UNIVERSITÀ
DI SIENA
1240

DEPARTMENT OF BIOTECHNOLOGY, CHEMISTRY AND PHARMACY

PhD in Biochemistry and Molecular Biology (BiBiM 2.0)

Lipidomic analysis to identify markers of the beneficial effects of Pioglitazone treatment on fatty liver disease

Relatrice
Dott.sa Amalia Gastaldelli

Tesi di dottorato di
Marco Russo

Marco Russo

Firmato il 01/05/2022

Anno accademico 2021/2022

LIST OF CONTENTS

ACKNOWLODGEMENTS

During the years of my PhD, I have been guided, supported and encouraged by many people to whom I owe a debt of sincere gratitude.

I've been honoured to be supervised by an outstanding researcher and professor, Dr. Amalia Gastaldelli, who believed in my potential and provided me all the tools in order to work on my research.

I would like also to express a profound gratitude to the lab group; starting from the seniors of the lab: Fabrizia Carli, Melania Gaggini and Demetrio Ciociaro, phenomenally qualified, altruistic and kind people without which I would have never been able to reach the goals I've been proposed to; I want to thank also my former colleagues Chiara Barbieri, Samantha Pezzica, Martina Nassisi, Gabriele Mocciaro and Silvia Sabatini.

Special mention to Sara Guerra and Barbara Patricio, more than just colleagues also flatmates and dear friends, their support and friendship have been invaluable.

I also want to thank Clinical Medicine and Surgery, University Federico II group, in particular Prof. Angela Albarosa Rivellese, my former supervisor during my Master degree and member of the scientific committee of TOSCA.it trial.

Last but not least, I would like to thank my parents, my brother and my girlfriend Carla for their endless love and affection.

ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is a metabolic disease that starting from hepatic fat accumulation (NAFL) can progress into more severe forms, i.e., non-alcoholic steatohepatitis (NASH), cirrhosis, hepatocellular carcinoma.

The derangement in lipid metabolism, either synthesis and accumulation of hepatic TG and secretion of lipotoxic compounds, e.g., ceramides, drives the progression of NAFLD to more severe forms. The prevalence of NAFLD is estimated to be 25% in the general population but increases to more than 55% in subjects with type 2 diabetes. It is therefore important to study the impact antidiabetic drugs on the indexes of NAFLD and the lipidomic profile. Among the most commonly used diabetes medications are metformin (MET) and sulfonylureas (SUL). The PPAR gamma agonist pioglitazone (PIO) is the most suitable antidiabetic treatment to reduce lipotoxicity and glucotoxicity since it significantly reduces hyperglycemia and peripheral lipolysis promoting the increase of subcutaneous adipose tissue but significantly reducing visceral fat and liver steatosis. However, not many studies compared the effects of PIO vs sulphonylurea (SUL) on adipose tissue insulin resistance, lipid composition and metabolism and scores of NAFLD in patients with type 2 diabetes.

Thus, the goal of my thesis was to study the impact of hepatic steatosis of metabolic origin (NAFLD / MAFLD) on the circulating lipidome in a population of subjects with type 2 diabetes treated with metformin but with poor glycemic control and then study the metabolic effects of the correction of glycemic control by adding pioglitazone or sulfonylurea; in particular I have investigated the effects on NAFLD / NASH scores, on insulin resistance indices and on the components of the lipidomic profile. The analyses were performed in a group of subjects that participated to the “Thiazolidinediones Or

Sulfonylureas Cardiovascular Accidents - Intervention Trial" (TOSCA.IT). One-year treatment with pioglitazone even at low dosage significantly improved liver steatosis and inflammation, systemic and adipose tissue insulin resistance in patients with T2D. Only PIO improved the lipidomic profile of subjects with MAFLD at baseline. The beneficial effects of pioglitazone on NAFLD/MAFLD were independent of blood glucose control.

To further explore the mechanism of action of pioglitazone I studied the effect of pioglitazone on difference in lipidomic and *de novo* synthesis in different adipose tissues and liver of mice fed with high sugar diet. Remarkably, pioglitazone induces a reduction of de novo lipogenesis (DNL) and desaturation in mesenteric adipose tissue and triglycerides associated with DNL in liver.

In conclusion, by using lipidomics and fluxomics, we demonstrated that pioglitazone, even at low dosage, exerts positive effects on both glucotoxicity and lipotoxicity by ameliorating insulin resistance and inducing a remodelling of adipose tissue depots.

List of Abbreviations or Acronyms

ADIPO-IR – adipose tissue insulin resistance index

ALT - alanine transaminase

AST - aspartate transaminase

BMI – Body mass index

CER - ceramides

DAG - diacylglycerol

DNL – *de novo* lipogenesis

FFA – Free fatty acids

FLI – fatty liver index

HDL – high density lipoprotein

HF – high fat

HFG – High Sugar Diet

HFG+PIO – High Sugar Diet + Pioglitazone

HOMA-IR – Homeostasis model assessment: insulin resistance

IR – insulin resistance

LDL – low density lipoprotein

LysoPC – lysophosphatidylcholine

LysoPE – lysophosphatidylethanolamine

MAFLD – Metabolic Dysfunction-Associated Fatty Liver Disease

MUFA – monounsaturated fatty acid

NAFLD – non-alcoholic fatty liver disease

NASH – non-alcoholic steatohepatitis

NEFA – non-esterified fatty acid

NMR – Nuclear magnetic resonance

PC – phosphatidylcholine

PE – phosphatidylethanolamine

PIO – Pioglitazone

PPAR gamma - peroxisome proliferator-activated receptor δ

PUFA – polyunsaturated fatty acid

ROS – reactive oxygen species

SFA – saturated fatty acid

SM – sphingomyelin

SUL – Sulfonylurea

TAG – triacylglycerol

TZD – Thiazolidinediones

UFA – unsaturated fatty acids

List of Figures

Figure 12. Heatmap of the fold changes from baseline of single lipid species associated to cardiometabolic diseases analyzed in MAFLD and noMAFLD groups and per treatment (PIO vs SUL)

Figure 13. (A) Body weight after 4 weeks of treatment. (B) OGTT profile in controls, HFG e HFG+PIO *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 vs controls; ANOVA.

Figure 14. PUFA, MUFA, UFA and SFA in different adipose tissues between the controls, HFG and HFG+PIO diets.

Figure 15. A) Liver Total TAGs, **B)** Total Ceramides and **C)** and **D)** single species correlated to NAFLD.

Figure 16. Adipose tissue fluxomic measured by NMR: **A)** DNL and **B)** Desaturation and **C)** Glyceroneogenesis in different adipose tissues. Difference between the controls, HFG and HFG+PIO diets

Figure 17. Deuterium enrichment (M+2/M+0 expressed as %) of liver TAGs in different groups after 5 days of D2O. TAGs 48:0, 50:0 and 52:0 are associated with DNL.

List of tables

1. Chapter 1

INTRODUCTION

1.1 Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is an increasingly hepatic condition defined as a metabolic disease characterized by the presence of ≥5% of hepatic steatosis in absence of excess alcohol consumption.

The term NAFLD includes a spectrum of liver diseases which also may progress into more severe forms such as inflammation, ballooning that may progress to end-stage cirrhosis and liver failure[1]. Hence, NAFLD has become one of the most common causes of chronic liver diseases and, as a result, liver transplants. **Figure 1.**

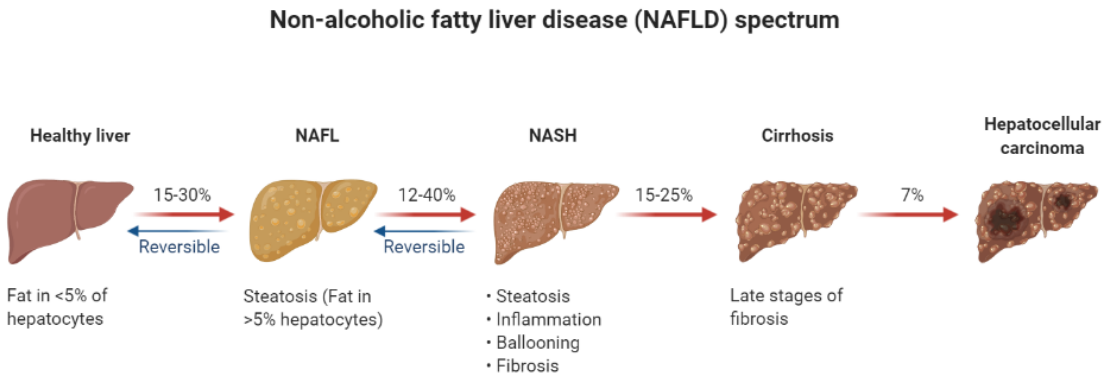


Figure 1. NAFLD progression from healthy liver to end stage irreversible state. (Figure was created with BioRender.com)

The global prevalence of NAFLD is estimated to range up to around 24%. Therefore, due to a subsequential increase of caloric intake associated with a raise of sedentary lifestyle the risk of NAFLD is significantly raising in the developed countries since it is highly correlated with diabetes type 2 (22.51%), obesity (51.34%) and hyperlipidemia (69.16%)

and metabolic syndrome (42.54%). The countries most affected by the emerging rates of NAFLD are South America and Middle East then Asia, USA, and Europe; meanwhile Africa has the lowest rate[2–5].

Originally NAFLD was considered as “the liver manifestation of the metabolic syndrome”[6] although more recent studies highlighted how NAFLD might be a strong predictor of T2D and metabolic syndrome[7] moreover recent studies have highlighted how hyperinsulinemia might be the consequence of NAFLD rather than the cause[8].

Back from 1998 it has been suggested a “two-hit” theory for the progression of NAFLD. The first “hit” would be an imbalance in hepatic lipid metabolism with an initial accumulation of lipids, mostly in the form of triglycerides, within hepatocytes. Therefore, the second “hit” suggested the activation of inflammatory pathways i.e., oxidative stress and a cytokine induction due to the imbalance itself that promotes the uptake and synthesis of fatty acids. Hence, those two hits would culminate into liver damage[9]. Nonetheless, more recent studies suggests that NAFLD is explained better by a "multiple hit” hypothesis where there is a reciprocal interaction between several factors (i.e., insulin resistance, hormones secreted from the adipose tissue, nutritional factors, gut microbiota and genetic and epigenetic) that predisposes the subject to NAFLD[10]. Eventually, a more specific model propose that fatty acids (FFAs) and its metabolites might be one of the causes of lipotoxicity that predisposes to NAFLD. The recruitment of FFAs caused by insulin resistance might eventually induce apoptosis and formation of reactive oxygen species. Hence, the cytochrome P450-dependent ω-oxidation of long chain FFAs caused either by T2D or FFAs overburden causes the induction of ROS and lipid peroxidation suggesting how FFAs accumulation might be one of the causes of NAFLD/NASH[11,12].

1.2 Pathogenesis of non-alcoholic fatty liver disease

The imbalance between the accumulation and catabolism of TG in the liver with the concomitant increase of availability and synthesis of hepatic FFAs might play a central role in the NAFLD pathogenesis even though the mechanisms are not fully understood. **Figure 2.** shows metabolic and pathophysiological processes that might contribute to NAFLD development[13].

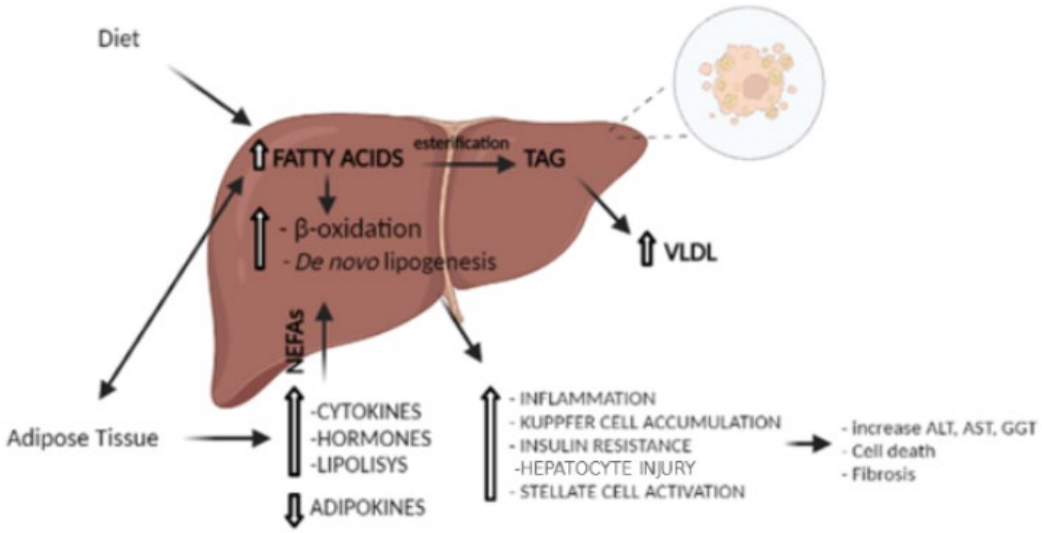


Figure 2. Schematic figure showing potential pathogenetic mechanisms involving intrahepatic lipid accumulation (Figure was created with BioRender.com)

1.3 Lipids and NAFLD

An unbalanced period of assumption of nutrients, i.e., dietary fats, might lead to an allosteric overburden that might be the primary cause of obesity, IR, metabolic syndrome, diabetes type 2 and other comorbidities[14]. Nowadays, Western dietary pattern, characterised by a high intake of saturated fatty acids (SFA), trans fat, refined carbohydrates and processed foods, the physical inactivity contributes increment intrahepatic lipids and risk of NAFLD[15,16]. Dietary fats are ingested and absorbed by

the intestine, assembled into chylomicrons, and released into circulation; approximately 80% of chylomicrons are hydrolyzed by lipoprotein lipase releasing constituent fatty acids and the remains are transported to the liver[17]. Those are transported as TAG via chylomicrons and absorbed as chylomicron remnants[18].

DNL contributes to very-low-density lipoproteins (VLDL) formation and may also contribute between 2 and 5% of VLDL-TAG production in healthy patients and 20 to 30% in pathophysiological ones[19–21]. The insulin resistance has been described to play a major role in the contribution of DNL[22]; it also has been demonstrated that its increase rate contributes to NAFLD [23].

Moreover, adipose tissue plays a major role in the source of non-esterified fatty acids (NEFA) which accumulate in the liver and are around 60% of the cause of TAG accumulation [19].

1.4 Metabolic Dysfunction-Associated Fatty Liver Disease (MAFLD) new nomenclature for metabolic associated fatty liver disease

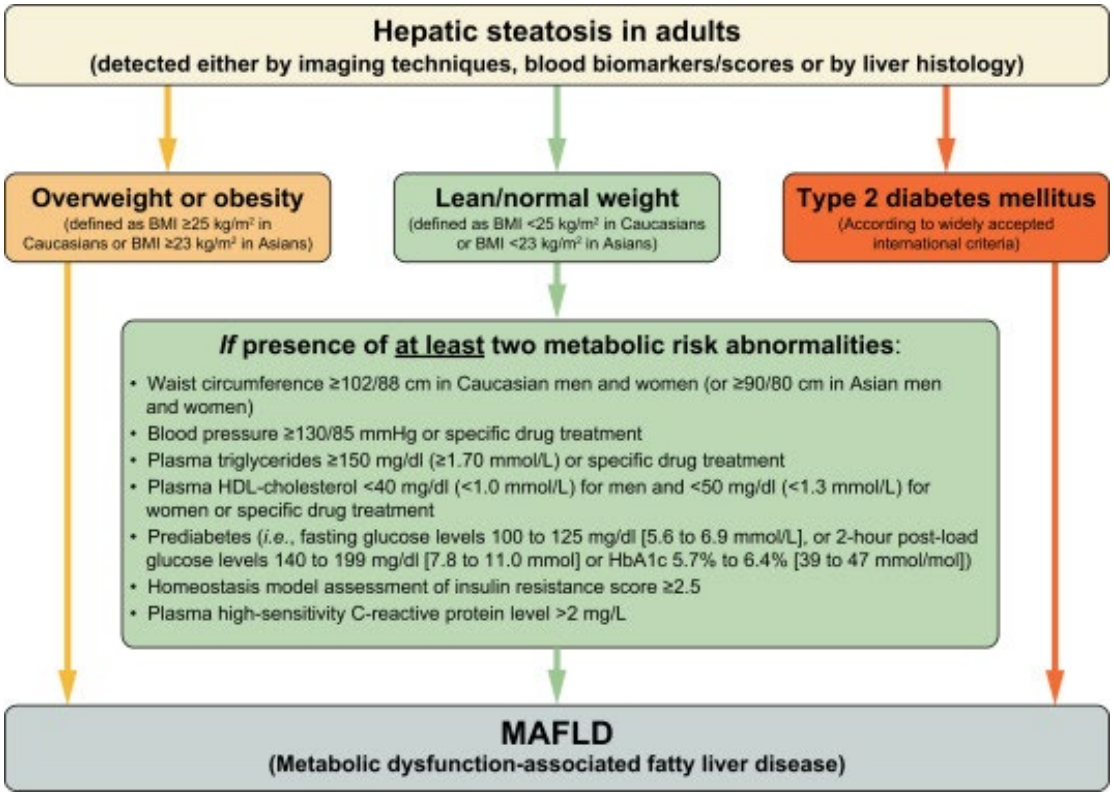
In 1980 Ludwig and colleagues formulated non-alcoholic fatty liver disease (NAFLD) term [24] in order to delineate the fatty liver disease deriving from a non-alcohol consumption circumstance.

Hence, alarming data leap to the eye from economic point of view. Therefore, Younossi at al. (2016) [25] estimated that in four Europe countries (Germany, France, Italy, and United Kingdom) there are more than 50 million of people affected with an economical burden of nearly €35 billion meanwhile in the US with an average of 60 million people the costs are around $103 billion.

Moreover, there is an increase trend of recognition of the pathology in youngers where obesity is a huge burden in recent years; *Wiegand, S., Keller, KM., Röbl, M. et al*[26] evidenced the association of NAFLD with BMI SDS and insulin in younger obese patients with a prevalence of 11%.

