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FACULTY OF HEALTH AND MEDICAL SCIENCES

PhD Thesis • 2017 • Karen Ekkelund Petersen

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LIFEPHARM
NOVO NORDISK & LIFE IN VIVO PHARMACOLOGY CENTRE

IN VIVO PHARMACOLOGY
GRADUATE PROGRAMME FOR IN VIVO PHARMACOLOGY & EXPERIMENTAL ANIMALS

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**Front page:** Pictures were provided by Novo Nordisk A/S

# Preface

This PhD project was performed in The Graduate Programme for In Vivo Pharmacology and Experimental Animals and was funded by the LIFEPHARM Centre for In Vivo Pharmacology (www.lifefarm.dk) together with Novo Nordisk A/S. The work has been carried out at the University of Copenhagen, Frederiksberg Campus, Denmark and Novo Nordisk A/S, Måløv, Denmark under the supervision of Jens Lykkesfeldt, Günaj Rakipovski and Kirsten Raun.

Animal studies were approved by the Animal Experiments Inspectorate, Ministry of Food, Fisheries and Agriculture, Denmark and carried out in accordance with the EU directive 2010/63/EU on the Protection of Vertebrate Animals used for Scientific Purposes, and the Danish Animal Experimentation Act (LBK 1306 from 23/11/2007 with 2011 amendments). The animal studies were carried out at Novo Nordisk A/S, Måløv, Denmark.

# Papers

The following papers are included in this thesis and are referred to by their Roman numerals:

**Paper I (I)**

**Does glucagon-like peptide-1 ameliorate oxidative stress in diabetes? Evidence based on experimental and clinical studies.**

Petersen KE, Rakipovski G, Raun K, Lykkesfeldt J.

Current Diabetes Reviews 2016; 12(2):1-28.

**Paper II (II)**

**The effect of liraglutide on oxidative stress induced by fluctuating hyperglycemia in Sprague Dawley rats.**

Petersen KE, Lykkesfeldt J, Raun K, Rakipovski G.

*Manuscript.*

**Paper III (III)**

**Plasma lipid oxidation predicts atherosclerotic status better than cholesterol in diabetic apolipoprotein E deficient mice.**

Petersen KE, Lykkesfeldt J, Raun K, Rakipovski G.

*Experimental Biology and Medicine 2017; 242(1):88-91*

**Paper IV (IV)**

**High dietary cholesterol improves glucose tolerance in low-density lipoprotein receptor deficient mice – comparison of two standard high fat diets.**

Petersen KE, Lykkesfeldt J, Kirk R, Raun K, Rakipovski G.

*Submitted to British Journal of Nutrition.*

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At Novo Nordisk I want to thank Louise Justesen and Jane Larsen for great assistance in the performance of animal studies and for always having the time to answer questions. Kent Pedersen, thank you for remarkable technical support with Accusampler and computer problems. Karen Juhl and Elene Carlsen, thank you for helping me solve both practical and technical issues. Helle Andersen, thank you for always having time for any questions and for the many good talks in the locker rooms. I am very grateful to Niels Lykke Munksgaard Rasmussen for being a mentor during qPCR analyses and for having a positive attitude to my initiatives in your laboratory. At the University, I would like to thank Annie B. Kristensen, Joan Frandsen and Belinda Bringtoft, who have provided excellent technical assistance in several analyses.

My fellow PhD colleagues and office buddies Søs, Bill, Trine, Marina, Ida, Victoria, Søren, David, Stine, Maiken and Vivi; all of you have added value to my time as a PhD student. We have had great scientific discussions and personally I have enjoyed a lot of great times with you all. Bill, two hours of daily biking have given us time for scientific discussions, which have been of great value to me. I have always been able to count on your support throughout my PhD – your friendship means a lot to me. Thank you for your constructive criticism and feedback throughout my PhD project and in relation to my thesis. Søs, even though you practically live on the other side of the world you have been able to be an important part of my life and you always will be.

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# Table of contents

# Summary

Diabetes is a serious threat to affected patients as the disease is associated with complications that compromise quality of life and increase mortality. The huge impact on global health is underlined by the fact that 415 million people are estimated to have diabetes worldwide. In diabetes the complications include both microvascular (retinopathy, neuropathy and nephropathy) and macrovascular (cardiovascular diseases) diseases. The latter is the major cause of death in diabetic patients.

Oxidative stress occurs when excess reactive oxygen species are present relative to the level of antioxidant. Lipid oxidation may be initiated when oxidants attack lipids, leading to the formation of lipid oxidation end-products, such as malondialdehyde and isoprostanes. In both type 1 and type 2 diabetes, hyperglycemia, reduced glucose tolerance and dyslipidemia may increase oxidative stress, potentially resulting in lipid oxidation. Impairment in the control of blood glucose, and the accompanying variations in blood glucose levels, has shown to increase oxidative stress. This suggests a link between diabetes and the development of oxidative stress. Oxidative stress and lipid oxidation play a role in the progression of diabetic complications, including the macrovascular complications such as atherosclerosis. The degree of oxidative stress in various animal models of diabetes was investigated in this thesis.

The connection between oxidative stress and diabetic complications implies that therapies aimed at ameliorating reduction oxidation imbalance might benefit patients with diabetes, potentially by reducing progression of diabetic complications. Glucagon-like peptide-1 therapy corrects hyperglycemia, hyperglycemic excursions, improves glucose tolerance and could thus lower oxidative stress level. Beneficial glucose-independent effects of glucagon-like peptide-1 have also been indicated in various studies. Recently, the investigation of these effects have become even more warranted as the glucagon-like peptide-1 analogue, liraglutide, has shown to significantly reduce the risk of cardiovascular events in a large clinical trial following 9340 type 2 diabetic patients in high risk of developing cardiovascular disease. Consequently, Study I examined the effect of glucagon-like peptide-1 on oxidative stress in Sprague Dawley rats. Unfortunately, it was not possible to successfully induce oxidative stress or lipid oxidation. Hence, no conclusions on the effects of liraglutide on oxidative stress or lipid oxidation could be made.

Study II was set up to investigate the relationship between lipid oxidation and plaque formation in mice developing atherosclerosis. In this study the angiotensin converting enzyme inhibitor, enalapril was used as a pharmacological tool hypothesized to decrease the development of oxidative stress and atherosclerosis. A positive correlation between plasma malondialdehyde concentration

and aortic plaque area was found in apolipoprotein E deficient mice, but inhibiting angiotensin converting enzyme did not significantly decrease neither oxidative stress nor atherosclerosis. Study III investigated the effect of dietary cholesterol on diabetes, lipid oxidation and atherosclerosis in low density lipoprotein receptor deficient mice. Dietary cholesterol has been suggested to impact disease development in both human and pre-clinical models and a more detailed understanding of the interplay between diet and disease development is needed. The cholesterol enriched diet used in Study III increased atherosclerotic development, hepatic steatosis, and hepatic fibrosis, but the animals fed this high cholesterol diet had lower lipid oxidation status and better glucose tolerance than those fed a low cholesterol diet. The latter was potentially induced by regulation of hepatic genes involved in glucose and lipid metabolism.

Overall, the main conclusion of this thesis is that lipid oxidation is elevated in diabetes and can play a role in the development of atherosclerosis. Worth noting is that this thesis stresses the importance of the diabetic status, dyslipidemic status, choice of animal model and diet when analysing the relationship between atherosclerosis and lipid oxidation.

# Sammendrag (dansk)

Diabetes er en alvorlig trussel mod de berørte patienter, da sygdommen er forbundet med komplikationer, der kompromitterer livskvaliteten og øger dødeligheden. Den enorme indvirkning på den globale sundhed understreges af det faktum, at 415 millioner mennesker skønnes at have diabetes på verdensplan. Diabetiske komplikationerne omfatter mikrovaskulære (retinopati, neuropati og nefropati) og makrovaskulære (kardiovaskulære sygdomme) konplikationer, med den sidstnævnte som den hyppigste dødsårsag hos diabetespatienter.

Oxidativt stress opstår, når reaktive oxygen species er i overskud i forhold til antioxidanter. Lipid-oxidation kan opstå når oxidanter angriber lipider, hvilket leder til dannelsen af lipid-oxidations-slutprodukter, såsom malondialdehyd og isoprostaner. I både type 1 og type 2 diabetes er hyperglykæmi, nedsat glukosetolerance og dyslipidæmi faktorer, der kan øge oxidativt stress og potentielt resultere i øget lipid-oxidation. Forringet kontrol af blodglukose, og deraf følgende variationer i blodglukose-koncentrationer, har vist sig at øge oxidativt stress. Det tyder altså på at der findes et link mellem diabetes og oxidativt stress. Da oxidativt stress og lipid-oxidation har vist sig at spille en rolle i udviklingen af diabetiske komplikationer, inklusiv de makrovaskulære komplikationer, såsom åreforkalkning, bliver graden af oxidativt stress i forskellige diabetiske dyremodeller undersøgt i denne afhandling.

Forholdet mellem oxidativt stress og diabetiske komplikationer antyder at behandlinger rettet mod at reducere reduktions-oxidations-ubalance kan være en fordel for patienter med diabetes og potentielt reducere udviklingen af diabetiske komplikationer. Glukagonlignende peptid-1-terapi korrigerer hyperglykæmi, hyperglykæmiske fluktuationer, forbedrer glukose-homeostase og kan potentielt nedsætte oxidativt stress niveau. Fordelagtige glukose-uafhængige effekter af glukagonlignende peptid-1 er blevet indikeret i flere studier. For nylig er undersøgelse af disse gavnlige effekter blevet berettiget i højere grad, da glukagonlignende peptid-1-analogen, liraglutid har vist sig at sænke risikoen for tilfælde af hjertekarsygdomme signifikant i et stort klinisk forsøg, som har fulgt 9340 type 2 diabetikere i høj risiko for at udvikle kardiovaskulær sygdom.

Derfor blev der i Studie I i denne afhandling undersøgt effekten af glukagonlignende peptid-1 på oxidativt stress i Sprague Dawley rotter. Men i den aktuelle undersøgelse var det ikke muligt at fremkalde oxidativt stress eller lipid-oxidation, og dermed kunne der ikke drages konklusioner på virkningen af liraglutid. Studie II blev sat op for at undersøge forholdet mellem lipid-oxidation og dannelsen af åreforkalkning i mus, som udvikler sådanne forandringer. I dette studie blev angiotensin-konverterende-enzym-inhibitoren, enalapril brugt som et farmakologisk redskab, ud fra en hypotese om at enalapril kunne sænke oxidativt stress niveau og åreforkalkning. En positiv

korrelation mellem plasma malondialdehyd-koncentration og åreforkalkning i aorta blev fundet hos apolipoprotein E-deficiente mus, men inhibitionen af angiotensin-konverterende-enzym gav hverken et signifikant nedsat oxidativt stress niveau eller grad af åreforkalkning. Studie III undersøgte effekten af kostens indhold af kolesterol på diabetes, lipid-oxidation og åreforkalkning i low density lipoprotein receptor-deficiente mus. Det er blevet foreslået at kostens indhold af kolesterol har en betydning for udvikling af sygdom i humane og prækliniske modeller. En mere detaljeret forståelse af samspillet mellem diæt og sygdomsudvikling er påkrævet. Den kolesterol-berigede diæt brugt i Studie III øgede udviklingen af åreforkalkning, hepatisk steatose og hepatisk fibrose, men dyrene der blev fodret med denne kolesterol-berigede diæt havde lavere lipid-oxidation-status og bedre glukose-tolerance end dyrene der blev fodret med kolesterol-fattig diæt. Det sidstnævnte kunne potentielt skyldes en regulering af hepatiske gener involveret i glukose- og lipid-homeostase.

En samlet konklusion på denne afhandling er at lipid-oxidation er øget i diabetes og kan spille en rolle i udviklingen af åreforkalkning. Men den diabetiske status, dyslipidæmiske status, valg af dyremodel og kost er meget relevante parametre for resultatet og vigtige at tage højde for når man analyserer på forholdet mellem åreforkalkning og lipid-oxidation.

# Abbreviations

| | |
|---|---|
| ACCORD | Action to Control Cardiovascular Risk in Diabetes |
| APOB | Apolipoprotein B |
| APOB-48 | Apolipoprotein B-48 |
| APOB-100 | Apolipoprotein B-100 |
| APOE | Apolipoprotein E |
| $APOE^{(-/-)}$ | Apolipoprotein E deficient |
| ATP | Adenosine triphosphate |
| AUC | Area under the curve |
| $BH_2$ | Dihydrobiopterin |
| $BH_4$ | Tetrahydrobiopterin |
| CVD | Cardiovascular disease |
| DIO | Diet-induced obese |
| DCCT | Diabetes Control and Complications Trial |
| EDIC | Epidemiology of Diabetes Interventions and Complications |
| eNOS | Endothelial nitric oxide synthase |
| FFA | Free fatty acids |
| FGF15 | Fibroblast growth factor 15 |
| FGFR4 | Fibroblast growth factor receptor 4 |
| GLUT2 | Glucose transporter 2 |
| GLP-1 | Glucagon-like peptide-1 |
| GLP-1R | Glucagon-like peptide-1 receptor |
| G6Pase | Glucose 6-phosphatase |
| $HbA_{1c}$ | Glycated hemoglobin percentage |
| HDL | High density lipoprotein |
| HFD | High fat diet |
| $HRO_2^{\bullet-}$ | Hydroperoxyl |
| $H_2O_2$ | Hydrogen peroxide |
| IDL | Intermediate density lipoprotein |
| LDL | Low density lipoprotein |
| LDLR | Low density lipoprotein receptor |
| $LDLR^{(-/-)}$ | Low density lipoprotein receptor deficient |
| LXR | Liver X receptor |
| MDA | Malondialdehyde |
| NAFLD | Non-alcoholic fatty liver disease |
| NASH | Non-alcoholic steatohepatitis |
| NO | Nitric oxide |
| NOS | Nitric oxide synthase |
| NOX | Nicotinamide adenine dinucleotide phosphate-oxidase |
| $ONOO^-$ | Peroxynitrite |
| oxLDL | Oxidized low density lipoprotein |
| $O^{2\bullet-}$ | Superoxide |
| $^{\bullet}OH$ | Hydroxyl |
| PEPCK | Phosphoenolpyruvate carboxykinase |
| PGC-1α | Peroxisome proliferator-activated receptor γ coactivator-1α |
| PROactive | PROspective pioglitAzone Clinical Trial In macroVascular Events |
| PUFA | Polyunsaturated fatty acids |
| Redox | Reduction oxidation |
| ROS | Reactive oxygen species |
| $^{\bullet}RO_2$ | Peroxyl |
| SD | Sprague Dawley |
| SREBP-1c | Sterol-regulated element binding protein 1c |
| STZ | Streptozotocin |
| TBA | Thiobarbituric acid |
| TBARs | Thiobarbituric acid reactive substances |
| TG | Triglycerides |
| T1D | Type 1 diabetes |
| T2D | Type 2 diabetes |
| UKPDS | United Kingdom Prospect Diabetes Study |
| vLDL | Very low density lipoprotein |
| 4-HNE | 4-hydroxynonenal |
| 8-iso-$PGF_{2\alpha}$ | 8-iso-prostaglandin F2α |

# 1. Introduction and objectives

Worldwide 415 million people are estimated to have diabetes mellitus (type 1 (T1D) or 2 (T2D)) and the estimated prevalence in 2040 is 642 million [1]. Obesity is a major risk factor for T2D development and is one of the most serious global health burdens. The worldwide number of overweight and obese people has increased reaching 2.1 billion people in 2013 [2, 3]. Diabetes is a chronic disease with devastating consequences such as the microvascular complications, retino- nephro- and neuropathy and the macrovascular complications, cardiovascular disease (CVD), all leading to reduced quality of life and increased risk of mortality [4]. Atherosclerosis is the major cause of CVD events, which is the most common source of death in humans and among diabetic patients the risk is even higher, making it essential to understand the pathophysiology of these disease complexes [5-9]. Over the past couple of decades the complexity of atherosclerosis has become apparent and factors such as inflammation and oxidative stress have gained focus in the pathogenesis [8, 10]. Oxidative stress denotes a situation with excess reactive oxygen species (ROS) in relation to the antioxidant status, which leads to a disruption of reduction oxidation (redox) balance and potentially molecular damage [11, 12]. Oxidative stress can lead to oxidation of macromolecules, including lipids. Lipid oxidation leads to the formation of end-products which can be used as measures of the oxidative stress status [13]. Hyperglycemia, the key characteristic of diabetes, and the fluctuations in blood glucose seen postprandially increase the production of ROS [14-17]. This increase can be the link between diabetes and the development of diabetic complications, such as atherosclerosis [14]. Another key contributor to the CVD development in diabetic patients is dyslipidemia, in terms of elevated serum triglyceride (TG) and low density lipoprotein (LDL) concentration and/or a reduced concentration of high density lipoprotein (HDL). Dyslipidemia may promote ectopic lipid deposition and facilitate atherosclerosis development [18]. Controlling hyperglycemia, dyslipidemia or oxidative stress in diabetes can have the potential to lower the risk of developing diabetic complications such as CVD [19]. In this context, glucagon-like peptide-1 (GLP-1) receptor agonists are interesting to investigate as they correct hyperglycemia and potentially also lowers oxidative stress through glucose-independent effects (reviewed in (I)).

Obesity, diabetes and the devastating complications associated with both are major threats to human health worldwide. Research of the mechanisms involved in the development of diabetic complications, such as atherosclerosis, is necessary to help minimize the development of CVD. Investigations of the relationship between atherosclerosis and oxidative stress or lipid oxidation status are warranted. Carrying out this investigation in various animal models could help clarify this

relationship under different diabetic or dyslipidemic conditions. The current PhD project was conducted to investigate the role of lipid oxidation in animal models of diabetes and dyslipidemia.

The main objective in this project was to investigate oxidative stress, especially lipid oxidation, in connection to dyslipidemia, diabetes and atherosclerosis. Previous studies have found increased levels of oxidative stress in rats with fluctuating hyperglycemia compared to rats with constant hyperglycemia [20, 21]. Based on a thorough review on the effects of GLP-1 on oxidative stress (I), Study I examined the ability of the GLP-1 analogue, liraglutide to decrease oxidative stress in rats in the setting of fluctuating hyperglycemia. In Study II the relationship between lipid oxidation and atherosclerosis in diabetic, diabetic enalapril (angiotensin converting enzyme inhibitor) treated and control apolipoprotein E deficient mice was investigated. As cholesterol is an important factor for plaque progression, Study III investigated the relationship between lipid oxidation and atherosclerosis in low density lipoprotein receptor deficient mice fed two standard high fat diets mainly varying in cholesterol content. The effect of dietary cholesterol was also evaluated in regard to glucose tolerance and hepatic expression of genes involved in lipid and glucose metabolism. The specific aims and hypotheses of the PhD project were:

Aim of Study I: To investigate the effect of glucagon-like peptide-1 analogue, liraglutide, on oxidative stress induced by fluctuating hyperglycemia in a previously developed rat model.

- Hypothesis 1: Liraglutide reduces oxidative stress induced by fluctuating blood glucose.

Aim of Study II: To investigate the relationship between lipid oxidation and atherosclerosis in diabetic and non-diabetic apolipoprotein E deficient mice. Enalapril was included as a pharmacological tool hypothesized to lower oxidative stress and potentially the plaque burden.

- Hypothesis 2: Lipid oxidation correlates with atherosclerotic lesions in apolipoprotein E deficient mice.

Aim of Study III: To investigate the relationship between lipid oxidation, glucose tolerance and atherosclerosis in low density lipoprotein receptor deficient mice fed two standard high fat diets, mainly varying in cholesterol content. In addition to investigate effects of dietary cholesterol on glucose tolerance and hepatic gene expression related to lipid and glucose metabolism.

- Hypothesis 3: Impaired glucose tolerance is associated with increased lipid oxidation and dietary cholesterol increases glucose tolerance by altering hepatic glucose/lipid metabolism.

# 2. Pathophysiology of diabetes

## 2.1. The pathogenesis of diabetes

Diabetes is defined as a condition where the production or the effect of insulin is insufficient, resulting in elevated blood glucose [22]. T1D usually develops in childhood or young adulthood and is characterized by hyperglycemia and insulin-dependency due to autoimmune destruction of pancreatic β-cells. Insulin deficiency leads to hyperglycemia caused by increased hepatic glucose output and decreased peripheral glucose utilization [22]. T2D is often seen in, though not restricted to, obese individuals and can occur at any age. But most often develops in adults. The characteristics in T2D are, besides obesity and hyperglycemia, insulin resistance and dyslipidemia in combination with loss of β-cell mass and function [23]. The current most commonly believed pathogenesis behind the hyperglycemia in T2D is described in figure 1.

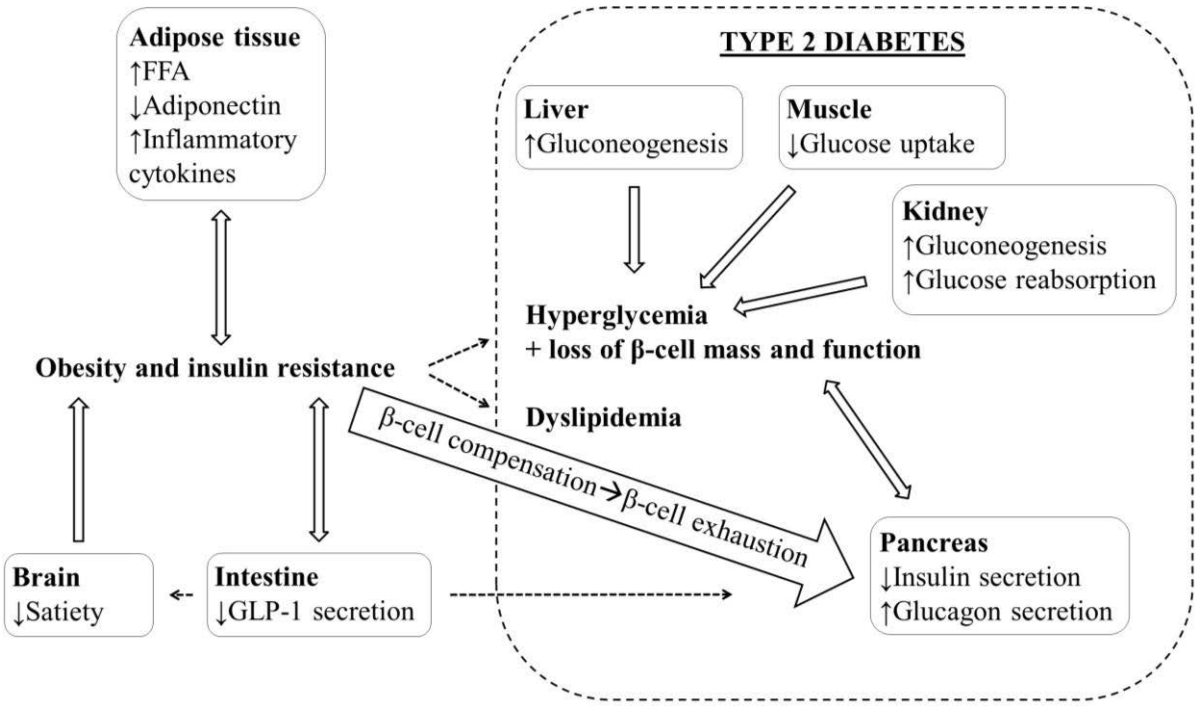


**Figure 1. The pathogenesis of type 2 diabetes.**

Insulin resistance is associated with additional metabolic changes such as hyperinsulinemia, hypertriglyceridemia, reduced HDL and hypertension, what presents first in this relationships is debated [23-26]. The risk factors and clinical features associated with diabetes development are also risk factors of CVD, providing a basis for exploring a shared pathophysiology [18, 22, 27, 28]. A result of insulin resistance and lipotoxicity can be β-cell dysfunction and destruction. The result of this β-cell failure is inability to maintain a sufficient insulin secretion to control blood glucose [25, 29]. Homeostasis of glucose metabolism is sustained by many elements such as absorption of

glucose from the intestines, uptake of glucose in muscle and adipose tissue and hepatic storage and glucose release [30]. Various organs are involved in glycemic control, including pancreas, brain, liver, gut, kidneys, adipose tissue and skeletal muscle. Variation between feeding and fasting gives the body a challenge maintaining blood glucose concentrations. However, in healthy subjects the blood glucose concentration is kept within a physiological range of 4-7 mmol/l [30]. Glucose homeostasis is governed by two main hormones; insulin and glucagon, secreted by β-cells and α-cells of the pancreas, respectively. After consuming a meal, blood glucose increases and insulin is secreted. This promotes uptake of glucose in muscle and adipose tissue via the insulin-dependent glucose transporter 4. In the liver, insulin induces glycogenesis and lipogenesis, while preventing glucose production and release (figure 2). In contrast, glucagon promotes breakdown of glycogen and glucose release from the liver during fasting [31, 32].

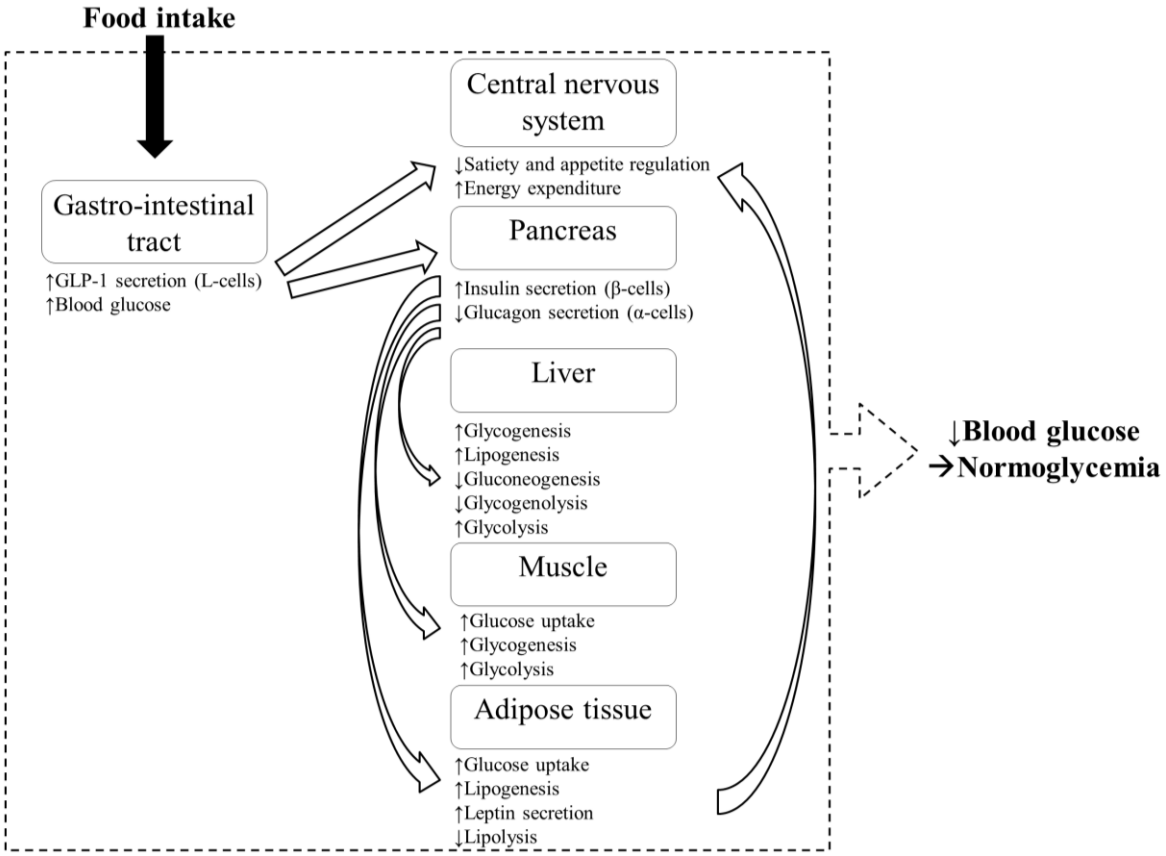


**Figure 2. Normal response to food intake to maintain glucose homeostasis.**

Excess dietary intake of calories induces obesity and decreases insulin sensitivity [23]. To compensate, the pancreatic β-cells increases insulin secretion, resulting in hyperinsulinemia [23]. In a state of insulin resistance, insulin is not able to promote uptake of glucose in muscles and adipose tissue or suppress hepatic glucose production [31, 32]. Ultimately, this results in hyperglycemia. Insulin resistance also results in increased release of free fatty acids (FFA) from the adipose tissue,

as insulin-mediated regulation of lipolysis is compromised, resulting in dyslipidemia [33]. Due to nutrient-overload, the adipose tissue may become dysfunctional and potentially increases insulin resistance and the diabetic state [34]. Dysregulation of the adipokine, leptin also plays a role in the pathophysiology of obesity and diabetes. The hormone reduces appetite and increases energy expenditure via neurons of the hypothalamus and regulates glucose homeostasis via the autonomous nervous system [35-37] (figure 2). Leptin has peripheral effects as well, as leptin regulates glucose homeostasis by activating peripheral leptin receptors [37].

Oral glucose intake stimulates release of incretins, e.g. GLP-1 from intestinal L-cells. GLP-1 decreases appetite and lowers blood glucose by stimulating glucose-dependent insulin release and suppressing the release of glucagon [38, 39] (figure 2). In obese and T2D patients, reduced circulating levels of GLP-1 and reduced release of GLP-1 in response to food intake alongside a blunted response to the effect of GLP-1, potentially increases obesity and hyperglycemia [39-41] (figure 1).

To sum up glucose homeostasis is maintained through interplay of various organs and hormones. As described this includes the pancreas, intestines, liver, adipose tissue and the hormones insulin, glucagon, GLP-1 and leptin.

#### 2.1.1. Oxidative stress and lipid oxidation

The formation of ROS such as superoxide ($O_2^{\bullet-}$), hydroxyl ($^{\bullet}OH$), peroxyl ($^{\bullet}RO2$), hydroperoxyl ($HRO_2^{\bullet-}$), hydrogen peroxide ($H_2O_2$), nitric oxide (NO) and peroxynitrite ($ONOO^-$) is a consequence of aerobic metabolism [42, 43]. Although having important physiological functions, high concentrations of ROS cause damage and contribute to the etiology of several diseases [44, 45]. One of the most important protective mechanisms against oxidative stress is the antioxidants and imbalance of ROS and antioxidants may therefore cause oxidative stress as illustrated in figure 3 [42].

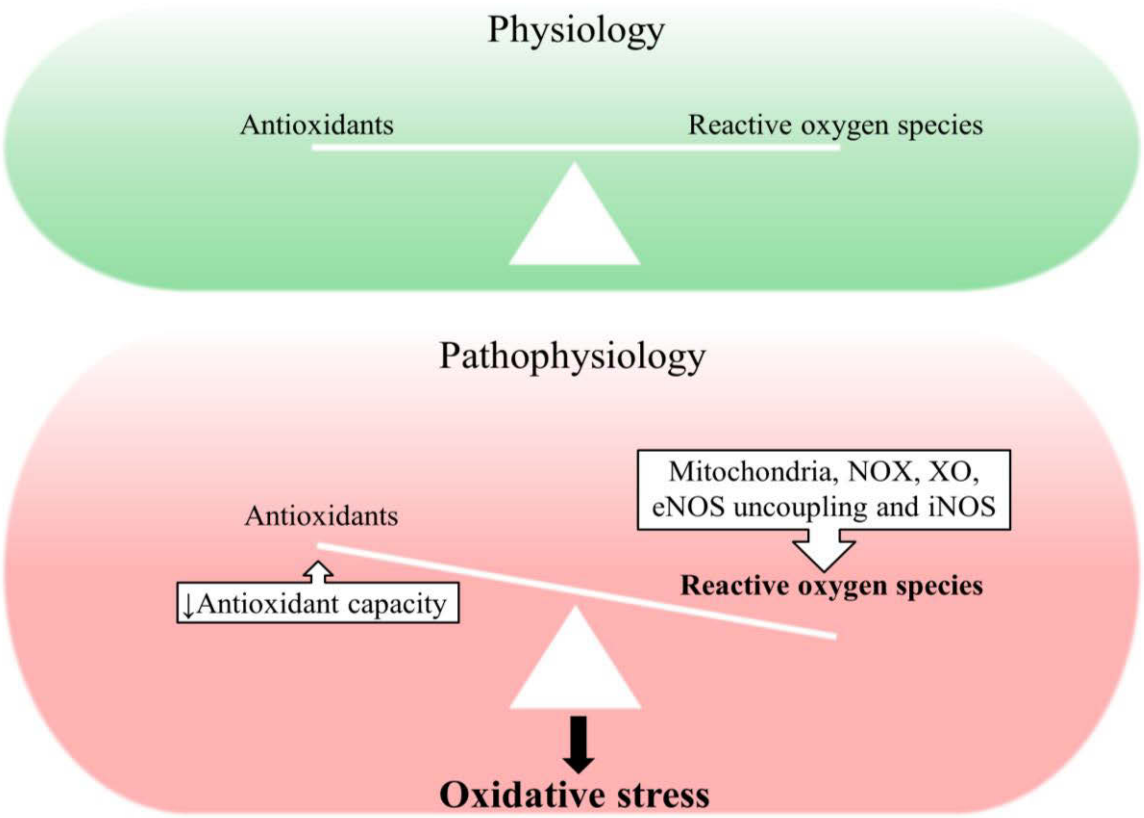


**Figure 3. Schematic presentation of oxidative stress.**

ROS can oxidize and damage macromolecules, namely DNA, RNA, proteins and lipids, which leads to the formation of damaged end-products that can be used as a measure of the oxidative stress status (illustrated in figure 1 of (I)) [46-49]. Some end-products of oxidative damage have the potential to induce damage themselves [47]. Surrogate markers are often used as measures of oxidative stress, as direct measures of ROS are complicated due to their volatile nature [46]. For surrogate markers to be used as biomarkers, factors such as correlation with severity of disease, stability, being able to accumulate to detectable concentrations and reflecting specific oxidation pathways are important [47].

Lipid oxidation or peroxidation is the process where oxidants including free radicals react with lipids containing carbon-carbon double bonds. A primary target of lipid oxidation is polyunsaturated fatty acids (PUFA) as these are rich in double bonds [13]. Lipid oxidation occurs in lipids of cell membranes and lipoproteins. Oxidation of lipoproteins is hypothesized to primarily occur within the extracellular space of vascular walls. The oxidation process might continue in macrophagal lysomes after uptake to these [50]. Oxidation of lipoproteins results in the formation of oxidized low density lipoprotein (oxLDL), which can be measured as a marker of oxidative stress level [50]. Lipid oxidation consists of multiple stages, initiation, propagation and termination [13].

Oxidation of lipids, including lipids in lipoproteins, leads to the formation of hydroperoxides. These are relatively unstable and decompose to different aldehydes, including malondialdehyde (MDA), propanal, hexanal, and 4-hydroxynonenal (4-HNE). MDA is one of the most abundant and studied aldehydes, which is documented as the primary biomarker of lipid damage caused by free radicals [51-53]. Aldehydes are relatively stable, but can still form adducts with different macromolecules including lysine residues of apolipoprotein B-100 (APOB-100) [13, 47, 54, 55].

Isoprostanes are formed from oxidation of arachidonic acid; they are stable and are considered to be reliable markers of lipid oxidation [56]. Isoprostanes containing a F-type prostane ring are termed $F_2$-Isoprostanes and these are categorized into four series. 8-iso-prostaglandin F2α (8-iso-$PGF_{2\alpha}$) is an example of a bioactive isoprostane, which is often used as a marker of lipid oxidation [56]. In plasma 8-iso-$PGF_{2\alpha}$ is found both esterified to plasma lipids and free, with the most abundant form being the first [47]. Even though MDA is less stable than isoprostanes, MDA has often been shown to be correlated to isoprostane concentration, when using these as biomarkers of oxidative stress and lipid oxidation status [57].

To sum up, the markers of lipid oxidation can be used as indirect measures of the oxidative stress level, by indicating the damages of ROS. Additionally, the reactivity and toxicity of the products of lipid oxidation themselves can reflect the potential for further damage.

#### 2.1.2. Oxidative stress and lipid oxidation in diabetes

Hyperglycemia can lead to increased oxidative stress and lipid oxidation, which may be involved in the pathogenesis and development of complications, such as CVD [14, 58, 59]. Chronic over-nutrition and obesity can increase oxidative stress, including lipid oxidation as well [60-62]. Oxidative stress may be involved in the development of both T1D and T2D, or be a consequence of these diseases [63, 64]. During hyperglycemic conditions the resulting intracellular glucose concentration leads to increased oxidation of glucose and potentially enhanced $O_2^{\bullet-}$ production [65]. Similar to glucose, oxidation of FFA, which are elevated in diabetic patients, can lead to generation $O_2^{\bullet-}$ [65-68]. $O_2^{\bullet-}$ produced by the mitochondria may damage and impair mitochondrial function, inducing mitochondrial dysfunction and generation of additional ROS. This has been associated with further development of insulin resistance and higher blood glucose levels [69]. This induces a vicious circle of ROS formation leading to additional ROS formation. The role of mitochondria in formation of ROS during diabetes is not entirely clear, but mitochondrial generated ROS may activate additional ROS producing enzymes, e.g. nicotinamide adenine dinucleotide phosphate-oxidase (NOX) and xanthine oxidase plus uncoupling of NO synthase (NOS) [70, 71]. The produced ROS can lead to lipid oxidation as described previously. ROS formation can lead to tissue

damage by activation of five different pathways described in (I) (illustrated in figure 2 of (I)). The connection between oxidative stress and tissue damage in various diseases has been recognized for a long time, including diabetes and its complications [72, 73]. Retina, glomeruli of the kidneys, peripheral nerves and arterial walls can suffer damage from oxidative stress in diabetes and during progression of these damages inflammatory processes are involved [71].

Poorly controlled diabetes can increase the risk of developing diabetic late complications, and major clinical trials have investigated the effects of intensive diabetes management, focusing on reduction of glycated hemoglobin percentage ($HbA_{1c}$) [74-78]. This intensive diabetes management lowers the risk of developing microvascular complications, but it has not been shown to have the same convincing effect on the macrovascular complications [74-81]. The lack of effect of these therapies focusing on reducing $HbA_{1c}$ on CVD in T2D has been underlined at multiple occasions [80-84]. These results and the beneficial effect of $HbA_{1c}$ reduction on microvascular complications are summarized in table 1.

**Table 1. Clinical trials of intensive glucose management and effect on development of diabetic complications.**

| Trial | Patients | Treatment | Observations | Comments |
|---|---|---|---|---|
| DCCT [74] | T1D (n=1.441) | Insulin (frequent injections or pump) | ↓$HbA_{1c}$<br>↓Retinopathy + neuropathy<br>↑Risk of hypoglycemia | |
| EDIC [85, 86] | T1D (n=1.229* and 1.340)** | Follow-up on DCCT | ↓Intima-media thickness*<br>↓Risk of CVD** | |
| UKPDS 33-35 [75-77] | T2D (n=753 to 3.867) | Insulin, metformin and sulphonylurea | ↓$HbA_{1c}$<br>↓Microvascular endpoints<br>↑Weight and risk of hypoglycemia<br>↓CVD for metformin in UKPDS 34 | Newly diagnosed patients. UKPDS 34 (subset of overweight individuals): No weight gain and had fewer hypoglycemic events than the sulphonylurea and insulin treated group. |
| UKPDS follow-up [87] | T2D (n=1.525) | Follow-up on UKPDS | ↓Diabetes related death<br>↓All-cause mortality<br>↓Myocardial infarction<br>↓Microvascular disease for sulphonylurea | |

| | | | | |
|---|---|---|---|---|
| ACCORD [82] | T2D (n= 10.251) | Insulin, metformin, sulphonylurea, α-glucosidase inhibitor, exenatide and Thiazolidinedione | ↓$HbA_{1c}$<br>↑All-cause mortality<br>↑Weight and hypoglycemic events | No difference in CVD. |
| PROactive [80] | T2D (n=5.238) | Thiazolidinedione | ↓$HbA_{1c}$<br>↓All-cause mortality, myocardial infarction, stroke<br>↑Weight and hypoglycemic events | No effect on primary composite end-point: All-cause mortality, myocardial infarction, stroke, acute coronary syndrome, leg amputation, coronary and peripheral revascularization. |
| ADVANCE [83] | T2D (n=11.140) | Insulin, metformin, sulphonylurea, α-glucosidase inhibitor, and thiazolidinedione | ↓$HbA_{1c}$<br>↓Microvascular events<br>↑Weight and hypoglycemic events | No difference in CVD. |
| VADT [81] | T2D (n=1.791) | Insulin, metformin, sulphonylurea, thiazolidinedione | ↓ $HbA_{1c}$<br>↑Progression of albuminuria<br>↑Weight and hypoglycemic events | No difference in CVD. The participants had suboptimal response to standard T2D treatment. |

* or ** refers to the same symbols within the same row only. ACCORD, Action to Control Cardiovascular Risk in Diabetes; ADVANCE, Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation; CVD, Cardiovascular disease; DCCT, Diabetes Control and Complications Trial; EDIC, Epidemiology of Diabetes Interventions and Complications; $HbA_{1c}$, Glycated hemoglobin percentage; PROactive, PROspective pioglitAzone Clinical Trial In macroVascular Events; UKPDS, United Kingdom Prospect Diabetes Study; VADT, Veteran Affairs Diabetes Trial.

Blood glucose levels may fluctuate in diabetic patients, especially in the postprandial phase, and the elevations are higher and faster than in healthy subjects [15]. Intensive therapy does not rule out the risk of postprandial hyperglycemia and these fluctuations may not be adequately reflected by $HbA_{1c}$ [88, 89]. Fluctuations in blood glucose can induce oxidative stress, which can lead to the development of diabetic complications, including CVD [16, 17, 88, 90]. Reduction in antioxidant capacity has been suggested to be implicated in the oxidative stress induced by fluctuations in blood glucose [90]. *In vitro* studies show that variation between high and low glucose concentrations in the media can increase oxidative stress. Acute hyperglycemia has shown to increase plasma 8-iso-$PGF_{2\alpha}$ in T2D patients and healthy subjects [91-93]. Fluctuating hyperglycemia has shown to lead to increased nitrotyrosine (a marker of protein nitration by $ONOO^-$) concentration, endothelial

dysfunction at the spikes of the fluctuations and increased urinary 8-iso-$PGF_{2\alpha}$ in both healthy subject and T2D patients [94]. *In vivo* and clinical studies have found that blood glucose fluctuations can increase oxidative stress, even when the overall glycemic exposure is lower than sustained high blood glucose concentrations [20, 21, 94].

Measuring and lowering fluctuations in blood glucose and oxidative stress status could be more useful in the cardiovascular risk assessment and treatment than $HbA_{1c}$. However, the hyperglycemia and its effect after acute myocardial infarction on cardiovascular outcomes in patients with T2D trial, also known as HEART2D, of T2D patients surviving acute myocardial infarction did not show a difference in CVD events between a group treated with the long-acting insulin to reduce basal fasting blood glucose and a group treated to specifically target postprandial glucose fluctuations with fast-acting insulin [95]. This could be due to a failure in achieving the intended difference in postprandial blood glucose between the groups and an advanced stage of CVD when starting the treatment regimes. In 2011 Standl *et al* summarized epidemiological studies and meta-analyses on the connection between postprandial hyperglycemia and CVD risk in healthy subjects and diabetic patients. This paper indicated that postprandial hyperglycemia is an important CVD risk factor. Prospective intervention studies targeting postprandial hyperglycemia are warranted to support the use of therapy targeting postprandial hyperglycemia [96].

#### 2.1.3. Dyslipidemia and diabetes

Dietary lipids are absorbed from the intestines and transported in the blood stream in the large lipoproteins, chylomicrons [97]. From here muscle and adipose tissue take up FFA from the chylomicrons [98]. The remaining chylomicrons are termed chylomicron remnants and these are taken up into the liver by apolipoprotein B-48 (APOB-48)- or apolipoprotein E (APOE)-receptor-mediated uptake. Lipids obtained from these and TG formed from de novo lipogenesis are then secreted from the liver as very low density lipoprotein (vLDL) [99]. In the blood stream vLDL is depleted of TG by lipoprotein lipase and becomes cholesterol-rich LDL [97]. The LDL particle uptake is mediated by the LDL receptor (LDLR), which is expressed in all peripheral cells [100, 101]. Ligands for LDLR are APOB-100 and APOE which are found in vLDL, intermediate density lipoproteins (IDL) and LDL. Conversely, cholesterol may be removed from cells by HDL in a process termed reverse cholesterol transport [97, 102]. HDL also exerts anti-atherogenic effects e.g. by inhibiting inflammation and lipid oxidation; these effects are however not as well established as the cholesterol scavenging potential of HDL [103].

T1D and T2D have different pathophysiology also in connection to dyslipidemia. In well-regulated T1D patients plasma lipid concentrations can be close to normal, depending on the

success of glycemic control, whereas poorly regulated T1D patients have increased chylomicron and vLDL concentrations due to insulin deficiency [104]. One of the primary defects in obesity is dysfunctional adipocytes. These release increased amounts of FFA and inflammatory cytokines (e.g. interleukin-6 and tumor necrosis factor α), as well as decreased amounts of anti-inflammatory adipokines such as adiponectin [34]. FFA concentration is increased as a result of impaired storage of FFA and increased lipolysis associated with insulin resistance in adipose tissue [33] (figure 1). The dyslipidemia in T2D is characterized by decreased HDL, increased small dense LDL particles and elevated TG which induce an overproduction of vLDL [105]. The release of vLDL can be a protective mechanism to prevent lipid accumulation in the liver, and thus steatosis. However, an oversupply of FFA to the liver during insulin resistance in combination with the systemic inflammation and oxidative stress set the stage for the development of non-alcoholic fatty liver disease (NAFLD) that may progress to non-alcoholic steatohepatitis (NASH) [106, 107].

#### 2.1.4. Hepatic glucose and lipid metabolism is dysregulated in diabetes

The liver plays a key role in glucose and lipid homeostasis by orchestrating carbohydrate, lipid and protein metabolism [108]. Glucose is taken up by the liver through the insulin-independent glucose transporter 2 (GLUT2) before it is phosphorylated, forming glucose-6-phosphate, by glucokinase (GK) [31]. When energy is needed, glucose-6-phosphate is used to form adenosine triphosphate (ATP) in the processes of glycolysis, the tricarboxylic acid cycle and electron transport chain. In situations of excess glucose, glucose-6-phosphate is used for glycogenesis and *de novo* lipogenesis. During low plasma glucose concentrations, glucagon stimulates glucose release from glycogen by glycogenolysis or from non-carbohydrate precursors by the process of gluconeogenesis [108]. The final step of glucose release is controlled by glucose 6-phosphatase (G6Pase), forming free glucose to be released to the circulation. Thus, controlling the hepatic glucose output [31]. Insulin suppresses glycogenolysis and gluconeogenesis, while promoting *de novo* lipogenesis [31, 32].

In diabetes, the regulation of glucose homeostasis is altered; insulin-dependent glucose uptake and regulation of glucose output from the liver is impaired. During insulin resistance hyperinsulinemia may still promote hepatic lipogenesis. This is done through activation of the transcription factor sterol-regulated element binding protein 1c (SREBP-1c) [109, 110]. Activation of SREBP-1c promotes activation of both glycolytic and lipogenic genes such as GK, liver pyruvate kinase, fatty acid synthase, acetyl-CoA carboxylase and S14 [111-113]. This selective insulin resistance is a contributing factor to increases in FFA and TG biosynthesis observed in T2D [109]. Activation of liver X receptor (LXR) can suppress gluconeogenic hepatic pathways as peroxisome

proliferator-activated receptor γ coactivator-1α (PGC-1α), phosphoenolpyruvate carboxykinase (PEPCK), G6Pase, promote transcription of SREBP-1c together with an increased expression of the glycolytic GK in mice [114, 115]. Cholesterol metabolites (oxysterols) serve as ligands for LXR, and dietary cholesterol has shown to increase expression of SREBP-1c in a LXRα-dependent manner [116, 117]. Potentially dietary cholesterol can improve glucose tolerance by activation of LXRα. Additionally, dietary cholesterol increases the synthesis of bile acids, which has been shown to have hormonal functions in metabolic processes [118, 119]. E.g. bile acids have shown to improve glucose homeostasis through an increased secretion of GLP-1 both *in vitro* and *in vivo* [120-122]. Additionally, bile acids improve glucose homeostasis and glucose tolerance by activation of hepatic farnesoid X receptors, which increases glycogenesis via inhibition of liver pyruvate kinase [119].

Leptin signaling in isolated rat livers and hepatocytes have shown to reduce glucose release [123-125]. *In vivo* peripheral actions of leptin can be mediated through decreased glucagon signaling and release [37]. Resistance to leptin is related to obesity and diabetes. This resistance can be involved in impaired insulin signaling, increased hepatic glucose output, hyperglycemia and hyperlipidemia [36] (figure 2).

In summary, the liver plays and important role in the homeostasis of glucose and lipids, but the effects of cholesterol on hepatic metabolism is not yet fully elucidated. Therefore, Study III investigated the impact of dietary cholesterol on hepatic expression of genes involved in lipid and glucose metabolism.

