entner Doudoroff pathway. "3" represents methyl glyoxal bypass/glyoxilate system. Adapted from Pronk et al , [1996,135] 10
Figure 4 Overview of the diet´s effect on the intestine and it´s inflammatory properties. Adapted from Burcelin et al [11]... 12
Figure 5 Overview of the TLR4 activation pathway by LPS recognition. Adopted from Jerala et al [156] 14
Figure 6 General representation of the PPAR pathways 17
Figure 7 General representation of PPAR activation [84]... 19
Figure 1 Schematic representation of the BCA protocol. Adapted from Thermo Scientific Pierce's "Protein Assay Technical Handbook Version 2" 25
Figure 2 Weight control of mice from the two diet groups for 10 weeks (N=6 per group) 29
Figure 3 Representation of the intestinal leakage of FITC-Dextran through time with standard error mean. The number of animals used per group were 6. 30
Figure 4: Representative Western blotting (six different mice from each group) showing the hepatic protein content of PPARα. Densitometry analysis of specific bands from all mice studied is shown. Values are expressed as a percentage relative to Dextrose (100 %) and are means versus SD. Equal loading of the gels was as demonstrated by probing the membranes with an anti-β-actin polyclonal antibody. P value was 0,0290 for unpaired t-test. Proteins bands are shown at the right of the figure. 31
Figure 5: Representative Western blotting (six different mice from each group) showing the hepatic protein content of PPARβ. Densitometry analysis of specific bands from all mice studied is shown. Values are expressed as a percentage relative to Dextrose (100 %) and are means versus SD. Equal loading of the gels was as demonstrated by probing the membranes with an anti-β-actin polyclonal antibody. P value was 0,0076 for unpaired t-test. Proteins bands are shown at the right of the figure 32

Figure 6: Representative Western blotting (six different mice from each group) showing the hepatic protein content of TNF-α. Densitometry analysis of specific bands from all mice studied is shown. Values are expressed as a percentage relative to Dextrose (100 %) and are means versus SD. Equal loading of the gels was as demonstrated by probing the membranes with an anti-β-actin polyclonal antibody. P value was 0,0002 for unpaired t-test. Proteins bands are shown at the right of the figure. 33
Figure 7 Representative Western blotting (six different mice from each group) showing the hepatic protein content of GLUT2. Densitometry analysis of specific bands from all mice studied is shown. Values are expressed as a percentage relative to Dextrose (100 %) and are means versus SD. Equal loading of the gels was as demonstrated by probing the membranes with an anti-β-actin polyclonal antibody. P value was 0,0055 for unpaired t-test. Proteins bands are shown at the right of the figure 34
Figure 16 Representation of what is thought to happen in a fructose rich diet on the gut and the liver. 41

Index of tables:

Table 1: Depiction of the PPAR types, location and function…………………………16

Table 2: Analysis of each protein expression along with the causes and effects of different expressions……………………………………………………………………38

Abstract

A fructose rich diet has been known to have nefarious effects on both mice and humans. However, the effects of this diet upon the intestine and surrounding tissues are still unclear. The intestine and the liver are the only organs that absorb fructose directly into metabolic processes, and are tightly connected via bile duct and hepatic portal vein, which means they both influence each other in the process, forming the intestine-liver axis. This connection makes this axis susceptible to inflammatory and metabolic changes. The present work intends to show how a fructose rich diet can affect the intestine's permeability, and how a change in permeability can affect the expression of certain proteins in liver in mice.

In this study we compared a fructose rich diet versus normal chow over the course of three months. Intestinal permeability was evaluated by gavaging a fluorescent probe (FITC-dextran) into fasting mice. The amount of probe present in blood was correlated with permeability. Protein expression levels in liver were quantified by Western blot, in which peroxisome proliferator associated receptor alpha and beta, tumor necrosis factor alpha and glucose transporter 2 were measured.

Intestinal permeability in high fructose fed mice was consistently and significantly higher than in the control mice in all time points. The data also suggests that there is an adaption of the intestine to the diets, as both control and experimental group have an increase in permeability until week 6, but both had a significant reduction in this value at week 12, which would mean that the intestine recovered some of its integrity. Western blot in the liver revealed an increase in cytokine expression (tumor necrosis factor alpha) as well as fructose associated transporters (glucose transporter 2). Expression of anti-inflammatory nuclear receptor peroxisome proliferator associated receptor alpha was decreased while its beta form was unchanged.

In this work it was possible to conclude that a fructose rich diet damages the intestine, which leads to a higher intestinal leakage than a regular diet would. Such increased leakage releases toxins and metabolites into the blood stream, directly affecting the liver, resulting in an increase in inflammation and fructose transport.

Keywords: fructose rich diet; intestinal permeability; hepatic nuclear receptors;

Resumo

A dieta rica em fructose é conhecida por causar efeito nefastos em ratos e em humanos. Contudo, os efeitos desta dieta no intestino e nos orgãos circundantes são ainda pouco claros. O intestino e o fígado são os únicos orgãos que absorvem fructose directamente para os seus processos metabólicos, e estão intrisecamente ligados entre eles pela vesícula biliar e pela veia portal hepática, o que significa que ambos se influenciam um ao outro no processo, formando assim o eixo intestino-fígado. Esta conecção torna este eixo susceptível a mudanças inflamatórias e metabólicas. O presente trabalho predispõe-se a mostrar de que forma é q uma dieta rica em fructose pode afectar a permeabilidade intestinal, e como é que uma mudança de permeabilidade pode afectar a expressão de certas proteínas nos fígado de ratinhos.

Neste estudo comparou-se uma dieta rica em fructose contra uma dieta normal no decorrer de três meses. A permeabilidade intestinal foi avaliada através da gavagem de uma sonda fluorescente (FITC-dextran) em ratinhos em jejum. A quantidade de sonda presente no sangue foi correlacionada com permeabilidade. Os níveis de expressão de proteína foram quantificados por Western Blot, nos quais foram medidos os níveis dos receptores associados a proliferação de peroxissoma alfa e beta, factor de necrose tumoral alfa e o transportador de glucose 2.

A permeabilidade intestinal dos ratinhos alimentados a dieta de fructose foi consistemente e significativamente mais alta que a do grupo controlo em todos os pontos temporais. Estes dados sugerem que há adaptação do intestino às dietas, visto que tanto o grupo de controlo como o grupo experimental tiveram a sua permeabilidade aumentada até à semana 6, mas ambos tiveram uma redução significante desse valor na semana 12, o que significa que o intestino recuperou alguma da sua integridade. Western blots no fígado revelaram que houve um aumento na expressão de citoquinas (factor de necrose tumoral alfa), tal como no transportador associado a fructose (transportador de glucose 2). A expressão do receptor nuclear anti-inflamatório associados a proliferação de

peroxissoma alfa foi reduzida, enquanto que os valores da sua forma beta se mantiveram inalteráveis.

Neste trabalho foi possível concluir que uma dieta rica em fructose danifica o intestino, o que vai levar a mais “vazamento” intestinal do que uma dieta normal. Tal aumento de permeabilidade vai corresponder a um aumento de toxinas e metabolitos na corrente sanguínea, afectando directamente o fígado, resultando num aumento em inflamação e em transporte de fructose.

Palavras chave: dieta rica em fructose; permeabilidade intestinal; receptores nucleares hepáticos;

1. Introduction

1. Introduction

1.1 Fructose in diet & Consumption

Fructose is a sugar that is naturally present in fruit and is also widely used in preserved food and sweetened beverages. Due to the increased availability and use of high-fructose corn syrup, fructose consumption in Western countries is rising at an alarming rate. A diet rich in fructose has been shown to provoke adverse alterations in carbohydrate and lipid metabolism in both animal models and humans. To date, the adverse effects of high fructose consumption have been explained in terms of its direct effects on hepatic metabolism. However, high fructose consumption may also indirectly influence hepatic metabolism through hitherto undefined effects on the intestinal microflora.

High fructose corn syrup is a cornstarch derived product. Glucose results from hydrolysis of starch and after isomerization of glucose, it is possible to obtain fructose. Before this syrup is complete it goes through chromatographic fructose enrichment, which will make this 90 % of this syrup rich in fructose[1,2].

Nowadays, fructose is present in many types of food as a sweetener and its usage contributes to higher sugar diets. In the United States of America from 1978 to 2003 [3] the consumption of fructose increased 17%, replacing sucrose as one of the main sweeteners (sucrose availability in food supply decreased about 32%). It is usually added in non-alcoholic beverages, which are the main source of fructose intake, particularly in children and young adults. In weight terms, this translates into an amount of 69 kg of fructose consumed per capita per year[4–6] . Fructose and sugar sweetened beverages have a high impact on health , and can lead to hypertriglyceridemia and other metabolic syndromes[7–9].

1.2. NAFLD: an important outcome of high fructose consumption

Nonalcoholic fatty liver disease (NAFLD) is a chronic liver disease which is characterized by a diagnosis of excessive triglyceride accumulation in the liver as measured by liver biopsy or magnetic resonance spectroscopy [10–12]. The incidence of NAFLD has risen in line with a steep rise in fructose consumption, hence excessive dietary fructose intake is highly implicated as a risk factor. NAFLD is also associated with both obesity and insulin resistance[13–15] and is considered to be an early milestone toward development of Type 2 diabetes.

The overall incidence of NAFLD in obese and diabetic individuals is between 60 and 80 %, rising to near 100% in morbidly obese patients[16]. Both NAFLD and obesity are associated with systemic and hepatic insulin resistance[17,18]. Peripheral tissues contribute to hepatic lipid overload and insulin resistance by increased release of non-esterified fatty acids and pro-inflammatory factors into the blood stream [19,20,21] . NAFLD can also act as a gateway to more severe liver disease, and can eventually progress to nonalcoholic steatohepatitis (NASH), which is a condition in which there is lobular inflammation as well as elevated fat deposits of the liver[22], which can be diagnosed via biopsy[23]. NASH can further progress into cirrhosis, which is characterized by extensive fibrosis of the liver and overall loss of hepatic function[24].

While NAFLD disease presents similar symptoms to the initial stages of alcoholic fatty liver disease [25,26], it is considered to be independent of alcohol intake[27]. However, despite an absence of ethanol intake, it is possible that the liver could be exposed to intestinal ethanol generation via bacterial fermentation (see later section). The extent to which this could contribute to NAFLD pathology is not known.

Excessive fructose intake can have negative effects on the liver through direct and indirect ways[27]. Direct mechanisms include the generation of toxic intermediary metabolites, and a temporary decrease in adenosine-triphosphate (ATP) levels by fructokinase and triokinase activites[28]. As will be subsequently discussed, indirect mechanisms include an increased inflammatory response that may originate from alterations in intestinal microflora and their interactions with enterocytes. Also, high fructose intake (coupled with intestinal malabsorption) may result in altered production of fermentative metabolites by intestinal bacteria, including possibly ethanol. Metabolite and inflammatory factors act synergistically to promote hepatic lipid accumulation and insulin resistance.