Therefore, since the huge heterogeneity of the phenotype of NAFLD/NASH patients an international consensus committee highlighted the need to redefine non-alcoholic fatty liver disease (NAFLD) terminology and suggested to be more appropriate to call it Metabolic Dysfunction-Associated Fatty Liver Disease (MAFLD) since the new terminology will better reflect its causes and improve public health initiatives[27].

Figure 3. Schematic figure showing potential pathogenetic mechanisms leading to MAFLD



1.5 The TOSCA.IT trial

Metformin has been recommended as the first-choice therapy for the treatment of patients with type 2 diabetes mellitus [28–32]. Meanwhile, considerable uncertainty exists in the choice of the second drug to be added in subjects with an inadequate compensation with metformin monotherapy. Hence, the addon of sulfonylurea (SUL) or pioglitazone (PIO) are therapeutic options equally effective in improving glycemic compensation [33]. However, these drugs differ greatly in the mechanism of action, side effects, and impact on cardiovascular (CV) risk factors. Therefore, SUL class (glibenclamide, glimepiride, gliclazide) have been on the market for several years and are the cheapest class of hypoglycemic drugs. These molecules work by stimulating insulin secretion by binding to ATP-sensitive potassium channels in pancreatic beta cells. Meanwhile, Thiazolidinediones (TZDs) are insulin-sensitizing agents which improves insulin sensitivity. TZDs exerts their effects through the activation of PPAR γ, a nuclear receptor involved in several metabolic process i.e., glucose and lipid metabolism [34].

The TOSCA.IT trial is a pragmatic study designed in 2006 and finished in 2016 with its first aim was to evaluate the effects of the two combinations of hypoglycemic treatments with metformin on the mortality rate, cardiovascular risk factors and the incidence of fatal and non-fatal cardiovascular events.

The main results were published in Lancet Diabetes Endocrinol in September 2017[35].

2. Chapter 2

Pioglitazone, even at low dosage, improves NAFLD in type 2 diabetes: clinical and pathophysiological insights from a subgroup of the TOSCA.IT randomised trial.

2.1 Introduction

Non-alcoholic fatty liver disease (NAFLD) is an ominous condition encompassing a wide range of liver histologic abnormalities, varying from simple triglyceride accumulation in hepatocytes (liver steatosis), non-alcoholic steatohepatitis (NASH), liver fibrosis, cirrhosis, and hepatocellular carcinoma [36]. NAFLD is a risk factor for type 2 diabetes (T2D) [37] and cardiovascular disease [38]. In addition, almost the totality of people with T2D has NAFLD [39,40], often in its most severe forms[41]. This epidemiological association reflects the fact that NAFLD and T2D share causative factors, pathophysiological mechanisms, and likely, possible therapeutic strategies [42].

Beyond weight loss and dietary advice focused on the reduction of simple sugars and saturated fats[43], no pharmacological treatment is currently recommended for NAFLD [44]. However, there is evidence that some glucose-lowering drugs are effective in reducing liver fat content and improving NASH [42].

Large, randomized placebo-controlled trials with an up 3-year follow-up have shown that high doses of pioglitazone (30-45 mg) reduce liver steatosis and ameliorate the histological abnormalities of NASH in ~ 60% of participants with T2D and biopsy-proven liver steatosis and inflammation [45–48]. Other authors report that pioglitazone reduces

fibrosis and prevents fibrosis progression in patients with T2D [49]. These beneficial effects are likely related to the insulin-sensitizing properties of pioglitazone that reduces insulin resistance in adipose tissue, muscle, and liver [50–52]. Despite these encouraging data, pioglitazone is not as widely used, probably due to safety issues concerning weight gain [45–48], congestive heart failure [53], pathological fractures [[54], and bladder cancer [55]. An accurate selection of the patients and the use of a low dose may minimize these risks [56–58]. However, the efficacy of low-dose pioglitazone on the progression of NAFLD are not known.

To explore possible effects of pioglitazone on NAFLD and related pathophysiological mechanisms we take advantage of data collected within the framework of the "Thiazolidinediones Or Sulphonylureas and Cardiovascular Accidents Intervention Trial (TOSCA.IT NCT00700856), a pragmatic trial designed to explore the long-term effects on cardiovascular events of add-on pioglitazone or a sulphonylurea, in patients with T2D insufficiently controlled with metformin [35].

Pioglitazone and sulphonylureas lowered blood glucose by a similar degree but target different mechanisms of hyperglycaemia. Indeed, sulphonylureas stimulate insulin secretion, while pioglitazone improves blood glucose control by ameliorating insulin-resistance. The randomized study design and the use of glucose-lowering drugs with profoundly different mechanisms of action make the TOSCA.IT trial a unique opportunity to test the efficacy of pioglitazone and its dose-effect on NAFLD compared with sulphonylureas and to study the role of insulin-resistance and blood glucose control as possible mechanisms.

On this background the aims of the present study are to evaluate in people with T2D 1) the effects of 1-year treatment with pioglitazone -even at low-dose -or sulphonylureas

on the evolution of NAFLD evaluated with indirect indices, and 2) the specific role of insulin-resistance and glucotoxicity in determining these effects.

The results of this chapter have been published:

Della Pepa G, Russo M*, Vitale M, Carli F, Vetrani C, Masulli M, et al. *Pioglitazone even at low dosage improves NAFLD in type 2 diabetes: clinical and pathophysiological insights from a subgroup of the TOSCA.IT randomised trial.* Diabetes Res Clin Pract 2021;178:108984.

2.2 Methods

2.2.1 Participants and study design

This study was conducted at the University Hospital of the Federico II University of Naples, one of the 57 centers participating in the TOSCA.IT trial, a multicenter randomized controlled trial designed to evaluate the cardiovascular effects of second line glucose-lowering drugs.

The study protocol and the main results have been published [35,59].

Briefly, patients with T2D, and without any acute or chronic hepatic disease [59], history of alcohol intake exceeding 30 g/day in men and 20 g/day in women [60], in the age range 50-75 years, insufficiently controlled (HbA1c 53–75 mmol/mol 7.0%–9.0%) with metformin at the dosage of 2 g/day were randomly allocated (1:1) to add-on pioglitazone or a sulphonylurea. The metformin dose remained unchanged throughout the study whereas the add-on drugs were initiated at the lowest effective dose and then titrated according to a standard protocol based on home glucose monitoring and HbA1c values. Drug compliance was assessed at each visit. Doses of the drugs taken, temporary or permanent discontinuation of the study drugs, and the reasons for discontinuation were reported in the study records on the basis of a patient's interview. HbA1c was

measured every 6 months. The study protocol (NCT00700856) was approved by Federico II University Ethics Committee, all participants provided written informed consent before entering the study.

For the purposes of the present work, the study population consists of 195 patients enrolled at Federico II University Hospital with complete data set at one year of follow-up.

2.2.2 Measurements

Anthropometric measures (weight, height, and waist circumference) were taken at baseline and at 1-year follow-up according to standardized procedures.

Fasting blood samples were collected by an antecubital vein. Plasma lipids and HbA1c were measured at centralized laboratory. Plasma glucose and liver enzymes (aspartate aminotransferase AST, alanine aminotransferase ALT, and gamma-glutamyl-transpeptidase GGT, Beckman Coulter), free fatty acids (WAKO) and insulin (Roche Diagnostics, Germany), were measured at CNR laboratory in Pisa on frozen plasma collected at baseline, prior to randomization, and at 1-year follow-up and kept a t – 70°C.

Indirect indices of NAFLD/NASH were calculated according to the following formulas:

- Liver Fat Equation (LFE): 10 (-0.805 + 0.282 × metabolic syndrome (yes = 1 / no = 0) + 0.078 × T2D (yes =2 / no =0) + 0.525 × log fasting serum insulin (mU/L) + 0.521 × log fasting serum AST (U/l) – 0.454 × log (AST/ALT) [61].
- Hepatic Steatosis Index (HSI): 8 × ALT/AST ratio + BMI (+2, if diabetes mellitus; +2, if female), with values <30 ruling out and values >36 ruling in steatosis [62].
- Index Of NASH (ION):1.33 waist to hip ratio + 0.03 × triacyclglycerols (mg/dL) + 0.18 × ALT (U/l) +8.53 × HOMA – 13.93 for men; 0.02 × triacyclglycerols (mg/dL)

+ 0.24 × ALT (U/l) + 9.61 × HOMA – 13.99 for women. An ION score of ≥ 50 used to identify NASH from simple steatosis provided a sensitivity of 92 and a specificity of 60[63].

Indirect indices of systemic insulin resistance (HOMA-IR), adipose tissue insulin resistance (ADIPO-IR) and Visceral Adiposity Index (VAI) were also calculated according to the following formulas:

- HOMA-IR: HOMA2 Calculator, based on fasting plasma glucose and fasting plasma insulin (http://www.dtu.ox.ac.uk/homacalculator).
- ADIPO-IR: fasting plasma non-esterified fatty acids × fasting plasma insulin [64].
- VAI: WC/[39.68+(1.88 × BMI)] × (triacyclglycerols/1.03) × (1.31/HDL) for men and WC/[36.58+(BMI × 1.89)] × (triacyclglycerols /0.81) × (1.52/HDL) for women [65].

2.2.3 Statistical analysis

Data are expressed as means±SD for continuous variables and frequencies (or percentages) for categorical variables unless otherwise stated. Between-treatments differences were evaluated by ANCOVA general linear model taking variables changes (1-year minus baseline) as dependent variables and treatment as fixed factor. Due to baseline differences in gender distribution between groups, gender was added in the model as covariate. Within groups, before–after intervention differences were evaluated by t-test for paired samples. Differences between groups at baseline were evaluated by ANCOVA general linear model taking the variable of interest (i.e., waist circumference, BMI, etc.) as dependent variable, treatment as fixed factor, and gender as covariate. For NAFLD-status (yes/no according to the diagnostic cut-offs of the indirect indices), the Wilcoxon signed-rank test was used to evaluate changes between

the baseline and post treatment status and the Wilcoxon-rank sum test for the differences between groups.

To evaluate possible influence of pioglitazone dosage, participants in the pioglitazone groups were divided in 3 subgroups according to the dose taken (15, 30, 45 mg/day) at least for the last three months before the follow-up measurements. Differences in between-subgroups baseline characteristics of participants and in liver indices changes were evaluated by one-way ANOVA and LSD post hoc analysis.

To explore the possible impact of blood glucose control status, participants allocated to pioglitazone or sulphonylureas were divided in subgroups according to the median changes (1-year minus baseline values) in HbA1c (-0.5%). Differences between-subgroups in variables changes were evaluated by t-test for independent samples.

A p value < 0.05 was considered statistically significant. Statistical analysis was performed using the SPSS software 26.0 (SPSS/PC; IBM, Armonk, NY, USA).

2.3 Results

2.3.1 General characteristics of participants at baseline and after 1-year of follow-up.

Ninety-eight participants were randomized to pioglitazone and 97 to sulphonylureas: either glibenclamide, gliclazide, or glimepiride could be used, based on the judgement of the treating physician. Average dose during the study was 26 mg/day for pioglitazone, 5 mg/day for glibenclamide, 36 mg/day for glicazide, and 2.6 mg/day for glimepiride. The proportion of men was significantly higher in pioglitazone than in sulphonylurea group (**Table 1**). The two study groups were comparable for all other variable, age,

anthropometric and metabolic characteristics, percentage use of anti-hypertensive and lipid-lowering drugs (**Table 1**).

Table 1. Anagraphic, anthropometric, and metabolic parameters at baseline and 1-year follow-up in the Pioglitazone and Sulphonylurea intervention groups.

	Pioglitazone (N=98)		Sulphonylurea (N=97)		†*p*
	Baseline	1-year	Baseline	1-year	
Gender (women/men)	37/61		50/47§		
Age (years)	61±7		62±6		
Body weight (kg)	79.0±12.1	80.0±13.1*	80.5±13.5	81.8±13.7*	0.642
Body Mass Index (kg/m^2)	29.5±4.2	29.9±4.6*	30.9±4.2	31.5±4.5*	0.553
Waist circumference (cm)	102±11	104±11*	106±11	107±10	0.426
Duration of diabetes (years)	10±6	11±6	9±6	10±6	0.853
Fasting plasma glucose, (mmol/l)	9.9±2.2	8.8±2.1*	9.3±1.7	7.9±2.0*	0.410
Fasting plasma insulin, (µU/ml)	14±8	13±9	13±7	15±10*	0.002
HbA1c, mmol/mol	62±6	57±9*	61±6	55±9*	0.439
HbA1c, %	7.8±0.5	7.4±0.8*	7.7±0.5	7.2±0.8*	0.439
Plasma total Cholesterol (mmol/l)	4.6±0.8	4.5±0.8	4.6±0.9	4.4±0.8	0.928
Triglycerides (mmol/l)	1.7±0.9	1.6±0.9*	1.6±0.7	1.6±0.8	0.160
HDL-cholesterol (mmol/l)	1.2±0.3	1.3±0.4*	1.2±0.3	1.2±0.3	0.015
LDL-cholesterol (mmol/l)	2.6±0.7	2.5±0.7	2.7±0.9	2.5±0.6*	0.966
Pioglitazone doses (mg/day)	-	26 ± 12	-	-	
Glibenclamide (mg/day)	-	-	-	5 ± 0	
Gliclazide (mg/day)	-	-	-	36 ± 15	
Glimepiride (mg/day)	-	-	-	2.6 ± 1.1	
Antihypertensive drugs, *n* (%)	67 (68)	75* (76)	69 (71)	73 (75)	0.172
Lipid lowering drugs, *n* (%)	58 (59)	65* (66)	64 (66)	70* (72)	0.261
Anti-platelet drugs, *n* (%)	32 (33)	57* (58)	37 (38)	53* (55)	0.316

Data are means (SD) or frequency (percentage). *p < 0.05 *vs.* baseline; § p < 0.05 *vs.* pioglitazone baseline; †p for between-treatments differences in variables changes (1-year *minus* baseline) by ANCOVA model with gender as covariate.

After 1-year treatment, BMI, and waist circumference slightly increased in both study arms, blood glucose control improved with a reduction in fasting blood glucose of about

1.7 mmol/l and HbA1c of 0.5%, in both groups equally. Fasting plasma insulin increased significantly with sulphonylureas compared to pioglitazone (**Table 1**).

A significant reduction for LDL-cholesterol in the sulphonylurea group and triacylglycerols in the pioglitazone group was observed without differences between groups. HDL-cholesterol increased significantly more in the pioglitazone than in the sulphonylurea group (**Table 1**). Changes in the use of lipid-lowering and anti-hypertensive drugs were not different between the two groups (**Table 1**).

2.3.2 Effects of pioglitazone or sulphonylureas on liver enzymes, indices of NAFLD and insulin resistance.

Baseline average concentrations of liver enzymes were within normal range in both groups (**Fig. 1**). A significant reduction of all liver enzymes concentrations was observed in the pioglitazone group. This reduction was significantly greater in the pioglitazone arm for ALT and GGT compared to the sulphonylurea arm (**Fig. 4**).

At baseline, LFE, HSI, and ION were not different between the two groups, and on average above the threshold for diagnosis of liver steatosis and steatohepatitis in both groups (Fig. 1). According to these cut-offs, baseline prevalence of NAFLD/NASH was equally high in the pioglitazone or sulphonylurea groups: LFE 67% vs. 71%; HSI 77% vs. 85%; ION 41% vs.30%, respectively (**Table 2**).

All indices of NAFLD improved after one year of treatment with pioglitazone but not with sulphonylureas. Statistically significant differences between changes (1-year minus baseline) were observed for LFE (-1.76±3.84 vs. 0.28±3.75), HSI (-1.35±2.78 vs. -0.27±2.63), and ION (-9.75±43 vs. 3.24±31); $p < 0.05$ for all (**Fig. 4**). According to LFE and HSI cut-offs, the prevalence of liver steatosis was significantly reduced by pioglitazone

treatment while did not change in the sulphonylurea group with a significant difference between treatments (**Table 2**). According to ION, the prevalence of NASH decreased in the pioglitazone arm, while tended to increase in the sulphonylurea arm with a significant difference between treatments (**Table 2**).

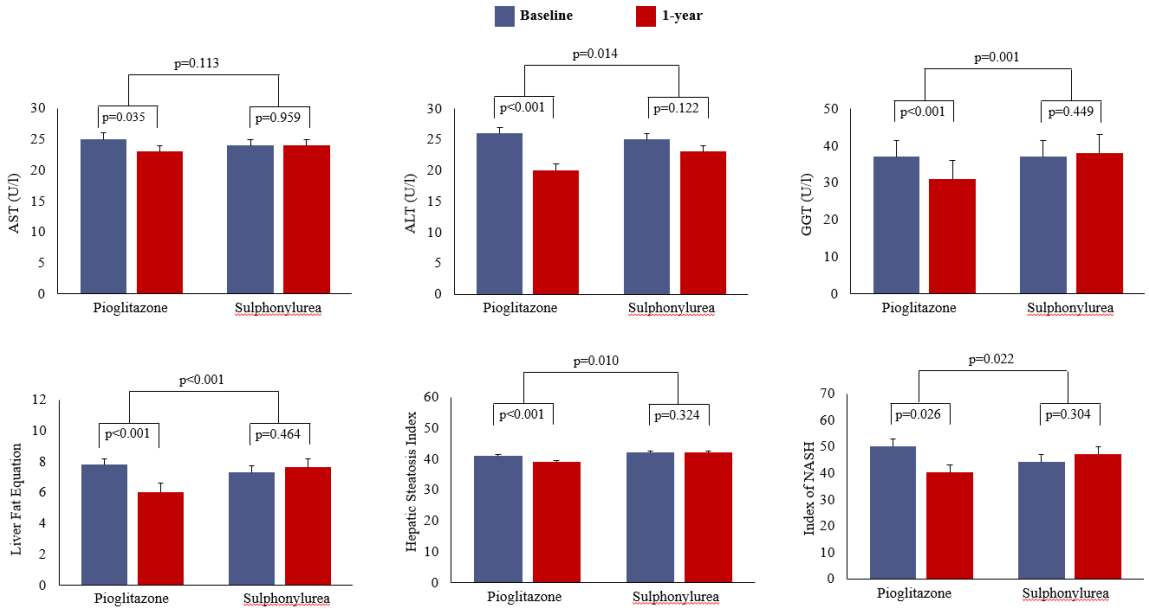


Figure 4. Liver enzymes and indirect indices of NAFLD at baseline and 1-year follow-up in the Pioglitazone and Sulphonylurea intervention groups. Between-treatments differences were evaluated by ANCOVA. Within groups differences were evaluated by t-test for paired samples.