## 2.2. The pathogenesis of atherosclerosis

Atherosclerosis is the leading cause of heart disease and stroke. The disease is characterized by accumulation of lipids and connective tissue in the large arteries in combination with inflammatory processes [8]. The composition of plasma lipoproteins and dyslipidemia are important risk factors for CVD and for a long time high HDL concentrations have been recognized as powerful indicators of low CVD risk in humans [126-128]. Several outcome studies have shown that APOB provides even better prediction of cardiovascular risk than non-HDL cholesterol and LDL cholesterol [129]. People with diabetes have been reported to have an approximately two-fold higher risk of developing CVD compared to healthy subjects [130-132]. The pathogenesis of atherosclerosis is illustrated in figure 4.

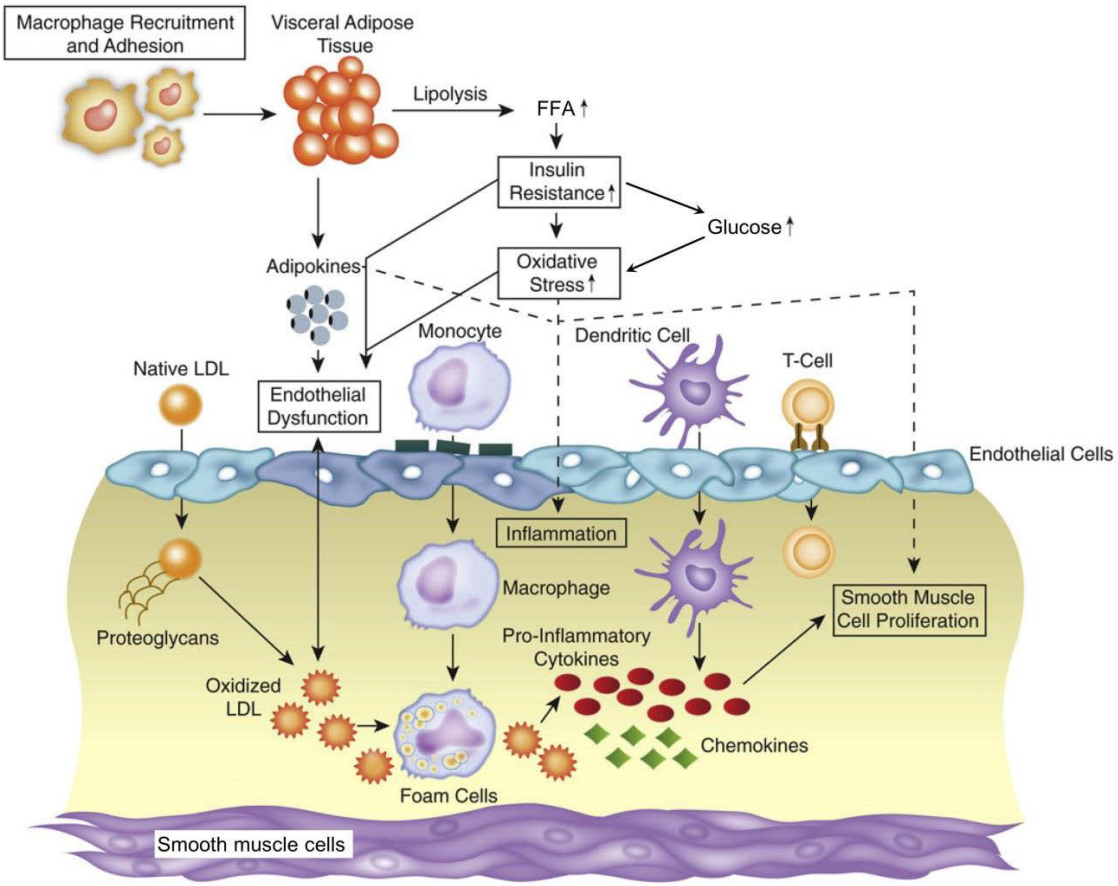


**Figure 4. The pathogenesis of atherosclerosis.** Modified from Hajjar, DP *et al* 2013 [10].

Briefly, atherosclerosis develops as macrophages in artery walls accumulate lipids forming foam cells, arranging in so-called ~~–f~~atty streaks". Dendritic cells and T-cells are recruited to the lesion, contributing to an inflammatory process [10]. When smooth muscle cells migrate to the intima and proliferate the more advanced plaque lesions are formed. Here the smooth muscle cells and collagen-rich connective tissue form a cap around a necrotic core consisting of foam cells and lipid-rich necrotic debris [8, 133, 134]. This atheroma can then become even more advanced by calcification and cause clinical manifestation by occluding the artery. More commonly vulnerable plaques rupture or erode, which can lead to emboli occluding smaller vessels. The formation of vulnerable plaques results from breakdown of the fibrous cap by matrix metalloproteinases [8]. Differences between humans and mice models of atherosclerosis are very apparent in these advanced stages, as the lesions in the models hardly ever progress to rupture [135].

### 2.2.1. Oxidative stress and atherosclerosis

Oxidative stress and inflammation are important processes in atherosclerotic development (figure 4) [8, 9]. The risk factors of atherosclerosis such as hypercholesterolemia, diabetes, hypertension, smoking and aging share a common feature; they are associated with increased

production of ROS, which oxidize lipids, induce expression of adhesion molecules, stimulate vascular smooth muscle cell proliferation and migration besides altering vasomotor activity [136]. Inhibition of NOX, the central producer of $O_2^{\bullet-}$ in endothelial cells, has been shown to improve endothelial cell function in both rat and human vessels [136, 137]. Oxidative stress decreases the bioavailability of the important regulator of vascular tone, NO and the cofactor involved in synthesis of NO, tetrahydrobiopterin ($BH_4$) [138]. $BH_4$ can be oxidized to dihydrobiopterin ($BH_2$) by $ONOO^-$. $BH_2$ can bind to endothelial NOS (eNOS) resulting in eNOS uncoupling, production of $O_2^{\bullet}$ instead of NO and endothelial dysfunction [139, 140]. Accordingly, supplementation of $BH_4$ to apolipoprotein E deficient (B6.129P2-*Apoe*$^{tm1Unc}$ N11 or J) (APOE$^{(-/-)}$) mice has been shown to reduce development of atherosclerosis [141]. Endothelial cells in the vasculature might be more susceptible to damage in diabetes as they do not seem to regulate uptake of glucose as other cell types, potentially increasing their vulnerability to hyperglycemia [142, 143].

Extensive investigation of the role of oxidized lipids in CVD has been performed since Steinberg *et al* presented the oxidation hypothesis of atherosclerosis in 1989. This hypothesis suggested oxidative modifications of LDL to be the primary cause of foam cell formation and development of atherosclerosis [144]. Since then substantial amounts of data supporting this have been collected [50]. Aldehydes, including MDA, can modify LDL by reacting with amino acid residues in APOB-100 forming MDA-LDL. This is part of the broader group oxLDL, which refers to various oxidative modifications of LDL [145]. LDL is susceptible to oxidation and modifications by oxidation end-products. This can be explained by the fact that half of the lipids in LDL are PUFA, aldehydes bind to APOB-100 and LDL has a long circulating half-life when compared to IDL and vLDL [146]. The formation of oxLDL is a major pathway for induction of oxidative stress induced endothelial dysfunction in dyslipidemia, and atherosclerotic lesions contain high amounts of oxLDL, as macrophages readily phagocytize oxLDL (figure 4) [55, 147]. These macrophages can develop into foam cells and in this way end-products of lipid oxidation, such as MDA, can be involved in the process of atherosclerotic development [148, 149]. MDA has the ability to react with a variety of proteins and as commercially available antibodies for MDA adducts cross react with MDA and MDA adducts, it could indicate that MDA adducts are less favorable as markers of atherosclerosis than total MDA [150, 151]. MDA can play a role in the development of atherosclerosis in an additional manner by reaction with collagen. This can lead to stiffening of the vascular wall [152]. Thiobarbituric acid reactive substances (TBARs), a crude measure of MDA, have shown to predict CVD events and atherosclerosis [153]. MDA modified LDL adducts have been suggested to be directly involved in starting the inflammatory process in atherosclerosis by activating the complement system [154]. Oxidative stress and oxLDL may also be involved in

adipose tissue dysfunction and could thus contribute indirectly to the formation of atherosclerosis through abnormal adipokine secretion and release of pro-inflammatory cytokines (figure 4) [10, 34]. Late-stage atherosclerotic plaques in humans have shown to contain oxidatively modified proteins, amino acids, lipids, and F2-isoprostanes [155-159]. A stage-dependent increase in hydroperoxides and end-products of lipid oxidation has been found in atherosclerosis in humans e.g. MDA and isoprostanes and isoprostanes have been suggested to be markers of atherosclerosis [158, 160].

Further investigation of oxidative stress and lipid oxidation in diabetes, dyslipidemia and atherosclerosis is warranted as data indicate a relationship between these conditions. In the three studies of this thesis this was the main focus.

## 2.3. Animal models of diabetes, dyslipidemia and atherosclerosis

Animal models can help clarify underlying mechanisms and pathophysiology of diseases and potentially be useful in the development of treatment therapies. In the following sections, relevant models for this thesis will be presented. These are rodent animal models of T1D, T2D, insulin resistance and atherosclerosis.

### 2.3.1. Inducible rodent models of diabetes

A commonly employed animal model of diabetes uses toxic glucose analogues such as streptozotocin (STZ) and alloxan to induce diabetes [161]. The following section will focus on STZ, which is used in Study II of this thesis. Accumulation of cytotoxic STZ occurs preferentially in pancreatic β-cells through GLUT2 uptake, resulting in β-cell-cytotoxicity through DNA damage and subsequently hyperglycemia is induced as insulin production and secretion is diminished [162]. Nonspecific toxicity of STZ can occur in other tissues expressing GLUT2, especially kidneys and liver [161, 163]. STZ-induced diabetes is associated with oxidative stress from STZ alone as well as from the induced hyperglycemia [162, 163].

Multiple low doses of STZ can be as effective as a single high dose of STZ in terms of inducing hyperglycemia. Multiple low doses are used to attempt to minimize the toxic effects on other tissues. In addition, the multiple doses also work by activating the immune system, by inducing a lymphocyte infiltration, which can increase damage to the β-cells in these lower dosing regimens [163-166]. The success rate of STZ-induced diabetes varies between ages, genders, species and strains. A 95 % response rate (blood glucose > 19.4 mmol/l) has been observed in $APOE^{(-/-)}$ mice treated with multiple low doses, indicating that it is a good model for STZ-induced hyperglycemia [162, 167, 168].

#### 2.3.2. Induction of fluctuating hyperglycemia

To induce fluctuations in blood glucose different approaches can be used; administration of glucose in different intervals and by different routes (e.g. oral, intravenous) or STZ-induction of diabetes, followed by multiple low doses of fast-acting insulin to lower blood glucose, which will then rise quickly again. The latter method has been used in Wistar rats, showing an increased endothelial dysfunction in animals undergoing fluctuations in blood glucose, even when compared to untreated diabetic animals. The authors hypothesized the induction of endothelial dysfunction to be attributed in part to oxidative stress [169]. In regard to induction of diabetic complications from these fluctuations, it has been shown that oral administration of maltose to $APOE^{(-/-)}$ mice accelerate atherosclerotic development [170]. However, the same result on atherosclerotic development was not obtained in a later study, where oral glucose was administrated to the animals actually leading to higher plasma glucose concentrations than the previously mentioned study [171]. The fluctuations induced in blood glucose by infusion of glucose through permanent catheters together with automatic blood sampling have been used in rats [20, 21]. Intravenous administration of multiple doses of glucose is facilitated by the use of permanent catheters as these minimize risk of perivenous administration, handling during administration and pain by repeated injections [172]. This technique was used in Study I to minimize stress and make dosing more reliable.

#### 2.3.3. Diet-induced models of insulin resistance, dyslipidemia and atherosclerosis

The diet-induced obese (DIO) models have the potential to better mimic aspects of the complex metabolic diseases observed in human obese individuals, than the genetically modified murine models [173]. High fat diets containing high amounts of fat and cholesterol mimic the human —western diet" and are often used to induce obesity, dyslipidemia and atherosclerosis [174]. However, the interplay between diet and disease development is not fully elucidated. Cholesterol is believed to have metabolic effects, though these are poorly understood. In 1951 Gould *et al* discovered that diets rich in cholesterol suppressed the synthesis of cholesterol in the liver [175, 176]. Some focus has since then been put on the investigation of dietary cholesterol effects on hepatic gene expression, but not all effects are known. Regulation and synthesis of bile acids are important elements of cholesterol turnover and could be a potential pathway by which dietary cholesterol affects hepatic gene expression, together with regulation of other hepatic genes involved in lipid and glucose metabolism [118, 177].

The effect of high fat diet (HFD) on obesity, glucose tolerance and insulin resistance differs when examining different strains, genders and ages of the animals. In addition, the dietary composition and length of dietary intervention is important for the outcome [173]. The different

substrains and stocks of C57BL/6 mice and Sprague Dawley (SD) rats are among the most frequently used DIO models and they both become obese, hyperglycemic and hyperinsulinemic on HFD [178-182]. Low density lipoprotein receptor deficient (B6.129S7-Ldlr$^{tm1Her}$/J (LDLR$^{(-/-)}$)) and APOE$^{(-/-)}$ mice are often fed HFD to promote the development of atherosclerosis, but the response of these diets on their metabolic status differs. The LDLR$^{(-/-)}$ mouse is more prone to develop obesity, hyperglycemia and hyperinsulinemia when fed a HFD and is considered a model of atherosclerosis in metabolically challenged animals, whereas the APOE$^{(-/-)}$ mouse is insulin sensitive and quite resistant to diet induced obesity [183-187]. How the exact dietary composition affects the outcome of studies of DIO animals is not yet fully understood, and Study III was set up to elucidate especially the effect of cholesterol on the outcome of such studies.

#### 2.3.4. Mouse models of dyslipidemia and atherosclerosis

The ideal model of human atherosclerosis mimics all aspects of the human disease, both pathophysiologically and metabolically. However, no model has all the traits of this multifactorial disease. Thus, models must be chosen with the particular study in mind [188]. In general mice are resistant to the development of atherosclerosis, partly due to their HDL dominant lipoprotein profile and therefore transgenic models are used in atherosclerosis research [188-190]. APOE$^{(-/-)}$ and the LDLR$^{(-/-)}$ mice are the most widely used mouse models for studying dyslipidemia and atherosclerosis [191-194]. APOE deletion results in reduced clearance of APOE-containing lipoproteins (chylomicrons, chylomicron remnants, IDL, vLDL and some HDL lipoproteins) leading to dyslipidemia [97, 192].

When fed standard chow diets, APOE$^{(-/-)}$ mice have plasma cholesterol concentrations of >10 mmol/l, located in chylomicron remnants and vLDL, and the mice develop atherosclerotic lesions [133, 174, 189, 192, 195-197]. When these mice are fed high fat cholesterol-enriched diets the plasma cholesterol concentrations will increase four to five times compared to chow or low fat fed APOE$^{(-/-)}$ mice [133, 174, 189, 192, 197]. The insulin sensitivity of APOE$^{(-/-)}$ mice can be caused by reduced lipid transport to insulin sensitive tissues, which causes resistance to diet induced obesity [186, 187].

LDLR$^{(-/-)}$ mice lack a functional LDLR, which clears APOB-100-containing lipoproteins and APOE-containing lipoproteins. The mouse is dyslipidemic and develops obesity, insulin resistance, inflammation and atherosclerosis when fed diets containing cholesterol [183, 193, 198, 199]. On chow diets the plasma cholesterol concentration is approximately 5 mmol/l, which slowly increases to 9 mmol/l with age [200]. On western diets the concentrations can be above 25 mmol/l [188]. In contrast to the APOE$^{(-/-)}$ mouse the LDLR$^{(-/-)}$ mouse only develops plaques on chow diet when

reaching about one year of age, and even then the lesion formation is limited [201]. When fed western diets for long periods of time, changes in fur and skin integrity can occur, which is also seen in APO$^{(-/-)}$ mice [200]. This is a limiting factor in regard to length of studies in both mouse models [202]. The main cholesterol carrying lipoprotein in LDLR$^{(-/-)}$ mice is LDL. APOB-100 is the predominate apolipoprotein in contrast to the APOB-48-isoform in vLDL and chylomicrons that is predominate in APOE$^{(-/-)}$ mice [203]. The LDLR$^{(-/-)}$ mice is also used as a model of NAFLD and NASH as they accumulate lipids in the liver developing disorders resembling the human diseases [204-206]. An important difference between APOE$^{(-/-)}$ and LDLR$^{(-/-)}$ mice is the type of atherosclerotic lesions they develop. The LDLR$^{(-/-)}$ mouse develop plaques characterized by fatty streaks containing foam cells only progressing to a fibroproliferative stage, whereas the APOE$^{(-/-)}$ mice develop lesions with traits of more advanced plaque findings, such as necrotic cores, proliferation of smooth muscle cells and extracellular matrix [133, 135, 196]. These are traits seen in human atherosclerosis [8]. However, neither APOE$^{(-/-)}$ or LDLR$^{(-/-)}$ mice develop plaques that are prone to rupture without a physical stimuli [135].

## 2.4. GLP-1 therapy

The potentials of GLP-1 therapy have gained focus since the GLP-1 hormone was discovered in 1986. GLP-1 has a half-life of less than two minutes and therefore glucagon-like peptide-1 receptor (GLP-1R) agonists are constructed to avoid the rapid degradation by dipeptidyl peptidase 4 [207]. The class of GLP-1R agonists used as antidiabetic drugs already comprises several agonists used for patients. Liraglutide provides the best effect on lowering $HbA_{1c}$ and body weight compared to other marketed GLP-1R agonists [208].

Today multiple effects besides the first discovered glucose-dependent insulin releasing effects of the hormone have been proposed, including beneficial effects on diabetic complications and NAFLD/NASH [209-211]. GLP-1 based therapy has proposed to protect against the development of diabetic macrovascular complications [210]. The Liraglutide Effect and Action in Diabetes: Evaluation of cardiovascular outcome Results trial, also known as LEADER® was initiated in 2010 and followed patients on liraglutide for up to five years evaluating the effect of diabetic complications, particularly CVD. The trial has followed 9340 adults in risk of major adverse cardiovascular events [212]. Liraglutide reduced the occurrence of these major adverse cardiovascular events [213]. This is supported by animal studies showing reduction of atherosclerosis by GLP-1 intervention [214-216]. Thus, GLP-1 therapy can have the potential to decrease the development of atherosclerosis. However, more studies are warranted, especially studies to help differentiate glucose-dependent effects from glucose-independent effects.

#### 2.4.1. GLP-1 therapy and oxidative stress under fluctuating hyperglycemia

GLP-1 therapy opens a new possibility to reduce blood glucose fluctuations, and the therapy is associated with a limited risk of hypoglycaemia [217]. GLP-1R agonist, exendin-4 has been found effective in the reduction of postprandial glucose fluctuations [218]. Liraglutide has been shown to reduce mean amplitude of glycemic fluctuations in T2D patients and thus has the ability to reduce postprandial glucose fluctuations [219].

As reviewed in detail in (I), GLP-1 seems to be able to lower oxidative stress status *in vitro*, *in vivo* and clinically. The review discusses the observed effects of GLP-1 in different study designs and aims to separate the known glucose-dependent effects from the glucose-independent effects. The oxidative stress lowering effects of GLP-1 are mediated through different mechanisms. As GLP-1 reduces blood glucose and blood FFA this pathway is the most obvious [220]. By this mean GLP-1 can reduce the mitochondrial ROS-production and decrease the activation of the five pathways described in above. The mechanism behind glucose-independent effects of GLP-1 on oxidative stress could be stimulation of the G-protein coupled GLP-1R [221, 222]. As described in (I) GLP-1R stimulation can decrease NOX activity and expression of NOX subunits through different pathways and increase antioxidant capacity. GLP-1 can decrease expression of advanced glycation end-product receptors, which decreases production of ROS by xanthine oxidase, NOX and mitochondria. Effects of GLP-1 can be decreased ROS-production and increased antioxidant capacity. Additional *in vivo*, *in vitro* and clinical studies have been performed in this area after (I) was published. These support that liraglutide can decrease ROS production and lower concentration of ROS *in vitro* potentially by GLP-1R stimulation [223, 224]. *In vivo* studies have shown liraglutide to decrease superoxide and reduce TBARs in tissue [224, 225]. Clinical studies of liraglutide in T2D patients have shown a decrease in derivatives of reactive oxygen metabolites in plasma [226]. The conclusion from these studies and (I) is that liraglutide definitely has the potential to lower oxidative stress. This is seemingly, to some extent, carried out glucose-independently by activation of specific pathways (I). Study I was carried out to investigate if liraglutide could lower oxidative stress induced by fluctuating hyperglycemia, glucose-independently.

# 3. Evaluation of lipid oxidation in animal models of diabetes and dyslipidemia

During the current PhD project lipid oxidation was investigated in three different animal models of diabetes and dyslipidemia; High fat fed NTac:SD rats subjected to fluctuating hyperglycemia and liraglutide intervention, STZ-induced diabetic APOE$^{(-/-)}$ (B6.129P2-Apoe$^{tm1Unc}$/N11) and LDLR$^{(-/-)}$ (B6.129S7-Ldlr$^{tm1Her}$/J) mice fed two standard HFD primarily varying in cholesterol content. The three studies are summarized in table 2.

**Table 2. Overview of the studies of the thesis.**

| Study | Model | Main results | Conclusion |
|---|---|---|---|
| **I** | Male NTac:SD on HFD. Glucose infused to permanent catheters to induce fluctuating hyperglycemia in two rounds of 96 hours separated by 30 days of liraglutide intervention. | No significant changes in oxidative stress were observed between the groups. | As oxidative stress was not induced, conclusions on the effect of liraglutide on oxidative stress induced by fluctuating hyperglycemia were not possible to reach. |
| **II** | Male APOE$^{(-/-)}$ mice fed chow diet were injected with STZ to induce diabetes. The mice were studied for 20 weeks. | A correlation was found between atherosclerotic plaque area in the aorta of the mice and plasma MDA concentration. | Plasma MDA is correlated to plaque area in APOE$^{(-/-)}$ mice and could provide additional information to cholesterol concentration when estimating plaque area in these mice. |
| **III** | Male LDLR$^{(-/-)}$ mice were fed either a high fat diet with low or high cholesterol content for 16 weeks. | Plasma MDA and 8-iso-PGF$_{2\alpha}$ were lowest in the group fed the high cholesterol diet, this group had the largest atherosclerotic plaque area and the best glucose tolerance. Expression of genes involved in lipid and glucose metabolism was different between the two groups. | Lipid oxidation is associated with glucose tolerance in LDLR$^{(-/-)}$ mice and dietary cholesterol is important for these mice to develop atherosclerosis. Additionally, dietary cholesterol can improve glucose tolerance potentially by changing hepatic gene expression. |

## 3.1. The effect of liraglutide on oxidative stress induced by fluctuating hyperglycemia in Sprague Dawley rats (paper II; manuscript)

The aim of this study was to evaluate potential oxidative stress-lowering effects of liraglutide in a model of oxidative stress induced by fluctuating hyperglycemia. Previously, Rakipovski 2016 [21] has described such a model: the NTac:SD rats fed a HFD showed increased plasma 8-iso-

$PGF_{2\alpha}$ and MDA besides aorta MDA when subjected to fluctuating hyperglycemia reaching 20-22 mmol/l. The goal of the present study was to reach a similar fluctuating hyperglycemia range, as this could be important for increasing oxidative stress. The study was set up as a pilot study to evaluate an effect of liraglutide in this demanding study before setting up a larger scale study. Four groups were included in the study; continuously saline vehicle, continuously saline liraglutide, fluctuating glucose vehicle and fluctuating glucose liraglutide. The liraglutide intervention was placed in-between the two infusion rounds to try to avoid direct glucose-lowering effects of liraglutide on oxidative stress.

All data are presented in (II) and analyses are presented in appendix 1. The Accusampler used for automatic blood sampling in this study has high demands to the patency of catheters. Lack of patency was the major cause for exclusion of animals from the study and the number of rats finalizing the study was 12, with two to four animals in each group. The blood glucose concentration in the fluctuating groups reached 12.5±0.4 mmol/l during infusions of glucose. No differences were observed in either blood glucose or plasma insulin between the two rounds of infusions. The MDA content in aortas of the rats did not differ significantly between the groups. Repeated measurement ANOVA showed no difference in plasma MDA or $BH_2/BH_4$ ratio between the groups, infusion round or over time. Thus, liraglutide treatment, infusion type and time did not affect plasma MDA or $BH_2/BH_4$ ratio.

### 3.2. Plasma lipid oxidation and atherosclerosis in diabetic APOE-deficient mice (paper III)

We expected MDA to have an important role in the development of diabetic complications, using this end-product of lipid oxidation as a marker of oxidative stress status. Thus, the second study was set up to evaluate how MDA is correlated with atherosclerosis in a model of diabetes. The ideal model for this study would 1) develop high blood glucose concentrations, 2) develop an atherosclerotic phenotype, making it possible to evaluate the atherosclerotic status and 3) have a reduced complexity in order to limit the risk of inconclusive results. The chosen model for this study was STZ-treated $APOE^{(-/-)}$ mice (from the Taconic). Three groups were included in the study; a control group (non-STZ treated $APOE^{(-/-)}$ mice)(CTRL), a diabetic group (DIAB) and a diabetic group treated with enalapril (10 mg/kg/day)(ENAL).

Details are described in (III) and a detailed description of the analyses is given in appendix 1. 9 out of 17 animals in the DIAB group and 10 out of 17 in the ENAL group were included as they had blood glucose concentrations above 15 mmol/l three weeks after STZ treatment (success rate of STZ treatment = 56 %). The mice were hyperglycemic for the entire study period and no difference

between the diabetic groups was observed. Both diabetic groups had significantly higher blood glucose concentrations than the CTRL group ($p<0.001$). Plasma concentrations of total cholesterol, vLDL and LDL, were significantly higher ($p<0.001$) and plasma HDL were significantly lower ($p<0.05$) in the two diabetic groups when compared to the CTRL group. Aortic plaque area and plasma MDA concentration were significantly higher in the DIAB group when compared to the CTRL group ($p<0.01$ and 0.05 respectively). The ENAL group was not significantly different from the CTRL or the DIAB group. No differences were shown in aorta MDA concentration. The aortic plaque lesion area and plasma MDA were positively correlated ($y=2.7x+6.6$, $p=0.0076$, $R^2=0.2713$). A stepwise multiple regression analysis with backward elimination including total cholesterol, LDL, vLDL, total cholesterol/HDL ratio and plasma MDA as covariates showed that plasma MDA was the variable describing the outcome, aortic plaque lesion area the best. Inclusion of $HbA_{1c}$ in this regression analysis showed the same result.

### 3.3. Effects of dietary cholesterol on plaque formation, lipid oxidation and glucose tolerance in LDLR-deficient mice (paper IV; manuscript)

The third study included in this thesis was carried out in $LDLR^{(-/-)}$ mice. The choice of model in this study was based on the ability of this mouse to develop an insulin resistant state when fed a HFD. It was possible to investigate lipid oxidation in relation to atherosclerosis in animals not subjected to STZ-injections. The focus was to investigate the relationship between atherosclerosis, lipid oxidation and glucose tolerance and how dietary cholesterol content affected these measures. To underline how choice of diet has an impact on study outcome, two commonly used standard HFDs were employed. The hepatic expression of genes involved in lipid and glucose metabolism were quantified by qPCR. The study was performed in two groups, a group fed a HFD with low cholesterol content (LCD) and a group fed a HFD with high cholesterol content (HCD).

Details are described in (IV) and analyses are described in appendix 1. The HCD group had a significantly lower fasting blood glucose ($p<0.01$) and lower area under the curve (AUC) than the LCD group in the glucose tolerance test, also when adjusting for baseline ($p<0.001$). The HCD group had significantly lower concentrations of plasma MDA and 8-iso-$PGF_{2\alpha}$ ($p<0.05$), the largest aortic plaque area ($p<0.001$) and highest hepatic accumulation of lipids and collagen ($p<0.001$) when compared to LCD. Evaluation of 14 hepatic genes showed that the HCD group had a significant higher expression of SREBP-1c, ATP-binding cassette transporter A1 and the leptin receptor and significantly lower expression of PEPCK, PGC-1α, fibroblast growth factor receptor 4 (FGFR4), β-klotho and GLUT2 when compared to LCD ($p<0.01$ for all) (table 4 and figure 4 in (IV)).

# 4. Discussion

The main aim for this project was to investigate oxidative stress, especially lipid oxidation, and its relationship to dyslipidemia, diabetes and atherosclerosis. For this investigation, different animal models with different control of glucose homeostasis, states of dyslipidemia and predisposition to develop atherosclerosis, were employed. The overall results of this thesis indicate that lipid oxidation is elevated in diabetes and seems to play a role in the development of atherosclerosis. Nevertheless, dietary content, of for example cholesterol, has important effects on the development of atherosclerosis in certain animal models.

Prior to Study I, a literature study was performed to evaluate the effects of GLP-1 on oxidative stress. This is summarized and published in (I). The aim of Study I was to investigate the effect of liraglutide on oxidative stress, which was unfortunately not possible as the expected increase in oxidative stress was not shown. Drawing any conclusions from Study I is however complicated by the small sample size, which was caused by the large drop out. This small sample size even compromises the use of the study as a pilot study, as the power calculations have a high degree of uncertainty with this small sample size. From the data obtained in the current study the power is 7 % and the sample size should have been 57 in the fluctuating glucose vehicle and liraglutide group respectively, to show a significant difference between the groups in regard to plasma MDA concentration with the variation and mean values seen in Study I. The difference and power is small and setting up a new study might not be relevant due to the high number of animals indicated to be needed to show a difference from this calculation. Taking the potential large drop out of animals into account would require an even higher number of animals. To show a significant difference between the groups with these small sample sizes the difference should have been at least 0.2 μM. This is within a physiologically possible spectrum for plasma MDA [21]. The calculations were made using the last measure of plasma MDA, a power level of 0.80 and the assumption that standard deviations are the same in the groups. Due to the low number of animals, the certainty on the standard deviation could be quite low and basing future study designs on these calculations would be hazardous. Placing the liraglutide intervention in between the two infusion rounds was successful in regard to avoiding a direct effect of liraglutide during the infusion rounds on blood glucose concentration and plasma insulin concentration, as no difference was shown between round one and two. However the outcome and limitations of Study I led to other models being considered for the next studies of the PhD project.

Study II investigated MDA in diabetic atherosclerosis, and Study III further investigated lipid oxidation in association with glucose tolerance and atherosclerosis. Results from both Study II and

III showed elevated lipid oxidation in both overt diabetes and in situations of impaired glucose tolerance, which supports the existing literature. Using the APOE$^{(-/-)}$ and LDLR$^{(-/-)}$ mice made it possible to study pathological outcomes of interventions due to their ability to develop atherosclerosis. The APOE$^{(-/-)}$ mice are insulin sensitive and rarely develop obesity [183, 227]. However they have been shown to develop obesity and insulin resistance when fed a diet with 60 kcal% from fat [228]. STZ was used to induce diabetes in the APOE$^{(-/-)}$ mice of Study II to avoid the use of extreme fat content in the diet. The use of STZ had a relatively low success rate (56 %) when compared to previous studies of APOE$^{(-/-)}$ mice [168]. The use of STZ for induction of diabetes has limitations; STZ has side effects and potentially induces oxidative stress [162]. Differentiation of glucose-induced effects from the direct effects on oxidative stress status can be difficult. However, the direct effects are speculated to be in the acute phase after STZ injections [162, 163]. As oxidative stress was measured 20 weeks after STZ injection in Study II, the oxidative stress induced acutely after STZ injections might not affect the outcome of the study.

The LDLR$^{(-/-)}$ mouse readily develops obesity, insulin resistance and glucose intolerance on HFD, which was an advantage in Study III as STZ was avoided [183]. However, the diabetic status of the mice of Study II and III were very different, with the mice in Study III having mild hyperglycemia when compared to the mice in Study II. LDLR$^{(-/-)}$ mice effectively clear chylomicrons and TG-enriched lipoproteins from circulation in contrast to APOE$^{(-/-)}$ mice [229]. LDLR$^{(-/-)}$ mice express APOE which facilitates uptake of FFA into adipose tissues, resulting in expansion of this tissue and obesity [230]. A well-documented association between obesity, hypertriglyceridemia and T2D exists, thus making the LDLR$^{(-/-)}$ mouse attractive in the study of atherosclerosis under these conditions [231-234]. Study III showed that the LCD group had the most impaired glucose tolerance and highest fasting blood glucose when compared to the HCD group. The HCD group did however show the largest plaque area. Thus, dietary cholesterol content can affect the potential for using these mice as models of diabetic atherosclerosis. The difference in the ability to develop metabolic disease could be induced by regulation of hepatic genes involved in lipid and glucose homeostasis. Pathways could be down-regulation of FGFR4 and β-klotho, which can decrease the inhibition of bile acid production, as this is induced by activation of FGFR4 [235, 236]. Another mechanism could be oxysterols activating LXRα leading to suppression of gluconeogenesis [114]. Cholesterol may be an important factor in the regulation of glucose homeostasis while being implicated in the development of atherosclerosis.

### 4.1. Reproducibility of studies

Reproducibility of experiments is a cornerstone in scientific research and the absence of reproducibility is a topic raising concern and debate [237]. Two different studies of reproducibility of research have shown a 20-25 % and 11 % success rate, respectively [238, 239]. Based on international state-of-the-art research and previous results from the model used in Study I, fluctuating hyperglycemia was expected to induce oxidative stress [20, 21]. Study I did not show this increase in oxidative stress, which could be a result of lower glucose concentrations during peaks in the induced fluctuations, only reaching 12.5±0.4 mmol/l compared to 20-22 mmol/l in the previous study [21]. The lack of reproducibility may be caused by multiple factors. Besides the liraglutide intervention the setup was as similar as possible to the previous study, however, small differences were inevitable. The rats were 29-32 weeks old at the end of the experiment, which corresponds to the 27-31 weeks of age of the rats in the previous experiment by Rakipovski *et al* [21]. The first round of infusion was however performed at an age of 22-25 weeks after 13-14 weeks on HFD in contrast to 20 weeks on HFD in the previous study. These differences could explain some of the differences between the two studies, but it is unlikely to be the entire explanation, as lean rats on chow diet has shown to reach 18-20 mmol/l when infused with 14.6 g of glucose per kg/day [20]. The rats in Study I were infused with 16.4 g of glucose per kg/day (II).

Changes in populations of animals could hold some of the explanation to the lack of induction of oxidative stress in Study I. Genetic drift can occur, which causes differences within the same strain or stock of rats [240, 241]. Genetic drift is well studied in inbred strains, but also arises in outbred stocks, where it can occur due to mutation, selection, or random drift associated with a restricted population size [240]. By personal communication with Taconic Denmark, after the results from the present study were obtained, information was acquired about changes in the colony of NTac:SD rats. In 2012, the American NTac:SD stock used by Taconic in USA was phased in at Taconic in Denmark. The change in population was made in between the study by Rakipovski *et al* (2016) and the present study. Different stocks of Sprague Dawley rats can show differences in metabolism studies e.g. a study of Hsd:SD and Crl:SD has shown a significantly different effect of intra uterine growth retardation on fat mass and glucose tolerance one year after birth between the two stocks [242]. Changes at one vendor or differences between two vendors could be of non-genetic nature. Feeding high-fat diets to 129S6/SvEvTac mice and 129S1/SvImJ has resulted in different responses in regard to weight gain, glucose tolerance, insulin resistance and hepatic steatosis. However, the effects of HFD on these parameters became nearly identical when the animals where housed together under identical conditions for three generations, indicating that the microbiota could have caused the observed differences [243]. The change in the NTac:SD stock by

changing from the Danish to the American stock at Taconic Denmark could potentially explain some the difference observed in glucose concentration, either by changes in genetics, microbiota or both. Emphasis should be placed on securing the same genetic background and to the extent possible the same microbial status of the animals when reproducing studies.

## 4.2. The connection between lipid oxidation and diabetes, dyslipidemia and atherosclerosis

### 4.2.1. Correlation between aortic plaque area and malondialdehyde

The hypothesis in Study II was that lipid oxidation is correlated to atherosclerotic development in APOE$^{(-/-)}$ mice, which was the finding of Study II. The initial full set of explanatory variables used for the stepwise multiple regression analysis with backward elimination was based on the assumption that the variables included were important for the development of atherosclerosis [244]. The criterion for exclusion was the p-value, excluding the variable with the largest p-value at each step, thus including the variables that contribute the most to $R^2$. An analysis like this is based on a solid selection of variables, which strengthens the model. From this analysis, plasma MDA was found to have the strongest correlation to atherosclerotic status in APOE$^{(-/-)}$ mice. The use of stepwise multiple regression has limitations and the use of the method is debated. Coincidence can affect the outcome of the analyses especially if covariates are correlated [245]. Including plasma MDA and total cholesterol as covariates in linear regression analyses, with aortic plaque area as the outcome, showed that plasma MDA provide additional information to the analysis, as the analysis resulted in a lower p value for MDA ($p=0.05$) than for total cholesterol ($p=0.20$). By analysing the MDA and cholesterol one by one, the results showed a lower root mean square error for MDA (6.89) when compared to the root mean square error of cholesterol (7.24) meaning that MDA can estimate aortic plaque area better than cholesterol. As for most animal experiments the number of animals is low. Extrapolation of results from such data to entire populations is not possible. The data can however be used as indicators of connections worth investigating further.

In Study II, aortic plaque area was elevated after 20 weeks of diabetes, which was similar to the findings of a previous study in STZ-induced diabetic APOE$^{(-/-)}$ mice lasting 12 weeks [246]. The study showed a rise in plasma TBARs concentration, a drop in erythrocyte-reduced glutathione/oxidized glutathione ratio and an up-regulation of glutathione peroxidase gene expression in the aorta. 4-HNE concentration in aorta and kidney tissue was increased in this study as well [246]. The 4-HNE increase in the aorta could indicate that the time course of plaque development gives rise to different levels of lipid peroxidation markers in the aorta, when measured

after 12 or 20 weeks, or that 4-HNE and MDA is accumulated and degraded differently in the aortic tissue as MDA was not elevated in the aorta in Study II. 4-HNE and TBARs being elevated does however support the fact that lipid peroxidation is implicated in the development of atherosclerosis in diabetes and thus supports the findings in the present study [247]. In high fat, high cholesterol fed APOE$^{(-/-)}$ mice, polyphenol compounds of red wine has been shown to reduce development of atherosclerosis, without affecting 8-iso-$PGF_{2\alpha}$ and hydroxyeicosatetraenoic acids [248]. This indicates that the correlation between lipid oxidation and atherosclerosis might only be relevant in the presence of diabetes. A connection between lipid oxidation and diabetic status has been shown in STZ-induced diabetic rats and, in addition, control of diabetes by insulin intervention has been shown to decrease lipid oxidation [249]. However, enalapril has been shown to reduce aortic MDA concentration and atherosclerosis in non-diabetic APOE$^{(-/-)}$ mice, indicating that lipid oxidation can be modified in non-diabetic APOE$^{(-/-)}$ mice and that plaque area can concurrently be decreased in a non-diabetic setting [250]. This has also been shown to be the case for nitrotyrosine in aorta and aortic atherosclerosis [251]. We aimed at using pharmacological intervention to reduce lipid oxidation and atherosclerosis in diabetic mice, as this would strengthen the use of MDA as a biomarker of atherosclerosis. However, we did not find a significant lowering of MDA or atherosclerosis in the ENAL group, which could be attributed to enalapril being unable to alter this effect of diabetes in this setup. However, a dose of 0.5 mg/kg/day has previously been shown not to reduce plaque formation in APOE$^{(-/-)}$ mice, while 30 mg/kg/day did significantly reduce plaque formation [250, 252]. Thus, the 10 mg/kg/day used in Study II could be too low to reduce atherosclerosis and lipid oxidation and a higher dose might have resulted in an effect of enalapril.

In the studies of this thesis both MDA and isoprostane concentration could have been normalized to the concentration of the molecules from which they are formed, thus PUFA for MDA and arachidonic acid for isoprostanes. This would lower the risk of reaching conclusions on the degree of oxidative stress that could have been caused by differences in substrate. These were however not specifically measured in the studies of this thesis. As MDA can bind to APOB-100-containing lipoproteins forming adducts that can be involved in the development of atherosclerosis, normalizing MDA to IDL, LDL and vLDL could be useful in future studies. Especially, LDL would be interesting to normalize due to the susceptibility to oxidation and modification by MDA [146].

The relationship between lipid oxidation and atherosclerosis was not found to be similar in Study II and III. Free MDA has a short half-life of approximately two hours in rats, whereas *in vitro* studies have shown MDA bound to lysine to have a half-life of approximately two days [253, 254]. The MDA bound to lipoproteins could be affected by the half-life of the lipoproteins, which can vary from hours to days in humans for vLDL and LDL, respectively [255]. As APOB-100 can bind

MDA, differences in clearance of MDA containing lipoproteins could vary between the mouse models used in this thesis. The APOE$^{(-/-)}$ mice have increased concentrations of APOB-48 containing lipoproteins in contrast to increased concentrations of APOB-100 containing lipoproteins in the LDLR$^{(-/-)}$ mice [203]. The increase in lipoproteins in the APOE$^{(-/-)}$ mice are mainly in the short-lived vLDL whereas LDLR$^{(-/-)}$ mice mainly has increased LDL, which has a long half-life and is more susceptible to binding MDA [146]. This potentially increases the half-life of MDA containing lipoproteins in the LDLR$^{(-/-)}$ mice when compared to the APOE$^{(-/-)}$ mice. Extensively modified LDL molecules may however have a shorter half-life in plasma as they are taken up by macrophages e.g. in the endothelium of the arteries [145]. These differences in composition of lipoproteins, in the two mouse strains used in Study II and III, could indicate that the LDLR$^{(-/-)}$ mice are more prone to atherosclerotic development due to the susceptibility to oxidative modifications, this is however not the case. The fact that the LDLR$^{(-/-)}$ mice are less prone to develop atherosclerotic lesions is a drawback to this model. However, the model has a huge advantage in comparison to the clinic, as humans with atherosclerosis have high plasma concentrations of APOB-100 containing lipoproteins [256]. Differences in the size of LDL can be a factor affecting the atherogenic nature of these lipoproteins [256]. A detailed understanding of the effect of lipoprotein composition and lipid oxidation in models of atherosclerosis is essential to understand the pathogenesis of the disease and the relationship between lipid oxidation and atherosclerosis.

#### 4.2.2. Dependency of dietary cholesterol for plaque formation

The results of Study III underline the fact that dietary cholesterol plays an important role in the development of atherosclerosis in LDLR$^{(-/-)}$ mice [198, 257]. In addition, the study revealed that dietary cholesterol can reduce the development of glucose intolerance induced by high fat feeding. The two diets used in Study III vary in other contents than cholesterol. This is a limitation to the study which is discussed in (IV). The hypercholesterolemia induced by the HCD diet in Study III and its connection to the increase in atherogenesis could explained by a redistribution of lipoproteins, as cholesterol feeding has been shown to decrease HDL and increase vLDL cholesterol in LDLR$^{(-/-)}$ mice [198]. The role of dietary cholesterol in the development of atherosclerosis in the LDLR$^{(-/-)}$ mice has been shown to be attributed to inflammatory effects induced by cholesterol in addition to the inflammatory effects induced by obesity [198]. This is possibly explained by serum amyloid A induced mechanisms and thus suggests a chronic inflammatory process [198, 257].

A study in LDLR$^{(-/-)}$ mice has shown that when fed either a HFD or high-fructose diet, both containing 0.15 % cholesterol, both groups develop atherosclerosis [199]. This indicates that dietary cholesterol has an important role in the development of atherosclerosis in these mice, when

compared to fat and fructose. In (IV), the high plasma cholesterol is connected to the development of atherosclerosis, whereas the lipid oxidation level is hypothesized to follow the glucose tolerance and fasting blood glucose. The APOE$^{(-/-)}$ mice in in Study II did however show a correlation between lipid oxidation and plaque area. The plaque formation in APOE$^{(-/-)}$ mice is not as dependent on dietary cholesterol. These mice develop atherosclerosis on low fat, low cholesterol diets from an age of 10 weeks, which is shown in Study II where the mice were fed chow diets [133, 174, 192, 195-197]. Endogenous serum amyloid A deficiency has shown not to affect plaque formation in APOE$^{(-/-)}$ mice [258]. This possibly explains some of the difference in the importance of dietary cholesterol in the development of atherosclerosis in the two strains. This difference is underlined by the findings in Study II and III of the thesis.

The mice in the HCD group of Study III had the highest accumulation of TG and collagen content in the liver. This has been shown to be connected to impaired glucose tolerance in both human and animal studies [259-261]. However, the HCD group was the group with the best glucose tolerance in the current study. Dissociation between hepatic lipid accumulation and glucose tolerance or insulin resistance has been observed previously, in various mouse models and humans with mutations leading to increased hepatic accumulation of TG [262-265]. The present study thus supports the previous findings and indicates that cholesterol feeding in LDLR$^{(-/-)}$ mice can protect against development of glucose intolerance despite of induction of hepatic steatosis and fibrosis. In future studies, further investigation of hepatic lipid oxidation in this model could be interesting, as oxidative stress and lipid oxidation is implicated in the development of hepatic steatosis and fibrosis [259].

#### 4.2.3. Cholesterol feeding and glucose tolerance

Eight of the 14 hepatic genes investigated in Study III had significantly different expression levels between the two groups. A thorough discussion is presented in (IV). In short, dietary cholesterol increases SREBP-1c, which could be the mechanism leading to the decreased expression of PEPCK [266]. PGC-1α expression was decreased as well, which together with the decreased expression of PEPCK could lead to a decrease in gluconeogenesis, and thus result in a lower fasting glucose concentration and better glucose tolerance in the HCD group [267]. Lower fasting blood glucose concentrations together with higher plasma TG and cholesterol concentrations have been observed in a study of double LDLR- and lechitin-cholesterol acyltransferase-deficient mice, when compared to LDLR$^{(-/-)}$ mice. Here, the double knockout mice had higher expression of SREBP-1c and lower PEPCK expression compared to LDLR$^{(-/-)}$ mice. This was seen together with a significantly lower plasma insulin concentration indicating that the double knockout mice were

more sensitive to insulin [268]. Together with our data, this indicates how a plasma lipid profile with high TG and cholesterol concentrations could be connected to the better metabolic profile in LDLR$^{(-/-)}$ mice, through regulation of SREBP-1c and PEPCK. Increased expression of hepatic leptin receptors can play a role in the better glucose tolerance observed in the HCD group [269]. Together with lower plasma leptin concentrations in the HCD, this could indicate a lower degree of leptin resistance in this group. A very plausible explanation for the differences observed in glucose tolerance between the two groups could be an increase in bile acid production and concentration, induced by dietary cholesterol [118, 119]. Activation of FGFR4, which is enhanced in the presence of β-klotho, is a part of a negative feed-back loop down-regulating the production of bile acids [235, 236]. FGFR4 is activated by fibroblast growth factor 15 (FGF15) in mice. FGF15 is expressed in the ileum in a farnesoid X receptor-dependent manner, when bile acids are released to the intestines, thereby initiating the negative feed-back loop just mentioned [119]. In the HCD group of the Study III, FGFR4 and β-klotho were expressed at a lower level. The lower expression of FGFR4 and β-klotho could indicate a higher production of bile acids, if this negative feedback loop is not activated in the same degree as in the LCD group. The effect of bile acids on the difference in glucose tolerance in the LDLR$^{(-/-)}$ mice of this study could be investigated in future studies by including measures of serum and feces bile acids, endogenous GLP-1 concentration and FGF15 expression in the ileum. The fact that the LCD group was less glucose tolerant in Study III could have been a contributing factor to the increased lipid oxidation observed in this group.