1.3 Fructose uptake & metabolism

Fructose is initially absorbed passively into the enterocytes (eukaryotic cells that share the intestine with bacteria and fungi). The enterocytes are the intestine's epithelial cells whose function is to absorb nutrients and to send them to the bloodstream, through the intestine-blood barrier[29]. Fructose is carried to the blood stream via glucose/fructose facilitated transporter 5 (GLUT5) which is a low affinity high capacity bi-directional fructose transporter, and GLUT2, another hexose transporter, also present in the membrane of enterocytes[30,31]. Under-expression of intestinal GLUT5 is associated with malabsorption of fructose[30]. Fructose can also be metabolized in the intestine prior to intestinal absorption and may 1) result in increased levels of potentially harmful bacterial fermentation products such as ethanol and 2), alter bacterial numbers and species distribution that constitute the intestinal microflora. Since the intestine is one of the primary nutrient absorption source [32], the type of nutrients that are absorbed affect the whole human body [33–38].

The hepatic portal vein drains the intestinal bed and directly delivers the absorbed nutrients to the liver. Absorbed fructose is taken up by the liver via GLUT5 transporters. Following absorption, fructose is phosphorylated by fructokinase to make fructose-1-phosphate. Expression of this enzyme is up-regulated by fructose, so an increase in fructose uptake results in increased fructokinase activity. Fructose-1-phosphate is metabolized by aldolase to make dihydroxyacetone phosphate and glyceraldehyde. This latter metabolite can be phosphorylated via triokinase to form glyceraldehyde 3-phosphate and enter glycogenesis. Both glyceraldehyde and dihidroxyacetone phosphate will form of these will form a pool of triose phosphate that can be converted to pyruvate, which in turn is be able to form acetyl-CoA (which can be further metabolized in the Krebs cycle) and citrate inside the mitochondria .

Unlike glucose metabolism, the uptake of fructose in the liver bypasses two of the main rate-determinant enzymes in hepatic glycolysis, glucokinase and phosphofructokinase. Glucokinase is an insulin sensitive high flux controller for gluconeogenesis in the liver[39], that can also mediate the coupling of glycolysis to

mitochondria at low glucose levels [40]. This kinase converts glucose to glucose-6-phosphate. Its activity is not affected by the presence of glucose-6-phosphate and it has low affinity for glucose.[41] Phosphofructokinase is an unidirectional enzyme which converts fructose-6-phosphate, that comes from normal glycolytic metabolism, into fructose-1, 6-biphosphate. This reaction is crucial in glycolysis, and can be inhibited by ATP and citrate[42] . Phosphofructokinase has also been reported to interact indirectly with glucokinase, as fructose-6-phosphate can repress this enzyme, through interaction with glucokinase receptor protein[43]. Another important factor that distinguishes fructose metabolism in the liver from the glucose one is the different insulin dependence, since insulin is the main inducer of phosphofructokinase and glucokinase [36,44,45].

The uptake of fructose by the liver can also lead to increased hepatic gluconeogenic and lipogenic fluxes, which can disrupt the liver's function in regulating blood glucose and triglyceride levels. Gluconeogenesis is sustained by the inflow of triose phosphate products of fructose catabolism[46].Thus, following a bolus of fructose, plasma glucose levels show a rapid increase, reflecting an increase in endogenous glucose production that is fuelled by gluconeogenesis from fructose[36,47]

Fructose can also contribute to the hepatic glycogenesis through an indirect pathway, which initially involves phosphorylation of fructose into fructose-1-phosphate. Fructose-1-phosphate will be cleaved into glyceraldehyde and dihydroxyacetone phosphate. After glyceraldehyde is phosphorylated to glyceraldehyde-3-phosphate, it will be joined with dihidroxyacetone to make fructose-1,6-biphosphate. This reaction will be followed by formation of glucose-6-phosphate and end in the formation of glycogen via glucose-1-phosphate and UDP-glucose[46] .

The stimulation of hepatic de-novo lipogenesis is another important change that is attributed to high fructose consumption. Metabolism of its triose phosphate intermediates to acetyl-CoA is postulated to increase the supply of acetyl-CoA precursors for the de novo lipogenesis pathway. In addition to supplying the carbons for de novo lipogenesis, alterations in hepatic sugar phosphate intermediates and other metabolites resulting from fructose metabolism may also upregulate the transcription of novo lipogenesis enzymes[36,48]. Increased lipogenic activity is coupled to increased export of very low density lipoproteins (VLDL) resulting in a rise of plasma VLDL levels[49–51]. VLDL are TAG-rich lipoproteins that are highly atherogenic and are well known risk

factors for cardiovascular disease. Increased VLDL flux is also associated with the accumulation of triglycerides in tissues other than adipocytes, such as skeletal muscle and pancreas. These so-called ectopic lipid pools are characteristic of dyslipidemia and are tightly associated with the development of insulin resistance in the afflicted tissue.

Based on its effects on hepatic intermediary metabolism, an excess of fructose is expected to increase hepatic triglyceride and VLDL production. Given this, it is plausible to hypothesize that high fructose consumption induces insulin resistance in part via alterations in hepatic and systemic lipid metabolism[52,31,36,47,53,54].

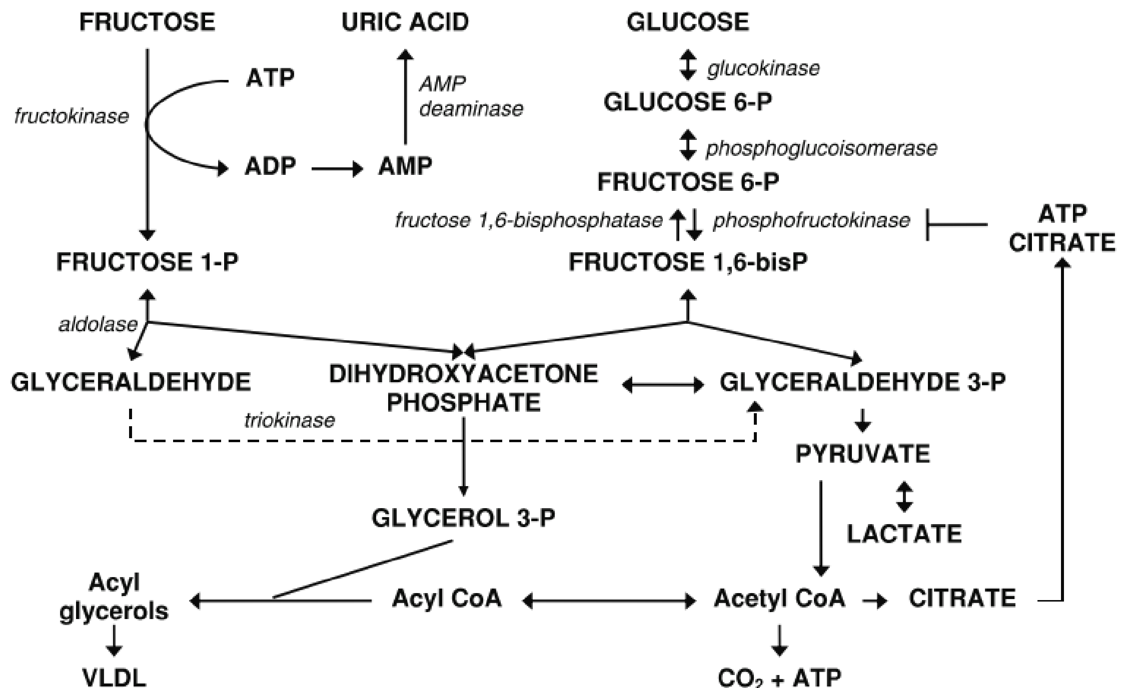


Figure 1: Overview of the reactions that fructose can partake in the cell. Adapted from Tran et al, 2009 [55].

Insulin resistance may be conferred by hereditary and by environmental factors. Environmental mechanisms include epigenetic modifications of gene expression [56] and over-nutrition [34,35,57,58]. Altered nutrient intake may also induce changes in the species

distribution and metabolic activity of intestinal microbiota which may in turn result in exposure of the liver to agents that promote the development of insulin resistance [32,38,59–62]. Since the focus of this thesis is to study the promotion of fructose-induced hepatic insulin resistance via mechanisms likely related to intestinal microbial activity, intestinal microflora and their interactions with intestinal function will be discussed in more detail.

The intestine can be considered as a system composed of two main metabolic parts: enterocytes and microbial flora. Each has a specific type of metabolism, and both have access to a wide range of nutrients. The intestine's commensal flora and the enterocytes are separated by a mucus layer that protects the enterocytes from bacterial endotoxin, which among other things, are highly pro-inflammatory. The interplay between flora and enterocytes can dictate our susceptibility to diet-induced complications such as insulin resistance (Figure 2).

Enterocytes are polarized epithelial cells connected between each other by tight junction, whose main function is to transfer nutrients including fructose[30].as well as fasting inducing factors into the blood stream.. Absorption is mediated by two different mechanisms: passive diffusion and protein-mediated transport[63].

Since the intestine is the gateway for nutrient absorption into the bloodstream, it is hypothesized that intestinal microbial metabolic activity can have effects on metabolism of the host tissues and organs. For example, short-chain fatty acids such as acetate and butyrate that are generated by microbial fermentation contribute to muscle energy metabolism; acetate can also potentially sustain hepatic lipogenesis, while other products (like L-α -Lysophosphatidylinositol[64]) may activate G coupled protein receptors (GPR) that are connected with obesity and insulin resistance [60,64,65].

An output "burst" of fatty acids from the intestine can also lead to the production of cytokines like fasting induced adipose factors (FIAF), which are involved with energy homeostasis[66,67] through the regulation of fat storage. The intestine also receives lipid products from whole body catabolism of sterols via the bile circulation. In addition to serving as emulsifying agents for solubilizing dietary triglycerides, these products can also be metabolized by intestinal bacteria to form conjugated bile acids, which can be recirculated into the host.

Bile is synthetized in hepatocytes and is stored in the bile ducts before being excreted into the gallbladder, where it stays until the gallbladder contracts after meals to pump bile acids into the intestine through the common ducts to mix with the food present there. Bile is a micellar aqueous solution of salts, bilirubin, bile acids and proteins, phospholipids and cholesterol. Bile acid salts are reabsorbed in the small distal bowel, directly into the portal vein to be taken up by the liver[68]. It is important to note that bile can help break down cholesterol into cholic and chenodeoxycholic acid, which can be used to produce more bile, but its main function is to act as physiological detergent for transport and absorption of nutrients[69].