Changes in indirect indices of insulin-resistance are shown in **Fig. 5**. HOMA-IR and ADIPO-IR significantly decreased after pioglitazone while tended to increase after sulphonylurea treatment with a significant difference between groups. VAI significantly decreased after pioglitazone, while did not change after sulphonylurea treatment with borderline significance (p = 0.074) for differences between groups.

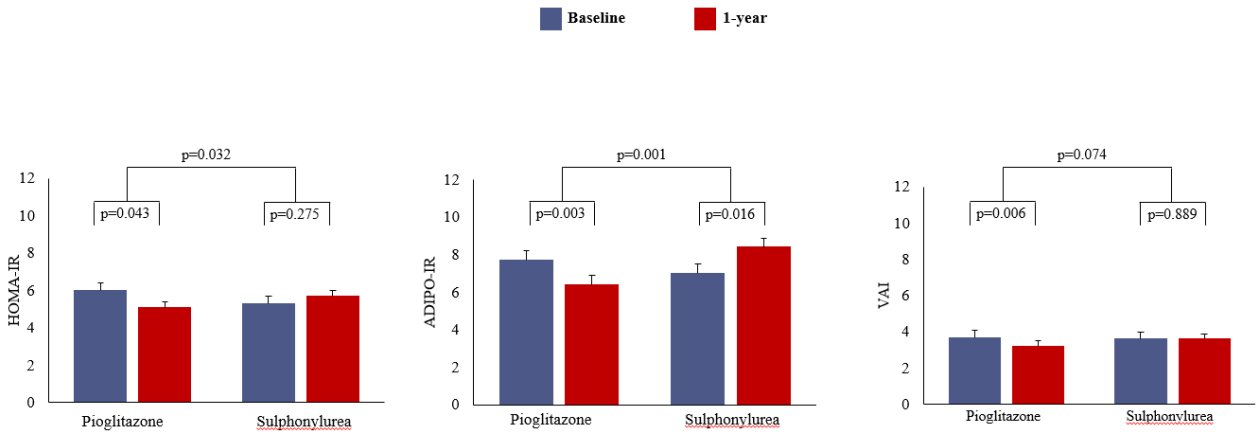


Figure 5. Indices of insulin resistance at baseline and 1-year follow-up in the Pioglitazone and Sulphonylurea intervention groups. Between-treatments differences were evaluated by ANCOVA. Within groups differences were evaluated by t-test for paired samples.

Table 2. NAFLD-status (yes or no according to diagnostic cut-off) at baseline, 1-year follow-up, and changes in the Pioglitazone and Sulphonylurea intervention groups

	Pioglitazone (N=98)			Sulphonylurea (N=97)			$p^{\dagger}$
	Baseline	1-year	p	Baseline	1-year	p	
LFE							
< 5 *n* (%)	33 (33.7)	53 (54)		28 (29)	29 (29.9)	0.001*	
≥ 5 *n* (%)	65 (66.7)	45 (46)		69 (71)	68 (70.1)		
Improvement (%)		24	0.000§		10	0.819§	0.017
Worsening (%)		4			9		
HSI							
< 30 *n* (%)	2 (2.1)	6 (6.1)		1 (1.1)	0		
30-35 *n* (%)	21 (21.4)	29 (29.6)		14 (14.4)	16 (16.5)	0.002*	
≥ 36 *n* (%)	75 (76.5)	63 (64.3)		82 (84.5)	81 (83.5)		
Improvement (%)		19	0.001		5	1.0	0.009
Worsening (%)		3			5		
ION							
<50 *n* (%)	58 (59.2)	74 (75.5)		68 (70.1)	60 (61.9)	0.045*	
≥50 *n* (%)	40 (40.8)	24 (24.5)		29 (29.9)	37 (38.1)		
Improvement (%)		21	0.002		7	0.088	0.002
Worsening (%)		5			15		

Data are frequency (percentage). § p <0.05 changes between the baseline and post-treatment status; † p <0.05 for between-treatment changes; * p <0.05 between frequency status at 1-year.

2.3.3 Effects of different doses of pioglitazone on liver enzymes and indices of NAFLD.

To explore the dose effect of pioglitazone the participants allocated to the pioglitazone group were divided in 3 subgroups according to the dosage (i.e.,15, 30, or 45 mg/day) taken in the three months preceding the follow-up visit.

The anthropometric and metabolic parameters of the 3 subgroups at baseline and 1-year follow-up are shown in **Table 3**. At baseline, participants needing the highest pioglitazone dose (45 mg/day) had lower BMI, waist circumference, and fasting insulin levels and worse blood glucose control compared with participants taking 15 or 30 mg/day. After 1 year blood glucose control improved at any dosage while triacylglycerols decreased only in the 45 mg/day subgroup without significant differences between the three groups. Similarly, HOMA-IR decreased significantly in the low-dosage group and ADIPO-IR and VAI in the high dosage groups without significant differences between the three groups (**Table 3**).

Liver enzymes and indices of NAFLD decreased after all dosages of pioglitazone treatment with not statistically significant differences in 1-year minus baseline changes among subgroups, except for HSI decreasing only at 30 and 45 mg/day dose (**Fig. 6**).

2.3.4 Impact of changes of blood glucose control on indices of NAFLD.

Anthropometric, metabolic parameters, liver enzymes and indirect indices of NAFLD and insulin resistance at baseline and 1-year follow-up in participants divided in subgroups according to changes in HbA1c (above or below the median change of -0.5%) in the context of pioglitazone and sulphonylurea groups are shown in **Table 4.**

Table 3. Anthropometric and metabolic parameters at baseline and 1-year follow-up in the Pioglitazone group divided according to the Pioglitazone dosage taken at 1-year follow-up.

	Pioglitazone 15 mg/day (N=47)		Pioglitazone 30 mg/day (N=31)		Pioglitazone 45 mg/day (N=20)		†*p*
	Baseline	1-year	Baseline	1-year	Baseline	1-year	
Gender (women/men)	15/32		16/15		7/13		0.327
Body weight (kg)	81.7±12.6	82.6±13.2*	77.7±11.1	78.8±12.9	74.8±11.5	75.8±12.1	0.976
Body Mass Index (kg/m^2)	30.1±4.0	30.5±4.4*	29.5±4.5	29.9±5.1	28.2±4.1	28.5±4.1	0.980
Waist circumference (cm)	103.8±9.6	105.6±9.7	102.8±11.1	103.5±11.4	96.9±10.3§	98.6±11.1	0.673
Fasting plasma glucose, (mmol/l)	9.1±1.7	8.3±1.7*	9.8±1.8	8.7±1.8*	11.9±2.5$^{\neq}$	10.3±2.5*	0.996
Fasting plasma insulin, (µU/ml)	15±9	13±7	14±7	13±12	9.9±5.6§	9.8±7.6	0.605
HbA1c, mmol/mol	58±5	54±9*	62±5	57±1*	66±5$^{\neq}$	62±6*	0.310
HbA1c, %	7.5±0.5	7.1±0.8*	7.8±0.5	7.4±0.9*	8.2±0.5$^{\neq}$	7.8±0.6*	0.310
Plasma total Cholesterol (mmol/l)	4.5±0.8	4.5±0.8	4.8±0.9	4.6±0.9	4.3±0.8	4.2±0.8	0.683
Triacylglycerols (mmol/l)	1.7±0.9	1.6±0.8	1.8±1.1	1.7±1.1	1.5±0.5	1.3±0.6*	0.713
HDL-cholesterol (mmol/l)	1.2±12	1.3±0.4*	1.2±0.3	1.2±0.3	1.2±0.4	1.3±0.4*	0.659
LDL-cholesterol (mmol/l)	2.6±0.6	2.5±0.6	2.8±0.8	2.6±0.7	2.4±0.6	2.3±0.7	0.654
HOMA-IR	6±4	5±3*	6±4	6±6	5.3±3.0	4.9±4.8	0.765
ADIPO-IR	8±5	7±4	8±5	7±7	6±4	4.0±2.2*	0.531
VAI	3.7±2.5	3.3±2.6	4.2±3.2	3.6±2.7	3.0±1.6	2.4±1.7*	0.940

Data are means (SD). *p < 0.05 vs. baseline; §p < 0.05 vs Pioglitazone 15 mg/day; $^{\neq}$p < 0.05 vs Pioglitazone 15 mg/day and 30 mg/day; †p for differences in between-groups by *post-hoc* one-way ANOVA.

In the pioglitazone group, participants with reduction of HbA1c greater of -0.5% or more had a significant decrease in all indices of insulin-resistance, liver enzyme concentrations and NAFLD indices; in participants with a reduction of HbA1c of less than -0.5%, ADIPO-IR, ALT, LFE and HSI significantly decreased with no changes in other parameters. In any case, differences in changes (1-year minus baseline) between subgroups with a HbA1c reduction above and below -0.5% were statistically significant only for VAI and GGT with a greater decrease in the pioglitazone group with better glucose control.

Table 4. Anthropometric and metabolic parameters, liver enzymes and indices of NAFLD and insulin resistance at baseline and 1-year follow-up according to changes in blood glucose control

	Pioglitazone					Sulphonylurea				
	Δ HbA1c ≤-0.5% N=46		Δ HbA1c >-0.5% N=52		†p	Δ HbA1c ≤-0.5% N=53		Δ >HbA1c -0.5% N=44		†p
	baseline	1-year	baseline	1-year		baseline	1-year	baseline	1-year	
BMI (kg/m^2)	30±4	31±4*	29±4	29±5	0.363	31±4	31±5	31±4	32±4*	0.001
Waist (cm)	103±10	104±10	101±11	103±11*	0.312	107±11	107±10	105±11	106±10*	0.043
Glucose (mmol/l)	10.2±2.5	8.3±1.9*	9.8±1.9	9.3±2.1	0.000	9.4±1.7	7.2±1.7*	9.1±1.8	8.8±2.1	0.000
HbA1c (mmol/mol)	64±5	53±5*	60±5	61±1	0.000	62±5	51±6*	61±5	62±7	0.000
Insulin (μU/ml)	14±9	13±11	13±8	12±7	0.672	13±8.2	14±8.1	13±6	18±11*	0.002
HOMA-IR	6.4±3.9	4.8±5.1	5.7±3.9	5.3±4.0	0.201	5.6±3.7	4.6±3.4*	5.1±2.5	7.0±4.7*	0.000
VAI	3.8±2.5	2.8±1.9*	3.7±2.8	3.5±2.9	0.024	3.6±2.1	3.5±2.7	3.6±2.2	3.8±2.2	0.424
ADIPO-IR	7.9±5.3	6.3±5.5*	7.4±4.5	6.5±4.3*	0.420	7.1±5.1	7.2±4.7	6.9±4.1	9.8±7.3*	0.010
GGT	37±32	27±23*	37±28	35±34	0.010	40±71	40±72	32±32	35±32	0.336
AST	25±10	22±6*	26±12	25±8	0.240	25±11	24±8	23±7	25±9*	0.046
ALT	26±15	17±9*	27±15	22±13*	0.238	26±18	22±11	24±10	24±13	0.114
Liver fat %	7.9±4.8	5.6±4.3*	7.7±4.4	6.4±4.2*	0.258	7.5±5.2	7.0±4.2	7.1±3.3	8.2±5.6	0.024
HSI	41±5	40±5*	40±6	39±6*	0.359	42±5	42±6	42±5	42±5	0.105
ION	53±37	37±49*	47±35	43±36	0.173	46±36	37±33*	41±25	59±44*	0.000

Data are means (SD). *p < 0.05 vs. baseline by paired sample t-test; †p for differences in between-group changes by ANCOVA model.

In the sulphonylurea group participants with a HbA1c reduction greater than -0.5% had a significant decrease in HOMA-IR and ION and a modest, and non-statistically significant trend to reduction in LFE; in participants with a HbA1c reduction lesser than -0.5% fasting plasma insulin, HOMA-IR, ADIPO-IR, AST, and ION significantly increased, similarly a modest and non-statistically significant trend in LFE increase was observed. Differences in changes between subgroups with a HbA1c reduction above and below -0.5% were statistically significant for fasting plasma insulin levels, HOMA-IR, AST, ADIPO-IR, LFE and ION.

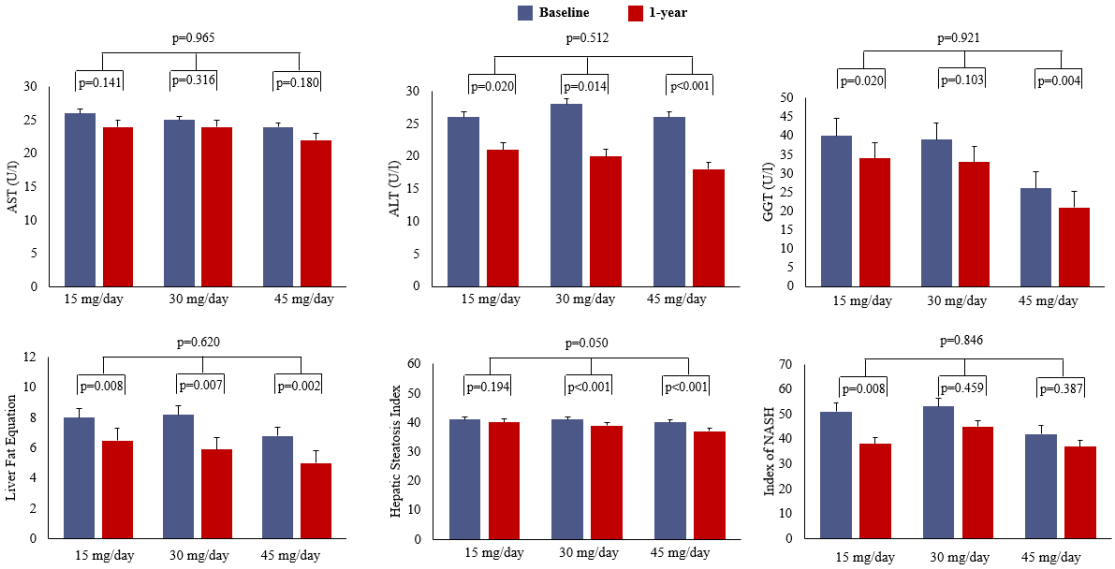


Figure 6. Liver enzymes and indices of NAFLD at baseline and 1-year follow-up in the Pioglitazone groups divided according to the Pioglitazone dosage taken at 1-year follow-up (15 mg, n=47; 30 mg, n=31; 45 mg, n=20). Between-treatments differences were evaluated by ANCOVA. Within groups differences were evaluated by t-test for paired samples.

2.4 Discussion

This study showed, for the first time, that, compared to sulphonylureas, pioglitazone, even at a low dose, is effective in improving indirect indices of liver steatosis and inflammation and systemic and adipose tissue insulin resistance in patients with T2D over a 1-year of follow-up. Moreover, the beneficial effects of pioglitazone on NAFLD were independent of blood glucose control.

In our study, an average dose of pioglitazone of 26 mg/day for one-year induced a significant reduction of indexes of liver steatosis, liver enzymes, and hepatic inflammation. These data were not only statistically significant but of clinical relevance; in fact, changes in indices of liver steatosis and inflammation indicate a resolution of the disease in ~ 20% of participants.

These effects came together with improved glucose control, a less atherogenic lipid profile, and relevantly, without a significant increase in body weight.

These findings were consistent for all pioglitazone doses used, indicating that the lowest pioglitazone dose is as effective as the highest on the whole metabolic profile of patients with T2D.

This is in line with dose-response studies on effects of pioglitazone on blood glucose control showing that even at low doses pioglitazone holds its beneficial effects with a reassuring safety profile [56,57].

It should be noted that participants allocated to the highest pioglitazone dose subgroup (45 mg) had a different metabolic phenotype compared with subgroups allocated to lower doses. They had higher blood glucose, were leaner and had lower plasma insulin levels. This subgroup was likely selected by the titration protocol of pioglitazone driven by response of glucose control to anti-hyperglycemic therapy. Keeping in mind the limits due to the small size of the dose subgroups, it could be hypothesized that the relative contribute of hyperglycaemia and insulin resistance in determining NAFLD in subgroup at 45 mg could have been different from the other subgroups. This could be also supported by the different impact of low and high doses of pioglitazone, respectively, on index of whole-body insulin resistance (HOMA-IR) and indices of visceral (VAI) and adipose tissue insulin resistance (ADIPO-IR).

Pioglitazone also significantly improved whole-body and adipose tissue insulin-resistance compared with sulphonylureas, while similarly improving blood glucose control.

The beneficial effects of pioglitazone on liver steatosis and inflammation were independent of blood glucose control, while insufficient glucose control in the sulphonylurea group was associated with worsening of liver steatosis and inflammation.

This indicates that improvement of NAFLD induced by pioglitazone is essentially mediated by the reduction of insulin-resistance, particularly in the adipose tissue, which in our cohort of patients with T2D may be the primum “movens” in determinism of NAFLD. The persistence of beneficial effects of pioglitazone in the subgroup with insufficient glucose control suggests that pioglitazone, unlike sulphonylureas, is able to counteract not only the deleterious effects of lipotoxicity but also glucotoxicity. This could be related with anti-inflammatory and anti-oxidative properties of pioglitazone at the hepatic level but also to its regulatory action on hepatic lipid metabolism [66].

The effects of pioglitazone on NAFLD are in line with data from new PPAR drugs, both PPAR-pan agonists and selective PPAR-gamma modulators [67,68].

Our study has some strengths and limitation. This is the first report indicating that low doses of pioglitazone may improve indices of NAFLD in people with T2D in the medium term. This is a clinically relevant information as it offers a safe and affordable therapeutic option for a currently untreatable condition carrying a huge burden of morbidity and mortality tied to end-stage liver disease, but also to cardiovascular accidents. Of note, our cohort included elderly people for whom NAFLD prognosis is worse than that observed in other patient groups [69]. Therefore, our data support pioglitazone as an optimal therapeutic option also in fragile patients.