### 4.3. Translation to clinical studies

In both the APOE$^{(-/-)}$ and the LDLR$^{(-/-)}$ mice, it is difficult to separate glycemic effects from the effects of high plasma cholesterol concentrations, and choice of dietary intervention might play an important role in these models as seen in Study III. However, they are still valuable in the investigation of lipid oxidation in diabetes, dyslipidemia and atherosclerosis [184, 270]. Uncovering specific connections between diabetes, control of diabetes, dyslipidemia and oxidative stress by using mouse models combined with investigations in humans could provide valuable insight and knowledge. In time, this could be used to lower the incidence of CVD in diabetes. Study II indicates the potential for MDA to be used as a biomarker of atherosclerosis by showing a correlation between MDA and aortic plaque area and in this study, MDA added information to the analysis when compared to performing a linear regression of aortic plaque area and cholesterol. In humans, MDA has been used as a biomarker for oxidative stress and lipid peroxidation, and has been associated with atherosclerotic development [271, 272]. In a small case control study including 51 male cases with increased carotid intima-media thickness and 51 male controls, urinary MDA, 8-

hydroxy-2'-deoxyguanosine and 8-iso-$PGF_{2\alpha}$ were associated with increased carotid intima-media thickness even when adjusting for conventional risk factors of CVD [273]. Plasma MDA measured by thiobarbituric acid (TBA) assays level has been shown to be elevated in hypertensive patients, when compared to healthy controls [274]. This has been seen in conjunction with a decrease in catalase activity in hypertensive pregnant women [275]. A correlation between MDA measured by TBA assay in plasma and the atherogenic index ((Total cholesterol-HDL cholesterol)/HDL cholesterol) has previously been shown in hyperlipidemic subjects, ischemic heart disease patients, ischemic heart disease risk patients and control subjects [276, 277]. The latter mentioned studies used the nonspecific TBA assay, and it is not clear from these results how much of the measured TBARs is indeed MDA, other products of lipid peroxidation or substances produced during the analytic procedure [47, 278]. However, the studies together with the results from Study II indicate that MDA is an interesting focus as a biomarker for CVD. An important aspect to investigate in humans is whether markers of lipid oxidation provide additional information when compared to the markers currently used to asses CVD risk. Studies investigating the connection between markers of lipid oxidation and atherosclerosis in different animal models of diabetes could be important to clarify in which situations the two are correlated and which mechanisms exist. The findings of Study III were in contrast to the findings of Study II in the current thesis, as the $LDLR^{(-/-)}$ mice with the highest amount of plaque formation had the lowest degree of lipid oxidation. This could be a consequence of differences between the models or a consequence of the difference in diabetic status in the two models. Even though various rodent models of dyslipidemia exist, no perfect model of human atherosclerosis is available [191]. One of the main obstacles is the fact that transgenic or knockout approaches are needed to induce dyslipidemia and atherosclerosis. This can induce unwanted changes in metabolic pathways which might result in a model differing additionally from the human situation, than the differences already existing between mice and man [279]. Besides the mentioned differences in plasma lipoprotein profiles and the likelihood of plaque rupture, mice and man have basic differences in immunology, which is an important player in atherosclerotic development [280].

As in Study III, the effect of dietary cholesterol in humans is interesting to investigate. Cholesterol has been shown to be elevated in both diabetic and CVD patients and is often associated with adverse effects [18, 281]. This is also the case in regard to development of atherosclerosis in the $LDLR^{(-/-)}$ mice of Study III. However, glucose tolerance was higher and lipid oxidation was lower in the HCD group. In T2D patients, high cholesterol diets with eggs as a source, has shown no to alter plasma glucose, but lower inflammatory markers without increasing plasma lipids and atherogenic lipoproteins when compared to an oatmeal breakfast [282]. This could however be

explained by the ability of eggs to increase plasma carotenoids and the anti-inflammatory and antioxidative properties of these [282]. Eggs did not increase plasma cholesterol when compared to the group eating oatmeal and other contents of eggs could affect the outcome of such a study e.g. by lowering plasma cholesterol. The results from Study III cannot be directly extrapolated to humans as multiple differences exists e.g. in bile acid synthesis and regulation [283].

### 4.4. Conclusion and perspective

No animal model mimics the entire pathogenesis of neither diabetes nor atherosclerosis. The three different models used in this thesis were set up to investigate the involvement of oxidative stress and lipid oxidation in diabetes, dyslipidemia and atherosclerosis. In Study I and II, potential therapeutic possibilities were investigated as well. In regard to diabetes, three distinct situations were studied; fluctuating hyperglycemia, overt diabetes or glucose intolerance. Study I did not find an increase in oxidative stress induced by fluctuating hyperglycemia. Study II and III highlight the importance of choice of model. They showed a difference between $APO^{(-/-)}$ and $LDLR^{(-/-)}$ mice in the relationship between lipid oxidation and atherosclerosis, as a positive correlation between atherosclerosis and lipid oxidation was found in Study II in contrast to Study III. The study design, e.g. feeding regimes and treatments, were nevertheless different in the two studies.

In perspective, results from this PhD project can be used to understand parts of the relationship between lipid oxidation, diabetes, dyslipidemia and atherosclerosis. Obtaining a thorough understanding of this relationship could potentially be used in the development of treatments or prevention strategies of diabetic atherosclerosis. Investigating the effects of liraglutide on oxidative stress is still warranted. This could include human and animal observational studies and mechanistic studies, which could aid in separating glucose-dependent from glucose-independent effects. Unraveling the causal mechanisms could be done using cellular signaling studies combined with measures of expression and activity of ROS-producing enzymes and antioxidant gene expression and concentrations, together with the measures of lipid oxidation. Liraglutide has shown beneficial effects on CVD risk and elucidating the involvement of a potential oxidative stress lowering mechanism is interesting.

To further investigate the correlation between lipid oxidation and atherosclerosis, plasma MDA and atherosclerotic plaque area in diabetic $APOE^{(-/-)}$ mice could be measured at different time points instead of only one. This could help clarify if the correlation is present at the initial stages of atherosclerosis and during the different stages of atherosclerotic progression. Here, histological evaluation of the plaques would be helpful in a more accurate determination of the atherosclerotic stage. The diabetic $APOE^{(-/-)}$ mouse could be used as a model of fluctuating hyperglycemia to

investigate the relationship between lipid oxidation and atherosclerosis under these conditions. Fluctuations in blood glucose could be induced by multiple injections of short acting insulin to diabetic APOE$^{(-/-)}$ mice. The correlation between MDA and plaque percentage needs to be confirmed in such studies of both shorter and longer duration to substantiate the use of MDA as a biomarker of atherosclerosis and atherosclerotic progression. In the APOE$^{(-/-)}$ mice, MDA could be a simple secondary biomarker or potentially play a pathological role, the latter is supported by the role of MDA in oxLDL formation. For MDA to serve as a biomarker of atherosclerosis, details such as the biological half-life of MDA and its pathways of metabolism and excretion needs to be further investigated.

Whether the observations in Study III are applicable to the model only, or if it is a more general observation raises a demand for further studies. Cholesterol content in western type diets vary from 0.2-2 %, which make it relevant to thoroughly investigate the effect of different levels of cholesterol content in animal models fed these diets. Study III investigated hepatic regulation of genes involved in lipid and glucose homeostasis in response to dietary cholesterol. The mice were kept under metabolic distress in the model setting of Study III and multiple pathways were regulated, which complicated the exact evaluation of effects of dietary cholesterol on each pathway. The regulation of bile acid production could be a very interesting focus of future studies as this pathway naturally must be affected by the high cholesterol concentrations. To elucidate what is attributed to the LDLR$^{(-/-)}$ phenotype, other mice strains or a C57BL/6J control could be helpful. Adding additional dietary groups to the study of LDLR$^{(-/-)}$ mice could be interesting in the study of associations between lipid oxidation, glucose tolerance and atherosclerosis of LDLR$^{(-/-)}$ mice. This could include diets with different cholesterol content (0.2-2 %) and fat content, and include the use of more controlled purified diets in regard to obtaining the same composition of the additional content besides cholesterol. Such studies could help obtain data on which concentrations of cholesterol that is needed to induce the observed effects on glucose homeostasis. Effects of other differences than cholesterol content in the two standard HFDs used in Study III could be ruled out by using diets only varying in cholesterol content.

In summary, the primary objective of Study I was to study the effect of liraglutide on the oxidative stress induced by fluctuating hyperglycemia in rats. As fluctuating hyperglycemia did not induce oxidative stress in the rats, the potential of liraglutide to reduce oxidative stress could not be elucidated. In Study II, the correlation between MDA and atherosclerosis was evaluated and the study showed a correlation between MDA in plasma and the atherosclerotic plaque area in APOE$^{(-/-)}$ mice. In Study III, lipid oxidation was found to be highest in the group with impaired glucose tolerance but with the smallest aortic plaque area. Thus, the study supports that impairment in

glucose tolerance is associated with increased lipid oxidation. The study underlined that dietary cholesterol is important for the development of atherosclerosis in these mice. In addition, cholesterol showed a protective effect on HFD-induced glucose intolerance, which was possibly caused by a regulation of hepatic gene expression. It is still warranted to find suitable models for diabetic induced atherosclerosis as these will allow the performance of mechanistic studies. Animal models can be used to clarify the parts of the relationship between atherosclerosis and lipid oxidation in humans which is still unclear. The connection between atherosclerosis and dyslipidemia is well recognized. The connection between atherosclerosis and glucose homeostasis does however need further investigation, especially due to the lack of effect of glycemic control on cardiovascular end points. The LDLR$^{(-/-)}$ mice could have the potential for investigation of dyslipidemia, glucose homeostasis, atherosclerosis and lipid oxidation. This will however demand a thorough investigation of the effect of dietary composition, as the mice in Study III showed an increase in atherosclerotic development in the group with the best glucose homeostasis and lowest concentration of plasma lipid oxidation end products.

From the results of this thesis, the overall conclusion is that lipid oxidation is elevated in diabetes and it can play a role in the development of atherosclerosis. However, the dyslipidemic status, choice of diet and animal model also play an important role for the outcome of studies of lipid oxidation performed in animal models of diabetes and dyslipidemia.

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# 6. Appendices

## Appendix 1. Analyses (Study I-III)

*Blood glucose*

For blood glucose determination 5 µl of whole blood were diluted in 250 µl glucose/lactate system solution (EKF Diagnostics, Barleben, Germany) and analyzed in the Biosen S line glucose analyzer (EKF Diagnostics, Barleben, Germany).

*$HbA_{1c}$*

HbA1c was determined by collecting 10 µl whole-blood in a 10 µl Na-Heparinized capillary tube (Vitrex Medical A/S, Herlev, Denmark), subsequently it was stabilized in Hemolyzing Reagent (Roche/Hitachi, Mannheim, Germany) before it was analyzed in the Cobas 6000 c 501 (Roche Diagnostic Systems, Bern, Switzerland).

*Plasma insulin*

Plasma for insulin determination (5 µl) was analyzed by Luminescence Oxygen Channeling Immunoassay (LOCI) [284, 285] using a sandwich immunoassay. The assay was based on two different latex beads, streptavidin coated (donor) beads and europium coated (acceptor) beads. The donor beads with a biotinylated antibody and the acceptor bead covalently coated with anti-insulin antibodies, thus making the sandwich around insulin, if it was present. During the assay the three reactants combine with analyte to form a bead-aggregate-immune complex. The analysis was performed in two steps. Illumination at 680 nm of the complex released singlet oxygen from the light sensitive donor beads. This channels into the acceptor beads and triggers chemiluminescence which was measured in the EnVision plate reader. This only occurs if the two beads (donor and acceptor) were in close proximity, which required to presence of insulin. The amount of light generated was proportional to the concentration of insulin. This method has shown to be superior to the traditional ELISA, e.g. in regard to precision and volume needed [285]. Lower limit of quantification (LLOQ) was 20 pmol/l and upper limit of quantification (ULOQ) was 3000 pmol/l.

*Plasma leptin*

Plasma leptin concentration was measured using Mouse Leptin AlphaLISA kit (Perkin Elmer, Boston, Massachusetts, USA) on a LOCI platform with only a few exceptions from the manufacturer's manual. Calibrators were prepared in charcoal treated rat plasma (Bioreclamation, Baltimore, Maryland, USA). The charcoal treatment includes adding 30 mg/ml charcoal to plasma, stirring overnight at room temperature followed by filtering on the following day. When sample dilution was necessary it was done using charcoal treated rat plasma. The calibrator and sample

volumes were changed from 5 µl to 1.25 µl in 3.75 µl of charcoal treated rat plasma. The plates were read in an Envision plate reader with excitation at 680 nm and emission measured at 615 nm. The calibrators were measured in quadruplicates, and samples in duplicates. LLOQ was 0.4 ng/ml and OLOQ was 400 ng/ml.

*Plasma total cholesterol, lipoprotein fractions and triglycerides*

The analysis of total cholesterol and lipoprotein fractions was performed at the Department of Pathology/Lipid Sciences, Wake Forrest University School of Medicine Winston-Salem, North Carolina, USA as previously described [286]. Total cholesterol was determined using the enzymatic kit Cholesterol/HP (Roche Diagnostics, Indianopolis, Indiana, USA) following the manufacturer's manual, and results were obtained using a SpectraMAX 250 plate reader (Molecular Devices, Sunnyvale, California, US). Cholesterol fractions (LDL, vLDL and HDL) were determined using 30 µl of plasma for high-performance liquid chromatography (HPLC) followed by fast protein liquid chromatography (FPLC). Plasma was diluted 1:1 in phosphate buffered saline (PBS) injected into Superose 6 10/300 GL columns (GE Healthcare, Pittsburg, Pennsylvania, USA) and run at 0.5 min/l in an Agilent 1100 HPLC automatized system (Agilent Technologies, Santa Clara, California, USA). After this the cholesterol fractions were measured using a total cholesterol enzymatic reagent, Infinite (Thermo Fischer Scientific, Waltham, Massachusetts, USA). Signal was read at 37°C and at an absorbance of 546 nm before it was integrated using Chrom Perfect Spirit software (Justice Laboratory Software). The signal from each of the lipoprotein fractions was used to determine the cholesterol fraction concentration, by multiplying them with the total cholesterol concentration. Total cholesterol/HDL ratio was calculated as this is believed to be a predictor of CVD risk [244]. Plasma triglycerides were analyzed on Cobas 6000 c 501 (Roche Diagnostic Systems, Bern, Switzerland) using commercially available enzymatic assays (Roche diagnostics, Bern, Switzerland) according to the manufacturer's manual.

*Plasma and aorta malondialdehyde (MDA)*

MDA was analyzed by the HPLC method, which has been described previously [287]. In summary, 10 µL of plasma or aorta homogenat (aorta was homogenized in 500 µl of lysis buffer (RTL, Qiagen GmbH, Hilden, Germany)) were added to a microcentrifuge tube. This was mixed gently with 500 µL of 42 mmol/L $H_2SO_4$ and then 125 µL of 100 g/L phosphotungstic acid. The mixture was incubated for five minutes at room temperature before it was centrifuged (3 min, 16000 rpm) at room temperature as well. As MDA is associated with lipoproteins it is contained in the pellet. The supernatant was discarded, and the precipitate was re-suspended in 300 µL of 42 mmol/L $H_2SO_4$

and 45 µL of 100 g/L PA, before centrifuging the sample again (3 min, 16000 rpm) at room temperature. The supernatant was again discarded, after which the pellet was re-suspended in 350 µL of $H_2O$, 50 µL of 0.7 mmol/L 2,6-tert-butyl-4-methylphenol in 200 µL/L 96 % ethanol, and 100 µL of thiobarbituric acid (TBA) reagent (6.7 g/L TBA in $H_2O$ diluted 1:1 with glacial acetic acid). Immediately after the re-suspension the mixture was heated to 95 °C for 60 minutes followed by cooling on ice. $MDA(TBA)_2$ adduct was then extracted by adding 500 µL of n-butanol and the layers were separated by centrifugation (3 min, 16 000 rpm) at room temperature. Calibration curves were constructed using 0.1–5.0 mM tetramethoxypropane for plasma and 2.5–50 mM tetramethoxypropane for aorta homogenates. The $MDA(TBA)_2$ adducts in the organic phase was determined on an automated HPLC gradient system by fluorescence detection (excitation, 515 nm; emission, 553 nm) as described in details in [287]. In addition to MDA in aorta, the protein concentration was determined by the bicinchoninic acid assay (Merck Millipore Corporation, Darmstadt, Germany) as described in [288].

*Plasma 8-iso-$PGF_{2\alpha}$*

Plasma 8-iso-PGF2α concentration was measured by the 8-Isoprostane ELISA kit (Cayman Chemical, Ann Arbor, Michigan, USA). In short, 40 µl of plasma were purified from proteins and phospholipids using the Waters Ostro 96-wells plate 25 mg (Waters Inc., Milford, Massachusetts, USA). Subsequently all samples were air dried using Heto-Vac (Biostadt, Denmark) and re-suspended with 120 µl enzyme immunoassay buffer supplied by the assay kit manufacturer. The samples were analyzed in duplicates with 50 µl in each of the 96 wells of the pre-coated plates also supplied by the assay manufacturer. Plates were read at 405 - 420 nm on a SpectraMax ELISA reader (Molecular Devices Inc., California, USA). The samples are hydrolyzed prior to analyses to measure both free and esterified 8-iso-PGF2α.

*Plasma dihydrobiopterin/tetrahydrobiopterin ($BH_2/BH_4$)*

The dithioerythritol-stabilized plasma was used for analysis of $BH_2/BH_4$ and it was performed based on the principle described in [289]. A 4% dithioerythritol solution (Sigma-Aldrich, Brøndby, Denmark) was used for the stabilization of plasma. The analysis was performed using HPLC with fluorescence detection using iodine oxidation. Trichloroacetic acid (Bie&Berntsen A/S, Herlev, Denmark) was used to precipitate protein. 50 µl and 70 µl of plasma were used oxidized with iodine (Sigma-Aldrich, Brøndby, Denmark) in either acid or base, respectively. Oxidation in acid was used to determinate $BH_4$, $BH_2$ and biopterin, while the oxidation in base was used to determine $BH_2$ and biopterin. After the oxidation, hydrochlroric acid (Shircks Laboratories, Jena, Switzerland) was

added to the alkaline sample, and ascorbic acid (Bie&Berntsen A/S, Herlev, Denmark) was added to both samples to remove excess iodine. The samples are centrifuged and analysed by HPLC (excitation, 275 or 350 nm; emission, 442 nm) (Agilent, Hørsholm, Denmark).

Real time quantitative PCR

30 mg of frozen liver tissue from the right lateral lobe was weighed for RNA purification using RNAeasy Mini Kit (Qiagen GmbH, Hilden, Germany) and cDNA was synthesized with SuperScript VILO cDNA Synthesis Kit (ThermoFischer Scientific, Waltham, Massachusetts, USA) both following manufacturer's manual. RNA quantity and quality were assessed by Nanodrop 1000 Spectrophotometer (ThermoFischer Scientific, Waltham, Massachusetts, USA) and Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany) using Agilent RNA 6000 Nano Kit (Agilent Technologies, Waldbronn, Germany). TaqMan primers and probes were obtained from Applied Biosystems (ThermoFischer Scientific, Waltham, Massachusetts, USA). Real-time quantitative polymerase chain reaction (RT-qPCR) was performed on QuantStudio 12K Flex Real-Time PCR System (ThermoFischer Scientific, Waltham, Massachusetts, USA) using TaqMan Fast Universal PCR Master Mix (ThermoFischer Scientific, Waltham, Massachusetts, USA) according to the manufacturer's manual. Each amplification mixture contained 40 ng cDNA in 4µl of sample or standard and 6 µl master mix including target specific TaqMan primers and probes. RT-qPCR thermocycling parameters were; hold stage of 50°C for 2 min and 95°C for 10 min, and then 40 cycles of 95°C for 15 sec and 60°C for 1 min. Standards for each gene were made from 1:4 dilution series with five concentrations. Three negative controls were used; one containing cDNA but no DNA polymerase, one with cDNA but no master mix and one without cDNA but with master mix. Three reference genes (TATA-box-binding protein (TBP), GAPDH and β-actin (ACTB)) were run together with the samples on each plate. The geometric means of the three reference genes were used to normalize data from the qPCR reactions of each outcome gene (table 11). Geometric means of multiple reference genes were used as this method is more robust and the results more accurate [290]. Selection of reference genes were made based on the literature [291-293]. All samples and standards were measured in duplicates.

Scanning

In conscious animals, body composition was evaluated by quantitative nuclear magnetic resonance spectroscopy (EchoMRI 3-in-1 Animal Tissue Composition Analyzer; Echo Medical Systems, Houston, Texas, USA). Before the measurement, the system was tested and the EchoMRI was calibrated by scanning a tube with a known amount of fat.

Histology

For visualization and measurement of lipid and triglyceride content of the livers, Oil Red O staining was applied. Liver tissue fixated in 10 % buffered formalin was transferred to 20 % sucrose until the tissue dropped to the bottom of the vial. Hereafter, the liver tissue was embedded in Tissue-Tek® OCT compound (#25608-930, Sakura®Finetek, Copenhagen, Denmark) and frozen before cutting 10 µm thick sections for staining. Hepatic fibrosis was visualized and measured using Picrosirius Red staining. Formalin fixed liver tissue was processed in a tissue processor (Leica ASP300S, Ballerup, Denmark), embedded in paraffin and 3 µm sections was cut. For both staining techniques two liver sections on each glass slide were stained with either Oil Red O (#O-0625, Sigma-Aldrich, Saint Louis, MO, USA) or Picrosirius Red Stain Kit according to the manufacturer's instructions (#ab150681, Abcam, Cambridge, United Kingdom). The slides were scanned on a slide scanner (Hamamatsu NanoZoomer 2.0 HT, Hamamatsu, Shinmiyakoda, Japan) on 40x magnification. For analysis of the images, the scanned slides were imported to a software program (Visiopharm A/S, Hørsholm, Denmark), in which the area of Oil Red O or Picrosirius Red positive staining was quantified and data was reported as the percentage of the whole area of two liver sections that was Oil Red O or Picrosirius Red positive.

# 7. Paper I-IV

## Paper I

**Does glucagon-like peptide-1 ameliorate oxidative stress in diabetes? Evidence based on experimental and clinical studies.**

Petersen KE, Rakipovski G, Raun K, Lykkesfeldt J.

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REVIEW ARTICLE

# Does Glucagon-like Peptide-1 Ameliorate Oxidative Stress in Diabetes? Evidence Based on Experimental and Clinical Studies

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**Abstract:** Glucagon-like peptide-1 (GLP-1) has shown to influence the oxidative stress status in a number of *in vitro*, *in vivo* and clinical studies. Well-known effects of GLP-1 including better glycemic control, decreased food intake, increased insulin release and increased insulin sensitivity may indirectly contribute to this phenomenon, but glucose-independent effects on ROS level, production and antioxidant capacity have been suggested to also play a role. The potential 'antioxidant' activity of GLP-1 along with other proposed glucose-independent modes of action related to ameliorating redox imbalance remains a controversial topic but could hold a therapeutic potential against micro- and macrovascular diabetic complications. This review discusses the presently available knowledge from experimental and clinical studies on the effects of GLP-1 on oxidative stress in diabetes and diabetes-related complications.

**Keywords:** Glucagon-like peptide-1, oxidative stress, diabetes, diabetic complications.

## 1. INTRODUCTION

Diabetes can lead to the development of late complications described as microvascular complications including retinopathy, nephropathy, neuropathy and macrovascular complications comprising cardiovascular diseases (CVD) [1], the latter being the leading cause of death among diabetic patients [2, 3]. The mechanisms underlying the development of complications in diabetes are still not completely understood. However, a hypothesis involving oxidative stress (OS) and inflammation as potent causative initiators of diabetic complications in general has been put forward [4, 5].

Glucagon-like peptide-1 (GLP-1) constitutes a relatively recent therapeutic option for type 2 diabetic patients. GLP-1 receptor agonists, as the approved pharmaceuticals liraglutide, exenatide, lixinatide, albiglutide and dulaglutide are designed to be more resistant to degradation than endogenous GLP-1 that with a half-life of less than two minutes has little therapeutic potential [6]. Pharmacological levels of these GLP-1 analogues are used to mimic incretin effects [7], i.e. glucose lowering, stimulation of glucose-dependent insulin secretion, and decrease of glucagon release [8, 9]. GLP-1 can to some extent preserve β-cell function and inhibit apoptosis [10, 11]. In rodents, GLP-1 analogues have been shown to stimulate proliferation of -cells, but it is still debated whether this occurs in humans [10, 12]. The clinical effects include decreased appetite and induction of body weight loss [10, 13]. Moreover, GLP-1 and its agonists have the potential to lower postprandial glucose excursions, which may otherwise be associated with endothelial dysfunction, inflammation and OS [14, 15]. Lately, a beneficial role of GLP-1 in inflammation has gained increasing interest as reports have shown that it is released in an interleukin-6 (IL-6) dependent manner [16, 17]. GLP-1 levels has been found to be increased in both atherosclerosis [18] and patients with ventricular systolic dysfunction [19] indicating that GLP-1 could be induced as a compensatory mechanism during disease progression where OS may also play a role. Experimental [20-22] and clinical [23, 24] studies have indicated, that GLP-1 can reduce OS under a variety of conditions making it important to investigate if these effects are dependent or independent of GLP-1's effects on glucose homeostasis (insulinotropic and glucose-lowering effects). The present review discusses the available literature on the role of GLP-1 as a redox modulator.

## 2. DIABETES AND OXIDATIVE STRESS

The "unifying theory" proposed by Brownlee [4] in 2001 suggests that hyperglycemia - a hallmark of both type 1 and type 2 diabetes (T1D and T2D) - can lead to increased OS if the antioxidant capacity is exceeded. OS is characterized as an imbalance between produced reactive oxygen species (ROS)/reactive nitrogen species (RNS) and the antioxidant capacity. According to the hypothesis, OS plays a pivotal role in the development of diabetes related late complications in both T1D and T2D [4, 25, 26]. It should be

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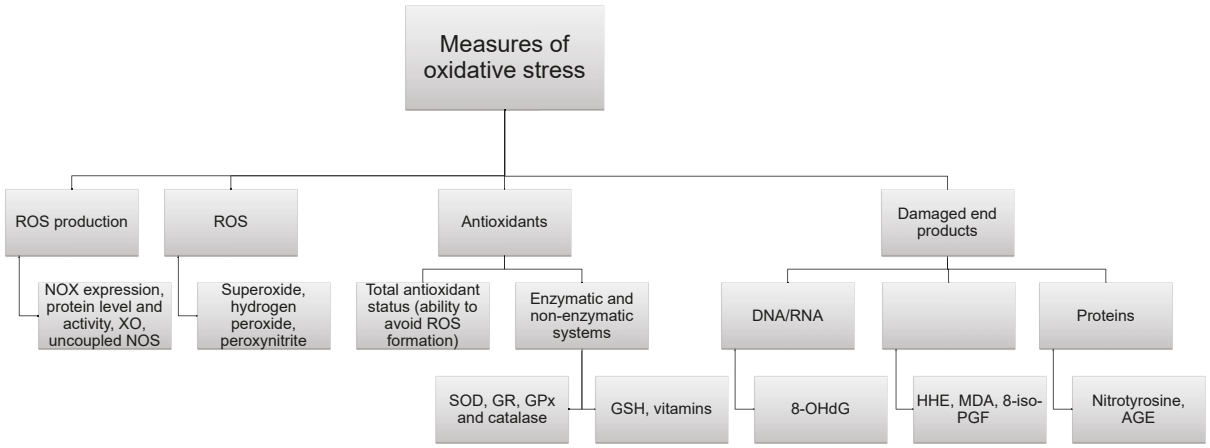


**Fig. (1).** ROS production/level or antioxidant capacity. OS status can be assessed by different means including the measurement of amounts and activities of ROS producing enzymes, ROS levels per se, antioxidant levels or damaged end products and adducts.

mentioned that OS may also play an important role in the development of both types of diabetes (reviewed in [27]). In short, -cells are vulnerable to injury from high levels of ROS due to a comparatively low expression of antioxidant enzymes [28] and consequently, elevated OS conditions are likely to cause -cell loss [28]. Importantly, loss of these cells cannot readily be restored because of their long lifespan and low proliferation rate [29, 30]. The loss of K-cells in turn results in further increasing blood glucose, OS and insulin resistance, thus starting a vicious circle (discussed in 2.1.).

### 2.1. Sources of Oxidative Stress in Diabetes

OS as measured both directly as ROS and by a large variety of biomarkers (Fig. **1**) has consistently been shown to be elevated in diabetes [4, 14]. Mitochondrial production of the ROS, superoxide and increased activation of the protein kinase C (PKC) pathway in diabetic vascular tissue have been hypothesized to be major contributors to excess ROS production together with enzymatic systems such as nicotinamide adenine dinucleotide phosphate-oxidase (NOX), which is a membrane-bound superoxide producing enzyme [4]. Elevated plasma concentrations of glucose and free fatty acids (FFA), which is observed in T2D and poorly controlled T1D subjects seems to be the main drivers of ROS production [5, 31-34]. This is also the case in other disorders with elevated FFA's as dyslipidemia and obesity (reviewed in [35]). Proinflammatory cytokines and excessive levels of nutrients can increase expression of phagocytic-like NOX in rat -cells [36]. Thus, OS is an important factor in glucotoxicity and lipotoxicity as well as the combined glucolipotoxicity [37]. Superoxide produced under these conditions has shown to damage and impair mitochondrial integrity and function *in vivo* and *in vitro* [38, 39]. Mitochondrial dysfunction also leads to increased ROS leakage from the electron transport chain, and has been associated with induction of insulin resistance resulting in higher blood glucose levels [40]. Fig. (**2**) summarizes the putative mechanism leading to OS in diabetes.

### 2.2. Oxidative Stress and Diabetic Late Complications

Diabetes induced OS has been suggested to lead to both micro- and macrovascular complications. ROS may induce wide spread damage to cells including *e.g.* endothelial, retinal, mesangial and neural cells leading to the development of diabetic complications [5].

Damage is collectively caused by both direct effects on cellular components and damaging cells [41, 42] and indirectly through activation of the above mentioned pathways subsequently affecting cellular function (Fig. **2**) and through induction of inflammation [4, 43]. Circulating ROS affect endothelial function and initiates inflammatory processes, both of which are thought to play an essential role in early atherogenesis (with atherosclerosis being the very core of diabetic macrovascular complications) as well as in hypertension and heart failures [4, 44-46].

Endothelial dysfunction, characterized by an imbalance between relaxing and contracting factors released by the endothelium, is seen in the early stages of atherosclerosis [56-58]. The vasodilator nitric oxide (NO) is produced by endothelial NO synthase (eNOS) [59]. OS contributes to a decreased NO bioavailability in several ways. OS lowers the production of NO through uncoupling of eNOS, turning production towards superoxide (Fig. **2**). Degradation of NO is enhanced, as NO reacts efficiently with superoxide forming the deleterious peroxynitrite and cellular damage is induced [60, 61]. ROS can inactivate eNOS and other anti-atherosclerotic enzymes such as prostacyclin synthase and increase proatherosclerotic events by affecting vascular tone (through activation of PKC, increased flux in the polyol pathway and increased levels of AGE) [44, 62]. Monocytes infiltrate the vascular wall and differentiate into macrophages, which can accumulate oxidized low density lipoproteins (oxLDL) leading to the formation of foam cells [63]. These events in turn stimulate macrophage proliferation, T-lymphocyte attraction, smooth muscle cell proliferation and collagen accumulation, collectively resulting in plaque formation. OxLDL is able to activate NOX and appear to contribute to the development of atherosclerosis through this mechanism as well [55].

Diabetes has been diagnosed in approximately 400 million people worldwide [64] and diabetic late complications is likely to affect a large proportion of these. Trials investigating the relationship between blood glucose status and devel-

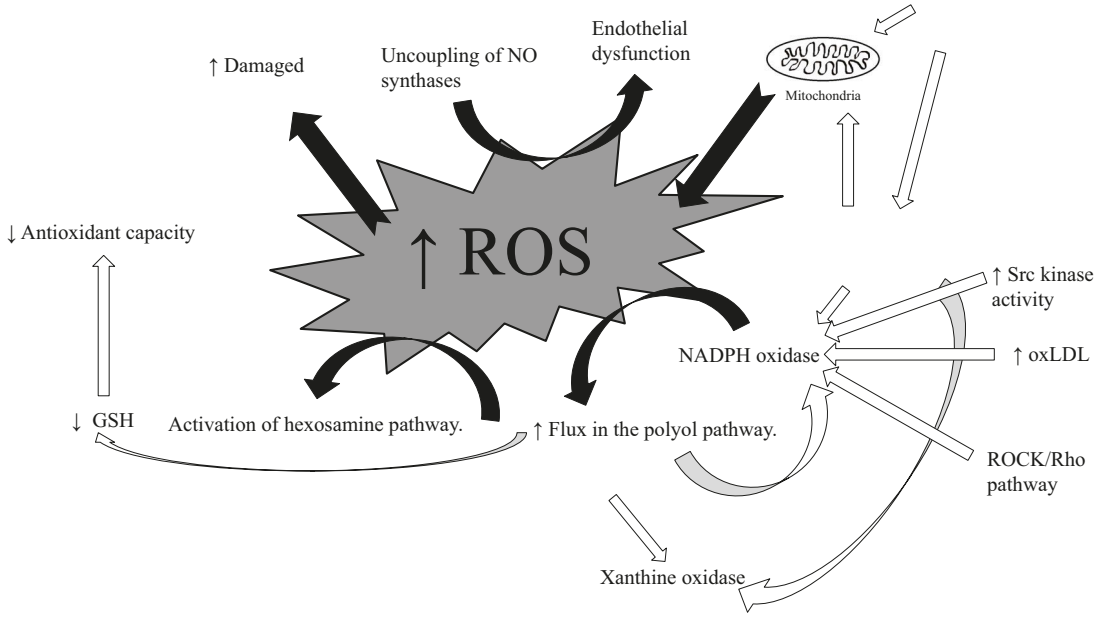


**Fig. (2).** Putative mechanisms leading to OS in diabetes. Five pathways are potentially activated, 1) Enlarged flux of monosaccharides through the polyol pathway [5]. 2) Increased formation of advanced glycation end-product (AGE) and 3) expression of advanced glycation end-product receptors (RAGE) [47]. 4) Activation of PKC [5] and 5) increased flux in the hexosamine pathway [5]. These are all downstream pathways from one common event, the production of ROS [5]. Damages through activation of the polyol pathway are conducted as NADPH is degraded, a process leading to a lower level of glutathione (GSH), thus decreasing antioxidant capacity. In addition, the pathway increases the level of the NOX substrate, NADH, thus favoring ROS production [48]. AGEs are formed when proteins or lipids are glycated in contact with for example glucose or other aldose sugars [49], and when these bind to RAGE the result can be production of ROS through activation of NOX [50, 51], xanthine oxidase (XO) and the mitochondrial electron transport chain [51]. The protein kinase C (PKC) pathway is stimulated when diacylglycerol (DAG) levels are elevated during hyperglycemia. The increased activity of PKC has many different effects on various genes, leading to changes in blood flow, increased inflammation and ROS production (by NOX) [52, 53]. A consequence can be an increase in NF- and release of inflammatory cytokines and growth factors in macrophages or mesangial cells [5]. Elevated glucose and FFA concentrations increase flow through the hexosamine pathway as well as induce OS [54]. The activation of these pathways initiates further ROS production. ROS can damage macromolecules including LDL. The resulting oxidized LDL (oxLDL) can lead to further NOX activation and thus further superoxide production [55]. The increase in ROS can uncouple eNOS switching eNOS from NO to superoxide production. This can lead to endothelial dysfunction (further described in section 2.2). The association between hyperglycemia, elevated FFA levels, mitochondrial dysfunction and ROS constitutes a vicious circle, in which the production of ROS during hyperglycemia worsens insulin resistance and thus further induce redox imbalance. These mechanisms are in particularly activated in cell types that are involved in the development of diabetic complications, as they are susceptible to hyperglycemic damage. These cells have a low capacity to reduce intracellular hyperglycemia, and include *e.g.* endothelial cells, mesangial cells, neurons and Schwann cells in peripheral nerves.

opment of diabetic complications have shown that intensive glucose lowering therapy can decrease the risk of developing in particular microvascular late complications in both T1D and T2D [65, 66], whereas regulation of blood glucose *per se* does not seem to have as beneficial an effect on the risk of developing macrovascular complications [65, 67]. Even though insulin resistance seems to play a role in the development of macrovascular complications in T2D [68], the mechanism is not fully understood but inflammation and OS could be part of the sequence of events. The effect of FFA's on OS status should be noted as well. The recognition of these aspects has further prompted the interest in new therapies targeting the redox imbalance associated with diabetes to try to reduce the development of diabetic late complications by lowering the OS.

### 2.3. GLP-1 and Oxidative Stress

The anti-inflammatory action of GLP-1 has recently gained considerable interest as endogenous GLP-1 has been found to be increased in patients with chronic inflammatory diseases, where OS is implicated, as systolic heart failure (bearing in mind that atherosclerosis can cause heart failure) and to be associated with coronary plaque burden in patients [18, 19, 70]. Moreover, GLP-1 intervention has been shown to decrease inflammation in both preclinical *in vivo* [21, 71-74] and clinical studies [75-77]. While some studies have incorporated various measures of OS, this area deserves more focused attention. The present review discusses the current literature on GLP-1 with particular focus on the putative effects on OS status beyond anti-hyperglycemia and appetite regulation. The G-protein coupled GLP-1 receptor (GLP-1R) is found in a wide variety of cells. In pancreatic islets, activation of this receptor increases intracellular $Ca^{2+}$, stimulates adenylate cyclase (increasing production of cAMP) and phospholipase C, and activates a number of pathways including protein kinase A (PKA), PKC, cAMP response element-binding protein (CREB), phospatidylinositol-3 kinase, exchange protein activated by cAMP 2 (Epac2), and mitogen-activated protein kinase pathways ([78] and reviewed in [79]). Both cAMP and PKA are inhibitors of

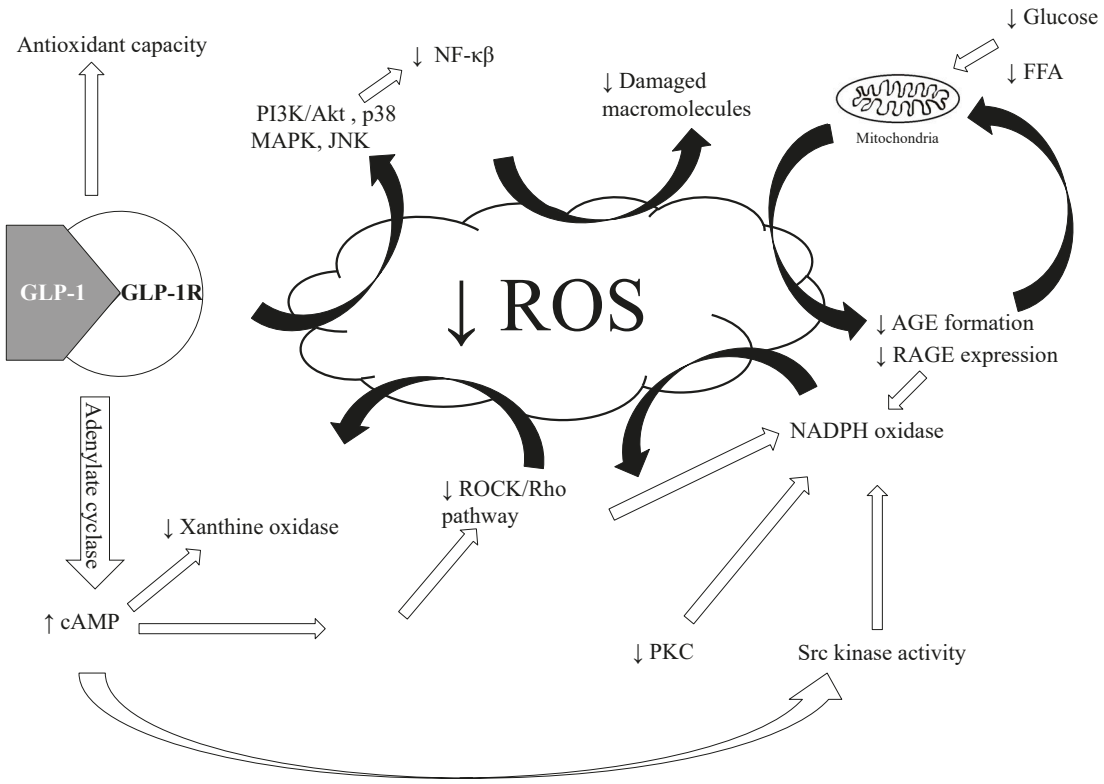


**Fig. (3).** Putative mechanisms by which GLP-1 affects OS status by glucose independent mechanisms in cells expressing the GLP-1R and glucose dependent mechanisms in cells ± GLP-1R expression. Black arrows indicate glucose dependent mechanisms, white arrows glucose independent and grey indicates pathways that can be affected by both glucose dependent and glucose independent GLP-1 effects. Black/white arrow indicates that mechanism has not been clarified. GLP-1 lowers OS status through different mechanisms with the glucose dependent effects being the most apparent through reduced food intake, blood glucose and FFA. This reduces the ROS formed by the mitochondria which can decrease the activation of the five pathways outlined in Fig. (**2**). Glucose independent effects are mediated through GLP-1R stimulation, which can affect pathways involved in ROS formation. Stimulation of GLP-1R leads to increased levels of cAMP in the cell, which results in lower levels of ROS being produced by NOX and XO. The decrease in NOX activity can also be obtained by GLP-1 inactivation of PKC. Increased cAMP level by GLP-1R activation can increase PKA activity and by this route decrease the Rho/ROCK pathway leading to a decreased ROS production. The latter mentioned pathway can be activated by hyperglycemia *e.g.* though AGE formation (review in [69]) and can thus both be affected by glucose dependent and independent pathways. Decreases in AGE/RAGE interaction can lower XO, NOX and mitochondrial ROS production. Increased cAMP can lower the activity of Src kinase, which is an activator of NOX. This results in decreased NOX activity and ultimately lower superoxide production. Increased antioxidant capacity favors redox homeostasis and has been shown to be induced by GLP-1R activation and/or increased Nrf2 concentration or expression by GLP-1. A decreased ROS production can affect various pathways as PI3K/Akt and p38 MAPK and JNK activities resulting in a decrease in inflammatory (by decreasing NF- activity) and apoptotic pathways as discussed in the text.

NOX [80-83]. However, some of these pathways vary from cell type to cell type, *e.g.* isoforms of PKC activates NOX in vascular tissues, where GLP-1 has here been shown to inactivate PKC and thus ROS production by NOX [84]. The role of GLP-1 in different cell types is under investigation and depends on various factors, such as stimuli (*e.g.* nutrient affecting the cells), species and the expression of the GLP-1R which has to be investigated with sensitive and specific antisera and detected in full length by PCR analysis [85]. Moreover, GLP-1 has been suggested to increase antioxidant capacity. Fig. (**3**) summarizes potential pathways through which GLP-1 may affect OS status and the following chapters discuss the *in vitro, in vivo* and clinical literature on GLP-1 and OS.