Excessive fructose may lead to higher lipid output from the liver to the gallbladder, which in turn will emulsify these lipids (with bile) and increase the efficiency of intestinal lipid absorption [36,44,70]

1.5 Intestinal microflora

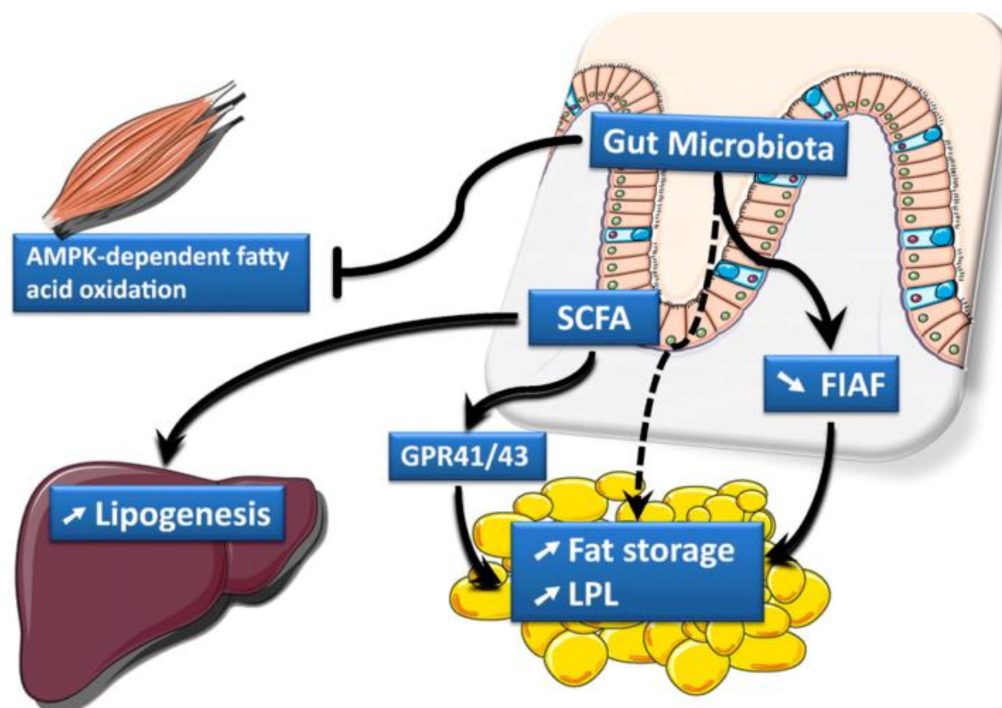


Figure 2 Schematic representation of how the muscle, liver and adipocytes are dependent on the intestinal metabolic reactions. SCFA stands for short chain fatty acids, and FIAF stands for fasting induced adipose factors Adapted from Cani et al, 2011, [49.]

Intestinal microorganisms account for nearly 90 % of the total body cell number [61] .The gastrointestinal organisms have different distributions in this system, as each organ has a different micro environment and different metabolic function. The stomach has the least bacteria, with 10^3 colony forming units (CFU) per gram of gastric juice. The small (or upper) intestine (duodenum and jejunum) also have a low count of bacteria, with about 10^2-10^4 CFU per gram of content, while increasing greatly in number as you reach the end of the gastrointestinal system, with ileum accounting for 10^{10} CFU per gram and the colon harbouring around 10^{10}-10^{12} CFU per gram[71] .If there is an increase in the number of bacteria in the small intestine it can cause small intestine bacterial overgrowth, which is an unhealthy condition associated with malabsorption[72]. This condition can happen in cases where fructose cannot be absorbed correctly in the small intestine so colonic bacteria have to metabolize more fructose, since metabolization of fructose by bacteria will make hydrogen, carbon dioxide and short chain fatty acids [73,74]. These bacterial products will cause enterocyte luminal distention and contribute to the nausea, abdominal discomfort and gas formation that are felt in small intestinal bacterial overgrowth.

Their principal sources of energy include simple and complex carbohydrates. For clearance of simple carbohydrates such as fructose and glucose, intestinal absorption competes with microbial metabolism. Impaired absorption of carbohydrate is therefore expected to increase its availability to intestinal bacteria. In the case of fructose, intestinal GLUT5 deficiency can limit its absorption and potentially provide intestinal bacteria with fructose for fermentation. This may explain symptoms of discomfort and excessive gas production in GLUT5-deficient individuals [30]. Complex carbohydrates are not digested by endogenous intestinal enzymes, nor are they transported into the bloodstream.

Complex carbohydrates are fermented by colonic bacteria to make SCFA, of which butyric acid, propionic acid, and acetic acid are the main products[75]. Acetic acid can cross the intestinal-blood barrier and reach the portal vein, where it can be absorbed into the liver to be used in the form of acetyl-CoA[76]. This and other SCFA derived products can then be used in the liver as precursors for Krebs cycle oxidation, lipogenesis[77], and gluconeogenesis [78].

For simple carbohydrates, the Crabtree[79] effect happens whenever bacteria[80,81], yeast[82–84] and even eukaryotic cells[85,86] have access to high levels of sugar , and metabolize them to ethanol.

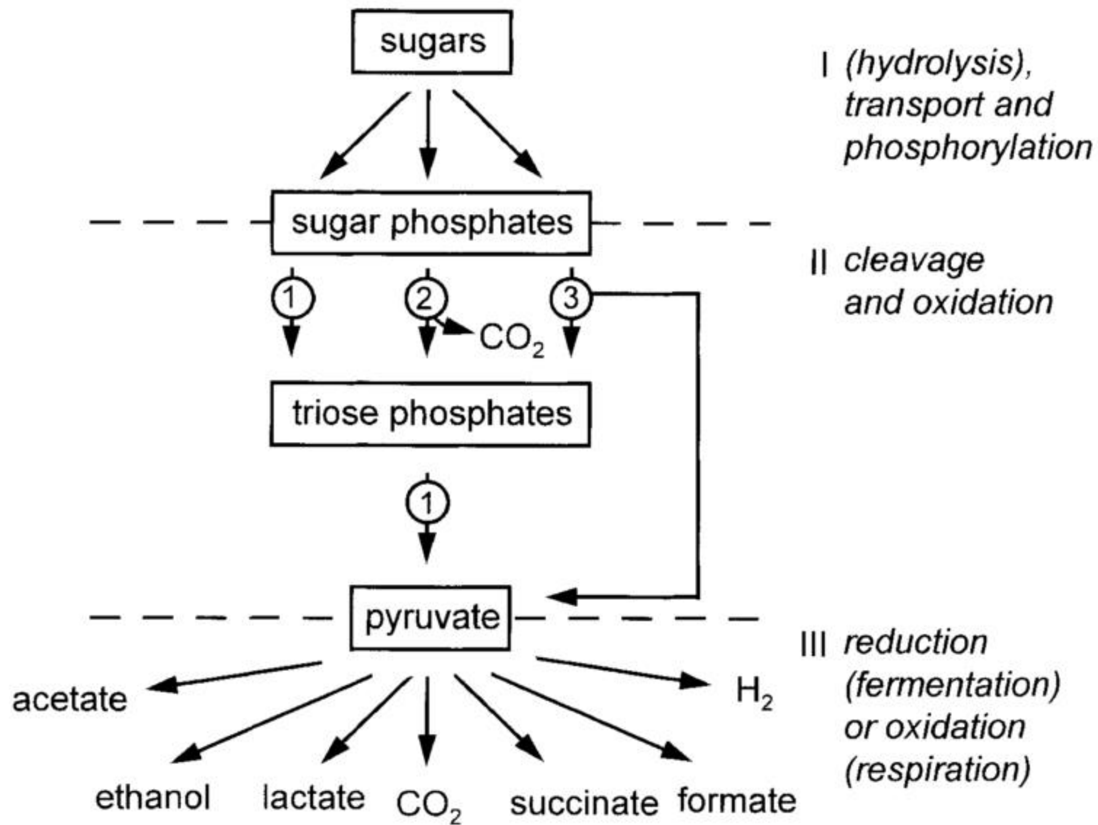


Figure 3 Schematic representation of the Crabtree effect in microorganisms. "1" represents glycolysis pathway. "2" represents Entner Doudoroff pathway. "3" represents methyl glyoxal bypass/glyoxilate system. Adapted from Pronk et al , [1996,135]

In addition to glycolysis, bacterial also possess the Entner Doudoroff and glyoxal bypass pathways (Figure 3). The Entner Doudoroff[87,88] pathway is a prokaryote exclusive pathway that acts as an alternative way (for some bacteria) to convert glucose to pyruvate. This pathway is usually associated with bacteria that lack some of the glycolytic enzymes, and can allow bacteria such as E. coli to use fructose as sole carbon source[89]. The methyl glyoxal pathway is also a glycolysis bypass[90], which is mostly found in prokaryotes.

The intestine is mostly filled with gram negative bacteria, its main strains are specifically *Bacterioidetes* and *Firmicutes* (this type has gram positive and negative strains). These two strains have an impact on how the gastrointestinal system behaves because they are envolved with intestinal mucosa immunity development and help the intestine in controlling bacteria by stimulation of the mucosa to produce immunoglobins[91]. It has also been reported that gram-negative bacteria are the main source of endotoxin from the intestine[92]. Due to the great number of gram negative bacteria in this system and due to the tight control of the balance between inflammation and symbiosis, any alteration in bacterial or enterocyte metabolism will have repercussions on the intestine itself.

1.5 Intestinal permeability

Intestinal permeability is now considered as an important factor in the development of hepatic insulin resistance. The quantity and type of leaked microbiota (species, metabolites and toxins) may trigger inflammatory and other processes in both enterocytes and adjacent tissues and organs, such as visceral fat and liver.. There have already been some studies on the effects of high-fat diet on inflammation of the intestine and surrounding tissues in mice [33–35,37,38,57,93]. They indicate that high fat feeding *per se* does not completely explain the observed inflammation, However it is implied that altering the intestinal permeability conjugated with endotoxin leakage should help justify the low grade inflammation set on metabolic diseases like NAFLD[26,94–96] and insulin resistance [32,62,97–102].

Non-invasive intestinal permeability assays have been used since the 1970's and [103,104,105]. All these tests use the same principle: oral administration of a large-molecular weight substance that is not metabolized in the intestine and is not transported by the normal enterocyte nutrient transporters, followed by its detection in blood or urine. The substance may be tagged with a fluorescent group to aid its detection by fluorescence emission spectroscopy, for example FITC-dextran[59,105] Alternatively, the substance may be identified and quantified by HPLC or NMR spectroscopy[106]. In any case, the higher the levels of the substance measured in blood or urine, the greater the degree of intestinal

permeability. While these methods do not provide absolute values of intestinal permeability, their non-invasive nature means that they can be used to assess relative changes in permeability following dietary or other interventions.

Endotoxin leakage and generation of an inflammatory response in surrounding sites known to be influenced by diet [65,94,107]. One possible explanation is that a particular diet modifies the species and distribution of bacteria that in turn results in an increased permeability to endotoxin leakage [59,62,108].