Strength of our information is limited by the fact that the TOSCA study was not primarily designed to evaluate the effects of pioglitazone on NAFLD. A further limitation is represented by the study subgroup population and the 1-year treatment duration. In addition, the pragmatic study design and the large number of subjects enrolled did not allow to perform invasive and/or high costly procedures. Therefore, no liver biopsies or imaging data are available. This implies, especially for steatohepatitis, often the only

feature of liver damage in type 2 diabetes [70], that we could have misclassified NAFLD status in some cases. For the same reason, body composition was not measured in our patients; thus, according to previous studies [71,72], we can only speculate that pioglitazone increases subcutaneous adipose tissue, without changes in total body water and lean mass, and with an improvement in muscle insulin resistance and mitochondrial function. However, the rigorous methodology of a multicentre randomized trial preserved us from relevant biases in the interpretation of results. In addition, our findings based on indirect indices of NAFLD, currently accepted by NAFLD guidelines [43], consistently reproduced results obtained in clinical trials in which biopsy proven NAFLD patients were enrolled [44–47]. This not only endorses our findings but also indicates that indirect indices of NAFLD are reliable tools in both clinical and experimental contexts. In conclusion, our results show that low doses of pioglitazone may be a therapeutic option to improve NAFLD. These effects are independent from changes in blood glucose control suggesting that improvement of insulin-resistance is the main pathway of the beneficial effects of pioglitazone. Our findings provide some insights for trials designed ad hoc to explore effects of low dose of pioglitazone also in people with more severe forms of NAFLD.

3. Chapter 3

Treatment with pioglitazone, but not sulfonylurea, improves lipidomic profile of patients with diabetes and MAFLD: results from the TOSCA.IT study.

3.1 Introduction

Non-alcoholic fatty liver disease (NAFLD) is a metabolic disease that starting from hepatic fat accumulation (NAFL) in absence of excess alcohol consumption can progress into more severe forms, i.e., non-alcoholic steatohepatitis (NASH), cirrhosis, hepatocellular carcinoma. The prevalence of NAFLD is estimated to be 25% in the general population but increases to more than 55% in subjects with type 2 diabetes and/or obesity)[73]. The derangement in lipid metabolism, either synthesis or accumulation of hepatic TG and secretion of lipotoxic compounds, es. ceramides, drives the progression of NAFLD to more severe forms. Thus, it is important to study the impact of antidiabetic drugs on NAFLD and on the lipidomic profile. Despite the metabolic characterization of NAFLD, metabolic alterations are not considered in the diagnosis. Thus, it has been proposed to move forward and include metabolic alterations in consideration with new criteria for the diagnosis of fatty liver disease, i.e., metabolic associated fatty liver disease (MAFLD)[74]. MAFLD is diagnosed when subjects with hepatic steatosis have the following metabolic conditions: obesity/overweight, diabetes, and metabolic dysregulation, either alone or in combination, making the

diagnosis MAFLD very simple. The global prevalence of MAFLD and NAFLD are very similar and very high, confirming that about half of the overweight/obese adults have MAFLD[75]. NAFLD is one of the comorbidities of diabetes[76] although the current ADA guidelines still do not consider NAFLD one of the complications at risk for patients with diabetes[77]. Among the drugs most commonly used for the treatment of diabetes there are metformin (MET) and sulfonylureas (SUL). The PPAR gamma agonist pioglitazone (PIO) is the most suitable treatment to reduce lipotoxicity and glucotoxicity since it significantly reduces hyperglycaemia and peripheral lipolysis promoting the increase of subcutaneous adipose tissue but significantly reducing visceral fat and liver steatosis[78]. However, not many studies compared the effects of antidiabetic drugs on adipose tissue insulin resistance, lipid composition and metabolism and scores of NAFLD; moreover, while it is known the effect of PIO on lipid metabolism, it is not known if SUL has any impact on the lipidomic profile.

Thus, the goal of this study was to evaluate the impact of NAFLD / MAFLD on the circulating lipidome in subjects with type 2 diabetes and poor glycemic control and then study the effect of 1 year treatment with pioglitazone or sulfonylurea.

3.2 Methods

3.2.1 Subjects

We studied 192 patients with T2D, age 50-75 years, poorly controlled with metformin (MET) 2 g/day participating to the multicenter “Thiazolidinediones or Sulfonylureas Cardiovascular Accidents - Intervention Trial” (TOSCA.IT) and enrolled at the University

Federico II in Napoli. The clinical characteristics of this cohort have been presented and discussed in Chapter 2.

After evaluation of baseline conditions, i.e., fasting blood glucose, HbA1c, insulin, liver enzymes (AST, ALT and GGT), lipid profile (total triglycerides, total cholesterol, LDL and HDL), subjects were randomized to receive, in addition to metformin, either pioglitazone (15–45 mg, n = 98) or sulfonylurea (5–15 mg glibenclamide, 2–6 mg glimepiride or 30–120 mg gliclazide, n = 97) [79].

3.2.2 Lipidomic profile

In all subjects we evaluated the lipidomic profile at baseline and after 1-year of treatment with either MET+PIO or MET+SUL. Non-esterified fatty acids (FFA) concentration was measured by spectrophotometry (FUJIFILM Wako Chemicals Europe GmbH, Germany) and composition (myristic, palmitoleic, palmitic, linoleic, oleic, stearic and arachidonic) by Gas Chromatography Mass Spectrometry (GC/MS, Agilent, Santa Clara ,CA) adding in 20ul of plasma sample extracted using Folch's method (using heptadecanoic acid (Merck, Darmstadt, Germany) as internal standard) and derivatized with N, O-Bis(trimethylsilyl)trifluoroacetamide + 1%TMCS (Merck, Darmstadt, Germany).

Lipidomic composition was analyzed by Liquid Mass Spectrometry coupled with a Quadrupole Time-Of-Flight Mass Spectrometry QTOF (UHPLC 1290- QTOF-MS 6540, Agilent, Santa Clara CA) equipped with a C18 column (ZORBAX Eclipse Plus C18 2.1 × 100 mm 1.8-Micron, Agilent, USA) and electrospray ionization (ESI) set in positive mode. Briefly, 10 uL of plasma were used and 10uL of an internal standards mixture were

added; samples were then deproteinized with 150uL of cold methanol, centrifuged for 20min at 14000 rpm and transfered to a vial for the analysis. The internal standard solution contained the following compounds: 1,2-diheptadecanoyl-sn-glycero-3-phosphoethanolamine (PE (17:0/17:0)), N-heptadecanoyl-D-erythro-sphingosylphosphorylcholine (SM(d18:1/17:0)), N-heptadecanoyl-D-erythro-sphingosine (Cer(d18:1/17:0)), 1,2-diheptadecanoyl-sn-glycero-3-phosphocholine (PC(17:0/17:0)), 1-heptadecanoyl-2-hydroxy-sn-glycero-3-phosphocholine (LPC(17:0)) and 1-palmitoyl-d31-2-oleoyl-sn-glycero-3-phosphocholine (PC(16:0/d31/18:1)), (Avanti Polar Lipids Inc., Merck, Germany). In addition, the mixture also contained triheptadecanoin (TG (17:0/17:0/17:0), Larodan, Sweden).

One microliter of the deproteinized solution was injected and chromatographic separation was obtained with organic phase, isopropanol: acetonitrile (1:1) and inorganic phase, water (MilliQ) + 0.1% formic acid (Merck, Darmstadt, Germany). Injection volume was 1 ul. Flow rate was 0.4 mL/min for the first 9 minutes and 0.6 mL/min for the last 7 minutes. Gradient used for lipids separation was: 0 min 35% B; 0-2 min 80% B; 2-9 min 100% B; 9-6 min 100% B. Post run was set up at 6 minutes. For the quantification of the lipidome, the spectra were analyzed using the MassHunter Profinder software (Agilent, Santa Clara CA) by processing a single adduct for each lipid species: i.e., CER, LPC, LPE, PC, PE, and SM with [M+H]+ while TAG and DAG with [M+Na]+.

3.2.3 Calculations

Insulin resistance was evaluated as HOMA-IR (Glu x Ins/22.5) and Adipo-IR (FFA x Ins) [80]. Presence of hepatic steatosis at baseline was diagnosed using validated indexes, i.e., the fatty liver index (FLI) greater than 60 [81]. and the NAFLD liver fat score (Liver Fat%) greater than 6% [61].

- Liver Fat %: 10 (-0.805 + 0.282 * metabolic syndrome (yes = 1 / no = 0) + 0.078 * type 2 diabetes (yes =2 / no =0) + 0.525 * log fasting serum insulin (mU/L) + 0.521 * log fasting serum AST (U/L) – 0.454 * log (AST/ALT).

- Fatty Liver Index (FLI) = (e0.953*loge (TG) + 0.139*BMI + 0.718*loge (GGT) + 0.053*waist circumference - 15.745) / (1 + e0.953*loge (TG) + 0.139*BMI + 0.718*loge (GGT) + 0.053*waist circumference - 15.745) x 100.

Presence of MAFLD was diagnosed if both FLI and Liver Fat% were above the cut-offs.

3.2.4 Statistical analysis

Data are gives as mean ± SEM, an ANOVA test was performed to identify differences between the MAFLD and no-MAFLD groups.

Difference between treatment groups is represented as Log2(Post/Basal). Statistical comparison was performed with One sample T-test.

Statistical analysis was accomplished using Statview 5.0.1. The volcano plot was realized using Metaboanalyst. Bar charts were performed with Prism-Graphpad 9.0.

3.3 Results

3.3.1 Baseline characteristics

The clinical characteristics of patients at study entry are shown in **Table 5**. At baseline, the great majority of subjects had MAFLD (n=146 vs n=45 no-MAFLD).

Table 5: Demographic and Clinical Characteristics of the Patients at Baseline

	MAFLD (n=146)	**no-MAFLD (n=45)**	***p***
Gender (Females/Males)	68/78	17/28	
Age (years)	61±1	63±1	
Body weight (kg)	83.4±1	68.8±1.3	<0.0001
Height (m)	1.6±0.0	1.6±0.0	n.s.
Body Mass Index (kg/m^2)	31.6±0.3	26.0±0.4	<0.0001
Waist circumference (cm)	107.6±0.8	92.5±0.9	<0.0001
Hip (cm)	106.8±0.7	97.6±0.9	<0.0001
SBP (mmHg)	126.6±1.0	128.6±2.8	n.s.
DBP (mmHg)	76.8±0.6	74.7±1.3	n.s.
Glucose (mg/dl)	172.2±2.8	175.4±6.3	n.s.
HbA1c (%)	7.8±0.0	7.8±0.1	n.s.
Insulin (mg/dl)	15.4±0.6	6.9±0.4	<0.0001
HOMA-IR	6.6±0.3	3.0±0.2	<0.0001
ADIPO-IR	8.4±0.4	4.0±0.4	<0.0001
AST (U/l)	26.0±0.9	21.1±1.2	<0.0005
ALT (U/l)	28.6±1.3	16.5±1.2	<0.0001
GGT (U/l)	42.0±4.1	20.9±20	0.0058
CHO tot (mg/dl)	179.7±2.9	167.4±4.6	0.0307
HDL (mg/dl)	44.3±0.9	50.3±1.8	0.0035
LDL (mg/dl)	104.1±2.6	95.5±4.1	n.s.
TG tot (mg/dl)	157.9±6.1	108.2±7.3	<0.0001
FFA tot (µM)	561±16	555±35	n.s.
BOH (µM)	72.1±5.2	96.4±20.9	n.s.

As expected, MAFLD group has higher BMI and waist circumference, concentrations of hepatic enzymes, ALT, AST and GGT, total cholesterol and triglycerides (TG), (all p<0.03-0.0001). There was no difference in glucose concentrations and HbA1c (p=n.s.) while given the high insulin concentrations in MAFLD (being 3 times higher in MAFLD, the insulin resistance indexes HOMA-IR and ADIPO-IR, were also significantly higher in MAFLD group (p<0.0001).

3.3.2 Lipid profile

The total FFAs were similar in the two groups, as well as FFA composition.

Saturated or partially saturated TAGs (0 to 3 double bonds) were remarkably higher in the MAFLD group (p<0.0001) compared to the no-MAFLD group **(Figure 7).**

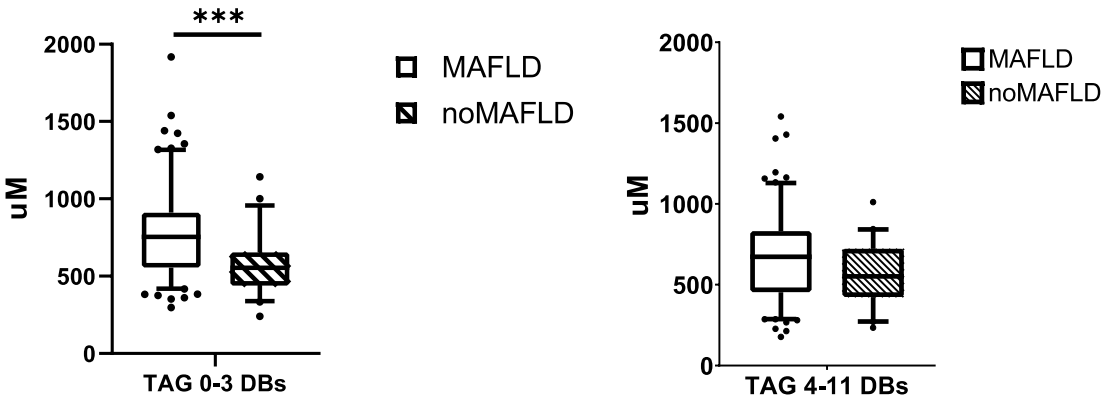


Figure 7. Basal concentration of **s**aturated or partially saturated (0-3 double bonds) and unsaturated (4-11 double bonds) TAGs in MAFLD vs noMAFLD groups, ***p ≤ 0.001

Total plasma concentrations of TAG, DAG and LPC were significantly higher (p<0.0001) in MAFLD group whilst there were no differences in total CER, SM, LPE, PC and PE. (**Figure 8A**)

Hence, among the single lipid species highly associated both to the presence of NAFLD and increased risk to develop T2D we found the TAG (48:0), TAG (48:1), TAG (50:0), TAG (50:0), TAG (50:1), TAG (50:2), PC C28:0, PC 40:5, PC 40:6 and LPC 16:0 concentration was higher in MAFLD group compared to no-MAFLD one.

Among the single ceramide species correlated with lipotoxicity and progression of liver disease several dehydroceramides were significantly higher (>1.5FC) in the MAFLD group i.e., Cer(d18:0/18:0), Cer(d18:0/20:0), Cer(d18:0/22:0) and Cer(d18:0/24:0) (**Figure 8B**)

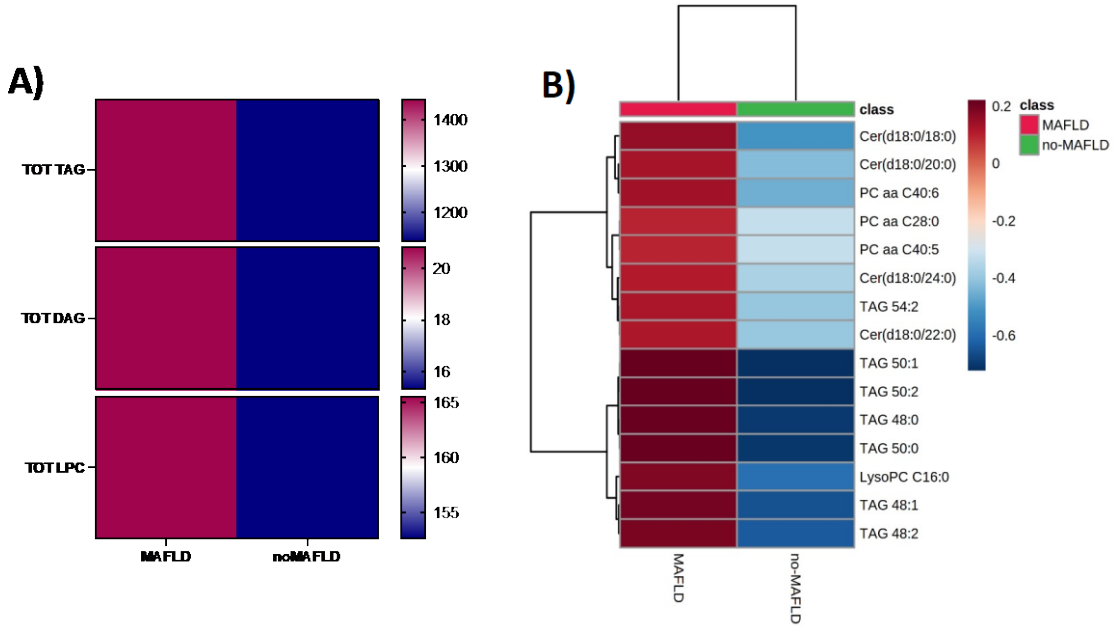


Figure 8. Basal plasma lipidomic concentration **A**) total TAG, DAG and LPC between MAFLD and noMAFLD group **B**) lipid species highly associated both to the presence of NAFLD and increased risk to develop T2D.

The volcano plot in **Figure 9** shows the lipid species with fold change threshold >1.5 and t-tests threshold 0.1. Hence, twenty-two most significant lipids are highlighted that are respectively: TAG 50:1, TAG 50:0, TAG 48:0, TAG 46:0, TAG 52:1, TAG 50:3, TAG 48:1, TAG 48:2, TAG 46:2, DAG 32:0, TAG 48:3, PC C32:1, TAG 52:0, DAG 36:1, Cer (d18:0:18:0), TAG 44:1, TAG 54:1, TAG 42:0, Cer (d18:0/20:0) , Cer (d18:0/22:0), TAG 54:0, DAG 36:0.