## 3. GLP-1 AND OXIDATIVE STRESS *IN VITRO*

Numerous *in vitro* studies have shown that GLP-1 and GLP-1 agonists reduces ROS and protects against apoptosis in several cell types when exposed to different stress factors *e.g.* high glucose levels or hydrogen peroxide [20, 21, 86-90] (Table **1**). However, the observed effects on apoptosis in a number of these studies may not strictly be attributed to effects *via* OS, as GLP-1 per se activates antiapoptotic genes and thus independently decreases apoptosis [91]. But in general, *in vitro* studies have reported decreased levels of ROS [20, 21, 76, 84, 88-90, 92-99], NOX subunits or activity [20, 21, 84], malondialdehyde (MDA) [90, 95, 100] and increased levels of antioxidants and/or genes involved in antioxidant activity [21, 86, 87, 90, 92, 95, 100-103] following GLP-1 exposure. Whether these observed effects are GLP-1R dependent or independent can be investigated by using cleavage products of GLP-1 known not to interact with the receptor. The GLP-1 cleavage product, GLP-1(9-36) is not involved in glucose homeostasis [104], but has been shown to have cytoprotective effects on mouse cardiomyocytes exposed to hydrogen peroxide [105] and to decrease super

**Table 1.** ***In vitro*** **studies of GLP-1 and oxidative stress.**

| Species | Cell Type | Study Design: Intervention (I), Duration (D) | Effect of GLP-1 Intervention on Oxidative Stress Markers | Survival/Apoptosis | Mechanisms | Comments |
|---|---|---|---|---|---|---|
| Mouse | Isolated mouse (C57BL/6 ± DIO) hepatocytes + H4IIE | **I:** GLP-1(28-36) amide (10 or 100 nmol/l) + tBHP (0.5 mmol/l). **D:** 24 hours prior to tBHP for 1 hour. | GLP-1(28-36)amide: ↓ ROS (CM-$H_2$DCFDA by spectrofluorometer) (hepatocytes: $p<0.028$ (for both ± DIO), H4IIE: $p<0.006$ (100 nmol/l) and $p<0.002$ (10 nmol/l)). | | | [98] |
| | Atrial HL cardiomyocytes | **I:** GLP-1 (100 or 200 nmol/l), palmitate (750 μM). **D:** 30 minutes preincubation ± GLP-1 (7-36) then 6 hours + palmitate. | GLP-1 (7-36): ↓ XO activity ($p<0.05$). ↓ ROS and RNS ($p<0.05$) (dichlorodihydrofluorescin DiOxyQ). ↑ GSH/GSSG ratio (200 nmol/l) ($p<0.05$). ↓ SOD activity (200 nmol/l) ($p<0.05$). | | | [116] |
| | Mouse pancreatic islet | **I:** GLP-1 (10 nmol/l) + glucose (20.8 mmol/l). **D:** 90 minutes. | GLP-1: ↓ Superoxide level (nitroblue tetrazolium assay) ($p<0.05$). | | | ROS may play an antagonistic role in the adenylate cyclase/cAMP/PKA pathway of insulin secretion [97]. |
| Hamster | Hamster pancreatic -cell line (HIT-T15) | **I:** GLP-1 (10 nmol/l) in GS containing medium. **D:** 5 days. | GLP-1: ↓ ROS ($p<0.01$) (DCFH-DA probe). ↑ GR protein expression, thus only significant ($p< 0.05$) in medium control cells. | | GLP-1: ↑ Nrf2 protein expression ($p<0.01$). ↑ MafA and PDX-1 protein expression ($p<0.05$). | [92] |
| | Hamster pancreatic -cell line (HIT-T15) | **I:** GLP-1 (10 nmol/l) in GS containing medium. **D:** 5 days. | GLP-1: No effect on GSSG levels. ↑ GSH ($p<0.001$). | GLP-1 increased proliferation ($p<0.01$, when compared with control medium, $p<0.001$ when compared to GS medium) rate and restored LDH release + caspase-3 activity to control cell levels. | GLP-1: ↓ RAGE expression ($p<0.05$). | [87] |

**Table 1. contd….**

| Species | Cell Type | Study Design: Intervention (I), Duration (D) | Effect of GLP-1 Intervention on Oxidative Stress Markers | Survival/Apoptosis | Mechanisms | Comments |
|---|---|---|---|---|---|---|
| Rat | Cardiomyoblasts (H9c2) | **I:** Exenatide treatment (1 nmol/l) prior to $H_2O_2$ (200 µmol/l) exposure. **D:** 30 minutes | Exenatide: ↓ ROS (DCFH-DA and flow cytometry) and ↓ MDA ($p<0.05$). ↑ T-SOD ($p<0.05$). | Exenatide: ↑ viability ($p<0.05$) ↓ LDH and CK-MB release ($p<0.05$). | | [90] |
| | Neonatal rat ventricular myocytes (NRVM) | **I:** Exendin-4 (30 mmol/l) ± D-glucose (5 or 33 mmol/l) or $H_2O_2$ (250 µmol/l). **D:** 24-48 hours. | Exendin-4: No effect on ROS level (DHR123 flourometri). | Exendin-4: ↓ cell death ($p<0.05$) (trypan blue). No change in ROS level. | Exendin-4: ↓ Expression of markers of ER stress (CHOP, GRP78 and PDI). | Protective effects of exendin-4 on glucose or $H_2O_2$ induced cell death might be downstream from OS. PKA dependent effect associated with activation of SERCA2a [109]. |
| | Cardiac microvascular endothelial cells | **I:** GLP-1 ($10^{-10}$, $10^{-9}$, $10^{-8}$ or $10^{-7}$ mol/l) ± glucose (25 mmol/l). **D:** 24 hours. | GLP-1 in high-glucose-medium: ↓ superoxide (lucigenin-enhanced chemiluminescence assay and DHE staining) ($p<0.05$ and 0.01 for $10^{-8}$ mol/l). ↓ NOX activity (lucigenin-enhanced chemiluminescence assay) ($p<0.05$ and 0.01 for $10^{-8}$ mol/l). ↓ $p47^{phox}$, $gp91^{phox}$, $p22^{phox}$ and $p40^{phox}$ protein level ($p<0.05$). | GLP-1 in high-glucose-medium: ↓ apoptosis ($p<0.05$) (terminal deoxynucleotidyl TUNEL assay). ↓caspase-3 ($p<0.05$). | ↓ Rho ($p<0.05$). ↓ ROCK ($p<0.05$). Fausudil and H89 reduced the effects of GLP-1. | GLP-1 effects might be through cAMP/PKA/Rho-dependent mechanisms. + *in vivo* study. [20] |
| | Neonatal rat ventricular myocytes (NRVM) | **I:** Exendin-4 (0.1-10 nmol/l) + high glucose exposure (25 mmol/l). **D:** 12 hours. | Exendin-4: ↓ MDA concentration ($p<0.05$ for 1 and 10 nmol/l). ↑ SOD activity ($p<0.05$ for 1 and 10 nmol/l). | Exendin-4: ↓ LDH ($p<0.05$ for 1 and 10 nmol/l). ↓ CK ($p<0.05$ for 1 and 10 nmol/l). | | ↓ TNF-α, IL-1 and HMGB1 ($p<0.05$ for 1 and 10 nmol/l). Hypothesis: (Hyperglycemia-induced) OS <-> inflammatory cytokine secretion [100]. |
| | PC12 | **I:** Methylglyoxal (1 mmol/l) ± GLP-1 (3.30 µg/ml). **D:** 30 min preincubation with GLP-1, then methylglyoxal for 24 hours. | GLP-1: ↑ GSH ($p<0.05$). ↑ GSH/GSSG ($p<0.001$). | GLP-1: ↓ apoptosis (DAPI staining) ($p<0.001$). | Rapamycin (mTOR inhibitor) reduced effects on GSH ($p<0.05$) and GSH/GSSG ($p<0.001$). | Pi3K/Akt/mTOR/GCLc pathway. [86] |

**Table 1. contd….**

| Species | Cell Type | Study Design: Intervention (I), Duration (D) | Effect of GLP-1 Intervention on Oxidative Stress Markers | Survival/Apoptosis | Mechanisms | Comments |
|---|---|---|---|---|---|---|
| | Rat insulinoma cells (INS-1) | **I:** Exendin-4 (10 or 100 nmol/l) + IFN-γ (1 ng/ml) and IL-1 (4 ng/ml).<br>**D:** 5 hour preincubation with exendin-4 then 20 hours of IFN-γ and IL-1 exposure. | Exendin-4:<br>↓ ROS ($p<0.01$ (10 nmol/l) and $p<0.001$ (100 nmol/l)) (DCFH-DA probe). | Exendin-4:<br>↓ caspase-4 activity ($p<0.001$). | Exendin-4:<br>Actions on different proteins in the electron transport chain, *e.g.* NADH-ubiquinone oxidoreductase 75 kDa SU ↓ ($p<0.05$).<br>↑ cAMP dependent PKA ($p<0.001$). | [88] |
| | Cardio-myoblasts (H9c2) | **I:** Exenatide (50-600 nmol/l) + H/R (hypoxia chamber).<br>**D:** 30 min prior to H/R. | Exenatide (200 nmol/l):<br>↓ ROS (DCFA-DA fluorescence) ($p<0.05$).<br>↑ T-SOD ($p<0.05$).<br>↓ MDA ($p<0.05$). | Exenatide:<br>↑ Viability ($p<0.05$ for 100, 200 and 400 nmol/l).<br>↓ Apoptosis ($p<0.05$ (200 nmol/l)).<br>↓ Caspase-3 (cleaved and activity) ($p<0.05$ (200 nmol/l)). | Exenatide:<br>↑ Mitochondrial membrane potential ($p<0.05$).<br>↓ mitochondrial $Ca^{2+}$ ($p<0.05$). | Improvement of mitochondrial function<br>[95]. |
| | Rat insulinoma cells. | **I:** Exendin-4 (20 nmol/l) + 0, 5, 20 and 40 mmol/l glucose.<br>**D:** 4 hours. | Exendin-4:<br>↓ TxNIP. | Exendin-4:<br>↓ capase-3. | Forskolin and PDE → similar results as Exendin-4.<br>Inhibitors of:<br>PKA or ePAC → attenuated effect of forskolin. | cAMP/PKA and/or ePAC and/or PDE pathway leading to ubiquitination and proteasomal degradation of TxNIP [103]. |
| | Goto-Kakizaki islet cells | **I:** Exendin-4 (100 µmol/l) + glucose (16.7 mmol/l after preincubation with 2.8 mmol/l for 20 minutes).<br>**D:** 60 minutes. | Exendin-4:<br>↓ ROS (CM-H2DCFDA) ($p<0.005$).<br>No effect on MnSOD activity. | | Src kinase inhibition by PP2 → ↓ ROS ($p<0.05$) (exendin-4 and PP2 did not further reduced ROS). Adenylate cyclase activator (forskolin) → ↓ ROS ($p<0.01$). Exendin-4 or forskolin + PKA inhibition (H-89) → no effect on the decreased ROS. General cAMP analog and ePAC specific cAMP analog → ↓ ROS ($p<0.001$).PI3K inhibitors (LY294002 and wortmannin) → ↓ ROS ($p<0.01$).<br>MAPK/ERK kinase inhibitor (PD98059) → no ROS effect.<br>EGFR kinase inhibitor (AG1478) → ↓ ROS ($p<0.001$). | The effects of exendin-4 and Src inhibitor (PP2) could not be produced in Wistar islet cells [112]. |

**Table 1. contd….**

| Species | Cell Type | Study Design: Intervention (I), Duration (D) | Effect of GLP-1 Intervention on Oxidative Stress Markers | Survival/Apoptosis | Mechanisms | Comments |
|---|---|---|---|---|---|---|
| | Rat INS-1E and rat pancreatic islet cells. | **I:** Exendin-4 (10 nmol/l) pretreatment ± cytokines (10 ng/ml IL-1 , 50 ng/ml TNF-α, 50 ng/ml IFN- ). **D:** Exendin-4 for 18 hours followed by cytokines for 18 hours. | Exendin-4: INS-1E: ↓ ROS (DCFH-DA probe) ($p<0.05$) in presence of cytokines. | Exendin-4: INS-1E: ↑ viability ($p<0.01$). INS-1E + islet cells: ↓ apoptosis and necrosis. | Exendin-4: ↑ phosphorylation of PKB (required for the protective effects shown). | CAT and MnSOD levels are mentioned to have been measured, but no data shown. Exendin-4 did not affect levels of either antioxidant. Conclusion: The reduction in ROS might be through lowering of ROS production rather that elimination. [94] |
| Human | Human umbilical vein endothelial cells (HUVECs) | GLP-1 (10 nmol/l) or exendin-4 + (30 mol/l). **D:** GLP-1 for 30 minutes prior to $H_2O_2$ for one hour. | GLP-1: ↑ expression of NQO1 and HO-1 ($p<0.05$). | GLP-1 or exendin-4: ↓ senescence ( -galactosidase staining) ($p<0.05$). | GLP-1: ↑ CREB ($p<0.05$). Receptor mediated: exendin(9-39) did not have any effect. Effects blocked by H89 or forskolin. | cAMP/PKA-dependent pathway involved. [102] |
| | HUVECs | **I:** GLP-1 (0.03 or 0.3 nmol/l) in glycated bovine serum albumin (100 μg/ml). **D:** 4 hours. | GLP-1: ↓ superoxide (DHE staining) ($p<0.01$). | | GLP-1: ↓ RAGE mRNA level ($p<0.01$) (in normal bovine serum albumin). This action was inhibited by siRNA's against GLP-1R and RAGE. 8-Br-cAMP had same effects on RAGE expression as GLP-1. | 0.3 nmol/l GLP-1 decreased VCAM-1 mRNA level and thus might reduce recruitment and adhesion of inflammatory cells. Actions of GLP-1 on OS might be through GLP-1R-cAMP axis, resulting in lower levels of RAGE expression [96]. |
| | Human aortic endothelial cells (HCAECs) | **I:** Liraglutide (30 nmol/l) and/or metformin (10 μM) treatment prior to high glucose exposure (25 mM). **D:** 48 hours. | Liraglutide or metformin: ↓ superoxide (DHE staining) ($p<0.01$). Liraglutide and metformin: ↓ superoxide (DHE staining) when compared to liraglutide or metformin alone ($p<0.01$). ↓ NOX activity ($p<0.05$). Prevention of $p47^{phox}$ translocation. | No effects on viability (> 94% in all samples). | Liraglutide: ↓ DAG ($p<0.01$) ↑ AMPK phosphorylation ($p<0.05$) ↓ PKC ($p<0.05$) | Combination of liraglutide and metformin further reduced ROS production. Both by inhibition of the PKC-NOX pathway. [84] |

**Table 1. contd….**

| Species | Cell Type | Study Design: Intervention (I), Duration (D) | Effect of GLP-1 Intervention on Oxidative Stress Markers | Survival/Apoptosis | Mechanisms | Comments |
|---|---|---|---|---|---|---|
| | HCAECs | **I:** Exendin-4 (0.1-10 nmol/l) during palmitate exposure (125 µmol/l).<br>**D:** 24 hours. | Exendin-4:<br>↓ ROS (DCFH-DA probe) ($p<0.05$). | Exendin-4:<br>↓ DNA fragmentation ($p<0.05$ for 10 nmol/l). | GLP-1 dependent pathway (effects eliminated by exendin (9-39)) and better effects of GLP-1 than exendin-4 (not significant). No effect of GLP-1 (9-36). | Incubation with $BH_4$ resulted in same reduction in ROS as exendin-4 ($p<0.05$). The effects on apoptosis might be through lower activation of PI3K/Akt pathway (which can be sensitive to ROS).<br>[89] |
| | HUVECs | **I:** Liraglutide (3, 30 or 300 nmol/l) + TNF-α (5 ng/ml).<br>**D**: Liraglutide for 30 minutes prior to TNF-α for 90 minutes to 24 hours. | Liraglutide:<br>↓ ROS (CM-$H_2$DCFDA by FACS calibur) ($p<0.01$ (30 nmol/l) and $p<0.05$ (300 nmol/l)).<br>↓ $gp91^{phox}$ (protein level and mRNA expression), $p22^{phox}$ (mRNA expression) ($p<0.05$).<br>↑ SOD-2 and CAT mRNA expression ($p<0.05$) (↑ SOD-1 and GPx (NS)).<br>↑ SOD-2, CAT ($p<0.05$) and GPx ($p<0.01$) protein level (↑ SOD-1 (NS)). | Liraglutide:<br>↓ Apoptosis ($p<0.05$ (3nmol/l) and $p<0.01$ (30 nmol/l)) (Annexin V-FITC apoptosis kit). | Liraglutide (30 nmol/l):<br>Eliminated PKC-α activation seen by TNF-α exposure. | Effects on ROS levels not blocked by exendin (9-36). NF- activity decreased by liraglutide.<br>[21] |
| | Human proximal tubular cells | **I:** GLP-1 (0.2 nmol/l) + 100 µg/l AGE.<br>**D:** 4 hours. | GLP-1:<br>↓ Superoxide (DHE staining) ($p<0.01$).<br>↓ ADMA levels ($p<0.01$). | | GLP-1:<br>↓ RAGE mRNA level ($p<0.01$). This action was inhibited by siRNA's against GLP-1R and RAGE. 8-Br-cAMP had same effects on RAGE expression as GLP-1. | RAGE gene suppression by GLP-1R-cAMP axis [93] |
| | Human mesangial cells | **I:** GLP-1 (0.03 or 0.3 nmol/l) + AGE-BSA (100 µg/ml).<br>**D:** 4 hours. | GLP-1:<br>↓ Superoxide (DHE staining) ($p<0.01$) (Br-cAMP had same effect). | | GLP-1:<br>↓ RAGE mRNA level ($p<0.01$) (in bovine serum albumin alone). This action was inhibited by siRNA's against GLP-1R and RAGE. 8-Br-cAMP had same effects on RAGE expression as GLP-1. | RAGE gene suppression by GLP-1R-cAMP axis. GLP-1 decreased MCP-1 ($p<0.01$) mRNA and protein. [99]. |

**Table 1. contd….**

| Species | Cell Type | Study Design: Intervention (I), Duration (D) | Effect of GLP-1 Intervention on Oxidative Stress Markers | Survival/Apoptosis | Mechanisms | Comments |
|---|---|---|---|---|---|---|
| | Human (T2D patients) peripheral blood mononuclear cells | **I:** Exendin-4 (50 nmol/l). **D:** 24 hours | Exendin-4: ↓ Superoxide (chemiluminescence) ($p<0.05$) | | | Activation of cytokine release → NOX activation. [76] |
| Rat, mouse and human | INS-1, mouse and human pancreatic islets | **I:** Exenatide (100 nmol/l). **D:** 24 hours. | Exenatide: ↓ TxNIP mRNA ($p<0.001$) and protein level. | Exenatide: INS-1: ↓ $H_2O_2$-induced apoptosis ($p<0.05$). Mouse and humans pancreatic islet cells: ↓ caspase-3 and bax mRNA level ($p<0.05$). | | [101] |

ADMA = asymmetric dimethyl arginine, AGE = advanced glycation end-products, AGE-BSA = advanced glycation end-product bovine serum albumin , AMPK = AMP-activated kinase, $BH_4$ = tetrahydrobiopterin, CHOP = cytosine-cytosine-adenosine-adenosine-thymidine/enhancer-binding homologous protein, CK = creatinine kinase, CK-MB = creatine kinase‑MB, CMECs = cardiac microvascular endothelial cells, CM-H2DCFDA = 2',7'-dichlorodihydrofluorescein diacetate, CREB = cAMP response element-binding protein, DAG = diacylglycerol, DAPI = 4',6-diamidino-2-phenylindole, DCFH-DA = 2',7'-dichloro-fluorescein diacetate, DHE = dihydroethidium, DHR123 = dihydrorhodamine 123, DIO = diet induced obese, EGFR = epidermal growth factor receptor, ePAC = exchange protein activated by cAMP, ER = endoplasmatic reticulum, FACS = fluorescence-activated cell sorting, fausudil = Rho kinase inhibitor, forskolin = activates the enzyme adenylyl cyclase and increases intracellular levels of cAMP, GCLc = catalytic glutamate-l-cysteine ligase, GRP78 = glucose-regulated protein-78, GR = glutathione reductase, GS = glycated serum, GSH = reduced glutathione, GSSG = oxidized glutathione, GPx = glutathione peroxidase, HAECs = Human aortic endothelial cells, HCAECs = Human Coronary Artery Endothelial Cells, HFD = high fat diet, HMGB1 = High-mobility group box 1, HO-1 = heme oxygenase-1, H/R = hypoxia/reperfusion, HUVECs = Human umbilical vein endothelial cells, H4IIe = rat hepatoma cell line, H89 = PKA inhibitor, LDH = lactate dehydrogenase, MafA = v-maf musculoaponeurotic fibrosarcoma oncogene homologue, MAPK/ERK = mitogen-activated protein kinase extracellular signal-regulated, MDA = malondialdehyde, mTOR = mammalian target of rapamycin, NADH = nicotine adenine dinucleotide, NF-κβ = nuclear factor kappa-light-chain-enhancer of activated B cells, NOX = NADPH oxidase, NSC = neuroblastoma spinal cord, Nrf2 = nuclear factor erythroid 2 p45-related factor 2, NRVM = Neonatal rat ventricular myocytes, NQO1= NADPH dehydrogenase quinone 1, PDE = phosphodiesterase, PDI = protein disulfide isomerase, PDX-1 = pancreatic and duodenal homeobox-1, Pi3K = phosphatidylinositol-3 kinase, PKA = protein kinase A, PKC = protein kinase C, RAGE = AGE receptor, ROCK = Rho-associated protein kinase, ROS = reactive oxygen species, SERCA2a = sarco/endoplasmic reticulum $Ca^{2+}$-ATPase, siRNA = small interfering RNA, SOD = sodium dismutase, SU = subunit, tBHP = tert-butyl hydroperoxide, T-SOD = total superoxide dismutase, TUNEL = terminal deoxynucleotidyl transferase dUTP nick end labeling, TxNIP = thioredoxin interacting protein, XO = xanthine oxidase.

oxide levels in hippocampal slices [106]. This suggests that the effects may not only be mediated through the glucose-lowering effects of GLP-1 or by binding and signaling through the GLP-1R. The following sections examine the available data on GLP-1 mediated effects on antioxidant level, ROS production, quenching of ROS, and protections against ROS induced damage.

### 3.1. GLP-1 Effect on Antioxidant Level

Intervention with GLP-1 has been shown to upregulate superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) activities and increase glutathione (GSH) concentrations in media containing stressors such as glucose or hydrogen peroxide [21, 86, 87, 90, 95, 100]. PKA activation following GLP-1R stimulation has been shown to protect endothelial cells against ROS through increased mRNA levels of NADPH dehydrogenase quinone 1 (NQO1) and heme-oxygenase 1 (HO-1) [102]. NQO1 and HO-1 both have some antioxidant activity [107, 108] and NQO1 also functions as a superoxide scavenger [107]. While these studies indicate an effect of GLP-1 on antioxidant capacity, increased levels of antioxidants could also result from adaptation to higher ROS levels, or decreased consumption through a lower ROS production and consequently, ROS levels and production should also be assessed [108].

### 3.2. GLP-1 Effect on ROS Level

GLP-1 is generally effective in lowering ROS levels *in vitro* [20, 21, 36, 76, 84, 88-90, 92-99]. However in one study, neonatal rat cardiomyocytes exposed to either high glucose concentrations or hydrogen peroxide showed that exendin-4 attenuated apoptosis without lowering ROS levels [109]. This result could suggest that effects of exendin-4 on apoptosis are downstream to that of OS, but differences in the study setup (cell type, glucose concentration and exposure time) may indicate otherwise [20, 84, 100]. A different technique was used for detection of ROS (dihydrorhodamine 123 (DHR123)), which primarily detects peroxynitrite, whereas neither hydrogen peroxide nor superoxide alone oxidizes DHR123 significantly [110]. If exendin-4 is able to reduce other reactive species than peroxynitrite, it would not be detected in this study. Thus, the technique used for measuring ROS levels is of major importance and overall conclusion on the levels of ROS cannot be done by only using techniques specific for only one type of ROS. Another important factor is the cell type. Potential glucose independent effects of GLP-1 is influenced by expression of the GLP-1R (as described in 2.4) and details in the expression of this in different cell types of the heart is still debated (reviewed in [111]. A study in Goto-Kakizaki rat islet cells exposed to high levels of glucose (16.7 mmol/l) showed a ROS concentration increase. Exendin-4 reduced this elevation in ROS without affecting MnSOD activity. Here different signaling

pathways of GLP-1 were investigated by adding a Src kinase inhibitor, an adenylate cyclase activator, cAMP and an ePAC-specific cAMP analog, a phosphatidylinositol-3 kinase inhibitor and an epidermal growth factor receptor kinase inhibitor, all of which led to decreased ROS concentrations, while PKA or mitogen-activated protein kinase extracellular signal-regulated (MAPK/ERK) kinase did not affect ROS concentrations. This study indicates that GLP-1 acts through suppression of Src kinase activity by increasing cAMP levels and PKA and MAPK/ERK kinase independently but that ePAC, phosphatidylinositol-3 kinase and epidermal growth factor receptor kinase are involved [112]. Src kinase is an activator of NOX, and the decrease in ROS concentration by Src kinase activity is thus a potential route of mechanism [113].

In human aortic endothelial cells (HAECs) exposed to palmitate, exendin-4 has been shown to lower the level of ROS and at the same time reduce the level of DNA fragmentation used as a measure of apoptosis [89]. ROS can stimulate p38 mitogen-activated protein kinase and Jun N-terminal kinase, which can activate NOX by translocation of $p47^{phox}$ and $p67^{phox}$ subunits leading to the production of superoxide [114, 115]. Adding palmitate to the medium markedly increased the phosphorylation (and subsequently activity) of the kinases, a process that was significantly decreased by co-incubating the cells with exendin-4 [89]. However, addition of trolox (a vitamin E analog) was not effective in decreasing palmitate-induced apoptosis even though it reduced ROS production, suggesting that the antiapoptotic effect of exendin-4 is mediated through lowering p38 MAPK and JNK activities. Another study of palmitate induced lipotoxicity in a cardiomyocyte cell line has shown a decrease in xanthine oxidase (XO) activity and ROS/RNS concentration and an increase in GSH/GSSG ratio. In this study, SOD activity was decreased [116]. The latter could be contributed to a decreased need of SOD as ROS production and concentrations were decreased.

### 3.3. GLP-1 Effect on ROS Production

GLP-1 and liraglutide has been shown to decrease NOX activity as well as protein and mRNA levels of NOX subunits [20, 21, 84]. By inhibition of Rho through the cAMP/PKA pathway, GLP-1 suppresses ROS production and expression of NOX subunits in cardiac microvascular endothelial cells [20]. High glucose-induced ROS production has been shown to be attenuated by liraglutide in endothelial cells [21, 84]. In a study by Batchuluun, B. *et al* [84], lower levels of superoxide (detected by DHE) was observed along with a decrease in the PKC phosphorylation, translocation of PKCβ2, translocation of $p47^{phox}$ and decreased activity of NOX [84]. As total diacylglycerol (DAG) was decreased and AMP-activated protein kinase (AMPK) phosphorylation increased by liraglutide stimulation, this study suggests that DAG and AMPK may be involved in the GLP-1 lowering effects on ROS production.

In diabetes, inflammatory conditions in the pancreatic β-cells play a central role, making *in vitro* studies with different inflammatory cytokines of interest. Preincubation with exendin-4 was found to decrease ROS levels and caspase-4 activity in rat insulinoma cells exposed to IFN-γ and IL-1β compared to controls [88]. Similar results were obtained when exposing HUVECs to TNF-α after liraglutide preincubation. Here, liraglutide resulted in decreased apoptosis, ROS production and expression NOX subunits ($gp91^{phox}$ and $p22^{phox}$). These observations support findings that NOX activity is reduced by liraglutide and other GLP-1 analogues. A simultaneous increase in mRNA levels of SOD-2 and CAT as well as protein levels of SOD-2, CAT and GPx was found [21]. These data support the hypothesis that GLP-1 analogues improve antioxidant status in pancreatic β-cells and endothelial cells in spite of lower levels of ROS and suggest a mechanism by which OS may potentiate cytokine-induced β-cell apoptosis.

*In vitro* studies have also shown that GLP-1 can decrease the expression of RAGE [87, 93, 96, 99], which can potentially lead to decreased ROS production from NOX [50, 51], XO and the mitochondrial electron transport chain [51]. The observed effect was accompanied by lower levels of superoxide and higher levels of GSH, indicating an advantageous redox status in the cells.

From these data it can be concluded that GLP-1 shows very positive effects *in vitro* both by decreasing the production of ROS but also by increasing the antioxidant capacity. These outcomes are seen together with an antiapoptotic effect thus to some extend indicating an effect of these effects on OS status.

## 4. GLP-1 AND OXIDATIVE STRESS *IN VIVO*

Most studies of GLP-1 in OS have been conducted in various disease models as outlined below. Thus, limited information is available on the effects of GLP-1 on OS status in healthy animals. However, one study examined the effect of exenatide on thioredoxin-interacting protein (TXNIP) [101]. In β-cells, TXNIP is a proapoptotic gene and expression of this gene is increased upon elevated levels of glucose, as seen in diabetes [117]. Expression of the gene inhibits the ROS scavenger, thioredoxin (TRx) and leads to increased ROS levels [118]. Exenatide injections for seven days in nondiabetic mice reduced the expression of TXNIP in pancreatic islets and suggested that this GLP-1 analogue is able to reduce apoptosis of mouse β-cells *in vivo* [101]. The available *in vivo* data are summarized in Table **2**.

### 4.1. Ischemia/reperfusion (I/R) Models

In mice subjected to acute left middle cerebral artery occlusion (MCAO), single injections of exendin-4 at different time points after reperfusion reduced 8-hydroxy-2'-deoxyguanosine (8-OHdG) and anti-4-hydroxy 2-hexenal (HHE) positive cells in the brain at both 24 hours, 72 hours and seven days after I/R [119]. This effect of GLP-1 on OS has been supported by a study showing that four days of liraglutide treatment, in a model of traumatic brain injury induced by cryolesion (probe cooled in liquid nitrogen), can decrease *in vivo* visualization of ROS/RNS in female C57BL/6 mice. The effect was accompanied by a reduction in α-spectrin, a marker of necrosis and apoptosis, indicating a reduction in these cellular events [120]. In CL57BL/6 mice, seven days of liraglutide treatment resulted in higher expression and protein concentration of Nrf2 and HO-1 in

the heart suggesting that liraglutide can increase antioxidant capacity [121].

In rats, the preventive effect of exendin-4 has also been examined in a MCAO model. Administration seven days prior to I/R resulted in significantly lower levels of MDA and higher levels of SOD and GSH [122]. This suggests that exendin-4 exerted its protective effect through increased antioxidant status capable of protecting against macromolecular damage induced by ROS. Similar results using exenatide were obtained in a model were the left coronary artery was ligated for 30 minutes followed by two hours of reperfusion. Here, exenatide administration for two weeks prior to I/R resulted in a significant decrease in MDA and increases in total SOD, CAT, and GPx [90]. Similarly, in a renal model of I/R, exenatide treatment for two weeks prior to I/R showed significant reductions in XO, MDA, combined with an increase in antioxidant capacity (GSH, GPx, SOD and CAT) [123]. In this study, a reduction in ROS producing enzymes was also observed, indicating that GLP-1 can prevent the production of ROS *in vivo*. Comparable effects of exenatide has been found after induction of diabetes with streptozotocin (STZ); thus, in kidney I/R induced after 10 days of exenatide, decreased kidney content of MDA and XO activity was observed and accompanied by increases in GSH concentration, GPx protein concentration, and SOD and CAT activities [124]. Collectively, these results suggest that prophylactic administration of GLP-1 analogues in I/R models may partially protect against OS both by induction of antioxidant capacity and by reduction of ROS producing enzymes. Therapeutic administration of GLP-1 in rats following I/R has been shown effective as well. A single administration of liraglutide acutely after reperfusion has been shown to reduce infarct volumes in a transient MCAO model [125]. Derivatives of reactive oxygen metabolites (d-ROMs) - a crude measure of peroxides [126] were significantly reduced in plasma in the liraglutide treated rats. Likewise, intrauterine growth retardation (IUGR) followed by six days of exendin-4 administration resulted in decreased thiobarbituric acid reactive substances (TBARS; a crude measure of lipid hydroperoxides with questionable specificity and quite inconsistent findings [127]) in the livers of the offspring, thus indicating a reduction in lipid peroxidation in newborn rats treated with exendin-4 [128]. Furthermore, antioxidant status was improved as shown by increased liver MnSOD and GSH/GSSG ratio.

The potential long term effect of a 6-day exendin-4 administration to newborn rats on cardiac OS status was examined by evaluating the cardiac response to *ex vivo* I/R after 4-6 weeks and 8-9 months [129]. Surprisingly, the study showed improved recovery in hearts from the exendin-4 treated groups, which was attributed to reductions in oxidative phosphorylation possibly through epigenetic alterations, as no differences between groups in TBARS and MnSOD activity were observed [129].

Two studies have been performed using porcine models of I/R. Exenatide intervention (started just prior to reperfusion and continued for two days after) in a cardiac I/R model resulted in higher activity of SOD and CAT as well as decreased 8-OHdG in the heart tissue [22]. In the same study, the exenatide group showed a 40 % reduction in infarction size. In the second experiment, treatment with human recombinant GLP-1 for four hours from one minute after reperfusion in a model of cardiac arrest resulted in lower levels of 8-iso-Prostagladin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) in plasma [130]. In contrast to the first study, the GLP-1 analogue did not have any effect on the SOD activity. The authors concluded that the decrease in the level of 8-iso-$PGF_{2\alpha}$ might be through non-enzymatic pathways, i.e. through a reduction in ROS [130]. Firstly, the decrease in 8-iso-$PGF_{2\alpha}$ could be due to changes in many other pathways as discussed through this review. Secondly, the decrease in 8-iso-$PGF_{2\alpha}$ could still be explained by SOD activity as the duration of these two experiments are different, maybe SOD is utilized to decrease superoxide levels and the amount of 8-iso-$PGF_{2\alpha}$, and the difference in the time allowed to increase SOD in these experiments are different. Catalase and SOD was only elevated at measurements after 72 hours. Unfortunately, the studies did not measure other antioxidants such as GSH or CAT. If the overall antioxidant capacity was indeed unchanged, the decrease in 8-iso-$PGF_{2\alpha}$ may be attributed to a decreased production of ROS by GLP-1, as GLP-1 has shown glucose independent effects on ROS production by down regulating NOX in both heart and kidney in *in vivo* studies [71, 131]. Importantly, the observation in Dokken *et al.* [130] shows that GLP-1 protects against I/R in isolated hearts, suggesting that the effects are independent of circulating insulin and blood glucose levels [130].

Collectively, these results support the hypothesis that GLP-1 can reduce tissue damage in I/R models (decrease infarct volume size, improve recovery, decrease DNA fragmentation etc.) [22, 119, 122, 124, 125, 129, 130] by lowering ROS production, oxidative damaged macromolecules, and/or increase antioxidant status. This indication of lower nuclear DNA damage and lipid peroxidation accompanied by increased intracellular cAMP supports the hypothesis that induction of cAMP signaling takes part in the protective effects of GLP-1. Further mechanistic details are needed to determine if the effects of GLP-1 are mediated through lowering the production of ROS or scavenging of ROS. It has already been summarized by Birnbaum *et al* (2012) that the protective effects observed by GLP-1 on injury after I/R are primarily due to GLP-1R activation, cAMP concentration increase intracellular and PKA activation, which leads to activation of several pathways as mentioned previously [132]. *E.g.* CREB has shown to be involved in reducing OS status both *in vitro* and *in vivo* potentially by activation of OS defense mechanisms [102, 119].

### 4.2. Obese and/or Spontaneously Diabetic Animal Models

As mentioned in the introduction, hyperglycemia may affect OS status in several ways. This issue becomes particularly important when studying effects of GLP-1 on OS in obese and diabetic animal models, as GLP-1's effects on body weight and blood glucose are easily mistaken for glucose independent effects, i.e. direct effects of GLP-1 on OS. Thus, body weight and blood glucose must be closely monitored, properly controlled for and taken into account when concluding on data from such experiments.

A 60-day exendin-4 treatment of ob/ob mice showed a reduction in TBARS in liver contents compared to untreated

animals [133]. The treated animals lost 14% of their body weight compared to vehicle controls, and as the experiment did not include a weight-matched control group, it cannot be ruled out that the effect of exendin-4 on TBARS in the liver may to some extent result from the reduction in body weight. Additionally normalization to liver lipids could help exclude the possibility that the observed effects on TBARS were due to the reduction in the liver lipid content. Measurement of antioxidant levels and ROS producing enzymes could be helpful on evaluation on potential glucose independent effects as well. Forty days of exendin-4 infusion has been shown to reduce the degree of cardiac remodeling in two different obese pre diabetic animal models, the hyperphagic, insulin resistance and dyslipidemic, KKay mouse and the diet induced obese (DIO) mouse [131]. Exendin-4 decreased the levels of cardiac superoxide and NOX4 in both models. In the DIO model - but not the KKay mouse - cardiac SOD-1 and GPx increased concurrently, showing that the choice of model often affects the readouts obtained in the evaluation of OS. Here, factors such as difference in obesity status and GLP-1R expression could play a role. In the same study, exendin-4 decreased both total cholesterol and triglycerides, systemically and in the heart but the potential indirect effect on ROS status caused by the improvement of dyslipidemia was not independently controlled for. Moreover, as NOX is down regulated by exendin-4, the GLP-1R activator might have a glucose independent effect on the ROS producing enzyme. Exendin-4 did not affect food intake (given as an individual average of the entire experimental course), body weight or blood glucose, but only the blood glucose during a challenge (oral glucose tolerance test) [131]. Even though the KKay model is generally considered insulin resistant [134], the effect of exendin-4 was significant in the oral glucose tolerance test, indicating that the treatment ameliorates glucose intolerance in the model, which was also the case in the DIO model. Similar difficulties in determining the independent effects of weight loss, food intake and blood glucose lowering were apparent in a study in db/db mice, in which two weeks of liraglutide intervention, in a relevant dosing regime in rodent models (200 μg/kg twice daily), resulted in increased GPx activity and a no significant increase in CAT in pancreatic tissue, while no effect on antioxidant gene expression was observed [135].

The first study on the effect of GLP-1 in the kidney was performed in db/db mice. Here, exendin-4 treatment for 8 weeks decreased urinary 8-OHdG and decreased immunohistochemical expression of 8-OHdG in the kidney. Exendin-4 did not decrease blood glucose, which could be due to a low number of -cells in db/db mice [136], but body weight was decreased by exendin-4 treatment making it difficult to rule out an effect of body weight on the OS status. However, the observed effects could be induced by increased GLP-1R and peroxisome proliferator-activator receptor- expression [137]. In a mouse model of diabetic nephropathy, the KK/Ta-Akita mouse, activation of the GLP-1R by liraglutide for four weeks has been shown to decrease NOX4 expression and its activity, a decrease in glomerular superoxide and an increase in glomerular NO, *via* an elevation of cAMP and PKA levels [138]. This resulted in lower levels of TBARS and a reduction in kidney damage as measured by decreased albuminuria, glomerular filtration rate, kidney hypertrophy, mesengial expansion score, fibronectin staining intensity, glomerular basement membrane and podocyte number. SOD-1 down-regulation in this mouse model plays an important role in the development of nephropathy [139] and thus the reduced superoxide production in the kidneys in the presence of liraglutide is likely to cause the attenuated kidney damage observed in this study. Importantly, no changes were observed in blood glucose or plasma insulin in the diabetic animals, as this mouse model has dysfunctional -cells [140]. The body weight was not affected either, suggesting that the observed protective mechanisms did not occur through the known metabolic effects of liraglutide.

### 4.3. Animal Models of Induced Diabetes

The acute effect of exendin-4 under hyperglycemic conditions (blood glucose = 20 mmol/l) has been investigated in one study using C57BL/7 mice undergoing hyperglycemic clamp studies, where blood glucose was fixed at a predetermined concentration. Intracerebral infusion of exendin-4 lowered ROS level in the hypothalamus and decreased the GSSG/GSH ratio. This study showed increased whole body glucose utilization in hyperglycemic mice [141]. The study indicates an improved OS status but glucose dependent effects cannot be ruled out as the intracerebral injection of exendin-4 could improve glucose homeostasis locally even though the systemic blood glucose is kept constant.

Prophylactic intervention with human recombinant GLP-1 seven days prior to STZ administration and 23 days after diabetes induction has been shown to decrease plasma and pancreatic levels of MDA, increased SOD and GPx (ns in pancreas) in Chinese Kun Ming mice [142]. Treatment with exendin-4 for 30 days to BALB/c mice in a post STZ administration regimen, resulted in a protection of -cells from apoptosis probably through increased antioxidant capacity [143]. Exendin-4 increased pancreatic CAT, GPx and SOD in diabetic but not in non-diabetic exendin-4 treated animals. In addition, exendin-4 reduced blood glucose and increased body weight, an effect that may be attributed to increased -cell mass and improved blood glucose regulation. Food intake was not recorded but effects of altered food intake would be expected to be most pronounced in the beginning of the study. Therefore, decreased blood glucose may at least in part explain the higher levels of antioxidants observed. Increased expression of Nrf2, a nuclear factor involved in expression of antioxidant genes and genes involved in the antioxidant defense [144], has been shown in a STZ induced T1D mouse model treated with exendin-4 after STZ injections [145]. MDA levels were decreased in the liver indicating a protective effect of exendin-4 through regulation of antioxidant genes.

In diabetic Wistar rats, ten weeks of exenatide treatment initiated five days after STZ exposure led to decreased blood glucose levels after four and eight weeks and improved glucose tolerance as measured by intraperitoneal glucose tolerance test (IPGTT) at the end of the ten weeks [146]. Immunofluorescence staining of the pancreas for CAT and glutathione reductase (GR) revealed an increase in treated animals (co-localized with insulin in the islets of Langerhans) together with an elevation in GPx gene expression. The results may suggest that exenatide increases antioxidant level

and gene expression, but an indirect effect through blood glucose lowering cannot be excluded. A similar study with GLP-1 yielded comparable results as ten weeks of GLP-1 treatment increased the percentage of GR containing cells in the pancreas and increased GR gene expression in STZ induced diabetic Wistar rats. The GLP-1 treated diabetic rats had a higher body weight and a lower blood glucose (both fasting and during an IPGTT), while GLP-1 did not affect body weight in nondiabetic rats. Moreover, the latter only showed a reduction in blood glucose at two time points during the IPGTT (no effect on fasting blood glucose) but still displayed increased GR gene expression [147]. This could indicate a glucose independent effect of GLP-1 on GR gene expression. Exendin-4 treatment of Sprague Dawley rats for eight weeks starting one week after STZ administration decreased urinary 8-OHdG, glomerular 8-OHdG content, expression of Nox-4 in cortex and Nox-4 content in kidney endothelial cells (detected by immunofluorescence staining) without any effect on blood glucose level, blood pressure, food intake or body weight [71]. These results indicate a glucose independent effect of exendin-4 on ROS induced DNA damage, a decrease in ROS production and a decrease in NF-κβ, implicating a favorable effect on inflammation [71]. However, these measurements were made at the end of the experiment (week 8) and the initial glucose dependent effects were not accounted for. Similar results were obtained in a four week liraglutide treatment study in Wistar rats, resulting in decreased mRNA expression of Nox-4, $gp91^{phox}$, $p22^{phox}$ and $p47^{phox}$ and protein levels of NOX4, $gp91^{phox}$ and $p47^{phox}$. Kidney superoxide levels were lower in the liraglutide treated group compared to controls and both urinary 8-OHdG and MDA were decreased. The observed effects were not accompanied by a change in food intake, body weight, blood glucose or blood pressure at day 28 [148]. In another study in Sprague Dawley rats, antioxidant enzyme activity (GR, SOD and CAT) was increased by four weeks of exenatide treatment together with a decrease in MDA, a result shown in a low dose and high dose regime (1.0 g/kg and 5.0 g/kg once daily) without returning blood glucose and insulin levels to normal in the low dose regime [149]. However, a significant decrease in blood glucose and an increase in serum insulin were observed. Even though their levels did not return to normal, the data do not seem to support the authors' conclusions of a glucose independent effect of exenatide on lipid peroxidation resulting from an improved oxidative status seemingly by increased antioxidant activity. In the same study a short term intervention with exenatide had the same effects on OS measurements, but as neither food intake or body weight was measured in these experiments, it is likely that the effect of exenatide on food intake (especially in the beginning of the treatment period) and on body weight could cause these effects. Shorter treatment regimens (two weeks) has also proven effective as *e.g.* a two-week exendin-4 administration by intraperitoneal infusion pump led to a reduction in urinary 8-OHdG content and increased body weight in male STZ treated diabetic Wistar rats [93]. In this study, RAGE expression was decreased and as depicted in Fig. (**3**), this could provide a mechanism explaining the effects of exendin-4 on ROS production and damage. Again, food intake was unfortunately not measured, which could have been interesting in this short treatment regimen.

Effects of GLP-1 treatment on body weight in STZ-induced diabetic animals are interesting as the animals both are hyperphagic and diabetic and when treatment increases body weight in some of these studies, it could indicate a remaining β-cell mass with the potential for GLP-1 to exert insulinotropic effects. C-peptide measures could be used to reveal the degree of β-cell function in such studies.

### 4.4. Animal Models of Cardiovascular Function and Disease

GLP-1 has shown protective effects on cardiovascular function in both apolipoprotein E (APO E) knockout mice and humans making the underlying mechanisms of these effects relevant to investigate [72, 150-152]. In APO E knockout mice, prophylactic intervention with human GLP-1 for four weeks (initiated simultaneously with a change to atherogenic diet) suppressed the formation of atherosclerotic lesions (surface area of atherosclerotic lesions, plaque size and macrophage infiltration) [150]. A reduction in oxLDL accumulation in isolated macrophages was also observed but without concurrent effects on plasma glucose or insulin when compared to control groups. However, as atherosclerotic development and OS status is highly dependent on food intake in this model [153], the duration of study becomes important as effects of lowered food intake (which are most pronounced in the beginning of GLP-1 intervention) may be misinterpreted as potential glucose independent effects of GLP-1 on atherosclerosis and OS. Administration of GLP-1 prior to change in diet can circumvent the most pronounced effect of GLP-1 on food intake and thereby on the intake of an atherogenic diet. Longer studies are needed both in prevention and treatment regimens to evaluate further on the latter effects of GLP-1.

In Wistar rats, seven days of exendin-4 injections prevented the induction of vascular endothelial dysfunction following streptozotocin treatment as evaluated by Ach-induced relaxation and integrity of endothelial lining [154]. The data suggest that the effect result from an increased production of eNOS by activation of Akt, as the effects of exendin-4 were attenuated by the eNOS inhibitor L-NAME. A significantly reduction in TBARS was observed in the exendin-4 treated when compared with control animals [154] implying effects on OS status. However, as a report showed that contractile force in aortic rings can be preserved by GLP-1 in STZ induced diabetic rats but not with exendin-4 while both treatments lead to reduced plasma MDA [155], the two effects may not be related. On the other hand, the lack of contractile effects of exendin-4 may be due to the low doses used, although they were clearly high enough to reduce lipid oxidation.

Collectively, the above results suggest that GLP-1 can lower the OS status in various animal models, both preventive and treatment studies, by lowering ROS producing enzymes and increasing antioxidant capacity, thus lowering the damage induced by high levels of ROS in the animals. Many different organ systems are involved and thus more detailed studies are needed to elucidate the effects of GLP-1 on OS

**Table 2.** ***In vivo*** **studies of GLP-1 and oxidative stress.**

| Species | Type | Study Design: Model (M), Intervention (I) and Treatment Duration (TD) | Effect of GLP-1 on Oxidative Stress Markers (Compared to Control Unless Otherwise Stated) | Pathological Changes (Compared to Control Unless OTHERWISE Stated) | Comments |
|---|---|---|---|---|---|
| Mice | I/R | **M:** C57BL/6 mice.<br>**I:** MCAO and exendin-4 0.1, 1, 10 or 50 g/100 L/mouse (iv).<br>**TD:** Single injection at different time points after I/R. | Brain:<br>8-OHdG and HHE (p<0,001). | Exendin-4 > 10 g reduced infarct area and volume (p<0.001), best effect when administrated at time 0 (p<0.001). | ↑ CREB, indicating actions through cAMP/CREB pathways [119]. |
| | | **M:** C57BL/6 mice (n=12).<br>**I:** Liraglutide 200 g/kg (ip twice daily.<br>**TD:** 7 days prior to MI. | Heart:<br>↑ Nrf2 expression and protein concentration (p<0.05).<br>↑ HO-1 expression and protein concentration (p<0.05). | Liraglutide:<br>Mice subjected to MI had improved recovery (left ventricular developed pressure) (p<0.01). | Nrf2 and HO-1 was measured prior to MI in other mice [121]. |
| | Acute clamp | **M:** C57BL/6 + GLP-1R (-/-) mice (n=62).<br>**I:** Catheters (lateral cerebral ventricle + left femoral vein) + ultrasonic flow probe. hyperinsulinemic-euglycemic (5.5 mmol/l) or hyperglycemic<br>(20 mmol/l) clamps. Intracerebral infusion of exendin-4 or exendin-9 (0.5 pmol/kg/min). $H_2O_2$ (2 or 20 nmol/min) was infused for the last hour.<br>**TD:** 3 hours. | Brain (hypothalamus):<br>ROS (CM-$H_2$DCFDA fluorescence) compared to hyperglycemic and normoglycemic controls (p<0.05).<br>GSSG/GSH ratio (20 nmol/l) (p<0.05). | | ↑ whole body glucose utilization (glucose infusion rate). Glucose dependent exendin-4 induced ROS reduction. Decreased eNOS activity during hyperglycemia. Hypothalamic ROS decrease was associated with reduction of femoral blood flow [141]. |
| | Cryo-lesion | **M:** C57BL/6 mice (n=20).<br>**I:** Traumatic brain injury induced applying a liquid nitrogen acclimatized probe to the skull. Liraglutide 200 g/kg twice daily (sc) ± Ex9.<br>**TD:** 4 days **(**from immediately after trauma) | Brain:<br>total and average ROS/RNS pr. pixel (p<0.05). | -spectrin cleavage pattern (marker of necrosis and apoptosis). | [120] |
| | Prevention | **M:** C3H/HeJ mice.<br>**I:** Exendin-4 24 nmol/kg (ip).<br>**TD:** 7 days. | Langerhans islets:<br>TxNIP expression (p<0.001). | Langerhans islets:<br>caspase-3 (p<0.05) and bax (p<0.001) mRNA level. | [101] |
| | | **M:** APO E (-/-) mice .<br>**I:** GLP-1(7-36) amide 2.2 nmol/kg/day (sc. pumps).<br>**TD:** 30 days. | Isolated peritoneal macrophages:<br>45% reduction in oxLDL induced cholesteryl ester accumulation (p<0.0001). | Oil red O staining and MOMA-2 staining of aorta.<br>Significant reduction in:<br>Surface area of atherosclerotic lesions (p<0.0001), plaque size (p<0.005) and macrophage infiltration (p<0.05). | oxLDL induced cholesteryl ester accumulation was assessed in cultured macrophages from treated mice. [150] |

**Table 2. contd…**

| Species | Type | Study Design: Model (M), Intervention (I) and Treatment Duration (TD) | Effect of GLP-1 on Oxidative Stress Markers (Compared to Control Unless Otherwise Stated) | Pathological Changes (Compared to Control Unless OTHERWISE Stated) | Comments |
|---|---|---|---|---|---|
| | | **M:** KK/Ta-Akita mice ♂ (n= 5 pr. group).<br>**I:** Liraglutide 200 g/kg/day (sc).<br>**TD:** 4 weeks. | Kidney:<br>↓ TBARS level (p<0.05).<br>↓ NOX activity (p<0.001).<br>↓ NOX4 expression (p<0.001).<br>↓ Superoxide level (DHE staining) (p<0.001).<br>↑ NO fluorescence intensity (p<0.0001). | Kidney:<br>↓ albuminuria (p<0.01).<br>↓ glomerular filtration rate (p<0.01).<br>↓ Kidney hypertrophy (p<0.05).<br>Kidney histology:<br>↓ Mesengial expansion score, fibronectin staining intensity, glomerular basement membrane (p<0.001).<br>↑ Podocyte number (p<0.001). | + Adenylate cyclase inhibitor (SQ22536) or PKA inhibitor (H-89) → eliminated effects of liraglutide.<br>No difference in blood glucose or plasma insulin. Experiments GLP-1R knockout mice supports that effects are mediated through the GLP-1R [138] |
| | Prevention + treatment | **M:** Chinese Kun Ming mice (n=24).<br>**I:** STZ (day 8-10) + rhGLP-1 24 nmol/kg (ip).<br>**TD:** 30 days from one week prior to STZ. | Plasma:<br>↓ MDA (p<0.05).<br>↑ SOD (p<0.05).<br>↑ GPx (p<0.05).<br>Pancreas:<br>↓ MDA (p<0.001).<br>↑ SOD (p<0.05). | Pancreas:<br>↑ Islet number/area (p<0.05).<br>↓ Histological changes. | [142]. |
| | Treatment | **M:** db/db and m/m ♂ mice.<br>**I:** Liraglutide 200 g/kg twice daily (sc).<br>**TD:** 2 weeks. | Pancreas:<br>db/db: ↑ GPx (p<0.01) after 2 weeks. | Pancreas:<br>db/db: ↓ Caspase-1, Caspase-3 and Cad (p<0.05) expression after 2 days and 2 weeks. ↑ Bcl2 (p<0.05) expression after 2 weeks (NS after 2 days). | ↓ ER stress (Xbp1 expression) (p<0.05) after 2 weeks. [135] |
| | | **M:** KKAy and DIO mice.<br>**I:** Exendin-4 24 nmol/kg/day (sc pumps).<br>**TD:** 40 days. | Heart:<br>↓ Superoxide (DHE staining) (p<0.05).<br>↓ Mfn1/Mfn2 (p<0.05).<br>↓ Mitochondria specific OS (MTR) (p<0.01).<br>PARKIN + PINK-1 (p<0.01 and 0.05).<br>Protein levels of:<br>↓ Nox-4 (p<0.01).<br>↑ SOD-1 (DIO) (p<0.05).<br>↑ GPx (DIO) (p<0.01).<br>Nox-2 and TRx no change. | Cardiac lipid accumulation ↓ (p<0.01) and fibrosis. | No weight loss observed. [131] |

**Table 2. contd…**

| Species | Type | Study Design: Model (M), Intervention (I) and Treatment Duration (TD) | Effect of GLP-1 on Oxidative Stress Markers (Compared to Control Unless Otherwise Stated) | Pathological Changes (Compared to Control Unless OTHERWISE Stated) | Comments |
|---|---|---|---|---|---|
| | | **M:** db/db (db/m as controls) ♂ (n=27).<br>**I:** Exendin-4 (0.5 or 1.0 nmol/kg) ip.<br>**TD:** 8 weeks. | Urinary (24 hours):<br>8-OHdG ($p<0.05$ for db/db vs. control db/db and $p<0.01$ for db/m vs. control db/db).<br>Kidney:<br>8-OHdG protein concentration in db/db mice (determined with immunostaining). | Kidney:<br>glomeruli lipid accumulation (oil red O).<br>collagen IV ($p<0.05$ and $p<0.01$).<br>TGF- ($p<0.05$ and $p<0.01$).<br>mesangial matrix expansion.<br>glomerular hypertrophy.<br>macrophage infiltration (F4/80 staining) ($p<0.05$ and $p<0.01$).<br>apoptotic cells (caspase-3 positive cells) ($p<0.05$ in 1.0 nmol/kg). | Induction of peroxisome proliferator-activator receptor- and GLP-1R expression. Decrease in GLP-1R positive cells in the glomeruli of db/db mice. Increased by exendin-4 treatment. No effect on food intake and blood glucose but decreased body weight in both db/db dose groups [137]. |
| | | **M:** Ob/ob mice.<br>**I:** Exendin-4 (10 g/kg for 14 days → 10 g/kg or 20 g/kg).<br>**TD:** 60 days. | Liver:<br>TBARS ($p<0.05$ for 20 g/kg). | Significant reduction in liver lipid content ($p<0.05$ for 10 g/kg and 0.01 for 20 g/kg ). | [133] |
| | | **M:** C57BL/6 ♂ (n=24).<br>**I:** STZ + exendin-4 (8 g/kg) sc ± omeprazole.<br>**TD:** From 14 days after first STZ injection and for 28 days. | Liver:<br>MDA ($p<0.01$). (+ omeprazole $p<0.001$).<br>↑ Nrf2 level ($p<0.01$). | Liver:<br>triglycerides ($p<0.01$).<br>(+ omeprazole $p<0.001$). | Nrf2 controls antioxidant genes and is important in protecting the liver against OS [145]. |
| | | **M:** BALB/c mice.<br>**I:** STZ + exendin-4 3 g/kg (sc).<br>**TD:** 30 days after STZ injection. | Pancreas (activity of):<br>↑ CAT ($p<0.05$)<br>↑ GPx ($p<0.01$)<br>↑ SOD ($p<0.05$) | Pancreas:<br>No observed effects on apoptosis (TUNEL assay and cleaved caspase-3). | No effect on CAT, GPx and SOD in nondiabetic mice. [143] |
| Rats | I/R | **M:** SD rat pups.<br>**I:** Exendin-4 (1.0 nmol/kg) sc. Isolation of heart at 4-6 weeks or 8-9 months and subjected to I/R ex vivo.<br>**TD:** 6 days from the day of delivery. | Heart homogenates:<br>No effect on TBARS and MnSOD activity in either group. Nor on nonenzymatic antioxidant capacity (Biovision kit). | Heart:<br>Improved recovery from I/R (percent recovery of left ventricular end diastolic pressure). | [129]. |
| | | **M:** Wistar rats ♂.<br>**I:** MCAO and liraglutide (1 ml, 700 g/kg (IP)<br>**TD:** Single dose one hour after reperfusion. | Peripheral blood:<br>d-ROMS (0.93) ($p<0.05$) | Significant reduction in infarct volume ($p< 0.05$). | [125] |