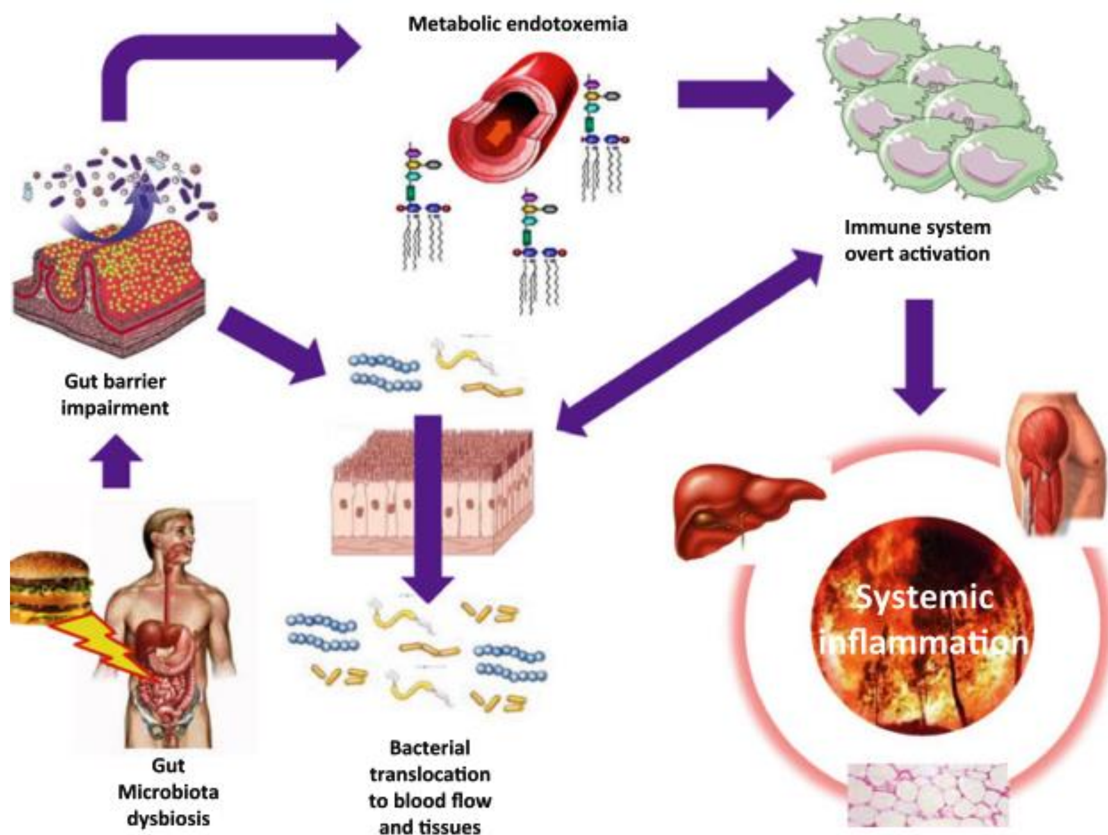


Figure 4 Overview of the diet´s effect on the intestine and it´s inflammatory properties. Adapted from Burcelin et al [11]

Figure 4 illustrates some proposed mechanisms that link diet-induced changes in intestinal bacteria to systemic inflammation. Increased intestinal permeability can allow translocation of endotoxin or even entire bacteria into surrounding tissues including liver, mesenteric fat and adipocytes thereby contributing to inflammation and metabolic

deregulation of these tissues[94,95,107,109–117]. While the extent of bacterial or endotoxin translocation and resulting systemic response are far below the catastrophic effects associated with sepsis or intestinal perforation, it is becoming increasingly clear that the sub-acute but chronic inflammation response is a key factor for promoting hepatic insulin resistance.

1.6 Endotoxin and inflammation mechanisms

The main endotoxin produced by the intestine´s microbiota is LPS. LPS is a powerful endotoxin produced by Escherichia coli and other gram negative bacteria [118],[119]. LPS is considered to be the main endotoxin excreted by the intestine´s flora and is known to induce Toll like receptor 4 (TLR4) [111,120–123] stimulates inflammation in various tissues such as adipocytes [96,114,115,124,125], kidney [111], pancreas[98,126–129] and is nowadays considered to contribute to development of insulin resistance [32–34,38,56,57,59,62,98–102,130,131], NAFLD[27,126,132] and cirrhosis[26,107,111]. The chronically elevated levels of circulating endotoxin constitutes a low-grade endotoxemia[119,126,133,134], and has been associated with over nutrition.

Toll Like Receptors (TLR) are pathogenic "sensors" found in vertebrates that modulate inflammatory response[62], and control the host´s innate response, which are usually present in macrophages and dendritic cells. TLR 4 is a specific receptor for LPS [62],[121], (and its co receptor CD14) (figure 5), that triggers release of cytokines, such as TNFα, IL-6, SOCS-3 and SOCS-6. TLR4 is expressed in adipocytes, hepatocytes[62] and Kupfer cells[135]

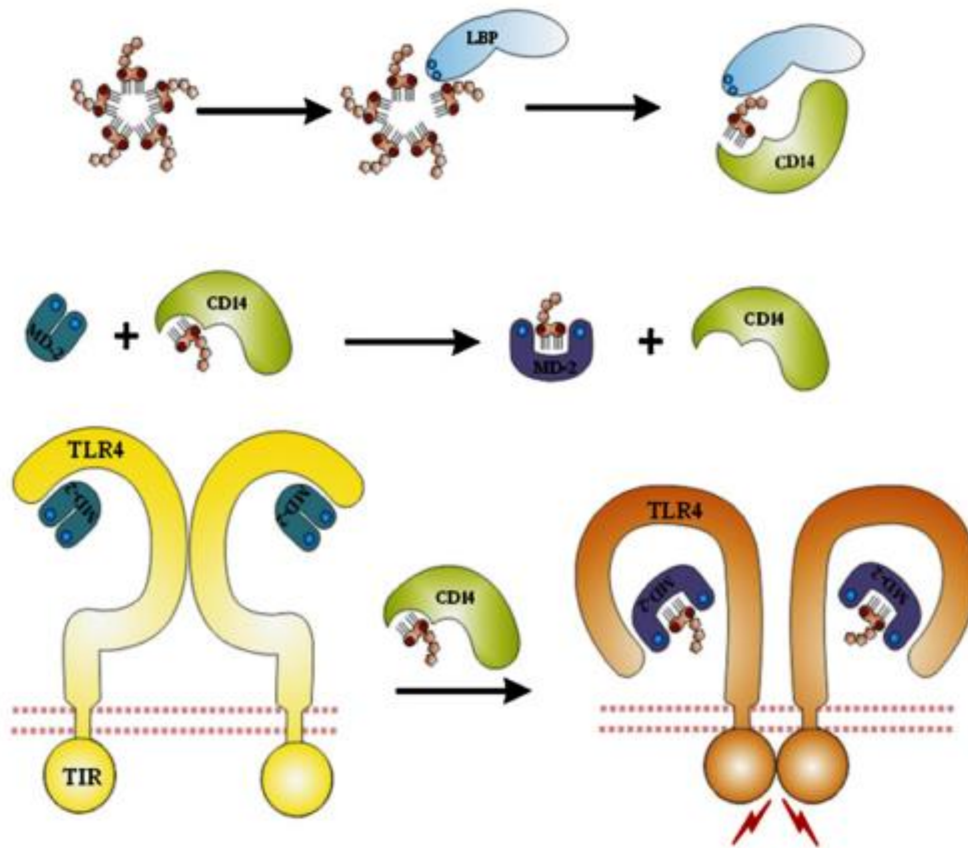


Figure 5 Overview of the TLR4 activation pathway by LPS recognition. Adopted from Jerala et al [156].

After its release from intestinal bacteria, LPS exists as multimolecular aggregates. Lipid binding proteins (LBP's) "dismantle" these aggregates and transfer individual LPS molecules to CD14 [136] and MD-2 (which binds exactly one LPS molecule per MD-2). It is important to note that LBP's are also involved with chylomicron metabolism, as both LPS and chylomicron have lipid A in their structure. There is also the possibility of a trimeric CD14:LPS:MD-2 complex as the final product of this pathway[118]. Activation of TLR4 is mediated by the CD14:LPS and MD-2:LPS complexes which leads to the inflammatory response.

The capacity of LPS to induce an immune response response is determined in part by the number of Lipid A "units" it contains. The higher the lipid A in LPS, the higher affinity it has to bind LBP [118],[137] (Figure 5) Theoretically, if there is excess lipid available, part of it may be converted into lipid A, which can be incorporated in LPS "assembly" thereby modifying its core and augmenting its inflammatory capacity. Toll like receptor

activation is also associated with recruitment of other inflammatory factors, mostly interleukins and tumor necrosis factor alpha TNF-α) [138]. The latter stands out since it is not dependent on macrophage activation to be produced (although macrophage activation also contribute). TNF-α, is associated with inflammation and is known to be involved with fructose induced metabolic syndromes [139]. However, since TNF-α secretion is triggered by a variety of factors, it is not known to what extent bacterial or endotoxin translocation contributes to elevated TNF-α levels in diet-induced insulin resistance.

1.3 Nuclear Receptors: a link between inflammatory factors and metabolism

Nuclear receptors are a class of molecules that act directly on the cell's nucleus and are tightly connected to metabolism. There are two main classes of these receptors: liver X receptors and peroxisome proliferator activated receptors. Liver X receptors (LXR) are nuclear receptors associated with regulation of cholesterol and lipid metabolism[140–146], They form heterodimers with Retinoic X receptors, and are also involved with peroxisome proliferator associated receptors[147,148]. However these receptors have a role that goes beyond metabolic regulation, as they are also implied in inflammatory response. The various isoforms of LXR [149], are known to be present in vital organs and cell types, specifically the liver, intestine, kidneys, adrenal glands, adipose tissue and macrophages [150]. This also contributes to the idea that nuclear receptors are important "players" in lipid metabolism, as all of the above deal with high access to lipids.

LXR activation[151] can happen by cholesterol and will lead to its efflux, until homeostasis is recovered. However in diseases like atherosclerosis this activation can be excessive in macrophages and lead to the downregulation of membrane cholesterol transporters and eventually lead to unhealthy lipid accumulation, this is what causes the formation of foam cells [140,152].

LXR's can a be activated via general inflammatory receptors, specifically interleukin receptors, toll like receptors and tumor necrosis factor receptor, the latter two are also activated by lipids [112,114,139,153,154]. LXR also doubles as an anti-inflammatory mediator when activated by these receptors [140,141,144–146,151,155]. This just shows that LXR as a nuclear receptor can adapt according to the activation it received, which ends up

being a double edged knife, because over stimulation can have different outcomes[156]. This can be seen in NAFLD, where it is known that LXR is expressed in abnormal levels [157].

1.3.1 Peroxisome Proliferator Activated Receptors

Peroxisome proliferator activated receptors (PPAR's) are orphan nuclear receptors, which are activated by free fatty acids[142,158], and much like LXR's they also deal with metabolism and inflammation. These receptors act as transcription factors that can induce changes in the expression of other genes when activated. It is known that there are different types of PPAR's and that each one has a different function, besides being present in a different site, in table 1 we can see more details on the matter.

Type of PPAR	Site of expression	Function	References
PPAR alpha	Liver, kidney, heart, endothelial cells, skeletal muscle, macrophage	Mediator of lipoprotein metabolism; anti-inflammatory effector, control lipid homeostasis in macrophages	149,159–161
PPAR beta/delta	Ubiquitously expressed	Regulates inflammatory response, lipid metabolism and cell proliferation, can modulate insulin sensitivity.	147,162–167
PPAR gamma	Adipose tissue, immune cells, dendritic cells, colon, spleen, smooth muscle, retina	Adipose differentiation, lipid metabolism homeostasis, modulator of insulin action, in charge of anti-inflammatory action	142,159,168–172

Table 1 Depiction of the PPAR types, location and function

Figure 6 shows a quick summary of how PPAR's work in the cell, when inflammatory bursts like TLR are laid out, but these are not the only events that trigger

the PPAR's. Lipid metabolism derivatives are one of the main activators of PPAR and each type of PPAR can be activated or repressed depending on what molecule binds to it. Leukotriene B4 and hydroxyeicosatetraenoic acid are known to successfully activate PPAR α, both these molecules come from arachidonic or linoleic acids after going through the cyclooxygenase and lipoxygenase pathways[173–176] and acyl-CoA's, which basically means that after going under oxidative metabolism these fatty acids may interact with PPAR, but most unsaturated fatty acids should be able to deal with at least one type of PPAR. PPARβ can be activated by 15-deoxy-prostaglandin and 15-HETE, prostacyclin, while PPAR γ is only known to be activated by 9-HODE (hydroxyoctadecadienoic acid) and 13-HODE. Co factor recruitment has also been described with the most studied one being rosiglitazone (an insulin sensitizer that also increases adiponectin[171]), that can act as an agonist for PPAR[170,177,178].