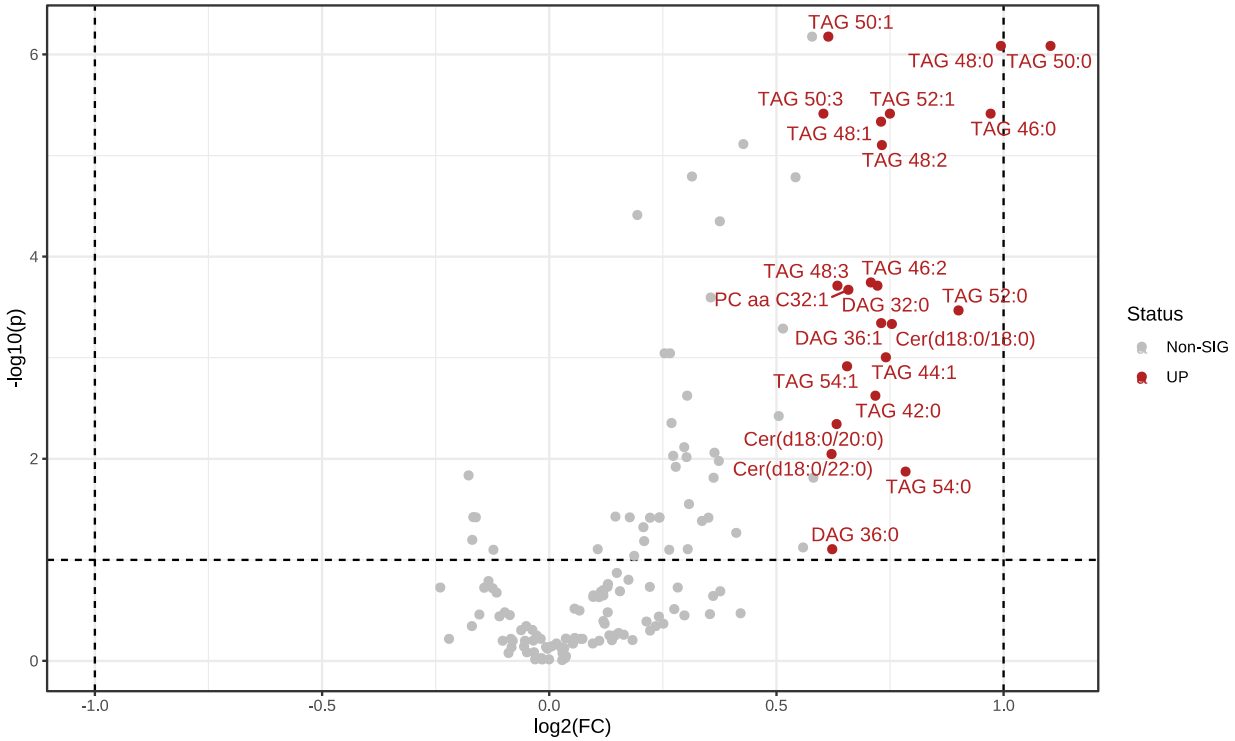


Figure 9. Volcano plot of lipid species with fold change threshold >1.5 and t-tests threshold 0.1

3.3.3 Effects of treatment

Both treatments equally improved fasting glucose and HbA1c (p≤0.01), and there were no significant differences in BMI, waist, hip, SBP and DBP between the two treatments **(Table 1)**. Concentration of ALT was significantly reduced vs baseline in MAFLD treated either with PIO or SUL groups, while AST and GGT decreased only in MAFLD treated with PIO **(Table 6)**. Indices of insulin resistance improved in both MAFLD and no-MAFLD after one year of treatment with pioglitazone but not with sulphonylureas **(Table 6)**.

Table 6: Demographic and Clinical Characteristics of the Patients: Changes from Baseline after 1 year of treatment

	PIOGLITAZONE		**SULFONYLUREA**		***p* value PIO vs SUL All**	***p* value PIO vs SUL *MAFLD***	***p* value PIO vs SUL noMAFLD**
	MAFLD (n=71)	**noMAFLD (n=25)**	**MAFLD (n=76)**	**noMAFLD (n=20)**			
Gender (F/M)	30/41	6/19	39/37	11/9			
Weight (kg)	1.5±0.4***	-0.2±0.6	1.2±0.3***	1.5±0.5*	n.s.	n.s.	.0456
BMI (kg/m^2)	0.6±0.1**	-0.1±0.2	0.5±0.1***	0.6±0.2**	n.s.	n.s.	.0198
Waist (cm)	1.3±0.5**	2.2±1.6	0.6±0.5	1.9±0.8	n.s.	n.s.	n.s.
Hip (cm)	0.8±0.5	1.0±1.1	0.8±0.4	1.3±0.8	n.s.	n.s.	n.s.
SBP (mmHg)	-2.8±1.8	-6.5±3.3	-0.7±1.7	0.9±4.5	n.s.	n.s.	n.s.
DBP (mmHg)	-1.6±1.1	-2.4±1.8	0.1±1.2	0.0±2.5	n.s.	n.s.	n.s.
Glucose (mg/dl)	-16.1±4.5***	-30.1±6.3**	-21.8±3.9***	-33.6±7.4**	n.s.	n.s.	n.s.
HbA1c (%)	-0.4±0.1***	-0.4±0.2**	-0.5±0.1***	-0.7±0.1***	n.s.	n.s.	n.s.
Insulin (mg/dl)	-1.4±1.1	-0.2±0.5	2.9±0.9**	1.7±0.8	.0002	.0012	n.s.
HOMA-IR	-1.0±0.6*	-0.6±0.3	-0.5±0.4	0.0±0.4	.0073	.0109	n.s.
ADIPO-IR	-1.4±0.5*	-0.9±0.6	1.4±0.6	1.1±1.1	.0003	.0009	n.s.
AST (U/l)	-3.1±1.3*	1.1±0.7	-0.5±1.0	1.6±1.4	n.s.	n.s.	n.s.
ALT (U/l)	-8.3±1.8***	-1.9±1.9*	-3.2±1.5*	2.5±0.9**	.0013	.0189	n.s.
GGT (U/l)	-7.3±1.7***	-0.9±1.8	-0.9±2.6*	1.5±0.7	.0001	.0004	n.s.
CHO tot (mg/dl)	-7.3±4.3	4.7±6.9	-5.7±3.2	-0.8±4.7	n.s.	n.s.	n.s.
HDL (mg/dl)	3.7±0.7**	2.6±2.1**	0.5±0.8	0.9±1.3	.0009	.0131	n.s.
LDL (mg/dl)	-7.3±3.7	2.0±6.7	-6.2±3.1	-1.4±3.7	n.s.	n.s.	n.s.
TG tot (mg/dl)	-19.6±7.9*	0.0±10.6	-0.2±7.2	-1.6±10.3	n.s.	n.s.	n.s.
FFA tot (μM)	-56±28	-73±43*	2±26	-36±61	n.s.	n.s.	n.s.
BOH (μM)	-1±10	-31±.29	1±13	-7±26	n.s.	n.s.	n.s.

*Significant changes from baseline, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 by non-parametric tests

Only patients with MAFLD treated with PIO had an improvement from baseline in the global lipid profile with a significant decrease in total TG (p≤0.01) **(Table 6)**.

No difference in total FFAs, an increase in HDL (p≤0.001) and a decrease in LDL (p≤0.05) despite the slight increase (p≤0.01) in weight in both groups apart from no-MAFLD PIO; FFA composition (saturated/unsaturated) was not modified by the treatments (p = ns vs baseline) **(Table 6).**

Lipidomic analysis showed that TAGs quantified by LC-MS were reduced significantly more in MAFLD treated with PIO than SUL (respectively p=0.0058, p=0.0515), while TAGs containing saturated fatty acids (i.e., with 0-3 double bonds) were decreased significantly only in the MAFLD group treated with PIO (p≤0.01, **Figure 10**).

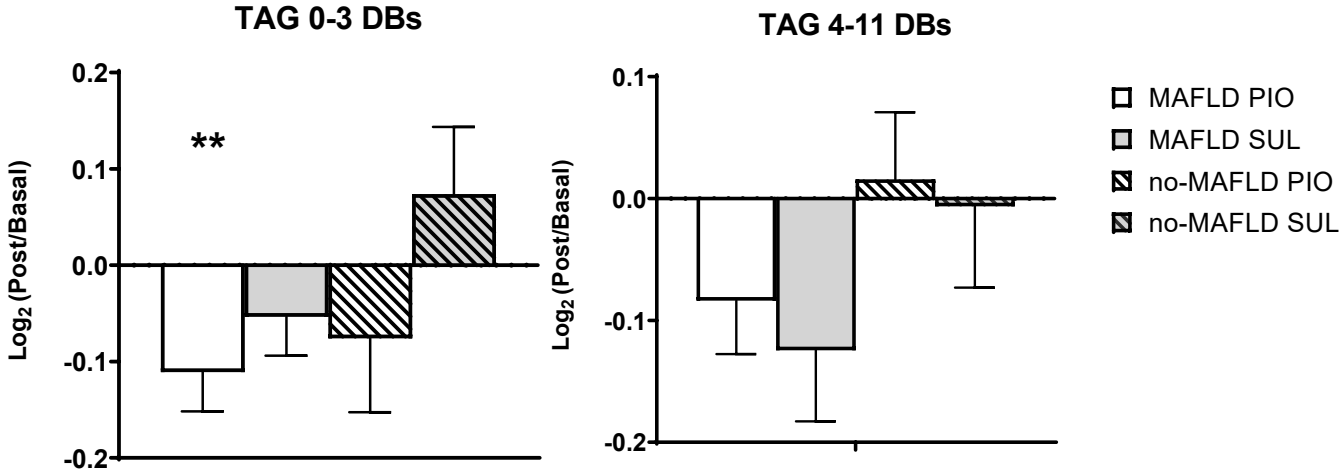


Figure 10. Changes of saturated or partially saturated (0-3 double bonds) and unsaturated (4-11 double bonds) TAGs in MAFLD vs noMAFLD groups within treatments; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 vs baseline

CER were significantly decreased in both treatment groups (p≤0.01); PC significantly in MAFLD PIO and SUL(p≤0.01); DAG significantly increased in both treatments, LPE increased in both MAFLD groups; no changes in total LPC and PE. **(Figure 11.)**

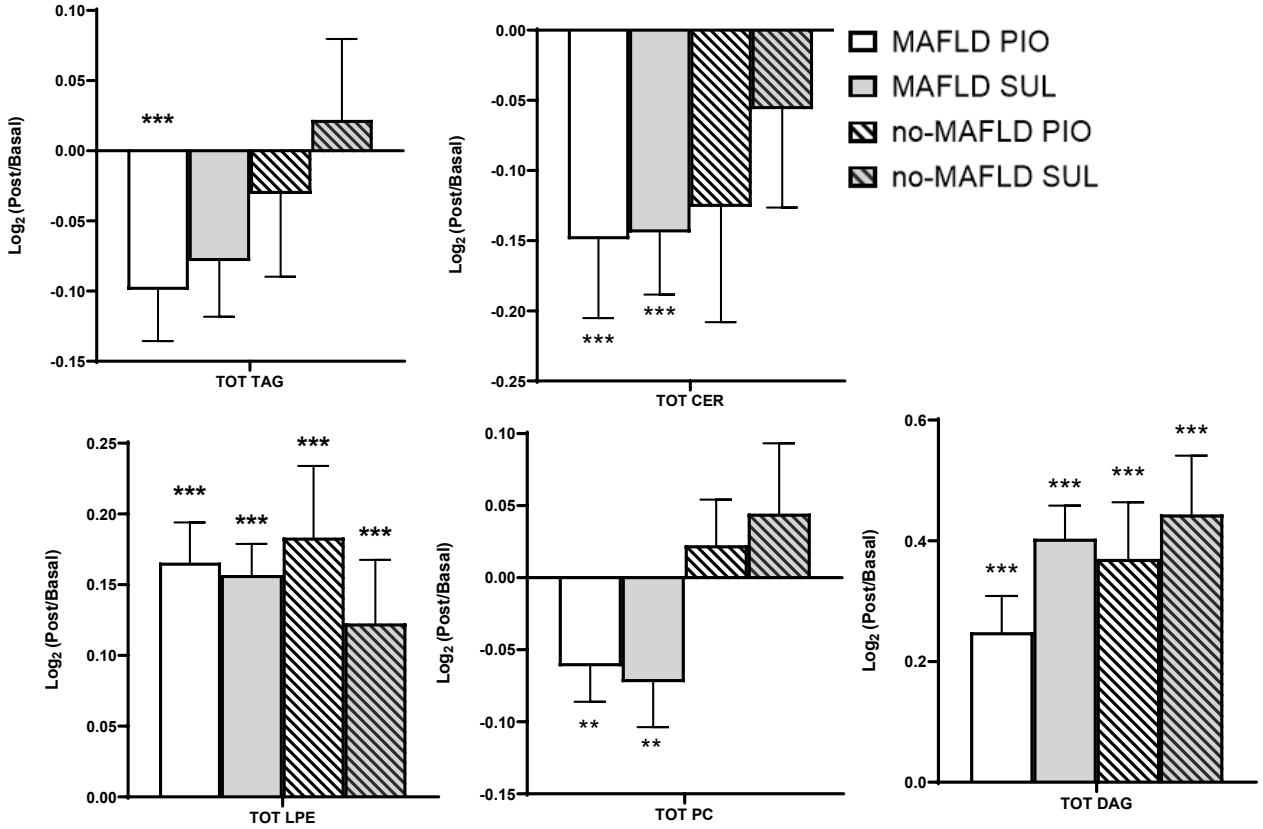


Figure 11. Changes in total lipids in MAFLD and noMAFLD groups within treatments; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 vs baseline

Meanwhile, going through single lipid species our analysis highlights a significant decrease in MAFLD PIO group in the TAGs often found correlated to cardiometabolic diseases TAG 48:0, 48:2, 50:0, 50:1, 50:2, 54:2 and 48:1. Furthermore, there was a significant decrease in both MAFLD group ceramides d18:0/18:0, d18:0/20:0, d18:0/22:0 and d18:0/24:0 one of the proinflammatory lipid classes that is also a determinant of lipotoxicity. **(Figure 12.)**

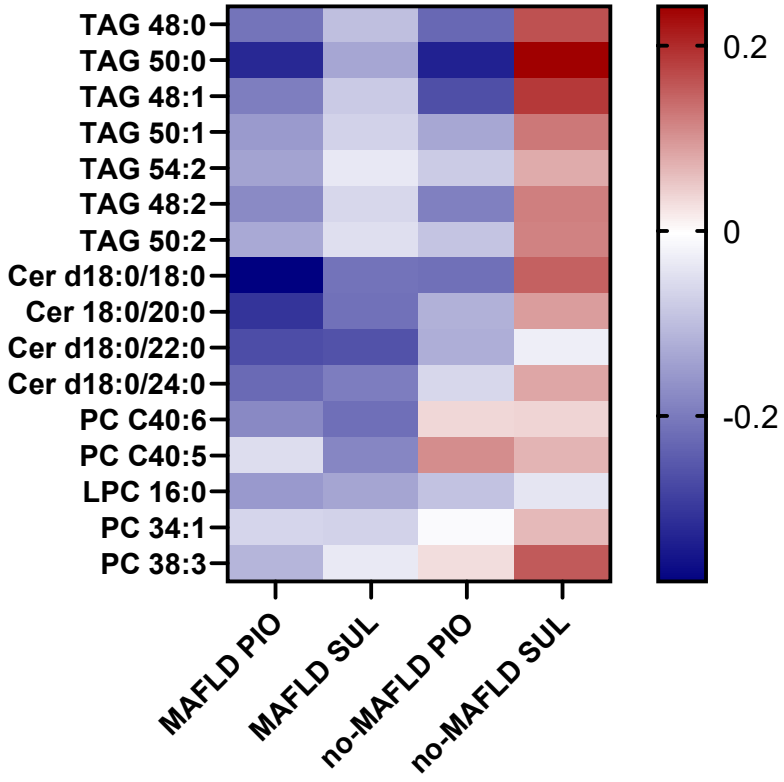


Figure 12. Heatmap of the fold changes from baseline of single lipid species associated to cardiometabolic diseases analyzed in MAFLD and noMAFLD groups and per treatment (PIO vs SUL)

3.4 Discussion

The results of this study clearly show that the great majority of subjects with poorly controlled diabetes have MAFLD. In our cohort, patients with diabetes and MAFLD and noMAFLD had similar glycemic levels and HbA1c at baseline, while insulin concentrations were approximately three times higher in the MAFLD group due to insulin resistance [82]. The hepatic enzymes were remarkably higher in the MAFLD group indicating liver disease. Further, as expected, patients with MAFLD have higher levels of total cholesterol and triglycerides. Moreover, lipidomic analysis showed that plasma lipid species that in literature are associated with NAFLD [83,84], i.e., TAG 48:0, TAG 50:0, TAG 48:1, TAG 50:1, TAG 48:2, TAG 50:2, TAG 54:2, Cer(d18:0/18:0),

Cer(d18:0/20:0), Cer(d18:0/22:0), Cer(d18:0/24:0) PC aa C28:0, PC aa C40:6, PC aa C40:5, LysoPC C16:0, were significantly higher in the MAFLD group compared to noMAFLD.

After 1 year treatment with either pioglitazone or sulphonylureas, these patients showed an improved glycemic control (fasting glucose and HbA1c, $p<0.0001$ vs baseline) with both treatments, while improvement from baseline in liver enzymes and in the lipidomic profile was observed only in patients with MAFLD treated with pioglitazone, confirming recent studies [85,86].

Our findings evidenced a different modulation of the lipidomic profile by the two treatments also influenced by the presence of MAFLD. Only the MAFLD group treated with PIO showed a reduction in insulin resistance (HOMA-IR and ADIPO-IR) and a significantly improvement in HDL, LDL, and total TGs, which were not seen in no-MAFLD PIO, MAFLD and no-MAFLD sulfonylurea one. This was somehow expected since thiazolidinediones (TZD) are among the most effective drugs for NAFLD/NASH treatment due to their ability to improve insulin resistance especially in the adipose tissue and to decrease lipolysis and FFA overflow to the liver while increasing FFA uptake[44]. This is in line with previous studies that showed how pioglitazone treatment was associated with an increase adipose tissue insulin sensitivity with a concomitant reduction of liver fibrosis in T2D patients [87].