**Table 2. contd…**

| Species | Type | Study Design: Model (M), Intervention (I) and Treatment Duration (TD) | Effect of GLP-1 on Oxidative Stress Markers (Compared to Control Unless Otherwise Stated) | Pathological Changes (Compared to Control Unless OTHERWISE Stated) | Comments |
|---|---|---|---|---|---|
| | | **M:** SD rats ♂.<br>**I:** MCAO and exendin-4 0.5 μg/kg twice daily (ip).<br>**TD:** 7 days prior to I/R. | Brain:<br>↓ MDA (0.75) ($p<0.001$)<br>↑ GSH (1.45) ($p<0.05$)<br>↑ SOD (1.67) ($p<0.01$). | Significant reduction in infarct volume ($p<0.001$). | [122] |
| | | **M:** Sprague Dawley rats ♂ (n=32).<br>**I:** Exenatide 10 g/kg/day (ip). Ligation of left coronary artery for 30 min followed by 2 h reperfusion.<br>**TD:** 2 weeks prior to MI/R. | Heart homogenates:<br>↓ MDA ($p<0.05$).<br>↑ T-SOD, CAT and GPx ($p<0.05$). | | [90] |
| | | **M:** Wistar rats ♂+♀.<br>**I:** STZ induced diabetes + surgical induced renal I/R. Exenatide 10 g twice daily (sc.).<br>**TD:** 2 weeks prior to I/R. | Liver:<br>↓ MDA ($p<0.001$)<br>↓ XO ($p<0.001$)<br>↑ GSH ($p<0.01$)<br>↑ SOD ($p<0.001$)<br>↑ GPx ($p<0.05$)<br>↑ CAT ($p<0.01$) | Significant reduction in ALT, AST and ALP after I/R ($p<0.01$). | [123] |
| | | **M:** Wistar rats ♂+♀.<br>**I:** STZ + renal I/R (after 10 days of exenatide (10 g twice daily (sc)) intervention)<br>**TD:** 2 weeks from 2 weeks after STZ. | Kidney:<br>↓ MDA ($p<0.001$).<br>↑ GSH ($p<0.05$).<br>↑ GPx ($p<0.001$).<br>↑ SOD activity ($p<0.05$).<br>↑ CAT activity ($p<0.001$).<br>↓ XO activity ($p<0.001$). | Kidney:<br>↓ DNA fragmentation.<br>Preservation of normal morphology. | After STZ but before I/R [124]. |
| | | **M:** neonatal SD rats ♂.<br>**I:** IUGR + exendin-4 1nmol/kg (sc).<br>**TD:** 6 days from day of life. | Liver:<br>↓ MnSOD ($p<0.05$).<br>↓ TBARS ($p<0.05$).<br>↑ GSH/GSSG ($p<0.05$). | | ↓ BW ($p<0.05$ in control+exendin-4, not significant in IUGR+exendin-4). No difference between control exendin-4 treated and control vehicle treated [128] |
| | Treatment | **M:** Wistar rats ♂+♀.<br>**I:** STZ or methionine + exendin-4 1 μg/kg (ip).<br>**TD:** 7 days. | Significant reduction in serum TBARS (STZ or methionine +/- exendin-4) ($p<0.05$). | Significant prevention of diabetes or hyperhomocysteinemia induced VED. | Inhibition of eNOS by L-NAME → no effect of exendin-4 [154] |
| | | **M:** Wistar ♂ rats.<br>**I:** STZ + Exendin-4 0.3 or 1.5 g/kg/h (ip pumps).<br>**TD:** 2 weeks. | ↓ Urinary 8-OHdG ($p<0.01$) (1.5 g/kg/h).<br>↓ ADMA level ($p<0.01$) (1.5 g/kg). | Kidney:<br>↓ glomerular area, macrophage infiltration in glomeruli, glomerular and tubulointerstitial fibrosis ($p<0.01$). | Inhibition of RAGE gene expression in both dose groups ($p<0.01$) [93]. |

**Table 2. contd…**

| Species | Type | Study Design: Model (M), Intervention (I) and Treatment Duration (TD) | Effect of GLP-1 on Oxidative Stress Markers (Compared to Control Unless Otherwise Stated) | Pathological Changes (Compared to Control Unless OTHERWISE Stated) | Comments |
|---|---|---|---|---|---|
| | | **M:** SD rats ♂.<br>**I:** STZ + exenatide (1.0 or 5.0 g/kg once daily) sc.<br>**TD:** 1 or 4 weeks from 4 days after STZ. | Heart:<br>↓ MDA ($p<0.05$).<br>↑ GR ($p<0.05$).<br>↑ SOD ($p<0.05$).<br>↑ CAT ($p<0.05$). | Plasma:<br>↓ markers of cell damage (LDH, total CK, absolute CK-MB and CK-MB relative index) ($p<0.05$). | Low dose treatment does not return blood glucose and serum insulin to normal, but still affects MDA, GR, SOD and CAT [149]. |
| | | **M:** Wistar rats ♂+♀.<br>**I:** STZ + GLP-1 (1 g/kg twice daily) or exendin-4 (0.1 g/kg/day) ip.<br>**TD:** 4 weeks. | Plasma:<br>↓ MDA in GLP-1 and exendin-4 treated groups when compared to controls ($p<0.001$) and diabetic controls ($p<0.001$). | Aortic rings:<br>GLP-1 reversed the decreased contractive force in diabetic rats. | [155]. |
| | | **M:** Wistar ♂ rats.<br>**I:** STZ + liraglutide (0.3 mg/kg twice daily) sc.<br>**TD:** 4 weeks from one week after STZ. | Kidney:<br>↓ Superoxide level (DHE in situ).<br>↓ Nox4 mRNA ($p<0.05$) and protein ($p<0.05$).<br>↓ $gp91^{phox}$ mRNA ($p<0.05$) and protein ($p<0.05$).<br>↓ $p22^{phox}$ mRNA ($p<0.05$).<br>↓ $p47^{phox}$ mRNA ($p<0.01$) and protein ($p<0.05$).<br>Urine:<br>↓ 8-OHdG ($p<0.01$).<br>↓ MDA ($p<0.01$). | Urine:<br>↓ albumin secretion ($p<0.05$). | Treatment [148]. |
| | | **M:** Sprague Dawley rats ♂ (n=23)<br>**I:** STZ + exendin-4 10 g/kg/day (ip).<br>**TD:** 8 weeks from one week after STZ. | Diabetic animals:<br>↓ Urinary 8-OHdG ($p<0.01$).<br>↓ Glomeruli 8-OHdG content ($p<0.001$).<br>↓ Cortex Nox-4 expression ($p<0.05$).<br>↓ NOX-4 content in kidney endothelial cells ($p<0.05$). | Kidney morphology:<br>↓ glomerular hypertrophy and mesangial matrix expansion.<br>Kidney microinflammation:<br>↓ expression of Cd14 ($p<0.001$), ICAM1 ($p<0.05$) and TGF- 1 ($p<0.05$) in cortex.<br>↓ level of ICAM1 ($p<0.001$), type IV collagen ($p<0.001$) and macrophage infiltration in glomeruli. | NF-κ p65 binding activity decreased by exendin-4 in diabetic group [71] |
| | | **M:** Wistar rats ♂.<br>**I:** STZ + exenatide 1 g/kg (ip).<br>**TD:** 10 weeks from day five after STZ. | Pancreas:<br>↑ CAT containing cells ($p<0.05$)<br>↑ GR containing cells ($p<0.05$)<br>↑ GPx gene expression ($p<0.05$) | Serum:<br>↓ alanine<br>and aspartic aminotransferases, cholesterol, triglyceride,<br>uric acids, blood urea nitrogen and creatinine. | Focus on insulin release.<br>[146] |
| | | **M:** Wistar rats ♂ (n=40).<br>**I:** STZ + GLP-1 50 ng/kg (ip).<br>**TD:** 10 weeks from day five after STZ. | Pancreas:<br>↑ GR containing cells ($p<0.05$)<br>↑ GR gene expression in both normal and diabetic animals ($p<0.01$). | Pancreas:<br>↑ heat shock protein-70 in both normal and diabetic animals ($p<0.05$).<br>↑ pancreatic duodenal homeobox-1 in both normal and diabetic animals ($p<0.05$). | [147] |

**Table 2. contd…**

| Species | Type | Study Design: Model (M), Intervention (I) and Treatment Duration (TD) | Effect of GLP-1 on Oxidative Stress Markers (Compared to Control Unless Otherwise Stated) | Pathological Changes (Compared to Control Unless OTHERWISE Stated) | Comments |
|---|---|---|---|---|---|
| Pigs | I/R | **M:** Pigs + (n=18). **I:** Cardiac arrest. Human rGLP-1 10pmol/kg/min (iv infusion pump). **TD:** 4 hours from one minute after resuscitation. | Plasma: 8-iso-$PGF_2$ (0.78) in coronary sinus ($p<0.05$). No effect on SOD activity. | Preservation of coronary microvascular function (compared to baseline). No decline in coronary flow reserve ($p<0.05$) (compared to controls). | No difference in blood glucose or plasma insulin [130]. |
| | | **M:** Pigs **I:** Cardiac I/R. Exenatide 10 g and 10 g (iv. and sc). **TD:** 5 min. before reperfusion and two days after (twice daily). | Heart (72 hours after reperfusion): 8-OHdg ($p<0.005$ (positive cells/$mm^2$)). ↑ SOD activity ($p<0.05$). ↑ CAT activity ($p<0.05$). | (40%) in infarct size ($p<0.05$). ↑ improved cardiac function. caspase-3 protein ($p<0.05$) (four hours after reperfusion). | [22] |

AGE = advanced glycation end-product, ADMA = asymmetric dimethyl arginine, ALT = alanine aminotransferase, ALP = alkaline phosphatase, AST = aspartate aminotransferase, APO E = Apolipoprotein E, Bax = BCL2-associated X protein, CAD = Caspase-activated DNase, CAT = catalase, CK = creatinine kinase, CK-MB = creatinine kinase-MB, CM-H2DCFDA = 2',7'-dichlorodihydrofluorescein diacetate, CREB = cAMP response element-binding protein, DIO = diet induced obese, d-ROMs = derivatives of reactive oxygen metabolites, GSH = reduced glutathione, GSSG = oxidized glutathione, GPx = glutathione peroxidase, GR = Glutathione reductase, HHE = 4-hydroxy 2-hexenal, ICAM-1 = intercellular Adhesion Molecule 1, I/R = ischemia reperfusion, IUGR = intrauterine growth retardation, LDH = lactate dehydrogenase, MCAO = middle cerebral artery occlusion, MDA = malondialdehyde, Mfn = mitofusin, MOMA-2 = Monocyte/Macrophage Marker Antibody-2, MI = myocardial infarction, MI/R = myocardial ischemia/ reperfusion, MTR = mitotracker red, NOS = nitric oxide synthase, Nrf2 = nuclear factor erythroid 2 p45-related factor 2, $PGF_2$ = prostaglandin 2 , PKA = protein kinase A, RAGE = advanced glycation end-product receptor, rhGLP-1 = recombinant human GLP-1, RNS = reactive ROS = reactive oxygen species, SOD = superoxide dismutase, STZ = streptozotocin, TBARS = thiobarbituric acid reactive substances, TGF- = transforming growth factor , TxNIP = thioredoxin interacting protein, TRx = Thioredoxin, TRxR = TRx reductase, T-SOD = total superoxide dismutase, TUNEL = terminal deoxynucleotidyl transferase dUTP nick end labeling VED = vascular endothelial dysfunction, XO = xanthine oxidase, 8-OHdg = 8-hydrodeoxyguanosine.

and mechanisms involved. Particularly, by assessing the balance between ROS, ROS production and antioxidant concentration and activity in all studies and trying to eliminate the confounding of GLP-1 on blood glucose, body weight and food intake. Moreover, it is important to investigate if and to what extent the effects observed in animal models may translate to humans bearing in mind the comparatively high doses used in animals compared to clinically relevant dosing regimens.

## 5. EFFECT OF GLP-1 ON OXIDATIVE STRESS IN CLINICAL STUDIES

The relationship between fasting GLP-1 concentration and OS has been investigated in a retrospective study where 72 T2D patients (65.3±8.6 yrs.) and 14 (63.4±8.7 yrs.) non-diabetic subjects were included. It revealed an inverse correlation between nitrotyrosine concentrations and GLP-1 independently of age, gender, BMI, HbA1c, antidiabetic treatment, and antihypertensive treatment. In this study, 8-OHdG was increased in the diabetic patients as well [116]. The effect of GLP-1 on OS status in humans has been investigated in both acute studies and longer term clinical studies. The available clinical data are summarized in Table **3**.

### 5.1. Acute Effects in Diabetic Patients

Ceriello and coworkers have performed several acute cross over experiments with hypo- or hyperglycemia in both T1D and T2D diabetic patients with focus on the effects of GLP-1 on OS [77, 156-159]. In one study, 30 male and female T1D patients, mean aged about 24 years and diagnosed for 7-8 years, were exposed to hypo- or hyperglycemic conditions [77]. Hypoglycemia has been shown to induce ROS production in an *in vivo* rat model in the CNS comparable to that of hyperglycemia [43, 160]. In the above study, two hours of hypo- or hyperglycemia and an simultaneous GLP-1 infusion to T1D patients decreased plasma 8-iso-$PGF_2$ and nitrotyrosine levels, although they remained significantly elevated compared to baseline. In a similar study of hyperglycemia in T2D patients, GLP-1 also reduced plasma 8-iso-$PGF_2$ and nitrotyrosine levels significantly [156]. The study was repeated after two months of optimized glycemic control of the diabetic patients. In these clamp experiments, which do not correspond to any physiological situation, two months of improved glycemic control *per se* decreased basal levels of 8-iso-$PGF_2$ and nitrotyrosine while the following GLP-1 treatment no longer improved the measures as it had done at baseline. The study suggests that the effect of GLP-1 on OS status is more pronounced when subjects are exposed to hyperglycemic conditions for longer periods of time, which may indicate that GLP-1 exerts a protective effect only under hyperglycemic conditions. However, these patients might have lost weight during the two months, and to evaluate on the effect of better glycemic control, a group of body weight and/or HbA1c matched patients should have been included, depending on whether the glycemic control aimed at lowering the HbA1c or fluctuations in blood glucose.

Effects of the antioxidant vitamin C and/or GLP-1 on OS status were examined in 20 T1D patients during acute hypoglycemia [158]. GLP-1 alone reduced both 8-iso-$PGF_2$ and nitrotyrosine significantly and vitamin C alone reduced the levels of these two biomarkers even more. Interestingly, simultaneous administration of both GLP-1 and vitamin C

resulted in levels of 8-iso-$PGF_2$ and nitrotyrosine not being different from baseline measurements. This can be contributed to different actions of vitamin C and GLP-1, vitamin C being a scavenger of ROS and GLP-1 acting through induction of intracellular antioxidant defenses, respectively. Similar results were obtained in T1D patients in a study of effects of GLP-1 and/or vitamin C on the glycemic changes during I/R like conditions, where hypoglycemic conditions were followed by either normo- or hyperglycemia [157]. During hypoglycemia, levels of both 8-iso-$PGF_2$ and nitrotyrosine increased, and these levels increased even more when hyperglycemia replaced hypoglycemia as compared to normoglycemia. GLP-1 lowered 8-iso-$PGF_2$ and nitrotyrosine but again, vitamin C had a more pronounced effect. In this case, simultaneous infusions of GLP-1 and vitamin C lowered 8-iso-$PGF_2$ and nytrosine to baseline level. The finding that GLP-1 is less effective than vitamin C could be caused by a reduction of sensitivity to GLP-1 in hypoglycemia as hyperglycemic conditions can increase the effects of GLP-1 on OS status as discussed above.

In the above experiments, the clamp technique was used and insulin was administrated. As insulin has effects on OS level and vasodilatory effects as well, the administration of insulin should be considered when evaluating on the effects of GLP-1 on OS [161-165]. It has been shown that combined administration of insulin and GLP-1 in such clamp studies can lower 8-iso-$PGF_2$ and nitrotyrosine levels more than GLP-1 by itself [159].

### 5.2. Short-Term Intervention Studies (< 6 Months)

A pilot study in 20 female and male T2D patients on metformin (57 ± 13 years) liraglutide treatment for two months lowered HbA1c significantly together with a decrease in blood concentrations of lipid peroxides (LOOH) and HO-1 and an increase in GSH and ghrelin. Body weight, fasting blood glucose and waist circumference were not affected in this study, and there was no correlation between LOOH and GSH concentrations, which implies a glucose independent effect of liraglutide on OS status [166]. Ghrelin has previously been associated with decrease in OS status in obese subjects [167]. Liraglutide induced ghrelin regulation could have an effect on OS status. The data on HO-1 in this study are not in line with the *in vitro* data of Oeseburg *et al* (2010), where HO-1 were increased by GLP-1, as it has antioxidant properties. T2D patients have increased levels of HO-1 [168] and the effect of liraglutide in this study could lower the need of HO-1 protection against OS and thus the concentration of HO-1. ROS was measured using DHE staining in this study, but no significant effect was found, which could support the hypothesis that antioxidant protection is present in diabetes. No metabolic studies were performed and only fasting blood glucose was measured, which together with the lowering of HbA1c makes it difficult to rule out that the effects on OS could be attributed in part to improved glycemic control.

In isolated mononuclear cells from obese T2D patients undergoing diabetes therapy, a 22 ± 9 % reduction in superoxide production was observed when the patients were treated with exenatide for 6 or 12 weeks prior to sampling. This was significantly different from baseline and from placebo, an effect seemingly independent of weight loss as no change in body weight was observed over the course of the study. The lack of weight loss could be due to the short duration of the study and that the patients were on insulin therapy throughout the study. An acute study was also performed; here a single injection of exenatide (5 g) reduced superoxide release from isolated mononuclear cells as well [24]. FFA was reduced in the single injection experiment but glucose and insulin were not measured. In the 12 week study fasting blood glucose, HbA1c and FFA were significantly lowered and insulin increased by exenatide treatment. Thus, regulatory effects on glucose, lowered FFA and increased insulin cannot be excluded to have an effect in this study. NF- binding and mRNA levels of inflammatory markers (TNF- , JNK-1, IL-1 , TLR-2, and TLR-4) were all decreased as well. The link between inflammation and OS has been suggested to be initiated by ROS activating NF- subsequently inducing proinflammatory cytokines. However, increased NF- may be induced by elevated blood glucose [169].

Reductions in inflammatory markers and 8-iso-$PGF_2$ have been shown in T2D patients following five months treatment with exenatide. Consistent with the expected effects of GLP-1 in T2D patients, these biochemical changes were accompanied by reduced body weights and BMIs, improved glucose profiles, reduced HbA1c, mean glucose levels and limited blood glucose excursions. To investigate the potential glucose independent effects of exenatide on 8-iso-$PGF_2$ , an analysis of covariance analysis was performed, showing that the effect of exenatide was independent of glycemic regulation and body weight [75]. Thus, this study supports that glucose excursions *per se* increase OS [170], since the glucose excursions measured in this study correlated with 8-iso-$PGF_2$ . However, as 8-iso-$PGF_2$ is decreased by exenatide, a glucose dependent effects of GLP-1 on OS status cannot be ruled out.

A comparison of the first line drug for prediabetic patients, metformin, and exenatide has been performed in male and female non-diabetic but obese and prediabetic patients with a mean age of 58.5±10.0 yrs. having either impaired fasting glucose, elevated HbA1c, or impaired glucose tolerance [171]. Three months of treatment resulted in no significant differences between exenatide or metformin groups on either oxLDL levels or endothelial function. The metformin group had a significantly lower oxLDL level after treatment than when compared to baseline. Unfortunately, the study had a small sample size and did not include a placebo group as baseline was used as control and albeit not significantly different, there was a difference in baseline data between the groups. Thus, the metformin treated group had a higher baseline concentration of oxLDL perhaps leaving a higher window of opportunity for effect of treatment. The effects of metformin could be due to altered antioxidant activity and antioxidant homeostasis [172, 173]. In another study, three months of exenatide treatment to obese children and adolescents did not reduce oxLDL levels even though BMI were significantly decreased [174]. These studies indicate that the effect of GLP-1 on OS may be restricted to or at least most pronounced when individuals are challenged with diabetes and *e.g.* postprandial glucose excursions. This is suggested

**Table 3. Clinical studies including measurements of oxidative stress level.**

| Duration of treatment | Study Design: Subjects (S), Intervention (I) and Treatment Duration (TD) | End point: Effect of GLP-1 Intervention on Oxidative Stress Markers (Compared to control Unless Otherwise Stated) | Comments |
|---|---|---|---|
| Hours | **S:** T1D (n=30). **I:** Hypo- (2.9 ± 0.1 mmol/l) or hyperglycemic (15 mmol/l) clamp ± GLP-1 infusion (0.4 pmol/kg/min). **TD:** 2 hours. | Plasma: 8-iso-$PGF_2$ + nitrotyrosine (hypo- and hyperglycemia) ($p<0.01$) but significantly higher than baseline ($p<0.01$). | [77] |
| | **S:** T2D (n=16) + controls (n=12). **I:** Hyperglycemic (15 mmol/l 0-1 hours and 12.8 mmol/l at 1-2 hours) clamp. Repeated after two months of better glycemic control (insulin therapy). **TD:** 2 hours. | Plasma: 8-iso-$PGF_2$ + nitrotyrosine ($p<0.05$) (after one and two hours in T2D, after one in controls). More pronounced effect after two months of better glycemic control. | [156] |
| | **S:** T1D (n=20). **I:** Hypoglycemic (2.9 ± 0.1 mmol/l) clamp. Vitamin C (30 mg/min) and/or GLP-1 (0.4 pmol/kg/min). **TD:** 2 hours. | Plasma: 8-iso-$PGF_2$ + nitrotyrosine (GLP-1 or vitamin C (more pronounced ($p<0.05$)). GLP-1 + vitamin C: 8-iso-$PGF_2$ and nitrotyrosine at one and two hours not significantly different from baseline measurements. | [158] |
| | **S:** T1D (n=15). **I:** Hypoglycemia (2.9 ± 0.1 mmol/l) superseded with either normoglycemia (4.5 mmol/l) or hyperglycemia (15 mmol/l) Vitamin C (30 mg/min) and/or GLP-1 (0.4 pmol/kg/min). **TD:** 4 hours (2 hours of hypoglycemia and 2 hours of hyper- or normoglycemia). | Plasma: 8-iso-$PGF_2$ + nitrotyrosine ($p<0.01$) (GLP-1 or vitamin C). Not significantly different from baseline when administrated together. | [157] |
| | **S:** T2D (n=24) receiving dietary or metformin intervention. **I:** Gr. 1: Normoglycaemic-normoinsulinaemic (blood glucose = 5 mmol/l) or normoglycaemic-hyperinsulinaemic (insulin infusion 0.1 mU/kg/min, blood glucose = 5 mmol/l) ± GLP-1 (0.4 pmol/kg/min). Gr. 2: Hyperglycaemic-normoinsulinaemic (blood glucose = 15 mmol/l) or hyperglycaemic-hyperinsulinaemic (insulin infusion 0.1 mU/kg/min, blood glucose = 15 mmol/l) ± GLP-1 (0.4 pmol/kg/min). **TD:** 2 hours. | Plasma: 8-iso-$PGF_2$ + nitrotyrosine ($p<0.05$), further decreased by hyperinsulinaemia. | [159] |
| < 6 months | **S:** T2D (n=20 + ) on metformin. **I:** Liraglutide (0.6 mg daily for two weeks then 1.2 mg daily). **TD:** 2 months. | Blood; LOOH ($p<0.05$). ↑ GSH ($p<0.01$). HO-1 ($p<0.05$). | Decreased HbA1c ($p<0.0001$) and increased ghrelin concentrations ($p<0.01$) [166]. |
| | **S:** T2D (n=12) on metformin. **I:** Exenatide (10 μg twice daily). Measuring superoxide release from isolated peripheral blood mononuclear cells. **TD:** 3 months. | Isolated peripheral blood MNCs: superoxide release (chemiluminescence) ($p<0.05$). | And acute study was performed as well testing superoxide generation in MNCs after a single dose of exenatide (5 g) → superoxide was reduced after 6 hours compared to baseline and placebo ($p<0.05$) [24]. |
| | **S:** Prediabetic patients (n=50) **I:** Exenatide (5 g for 4 weeks then 10 μg twice daily) or metformin (500 mg for 4 weeks then 1000 mg twice daily). **TD:** 3 months. | Plasma: oxLDL in metformin group ($p<0.05$) but no significant difference between the metformin and exenatide group. | [171] |
| | **S:** Obese but nondiabetic children and adolescents (age 9–16 years old) (n=12). **I:** Exenatide (5 g for 4 weeks then 10 g twice daily). **TD:** 3 months. | Plasma: No effect on oxLDL. | Significant BMI decrease [174] |

**Table 3. contd….**

| Duration of treatment | Study Design: Subjects (S), Intervention (I) and Treatment Duration (TD) | End point: Effect of GLP-1 Intervention on Oxidative Stress Markers (Compared to control Unless Otherwise Stated) | Comments |
|---|---|---|---|
| | **S:** T2D (n=23) on metformin and/or sulfonylurea. **I:** Exenatide (5 g for 4 weeks then 10 g twice daily). **TD:** 5 months. | Plasma: 8-iso-$PGF_2$ ($p<0.05$). | Covariance analysis indicated glucose independent effect on 8-iso-$PGF_2$ levels that was independent of HbA1c, mean SD, body weight, and BMI. [75] |
| > 6 months | **S:** T2D (n=65) (58.7±10.2 years) no more than 15 years of disease duration. n=32 on sulfonylurea. **I:** Liraglutide (started at 0.3 mg/day then 0.6-0.9 mg/day (mean dose 0.74 mg/day). **TD:** 8 months. | Blood: d-ROMs ($p<0.05$) (compared to baseline). No change in MDA. | Decreased HbA1c and blood glucose when compared to baseline ($p<0.01$). No effect on body weight [176]. |
| | **S:** T2D + (n=60) on metformin. **I:** Exenatide (5 g for 4 weeks then 10 g twice daily) or insulin glargine (starting at 10 U once daily). **TD:** 1 year. | Plasma: MDA (compared to baseline and insulin glargine group ($p<0.001$)). oxLDL/LDL in exenatide group (compared to baseline) ($p<0.05$). | Decrease in blood glucose ($p<0.001$) and triglycerides ($p<0.05$) both compared to baseline and insulin glargine group. No correlation between change in body weight and effects on blood glucose and triglycerides (body weight change not given) [23]. |

BMI = body mass index, d-ROMs = derivatives of reactive oxygen metabolites, HbA1c = hemoglobin A1c, HO-1 = heme oxygenease-1, GSH = glutathione, LDL = low density lipoprotein, LOOH = lipid hydroperoxide, MDA = malondialdehyde, MNCs = mononuclear cells, oxLDL = oxidized low density lipoprotein, SD = standard deviation, T1D = type 1 diabetes, T2D = type 2 diabetes, 8-iso-$PGF_2$ = 8-iso-prostaglandin $F_2$ .

by Tesauro *et al.* (2013) as well, as healthy obese subjects do not respond to GLP-1 therapy when measuring endothelial vasodilation [175]. But aspects such as duration of the studies, obesity level of the patients, and its effect on blood glucose should be evaluated more thoroughly in future studies, which could also include more biomarkers of OS as a single marker does not give a complete picture.

### 5.3. Longer Term Intervention studies (> 6 Months)

Very limited data are available on long-term effects of GLP-1 administration on OS in humans as only two studies have been performed. Here, one year of exenatide treatment resulted in improved postprandial glucose homeostasis and dyslipidemia in 60 metformin treated T2D patients subjected to a standardized meal after an overnight fasting [23]. Exenatide treatment lowered plasma MDA significantly both compared to baseline and to a parallel insulin glargine treatment group. The postprandial oxLDL/LDL ratio was significantly reduced from baseline measures in the exenatide group, but no significant difference was observed between the two treatment groups. The glucose lowering effects of both treatments may explain these findings. The treatment period of one year was followed by a period of five weeks off drug, after which the subjects had the standardized mixed meal and the above measurements were repeated, displaying a return of all parameters to their baseline values [23]. The study shows that exenatide lowers excursions in glucose and lipids after a meal and improved biochemical markers of oxidative damage to lipids but does not provide a lasting effect following discontinuation of treatment. Again measuring other biomarkers of ROS level, antioxidant capacity and damaged end products could help elucidate the mechanisms behind the observed effects. In another long-term study, eight months of liraglutide intervention (mean dose 0.74 mg/day) in T2D patients did not result in a reduction of blood MDA even though it lowered d-ROMs when compared to baseline [176]. Blood glucose and plasma insulin was significantly lower, which may explain the effect on d-ROMs. The absence of effect on MDA could be due to the low doses used in this study as the normal human dose is 1.2 mg/day in T2D. In the former mentioned exenatide study, the normal dosing regimen of exenatide was used.

Part of the evaluation of the effects of GLP-1 on diabetic late complications and in particular CVD will be done in the LEADER trial, which was initiated in 2010 and will follow patients for up to five years. This trial evaluates the effect of liraglutide on CVD [177]. Such a trial makes studying the mechanisms behind a potential effect even more warranted, as the clinical studies available so far have not been suitable for separating glucose dependent effects from glucose independent effects.

## CONCLUSION

Besides the well-established metabolic effects of GLP-1 that in several ways may positively affect redox homeostasis,

glucose independent effect on OS may also play a role and can be investigated by choosing other compounds exerting distinct parts of the GLP-1 activity complex. Better glycemic control is known to lower the risk of developing diabetic complications such as CVD [2]. Decreased food intake caused by diet restriction has been shown repeatedly to affect OS levels in rodents, primarily lowering oxidative damage and increasing GSH, but the effect on antioxidant status varies from tissue to tissue [178]. Moreover, caloric restriction is an effective way to extend lifespan in rodents supporting the role of OS in late onset disease development [179]. However, from the clinical studies available so far, it seems that lowering blood glucose may be the main driver and not body weight reduction in humans.

This review summarizes the effects of GLP-1 on OS status in different treatment or intervention regimes, both in *in vitro*, *in vivo* and in the clinical setting. The less pronounced effects of GLP-1 in clinical studies may possibly be explained by something as simple as the lower doses used, as *in vitro* and *in vivo studies* in general have used doses well above those approved clinically while the differences between "mouse and man" are not to be neglected. There is no clear evidence supporting a single mechanism driving the effects of GLP-1 on redox homeostasis, but it seems that both ROS production and antioxidant capacity can be affected in a positive direction, favoring lower OS status and lower levels of damaged end products. The binding of GLP-1 to the GLP-1R increases cAMP, activates PKA and downstream pathways from this, which can explain the glucose independent pathways of GLP-1 induced improvement of OS status.

Collectively, the available data demonstrates that GLP-1 can decrease OS but also that this effect is dependent on dose, diabetic status, obesity etc. This effect of GLP-1 may have therapeutic potential if OS is in fact causally related to the development of late-onset diabetic complications.

## CONFLICT OF INTEREST

KR and GR are employees at Novo Nordisk A/S. The authors declare no conflicts of interest regarding the present work.

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## Paper II

**The effect of liraglutide on oxidative stress induced by fluctuating hyperglycemia in Sprague Dawley rats.**

Petersen KE, Lykkesfeldt J, Raun K, Rakipovski G.

*Manuscript.*

# The effect of liraglutide on oxidative stress induced by fluctuating hyperglycemia in Sprague Dawley rats

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Short title: Effect of liraglutide on oxidative stress

Abbreviations: Analysis of variance, ANOVA; Cardiovascular disease, CVD; Diabetes Control and Complications Trial, DCCT; Dihydrobiopterin, $BH_2$; Endothelial nitric oxide synthase, eNOS; GLP-1, glucagon-like peptide-1; Glycated hemoglobin percentage, $HbA_{1c}$; High fat diet, HFD; Low density lipoprotein, LDL; Malondialdehyde, MDA; Nitric oxide, NO; Oxidized LDL, oxLDL; Standard error of the mean, SEM; Tetrahydrobiopterin, $BH_4$; United Kingdom Prospective Diabetes study, UKPDS; 8-iso-prostaglandin F2α, 8-iso-$PGF_{2\alpha}$;

**Abstract:**

Fluctuating hyperglycemia is a consequence of poorly controlled diabetes, which has been shown to increase oxidative stress in both humans and animals. In particular, fluctuating hyperglycemia increases oxidative stress in the cardiovascular system. As oxidative stress is implicated in the development of diabetic complications such as atherosclerosis and cardiovascular disease in general, it is interesting to find therapeutic means to lower the oxidative stress status. This study investigated if the glucagon-like peptide-1 analogue, liraglutide could decrease oxidative stress induced by fluctuating hyperglycemia. Male Sprague Dawley rats were randomized in to four groups: Fluctuating glucose liraglutide, fluctuating glucose vehicle, continuous saline liraglutide and continuous saline vehicle. Fluctuations in blood glucose were induced by infusing glucose through permanent catheters in the carotid artery and blood samples were drawn, using an automatic blood sampling system, through permanent catheters in the jugular vein. The rats were kept on high fat diets for 11 weeks prior to the surgery and during the rest of the study period. Rats were euthanized after 21 weeks. Fluctuations in blood glucose and plasma insulin were induced, reaching 12.5±0.4 mmol/l and 1995.0±106.6 pmol/l respectively. Aorta and plasma malondialdehyde and plasma dihydrobiopterin/tetrahydrobiopterin showed no significant differences between the groups, however, glucose fluctuations were not as high as in previous studies. In this study setup it was not possible to induce oxidative stress by fluctuating hyperglycemia and thus it was not possible to show if liraglutide has the potential to lower oxidative stress in this setting.

*Keywords: Fluctuating hyperglycemia, oxidative stress, lipid oxidation, liraglutide, Sprague Dawley rats, cardiovascular disease.*

**Introduction:**

Hyperglycemia is a characteristic of both type 1 and type 2 diabetes and can lead to increased oxidative stress and lipid oxidation (1-3). Patients with poorly controlled diabetes often experience fluctuating hyperglycemia, which constitutes an important source of oxidative stress (4-6). Tissue damage in various diseases and oxidative stress has been connected for a long time, including oxidative stress in diabetes and the tissue damage leading to diabetic complications, such as cardiovascular disease, nephropathy, neuropathy and retinopathy (7-9). Oxidative stress contributes to the development of endothelial dysfunction, which has been implicated as one of the initial events in the development of atherosclerosis (10, 11). The ratio of dihydrobiopterin ($BH_2$) and tetrahydrobiopterin ($BH_4$) ($BH_2/BH_4$) can be used as an indicator of oxidative stress status and endothelial function, as nitric oxide (NO) is formed in an enzymatic reaction involving cofactors such as tetrahydrobiopterin ($BH_4$) (12). Malondialdehyde (MDA) is an end-product of lipid oxidation, which can be used as a measure of the oxidative stress status. This aldehyde can also be involved in formation of atherosclerotic plaques, by forming adducts with low density lipoprotein (LDL) which is then taken up into the endothelium by scavenger-receptors and other receptors of macrophages. This leads to formation of foam cells one of the hallmarks of atherosclerotic development (13, 14).

Poorly controlled diabetes can increase the risk of developing diabetic late complications, and major clinical trials, including the Diabetes Control and Complications Trial (DCCT) and United Kingdom Prospective Diabetes study (UKPDS), have investigated the effects of intensive diabetes management, focusing on reduction of glycated hemoglobin percentage ($HbA_{1c}$) (15-19). Intensive diabetes management, with reduction of $HbA_{1c}$, lowers the risk of developing microvascular complications, but it has not been shown to have the same convincing effect on the macrovascular complications (15-22).

Fluctuations in blood glucose can induce oxidative stress (5, 6), which can lead to the development of diabetic complications, including cardiovascular disease (CVD) (23, 24). Reduction in antioxidant capacity has been suggested to be implicated in the oxidative stress induced by fluctuations in blood glucose (23). Intensive therapy does not rule out the risk of postprandial hyperglycemia and glycemic profiles can fluctuate without affecting $HbA_{1c}$ percentages (24, 25). Accordingly, postprandial hyperglycemia is an important CVD risk factor (26). Thus, combining measures of postprandial blood glucose and oxidative stress may be valuable in the evaluation of

cardiovascular risk. Glucagon-like peptide-1 (GLP-1) analogues such as liraglutide lowers postprandial glucose excursions, improves glucose homeostasis and potentially lowers oxidative stress in a glucose-independent manner (27-29). By these means GLP-1 analogues may protect against diabetes-induced CVD and investigations of this effect is warranted.

The present study, therefore, evaluated if liraglutide could have glucose-independent lowering effects on oxidative stress induced in our rat model of fluctuating hyperglycemia (30).

**Materials and methods:**

Animals and diet

The study was carried out at Novo Nordisk A/S, Måløv, Denmark and all procedures were performed in accordance with The Danish Animal Experimentation Act (LBK no 1306 of 23/11/2007). The animal procedures were approved by the Animal Experiments Inspectorate, Ministry of Food, Agriculture and Fisheries, Denmark. Under permission number: 2012-15-293400069.

6-10 weeks old male rats (NTac:SD) were acclimatized for period of at least one week, and then fed a HFD (D12451, 45 kcal% fat, Research Diets, Inc., New Jersey, USA) with free access to tap water until the end of the experiment (a total of 21 weeks). Rats were housed with a temperature of 22 ± 2 °C, humidity of 50± 20 % and a 12h/12h light/dark cycle (lights on at 6AM). The rats were housed two rats per cage. From two to three weeks prior to surgery and until the end of the study the rats were housed individually. Two days after this separation and for the rest of the experiment, the daily food intake (manual weighing) and body weight of the rats were registered. The mean caloric intake in the rats was calculated as the kcal each rat received normalized to body weight, when combining the infused glucose and the food intake of the rat. However, body weight was not registered during the actual infusion studies, as the system does not allow for a precise body weight registration.

The rats were randomized into four groups of either fluctuating glucose vehicle or liraglutide (FGV and FGL) and continuously saline vehicle or liraglutide (CSV and CSL) (figure 1, where the final n-values are given).

Surgical procedures

A system for automatic blood sampling, the Accusampler (VeruTech AB/DiLab, Lund, Sweden) was used in the experiment together with automatic infusion pumps (World Precision Instruments Inc. Sarasota, Florida, USA) to infuse glucose in a fluctuating pattern. To use these systems permanent catheters were implanted in the carotide artery and jugular vein in 24 rats. 12 automatic infusion pumps were available for the study and thus two rounds of infusions were planned, with 12 rats in each round. Thus, two rounds of surgery were performed over two rounds, both lasting three days.

After 11 weeks on HFD 27 rats (three rats in surplus) underwent surgery under microscope. 30 minutes prior to surgery, the rats received sc injections of 10 mg/kg enrofloxacin (Baytril®, Bayer AG, Leverkusen, Germany. 2 ml/kg) and 10-20 minutes prior to surgery the rats received sc injections of 0.05 mg/kg buprenorphin (Temgesic®, Reckitt Benckiser, Berkshire, England. 1.7 ml/kg). Anaesthesia was induced by placing the rat in an induction chamber with a flow rate of 0.3 l $O_2$ and 0.7 l $N_2O$ per minute with 4 % isoflurane (Baxter International Inc, Deerfield, Illinois, USA). After the induction, anaesthesia was maintained using a nose cone and a flow rate of 1 l $O_2$ per minute and 1.5-2.0 % isoflurane. Their eyes were moisturized with ophthalmic ointment just after induction of anaesthesia.

A 1-1.5 cm incision was made, the left carotid artery and the right jugular vein were found, and permanent catheters were placed in these. The catheters (Tygon Microbore Tubing, Best.nr.S-50-HL, Cole Parmer, Buch & Holm, Herlev, Denmark) had an inner diameter of 1.22 Fr., an outer diameter of 2.36 Fr. and the ends were cut bluntly and were filled with 100IU/ml heparin in 0.9 % NaCl. The carotid artery catheter had a total length of 16cm. It was placed approximately 5 cm in the left carotid artery and a piece of vlieseline was attached to the catheter with glue 6 cm from the tip of the catheter. The jugular vein catheter had a total length of 13 cm and was placed 2-3 cm into the right jugular vein. A piece of vlieseline was attached to the catheter with glue 4 cm from the tip of the catheter.

After completion of surgery the catheters were filled with 500IU/ml heparin (LEO Pharma Nordic, Ballerup, Denmark) in Haemaccel® (Piramal Healthcare UK Limited, Northumberland, England) and closed with a stopper made of fishline with a 0.45mm diameter. The catheters were made accessible at the neck of the rats via a midscapular incision (approximately 1 cm). The catheters

were led using a 14G cannula and led through the skin next to the wound using an 18G needle, having the catheters accessible through the skin and not through the wound. Before closing the wound in the neck a sc injection of 5 mg/kg carprofen (Rimadyl®, Pfizer, New York, USA. 1 ml/kg) were given to the rats. Subsequently the rats were hydrated with a sc injection of 10 ml/kg isotonic saline (Fresenius Kabi, Oslo, Norge) at two to three different sc sites. The wounds were closed with intradermal suturing using absorbable suture material (Vicryl® plus antibacterial 4/0, Ethicon).

During the surgery sterile techniques were used. Catheters and fishline were sterilized in ethylenoxid, instruments, suture and cotton swabs were autoclaved at 134˚C, the table was cleaned in DEC-CLEAN® (PMT Particle Measuring Technique Limited, Malvern Malvern Worcestershire, England) and covered in sterile drapping, the rat was shaved, the rat was wrapped in sterile draping, and the skin was rinsed in 70% ethanol, and surgical hand wash was performed before surgery and sterile gloves were used under the surgery. These were changed if contaminated.

Postoperative care

After surgery the rats were carefully observed. On the day of surgery, the rats were provided with wet chow (Altromin #1324. Altromin Spezialfutter GmbH & Co. KG, Germany) that had been soaked in water for 30-60 minutes and 15 grams of dried fruit (Fruity Gems, BioServ, Fleminton, New Jersey, USA). From postoperative day two only HFD was provided. The rats were injected sc with 0.025 mg/kg (0.85 ml/kg) buprenorphine on the first postoperative day together with 10 mg/kg enrofloxacin and 5 mg/kg carprofen. The two latter were also administrated on the second postoperative day. The rats had 8-9 days of recovery before they were placed in the room where the infusions and blood sampling by the Accusampler were performed. For the entire study period, the catheters were opened once a week by drawing the Haemaccel and heparin mixture from the catheters and flushing with saline before filling the catheters with Haemaccel and heparin mixture again. The rats that did not obtain pre-surgery body weight were excluded from the infusion study. 20 % body weight-loss was set a human endpoint and thus, an exclusion criterion. The rats were observed daily.

Automatic blood sampling and infusions

Three days prior to study start the rats were acclimated in the Accusampler cage (figure 2). Body weight of the rats was registered and the Haemaccel was drawn from the catheters before they were flushed with saline with 100u heparin per ml. The jugular catheters were connected to the infusion

pump and the carotid catheter to the Accusampler, and the rat was able to move around freely in the cage with catheters connected to the infusion pump and the Accusampler. Until the beginning of the infusions the Accusamplers were set to flush the catheters with 50 µl saline with 50u heparin per ml three times per hour to avoid clotting of the catheter.

*Glucose infusion*

The glucose infusion lasted for 96 hours. Five hours before study start, the rats were fasted, but had access to water. Two minutes prior to initiation of infusions a fasted blood sample was taken. At t=0 the infusions started with either glucose solution (50 % glucose, Fresenius Kabi, Oslo, Norge) or saline (Fresenius Kabi, Oslo, Norge). After the first infusion of the first infusion round and measures of glucose concentration the rates were adjusted if the glucose increase were not as high as in expected from the previous study (30). The good practice guide for infusions by Diehl *et al* from 2001 was taken into account for these adjustments (31). The final infusion rates and volumes are found in table 2 and 3. The infusion was performed using infusion pumps and software developed in house (InsulinExtravaganza, version 1.01). This software was developed in Labview (National Instruments™, USA). During the experiment, the rats had free access to bottled tap water and HFD.

*Blood sampling*

Blood for determination of glucose and insulin was sampled from the carotid artery catheter by the Accusampler. Blood samples were taken at -10, +2, +32 and +60 minutes in relation to the time points given for infusion rates given in table 2 and in connection to 8 of the 36 induced glucose spikes (the exact spikes are given in table 4). 50 µl of blood were taken and stabilized with tri-potassium ethylenediaminetetra-acetic acid (K3-EDTA). 5 µl of whole blood were taken in a capillary tube for glucose analysis. The rest of the blood was centrifuged (5 min, 4ºC, 8000 rpm). 30 µl plasma were pipetted into Micronic tubes (Micronic, Lelystad, Holland) and placed on dry ice before storage at minus 80°C

At the beginning of each of the two infusion rounds and at the end of these the following blood samples were taken. 100 µl of blood were collected for MDA analysis. The blood samples were stabilized with K3-EDTA solution followed by centrifugation (5 min, 4ºC, 8000 rpm). 50 µl of plasma were transferred to Micronic tubes, placed on dry ice and kept at minus 80°C until analysis. 400 µl of blood were collected for dihydrobiopterin/tetrahydrobiopterin ($BH_2$/$BH_4$) analysis. The

blood was stabilized immediately in 10 µl of a dithioerythriol (DTE) solution as described in (32). The DTE solution was 40 mg DTE dissolved in 1000 µl MQ-water (4 %) and was made fresh daily. The blood was centrifuged (1 min, 4°C, 15000 rpm) and 200 µl of plasma were transferred to Micronic tubes, placed on dry ice and kept at minus 80°C until analysis.

The haematocrit was measured before and after the infusion rounds to evaluate hemodilution, which was used to normalize the level of plasma MDA and $BH_2/BH_4$.

Liraglutide intervention

The liraglutide intervention was placed in between the two infusion rounds. The treatment was started at the first morning after the rats were taken out of the Accusampler systems. 100 µg/kg were dosed sc to the rats twice a day for 30 days. 31 days after the first infusion round the second infusion round began. The liraglutide treatment was located in-between the two infusion rounds to try to avoid direct glucose-lowering effects of liraglutide on oxidative stress in the second infusion round.

*Blood sampling*

Blood sampling during the period of liraglutide intervention was performed by sublingual vein puncture once a week. Blood was drawn to measure blood glucose, plasma insulin, plasma MDA and BH2/BH4. The volumes, stabilization of plasma and handling were done as described in Blood sampling under Automatic blood sampling and infusions.

Study termination

After the last infusion round the rats were anaesthetized in a mixture of 0.05/1.5 ug/g fentanyl/fluanison (Hypnorm, VetaPharma Limited, Leeds, United Kingdom) and 0.75 µg/g midazolam (Accord Healthcare, Copenhagen, Denmark). A midline incision was made and the thorax was opened. Cardiac perfusion with cooled isotonic saline (Fresenius Kabi, Oslo, Norge) was performed and organs were harvested and prepared for further examination (described in the next section).

The aorta was removed from the rats in a section from the base of the heart to the level of the seventh rib. The aorta was kept cold while removing fat from the surface, before it was snap frozen using liquid nitrogen, and stored at minus 80°C before further analysis.

Biochemical analyses

*Blood glucose*

For blood glucose determination 5 µl of whole blood were diluted in 250 µl glucose/lactate system solution (EKF Diagnostics, Barleben, Germany) and analyzed in the Biosen S line glucose analyzer (EKF Diagnostics, Barleben, Germany).