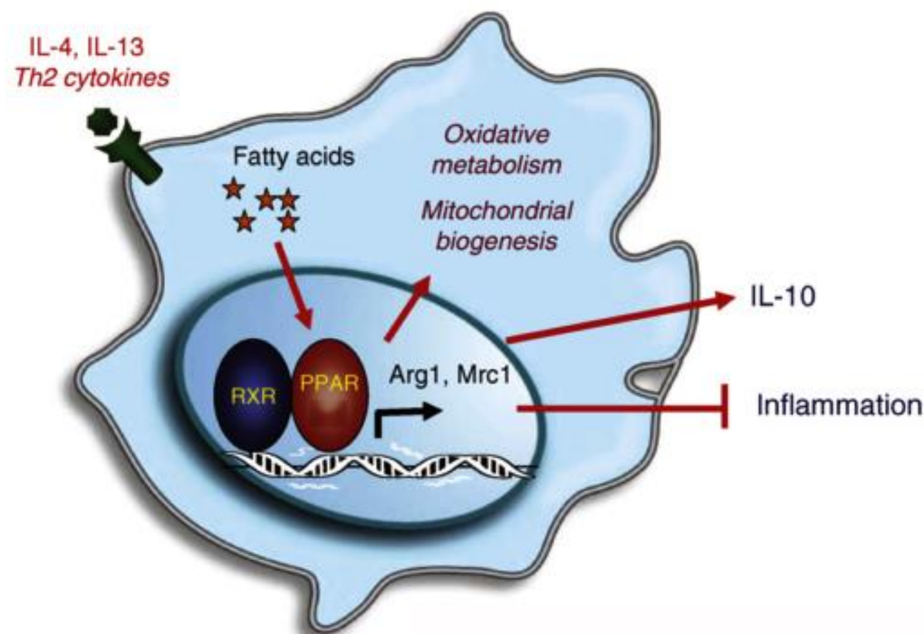


Figure 6 General representation of the PPAR pathways

Just like the liver X receptors, PPAR's also form heteromdimers with RXR, this will lead to some competion for binding sites and it has been reported that they can even downregulate each others expression[147]. After the PPAR has had stimulation it will proceed to bind with the peroxissome proliferator response element (PPRE) sequence (in

the nuclei) and depending on the PPAR form it will act on different regions and help express diferent proteins[179] (figure 7), PPAR β can even inhibit expression of the target genes for PPARα and PPARγ[180,181].

PPAR α is the most studied of this subfamily of nuclear receptors, and is known to promote expression of many proteins essencial for metabolic pathways, this can be particularly important in the liver. There are too many proteins regulated by this PPAR to mention here, but I will mention some of the most important. Regarding fatty acid metabolism , Acyl-CoA oxidase, one of the enymes that starts the β-oxidation process in acyl-CoA chains, ketogenic enzymes, fatty acid uptake genes (Abcd2 and Abcd3) , are related to PPAR α. Some mitochondrial uncoupling proteins and dehydrogenases are also expressed via PPAR α activation, as well as some lipogenic and some "major" fatty acid binding proteins like fatty acid binding protein 1 through 5. PPAR α can also help control lipoprotein pathways and lower fasting tryglicerydes and raise plasma high density lipids. Glucose and glycerol metabolism is also mediated by this receptor, as the genes for phosphoenolpyruvate kinase 1, pyruvate carboxylase and lactate dehydrogenase A are targets for PPAR α. These targets are just a small fraction of what PPAR α can interact with, it is also important to consider the regulation of bile acid production and amino acid metabolism are also a part of its many targets.

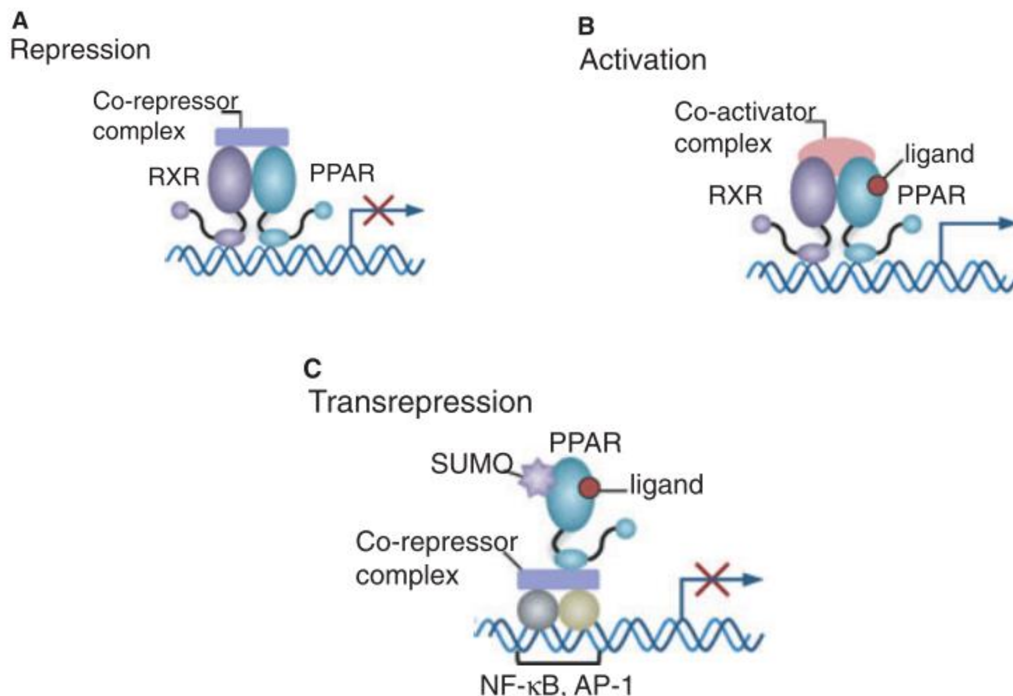


Figure 7 General representation of PPAR activation [84]

Just as LXR's, PPAR α has a strong connection with inflammatory targets, although its activity is far more anti-inflammatory than the remaining PPAR's. Just like the metabolical aspect of PPAR α only a part of the inflammatory genes will be highlighted, as this receptor also acts as an important regulation factor for many of these genes. C-jun and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) both transcription factors associated with inflammatory response to cellular stress [70,162,182] are inhibited by PPAR α. Fibronogen, C-protein, interleukin and TLR expression is also repressed by PPAR α activation[183–185]. As we can see PPAR is one of the best candidates for studying when considering diet induced liver damage, since it can stimulate fatty acid oxidation and downregulate hepatic inflammation.

PPAR β and γ action mechanisms are quite unclear at the moment, however there are some correlations that have already been made. PPAR β can act as a toxicity handler, and is known to protect the liver by regulating CD14, NF-kβ, calcium binding proteins, cyclin D1 and kinase signalling [162,186–189]. PPAR γ is even less studied, as only indirect evidence for its function has been provided yet, and little is known of its target genes. However, its connection with adipogenesis has been proved[168], as well as a relationship between this receptor and insulin sensitivity and beta-oxidation[172,188]. Thus, LXR's, PPAR's and cytokines work hand in hand during alterations in metabolic and inflammatory states. More generally, hepatic nuclear receptor expression feels the repercussions of altered intestinal microbiota.

1.8 Objectives / Scope of this work

As seen in previous sections there is justification in considering the effects of a fructose rich diet on the intestinal-liver axis as an important contributor to the development of fatty liver disease and insulin resistance. To date, indirect intestine-mediated mechanisms of hepatic injury from high fructose feeding have been much less studied in comparison to those attributed to direct modifications of hepatic intermediary metabolism. Therefore this study aims test the primary hypothesis that **high-fructose feeding results in and increased intestinal permeability** and the secondary hypothesis

that increased intestinal permeability is associated with **a pro-inflammatory expression of hepatic nuclear receptors and cytokines**.

2. Methods

2. Methods

2.1 Mice keeping and diet

In this study the experimental group mice were fed with 60 % fructose (the only sugar intake in this diet) and 40% of other nutrients for 12 weeks. This diet has a value of 3,6 kcal for gram, and for each 100 calories we have 20,2 calories of protein, 66,8 calories of carbohydrates and 12,9 calories of fat. After the 12 weeks mice were sacrificed, that time frame was used to evaluate metabolic and physiological changes, as it is common to have mice dieting for this long in diet based studies[26,93,96,105]. The control group was composed of mice fed with normal chow, which contains the following in g per kg of chow: casein, 200 g/kg; L-cysteine, 3 g/kg; corn starch, 397,486 g/kg; maltodextrin, 132 g/kg; sucrose, 100 g/kg; soybean, 70 g/kg; cellulose, 50 g/kg; mineral mix, 35 g/kg; vitamin mix, 10g/kg; choline bitartarate, 2,5 g/kg; tert-butylhydroquinone (antioxidant), 0,014 g/kg. The mix in normal chow corresponds to 3,8 kcal/g , and for 100 calories it has 18,8 calories from protein, 63,9 calories from carbohydrates and 17,2 calories from fat, which means that even though this diet is different from the high fructose diet, both have nearly the same amount of calories from fat, protein and carbohydrates. The diet in the control group is the standard rodent "baseline" for metabolic and nutritional studies.

2.2 Blood collection and Intestinal Permeability

The intestinal permeability assay used in this work, is based in the method described by Cani in 2009 [104]. It consists on using a fluorophore molecule, FITC-dextran, which has a high molecular weight (4.4 kDa) to acess whether or not there has been intestinal damage. The FITC-dextran was gavaged to mice, and one hour later blood was collected from the tip of the tail, to be treated with PBS buffer and read on a fluorometer. The fundament behind this assay is that FITC-dextran is not able to cross the intestinal barrier and leak out into the blood current in a healthy situation, at least not in a quantifiable way [104]. In a situation of intestinal leakage the probe will be able to get to the portal vein, and we are able to establish a relationship between fluorescence and leakage,

since higher concentrations of probe in the blood stream can be associated with higher leakage, which in turn correlate with a damaged intestinal mucosa.

The mice were gavaged with 6,5 µg of Fluorescein isothiocyanate-Dextran (FITC-Dextran), average molecular mass 4.4 kDa, after fasting for at least 4 hours. The mice rested for an hour before being subjected to infrared light (to cause vasodilation). With the aid of a surgical blade, the mice had their tail tip cut and 100 uL of blood was collected from it. The blood samples were centrifuged at 13,000 relative centrifuge force (RCF) for 10 minutes. Afterwards the supernatant plasma was recovered (50 µL) and added to equal volume of phosphate buffered saline (PBS) buffer.

These samples were transferred to a 96 well plate with clear bottom and read in the fluorometer. The excitation and emission wavelength were set at 425 nm and 535 nm respectively, for this is the setting used for fluorescence assays with FITC molecules [59,122,190–192]. The presence of FITC-dextran was quantified in each sample after obtaining a concentration curve of this molecule in blood (1:2 dilution), prepared according to literature[59]. Calculation of intestinal permeability was done by calculating the concentration of probe in the mice's blood, quantifying the probe for whole mice blood and then a comparison with the gavaged quantity.