In depth, we evaluated fatty acid composition in the circulating FFA and TAGs. While FFA composition was similar in MAFLD and noMAFLD at baseline and was not affected by either treatment, the saturated or partially saturated triglycerides (i.e., TAGs containing 0 to 3 double bonds) were significant reduced by pioglitazone in MAFLD patients. Several lipids previously founded increased in NAFLD were significantly reduced after

pioglitazone only in patients that were MAFLD at baseline (Figure 12). Pioglitazone significantly reduced principally lipids containing saturated fatty acids like palmitate or stearate, or their desaturated forms palmitoleic acid and oleate that are major cause of liver lipotoxicity [88–90]. Moreover, pioglitazone significantly reduced circulating ceramides, which are compounds derived from palmitate associated with apoptosis and insulin resistance. The reduction in ceramides species is highly connected with a reduction in lipotoxicity as recent studies highlights[94,95].

In conclusion, the prevalence of MAFLD among T2D patients with poor glycemic control in high. The PIO+MET treatment showed an improvement in the lipid and liver profile, associated with the improvement of other metabolic parameters mainly of the Adipo-IR and HOMA-IR independently from glycemic improvement which was superior to the improvement shown by sulphonylureas. Of note subjects with MAFLD at baseline showed a better improvement compared to noMAFLD.

4. Chapter 4

Effects of Pioglitazone on DNL in liver and Adipose Tissue depots of Mice Fed a High Sugar Diet

4.1 Introduction

Pioglitazone is member of Thiazolidinedione (TZD) class, significantly associated with an increase of insulin sensitivity, commonly used in T2D and NAFLD patients [72,7]. Pioglitazone is a PPAR-γ agonist so exert its effect through its selective binding [2]. Moreover, it has been proven to increase the hepatic and adipose tissue insulin sensitivity [52,92]. Furthermore, no drug has been approved yet for the treatment of NAFLD, PPAR- γ agonists (as single or multiple agonists) have been shown to improve both hepatic metabolism and histology [94].

There is a clear cross talk between the adipose tissue and the liver that drives the development and progression of NAFLD [91,95]. De novo lipid synthesis (DNL) occurs mainly in the liver but we cannot exclude that DNL occurs also in the adipose tissues, mainly in the mesenteric adipose tissue [52].

Considering that mesenteric adipose tissue is drained by the portal vein, hence any release of fatty acids directly impacts the liver [96]. It has been shown that Pioglitazone reduces visceral fat (mainly mesenteric) and this is associated to decreased hepatic fat and improved histology in subjects with NASH [78].We hypothesize that Pioglitazone might inhibit hepatic lipid accumulation not only by reducing but also by remodelling mesenteric adipose tissue triglyceride metabolism. To prove this, we have studied adipose tissue metabolism and composition in an animal model of NAFLD by NMR

spectroscopy, LC-mass spectrometry and isotope tracers (2H2O) for the measurement of DNL.

4.2 Methods

4.2.1 Animal protocol

Nineteen 12-week-old C57BL/6J mice were used in this study. Animals were fed with standard chow and maintained in a 12-h light cycle as well as controlled humidity, ventilated air, and temperature at the CEDOC-NOVA Medical School bioterium. At the beginning of the dark period, animals were injected intraperitoneally with 99.9% enriched 2H2O containing 0.9% NaCl (4 mL/100 g body weight). Five mice (CTRL) were kept at standard chow meanwhile for the remaining fourteen the drinking water was supplemented with 5% of 2H2O and glucose and fructose (HFG) were added (17.5 g of each sugar to 100 g water). Moreover, seven (HFG+PIO) of the fourteen ones were supplemented with Pioglitazone (30mg/Kg/day). The pioglitazone solution preparation has been made by mixing stock 6mg/mL in 0.5%methylcellulose+0.6% Tween80. Livers and adipose tissue depots were freeze clamped and stored at−80 °C until further processing for TG extraction and purification. Urine was also collected overnight for analysis of body water 2H-enrichment.

4.2.2 Liver TG extraction and purification

Liver TGs were extracted and purified as previously described (Matyash et al., 2008; Viegas et al., 2016). Briefly, livers were powdered under liquid nitrogen and then rapidly mixed with HPLC-grade methanol (4.6 mL/g) followed by methyl-tert-butyl ether (MTBE) (15.4 mL/g). The mixture was placed in a shaker for 1 h at room temperature then centrifuged at 13,000 x g for 10 min. The liquid fraction was collected, and phase

separation was induced by adding 4 mL of distilled water to the liquid fraction and letting it rest at room temperature for 10 min. The liquid was then centrifuged for 10 min at 1000 x g. The upper organic phase containing the lipids was carefully separated and dried under nitrogen gas in an amber glass vial. TGs from the dried organic fraction were purified with a solid phase extraction (SPE) process. Discovery DSC-Si SPE cartridges (2 g/12 mL) were washed with 8 mL of hexane/MTBE (96/4; v/v) followed by 24 mL of hexane. The dried lipids were re-suspended in 800 µL of hexane/MTBE (200/3; v/v) and loaded into the column after washing. The lipid vials were washed with a further 500 µL of solvent to quantitatively transfer the lipids to the column. TGs were eluted with 32 mL of hexane/MTBE (96/4; v/v), collected in 4 mL fractions. Fractions containing TGs were identified by thin-layer chromatography (Hamilton and Comai, 1988). A few microliters of the eluted fractions were spotted on the TLC plate alongside TG standards and the plate was developed with petroleum ether/diethyl ether/acetic acid (7.0/1.0/0.1; v/v/v). After drying, lipid spots were visualized under iodine vapor. The TG-containing fractions were pooled and dried under nitrogen gas and stored at −20 °C until NMR analysis. For analysis of adipose tissue TGs, 40–60 mg of frozen adipose tissue portions was placed for a few minutes in a 1 mL glass vial containing ~0.5 mL CHCl3 and gently shaken. The supernatant was pipetted away from the solid tissue and prepared for NMR analysis without any further purification.

4.2.3 NMR analysis

Purified TGs were dissolved in ~0.4 mL CHCl3. To these, as well as to the adipose tissue CHCl3 fractions, 25 µL of a pyrazine standard enriched to 1% with pyrazine-d4 and dissolved in CHCl3 (0.07 g pyrazine/ g CHCl3) and 50 µL C6F6 were added. 1H and 2H NMR spectra were acquired with an 11.7 T Bruker Avance III HD system using a

dedicated 5mm 2H-probe with 19F lock and 1H-decoupling coil as previously described (Viegas et al., 2016). 1H spectra at 500.1 MHz were acquired with a 90-degree pulse, 10 kHz spectral width, 3 s of acquisition time and 5 s of pulse delay. Sixteen free-induction decays (fid) were collected for each spectrum. 2H NMR spectra at 76.7 MHz were obtained with a 90-degree pulse, a 1230 Hz spectral width, an acquisition time of 0.37 s and a pulse delay of 0.1 s. Approximately 20,000 fid were acquired for each spectrum. Correction factors for partially-saturated 2H signals were obtained from a set of seven samples where for each sample, a spectrum was acquired with the described parameters and immediately followed by a spectrum acquired under the same parameters with the exception of the acquisition time and pulse delay, which were set to 1 s and 8 s, respectively. For analysis of body water 2H-enrichment, 10 µL of urine were mixed with 1 mL acetone and ~0.5 mL were loaded in a 5mm NMR tube to which 50 µL C6F6 were added. 2H NMR spectra of these samples were acquired with a 23° pulse angle, 922 Hz spectral width, 4 s acquisition time and 8 s pulse delay (Jones et al., 2001). Sixteen fid were collected for each spectrum and water 2H-enrichment was estimated from a calibration curve calculated from 2H-enriched water standards (Jones et al., 2001). For 13C isotopomer analysis by 13C NMR, dried TG samples were dissolved in 0.2 mL 99.96% enriched CDCl3 (Sigma-Aldrich) and placed in 3mm NMR tubes. 13C NMR spectra were acquired at 150.8 MHz with an Agilent V600 spectrometer equipped with a 3mm broadband probe. Spectra were acquired with a 70° pulse, an acquisition time of 2.5 s, and a 0.5 s pulse.

4.2.4 LC-MS analysis

Liver tissues were homogenized with 300 µL cold methanol (adding 20 µL of internal standard mix) on Precellys Evolution Homogenizer (Bertin Instruments, Frankfurt,

Germany) (3 cycles of 30 seconds at 5500 rpm, with 10 seconds pause between each cycle, at 4°C) using one titanium bead and two smaller ceramic beads. The homogenized sample was centrifuged at 14000 rpm for 20 min, and proteins were precipitated. The internal standard solution contained the following compounds: 1,2-diheptadecanoyl-sn-glycero-3-phosphoethanolamine (PE (17:0/17:0)), N-heptadecanoyl-D-erythro-sphingosylphosphorylcholine (SM(d18:1/17:0)), N-heptadecanoyl-D-erythro-sphingosine (Cer(d18:1/17:0)), 1,2-diheptadecanoyl-sn-glycero-3-phosphocholine (PC(17:0/17:0)), 1-heptadecanoyl-2-hydroxy-sn-glycero-3-phosphocholine (LPC(17:0)) and 1-palmitoyl-d31-2-oleoyl-sn-glycero-3-phosphocholine (PC(16:0/d31/18:1)), (Avanti Polar Lipids Inc., Merck, Germany). In addition, the mixture also contained triheptadecanoin (TG (17:0/17:0/17:0), Larodan, Sweden).

The supernatant was placed in a tube, and 600 µL of chloroform and 200 µL water were added for phase separation after 15 min centrifugation at 14000 rpm. The upper phase was collected and dried under gentle nitrogen flux and samples were resuspended in 10 µL of chloroform and 290 µL of methanol and placed in vials to be analyzed in LCMS.

TAGs and CER were quantified with Liquid Mass Spectrometry coupled with a Quadrupole Time-Of-Flight Mass Spectrometry QTOF (UHPLC 1290- QTOF-MS 6540, Agilent, Santa Clara CA) equipped with a C18 column (ZORBAX Eclipse Plus C18 2.1 × 100 mm 1.8-Micron, Agilent, USA) and electrospray ionization (ESI) set in positive mode. For the quantification of the lipidome, the spectra were analyzed using the MassHunter Profinder software (Agilent, Santa Clara CA).

Deuterium enrichment in the respective TAG species was measured with mass spectrometry (6545 QTOF, Agilent, Santa Clara, CA) and analyzed with software Agilent MassHunter Profinder 8.00. To quantify deuterium enrichment in the TAG species,

m + 1/m + 0 area ratio from a non-labelled standard reference was used to correct for background; TAGneogenesis was quantified by dividing the deuterium enrichment in each TAG species by the number of possible hydrogens exchanged with deuterium, and normalized by precursor water enrichment (5 %).

4.2.5 Metabolic test

All mice were subjected to metabolic tests, i.e., oral glucose tolerance test (OGTT) that was performed after overnight fasting by oral administration of 1 g of glucose/kg body weight. Blood was collected from the tail vein, and glucose concentration was determined using the One-Touch AccuChek Glucometer (Roche).

4.3 Results

Three weeks of HFG or HFG+PIO did not change glucose tolerance measured by OGTT even though HFG and HFG+PIO mice had higher glycemia at 15 ($p<0.05$) and 30 minutes compared to controls. (**Figure 13)**

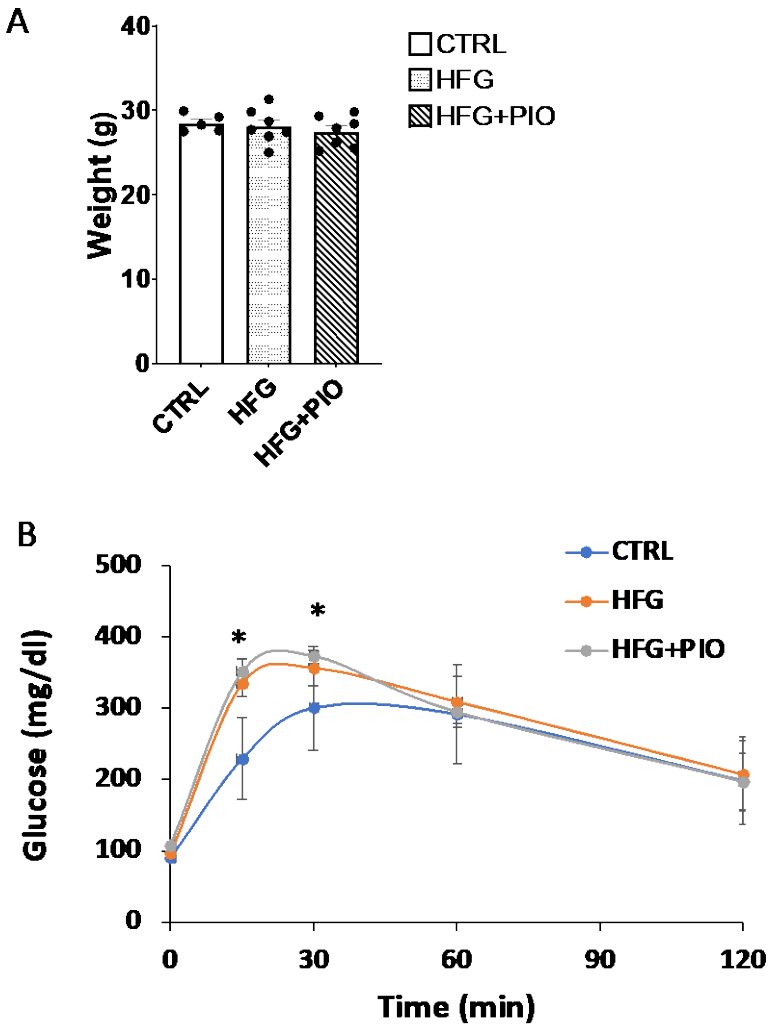


Figure 13. (A) Body weight after 4 weeks of treatment. **(B)** OGTT profile in controls, HFG e HFG+PIO *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 vs controls; ANOVA.

The qualitative (%) lipidomic analysis by NMR of mesenteric epididymal and subcutaneous adipose tissue highlighted several differences after HFG diet with/without PIO. HFG diet increased SFA and decreased UFA in the subcutaneous fat, mainly by decreasing PUFA, since MUFA were increased, while no significant change was observed in epididymal and mesenteric adipose depots (**Figure 14).** Addition of pioglitazone to HFG diet reduced SFA and increased UFA in mesenteric and while the effects in epididymal and subcutaneous fat were similar to HFG diet alone (**Figure 14**). No differences in linoleic and DHA.

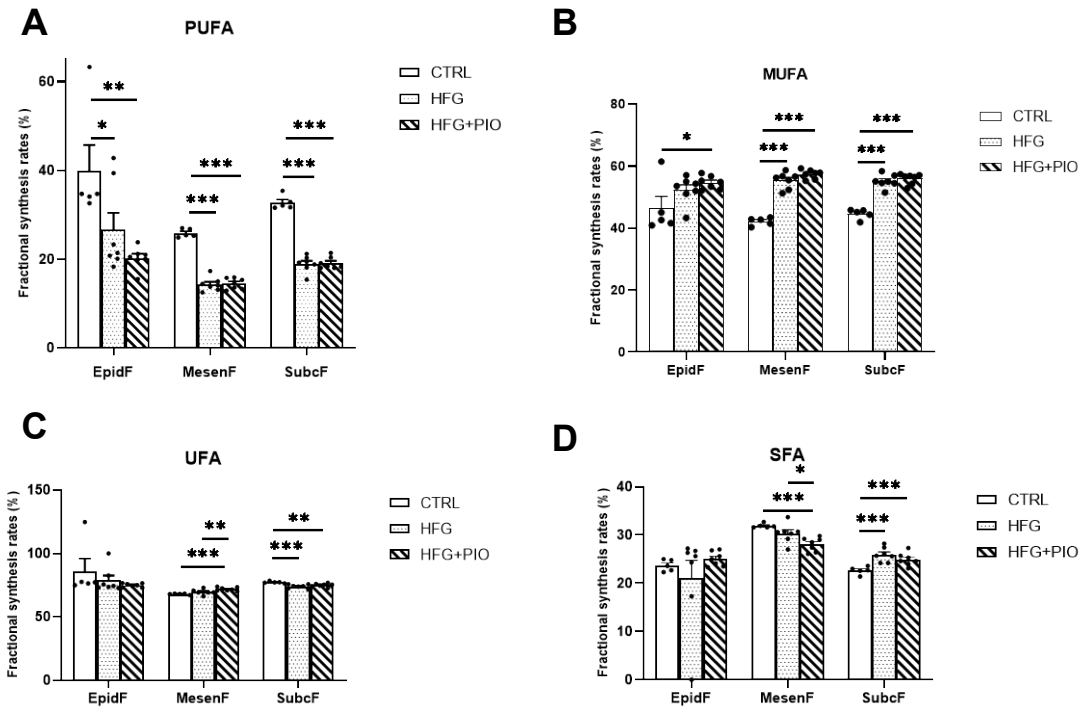


Figure 14. PUFA, MUFA, UFA and SFA in different adipose tissues between the controls, HFG and HFG+PIO diets; results are presented as Mean±SEM; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

Changes in hepatic lipid composition measured by LC-MS revealed a protection against TAG accumulation in HFG+PIO group (i.e., more similar to control diet than to the values observed in HFG group, **Figure 15 A**). The HFG diet reduced total hepatic ceramides and a further significant reduction was observed in HFG+PIO vs HFG (p≤ 0.001) (**Figure 15 B**). Hence, we analyzed single TAG and CER species highly associated with NAFLD. We found that PIO limited the increase in TAG 52:0, TAG 50:0 but not in TAG 48:0 in the HFG group (**Figure 15 C**). We observed that dihydroceramides, i.e., Cer(d18:0/XX), were decreased after HFG+PIO compared to control diet. Cer(d18:0/24:0) and Cer(d18:0/24:1) were significantly lower in HFG than control and significantly decreased in HFG+PIO group. Compared to control diet Cer(d18:0/22:0) was higher in HFG and lower in HFG+PIO

respectively. Cer(d18:0/18:0) was lower than control in HFG+PIO but unchanged in HFG diet (**Figure 15 D**).