*Plasma insulin*

Plasma for insulin determination (5 µl) was analyzed by Luminescence Oxygen Channeling Immunoassay (LOCI) (33, 34) using a sandwich immunoassay. This method has shown to be superior to the traditional ELISA, e.g. in regard to precision and volume needed (34). Lower limit of quantification (LLOQ) was 20 pmol/l and upper limit of quantification (ULOQ) was 3000 pmol/l.

*Plasma and aorta MDA*

MDA concentration was analyzed in 10 µL of plasma or aorta homogenat (aorta was homogenized in 500 µl of lysis buffer (RTL, Qiagen GmbH, Hilden, Germany)) by the high-performance liquid chromatography (HPLC) method, which has been described previously (35).

*Plasma $BH_2/BH_4$*

The DTE-stabilized plasma was used for analysis of $BH_2/BH_4$ and it was performed based on the principle described in (36). This method uses HPLC with fluorescence detection using iodine oxidation.

Statistical analyses

Statistical analyses were performed in SAS Enterprise Guide 4.3 (SAS, Cary, North Carolina, USA) and GraphPad Prism 6 (GraphPad Software Inc. La Jolla, California, USA) was used for graphical presentation. All data are presented as means ± standard error of the mean (SEM) and p-values under 0.05 were considered significant. Groups were compared by repeated measurements analysis of variance (ANOVA) using Tukey's posthoc comparisons for body weight, caloric intake, plasma MDA and $BH_2/BH_4$ concentrations. Comparison of glucose and insulin concentrations in the two infusion rounds was done by two-way and three-way ANOVA with Tukey's posthoc comparisons. The two-way ANOVA included infusion type and liraglutide intervention as parameters when comparing peak glucose or insulin concentration. While three-way ANOVA included infusion type,

liraglutide intervention and infusion round when comparing basal to peak differences of glucose or insulin concentration. Aorta MDA was compared between the groups using one-way ANOVA with Tukey's posthoc comparisons.

**Results:**

This study was performed to investigate if liraglutide could lower the oxidative stress induced by fluctuating hyperglycemia, which had been shown to increase in the same model previously (30). This rat model based on automatic infusions of glucose and automatic blood samples to allow multiple samples without handling the animals. The potential of liraglutide to lower oxidative stress, independently on the effects glucose homeostasis, could indicate a potential for decreasing the risk of developing diabetic complications, a development that is associated with oxidative stress. The fluctuations in this model did not lead to increases in oxidative stress. As presented below, the blood glucose concentration in the fluctuating groups did not reach the concentrations observed in the previous study by Rakipovski *et al* (2016).

*Model establishment and refinement*

By rehearsing the surgery prior to the study, the surgical technique was optimized bringing down surgery time pr. rat from an average of 60 minutes to 30 minutes. For the experiment only one rat was euthanized during surgery due complications and loss of blood (table 1).

The Accusampler system relies on high patency of the catheters. Over time the catheters in the experiment lost patency, resulting in rats being taken out of the experiment. Four rats were taken out of the experiment in the first infusion round due to technical problems; the catheters were damaged by the devices used for connecting the catheters to the Accusampler system. All rats taken out of the experiment were anaesthetized as described above using buprenorphine and isoflurane before they were injected with pentobarbital intracardially. This brought the total number of rats at the end of the experiment down to 12 rats with 2 to 4 in each group (figure 1 + table 1). The results will only be given for the rats that completed the entire experiment.

*Body weight and caloric intake*

No significant differences in body weight were found between the groups at any time point. (figure 3). The mean caloric intake was different between the groups at different time points (figure 4). At day 1, the caloric intake was significantly lower in the rats in the CSL group compared to rats in the

FGL group ($p<0.05$). At day 9, the CSL group had significantly lower caloric intake than the FGV group ($p<0.05$). At day 27, the FGV group had significantly lower food intake than the three other groups and day 28 the FGV group had significantly lower food intake when compared to the CSV group ($p<0.05$ for both).

*Blood glucose*

In the continuously saline infused groups, the blood glucose was constant at 5.3±0.1 mmol/l. This was significantly higher than in the fluctuating groups, where the baseline blood glucose was 4.9±0.1 mmol/l ($p<0.01$). In the fluctuating groups blood glucose reached 12.5±0.4 mmol/l during infusions and was 6.2±0.2 mmol/l 30 minutes after infusions of glucose was stopped.

In order to compare the two rounds, the average of the peak value at 32 minutes after infusions were started was used, creating one value for each rat. The same was done for the baseline measure 10 minutes before infusions were started. The two-way ANOVA investigating the effect of liraglutide treatment and infusion type on peak glucose from round one to two showed no effect of either liraglutide treatment or infusion type from round one to two. A three-way ANOVA was performed as well where the effect of liraglutide treatment, infusion type and round on baseline to peak concentration was investigated. The result of this analysis showed that the baseline to peak concentration of glucose was significantly increased in the fluctuating glucose groups ($p<0.001$), but not by treatment or round number. As liraglutide did not affect the blood glucose concentration the data are shown with the two fluctuating glucose groups and the two continuously saline infused groups combined in figure 5.

*Plasma insulin*

The plasma insulin concentration was 348.4±17.4 pmol/l in the group continuously infused with saline. This was significantly lower than in the fluctuating groups, where the baseline insulin concentration (between infusions) was 467.1±26.6 pmol/l ($p<0.001$). In the fluctuating groups plasma insulin reached 1995.0±106.6 pmol/l before concentrations falling to 647±38.2 pmol/l at 30 minutes after infusions of glucose were stopped.

The two-way ANOVA investigating the effect of liraglutide treatment and infusion type on peak insulin from round one to two showed an effect of infusion type. As for the difference in peak

glucose concentration, the liraglutide intervention did not affect the difference in insulin observed between the two rounds. However, the infusion type did affect the difference in peak insulin concentration ($p<0.05$). No interaction between treatment and infusion type was observed. A three-way ANOVA was performed as well where the effect of liraglutide treatment, infusion type and infusion round on baseline to peak concentration was investigated. Baseline to peak of plasma insulin concentration was significantly increased in the fluctuating glucose groups ($p<0.001$) and round ($p<0.05$) but not by liraglutide treatment. As liraglutide did not affect the plasma insulin concentration the data are shown with the two fluctuating glucose groups and the two continuously saline infused groups combined in figure 6. The significant effect of round shown in the two-way ANOVA together with figure 6 show that insulin concentration was lower in the second infusion round.

*Plasma MDA*

Plasma MDA did not differ between infusion rounds and liraglutide treatment, infusion type and time did not affect plasma MDA. Time spent in the Accusampler systems getting either continuous saline infusions or fluctuating glucose infusion seemed to increase plasma MDA an increase that seemed to decrease after the rats were taking out of the Accusamplers. The increase appeared most pronounced in round two.

*Aorta MDA*

The MDA content in aortas of the rats did not differ significantly between the groups (figure 8). A non-significant tendency towards lower MDA concentrations was observed in the two groups receiving continuously saline compared to the groups receiving fluctuating glucose.

*Plasma $BH_2/BH_4$*

The plasma $BH_2/BH_4$ ratio during the first infusion round, throughout the liraglutide intervention and in the second infusion round did not differ (figure 9). Thus, liraglutide treatment, infusion type and time did not affect plasma $BH_2/BH_4$ ratio. The periods the rats spent in the Accusampler systems did not affect the plasma $BH_2/BH_4$ ratio. However, concentrations at the first measure (day 14) after the first infusion round the groups, especially, CSL, CSV and FGL seemed to increase. The concentration did seem to decrease again before the next infusion round.

**Discussion:**

We hypothesized that liraglutide reduces oxidative stress induced by fluctuating glucose infusions. However, the expected increase in oxidative stress was not induced in the range of blood glucose reached in this experiment, which limits conclusions relating to the effect of liraglutide on oxidative stress induced by fluctuating hyperglycemia.

The glucose and insulin fluctuated in the fluctuating glucose groups, but the glucose concentrations were not elevated to the expected concentration of 20-22 mmol/l, which was seen in our previous study, despite an increase of the infusion rates after the first infusions of infusion round one. The present study aimed at reaching the same blood glucose concentrations as in the previous study during the fluctuating glucose infusions to induce oxidative stress. As this could be a prerequisite for the increase in oxidative stress shown in this model previously as a result of fluctuating hyperglycemia in both lean and DIO rats (30, 37). The lower blood glucose concentrations in the present study could explain the lack of effect on oxidative stress markers. As large infusion volumes can result in distress, the infusion rates were not increased additionally (31). In addition, the MDA concentrations were lower than in the previous study, even though the same experimental setup and technique for measuring MDA were used. In the present study, the highest values of plasma MDA were approximately 3 times lower and the highest values of aorta MDA were approximately five times lower than in the previous study (30). Comparing these values directly between different studies is of course not possible, but the difference is remarkable. It, therefore, seems as if a certain rise in blood glucose is needed to induce oxidative stress, measured by MDA and $BH_2/BH_4$. The glucose tolerance of the rats should be studied before starting the experiments, making sure that the potential window for increasing blood glucose is high enough with the intended infusion protocol.

A non-significant drop in body weight and food intake was observed in all rats after they were taking out of the Accusamplers. The drop in body weight and food intake did however look most pronounced in the two liraglutide treated groups. Thus, indicating that the liraglutide intervention had the expected effect on body weight and food intake (38, 39). All groups appeared to continue reducing their food intake the first days out of the Accusamplers, which could mask some of the well described effect of liraglutide on food intake (38, 39). The decline in body weight and food intake could be attributed to the stress the change of environment potentially introduces, as the rats were moved from the Accusampler rooms to the rooms normally used for housing the rats. As the rats were not weighed in the Accusamplers it is difficult to evaluate the direct effect of moving the

rats from the normal stables to the Accusampler rooms. The measure of food intake is sensitive to measuring errors, as the data are collected by weighing the food bowl. Thus, the differences observed may not be attributed huge importance due to the limitation in measurements of food intake.

The two-way ANOVA investigating the effect of liraglutide treatment and infusion type on peak glucose from round one to two showed no effect of either liraglutide treatment or infusion type between the two rounds. The observation that liraglutide did not affect the glucose peak between the two rounds is as hypothesized, as the liraglutide intervention was performed in between infusion rounds, to minimize the direct effects of liraglutide on glucose homeostasis. The result of the three-way ANOVA was that glucose infusion has an effect on the increase from baseline to peak glucose concentration, as expected. The two-way ANOVA investigating the effect of liraglutide treatment and infusion type on peak insulin from round one to two showed an effect of infusion type. As for difference in peak glucose concentration, liraglutide intervention did not affect the difference in insulin observed between the two rounds. Thus, a lower insulin concentration in round two seems to be explained by the infusion type and not liraglutide treatment. The conclusion from three-way ANOVA was that glucose infusion has an effect on the increase from baseline to peak insulin concentration, as expected. The statistical differences between the infusion rounds in insulin peak concentrations could be explained by some kind of adaptation effect to the glucose infusions resulting in different response to the same glucose infusion. Adaptation to glucose infusion has previously been observed in rats (40-42). Where a decrease in plasma insulin during infusions has been observed, which could be caused by an increase in insulin sensitivity (40, 42). The blood samples were drawn in a pattern to cover the circadian rhythm of the rats. This was done by always measuring the first pulsatile glucose infusion of the day and then every other spike after this. Especially in the first infusion round the glucose concentrations seemed to be lower in the two samples taken in the dark period. This could be explained by the stability of blood glucose values, as studies have demonstrated that hepatic expression of key glycolytic enzyme peaks at the onset of circadian night (43). Thus, these samples drawn at night time could be affected by these changes when compared to the samples drawn in the light phases.

Fluctuating blood glucose and poorly controlled diabetes resulting in fluctuating blood glucose causes oxidative stress. In type 2 diabetes patients and in healthy subjects plasma 8-iso-

prostaglandin F2α (8-iso-$PGF_{2\alpha}$) isoprostanes have shown to increase in response to acute hyperglycemia (44, 45). *In vitro* studies show that varying between high and low glucose concentrations in the media can increase oxidative stress, shown by increased nitrotyrosine and 8-hydroxydeoxyguanosine in human umbilical vein endothelial cells (46). *In vivo* and clinical studies have shown that blood glucose excursions can increase oxidative stress, even when the overall glycemic exposure is lower than sustained high blood glucose concentrations (30, 37, 47). In lean rats plasma MDA was significantly higher in animals exposed to fluctuations for 48 hours, with blood glucose reaching 18-20 mmol/l during the peaks, from a basal concentration of 5-6 mmol/l, when compared to rats exposed to sustained hyperglycemia of 22 mmol/l. After 72 hours the plasma MDA concentrations were the same in the groups exposed to fluctuations and sustained high blood glucose concentrations (37). In rats fed a high fat diet (HFD) fluctuating hyperglycemia (blood glucose reaching 20-22 mmol/l) for 96 hours increased aorta MDA and aorta oxidized LDL (oxLDL) while sustained increased in blood glucose (27 mmol/l) increased the liver MDA concentrations (30). Nitrotyrosine has shown to increase together with an observed endothelial dysfunction when either healthy subjects or type 2 diabetes patients were exposed to sustained hyperglycemia of either 10 or 15 mmol/l in blood glucose for 48 hours when compared to baseline. Endothelial dysfunction, which has strong association with obesity and insulin resistance, is an important feature of type 2 diabetes and cardiovascular disease, and oxidative stress is thought to be the shared link (48, 49). When comparing sustained hyperglycemia to oscillating hyperglycemia (from 5 mmol/l to 15 mmol/l two times doing the 48 hours) in both healthy and type 2 diabetes patients, the oscillating groups had higher nitrotyrosine concentration and more pronounced endothelial dysfunction at the spikes of the fluctuations, and a more pronounced difference were observed between the 10 mmol/l groups and the groups subjected to oscillations in blood glucose. In addition the oscillating groups had increased urinary 8-iso-$PGF_{2\alpha}$ (47). The model did not perform as expected in the present study, but it does not undermine the potential of the model, as two previous studies have been performed showing that fluctuations on blood glucose increased oxidative stress status in both lean and DIO rats (30, 37). In the current study the lack of reproducibility may be caused by multiple factors even though the setup was as similar as possible to the previous study, small differences were inevitable. But the study was made as similar to the previous as possible when introducing the liraglutide intervention. E.g. the rats were 29-32 weeks at the end of the experiment, which correspond to the age of the rats in the previous experiment by Rakipovski *et al*. Here the rats were 27-31 weeks (30). The first round of infusion was however performed at an age of 22-25 after 13-14 weeks on HFD in

contrast to 20 weeks in the previous study. These differences could explain some of the differences between the two studies, but we find it unlikely to be the entire explanation.

Reproducibility of experiments is often debated and lack of reproducibility is a major concern in preclinical studies (50). Changes in the animals could hold an explanation. Genetic drift can occur, which causes differences within the same strain or stock of rats (51, 52). By personal communication with Taconic Denmark, after the results from the present study were obtained, information was acquired about changes in the colony of SD rats. In 2012, the American SD strain used by Taconic in USA was phased in at the Danish Taconic. The change in population was made after the previous study by Rakipovski *et al* (2016) and before the present study.

The study had a high technical demand of the model and the reliance on the patency of catheters over long periods of time, here seven weeks, can be challenging. Acute effect of surgery seemingly not the problem as 96 % survived surgery. A potential reason for loss of patency of catheters can be fibrin sleeves forming around the tip of the catheters. These sleeves can form despite anticoagulant administration (53). Fibrin sleeves can develop into organized cellular connective tissue; a process that can decrease patency. Heparin-coating of catheters have shown to reduce the development of these more advanced lesions (54). The choice of catheter in this experiment was however made from the success from previous studies with these catheters (30, 37) and that the Tygon catheters are made of polyvinyl chloride (PVC), which is more flexible. Flexibility has shown to increase catheter patency and compared to the heparin coated polyutherane catheters, using the PVC catheters has shown a lower development of catheters that were hard to flush in a short, seven day study (53, 55). From the results of the present study, the longer time demand for catheter patency, when compared to the previous study by Rakipovski *et al*, did not seem to be the main problem. In both infusion rounds animals were taken out of the study due to loss of catheter patency. Thus, the problem was apparent after only the recovery period from surgery. This indicates a problem with the surgical technique rather than the long period demanded for patency. It is well known that the placement of the catheter tip is important for patency (53) and here genetic differences or small size differences could induce small anatomical differences. Thorough investigation of this issue could be done in advance of future studies. Other catheter materials could be used in future studies, using e.g. silastic catheters which have shown good results in regards to patency. However, these were flexible, which can complicate the surgical procedure (53).

The greatest limitation of the current study is the sample size. From the data obtained in the current study the sample size should have been 57 in the fluctuating glucose vehicle and liraglutide group respectively, to show a significant difference between the groups on regards to plasma MDA concentration. Thus a very high number of animals should have been used to show a significant difference between these two groups. The difference is thus small and setting up a new study might not be relevant due to the high number of animals indicated to be needed from this calculation. Taking the drop out of animals, the number would be even higher. To show a significant difference between the groups with these small sample sizes the difference should have been at least 0.2 μM. This is within a physiologically possible spectrum for plasma MDA (30). These calculations were made using the last measure of plasma MDA using a power level of 0.80 were used.

**Conclusion:**

The primary objective of this study was to study the effect of liraglutide on the oxidative stress induced by fluctuating hyperglycemia in rats. As fluctuating hyperglycemia did not induce oxidative stress in the rats, the potential of liraglutide to reduce oxidative stress could not be answered, but is still highly interesting. Especially with a focus on reducing CVD risk in diabetic patients.

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**Conflicts of interest:**

KR and GR are employees of Novo Nordisk A/S that produces insulin and the GLP-1 analogue, liraglutide. The authors declare no conflicts of interest that could influence the present work.

**Statement of author contributions:**

The study was designed by all authors and the experiments were carried out by KEP. Initially the data analysis was performed by KEP followed by data interpretation by all authors. The draft manuscript was written by KEP and subsequently edited by JL.

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**Tables and figures:**

**Table 1. Overview of rats undergoing surgery and the two infusion rounds.**

| Surgery time | Number of rats undergoing surgery | Number of rats after surgery | Number of rats completing 1st Accusampler round | Number of rats completing 2nd Accusampler round |
|---|---|---|---|---|
| Approximately 30 minutes | 27 | 26 (96 %) | 16 (59 %) | 12 (44 %) |

**Table 2. Infusion rates (ml/kg/h).**

| Group: | 0-2 min | 2-32 min | 32-152 min |
|---|---|---|---|
| Fluctuating glucose | 30.0 | 5.0 | 0 |
| Continuously saline | 1.2 | 1.2 | 1.2 |

**Table 3. Infusion volumes (ml/kg/day) and dose of glucose infused (g/kg/day).**

| Group: | Infusion volumes | Glucose |
|---|---|---|
| Fluctuating glucose | 31.5 | 16.4 |
| Continuously saline | 31.2 | 0 |

**Table 4. Blood sampling in connection to nine daily glucose spikes during the 96 hours of the infusion study.**

| Spike: | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Hours | | | | | | | | | |
| 0-24 | X | | X | | | | | | |
| 24-48 | X | | | | X | | | | |
| 48-72 | X | | | | | | X | | |
| 72-96 | X | | | | | | | | X |

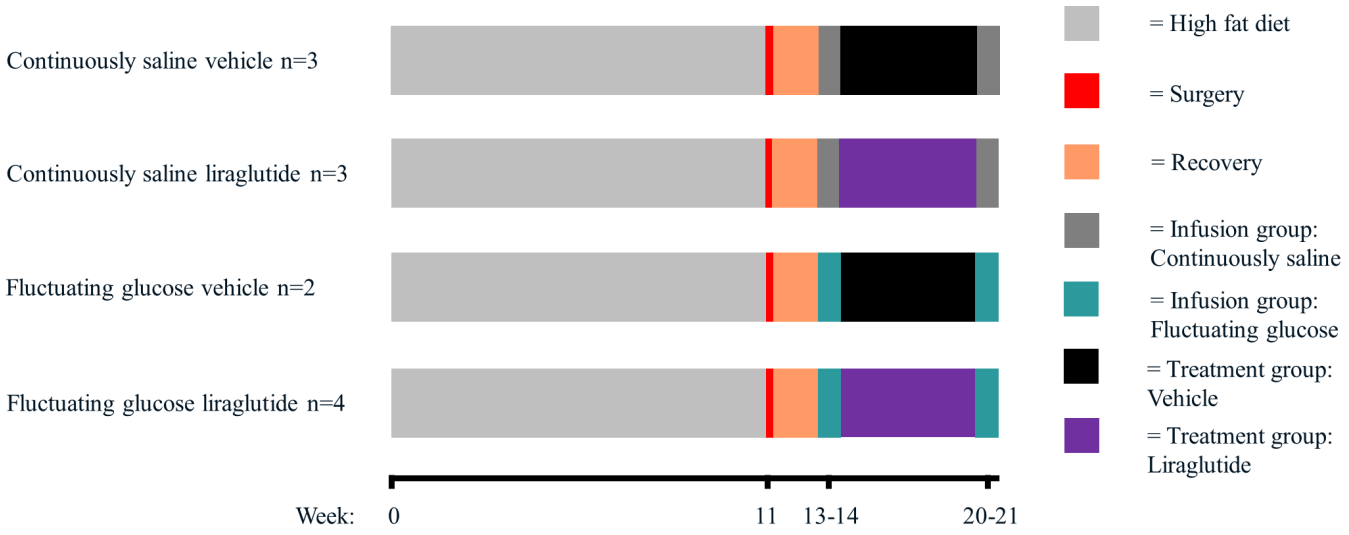


**Figure 1. The four groups with illustration of interventions.**

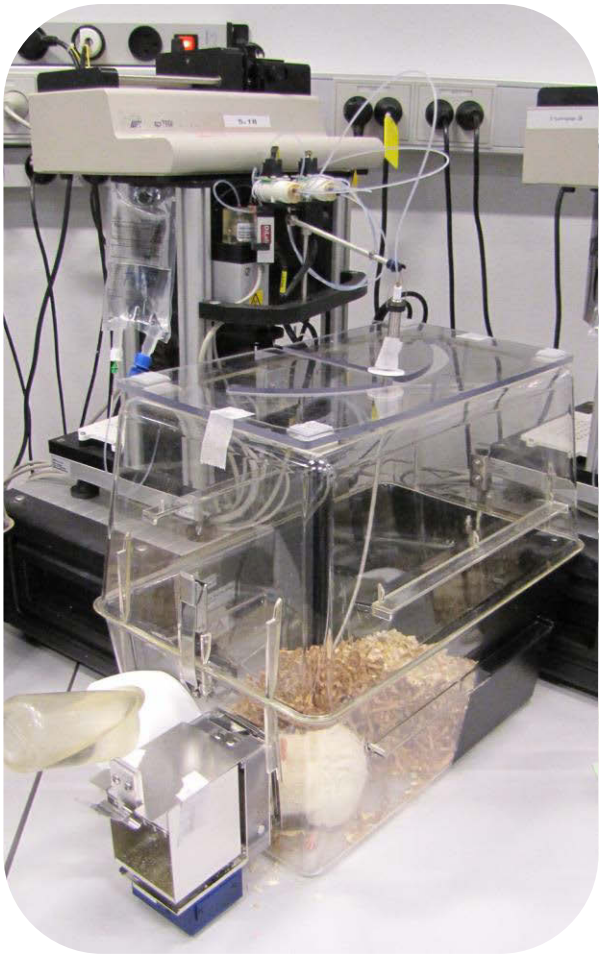

**Figure 2 Experimental setting for infusion and blood sampling in the Accusampler.** This was not the permanent housing of the rats, but an experimental setup.

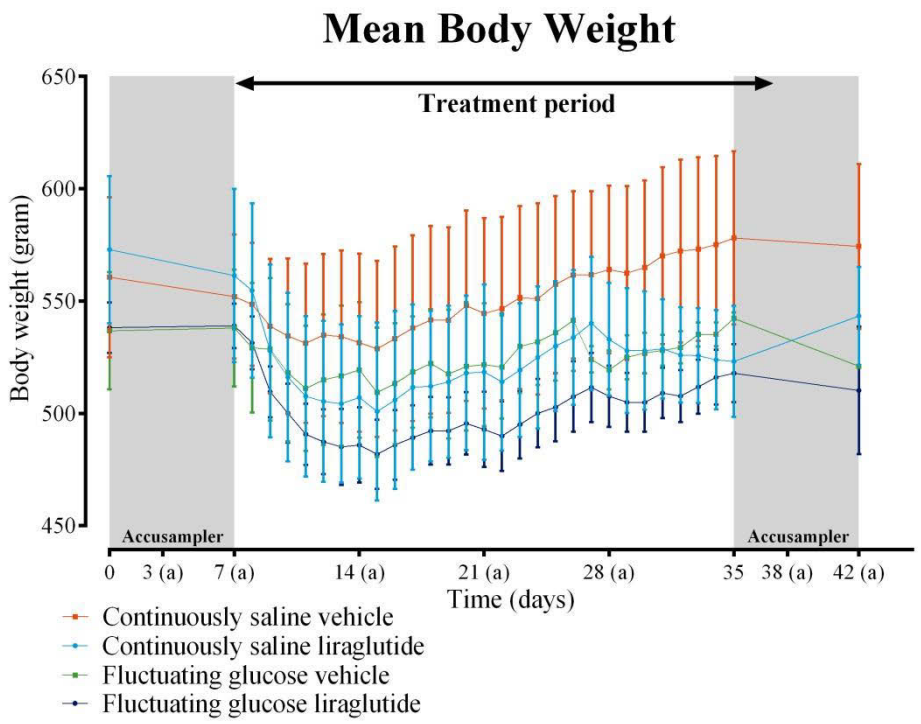


**Figure 3. Mean body weight during the two infusion rounds and the liraglutide intervention of 30 days in between.** a = times points blood sampling for malondialdehyde (MDA) and dihydrobiopterin/tetrahydrobiopterin ($BH_2/BH_4$). Grey coloured area indicates the time the rats were in the Accusampler system. Data are presented as mean ± SEM. (n-values: Continuously saline vehicle=3, continuously saline liraglutide=3, fluctuating glucose vehicle=2 and fluctuating glucose liraglutide=4).

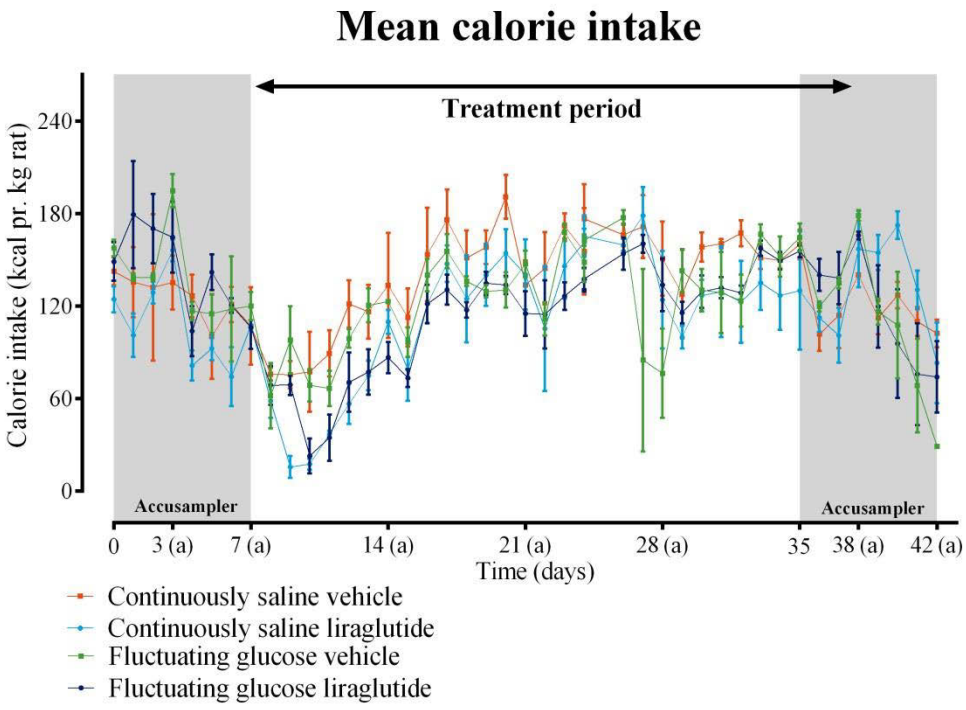

**Figure 4. Mean calorie intake during the two infusion rounds and the liraglutide intervention of 30 days in between.** a = times points blood sampling for MDA and $BH_2/BH_4$. Grey coloured area indicates the time the rats were in the Accusampler system. Data are presented as mean ± SEM. (n-values: Continuously saline vehicle=3, continuously saline liraglutide=3, fluctuating glucose vehicle=2 and fluctuating glucose liraglutide=4).

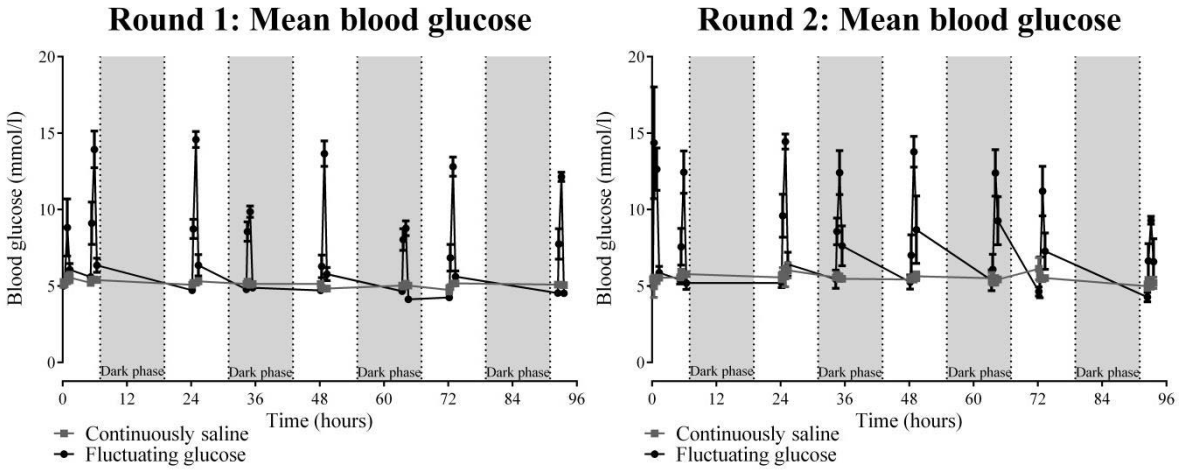


**Figure 5. Mean blood glucose during the 96 hours of accusampling and infusions, the 1st round is shown to the left and the 2nd round is shown to the right.** Grey coloured areas indicate dark phases in the experimental room. Data are presented as mean ± SEM. (n-values: Continuously saline vehicle=3, continuously saline=6 and fluctuating glucose=6).

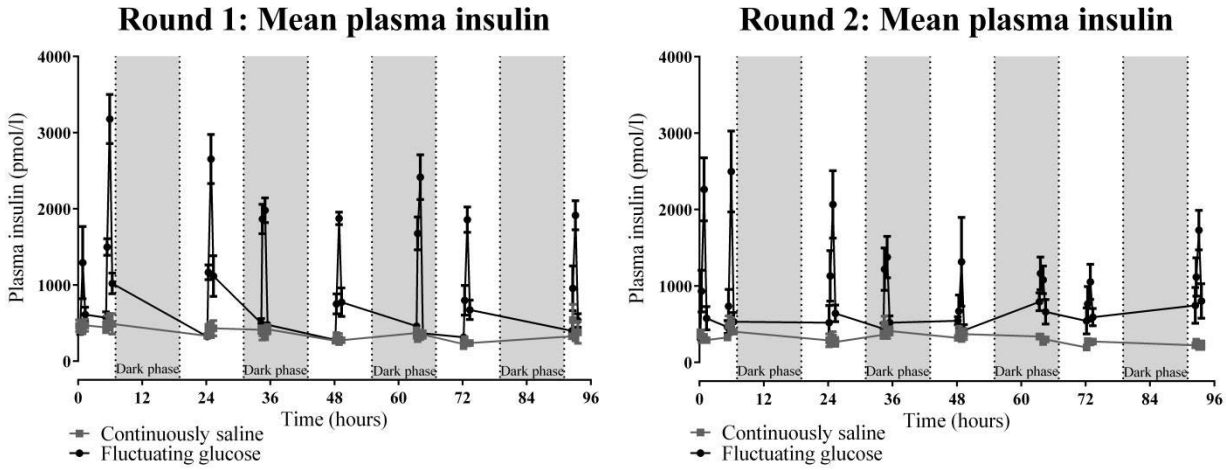


**Figure 6. Mean plasma insulin during the 96 hours of accusampling and infusions, the 1st round is shown to the left and the 2nd round is shown to the right.** Grey coloured areas indicate dark phases in the experimental room. Data are presented as mean ± SEM. (n-values: Continuously saline vehicle=3, continuously saline=6 and fluctuating glucose=6).

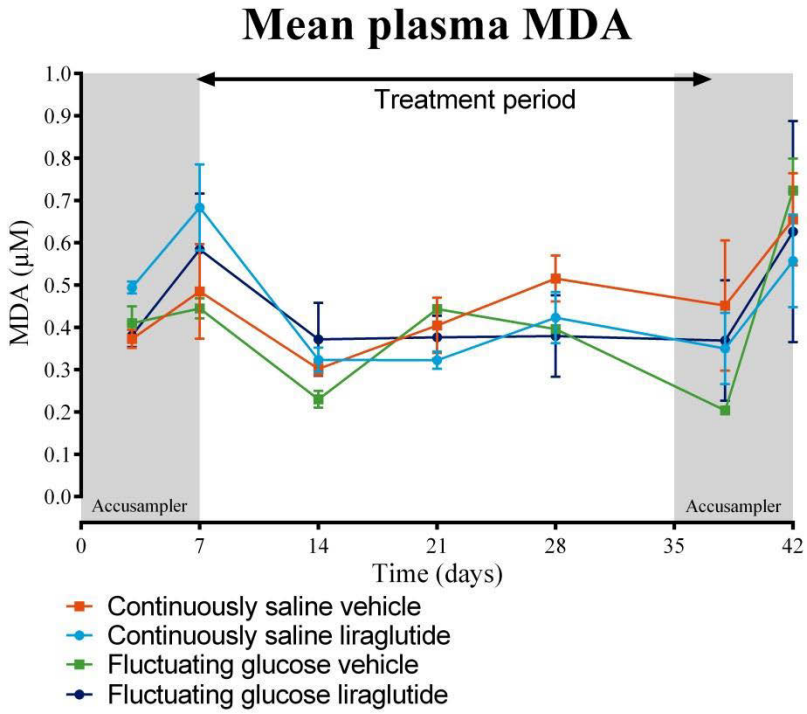


**Figure 7. Mean plasma malondialdehyde (MDA) during the two infusion rounds and the liraglutide intervention of 30 days in between.** Plasma MDA was measured at the beginning and the end of the infusion rounds and every week during the liraglutide intervention. Grey coloured area indicates the time the rats were in the Accusampler system. Data are presented as mean ± SEM. (n-values: Continuously saline vehicle=3, continuously saline liraglutide=3, fluctuating glucose vehicle=2 and fluctuating glucose liraglutide=4).

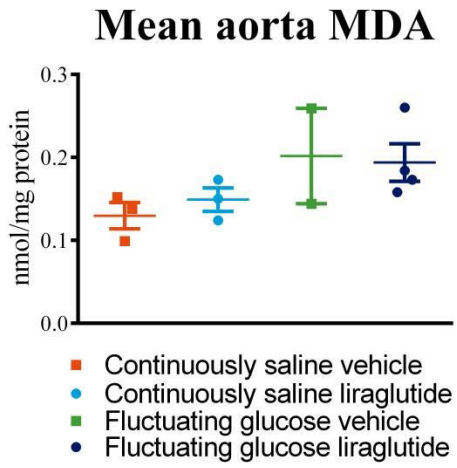


**Figure 8. Mean aorta malondialdehyde (MDA).** Aorta MDA was calculated as MDA in relation to total protein in the aorta homogenates. Data are presented as mean ± SEM. (n-values: Continuously saline vehicle=3, continuously saline liraglutide=3, fluctuating glucose vehicle=2 and fluctuating glucose liraglutide=4).

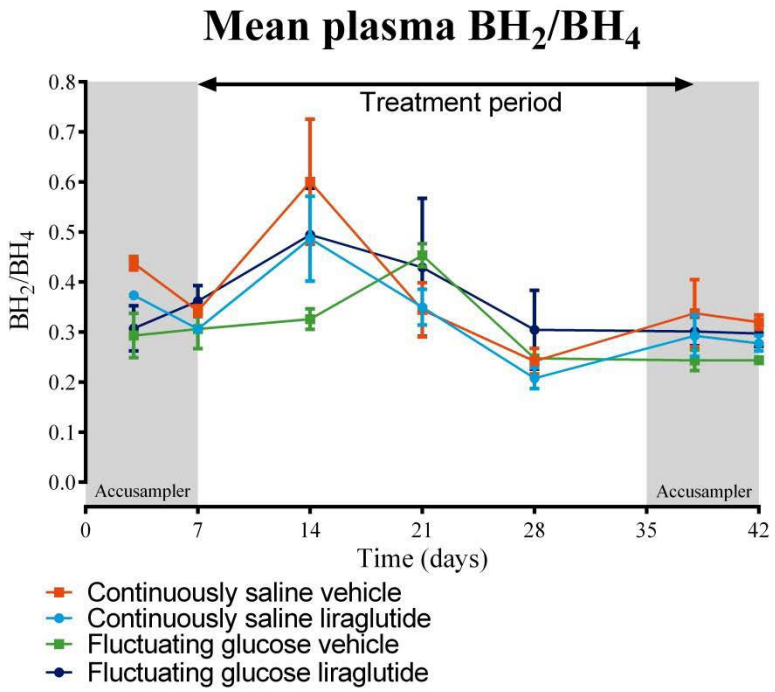


**Figure 9. Mean plasma $BH_2/BH_4$ ratio during the two infusion rounds and the liraglutide intervention of 30 days in between.** Plasma dihydrobiopterin/tetrahydrobiopterin ($BH_2/BH_4$) was measured at the beginning and the end of the infusion rounds and every week during the liraglutide intervention. Grey coloured area indicates the time the rats were in the Accusampler system. Data are presented as mean ± SEM. (n-values: Continuously saline vehicle=3, continuously saline liraglutide=3, fluctuating glucose vehicle=2 and fluctuating glucose liraglutide=4).

## Paper III

**Plasma lipid oxidation predicts atherosclerotic status better than cholesterol in diabetic apolipoprotein E deficient mice.**

Petersen KE, Lykkesfeldt J, Raun K, Rakipovski G.

*Experimental Biology and Medicine 2017; 242(1):88-91.*

# Brief Communication

# Plasma lipid oxidation predicts atherosclerotic status better than cholesterol in diabetic apolipoprotein E deficient mice

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## Abstract

Increased levels of oxidative stress have been suggested to play a detrimental role in the development of diabetes-related vascular complications. Here, we investigated whether the concentration of malondialdehyde, a marker of lipid oxidation correlated to the degree of aortic plaque lesions in a proatherogenic diabetic mouse model. Three groups of apolipoprotein E knockout mice were studied for 20 weeks, a control, a streptozotocin-induced diabetic, and a diabetic enalapril-treated group. Enalapril was hypothesized to lower oxidative stress level and thus the plaque burden. Both diabetic groups were significantly different from the control group as they had higher blood glucose, $HbA_{1c}$, total cholesterol, low-density lipoprotein, very low-density lipoprotein, together with a lower high-density lipoprotein concentration and body weight. Animals in the diabetic group had significantly higher plaque area and plasma malondialdehyde than controls. The two diabetic groups did not differ significantly in any measured characteristic. In summary, there was a positive correlation between plasma malondialdehyde concentration and aorta plaque area in apolipoprotein E knockout. Even though further investigation of the role of lipid oxidation in the development of atherosclerosis is warranted, these results suggest that biomarkers of lipid oxidation may be of value in the evaluation of cardiovascular risk.

**Keywords:** Diabetes, oxidative stress, lipid oxidation, atherosclerosis, $APOE^{(-/-)}$ mice, malondialdehyde

***Experimental Biology and Medicine* 2017; 242: 88–91. DOI: 10.1177/1535370216650520**

## Introduction

Oxidative stress is an imbalance between antioxidants and reactive oxygen species that is accelerated by hyperglycemia and has been suggested to play a pivotal role in the development of diabetic late complications.[1] Macrovascular diabetic complications include cardiovascular diseases (CVD), which are the leading cause of mortality among diabetes patients. These include atherosclerosis, which is an important element in CVD, and the estimation of atherosclerotic status may help assess the risk of cardiovascular events. Low-density lipoprotein (LDL) is considered a predictive risk marker of cardiovascular events but recently, several outcome studies have shown that ratios between cholesterol fractions and apolipoprotein concentrations may provide even better prediction of cardiovascular risk.[2] This underlines the complexity of biomarker-based CVD risk assessment and the need for more predictive markers. Oxidative modifications increase the atherogenic potential of LDL,[3–5] suggesting that assessment of oxidative stress status may improve predictivity of atherosclerosis.

The association between lipid oxidation and CVD has long been recognized (reviewed in Naito et al.[6]). Accordingly, malondialdehyde (MDA)—a biomarker of lipid oxidation—has been found to be associated with increased coronary intima-media thickness[7] and atherogenic index ((total cholesterol – HDL cholesterol)/HDL cholesterol).[8,9]

Lipid oxidation has also been investigated in animal models of diabetes and atherosclerosis, and studies have identified a relationship between increased lipid oxidation and atherosclerosis as well as arterial stiffening.[10–12]

In the present study, we investigated the correlation between MDA and aorta plaque burden in long-term diabetic apolipoprotein E knockout mice. Furthermore, we wanted to test if lowering oxidative stress attenuates plaque burden. Here, the angiotensin-converting enzyme inhibitor, enalapril, was used as a pharmacological tool. Enalapril lowers hypertension by reducing the formation of angiotensin II and thereby aldosterone production, both of which have been shown to increase oxidative stress *in vitro* and *in vivo*.[10,13] Thus, enalapril was expected to

decrease plaque formation in a cholesterol and glucose independent manner.

## Methods

### Animals

The experiment was approved by Danish Animal Experimentation Inspectorate (permission number: 2012-15-2934-00304). Five- to eight-week-old male apolipoprotein E knockout B6-129P2-*Apoe*$^{tm1Unc}$N11 (APOE$^{(-/-)}$) mice (Taconic, Ejby, Denmark) were housed under controlled conditions (temperature: 22 2 C; humidity: 50 20%; 12 h/12 h light/dark cycle). The animals were randomly divided into three groups: control (CTRL; n = 7), diabetic (DIAB; n = 9), and diabetic enalapril (ENAL; n = 10) with free access to water and chow (Altromin 1324, GmBH, Lage, Germany).

### Treatment protocols

Diabetes was induced in DIAB and ENAL animals by streptozotocin (STZ) (Sigma-Aldrich, Saint Louis, MO, USA) IP injections over a five-day period (55 mg/kg/day). No animals died from this treatment, but only animals with blood glucose > 15 mmol/L three weeks after STZ initiation were included in the study (the n values specified in ''Animals'' section are the final n values). From this time point onward, enalapril (Sigma-Aldrich, Saint Louis, MO, USA) was administrated to ENAL animals for 20 weeks through the drinking water (38 mg/L) calculated based on water intake to result in a dose of 10 mg/kg/day. During the study, samples for blood glucose (once a week—data are only shown from the last sample) and $HbA_{1c}$ (two weeks after STZ injections and six and 20 weeks after initiation of enalapril treatment—data not shown) were collected from a tail vein.

At termination, animals were anaesthetized using Hypnorm (VetaPharma Ltd, Leeds, United Kingdom)/ Midazolam (Accord Healthcare, Copenhagen, Denmark) (0.6, 19, and 9 μg/g of fentanyl/fluanisone/midazolam, respectively) and blood was collected by puncture of the orbital sinus. Abdomen and thorax were opened and animals were perfused with sterile isotonic saline (10 mL; 23 G cannula). The aortas were cut at the base of the heart and by the seventh rib and kept cold while cleansed (removal of fat), and opened for *en face*, where a picture was taken and analyzed for plaque content by morphometry (Visiomorph, Visiopharm A/S, Hørsholm, Denmark). Subsequently, aortas were stored at 80 C until homogenization in 150 μL RTL lysis buffer using TissueLyser II (Qiagen GmbH, Hilden, Germany).

### Biochemical analyses

For determination of blood glucose, 5 μL whole blood collected in Na-heparinized capillary tubes (Vitrex Medical A/S, Herlev, Denmark) was diluted in 250 μL glucose/lactate system solution (EKF Diagnostics, Barleben, Germany) and analyzed in a Biosen S-line glucose analyzer (EKF Diagnostics, Barleben, Germany). $HbA_{1c}$ was determined in 10 μL Na-heparinized whole blood stabilized with hemolyzing reagent using a Cobas 6000 (Roche/ Hitachi, Mannheim, Germany). Total plasma cholesterol and lipoprotein fractions were determined at Department of Pathology/Lipid Sciences, Wake Forrest University School of Medicine Winston-Salem, NC, USA by a method previously described.[14] In short, total plasma cholesterol concentration was determined using the enzymatic Cholesterol/ HP kit (Roche Diagnostics, Indianapolis, IN, USA). Cholesterol fractions were separated by fast protein liquid chromotography (FLPC) size exclusion following HPLC and subsequently measured colorimetrically using Infinite (Thermo Fischer Scientific, Waltham, MA, USA) and integrated using Chrom Perfect Spirit software (Justice Laboratory Software). The area percent distribution for each of the lipoprotein fractions was used to determine the cholesterol fraction concentration, by multiplying them with the total cholesterol concentration. Lipid oxidation was measured as MDA in plasma and aorta by HPLC as described previously.[15] Aorta protein concentrations were determined by bicinchoninic acid assay (Merck, Darmstadt, Germany).

### Statistical analysis

Statistical analysis was carried out by one-way ANOVA using Tukey's *post hoc* comparisons (SAS Enterprise 7.1, Cary, NC, USA). Stepwise multiple regression analysis was performed using total cholesterol, LDL, very low-density lipoprotein (vLDL), total cholesterol/high-density lipoprotein (HDL) ratio, and plasma MDA as explanatory variables and plaque area as dependent variable. All data are presented as mean SEM and p-values $< 0.05$ were considered significant.

## Results

As expected, STZ-treated animals became diabetic (30.3 1.4 mmol/L glucose versus 8.7 0.3 for CTRL, $p < 0.001$). Animals remained hyperglycemic for the entire study period and no difference between the diabetic groups was observed (Figure 1). $HbA_{1c}$ was 8.9 0.4 and 9.3 0.6% in DIAB and ENAL animals, respectively versus 4.0 0.1% among CTRLs. Plasma concentrations of total cholesterol, LDL, and vLDL were increased in diabetic versus CTRLs, while HDL was higher in CTRLs versus diabetic groups ($p < 0.05$; Figure 1). Total cholesterol/HDL ratio was approximately threefold higher in the diabetic versus CTRL animals. None of the measures differed significantly between DIAB and ENAL.

*En face* evaluation showed increased aortic plaque lesion area ($p < 0.01$) and higher plasma MDA ($p < 0.05$) in DIAB versus CTRL animals. ENAL animals were not significantly different from CTRL or DIAB. Aorta MDA concentrations did not differ between groups (Figure 1). A positive correlation was found between aortic plaque lesion area and plasma MDA ($p = 0.0076$, $R^2 = 0.2713$). As aortic plaque lesion area was also correlated to total cholesterol, LDL, and total cholesterol/HDL ratio, a stepwise multiple regression analysis with backward elimination was performed identifying plasma MDA as the variable describing aortic plaque lesion area the best.