2.3 Tissue Collection

After three months of dieting the mice were sacrificed for organ collection. The mice were sacrificed at the start of the light period and were therefore in the early postabsorptive phase. They were anesthetized with haloethanol and killed by cardiac puncture. Liver was collected in aseptic conditions to avoid bacteria leakage, fat was scraped from the samples collected to ensure the least metabolite and protein contamination. The livers collected were kept in -80º C.

2.4 Western Blots

Western blot was used to evaluate the quantity and to identify proteins present in samples[193–195], since it is a simple technique for obtaining intracellular protein expression levels, while quantifying them via fluorescence.

2.4.1 Cell lysate preparation

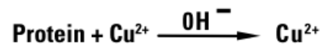
5 grams of liver for each sample were weighed and homogeneized in 400 uL of cold RIPA (radio immuno precipitation assay) Buffer. RIPA was prepared with 20 mM Tris HCl pH 7.4, 25 mM NaCl, 1% NP-40 (Nonidet P-40), 5 mM EDTA, 10 mM Sodium diphosphate (Na4P2O7), 10 mM Sodium Fluoride (NaF), 2 mM Sodium Vanadate Na3VO4, 10 µg ml-1 Aprotinin from bovine lung, 1 mM Benzamidine and 1 mM Phenylmethylsulfonyl fluoride (PMSF), (Sigma-Aldrich, St. Louise, MO, USA)) .

Cell lysates were homogeneized 3 times at 13500 rpm on a ULTRA-TURRAX® T 25 basic, IKA®-Werke (Staufen, Germany) homogeneizer, to disrupt cellular structure. The next step was to centrifuge the samples at 13,000 g at 4ºC. The supernatant was collect and centrifuged again. This procedure was repeated for 2 times to make sure the liver's lipids and other contaminants were properly discarded in the pellet.

2.4.2 Protein Quantification

The protein quantification of the lysates was assessed by the bicinchoninic acid assay[196–198] (BCA assay). This assay's principle revolves around copper ion reduction (Cu^{2+} to Cu^{+}) in an alkaline medium (figure 8). After copper was added to the protein in solution (in Cu^{2+} form), it made a blue colored compound, copper reduction occurred, and the Cu^{+} was detected by colometry via bicinchoninic acid.

Step 1.



Step 2.

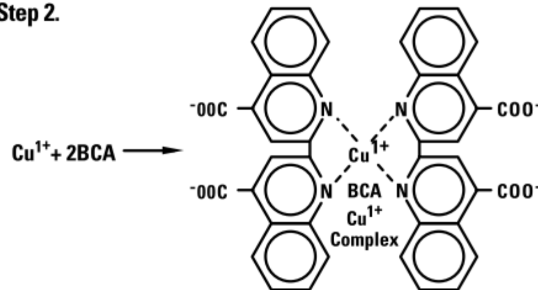


Figure 8 Schematic representation of the BCA protocol. Adapted from Thermo Scientific Pierce's "Protein Assay Technical Handbook Version 2"

Relative amount of protein expressed in the liver was quantified by Western Blot analysis. 20 ug of protein was used in each Western Blot, except when using GLUT2 antibody, in which was used 5 µg of protein.

2.4.3 SDS-PAGE

The Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a commonly used method for observing protein mass differences on an acrylamide gel. This method is based around the SDS compound, which when mixed with proteins in solution will charge every protein in sample negatively, and make them have the same charge density, i.e. every protein will have the same negative charge per mass. Afterwards, the proteins are added to a gel and "run" against an electric field, which makes them migrate from negative to positive pole (in the gel) and separates them by mass, due to the SDS properties.

Before performing this technique the liver had to be prepared in order to be added to the acrylamide gel. Samples were unfrozen and diluted in Sample Buffer (10% β-

mercaptoethanol, 4 % SDS, 250 mM Tris pH 7,4 , 20% glycerol , bromophenol,blue, pH 6,8) before being denatured at 95º C for 5 minutes.

In order to perform the SDS-PAGE protein separation a 12 % acrylamide gel was prepared (0,375 M Tris pH 8,8 ; 0,2 % SDS and 0,1 % 2-Acrylamido-2-methylpropane sulfonic acid (AMPS)) as a "running gel" and a 4 % acrylamide gel ("stacking" gel) (0,16 M Tris, 0,1 % SDS, 0,05 % AMPS). The selected electric current for the proteins was 130 V, and the gel ran vertically in the appropriate buffer (125 mM Tris, 0,96 M Glycine, 0,5% SDS, pH 8,3) for about 1 hour and a half, or until the bromophenol blue from the sample buffer had left the gel.

2.4.4 Electric Transfer to PVDF Membrane

Afterwards the samples were transferred from gels to polyvinylidene fluoride (PVDF) membranes. This transference lasted for 1 hour at 100 Volts, and was mediated by transference buffer (100 mM CAPS pH 11).

2.4.5 Antibody blocking and detection

The membranes were then washed with distilled water and blocked in 5% milk with PBS before incubating the primary antibody (GLUT2, PPARα and PPARβ diluted in 1:1000 while Tnf-α had a 1:500 dilution, all antibodies were in 5% milk) overnight in cold. Afterwards the membranes were washed, blocked and then incubated at room temperature with secondary antibody (1:5000 anti-rabbit)

Antibody binding was revealed with chemoluminescence, using ECF as the luminescent molecule. The secondary antibody had alkaline phosphatase attached to its terminal, this way when ECF is added luminescence can be observed in the form of a yellow shade on the membranes and was revealed via Versa Doc model 3000 by BioRad, which also allows for relative protein expression quantification.

2.5.4 Results analysis

All results are presented in a mean plus standard error of the mean (SEM) using Graph Pad, version 6 (GraphPad Software, San Diego, CA, USA). When comparing two groups (control vs. fructose rich diet) against the hypothesis that they are different the Mann-Whitney-Wilcoxon test was used. Differences were considered significant when $p < 0.05$.

3. Results

3. Results

3.1 Weight Gain

Mice weight was controlled throughout the study, it was measured once each two weeks. In figure 9 we can see how weight varied between normal chow and fructose rich diet fed mice for 10 weeks of dieting, it is important to note that all mice were used for this measurement.

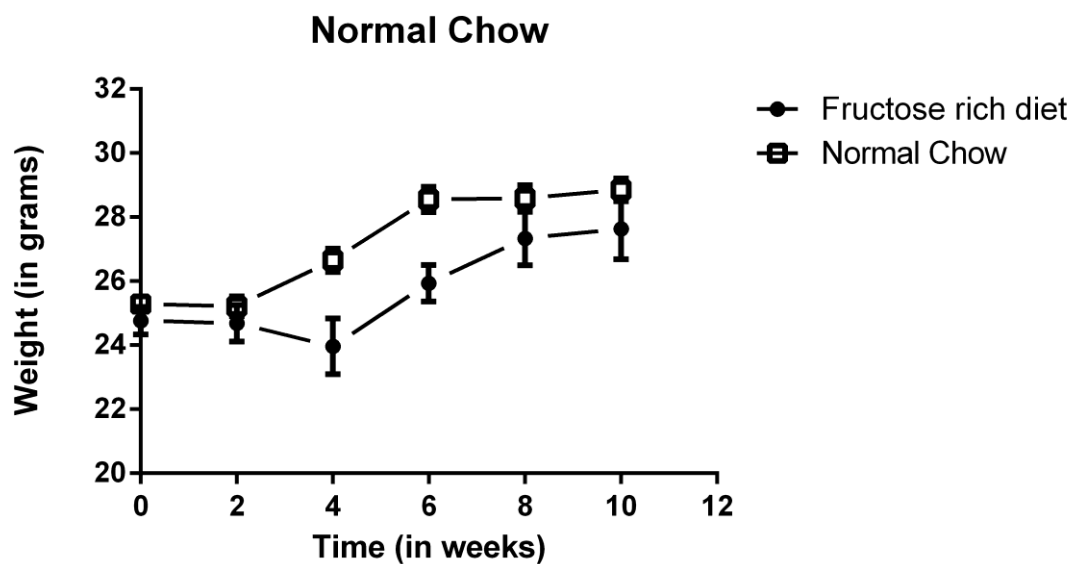


Figure 9 Weight control of mice from the two diet groups for 10 weeks (N=6 per group)

While overall weight gain was not significantly different between the two groups, the fructose fed mice had a tendency not to gain weight over the first four weeks, but then showed comparable rate of weight gain over the last four weeks. This difference may reflect some degree of metabolic adaptation to high fructose feeding.

3.2 Intestinal Permeability

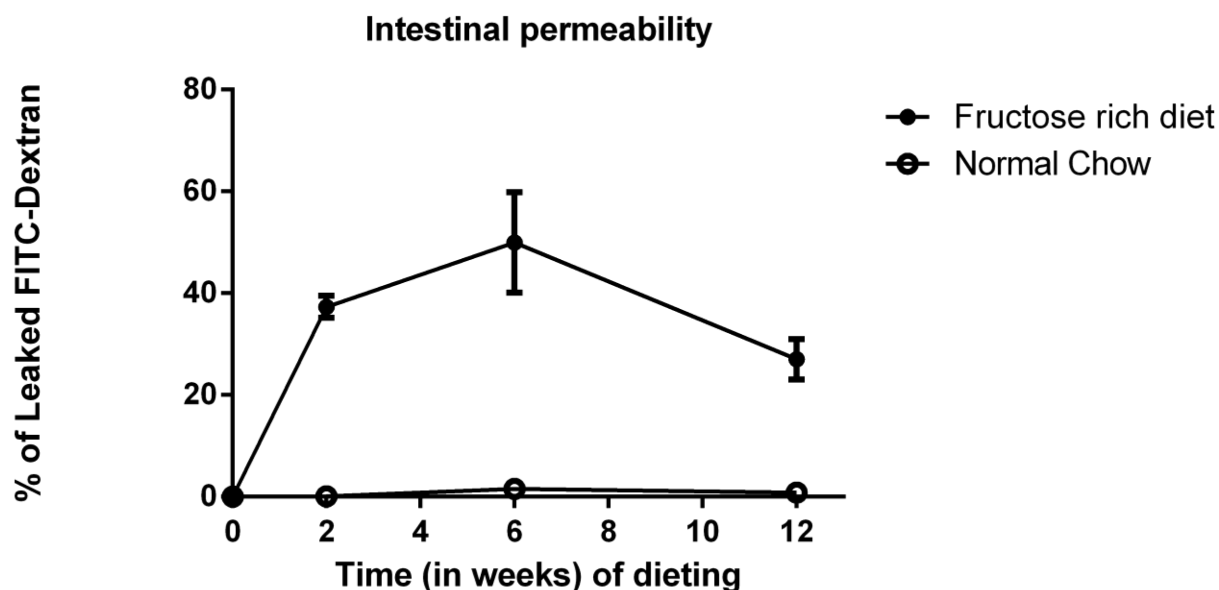


Figure 10 Representation of the intestinal leakage of FITC-Dextran through time with standard error mean. The number of animals used per group were 6.