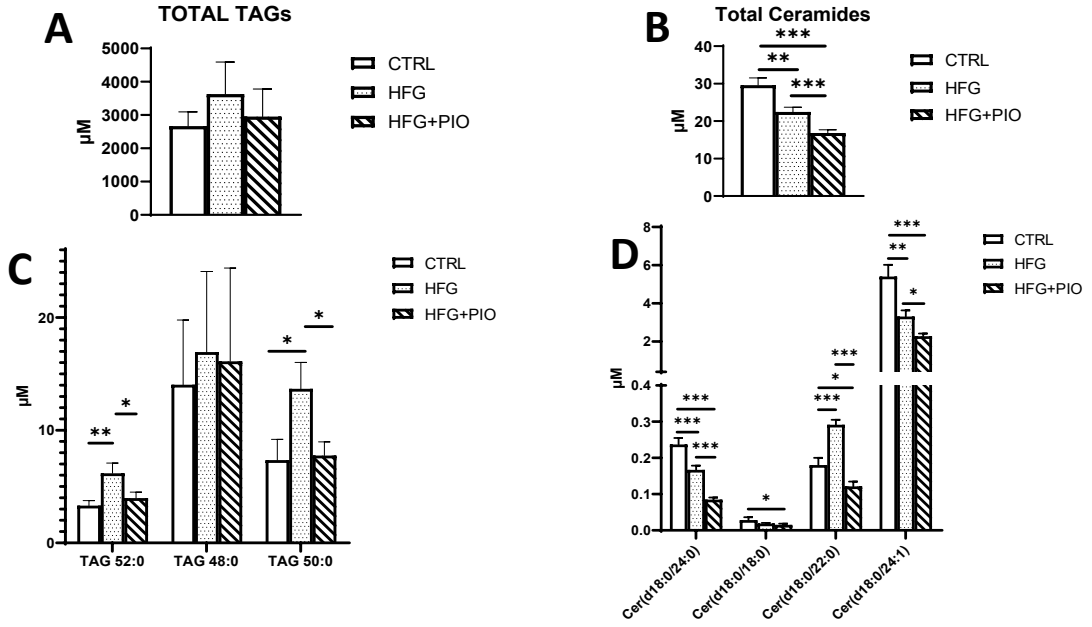


Figure 15. A) Liver Total TAGs, **B)** Total Ceramides and **C)** and **D)** single species correlated to NAFLD; results are presented as Mean±SEM; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001; ANOVA.

4.3.1 Fluxomic analysis

NMR data shows clearly that HFG increases DNL% and this occurred more in the mesenteric than other adipose tissues (**Figure 16A**). The addition of PIO to HFG limited the increase in DNL% in the mesenteric and epididymal adipose tissues, while it increased DNL% in subcutaneous fat compared to HFG and control diet (**Figure 16A**). Similarly, desaturation of fatty acids was increased after HFG in the mesenteric and subcutaneous fat (**Figure 16 B**) and PIO reduced desaturation in mesenteric AT

compared to HFG, while in subcutaneous fat % desaturated fatty acids were similarly elevated in HFG and HFG+PIO compared to control diet (**Figure 16 B**).

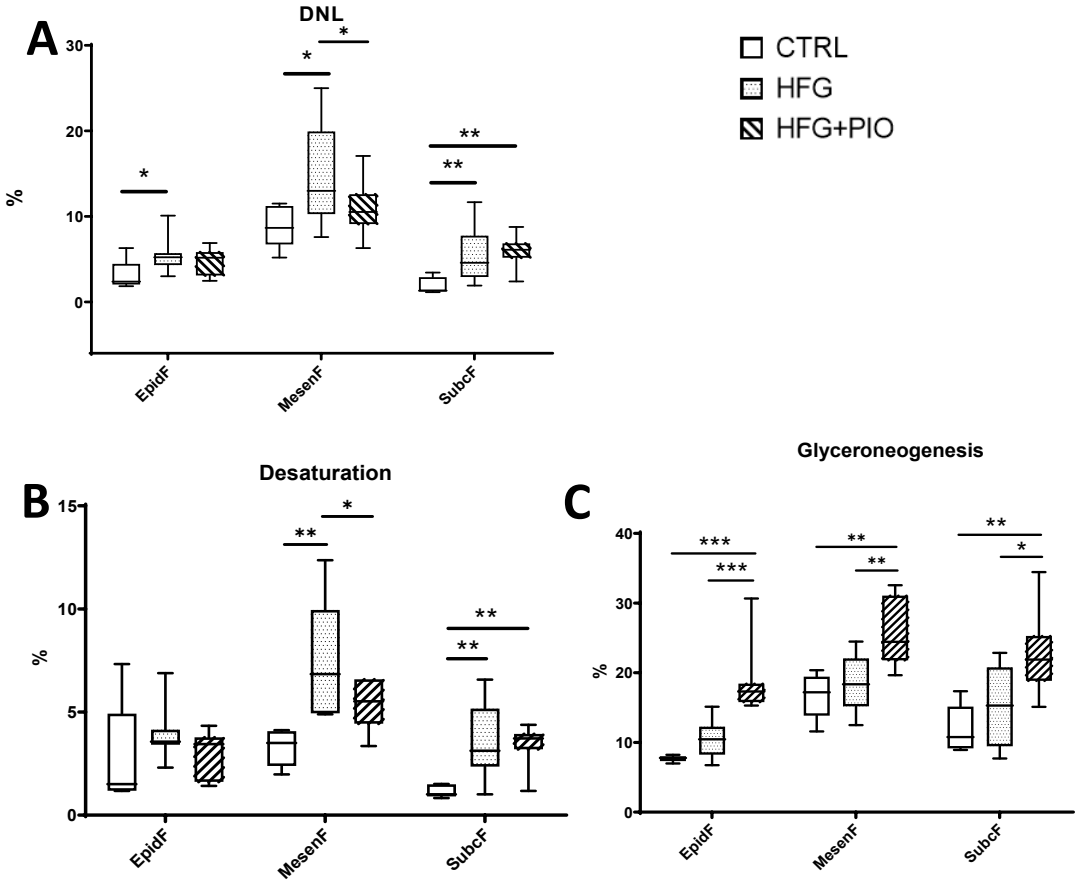


Figure 16. Adipose tissue fluxomic measured by NMR: **A)** DNL and **B)** Desaturation and **C)** Glyceroneogenesis in different adipose tissues. Difference between the controls, HFG and HFG+PIO diets. Results are presented as Mean±SEM; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

During the measurement of de novo synthesis of triglycerides it is important not only to measure the contribution of de novo synthesized fatty acids (mainly palmitate) but also to measure the rate of glyceroneogenesis. **Figure 16** shows the contribution of glyceroneogenesis to adipose tissue TAG-glycerol that was significantly higher in the HFG+PIO group than in the control or HFG groups in all three adipose tissues (EpidF p ≤ 0.001, MesenF p ≤ 0.01, SubcF p ≤ 0.01).

Regarding the liver analyses **Figure 17** shows the % deuterium enrichment of specific TAG species and included the contribution of both de novo fatty acid synthesis and glyceroneogenesis. Enrichment of TAG 48:0, TAG 50:0 and TAG 52:0 was increased after HFG compared to control diet, while the HFG+PIO group showed a significative reduction of TAGs correlated to DNL compared to HFG group, but not different from control group.

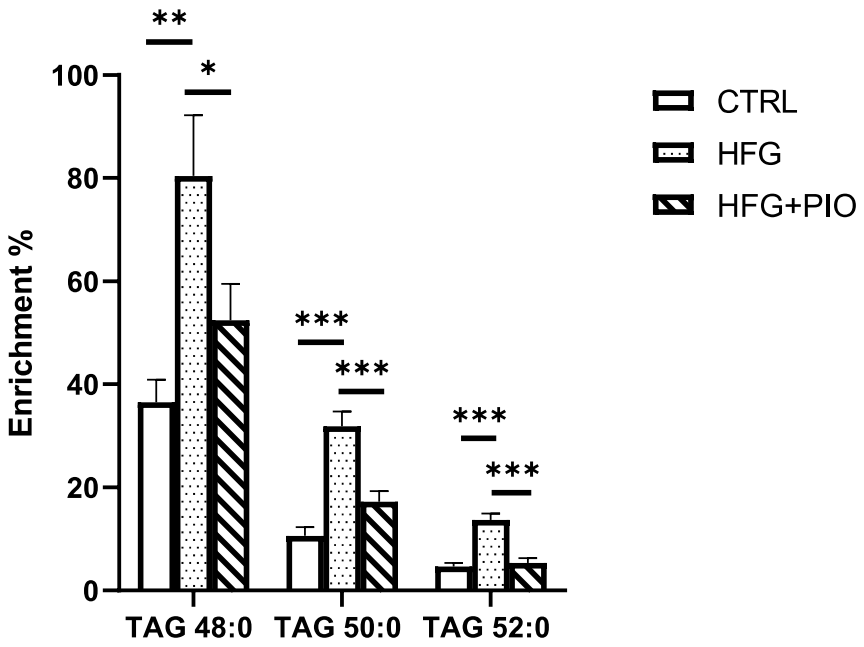


Figure 17. Deuterium enrichment (M+2/M+0 expressed as %) of liver TAGs in different groups after 5 days of D2O. TAGs 48:0, 50:0 and 52:0 are associated with DNL. Results are presented as Mean±SEM; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001; ANOVA.

4.4 Discussion

Non-alcoholic fatty liver disease still has no conclusively effective pharmacology treatment although Thiazolidinediones have proven to be one of the most successful [49,50,87], due to their ability to ameliorate IR and redistribute adipose tissue (AT) with

a reduction of visceral adipose tissue and an increase in subcutaneous adipose tissue [102–104]; still, most of the mechanisms behind metabolic improvements are unclear.

We have studied the impact of pioglitazone treatment on adipose tissue and liver lipid metabolism. Since mesenteric adipose tissue is drained by the portal vein, hence any release of fatty acids directly impacts the liver [105]. In this chapter we analyzed the effects of Pioglitazone on different adipose tissues and liver lipid composition and synthesis by DNL in an animal model of NAFLD, i.e., after HFG diet.

Glucose concentrations were similar in HFG and HFG+PIO, and glucose tolerance, i.e., 2-hour glucose concentrations during OGTT, was similar in the 3 groups. These results confirm that any improvement in lipid metabolism and in the lipidomic profile is independent of changes in glycemic control.

The analyses of adipose tissue fatty acid composition and saturation showed that HFG decreased PUFA and increased MUFA in all adipose tissue depots, but the addition of pioglitazone had no effect. However, in the mesenteric adipose tissue the addition of pioglitazone increased UFA and decreased SFA.

The composition of fatty acids of the adipose tissues derived from *de* novo lipid synthesis evidences an increase of DNL in the HFG group in all tissues, but higher in the mesenteric adipose tissue, in line with a recent study [101]. Interestingly, the HFG+PIO group shows a significant lower DNL compared to HFG in mesenteric adipose tissue and an increase in the subcutaneous adipose tissue highlighting its effect on fat remodeling and redistribution. In line with the results of DNL also the desaturation (%) shows the same trend. The synthesis of triacylglycerols depends on the availability of glycerol-3P that in the adipose tissue can be synthesized by glucose or as glyceroneogenesis. In the adipose

tissue depots pioglitazone significantly increased glyceroneogenesis vs controls and vs HFG, showing how pioglitazone contributes to promote the lipid-buffering function of subcutaneous adipose tissue.

The analysis of lipid composition indicates that pioglitazone ameliorates NAFLD with a significant reduction of TAGs correlated with DNL (i.e., TAG 52:0, TAG 50:0 and a trend in TAG 48:0) in line with a recent study[107]. Furthermore, the enrichment (%) analysis on the TAG 48:0, 52:0 and 50:0, i.e., TAGs that includes the saturated fatty acids derived primarily by DNL, shows how pioglitazone reduces hepatic DNL. Moreover, PIO reduced total ceramides and dehydroceramides that are markers of lipotoxicity and NAFLD[92,108,109].

In conclusion, our study highlights some of the mechanisms by which pioglitazone improves NAFLD, i.e., by the reduction of mesenteric adipose tissue triglyceride synthesis that might be directly related to reduced hepatic lipid accumulation; certainly, more in dept analysis should be done in order to definitely confirm the results.

Final remarks

This thesis addressed I have investigated the effects of Pioglitazone on lipid metabolism in two different settings: a) in patients with T2D and fatty liver disease where I have investigated the effects of PIO treatment vs SUL on insulin resistance, indexes of NAFLD, liver enzymes and circulating plasma lipidome (by GC-MS and LC-MS lipidomics); b) in an animal NAFLD model where I have investigated PIO effects on lipid synthesis (by NMR and LC-MS fluxomics) and composition (by NMR and LC-MS lipidomics) in different adipose tissue depots and liver.

The results of my thesis showed that most of T2D patients with poor glycemic control had MAFLD and that pioglitazone was effective in ameliorating MAFLD, by reducing hepatic enzymes, insulin resistance and improving lipidomic profile. This was evident even when lower doses of PIO (15 mg/day and 30 mg/day) were used to improve glycemic control, and regardless of changes in blood glucose control, suggesting that lipid improvement is also mediated by improved sensitivity.

Pioglitazone treatment reduced the concentrations of lipids associated with fatty liver disease i.e., TAG (48:0), TAG (48:1), TAG (50:0), TAG (50:0), TAG (50:1), TAG (50:2), PC C28:0, PC 40:5, PC 40:6, LPC 16:0 and several dihydroceramides; this was associated with the improvement of other metabolic parameters mainly the Adipo-IR and HOMA-IR, independently of glycemic improvement.

Furthermore, in an animal model of NAFLD we evidenced how Pioglitazone induces a different remodeling of adipose tissue depots by reducing DNL in the mesenteric adipose tissue (that has been proven to have increased rates of de novo lipid synthesis) and increasing it in the subcutaneous fat. Fluxomic data on liver that shows a reduction

in the TAGs correlated with DNL indicating that this might one of the mechanisms through which pioglitazone decreases hepatic lipid accumulation.

In conclusion, the results of this thesis add new knowledge on the effect of pioglitazone and its possible use for the treatment of NAFLD/MAFLD and suggest the use of this drug for the treatment of NAFLD/MAFLD.

REFERENCES

1 Younossi ZM, Koenig AB, Abdelatif D, *et al.* Global Epidemiology of Nonalcoholic Fatty Liver Disease-Meta-Analytic Assessment of Prevalence, Incidence, and Outcomes. Published Online First: 2015. doi:10.1002/hep.28431/suppinfo

2 Gaggini M, Morelli M, Buzzigoli E, *et al.* Non-Alcoholic Fatty Liver Disease (NAFLD) and Its Connection with Insulin Resistance, Dyslipidemia, Atherosclerosis and Coronary Heart Disease. *Nutrients* 2013;**5**:1544. doi:10.3390/NU5051544

3 Dai W, Ye L, Liu A, *et al.* Prevalence of nonalcoholic fatty liver disease in patients with type 2 diabetes mellitus: A meta-analysis. *Medicine* 2017;**96**. doi:10.1097/MD.0000000000008179

4 Younossi ZM, Koenig AB, Abdelatif D, *et al.* Global epidemiology of nonalcoholic fatty liver disease—Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology* 2016;**64**:73–84. doi:10.1002/HEP.28431/SUPPINFO

5 Younossi ZM, Marchesini G, Pinto-Cortez H, *et al.* Epidemiology of Nonalcoholic Fatty Liver Disease and Nonalcoholic Steatohepatitis: Implications for Liver Transplantation. *Transplantation* 2019;**103**:22–7. doi:10.1097/TP.0000000000002484

6 Kim CH, Younossi ZM. Nonalcoholic fatty liver disease: a manifestation of the metabolic syndrome. *Cleveland Clinic journal of medicine* 2008;**75**:721–8. doi:10.3949/CCJM.75.10.721

7 Ballestri S, Zona S, Targher G, *et al.* Nonalcoholic fatty liver disease is associated with an almost twofold increased risk of incident type 2 diabetes and metabolic syndrome. Evidence from a systematic review and meta-analysis. *Journal of gastroenterology and hepatology* 2016;**31**:936–44. doi:10.1111/JGH.13264

8 Vanni E, Bugianesi E, Kotronen A, *et al.* From the metabolic syndrome to NAFLD or vice versa? *Digestive and liver disease : official journal of the Italian Society of Gastroenterology and the Italian Association for the Study of the Liver* 2010;**42**:320–30. doi:10.1016/J.DLD.2010.01.016

9 Day CP, James OFW. Steatohepatitis: a tale of two “hits”? *Gastroenterology* 1998;**114**:842–5. doi:10.1016/S0016-5085(98)70599-2

10 Tilg H, Moschen AR. Evolution of inflammation in nonalcoholic fatty liver disease: The multiple parallel hits hypothesis. *Hepatology* 2010;**52**:1836–46. doi:10.1002/HEP.24001

11 Berlanga A, Guiu-Jurado E, Porras JA, *et al.* Molecular pathways in non-alcoholic fatty liver disease. *Clinical and experimental gastroenterology* 2014;**7**:221–39. doi:10.2147/CEG.S62831

12 Bechmann LP, Hannivoort RA, Gerken G, *et al.* The interaction of hepatic lipid and glucose metabolism in liver diseases. *Journal of hepatology* 2012;**56**:952–64. doi:10.1016/J.JHEP.2011.08.025

13 Buzzetti E, Pinzani M, Tsochatzis EA. The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD). *Metabolism - Clinical and Experimental* 2016;**65**:1038–48. doi:10.1016/J.METABOL.2015.12.012

14 Veena J, Muragundla A, Sidgiddi S, *et al.* Non-alcoholic fatty liver disease: need for a balanced nutritional source. *British Journal of Nutrition* 2014;**112**:1858–72. doi:10.1017/S0007114514002591

15 Nseir W, Hellou E, Assy N. Role of diet and lifestyle changes in nonalcoholic fatty liver disease. *World Journal of Gastroenterology : WJG* 2014;**20**:9338. doi:10.3748/WJG.V20.I28.9338

16 Mirmiran P, Amirhamidi Z, Ejtahed HS, *et al.* Relationship between Diet and Non-alcoholic Fatty Liver Disease: A Review Article. *Iranian Journal of Public Health* 2017;**46**:1007./pmc/articles/PMC5575379/ (accessed 20 Jan 2022).