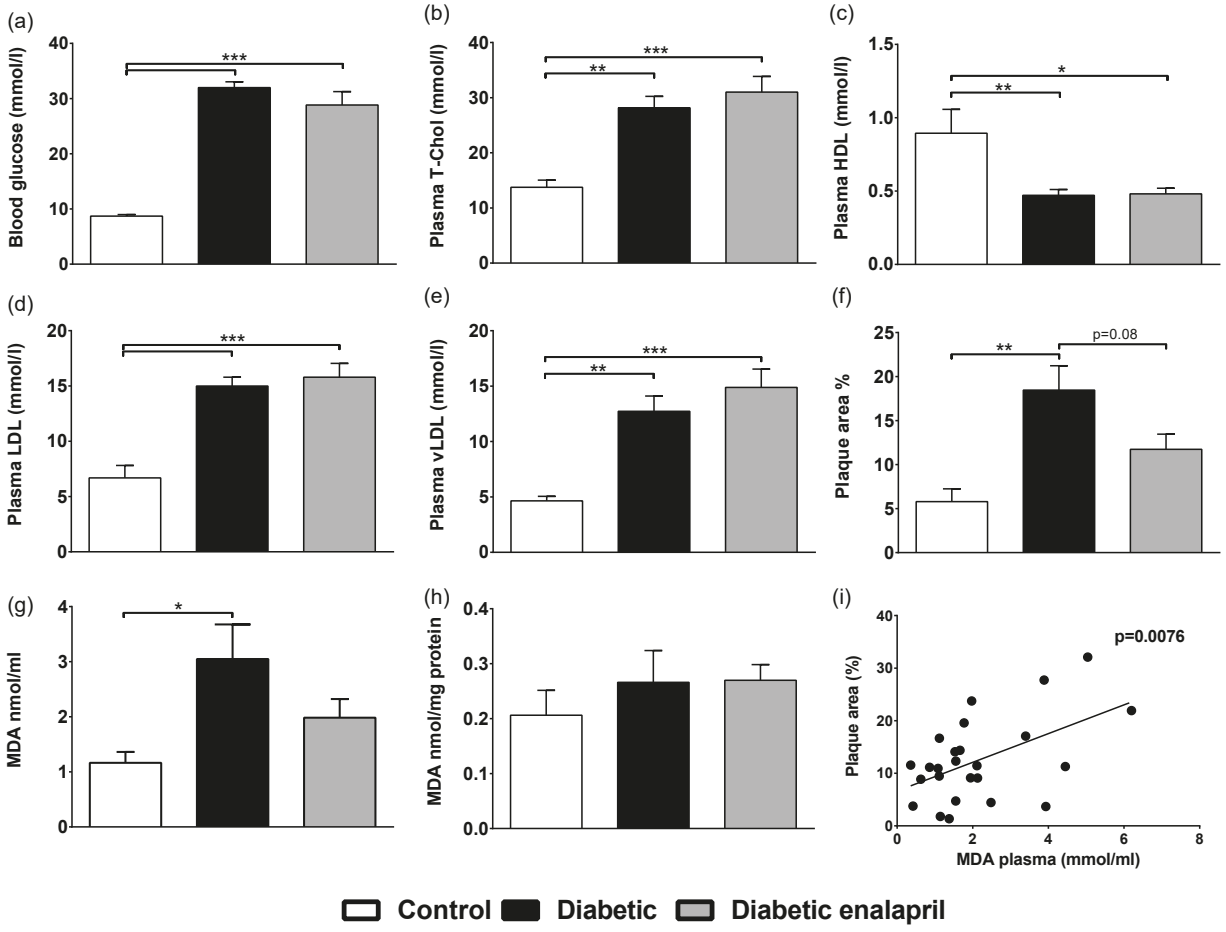


**Figure 1** Effects of diabetes and enalapril treatment in APOE$^{(-/-)}$ mice. (a) Blood glucose at the end of the experiment. Plasma total cholesterol (b), HDL (c), LDL (d), and vLDL (e) concentrations at the end of the experiment. (f) Plaque area in the aorta of the mice, determined by *en face*. MDA concentrations in plasma (g) and aorta homogenates (h) at the end of the experiment. (i) Correlation between MDA in plasma and plaque area in the aortas. Values are mean SEM. *P < 0.05, **P < 0.01, *** P < 0.001 compared to control. HDL: high-density lipoprotein; LDL: low-density lipoprotein; MDA: malondialdehyde; T-Chol: total cholesterol; vLDL: very low-density lipoprotein

## Discussion

In the present study, plasma lipid oxidation as measured by MDA was found to be a better predictor of atherosclerotic status than LDL and cholesterol in STZ-induced diabetic APOE$^{(-/-)}$ mice.

Both diabetic groups had higher total cholesterol, LDL, and vLDL concentrations combined with a concurrent decrease in HDL. The cholesterol profile supports the face validity of the model as compared to the human situation. Moreover, the increases in the well-established human risk factors for CVD, i.e. total cholesterol, LDL, vLDL, and total cholesterol/HDL ratio, in both diabetic groups are assumed to contribute to the atherosclerosis development.[16] Increased plaque formation has previously been shown in STZ-induced diabetic APOE$^{(-/-)}$ mice without the simultaneous shift in cholesterol fraction composition.[12] In a study in diabetic rats, treatment with statins lowered plasma and aorta thiobarbituric acid reactive substances (TBARS) level (an unspecific marker of lipid oxidation) and arterial stiffness without the expected effect on lipid profile, presumably due to the low dose used.[11] Thus, these studies indicate that lipid oxidation *per se* is important in CVD pathogenesis in diabetic animals and may contribute to the risk assessment in addition to the lipid profile. In humans, CVD events are not necessarily associated with high cholesterol concentrations, indeed supporting the need for a wider range of biomarkers for risk assessment.[17]

In long-term diabetic APOE$^{(-/-)}$ mice (four months), increased plaque areas were accompanied by a concurrent rise in plasma TBARS level, a drop in erythrocyte reduced-to-oxidized glutathione ratio, and an upregulation of glutathione peroxidase gene expression in the aorta.[12] Interestingly, aorta and kidney concentrations of 4-hydroxynonenal (a lipid oxidation marker; 4-HNE) were increased in the latter study in contrast to aorta MDA in the present study. This could indicate that the extended time course of the present study results in different levels of lipid oxidation or that 4-HNE and MDA are accumulated and degraded differently in aortic tissue. Regardless, the observed increase in 4-HNE does support the involvement of lipid oxidation in the development of atherosclerosis in diabetes and hence the findings of the present study. It should be noted, however, that atherosclerotic lesions from the APOE$^{(-/-)}$ mouse, rabbits, and humans have been shown to contain MDA-modified LDL and circulating autoantibodies against MDA modified LDL have been found in

APOE$^{(-/-)}$ mice.[3–5] Moreover, degradation of MDA in these lesions can result in MDA conjugated breakdown products, potentially not measurable by the HPLC methodology used in the present study. Thus, as we only measured unbound MDA, LDL-bound MDA could well be present in higher quantities in the aortas from the diabetic groups that have increased plaque area.

Enalapril treatment decreased lipid oxidation and aortic plaque lesion area compared to DIAB animals as expected, albeit these changes did not reach statistical significance. Histological evaluation could perhaps have separated the groups, as morphological investigations typically offer more detailed and precise information on plaque severity compared to a two-dimensional qualitative analysis such as the *en face* method.

The present study suggests a relationship between MDA and atherosclerotic status in diabetic APOE$^{(-/-)}$ mice. Whether MDA is a simple secondary biomarker or plays a pathological role has yet to be unraveled, although the latter could be implied by the data presented above. Furthermore, the biological half-life of MDA and its pathways of degradation and excretion have to be more clearly defined to make it a good candidate for the evaluation of atherosclerotic status in diabetes. Studies similar to the present but with additional and larger study groups euthanized at different time points assessing MDA in plasma and aorta, *en face* measurements, and histological examination of plaque morphology could help clarify if and how accurately measured MDA may be used to assess the level of atherosclerosis *in vivo*. Investigating known therapies of hyperglycemia and dyslipidemia could be useful as well to investigate the effect of dyslipidemia on atherosclerosis in this model. However, prospective cohort studies in large human populations of diabetic and non-diabetic patients with and without established atherosclerosis are needed to evaluate the value of plasma MDA as biomarker in human atherosclerosis.

**Author contributions:** The study was designed by all authors. The experiments were carried out by KEP and GR. The initial data analysis was performed by KEP followed by data interpretation by all authors. The draft manuscript was written by KEP and subsequently edited by all authors and all authors have approved the final version of the manuscript.

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#### DECLARATION OF CONFLICTING INTERESTS

The author(s) declared following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: KR and GR are employees of Novo Nordisk A/S that produces insulin.

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## Paper IV

**High dietary cholesterol improves glucose tolerance in low-density lipoprotein receptor deficient mice – comparison of two standard high fat diets.**

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# High dietary cholesterol improves glucose tolerance in low density lipoprotein receptor deficient mice – comparison of two standard high fat diets

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Abbreviations: ABCA1, ATP-binding cassette transporter; AUC, area under the curve; CVD, cardiovascular disease; CYP7A1, cholesterol 7 α-hydroxylase; CYP8B1, sterol 12 α-hydroxylase; FGF21, Fibroblast growth factor 21; FH, familial hypercholesterolemia; FGFR4, Fibroblast growth factor receptor 4; FXR, farnesoid X receptor; GK, glucokinase; GLP-1, glucagon-like peptide-1; GLUT2, glucose transporter 2; G6Pase, glucose-6-phosphatase; HCD, High cholesterol diet with cholesterol; INSR, insulin receptor; LCD, Low cholesterol diet; $LDLR^{(-/-)}$, low density lipoprotein receptor deficient; LEPR, leptin receptor; LXRα, liver X receptor α; MDA, malondialdehyde; OGTT, oral glucose tolerance test; PEPCK, phosphoenolpyruvate carboxykinase; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator 1α; PPARγ, peroxisome proliferator-activated receptor γ; SREBP-1c, Sterol regulatory element-binding protein 1c; TG, triglyceride.

**Abstract:**

Low density lipoprotein receptor-deficient mice are capable of developing atherosclerosis in combination with obesity, hyperglycemia and hyperinsulinemia. Cholesterol content is often used as inducer of atherosclerosis in these mice, however, little is known about the effect of dietary cholesterol *per se*. We wanted to more specifically investigate the effect of cholesterol on atherosclerosis development, glucose tolerance and hepatic expression of genes involved in lipid and glucose metabolism. Thus, two commonly used standard high fat diets primarily varying in cholesterol content was used in the present study.

Forty low density lipoprotein receptor-deficient mice were randomly divided into two groups and fed high fat diets with either low (LCD) or high (x10) cholesterol content (HCD) for 16 weeks. HCD animals developed significantly larger aortic plaque areas and displayed higher hepatic accumulation of both triglycerides and collagen than the low cholesterol animals ($p<0.001$ in all cases). However, high cholesterol animals showed lower fasting blood glucose ($p<0.01$) and better glucose tolerance than the LCD group ($p<0.001$) together with lower concentrations of oxidative stress markers malondialdehyde and isoporastanes ($p<0.05$). Changes in hepatic gene expression were observed as SREBP-1c, ABCA1, LEPR were all up-regulated while GLUT2, PEPCK, PGC-1α, FGFR4 and β-klotho were down-regulated in HCD vs. LCD animals ($p<0.01$ or less).

Our results suggest that high dietary cholesterol content can improve glucose tolerance and lipid oxidation status in low density lipoprotein receptor-deficient mice at the expense of increased plaque formation and hepatic steatosis and fibrosis, potentially through regulation of hepatic gene expression.

## Introduction

The number of people with obesity is increasing rapidly worldwide, from 857 million people in 1980 to 2.1 billion people in 2013[1]. Obesity is an important risk factor for development of other diseases such as type 2 diabetes, cardiovascular disease (CVD), non-alcoholic fatty liver disease and some types of cancer[2-5]. Increased caloric intake and a change in diet composition towards higher fat and sucrose content are considered important contributors to the increasing prevalence of obesity and its comorbidities[1,6]. For atherosclerosis and CVD, circulating cholesterol concentrations has long received much attention and the adverse effects and increased disease risk associated with particular plasma cholesterol profiles are well-established[7]. It is however debated how dietary cholesterol affects circulating cholesterol profiles[8]. Other risk factors for the development of atherosclerosis include obesity, dyslipidemia, diabetes, the metabolic syndrome and hepatic steatosis[9-12]. Oxidative stress is assumed to be an important early event in the development of atherosclerosis, and oxidative stress status can be evaluated by measuring markers of lipid oxidation, e.g. malondialdehyde (MDA) and 8-iso-prostaglandin F2α (8-iso-$PGF_{2\alpha}$)[13,14].

Diet-induced obesity in rodents is frequently used in *in vivo* studies to mimic human obesity and its related diseases. The low density lipoprotein receptor-deficient ($LDLR^{(-/-)}$) mouse develops obesity, hyperglycemia and hyperinsulinemia when fed a high fat diet with or without cholesterol[15-17]. The combination of its susceptibility to develop atherosclerosis when fed a cholesterol-enriched diet and the above metabolic complications gives the $LDLR^{(-/-)}$ mouse some face validity towards the disease complex related to human obesity, as correlations between atherosclerotic development in these mice and their plasma lipid profile have shown resemblance to the human situation[18-20]. Risk factors for the development of atherosclerosis are numerous, emphasizing the complexity of the disease. $LDLR^{(-/-)}$ mice are used as a model of hepatic lipid accumulation disorders, such as non-alcoholic fatty liver disease , nonalcoholic steatohepatitis and fibrosis. The latter is indicative of a more progressed stage of liver disease, which develops in connection to the metabolic syndrome as seen in humans[21-24].

In spite of the well-known effects of cholesterol in atherosclerotic development, the metabolic effects of dietary cholesterol and hypercholesterolemia are not well understood. Since Gould *et al* discovered that diets rich in cholesterol suppressed the synthesis of cholesterol in the liver[25,26], the investigation of dietary cholesterol effects on hepatic gene expression has gained focus. Regulation

and synthesis of bile acids are important elements of cholesterol turnover and could be a potential pathway by which dietary cholesterol affects hepatic gene expression, together with regulation of other hepatic genes involved in lipid and glucose metabolism[27,28].

In the present study, two groups of LDLR$^{(-/-)}$ mice were fed two standard high fat diets primarily different in cholesterol content (10-fold difference) for 16 weeks to investigate the effect of dietary cholesterol on atherosclerotic plaque burden, hepatic steatosis, hepatic fibrosis, glucose tolerance, plasma markers of lipid oxidation and hepatic gene regulation of genes involved in lipid and glucose metabolism.

## Materials and methods

### Animals

The experiment was performed by certified personnel and after approval from the Danish Animal Experimentation Inspectorate. Six to seven week old male LDLR$^{(-/-)}$ mice (genotype: B6.129S7-Ldlr$^{tm1Her}$/J) from Jackson Lab, USA were randomly divided into two groups. One group (HCD, n=20) was fed a high fat with cholesterol (D12079B, Research Diets, New Brunswick, New Jersey, USA) and the other group (LCD, n=20) was fed a high fat diet (D12451, Research Diets, New Brunswick, New Jersey, USA) (see table 1). The animals were housed under controlled conditions with a temperature of 22 ± 2°C, humidity of 50 ± 20% and with a 12h/12h light/dark cycle (lights on at 6AM). Water was provided ad libitum and the animals had free access food.

### Oral glucose tolerance test

The animals were fasted for five hours prior to the oral glucose tolerance test (OGTT), where a dose of 2g/kg (250 mg/kg glucose solution (Fresenius Kabi, Copenhagen, Denmark)) was dosed to the animals by oral gavage. Ten animals from each group underwent OGTT. During the OGTT, blood samples were collected in capillary tubes from the tail vein for blood glucose measurements at t=0 (prior to glucose dosing), 30, 60, 90, 120 and 180 minutes. See biochemical analyses for further details. Ten animals from each group were challenged with the OGTT. Area under the curve (AUC) was calculated for each group, both on data normalized to baseline and on the original data.

### Scanning

In conscious animals, body composition was evaluated by quantitative nuclear magnetic resonance spectroscopy (EchoMRI 3-in-1 Animal Tissue Composition Analyzer; Echo Medical Systems, Houston, Texas, USA). Before the measurement, the system was tested and the EchoMRI was calibrated by scanning a tube with a known amount of fat.

Study termination

Animals were anaesthetized using Isofluran (Baxter A/S, Søborg, Denmark) and blood samples were collected by puncture of the orbital sinus. After blood collection, the abdominal and thoracic cavities were opened and sterile isotonic saline was injected to their hearts for perfusion (10 ml with a 23G cannula). From the base of the heart to the level of the seventh rib the aortas were removed and pinned out with acupuncture needles on frozen oasis plates and cleansed by removing fat and connective tissue. The aortas were cut open and a picture was taken and analyzed (*en face*) by morphometry (Visiopharm A/S, Hørsholm, Denmark). The right lateral liver lobe was immediately snap frozen and stored at -80˚C before tissue were weighed for RNA purification (30 mg) and the rest of the liver was fixated in 10% buffered formalin for histological assessment.

Biochemical analyses

For determination of blood glucose, 5 µl of whole-blood were collected in a Na-Heparinized capillary tube (Vitrex Medical A/S, Herlev, Denmark) and diluted with 250 µl EBIO buffer (Eppendorf AG., Hamburg, Germany) before it was analyzed in the an Biosen S line glucose analyzer (Eppendorf AG., Hamburg, Germany). Plasma triglyceride (TG) was analyzed on Hitachi 912 analyzer (Roche A/S Diagnostics, Mannheim, Germany). Total cholesterol concentration was analyzed on Cobas c 501 (Roche Diagnostic Systems, Bern, Switzerland). Insulin was determined by Luminescence Oxygen Channeling Immunoassay (LOCI) as described previously[29]. Plasma leptin concentrations were measured by Mouse Leptin AlphaLISA (LOCI) kit (Perkin Elmer, Boston, Massachussets, USA) according to manufactures protocol. The distribution of cholesterol into the lipoprotein classes was determined at Department of Pathology/Lipid Sciences, Wake Forrest University School of Medicine (Winston-Salem, NC, USA) as described previously[30]. Lipid oxidation was evaluated by measuring MDA in plasma and aorta homogenates and 8-iso-$PGF_{2\alpha}$ concentration in plasma, measured by an 8-Isoprostane ELISA kit (Cayman Chemical, Ann Arbor, Michigan, USA). MDA was analyzed by HPLC described previously[31].

Histology

For visualization and measurement of lipids and triglycerides content of the livers, Oil Red O staining was applied. Liver tissue fixated in 10 % buffered formalin was transferred to 20 % sucrose until the tissue dropped to the bottom of the vial. Hereafter, the liver tissue was embedded in Tissue-Tek® OCT compound (#25608-930, Sakura®Finetek, Copenhagen, Denmark) and frozen before cutting 10 µm thick sections for staining. Hepatic fibrosis was visualized and measured using Picrosirius Red staining. Formalin fixed liver tissue was processed in a tissue processor (Leica ASP300S, Ballerup, Denmark), embedded in paraffin and 3 µm sections was cut. For both staining techniques two liver sections on each glass slide were stained with either Oil Red O (#O-0625, Sigma-Aldrich, Saint Louis, MO, USA) or Picrosirius Red Stain Kit according to the manufacturer's instructions (#ab150681, Abcam, Cambridge, United Kingdom). The slides were scanned on a slide scanner (Hamamatsu NanoZoomer 2.0 HT, Hamamatsu, Shinmiyakoda, Japan) on 40x magnification. For analysis of the images, the scanned slides were imported to a software program (Visiopharm A/S, Hørsholm, Denmark), in which the area of Oil Red O or Picrosirius Red positive staining was quantified and data was reported as the percentage of the whole area of two liver sections that was Oil Red O or Picrosirius Red positive.

Real-time quantitative PCR

Expression of SREBP-1c, LXRα, PPARγ, ABCA1, PGC-1α, PEPCK-C, GK, G6Pase, FGFR4, β-klotho, INSR, LEPR, GLUT2 and FGF21 was measured in the liver by real-time quantitative PCR. RNA was purified by RNAeasy Mini Kit (Qiagen GmbH, Hilden, Germany) and cDNA was synthesized with SuperScript VILO cDNA Synthesis Kit (ThermoFischer Scientific, Waltham, Massachusetts, USA). RNA quantity and quality was assessed by Nanodrop 1000 Spectrophotometer (ThermoFischer Scientific, Waltham, Massachusetts, USA) and Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany) using Agilent RNA 6000 Nano Kit. TaqMan primers and probes were obtained from Applied Biosystems (ThermoFischer Scientific, Waltham, Massachusetts, USA). Real-time quantitative PCR was performed on QuantStudio 12K Flex Real-Time PCR System (ThermoFischer Scientific, Waltham, Massachusetts, USA) using TaqMan Fast Universal PCR Master Mix (ThermoFischer Scientific, Waltham, Massachusetts, USA) according to manufacture manual with PCR thermocycling parameters of hold stage of 50°C for 2 min and 95°C for 10 min, and then 40 cycles of 95°C for 15 sec. and 60°C for 1 min. The geometric mean of three reference genes (TBP, GAPDH and ACTB) was used to normalize data from the qPCR

reactions of each outcome gene (table 2). Multiple reference genes were used as this makes the method more robust and the results more accurate[32]. Selection of reference genes were made based on the literature[33-35]. All samples were measured in duplicates.

Statistical analyses

For statistical analyses, SAS Enterprise Guide 4.3 (SAS, Cary, North Carolina, USA) was employed. Prism 6 (GraphPad Software Inc. La Jolla, California, USA) was used for graphical presentation and calculation of AUC. Groups were compared using two-tailed unpaired t-tests and permutation tests were used for the RT-qPCR results. The RT-PCR data were calculated on log2-transformed data, but data are represented as non-transformed data on semi-log graphs. All data are represented as means ± standard error of the mean. P-values less than 0.05 were considered significant. A power calculation was performed using data from a previous study of LDLR$^{(-/-)}$ mice on diets of either high or low cholesterol content[36]. The difference in atherosclerotic development was used for the calculations and a power of 0.80 and a two-sided significance level of 0.05. These calculations resulted in the need of a sample size of 20 animals per group.

**Results**

Impact of diet on body weight, caloric intake and plasma lipids

As shown in table 3, the animals on both diets developed obesity with a fat percentage of 32.7±0.9% and 30.7±09% in the LCD and the HCD group, respectively. There were no statistical differences between the two dietary groups on body weight, fat mass or caloric intake. Likewise, there was no statistical difference between the groups in plasma insulin concentrations, while leptin levels, surprisingly, were significantly higher in the LCD group when compared to the HCD group ($p<0.0001$). As expected, dietary intake of cholesterol increased plasma cholesterol levels significantly. Plasma TG concentrations showed a similar pattern.

Glucose tolerance

The glucose tolerance was evaluated by an OGTT. Fasting blood glucose was significantly lower in the HCD group (8.57±0.25 mmol/l vs. 10.53±0.40 mmol/l for LCD, $p<0.01$). The HCD group also showed a significant better glucose handling as judged by the AUC (1907±31.00 min*mmol/l vs. 2573±64.81 min*mmol/l for LCD, $p<0.001$), the difference was also significantly different when adjusting for baseline ($p<0.001$) (figure 1 shows data without the adjustment for baseline).

Aortic plaque lesions and oxidative stress

The HCD animals developed pronounced plaque lesions in the thoracic aorta. This effect was highly significant as compared to the LCD group (13.00±0.04% vs. 1.02±0.42% for LCD $p<0.001$). Lipid oxidation, as measured by plasma MDA and 8-iso-$PGF_{2\alpha}$ levels, was significantly lower in this group when compared to LCD (MDA: 2.61±0.14 nmol/ml vs. 3.04±0.11 nmol/ml for LCD, $p<0.05$. 8-iso-$PGF_{2\alpha}$: 33.75±3.39 pg/ml vs. 99.35±26.94 pg/ml for LCD, $p<0.05$) (figure 2).

Hepatic lipid accumulation, collagen content and gene expression of genes involved in lipid and glucose metabolism

The hepatic lipid content evaluated by Oil Red O stained tissue showed that HCD feeding resulted in a significantly higher lipid accumulation and collagen content in the liver (figure 3). To gain insight in the effect of the two different diets on hepatic gene expression of genes capable of changing glucose tolerance the gene expression of 14 different genes was investigated. When comparing the LCD and the HCD groups, there was a significantly higher expression of SREBP-1c, ABCA1 and LEPR and significantly lower expression of PEPCK, PGC-1α, FGFR4, β-klotho and GLUT2 in the HCD group (table 4 and figure 4). No significant difference in expression of LXRα, PPARγ, GK, G6Pase, INSR and FGF21 was found between the two groups.

**Discussion**

The present study gives new insight into the effects of dietary cholesterol in $LDLR^{(-/-)}$ mice. An overview is given in figure 5. Interestingly the HCD group showed a better regulated glucose metabolism both in terms of lower fasting blood glucose and improved glucose tolerance. Dietary cholesterol content led to significantly higher plasma cholesterol concentrations and TG concentrations, also when compared to humans, which confirmed what has been observed by others[37].

Depleting cholesterol from pancreatic islets *in vitro* by methyl-β-cyclodextrin increases insulin secretion from pancreatic islets isolated from both $LDLR^{(-/-)}$ and wild type mice, an effect that is enhanced when the islets are subjected to high glucose concentrations[38]. *In vivo* investigations of the $LDLR^{(-/-)}$ mouse have shown impaired glucose tolerance compared to wild type mice, likely because of high TG concentration in tissue of $LDLR^{(-/-)}$ mice and increased membrane cholesterol

content in pancreatic islet cells in these mice[38,39]. This contradicts human studies where patients with familial hypercholesterolemia have lower risk of developing diabetes, which is assumed to be the result of a lower uptake of cholesterol to pancreatic β-cells[40,41]. These indications of beneficial effects of cholesterol is supported by reports that statins may increase type 2 diabetes incidences, an effect that is, however, not observed in patients with familial hypercholesterolemia[42-44]. The latter could be due to part of the effect of statins being increased expression of LDLR, which would not be effective in patients with familial hypercholesterolemia[43]. Results from animal studies on the effect of dietary cholesterol on glucose homeostasis are not unambiguous. LDLR$^{(-/-)}$ mice fed two high fat diets, primarily varying in cholesterol content (0.2% vs. 0.05%), for 15 weeks showed no difference in glucose tolerance[45]. However, the mice were fasted for nine hours in the study by Funke *et al*, which could affect glucose homeostasis in a way mimicking starvation and could explain why no difference was observed as a result of dietary cholesterol content in this study[46]. In rats, dietary cholesterol has shown no effects on blood glucose after four weeks of feeding, while simvastatin has shown to result in impaired glucose tolerance[47,48]. In contrast, dietary cholesterol has been shown to result in impaired glucose tolerance in hamsters[49]. The effect observed in hamsters could be due to a difference in bile acid regulation and synthesis[50].

The liver serves as energy storage, being responsible for disposal of >50% of an oral glucose load. This includes both uptake and control of release, and the liver is by these means important for glucose handling[51]. The RT-PCR data revealed different expression patterns of some of the investigated genes; as SREBP-1c, ABCA1, LEPR were up-regulated and GLUT2, PEPCK, PGC-1α, FGFR4 and β-klotho were down-regulated in the HCD group when compared to the LCD group. Hepatic glucose uptake and output is facilitated by the bidirectional GLUT2 transporter[52]. A lower content of gluconeogenic enzymes, indicated by the lower expression of PEPCK and PGC-1 can be connected to the lower expression of GLUT2 in the HCD group and lower glucose concentrations in this group could be a contributing factor to the lower GLUT2 expression[52]. The lower GLUT2 expression could lead to a decreased release of glucose from the liver. Together with a decreased PEPCK expression - the rate limiting enzyme for liver glucose output - this may be involved in the lower fasting blood glucose and also the better glucose tolerance in this group[53-55]. Leptin has been reported to regulate PEPCK gene expression in different directions. In non-diabetic rats, leptin up-regulates hepatic PEPCK gene expression, whereas leptin reduces the expression in streptozotocin-induced diabetic rats, largely via the central leptin receptors[56-58]. In the present

study, we observed a higher gene expression of LEPR, but a lower leptin concentration and PEPCK gene expression in the HCD group. The difference in PEPCK gene expression could, in part, be controlled by the different systemic leptin concentrations.

Loss of peripheral leptin sensitivity can be due to decreased expression of hepatic LEPR, which in turn can impair insulin signaling[59,60]. The lower expression of LEPR in the LCD group together with the higher plasma leptin concentration implies a higher degree of leptin resistance in this group. This supports the finding of a more glucose intolerant state in the LCD group together with a lower LEPR expression. The higher expression of LEPR can explain some of the improved glucose homeostasis in the HCD group, as leptin can improve glucose homeostasis, in part by direct actions in hepatocytes[61].

Leptin can suppress SREBP-1c expression in mice livers and could explain the lower expression of SREBP-1c in the LCD group[62,63]. Hepatic leptin signaling has been connected to decrease accumulation of lipid, and resistance to leptin has been associated with increased SREBP-1c expression, but leptin signaling has shown to be essential for the development of fibrosis in steatotic livers[62,64-66]. Thus, the interplay between leptin and hepatic steatosis and fibrosis is complex, and the effect of cholesterol on SREBP-1c might be the most important pathway for its regulation in the current study[67,68]. Increased lipogenesis by increased SREBP-1c expression facilitates consumption of glucose and together with suppression of PEPCK, SREBP-1c has the potential to lower blood glucose and induce steatosis[69-73]. PGC-1α is important in modulation of hepatic gluconeogenesis and LXRα ligand-binding can decrease PGC-1α gene expression as well as glucose-6-phosphatase, but, the latter was not significantly decreased in the HCD group in the present study[74,75]. The decreased expression of PGC-1α in the HCD could be a contributing factor to the improved glucose homeostasis in these animals. PGC-1α has also been found to induce fatty acid β-oxidation and thus, lower gene expression may explain some of the steatosis observed in the HCD group[76].

LXRα activation has been observed to improve glucose tolerance in diet-induced obese C57BL/6J mice treated with a LXRα agonist[75]. Activation through binding of its natural ligands, oxysterols, which are generated under high cholesterol concentrations, could be a contributing factor in the observed up-regulation of SREBP-1c expression, as dietary cholesterol increased expression of

SREBP-1c has shown to be dependent on LXRα activation[67,68]. The lack of difference in expression of LXRα does not rule out its influence on the better glucose homeostasis observed in the HCD group in the present study. High plasma TG and cholesterol concentrations has previously been observed together with lower fasting blood glucose in lechitin-cholesterol acyltransferase-deficient mice with higher expression of SREBP-1c, lower PEPCK expression and without any difference in LXRα expression when compared to LDLR$^{(-/-)}$ mice. This was however, seen together with a significantly lower plasma insulin concentration also indicating that the double knockout mice were more sensitive to insulin[77]. Activation of LXRα can up-regulate ABCA1 gene expression, which is involved in cellular cholesterol efflux[78,79]. Higher expression of ABCA1 in the HCD group can be connected the higher degree of plaque formation in this group. Overexpression of hepatic ABCA1 has previously been investigated in LDLR$^{(-/-)}$ mice, showing that overexpression is connected to a higher degree of aortic atherosclerotic plaque lesions, which may be due to an observed increase of apolipoprotein B rich lipoproteins[80].

The production of bile acids plays an important role in the catabolism and turnover of cholesterol[28]. Dietary cholesterol increases bile acid synthesis, which achieves increasing focus for their hormonal functions in metabolic processes[27,81]. FGF19 and the mouse ortholog, FGF15 activate FGFR4. Although apparently enhanced by β-klotho, it is discussed whether this activation is completely or partially dependent on the presence of β-klotho[82,83]. The lower expression of both FGFR4 and β-klotho in the HCD group indicates a lower activation of the less expressed FGFR4 in this group and less signaling through the pathway. Signaling through this pathway functions as a postprandial negative feedback loop and decreases bile acid synthesis. FGF15/FGF19 is expressed in the ileum in a farnesoid X receptor (FXR) dependent manner when bile acids are released to the intestines activating FXR[84,85]. Pharmaceutical activation of FXR has been shown to decrease plasma TG and HDL and lower plaque formation in LDLR$^{(-/-)}$ mice[86].

Taken together, the lower expression of FGFR4 and β-klotho in the HCD group may contribute to a sustained production of bile acids and elimination of cholesterol in this state of extremely high plasma cholesterol. However, bile acids do facilitate absorption of dietary cholesterol[86]. FGFR4 and FXR activation suppresses expression of cholesterol 7 α-hydroxylase (CYP7A1) and sterol 12 α-hydroxylase (CYP8B1), which are the enzymes controlling bile acid pool and composition[81,87,88]. In several species, including mice, dietary cholesterol induces the expression of CYP7A1[89]. The

adverse effect of dietary cholesterol on glucose tolerance in hamster, mentioned previously, could be caused by a different regulation of bile acids synthesis in this species when compared to rats and mice[49,89,90]. Hamsters lack CYP7A1 induction by cholesterol that is seen in other species[89]. In this aspect, hamsters could resemble the human response, as the human CYP7A1 promoter does not have a LXRα binding site and LXRα binding to the CYP7A1 promoter is involved in the increase in expression of CYP7A1 induced by dietary cholesterol[91]. Generally, there are differences between mice and humans with respect to bile acid synthesis and regulation. Thus, extrapolation of effects on glucose tolerance should therefore be done with care, and studies should include suitable human model systems for investigating specific pathways of bile acid regulation[50].

Future studies incorporating measures of bile acid concentrations would be highly relevant. Bile acids can improve glucose homeostasis through an increased secretion of glucagon-like peptide-1 (GLP-1) from the intestine promoted by bile acids, which have been shown both *in vitro* and *in vivo*[92-94]. The role of FGF15/FGF19 and β-klotho in glucose metabolism is debated, but it has been shown that bile acids can increase insulin receptor signaling in rodent hepatocytes[95,96]. Restoration of signaling through hepatic FGFR4 in FGFR4 deficient mice (exhibiting hyperlipidemia, glucose intolerance and insulin resistance) can also help decrease plasma lipid concentration, together with an improvement of insulin sensitivity, but increase the development of steatosis[97], and FGF19 can increase hepatic β-oxidation, which improve glucose homeostasis[98]. FGF19 increases ERK phosphorylation in hepatocytes as well, which could contribute to increased insulin sensitivity[82]. FGF15/19 suppresses gluconeogenesis through reduction of PGC-1α expression[99]. FGF15/19 signaling pathway of the liver is an interesting target to investigate. Together with an evaluation of the non-hepatic FGF15/19 signaling in adipose tissue and pancreas, which might be the major site of the effect of FGF15/19 on glucose homeostasis, this could help reveal if FGF15/19, FGFR4, β-klotho and bile acid signaling plays a role in the improved glucose tolerance observed by high cholesterol feeding in the present study[100]. FGF21 does not activate FGFR4 but it has been shown that FGF21 can suppress gluconeogenesis in the liver and has been shown to improve glucose tolerance[101]. It has been suggested that FGF21 increases glucose utilization in brown adipose tissue e.g. by increased expression of uncoupling protein 1[102]. Although no difference in hepatic FGF21 mRNA levels between groups was observed in the present study, the presence of different systemic FGF21 concentrations cannot be ruled out. Additionally, β-klotho concentrations could be increased in the HFD group, even though hepatic expression was decreased at the end of the study. Increased

concentrations of FGF21 and/or β-klotho, which is a required co-factor for FGF21 activity, could potentially be part of the explanation for the better glucose tolerance and lower blood glucose concentration in the HFD group, and the concentrations should be measured in future studies.

The concentration of plasma markers of lipid oxidation was significantly lower in the HCD group, where the largest plaque area was found. This was surprising as oxidative stress has been suggested as an early event in the development of atherosclerosis[13]. The findings of the current study support the importance of dietary cholesterol for the development of atherosclerosis in LDLR$^{(-/-)}$ mice, as several animals in the LCD group had no plaques[36,103]. Cholesterol has been reported to have antioxidative properties, and the high cholesterol concentration in HCD group may, to some extent, contribute to the lower concentration of MDA and 8-iso-$PGF_{2\alpha}$ in this group[104-106]. In the LCD group, the impaired glucose tolerance and the higher blood glucose concentrations may explain the higher level of oxidative stress[107]. Oxidative stress has been discussed as a mechanism involved in the development of type 2 diabetes, primarily by lowering insulin secretion and action[108]. This could be a factor in worsening the glucose homeostasis in the LCD group. However, no differences in plasma insulin between the groups were observed in this study. Plasma insulin and leptin concentrations were very high in both groups when compared to e.g. C57BL/6J mice on LCD[109,110]. Plasma leptin concentrations were significantly higher in the LCD group when compared to the HCD group. Short-term treatment with leptin in rats have been shown to induce increased concentrations of MDA and isprostanes in the plasma and urine, respectively[111]. Whereas, longer term leptin treatment has shown to decrease expression of genes related to oxidative stress in the skeletal muscle of mice[112]. Investigations on how leptin affects oxidative stress is warranted and it should be elucidates whether the effect is dependent on leptin receptors, as well as how high leptin concentrations during leptin resistant states affects oxidative stress. This is however complicated by the complexity of leptin resistant states. The higher leptin concentrations in the LCD group could contribute to the higher MDA and 8-iso-$PGF_{2\alpha}$ concentrations in this group. The lack of difference in insulin concentration between the two groups indicates that the observed difference in glucose handling could be due to a lower response to insulin or a less efficient insulin independent glucose uptake. The liver is often described as an organ with insulin independent glucose uptake[113].

The choice of comparing two commonly used standard diets is a limitation to the present study as differences besides cholesterol content exists between the two diets. This is primarily the source of fat (table 1). The fat source of the LCD diet is lard and soy bean oil, while the fat sources of the HCD diet is anhydrous milk fat and corn oil. These differences leads to a difference in the fatty acid composition (appendix 1+2) including different distributions of fatty acids in regards to polyunsaturated fatty acids (PUFA), monounsaturated fatty acids (MUFA) and saturated fatty acids (SFA) (table 1). The LCD group has a higher percentage of PUFA, a lower percentage of SFA and a slightly higher percentage of MUFA. Dietary PUFA have been shown to decrease the accumulation of lipids in the liver of LDLR$^{(-/-)}$ mice[114]. In LDLR$^{(-/-)}$ mice it has further been shown that diets rich in SFA (30 kcal%) and PUFA (26 kcal%) results in smaller plaque lesion areas, while diets rich in MUFA (30 kcal%) and carbohydrates (62 kcal%), from corn starch and sucrose, results in larger plaque lesion areas, an effect that is not observed in APOE$^{(-/-)}$ mice[115]. It cannot be directly compared to the present study as the differences in these dietary components are not as extreme in this study as in the one performed by Merkel *et al*[115] (see table 1). PUFA are main targets of oxidative attack[116]. The higher amount of PUFA in the LCD group could be part of the explanation of the higher concentration of markers of lipid oxidation in this group. Diets rich in PUFA can increase hepatic concentrations of thiobarbituric acid-reactive subtances (TBARS; a crude estimate of lipid oxidation) in rats[117]. Another study in rats has shown that the diets with the lowest content of PUFA compared to SFA have the lowest hepatic TBARS concentration[118]. In this study the indicator of PUFA oxidation was used to evaluate the diets as well, showing that the lowest to middle degree resulted the lowest concentration of hepatic MDA, Feeding heated flaxseed oil which is rich on PUFA and contains more hydroperoxide and TBARS than fresh flaxseed oil, leads to increased concentrations of hepatic MDA in mice[119]. These studies underline a complex interplay, which is additionally dependent on the degree of oxidation of the diet prior to ingestion at least on the effects on lipid oxidation in the liver[117-119].

A study by Janssens *et al* has investigated how a diet using lard as a fat source differs from one with palm oil as a fat source, in regards to hepatic lipid metabolism and glucose tolerance in rats[120]. By an intraperitoneal glucose tolerance test (ipGTT) they found that both diets resulted in impaired whole body glucose tolerance, when compared to control. No significant difference was observed in hepatic glucose tolerance, shown by an ipGTT using deuterated glucose. However, the palm oil fed animals had an increase in fatty acid oxidative capacity in isolated liver mitochondria. This could

indicate less favorable effects of lard on metabolism, and was attributed to the content of the SFA, stearic acid which was three times higher in the lard-based diet[120]. The diets in the present study contained almost the same amount of stearic acid (LCD: 19.8 out of 202.5 g and HCD: 24.4 out of 196.6 g, appendix 1+2) indicating that the lard content in the diet in the LCD group might not be the reason for the impaired glucose tolerance in this group. However the n-6/n-3 ratio of the two diets in the present study could affect glucose tolerance as lower n-6/n-3 ratios are associated with better glucose tolerance[121]. The LCD diet has a n-6/n-3 ratio of 12.7 while HCD has a ratio of 3.6, which could explain some difference between the two groups. A study in LDLR$^{(-/-)}$ mice compared three diets, two diets with 21 % fat with milk fat as the fat source and one diet containing 36 % fat from lard. The group on 36 % fat diet had an impaired glucose tolerance when compared to the two groups on diets with 21 % fat[45]. The difference in glucose tolerance in this study cannot be concluded to be caused by fat source, but rather fat content[46]. In the present study the LCD group, fed the lard-based diet had an impaired glucose tolerance.

The sucrose content in the HCD diet is higher than the content in the LCD diet. In a study LDLR$^{(-/-)}$ mice were fed two high fat diets primarily varying in sucrose content (6.8 vs. 17.5 kcal%) for 20 weeks. At week 12, an ipGTT revealed a significantly impaired glucose tolerance at 15 minutes after injection in the group fed the highest amount of sucrose. A difference that was no longer present at the glucose tolerance test performed after 20 weeks of dietary intervention. The *en face* analysis of the aorta revealed that the high sucrose group had significantly higher plaque percentage at 16 weeks but not at 20 weeks[122]. Both diets in the current study contain high amounts of sucrose (17 and 29 kcal% for LCD and HCD respectively), with the kcal% in the LCD being the same as the content of sucrose in the high sucrose diet in Neuhofer *et al.* Neuhofer *et al* showed no difference in hepatic lipid content by Oil Red O staining at neither 16 nor 20 weeks, but the livers were steatotic at both time points. The high sucrose group had significantly higher total cholesterol, vLDL and LDL concentrations when compared to the low sucrose group at 16 weeks. The results of the current study indicate that dietary cholesterol affects glucose tolerance in LDLR$^{(-/-)}$ mice fed diets with high sucrose content. The plasma lipid profile in the two groups of the present study could have been additionally affected by the high sucrose content of the diets, with the most pronounced effect in the HCD group, as the content of the diet in this group was the highest. In addition the sucrose content could have an effect on atherosclerotic development in the HCD group.

Thus, the current study suggests that cholesterol concentration in the diet affects the glucose tolerance, at least when administrated in high fat diets rich on sucrose.

## Conclusion

We find that dietary cholesterol is important for the development of atherosclerosis in LDLR$^{(-/-)}$ mice. Moreover, we find that cholesterol-enriched diet improved glucose tolerance, possibly via changes in hepatic gene expression. The results emphasizes the importance of choosing the right dietary cholesterol level when designing studies in the LDLR$^{(-/-)}$ mouse model. The model can be used to answer scientific questions on atherosclerosis and conditions with impaired glucose tolerance, but to get the right response in the animals, the right dietary combination in the given situation should be used. E.g. to induce atherosclerosis, cholesterol should be in the diet, which could however give rise to an improvement of glucose tolerance. Studies focusing on glucose homeostasis in the animals could potentially benefit from avoiding high concentrations of cholesterol in the diet and maybe increase the length of the study to possibly induce some plaques. As the LDLR$^{(-/-)}$ mouse can actually, to a limited degree, develop plaques in long-term studies even on chow diet[123]. To underline the findings of this study a future study, using diets only varying in cholesterol content could be useful.

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## Conflicts of interest

KR, RK and GR are employees of Novo Nordisk A/S that produces insulin and the GLP-1 analogue, liraglutide. The authors declare no conflicts of interest that could influence the present work.

**Statement of author contributions**

The study was designed by all authors and the experiments were carried out by KEP and GR. Initially the data analysis was performed by KEP followed by data interpretation by all authors. The draft manuscript was written by KEP and subsequently edited by all authors and all authors have approved the final version of the manuscript.

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807 Table 1. Content in LCD and HCD.
808

| | LCD | | HCD | |
|---|---|---|---|---|
| | g% | kcal% | g% | kcal% |
| Protein | 24 | 20 | 20 | 17 |
| Carbohydrate | 41 | 35 | 50 | 43 |
| - Sucrose | 20 | 17 | 34 | 29 |
| Fat | 24 | 45 | 21 | 41 |
| - SFA | 7 | 13 | 12 | 24 |
| - MUFA | 8 | 15 | 6 | 12 |
| - PUFA | 7 | 14 | 1 | 3 |
| - Cholesterol | 0.015 | - | 0.21 | - |
| kcal/g | 4.7 | | 4.7 | |
| **Ingredient** | **G** | **kcal** | **G** | **Kcal** |
| Casein (80 mesh) | 200 | 800 | 195 | 780 |
| L-Cystine | 3 | 12 | - | - |
| DL-Methionine* | - | - | 3 | 12 |
| Corn Starch | 73 | 291 | 50 | 200 |
| Maltodextrin 10 | 100 | 400 | 100 | 400 |
| Sucrose | 173 | 691 | 341 | 1364 |
| Cellulose (BW200) | 50 | - | 50 | - |
| Soybean Oil | 25 | 225 | - | - |
| Corn Oil | - | - | 10 | 90 |
| Lard* | 178 | 1598 | - | - |
| Milk fat, anhydrous** | - | - | 200 | 1800 |
| Mineral Mix (S10001) | - | - | 35 | - |
| Mineral Mix (S10026) | 10 | 0 | - | - |
| DiCalcium Phosphate | 13 | 0 | - | - |
| Calcium Carbonate | 6 | - | 4 | - |
| Potassium Citrate ($1H_2O$) | 17 | - | - | - |
| Vitamin Mix (V10001) | 10 | 40 | 10 | 40 |
| Choline Bitartrate | 2 | - | 2 | - |
| Cholesterol | - | - | 1.5 | - |
| Ethoxyquin | - | - | 0.04 | - |
| **Total** | **858** | **4057** | **1001.54** | **4686** |

809 HCD, High cholesterol diet; LCH, Low cholesterol diet. All values were obtained from Research Diets product data
810 sheets and fatty acid content sheets for D12451 (LCD) and D12079B (HCD). Numbers are rounded to the nearest whole
811 number when possible. *Lard typically contains 0.72 mg/gram cholesterol, resulting in 0.015 % cholesterol in the diet.
812 **Milk fat typically contains 0.3 % cholesterol.
813
814

Table 2. Primers and hydrolysis probes:

| **Target gene:** | Assay ID: |
|---|---|
| SREBP-1c | Mm00550338_m1 |
| PGC-1α | Mm01208835_m1 |
| PEPCK-C | Mm01247058_m1 |
| G6Pase | Mm00839363_m1 |
| GK | Mm00439129_m1 |
| FGF21 | Mm00840165_g1 |
| β-klotho | Mm00473122_m1 |
| FGFR4 | Mm01341852_m1 |
| GLUT2 | Mm00446229_m1 |
| INSR | Mm01211875_m1 |
| LEPR-b | Mm01262070_m1 |
| LXRα | Mm00443451_m1 |
| ABCA1 | Mm00442646_m1 |
| PPARγ | Mm01184322_m1 |
| **Reference genes:** | |
| ACTB | Mm00607939_s1 |
| TBP | Mm00446971_m1 |
| GAPDH | Mm01175863_g1 |

ABCA1, ATP-binding cassette transporter; ACTB, Actin beta; FGF21, Fibroblast growth factor 21; FGFR4, Fibroblast growth factor receptor 4; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GK, glucokinase; GLUT2, glucose transporter 2; G6Pase, glucose-6-phosphatase; HCD, High cholesterol diet with cholesterol; INSR, insulin receptor; LCD, Low cholesterol diet; LEPR, leptin receptor; LXRα, liver X receptor α; PEPCK, phosphoenolpyruvate carboxykinase; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator 1α; PPARγ, peroxisome proliferator-activated receptor γ; SREBP-1c, Sterol regulatory element-binding protein 1c; TBP, TATA binding protein.

Table 3. Effects of diets on body weight, fat mass, insulin, leptin and plasma lipids.

| | LCD | HCD |
|---|---|---|
| Time (weeks) | 16 | 16 |
| Body weight (gram) | 45.3 ± 0.8 | 43.5 ± 1.0 |
| Fat mass (%) | 32.7 ± 0.9 | 30.7 ± 0.9 |
| Plasma insulin (pmol/l) | 1228 ± 126 | 1045 ± 129 |
| Plasma leptin (ng/ml) | 117.0 ± 6.2 | 72.3 ± 4.6*** |
| Plasma total cholesterol (mmol/l) | 23.05 ± 1.07 | 48.95 ± 1.82*** |
| Plasma triglycerides (mmol/l) | 4.90 ± 0.28 | 11.49 ± 0.70*** |

HCD, High cholesterol diet; LCD, Low cholesterol diet. ***P<0.001 compared to LCD. Data is represented as mean ± SEM.

Table 4. Hepatic gene expression in relative expression levels and difference between the two groups:

| **Gene:** | **LCD:** | **HCD:** | **Difference and confidence interval:** |
|---|---|---|---|
| **SREBP-1c** | 1.40 | 1.76*** | 1.26 [1.14; 1.39] |
| **LXRα** | 1.47 | 1.55 | 1.06 [0.89; 1.25] |
| **PPARγ** | 0.73 | 0.89 | 1.23 [0.61; 1.08] |
| **ABCA1** | 0.96 | 1.33** | 1.39 [1.12; 1.71] |
| **PGC-1α** | 1.39 | 0.95*** | 0.68 [0.58; 0.80] |
| **PEPCK-C** | 1.86 | 0.75*** | 0.40 [0.27; 0.60] |
| **GK** | 1.67 | 1.57 | 0.90 [0.83; 1.06] |
| **G6Pase** | 1.50 | 1.34 | 0.94 [0.64; 1.24] |
| **FGFR4** | 1.68 | 1.29** | 0.78 [0.65; 0.90] |
| **β-klotho** | 5.28 | 2.16*** | 0.77 [0.31; 0.55] |
| **INSR** | 1.44 | 1.42 | 0.98 [0.85; 1.13] |
| **LEPR** | 0.67 | 1.46*** | 2.18 [1.80; 2.65] |
| **GLUT2** | 1.51 | 1.18*** | 0.41 [0.70; 0.87] |
| **FGF21** | 1.36 | 1.23 | 0.89 [0.63; 1.30] |

ABCA1, ATP-binding cassette transporter; FGF21, Fibroblast growth factor 21; FGFR4, Fibroblast growth factor receptor 4; GK, glucokinase; GLUT2, glucose transporter 2; G6Pase, glucose-6-phosphatase; HCD, High cholesterol diet with cholesterol; INSR, insulin receptor; LCD, Low cholesterol diet; LEPR, leptin receptor; LXRα, liver X receptor α; PEPCK, phosphoenolpyruvate carboxykinase; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator 1α; PPARγ, peroxisome proliferator-activated receptor γ; SREBP-1c, Sterol regulatory element-binding protein 1c. ** = p<0.01 compared to LCD. *** = p<0.001 compared to LCD.