Intestinal permeability was evaluated in 3 time points in mice, after two, six and twelve weeks of dieting (figure 10). Intestinal leakage values for mice fed with control diet were residual, in agreement to results described by Cani in 2009 [59] for this intestinal permeability assay. In week 2 there was already a significant difference between control and high fructose diets, as the experimental group had values of leakage 37,32 ± 2,149% , while the control group had only 0,07448 ± 0,03576% ($P < 0{,}0001$). In week 6 this difference was more aggravated as the high fructose fed group had leakage in the 49,96 ± 9,884% , in contrast with the control group , whose leakage also rose, and had a value of 2,352 ± 0,01685% ($P=0{,}0029$). By week 12 the intestinal permeability of the high fructose diet had decreased, (27,01 ± 3,961%) but remained significantly higher than , the control group had a (0,8657 ± 0,4707% , $p< 0{,}0001$).

Changes in permeability appear to be associated with weight changes in mice. For instance, in week 2 we can see a slight decrease in the weight of both groups, but a significant change in leakage of FITC-dextran was observed in fructose fed mice at this time point, as well as in week 6 when leakage reached its maximum value, which

corresponds to a weight increase in both groups. However a decrease in leakage can be observed in week 12 to the lowest values in this study, which also matches the reach of a weight threshold in both groups.

3.3 Western Blots

All results for Western Blots are the result of a repletion of 4 different experiments with all mice (N=6) for each group (high fructose diet vs. normal chow), and were tested against "Mann-Whitney" statistical test and "Student t-test".

PPAR α

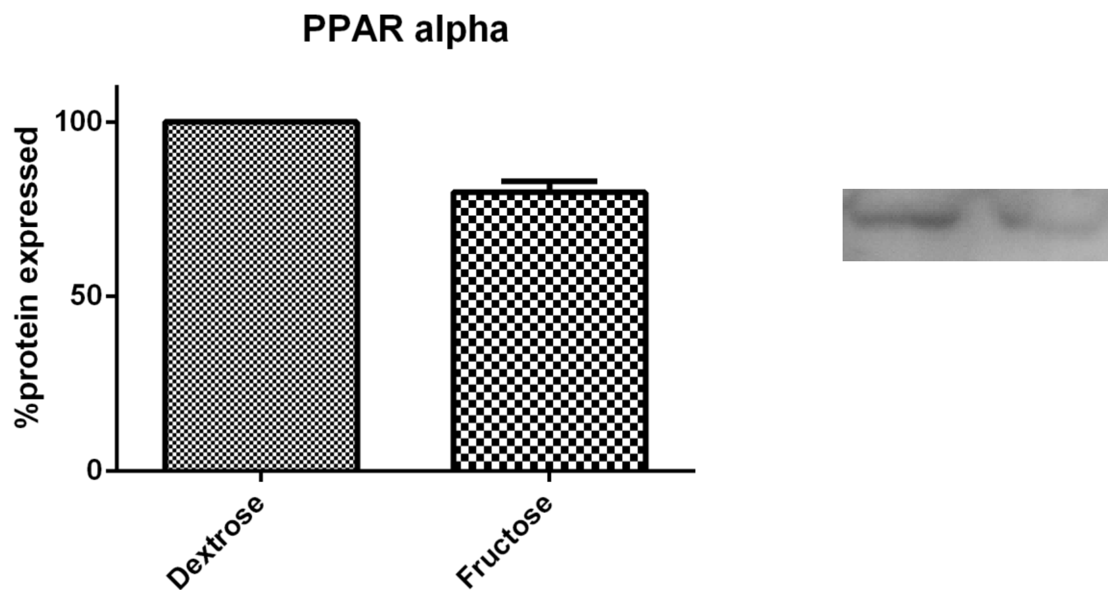


Figure 11: Representative Western blotting (six different mice from each group) showing the hepatic protein content of PPARα. Densitometry analysis of specific bands from all mice studied is shown. Values are expressed as a percentage relative to Dextrose (100 %) and are means versus SD. Equal loading of the gels was as demonstrated by probing the membranes with an anti-β-actin polyclonal antibody. P value was 0,0290 for unpaired t-test. Proteins bands are shown at the right of the figure.

Expression levels of PPAR α (Figure 11) were decreased in the liver of fructose rich diet fed mice when compared with the control. The levels obtained of PPAR- α in the experimental group were of 79,77 ± 3,120 (P=0,029)

PPARβ

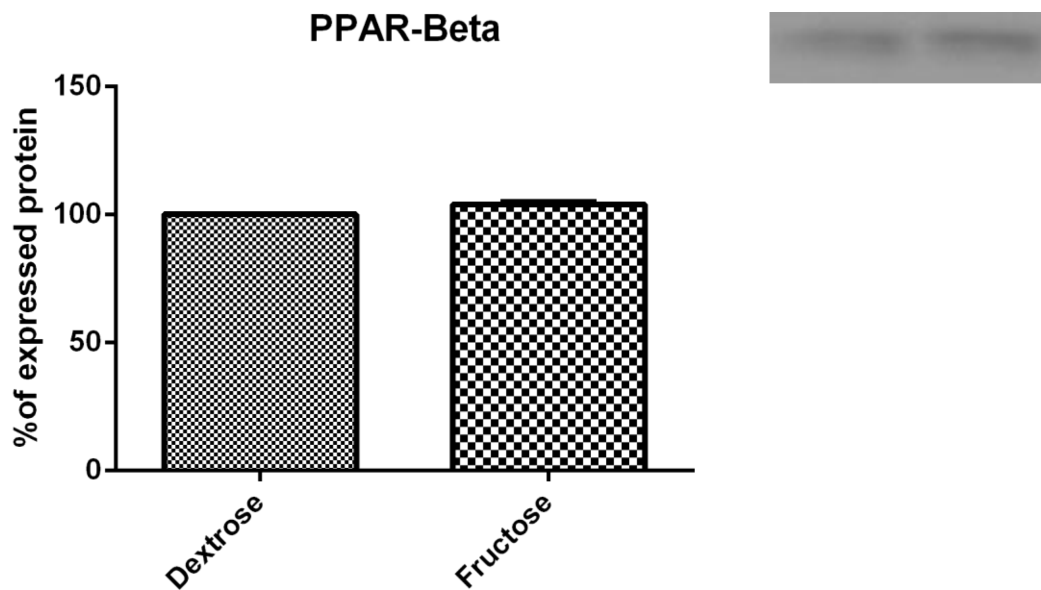


Figure 12: Representative Western blotting (six different mice from each group) showing the hepatic protein content of PPARβ. Densitometry analysis of specific bands from all mice studied is shown. Values are expressed as a percentage relative to Dextrose (100 %) and are means versus SD. Equal loading of the gels was as demonstrated by probing the membranes with an anti-β-actin polyclonal antibody. P value was 0,0076 for unpaired t-test. Proteins bands are shown at the right of the figure

Levels of PPAR-β in the liver of the test group showed small but significant differences from the control group, even though it has been described that it helps regulate

toxicity in the liver[186]. The experimental group had levels of 103,9 ± 0,9966 (P=0,0076) of PPAR- β (figure 12).

Tumor necrosis factor α (TNF-α)

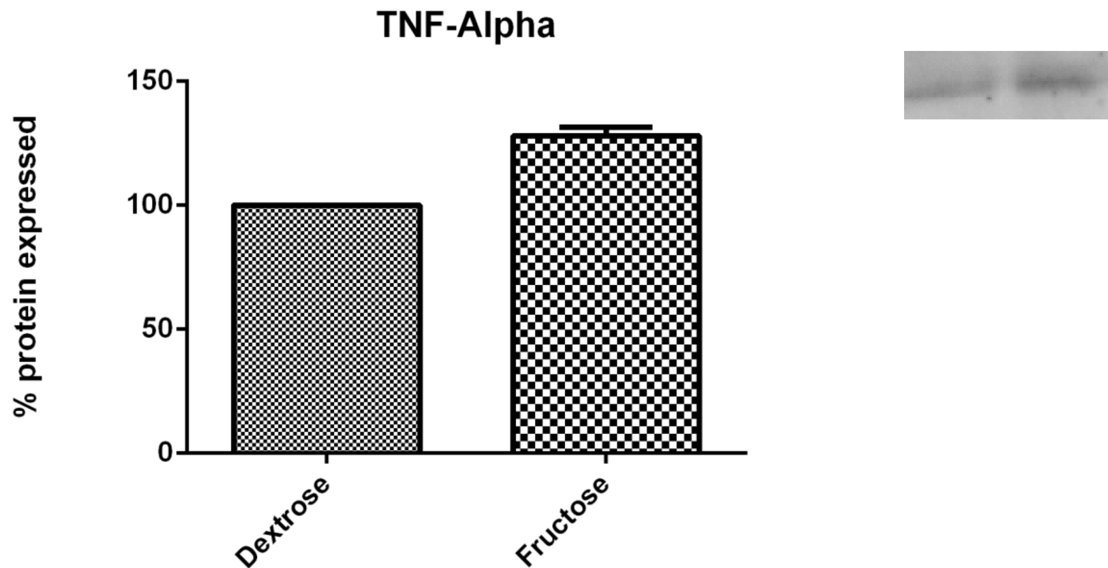


Figure 13: Representative Western blotting (six different mice from each group) showing the hepatic protein content of TNF-α. Densitometry analysis of specific bands from all mice studied is shown. Values are expressed as a percentage relative to Dextrose (100 %) and are means versus SD. Equal loading of the gels was as demonstrated by probing the membranes with an anti-β-actin polyclonal antibody. P value was 0,0002 for unpaired t-test. Proteins bands are shown at the right of the figure.

In figure 13 is shown that the expression of TNF- α in the liver of the test group has increased by nearly 30 % when compared to the control. Expression levels obtained for the experimental group were 127,9 ± 3,6 (P=0,0002).

Glucose transporter 2 (GLUT2)

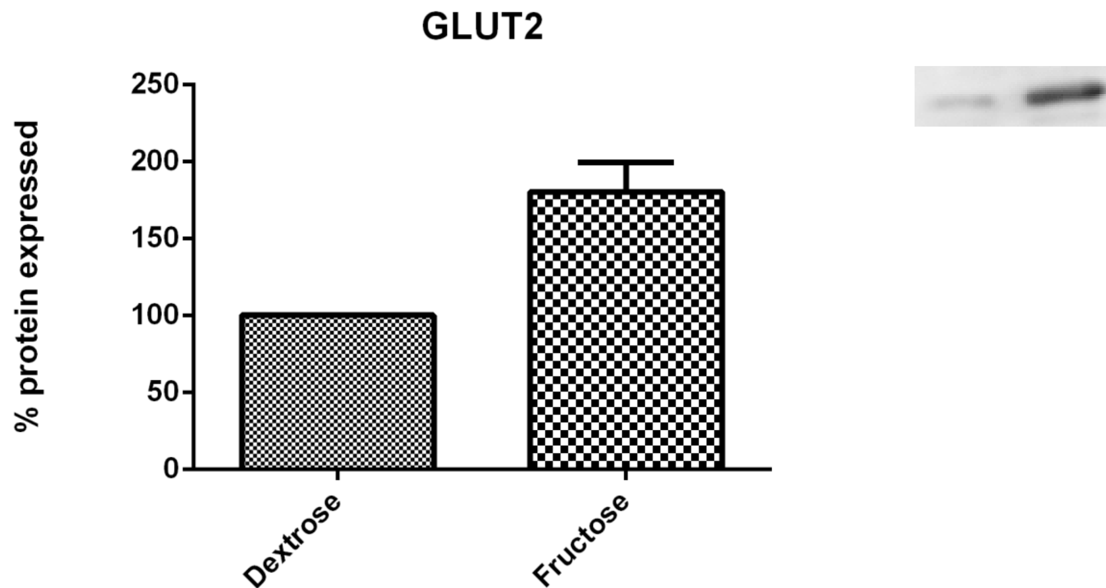


Figure 14 Representative Western blotting (six different mice from each group) showing the hepatic protein content of GLUT2. Densitometry analysis of specific bands from all mice studied is shown. Values are expressed as a percentage relative to Dextrose (100 %) and are means versus SD. Equal loading of the gels was as demonstrated by probing the membranes with an anti-β-actin polyclonal antibody. P value was 0,0055 for unpaired t-test. Proteins bands are shown at the right of the figure

GLUT2 expression in the liver (figure 14) was increased almost two fold in the fructose rich diet group, with its expression levels around 180,4 ± 19,0 (P=0,0055).

4. Discussion

4. Discussion

The difference in intestinal permeability between high fructose diet fed mice and normal chow fed mice is quite substantial, and it's clear that the experimental group had many times the amount of intestinal leakage that the control group at all times.

An increase in intestinal permeability with high fructose feeding has not been reported yet, even though there had already been some speculation on the matter [199,200]. The increase in permeability for the first six weeks can be assigned to the fact that fructose induces production of short-chain fatty acids, hydrogen and butyrate in the intestine, all of these metabolites were already know to be associated with luminal distension, and therefore could be associated with increasing intestinal leakage. The decrease in leakage in week 12 can probably be associated with host adaptation to the fructose rich diet, as the intestine's bacteria can respond to this diet by causing small intestine overgrowth[74] or by changing its species[201]. It is also implied that there could be fructose malabsorption related problems due to this fructose induced small intestine overgrowth[72], which can lead to a deficiency in fructose transporters in the enterocytes[30], this may as well contribute to a threshold in fructose absorption. Considering the impact a change in intestinal microbiota can have in the host[61,182] , and the fact that the host can adapt its mucosal immunity to this change[154] it is plausible for a decrease in leakage to occur in week 12.

The fact that intestinal leakage is increased in fructose rich diet has implications for the liver, as this increase means that bacterial translocation is made easier, and that there is an increase in the quantity of intestinal blood barrier crossing endotoxins and metabolites, all of these can easily reach the liver via portal vein. These pro-inflammatory agents, combined with the increased lipogenesis associated with high fructose ingestion, can lead to the development of NAFLD and NASH[10].

A decrease in PPAR α could reflect increased transport of fatty acids and triglycerides from the gut into the liver. This increase will likely induce the repressors for PPAR α, and down regulate anti-inflammatory proteins and factors, besides leading to increased synthesis of lipoproteins. A decrease in this anti-inflammatory receptor is

also in agreement with reports of increased inflammation (and higher production of cytokines) in NAFLD [157].

Since the production of cytokines can induce the production of mitogen activated kinase (MAPK), which can phosphorylate and repress PPAR-β[202,203] , it is possible that these levels of expression of PPAR-β result from a balance of increased levels due to toxicity (following intestine leakage[204]) against increased repression of PPAR-β via phosphorylation. This may lead to this maintenance of PPAR-β levels in the liver, even though PPAR-α levels are altered.

The increase in Tnf-α expression concurs with the data shown in figure 11 and 12, as an increase in levels of this cytokine was reported for inhibiting PPAR- α expression in the liver[205]. This increase likely reflects higher intestinal leakage of endotoxin and other bacterially-derived pro-inflammatory agents which activate the immune system and lead to Tnf- α production. This represents a mechanism that links toll like receptor activation and fructose induced steatosis [206].

The increase in GLUT2 expression is possibly due to the overproduction of hepatic glucose from gluconeogenic fructose metabolism. Increased GLUT2 expression would mediate a more effective export of hepatic glucose into the bloodstream[207]. This result is in agreement with older research in which fructose had been associated with upregulation of GLUT2 mRNA in a hepatocyte cell line[208].

In table 2 the expression and possible causes and effects of the measured protein expressions are summarized. The increase in Tnf-α[157] and the decrease in PPAR-α[209] are in agreement with the inflammatory effects felt in NAFLD, as well as some of its metabolic outcomes, like the increase in lipogenesis. PPAR β had not been described yet in this disease.

	Average expression level ± Standard Deviation	**Caused by**	**Effects**
PPAR-α	79,8 ± 3,1	Repressed by excess of fatty acids and TAG's;	Less anti-inflammatory factor expression ; Less lipid catabolism
PPAR-β	103,9 ± 2,0	Increase due to liver toxicity; Repression by cytokines	Higher expression of pro inflammatory factors:
Tnf- α	127,0 ± 7,2	Increased by excess of fatty acids and of bacterial byproducts from the intestine; Possible macrophage infiltration	Activation of inflammatory pathways;
GLUT2	180,0 ± 38,0	Increased by overload of fructose	Higher levels of fructose and glucose on the bloodstream and liver

Table 2 Analysis of each protein expression along with the causes and effects of different expressions

5. Conclusions

5. Conclusions

The effect of a high fructose diet on intestinal permeability is yet to be fully established, but the data on the present work are a step forward in unravelling what happens in this complex system. Furthermore, this also serves to show how important the intestine can be in forming metabolic diseases liver and how both can interact with each other. This also leads to the conclusion that fructose metabolism in the intestine has quite an impact on the mucosa itself, as leakage increased at a fast pace. The differences in the intestinal leakage between groups can point out to the importance that the intestine's bacteria can have as therapeutic target for NAFLD and NASH, as modulation of the flora affects what is leaked into the portal vein and may alter the outcome of liver disease.

Putting everything into perspective, a fructose rich diet was capable of altering the intestinal permeability of our mice after a short period of time (figure 10) to high levels of leakage. Besides giving information on how the intestine barrier is, this also points out to a possible increase in the production of hydrogen, carbon dioxide and short chain fatty acids by bacteria, after consuming fructose. This alteration will lead to a higher leakage of fatty acids, fructose and other metabolites into the blood stream, besides leaking toxins and bacterial by-products that may lead to an increased inflammatory response (figure 4). The hepatic portal vein delivers molecules to the liver before any other organs, therefore indicating that changes in the liver's metabolome and proteome are also linked to increased intestinal permeability. High amounts of fatty acids in the liver eventually leads to metabolic stress, causing PPAR alpha shutdown (figure 15). This will also contribute to higher TNF-alpha levels. High fructose access by the liver is indicated by the up regulation of GLUT2, which is overexpressed in order to regulate hepatocytes intracellular glucose levels and glucose homeostasis. All these changes will contribute to the development to NAFLD, and may even lead to NASH and cirrhosis in liver, besides a possibility for insulin resistance and small intestine overgrowth.

Concluding, there is still the need to look deeper into the intestine to figure out what bacteria species are associated with this increase in leakage in dietary changes, what is in fact the main product leaking through the intestinal blood barrier, but mostly what is more devastating for the host's liver, if the bacterial translocation or the toxins and metabolites that are responsible for this metabolic effects. For the last, it is also quite important to "pin down" the mechanisms behind fructose induced enterocyte tight junction destruction.

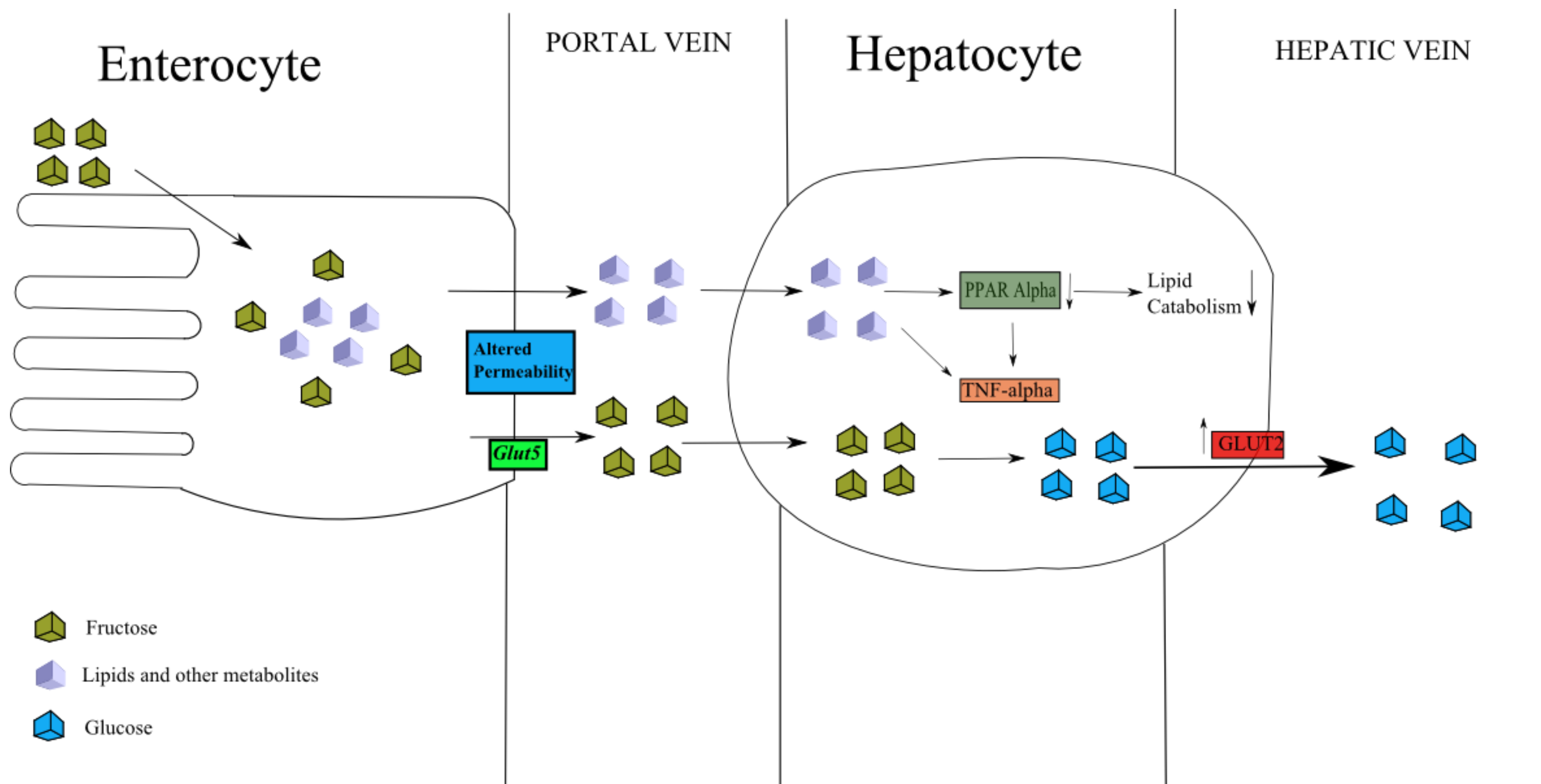


Figure 15 Representation of what is thought to happen in a fructose rich diet on the gut and the liver.

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