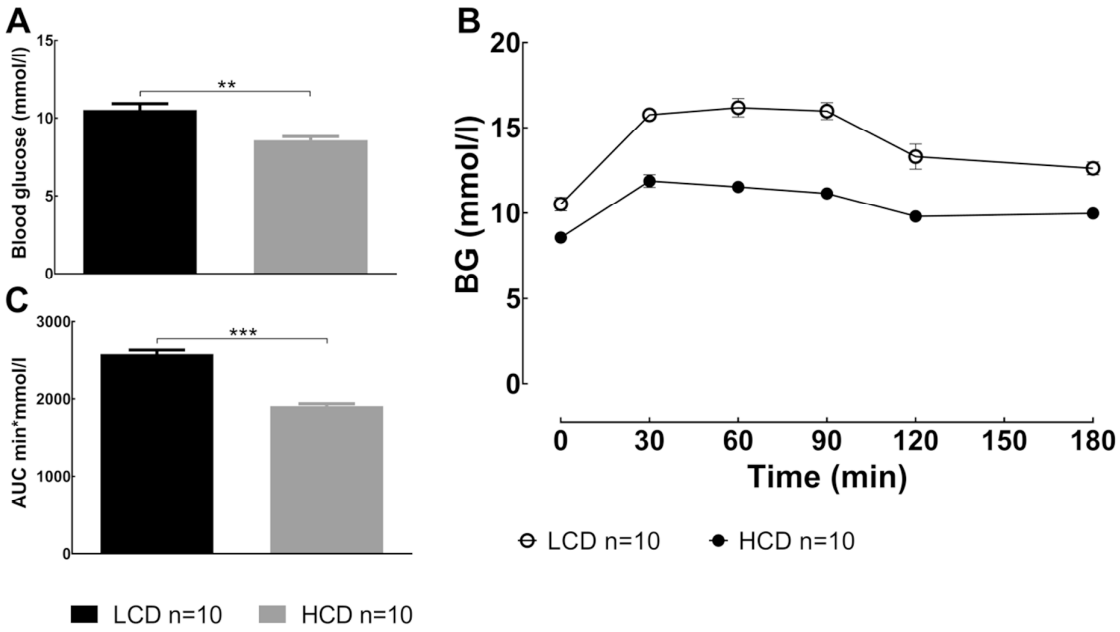
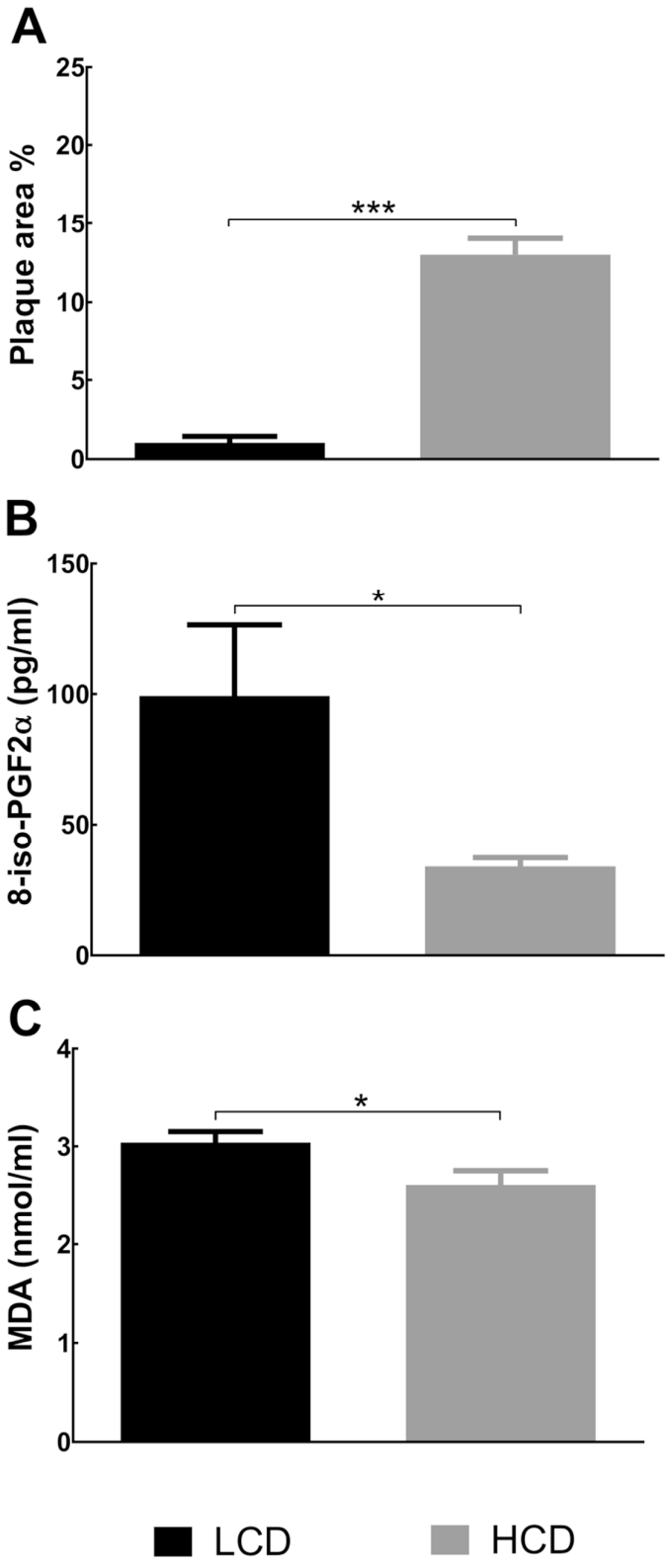



Figure 1. Dietary effect of two standard high fat diets on glucose metabolism in LDLR$^{(-/-)}$ mice after 16 weeks on either LCD or HCD. HCD, High cholesterol diet; LCD, Low cholesterol diet. Fasting blood glucose (A) was measured just prior to the beginning of the oral glucose tolerance test (OGTT) after a five hour fast. The OGTT (B) was performed by administration glucose to the animals (2 g/kg) and taking blood samples at 0 (prior to dosing), 30, 60, 90, 120 and 180 minutes after dosing. The area under the curve (AUC) (C) was calculated from the blood glucose measures from the OGTT. Data are compared using two-tailed unpaired t-tests and expressed as mean ± SEM, n= 10. **P<0.01, ***P<0.001.


A
Plaque area %
25
20
15
10
5
0
***
B
8-iso-PGF2α (pg/ml)
150
100
50
0
*
C
MDA (nmol/ml)
4
3
2
1
0
*
LCD
HCD

844

Figure 2. Effects of diet on plaque area and markers of lipid oxidation in plasma., HCD, High cholesterol diet; 8-iso-prostaglandin F2α, 8-iso-$PGF_{2\alpha}$; LCD, Low cholesterol diet; MDA, Malondialdehyde. Plaque area was determined by *en face* method and represented as percentage of aorta covered by plaques (A). Lipid oxidation was measured in plasma as malondialdehyde (MDA) (B) and 8-iso-$PGF_{2\alpha}$ (C). The liver was histologically evaluated by oil-red-o staining and data is given as the percentage of oil-red-o stained liver (D). Data are compared using two-tailed unpaired t-tests and represented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001.

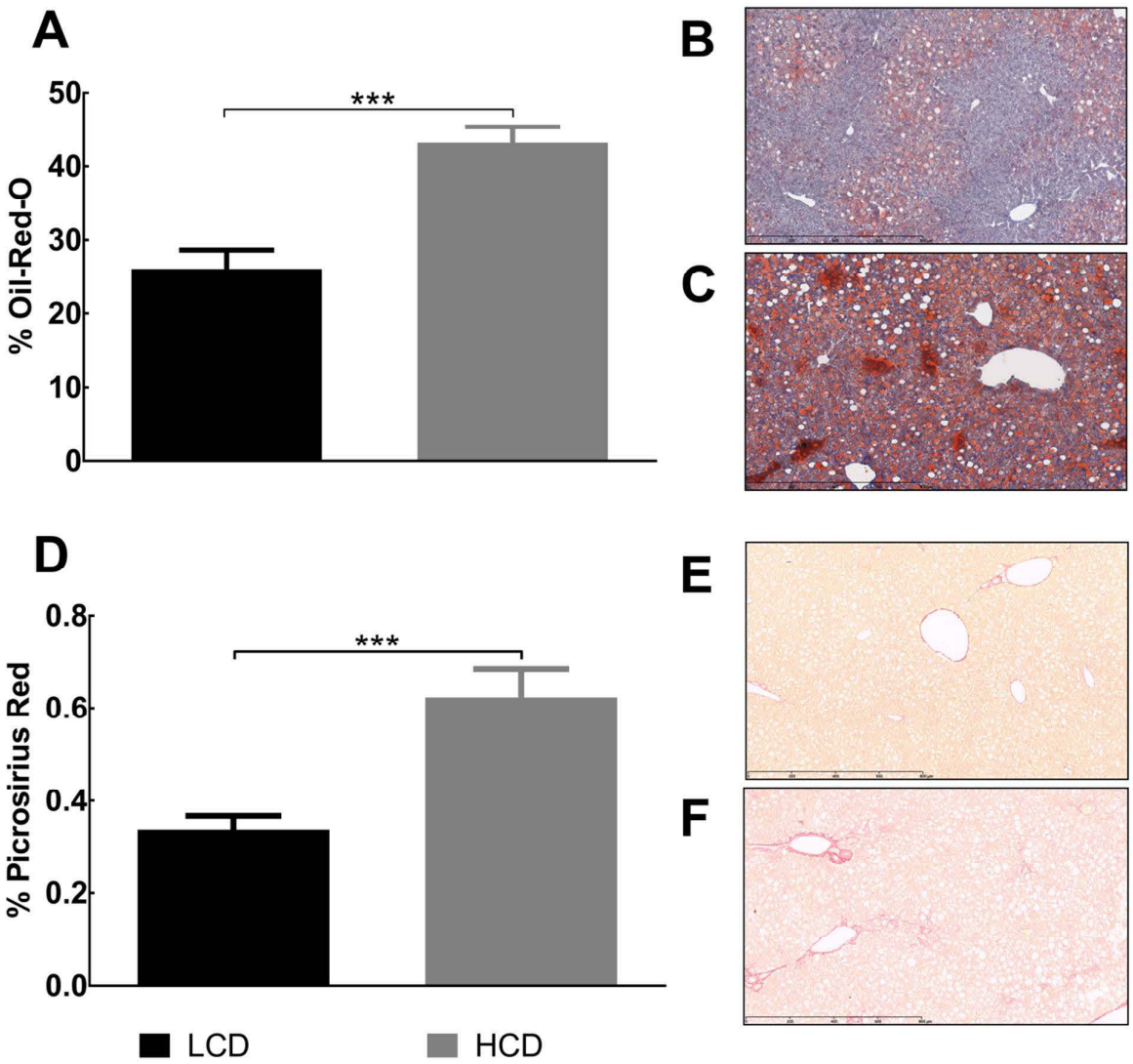
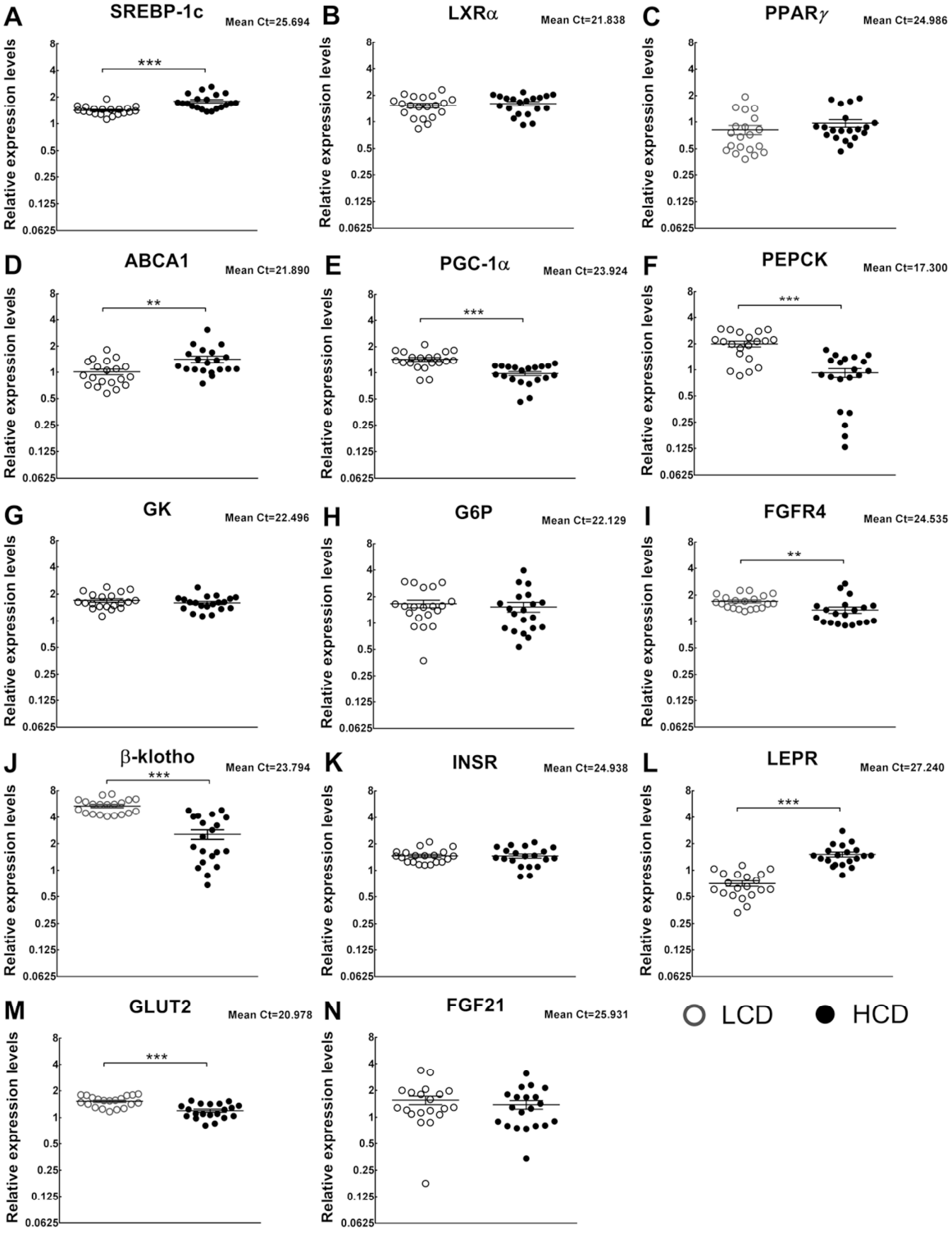



Figure 3. Hepatic lipid accumulation and collagen content. HCD, High cholesterol diet; LCD, Low cholesterol diet. The liver was histologically evaluated by Oil Red O staining and data is given as the percentage of Oil Red O stained liver (A). Histological pictures of the Oil Red O stained liver tissue from the LCD group (B) and the HCD group (C). Collagen content was evaluated by Picrosirius Red staining and evaluation of fibrosis was made from percentage of stained liver tissue (D). Histological pictures of the Picrosirius Red stained liver tissue from the LCD group (E) and the

858 HCD group (F). Scale bars for the histological pictures are from 0-800 µm. Data are compared using two-tailed
859 unpaired t-tests and represented as mean ± SEM. ***P<0.001.


A
SREBP-1c
Mean Ct=25.694
B
LXRα
Mean Ct=21.838
C
PPARγ
Mean Ct=24.986
D
ABCA1
Mean Ct=21.890
E
PGC-1α
Mean Ct=23.924
F
PEPCK
Mean Ct=17.300
G
GK
Mean Ct=22.496
H
G6P
Mean Ct=22.129
I
FGFR4
Mean Ct=24.535
J
β-klotho
Mean Ct=23.794
K
INSR
Mean Ct=24.938
L
LEPR
Mean Ct=27.240
M
GLUT2
Mean Ct=20.978
N
FGF21
Mean Ct=25.931
Relative expression levels
8
4
2
1
0.5
0.25
0.125
0.0625
***
**
LCD
HCD

860

Figure 4. Hepatic gene expression after 16 weeks of either LCD or HCD in LDLR$^{(-/-)}$ mice. ABCA1, ATP-binding cassette transporter; FGF21, Fibroblast growth factor 21; FGFR4, Fibroblast growth factor receptor 4; GK, glucokinase; GLUT2, glucose transporter 2; G6Pase, glucose-6-phosphatase; HCD, High cholesterol diet with cholesterol; INSR, insulin receptor; LCD, Low cholesterol diet; LEPR, leptin receptor; LXRα, liver X receptor α; PEPCK, phosphoenolpyruvate carboxykinase; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator 1α; PPARγ, peroxisome proliferator-activated receptor γ; SREBP-1c, Sterol regulatory element-binding protein 1c. RT-PCR was performed on liver tissue and the data from each outcome gene was normalized to three reference genes. Data are compared using permutation tests and presented as means±SEM. **P<0.01, ***P<0.001.

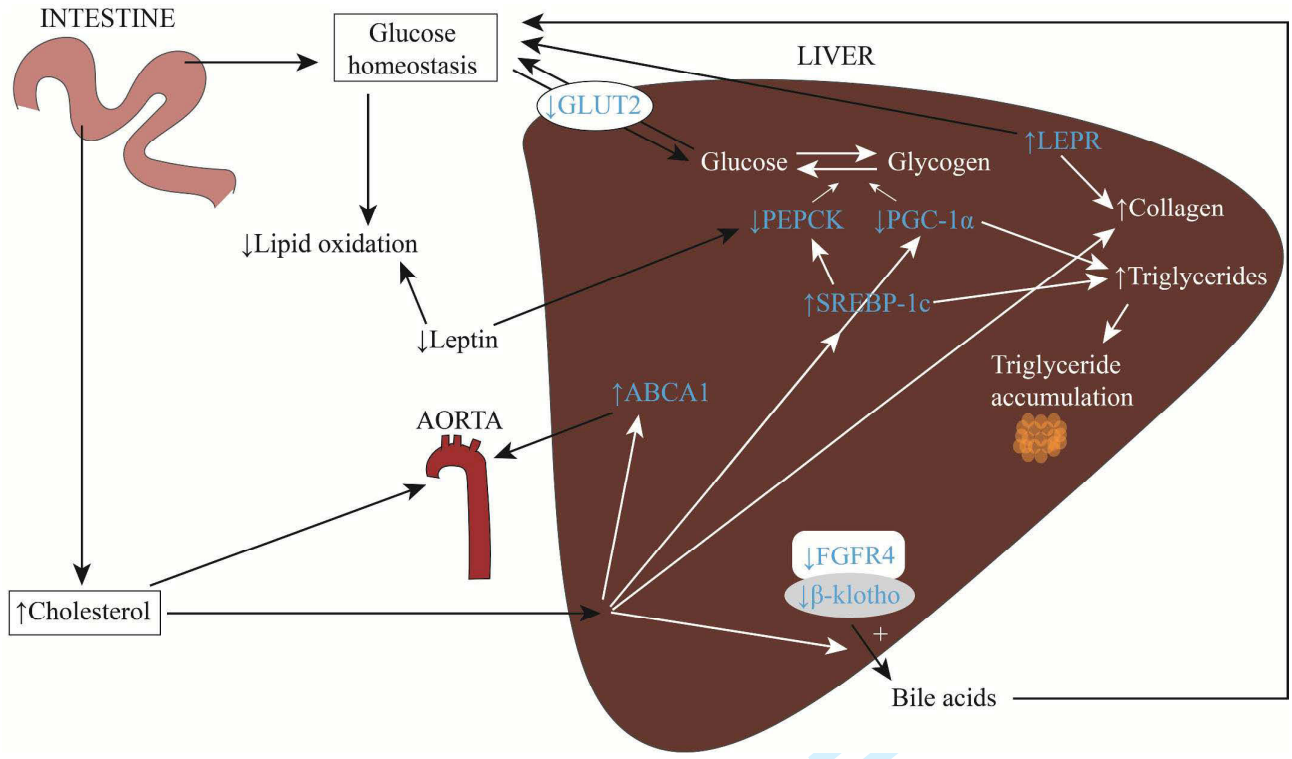



Figure 5. Effects of cholesterol in the HCD group. ABCA1, ATP-binding cassette transporter; FGFR4, Fibroblast growth factor receptor 4; GLUT2, glucose transporter 2; LEPR, leptin receptor; PEPCK, phosphoenolpyruvate carboxykinase; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator 1α; SREBP-1c, Sterol regulatory element-binding protein 1c. Glucose is taking up from the intestines and part of it is transported to the liver where it is taking up and stored as glycogen. Gluconeogenesis is important in the regulation of glucose homeostasis and dietary cholesterol can increase the expression of SREBP-1c, and by this mean down-regulate the expression of gluconeogenic genes (in the present experiment PEPCK and PGC-1α was down-regulated in the HCD group). The up-regulation of SREBP-1c and the down-regulation of PGC-1α can increase hepatic triglyceride accumulation. Cholesterol has been linked to fibrosis e.g. by accumulation of cholesterol in stellate cells or the presence of cholesterol crystals in lipid droplets leading to activation of Kupffer cells [124]. Increased leptin signalling could explain some of the fibrosis as well. As described in the text the degree of lipid oxidation is not reflecting the plaque degree in the present study, and the better glucose homeostasis in HCD group might explain the lower concentration of MDA and 8-iso-$PGF_{2\alpha}$ in this group,

where cholesterol might be responsible for the higher degree of plaque formation. ABCA1 expression is increased as well, which could contribute to the increased plaque formation. The role of bile acids in glucose metabolism is an interesting topic. In HCD group in this study the increased cholesterol together with lower expression of FGFR4 and β-klotho have the potential to increase bile acid production, and thus contribute to the better glucose homeostasis. Blue lettering indicates mRNA levels.

889 **Appendix 1: Fatty acid profile in the LCD diet (D12451)**

## DIO Series Fatty Acid Profile

Research Diets, Inc.
11/15/11

Typical Fatty Acid Composition of Fats used by Research Diets, Inc.

| | D12450B | D12451 | D12492 |
|---|---|---|---|
| Ingredient (gm) | | | |
| Lard | 20 | 177.5 | 245 |
| Soybean Oil | 25 | 25 | 25 |
| **Total** | **45** | **202.5** | **270** |
| C2, Acetic | 0 | 0 | 0 |
| C4, Butyric | 0 | 0 | 0 |
| C6, Caproic | 0 | 0 | 0 |
| C8, Caprylic | 0 | 0 | 0 |
| C10, Capric | 0.0 | 0.1 | 0.1 |
| C12, Lauric | 0.0 | 0.2 | 0.2 |
| C14, Myristic | 0.2 | 2.0 | 2.8 |
| C14:1, Myristoleic | 0 | 0 | 0 |
| C15 | 0.0 | 0.1 | 0.2 |
| C16, Palmitic | 6.5 | 36.9 | 49.9 |
| C16:1, Palmitoleic | 0.3 | 2.4 | 3.4 |
| C16:2 | 0 | 0 | 0 |
| C16:3 | 0 | 0 | 0 |
| C16:4 | 0 | 0 | 0 |
| C17 | 0.1 | 0.7 | 0.9 |
| C17:1 | 0 | 0 | 0 |
| C18, Stearic | 3.1 | 19.8 | 26.9 |
| C18:1, Oleic | 12.6 | 64.4 | 86.6 |
| C18:2, Linoleic, n-6 | 18.3 | 56.7 | 73.1 |
| C18:3, Linolenic, n-3 | 2.2 | 4.3 | 5.2 |
| C18:4, Stearidonic, n-3 | 0 | 0 | 0 |
| C20, Arachidic | 0.0 | 0.3 | 0.4 |
| C20:1, | 0.1 | 1.1 | 1.5 |
| C20:2 | 0.2 | 1.4 | 2.0 |
| C20:3, n-6 | 0.0 | 0.2 | 0.3 |
| C20:4, Arachidonic, n-6 | 0.1 | 0.5 | 0.7 |
| C20:5, Eicosapentaenoic, n-3 | 0 | 0 | 0 |
| C21:5, n-3 | 0 | 0 | 0 |
| C22, Behenic | 0 | 0 | 0 |
| C22:1, Erucic | 0 | 0 | 0 |
| C22:4, Clupanodonic, n-6 | 0 | 0 | 0 |
| C22:5, Docosapentaenoic, n-3 | 0.0 | 0.2 | 0.2 |
| C22:6, Docosahexaenoic, n-3 | 0 | 0 | 0 |
| C24, Lignoceric | 0 | 0 | 0 |
| C24:1 | 0 | 0 | 0 |
| **Total (g)** | **43.7** | **191.3** | **254.5** |
| Saturated (g) | 9.9 | 60.0 | 81.5 |
| Monounsaturated (g) | 13.0 | 68.0 | 91.5 |
| Polyunsaturated (g) | 20.7 | 63.3 | 81.5 |
| Saturated (%) | 22.7 | 31.4 | 32.0 |
| Monounsaturated (%) | 29.9 | 35.5 | 35.9 |
| Polyunsaturated (%) | 47.4 | 33.1 | 32.0 |

Research Diets, Inc.
20 Jules Lane
New Brunswick, NJ 08901 USA
info@researchdiets.com

DIO FA Prof 11-11

890 **Appendix 2. Fatty acid profile in the HCD diet (D12079B)**


# Fatty Acid Profile

RESEARCH DIETS INC.
www.ResearchDiets.com
20 Jules Lane
New Brunswick, NJ 08901
p: 732-247-2390 f: 732-247-2340
info@researchdiets.com

## Typical Fatty Acid Composition of D12079B

| | D12079B |
|---|---|
| **Ingredient** | |
| Butter, Anhydrous | 200 |
| Corn Oil | 10 |
| | |
| Total | |
| | |
| C2, Acetic | 0.0 |
| C4, Butyric | 6.4 |
| C6, Caproic | 3.8 |
| C8, Caprylic | 2.2 |
| C10, Capric | 5.0 |
| C12, Lauric | 5.6 |
| C14, Myristic | 20.0 |
| C14:1, Myristoleic | 3.0 |
| C16, Palmitic | 53.5 |
| C16:1, Palmitoleic | 4.6 |
| C18, Stearic | 24.4 |
| C18:1, Oleic | 52.7 |
| C18:2, Linoleic | 10.6 |
| C18:3, Linolenic | 2.9 |
| C18:4 | 0.0 |
| C20, Arachidic | 1.9 |
| C20:1, | 0.0 |
| C20:4, Arachidonic | 0.0 |
| C20:5, | 0.0 |
| C22, Behenic | 0.0 |
| C22:1, Erucic | 0.0 |
| C22:4, Clupanodonic | 0.0 |
| C22:5 | 0.0 |
| C22:6, | 0.0 |
| C24, Lignoceric | 0.0 |
| | |
| Total | 196.6 |
| | |
| Saturated (g) | 122.8 |
| Monounsaturated (g) | 60.3 |
| Polyunsaturated (g) | 13.6 |
| | |
| | |
| Saturated (%) | 62.4 |
| Monounsaturated (%) | 30.7 |
| Polyunsaturated (%) | 6.9 |
| | |
| Omega-6 Fatty Acid (gm) | 10.61 |
| Omega-3 Fatty Acid (gm) | 2.9 |
| Omega6:Omega 3 ratio | 3.6 |

D12079B FA content



891
892

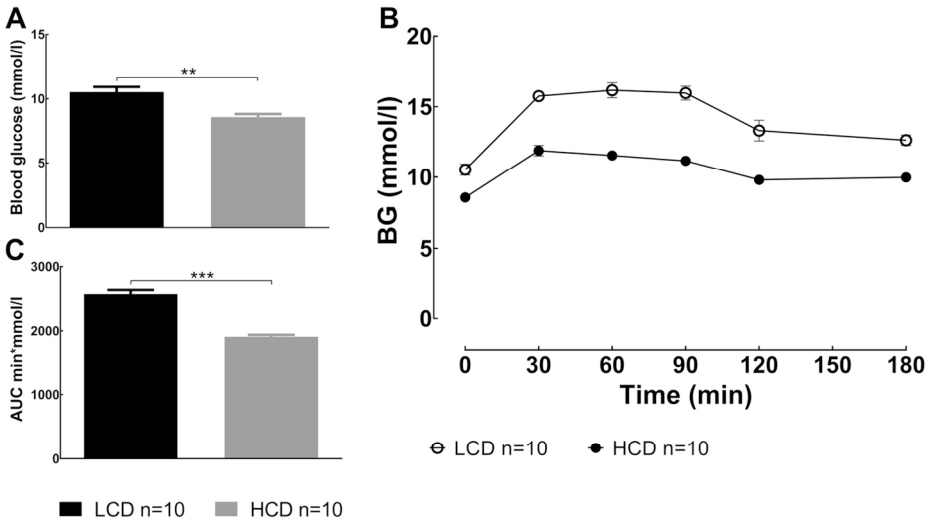



Figure 1. Dietary effect of two standard high fat diets on glucose metabolism in LDLR(-/-) mice after 16 weeks on either LCD or HCD. HCD, High cholesterol diet; LCD, Low cholesterol diet. Fasting blood glucose (A) was measured just prior to the beginning of the oral glucose tolerance test (OGTT) after a five hour fast. The OGTT (B) was performed by administration glucose to the animals (2 g/kg) and taking blood samples at 0 (prior to dosing), 30, 60, 90, 120 and 180 minutes after dosing. The area under the curve (AUC) (C) was calculated from the blood glucose measures from the OGTT. Data are compared using two-tailed unpaired t-tests and expressed as mean ± SEM, n= 10. **P<0.01, ***P<0.001.
figure 1
133x73mm (300 x 300 DPI)

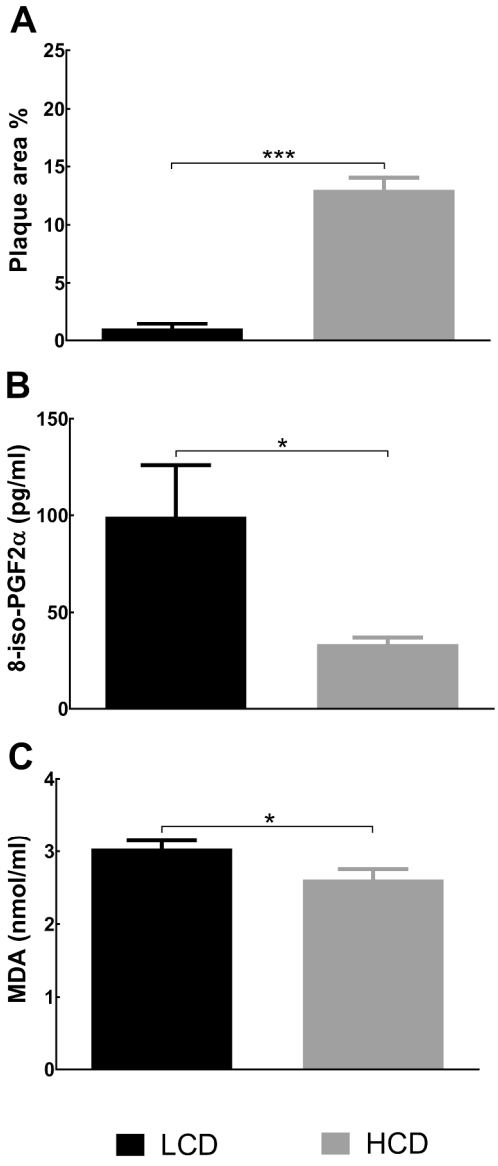



Figure 2. Effects of diet on plaque area and markers of lipid oxidation in plasma., HCD, High cholesterol diet; 8 iso-prostaglandin F2α, 8 iso-PGF2α; LCD, Low cholesterol diet; MDA, Malondialdehyde. Plaque area was determined by en face method and represented as percentage of aorta covered by plaques (A). Lipid oxidation was measured in plasma as malondialdehyde (MDA) (B) and 8 iso-PGF2α (C). The liver was histologically evaluated by oil red o staining and data is given as the percentage of oil red o stained liver (D). Data are compared using two-tailed unpaired t tests and represented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001.
figure 2
230x505mm (300 x 300 DPI)

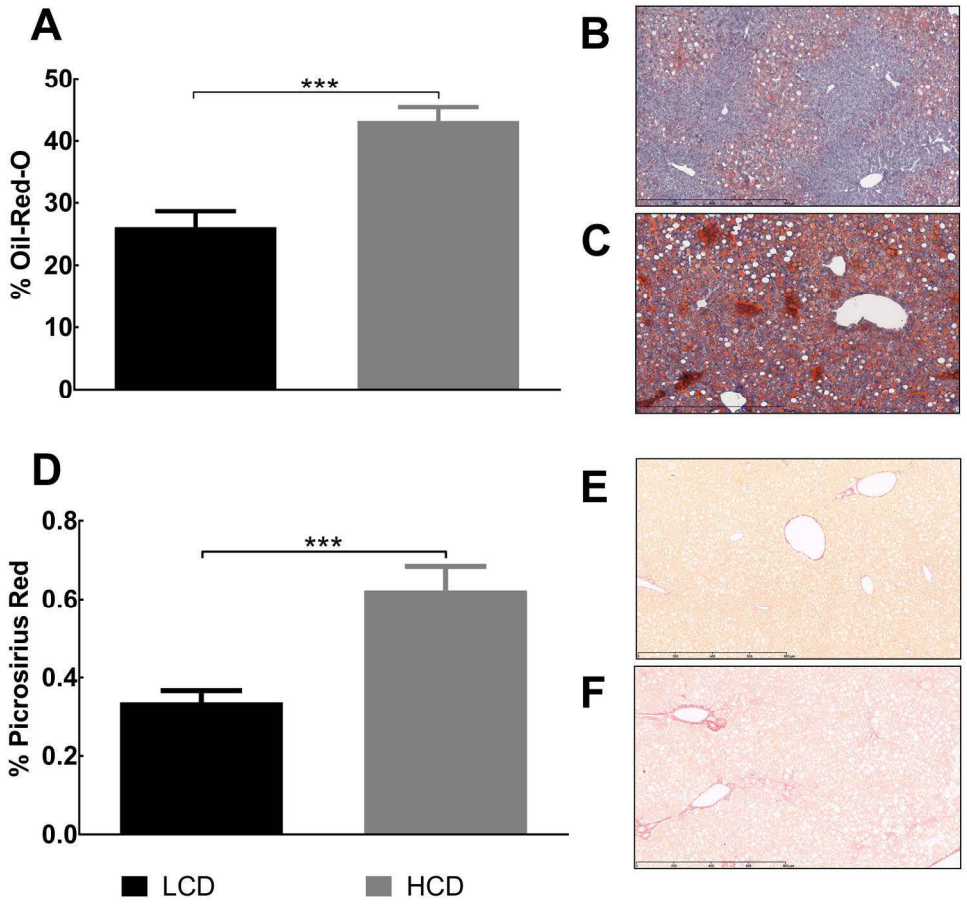



Figure 3. Hepatic lipid accumulation and collagen content. HCD, High cholesterol diet; LCD, Low cholesterol diet. The liver was histologically evaluated by Oil Red O staining and data is given as the percentage of Oil Red O stained liver (A). Histological pictures of the Oil Red O stained liver tissue from the LCD group (B) and the HCD group (C). Collagen content was evaluated by Picrosirius Red staining and evaluation of fibrosis was made from percentage of stained liver tissue (D). Histological pictures of the Picrosirius Red stained liver tissue from the LCD group (E) and the HCD group (F). Scale bars for the histological pictures are from 0-800 µm. Data are compared using two-tailed unpaired t-tests and represented as mean ± SEM. ***P<0.001.
figure 3
196x184mm (300 x 300 DPI)

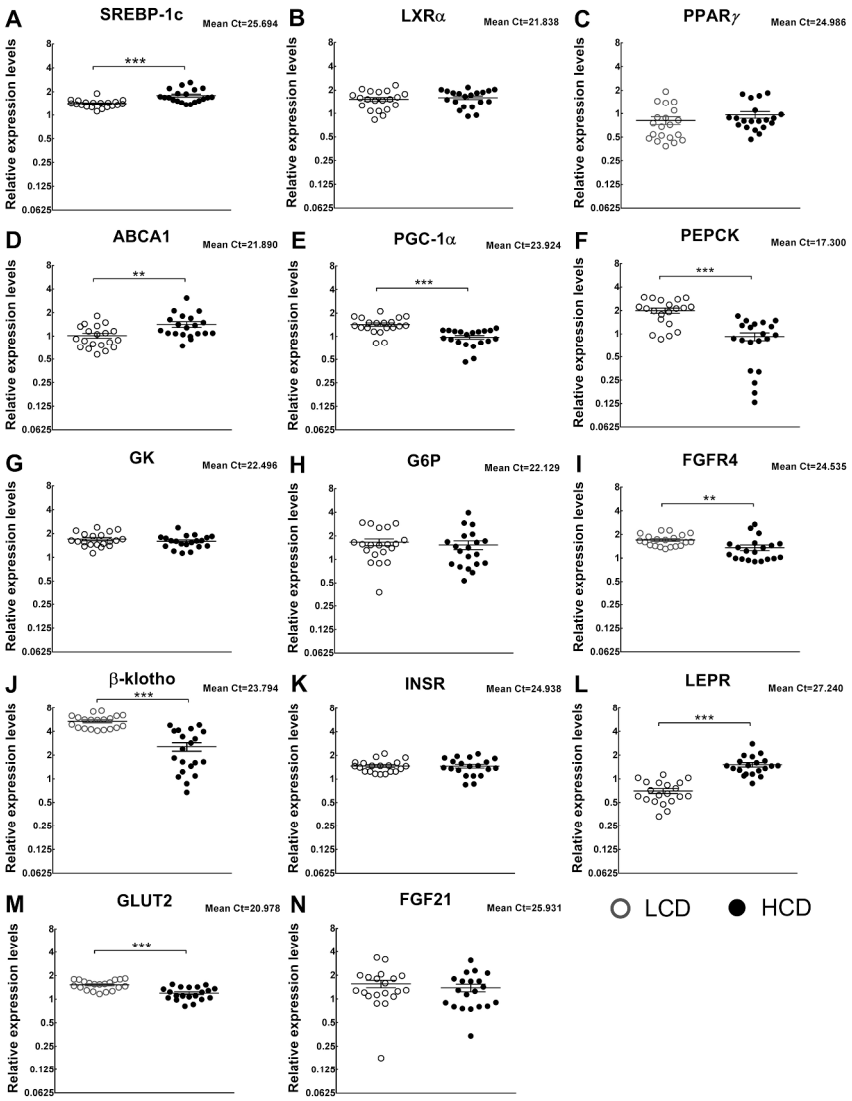



Figure 4. Hepatic gene expression after 16 weeks of either LCD or HCD in LDLR(-/-) mice. ABCA1, ATP-binding cassette transporter; FGF21, Fibroblast growth factor 21; FGFR4, Fibroblast growth factor receptor 4; GK, glucokinase; GLUT2, glucose transporter 2; G6Pase, glucose-6-phosphatase; HCD, High cholesterol diet with cholesterol; INSR, insulin receptor; LCD, Low cholesterol diet; LEPR, leptin receptor; LXRα, liver X receptor α; PEPCK, phosphoenolpyruvate carboxykinase; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator 1α; PPARγ, peroxisome proliferator-activated receptor γ; SREBP-1c, Sterol regulatory element-binding protein 1c. RT-PCR was performed on liver tissue and the data from each outcome gene was normalized to three reference genes. Data are compared using permutation tests and presented as means±SEM. **P<0.01, ***P<0.001.

figure 4
287x390mm (300 x 300 DPI)

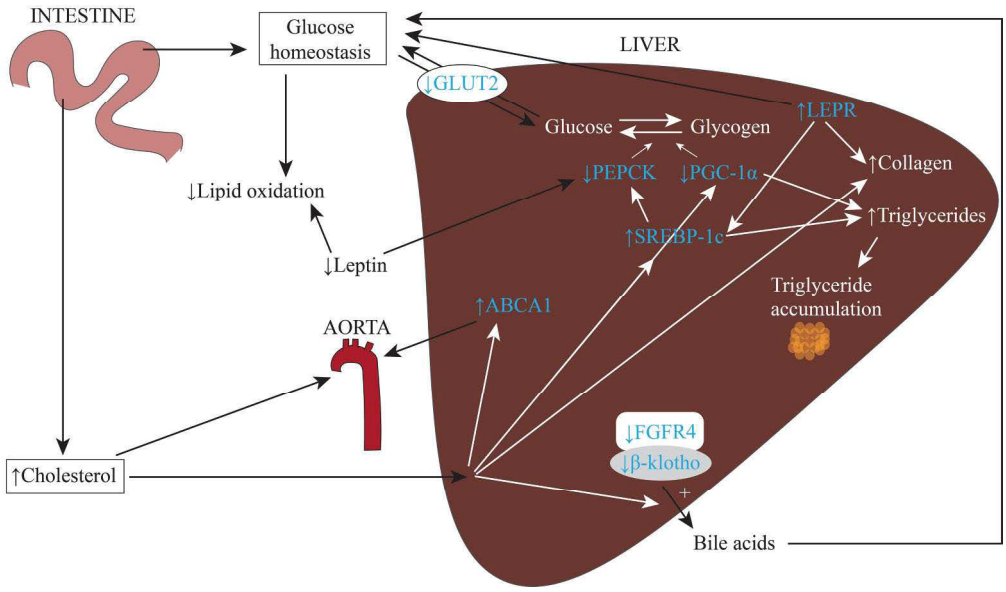



Figure 5. Effects of cholesterol in the HCD group. ABCA1, ATP-binding cassette transporter; FGFR4, Fibroblast growth factor receptor 4; GLUT2, glucose transporter 2; LEPR, leptin receptor; PEPCK, phosphoenolpyruvate carboxykinase; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator 1α; SREBP-1c, Sterol regulatory element-binding protein 1c. Glucose is taking up from the intestines and part of it is transported to the liver where it is taking up and stored as glycogen. Gluconeogenesis is important in the regulation of glucose homeostasis and dietary cholesterol can increase the expression of SREBP-1c, and by this mean down-regulate the expression of gluconeogenic genes (in the present experiment PEPCK and PGC-1α was down-regulated in the HCD group). The up-regulation of SREBP-1c and the down-regulation of PGC-1α can increase hepatic triglyceride accumulation. Cholesterol has been linked to fibrosis e.g. by accumulation of cholesterol in stellate cells or the presence of cholesterol crystals in lipid droplets leading to activation of Kupffer cells (124). Increased leptin signalling could explain some of the fibrosis as well. As described in the text the degree of lipid oxidation is not reflecting the plaque degree in the present study, and the better glucose homeostasis in HCD group might explain the lower concentration of MDA and 8-iso-PGF2α in this group, where cholesterol might be responsible for the higher degree of plaque formation. ABCA1 expression is increased as well, which could contribute to the increased plaque formation. The role of bile acids in glucose metabolism is an interesting topic. In HCD group in this study the increased cholesterol together with lower expression of FGFR4 and β-klotho have the potential to increase bile acid production, and thus contribute to the better glucose homeostasis. Blue lettering indicates mRNA levels.
figure 5
238x138mm (300 x 300 DPI)

## Appendix 1: Fatty acid profile in the LCD diet (D12451)

### DIO Series Fatty Acid Profile

Research Diets, Inc.
11/15/11

Typical Fatty Acid Composition of Fats used by Research Diets, Inc.

| | D12450B | D12451 | D12492 |
|---|---|---|---|
| | | | |
| Ingredient (gm) | | | |
| Lard | 20 | 177.5 | 245 |
| Soybean Oil | 25 | 25 | 25 |
| | | | |
| **Total** | **45** | **202.5** | **270** |
| | | | |
| C2, Acetic | 0 | 0 | 0 |
| C4, Butyric | 0 | 0 | 0 |
| C6, Caproic | 0 | 0 | 0 |
| C8, Caprylic | 0 | 0 | 0 |
| C10, Capric | 0.0 | 0.1 | 0.1 |
| C12, Lauric | 0.0 | 0.2 | 0.2 |
| C14, Myristic | 0.2 | 2.0 | 2.8 |
| C14:1, Myristoleic | 0 | 0 | 0 |
| C15 | 0.0 | 0.1 | 0.2 |
| C16, Palmitic | 6.5 | 36.9 | 49.9 |
| C16:1, Palmitoleic | 0.3 | 2.4 | 3.4 |
| C16:2 | 0 | 0 | 0 |
| C16:3 | 0 | 0 | 0 |
| C16:4 | 0 | 0 | 0 |
| C17 | 0.1 | 0.7 | 0.9 |
| C17:1 | 0 | 0 | 0 |
| C18, Stearic | 3.1 | 19.8 | 26.9 |
| C18:1, Oleic | 12.6 | 64.4 | 86.6 |
| C18:2, Linoleic, n-6 | 18.3 | 56.7 | 73.1 |
| C18:3, Linolenic, n-3 | 2.2 | 4.3 | 5.2 |
| C18:4, Stearidonic, n-3 | 0 | 0 | 0 |
| C20, Arachidic | 0.0 | 0.3 | 0.4 |
| C20:1, | 0.1 | 1.1 | 1.5 |
| C20:2 | 0.2 | 1.4 | 2.0 |
| C20:3, n-6 | 0.0 | 0.2 | 0.3 |
| C20:4, Arachidonic, n-6 | 0.1 | 0.5 | 0.7 |
| C20:5, Eicosapentaenoic, n-3 | 0 | 0 | 0 |
| C21:5, n-3 | 0 | 0 | 0 |
| C22, Behenic | 0 | 0 | 0 |
| C22:1, Erucic | 0 | 0 | 0 |
| C22:4, Clupanodonic, n-6 | 0 | 0 | 0 |
| C22:5, Docosapentaenoic, n-3 | 0.0 | 0.2 | 0.2 |
| C22:6, Docosahexaenoic, n-3 | 0 | 0 | 0 |
| C24, Lignoceric | 0 | 0 | 0 |
| C24:1 | 0 | 0 | 0 |
| | | | |
| **Total (g)** | **43.7** | **191.3** | **254.5** |
| | | | |
| Saturated (g) | 9.9 | 60.0 | 81.5 |
| Monounsaturated (g) | 13.0 | 68.0 | 91.5 |
| Polyunsaturated (g) | 20.7 | 63.3 | 81.5 |
| | | | |
| Saturated (%) | 22.7 | 31.4 | 32.0 |
| Monounsaturated (%) | 29.9 | 35.5 | 35.9 |
| Polyunsaturated (%) | 47.4 | 33.1 | 32.0 |

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info@researchdiets.com

DIO FA Prof 11-11

**Appendix 2. Fatty acid profile in the HCD diet (D12079B)**


# Fatty Acid Profile

RESEARCH DIETS INC.
www.ResearchDiets.com
20 Jules Lane
New Brunswick, NJ 08901
p: 732-247-2390 f: 732-247-2340
info@researchdiets.com

## Typical Fatty Acid Composition of D12079B

| | D12079B |
|---|---|
| **Ingredient** | |
| Butter, Anhydrous | 200 |
| Corn Oil | 10 |
| | |
| Total | |
| | |
| C2, Acetic | 0.0 |
| C4, Butyric | 6.4 |
| C6, Caproic | 3.8 |
| C8, Caprylic | 2.2 |
| C10, Capric | 5.0 |
| C12, Lauric | 5.6 |
| C14, Myristic | 20.0 |
| C14:1, Myristoleic | 3.0 |
| C16, Palmitic | 53.5 |
| C16:1, Palmitoleic | 4.6 |
| C18, Stearic | 24.4 |
| C18:1, Oleic | 52.7 |
| C18:2, Linoleic | 10.6 |
| C18:3, Linolenic | 2.9 |
| C18:4 | 0.0 |
| C20, Arachidic | 1.9 |
| C20:1, | 0.0 |
| C20:4, Arachidonic | 0.0 |
| C20:5, | 0.0 |
| C22, Behenic | 0.0 |
| C22:1, Erucic | 0.0 |
| C22:4, Clupanodonic | 0.0 |
| C22:5 | 0.0 |
| C22:6, | 0.0 |
| C24, Lignoceric | 0.0 |
| | |
| Total | 196.6 |
| | |
| Saturated (g) | 122.8 |
| Monounsaturated (g) | 60.3 |
| Polyunsaturated (g) | 13.6 |
| | |
| | |
| Saturated (%) | 62.4 |
| Monounsaturated (%) | 30.7 |
| Polyunsaturated (%) | 6.9 |
| | |
| Omega-6 Fatty Acid (gm) | 10.61 |
| Omega-3 Fatty Acid (gm) | 2.9 |
| Omega6:Omega 3 ratio | 3.6 |

D12079B FA content