17 Redgrave TG. Formation of cholesteryl ester-rich particulate lipid during metabolism of chylomicrons. *The Journal of clinical investigation* 1970;**49**:465–71. doi:10.1172/JCI106255

18 Cooper AD. Hepatic uptake of chylomicron remnants. 1997;**3**:2173–92. doi:10.1016/S0022-2275(20)34932-4

19 Donnelly KL, Smith CI, Schwarzenberg SJ, *et al.* Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *The Journal of clinical investigation* 2005;**115**:1343–51. doi:10.1172/JCI23621

20 Hodson L, Frayn KN. Hepatic fatty acid partitioning. *Current Opinion in Lipidology* 2011;**22**:216–24. doi:10.1097/MOL.0B013E3283462E16

21 Nassir F, Rector RS, Hammoud GM, *et al.* Pathogenesis and Prevention of Hepatic Steatosis. *Gastroenterology & Hepatology* 2015;**11**:167./pmc/articles/PMC4836586/ (accessed 20 Jan 2022).

22 Ameer F, Scandiuzzi L, Hasnain S, *et al.* De novo lipogenesis in health and disease. *Metabolism: clinical and experimental* 2014;**63**:895–902. doi:10.1016/J.METABOL.2014.04.003

23 Donnelly KL, Smith CI, Schwarzenberg SJ, *et al.* Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *Journal of Clinical Investigation* 2005;**115**:1343. doi:10.1172/JCI23621

24 Ludwig J, Viggiano TR, McGill DB, *et al.* Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. *Mayo Clinic proceedings* 1980;**55**:434–8.

25 Younossi ZM, Blissett D, Blissett R, *et al.* The economic and clinical burden of nonalcoholic fatty liver disease in the United States and Europe. *Hepatology (Baltimore, Md)* 2016;**64**:1577–86. doi:10.1002/HEP.28785

26 Wiegand S, Keller KM, Röbl M, *et al.* Obese boys at increased risk for nonalcoholic liver disease: evaluation of 16 390 overweight or obese children and adolescents. *International Journal of Obesity 2010 34:10* 2010;**34**:1468–74. doi:10.1038/ijo.2010.106

27 Eslam M, Sanyal AJ, George J, *et al.* MAFLD: A Consensus-Driven Proposed Nomenclature for Metabolic Associated Fatty Liver Disease. *Gastroenterology* 2020;**158**:1999-2014.e1. doi:10.1053/J.GASTRO.2019.11.312

28 Sanchez-Rangel E, Inzucchi SE. Metformin: clinical use in type 2 diabetes. *Diabetologia* 2017;**60**:1586–93. doi:10.1007/S00125-017-4336-X

29 Type 2 diabetes: The management of type 2 diabetes | Guidance | NICE. https://www.nice.org.uk/guidance/cg87 (accessed 17 Jan 2022).

30 A Publication of the Professional Sections of the Canadian Diabetes Association Canadian Diabetes Association 2008 Clinical Practice Guidelines for the Prevention and Management of Diabetes in Canada.

31 Rodbard HW, Jellinger PS, Davidson JA, *et al.* Statement by an American Association of Clinical Endocrinologists/American College of Endocrinology consensus panel on type 2 diabetes mellitus: an algorithm for glycemic control. *Endocrine practice : official journal of the American College of Endocrinology and the American Association of Clinical Endocrinologists* 2009;**15**:540–59. doi:10.4158/EP.15.6.540

32 IDF Clinical Guidelines Task Force Global guideline for type 2 diabetes. International Diabetes Federation, Brussels (2005).

33 Bruno G, de Micheli A, Frontoni S, *et al.* Highlights from "Italian Standards of Care for Diabetes Mellitus 2009–2010." *Nutrition, Metabolism and Cardiovascular Diseases* 2011;**21**:302–14. doi:10.1016/J.NUMECD.2010.08.009

34 Yki-Järvinen H. Thiazolidinediones. *http://dx.doi.org/101056/NEJMra041001* 2009;**351**:1106–18. doi:10.1056/NEJMRA041001

35 Vaccaro O, Masulli M, Nicolucci A, *et al.* Effects on the incidence of cardiovascular events of the addition of pioglitazone versus sulfonylureas in patients with type 2 diabetes inadequately controlled with metformin (TOSCA.IT): a randomised, multicentre trial. *The Lancet Diabetes & Endocrinology* 2017;**5**:887–97. doi:10.1016/S2213-8587(17)30317-0

36 Rinella ME. Nonalcoholic Fatty Liver Disease. *JAMA* 2015;**313**:2263. doi:10.1001/jama.2015.5370

37 Mantovani A, Byrne CD, Bonora E, *et al.* Nonalcoholic Fatty Liver Disease and Risk of Incident Type 2 Diabetes: A Meta-analysis. *Diabetes Care* 2018;**41**:372–82. doi:10.2337/dc17-1902

38 Targher G, Byrne CD, Tilg H. NAFLD and increased risk of cardiovascular disease: clinical associations, pathophysiological mechanisms and pharmacological implications. *Gut* 2020;**69**:1691–705. doi:10.1136/gutjnl-2020-320622

39 Portillo-Sanchez P, Bril F, Maximos M, *et al.* High Prevalence of Nonalcoholic Fatty Liver Disease in Patients With Type 2 Diabetes Mellitus and Normal Plasma Aminotransferase Levels. *The Journal of Clinical Endocrinology & Metabolism* 2015;**100**:2231–8. doi:10.1210/jc.2015-1966

40 Younossi ZM, Golabi P, de Avila L, *et al.* The global epidemiology of NAFLD and NASH in patients with type 2 diabetes: A systematic review and meta-analysis. *Journal of Hepatology* 2019;**71**:793–801. doi:10.1016/j.jhep.2019.06.021

41 Alexander M, Loomis AK, van der Lei J, *et al.* Risks and clinical predictors of cirrhosis and hepatocellular carcinoma diagnoses in adults with diagnosed NAFLD: real-world study of 18 million patients in four European cohorts. *BMC Medicine* 2019;**17**:95. doi:10.1186/s12916-019-1321-x

42 Gastaldelli A, Cusi K. From NASH to diabetes and from diabetes to NASH: Mechanisms and treatment options. *JHEP Reports* 2019;**1**:312–28. doi:10.1016/j.jhepr.2019.07.002

43 della Pepa G, Vetrani C, Lombardi G, *et al.* Isocaloric Dietary Changes and Non-Alcoholic Fatty Liver Disease in High Cardiometabolic Risk Individuals. *Nutrients* 2017;**9**:1065. doi:10.3390/nu9101065

44 Fonseca J, Nunes G, Fonseca C, *et al.* Comment to: "EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease." *Journal of Hepatology* 2017;**66**:465–6. doi:10.1016/j.jhep.2016.10.036

45 Belfort R, Harrison SA, Brown K, *et al.* A Placebo-Controlled Trial of Pioglitazone in Subjects with Nonalcoholic Steatohepatitis. *New England Journal of Medicine* 2006;**355**:2297–307. doi:10.1056/NEJMoa060326

46 Aithal GP, Thomas JA, Kaye P v., *et al.* Randomized, Placebo-Controlled Trial of Pioglitazone in Nondiabetic Subjects With Nonalcoholic Steatohepatitis. *Gastroenterology* 2008;**135**:1176–84. doi:10.1053/j.gastro.2008.06.047

47 Sanyal AJ, Chalasani N, Kowdley K v., *et al.* Pioglitazone, Vitamin E, or Placebo for Nonalcoholic Steatohepatitis. *New England Journal of Medicine* 2010;**362**:1675–85. doi:10.1056/NEJMoa0907929

48 Cusi K, Orsak B, Bril F, *et al.* Long-Term Pioglitazone Treatment for Patients With Nonalcoholic Steatohepatitis and Prediabetes or Type 2 Diabetes Mellitus. *Annals of Internal Medicine* 2016;**165**:305. doi:10.7326/M15-1774

49 Musso G, Cassader M, Paschetta E, *et al.* Pioglitazone for advanced fibrosis in nonalcoholic steatohepatitis: New evidence, new challenges. *Hepatology* 2017;**65**:1058–61. doi:10.1002/hep.28960

50 Gastaldelli A, Miyazaki Y, Mahankali A, *et al.* The Effect of Pioglitazone on the Liver. *Diabetes Care* 2006;**29**:2275–81. doi:10.2337/dc05-2445

51 Glass LC, Cusi K, Berria R, *et al.* ORIGINAL ARTICLE: Pioglitazone improvement of fasting and postprandial hyperglycaemia in Mexican-American patients with Type 2 diabetes: a double tracer OGTT study. *Clinical Endocrinology* 2010;**73**:339–45. doi:10.1111/j.1365-2265.2010.03811.x

52 Gastaldelli A, Harrison SA, Belfort-Aguilar R, *et al.* Importance of changes in adipose tissue insulin resistance to histological response during thiazolidinedione treatment of patients with nonalcoholic steatohepatitis. *Hepatology* 2009;**50**:1087–93. doi:10.1002/hep.23116

53 Dormandy JA, Charbonnel B, Eckland DJ, *et al.* Secondary prevention of macrovascular events in patients with type 2 diabetes in the PROactive Study (PROspective pioglitAzone Clinical Trial In macroVascular Events): a randomised controlled trial. *The Lancet* 2005;**366**:1279–89. doi:10.1016/S0140-6736(05)67528-9

54 Yau H, Rivera K, Lomonaco R, *et al.* The Future of Thiazolidinedione Therapy in the Management of Type 2 Diabetes Mellitus. *Current Diabetes Reports* 2013;**13**:329–41. doi:10.1007/s11892-013-0378-8

55 Filipova E, Uzunova K, Kalinov K, *et al.* Pioglitazone and the Risk of Bladder Cancer: A Meta-Analysis. *Diabetes Therapy* 2017;**8**:705–26. doi:10.1007/s13300-017-0273-4

56 Miyazaki Y, Matsuda M, DeFronzo RA. Dose-Response Effect of Pioglitazone on Insulin Sensitivity and Insulin Secretion in Type 2 Diabetes. *Diabetes Care* 2002;**25**:517–23. doi:10.2337/diacare.25.3.517

57 Aronoff S, Rosenblatt S, Braithwaite S, *et al.* Pioglitazone hydrochloride monotherapy improves glycemic control in the treatment of patients with type 2 diabetes: a 6-month randomized placebo-controlled dose-response study. The Pioglitazone 001 Study Group. *Diabetes Care* 2000;**23**:1605–11. doi:10.2337/diacare.23.11.1605

58 Vaccaro O, Riccardi G, Maggioni A pietro. Risk of heart failure in diabetic patients receiving sulfonylureas. *European Journal of Heart Failure* 2018;**20**:1371–2. doi:10.1002/ejhf.1238

59 Vaccaro O, Masulli M, Bonora E, *et al.* Addition of either pioglitazone or a sulfonylurea in type 2 diabetic patients inadequately controlled with metformin alone: Impact on cardiovascular events. A randomized controlled trial. *Nutrition, Metabolism and Cardiovascular Diseases* 2012;**22**:997–1006. doi:10.1016/j.numecd.2012.09.003

60 Vitale M, Masulli M, Rivellese AA, *et al.* Pasta Consumption and Connected Dietary Habits: Associations with Glucose Control, Adiposity Measures, and Cardiovascular Risk Factors in People with Type 2 Diabetes—TOSCA.IT Study. *Nutrients* 2019;**12**:101. doi:10.3390/nu12010101

61 Kotronen A, Peltonen M, Hakkarainen A, *et al.* Prediction of Non-Alcoholic Fatty Liver Disease and Liver Fat Using Metabolic and Genetic Factors. *Gastroenterology* 2009;**137**:865–72. doi:10.1053/j.gastro.2009.06.005

62 Lee J-H, Kim D, Kim HJ, *et al.* Hepatic steatosis index: A simple screening tool reflecting nonalcoholic fatty liver disease. *Digestive and Liver Disease* 2010;**42**:503–8. doi:10.1016/j.dld.2009.08.002

63 Otgonsuren M, Estep MJ, Hossain N, *et al.* A single non-invasive model to diagnose non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH). *Journal of Gastroenterology and Hepatology* 2014;**29**:2006–13. doi:10.1111/jgh.12665

64 Gastaldelli A, Gaggini M, DeFronzo RA. Role of Adipose Tissue Insulin Resistance in the Natural History of Type 2 Diabetes: Results From the San Antonio Metabolism Study. *Diabetes* 2017;**66**:815–22. doi:10.2337/db16-1167

65 Amato MC, Giordano C, Galia M, *et al.* Visceral Adiposity Index. *Diabetes Care* 2010;**33**:920–2. doi:10.2337/dc09-1825

66 Hong S-W, Lee J, Cho JH, *et al.* Pioglitazone Attenuates Palmitate-Induced Inflammation and Endoplasmic Reticulum Stress in Pancreatic β-Cells. *Endocrinology and Metabolism* 2018;**33**:105. doi:10.3803/EnM.2018.33.1.105

67 DePaoli AM, Higgins LS, Henry RR, *et al.* Can a Selective PPARγ Modulator Improve Glycemic Control in Patients With Type 2 Diabetes With Fewer Side Effects Compared With Pioglitazone? *Diabetes Care* 2014;**37**:1918–23. doi:10.2337/dc13-2480

68 Huan Y, Pan X, Peng J, *et al.* A novel specific peroxisome proliferator-activated receptor γ (PPARγ) modulator YR4-42 ameliorates hyperglycaemia and dyslipidaemia and hepatic steatosis in diet-induced obese mice. *Diabetes, Obesity and Metabolism* 2019;**21**:2553–63. doi:10.1111/dom.13843

69 Skladany L, Molcan P, Vnencakova J, *et al.* Frailty in Nonalcoholic Fatty Liver Cirrhosis: A Comparison with Alcoholic Cirrhosis, Risk Patterns, and Impact on Prognosis. *Canadian Journal of Gastroenterology and Hepatology* 2021;**2021**:1–10. doi:10.1155/2021/5576531

70 Masarone M, Rosato V, Aglitti A, *et al.* Liver biopsy in type 2 diabetes mellitus: Steatohepatitis represents the sole feature of liver damage. *PLOS ONE* 2017;**12**:e0178473. doi:10.1371/journal.pone.0178473

71 Balas B, Belfort R, Harrison SA, *et al.* Pioglitazone treatment increases whole body fat but not total body water in patients with non-alcoholic steatohepatitis. *Journal of Hepatology* 2007;**47**:565–70. doi:10.1016/j.jhep.2007.04.013

72 Berria R, Glass L, Mahankali A, *et al.* Reduction in Hematocrit and Hemoglobin Following Pioglitazone Treatment is not Hemodilutional in Type II Diabetes Mellitus. *Clinical Pharmacology & Therapeutics* 2007;**82**:275–81. doi:10.1038/sj.clpt.6100146

73 Younossi ZM, Golabi P, de Avila L, *et al.* The global epidemiology of NAFLD and NASH in patients with type 2 diabetes: A systematic review and meta-analysis. *Journal of Hepatology* 2019;**71**:793–801. doi:10.1016/j.jhep.2019.06.021

74 Eslam M, Newsome PN, Sarin SK, *et al.* A new definition for metabolic dysfunction-associated fatty liver disease: An international expert consensus statement. *Journal of Hepatology* 2020;**73**:202–9. doi:10.1016/j.jhep.2020.03.039

75 Liu J, Ayada I, Zhang X, *et al.* Estimating Global Prevalence of Metabolic Dysfunction-Associated Fatty Liver Disease in Overweight or Obese Adults. *Clinical Gastroenterology and Hepatology* Published Online First: February 2021. doi:10.1016/j.cgh.2021.02.030

76 Gastaldelli A, Cusi K. From NASH to diabetes and from diabetes to NASH: Mechanisms and treatment options. *JHEP Reports* 2019;**1**:312–28. doi:10.1016/j.jhepr.2019.07.002

77 4. Comprehensive Medical Evaluation and Assessment of Comorbidities: *Standards of Medical Care in Diabetes—2022*. *Diabetes Care* 2022;**45**:S46–59. doi:10.2337/dc22-S004

78 Gastaldelli A, Sabatini S, Carli F, *et al.* PPAR-γ-induced changes in visceral fat and adiponectin levels are associated with improvement of steatohepatitis in patients with NASH. *Liver International* 2021;**41**:2659–70. doi:10.1111/liv.15005

79 della Pepa G, Russo M, Vitale M, *et al.* Pioglitazone even at low dosage improves NAFLD in type 2 diabetes: clinical and pathophysiological insights from a subgroup of the TOSCA.IT randomised trial. *Diabetes Research and Clinical Practice* 2021;**178**:108984. doi:10.1016/j.diabres.2021.108984

80 Gastaldelli A, Gaggini M, DeFronzo RA. Role of Adipose Tissue Insulin Resistance in the Natural History of Type 2 Diabetes: Results From the San Antonio Metabolism Study. *Diabetes* 2017;**66**:815–22. doi:10.2337/db16-1167

81 Bedogni G, Bellentani S, Miglioli L, *et al.* The Fatty Liver Index: a simple and accurate predictor of hepatic steatosis in the general population. *BMC gastroenterology* 2006;**6**. doi:10.1186/1471-230X-6-33

82 Cerf ME. Beta Cell Dysfunction and Insulin Resistance. *Frontiers in Endocrinology* 2013;**4**. doi:10.3389/FENDO.2013.00037

83 Sanders FWB, Acharjee A, Walker C, *et al.* Hepatic steatosis risk is partly driven by increased de novo lipogenesis following carbohydrate consumption. *Genome biology* 2018;**19**. doi:10.1186/S13059-018-1439-8

84 Gorden DL, Myers DS, Ivanova PT, *et al.* Biomarkers of NAFLD progression: a lipidomics approach to an epidemic. *Journal of lipid research* 2015;**56**:722–36. doi:10.1194/JLR.P056002

85 Cusi K, Orsak B, Bril F, *et al.* Long-Term Pioglitazone Treatment for Patients With Nonalcoholic Steatohepatitis and Prediabetes or Type 2 Diabetes Mellitus: A Randomized Trial. *Annals of internal medicine* 2016;**165**:305–15. doi:10.7326/M15-1774

86 van Dalem J, Driessen JHM, Burden AM, *et al.* Thiazolidinediones and Glucagon-Like Peptide-1 Receptor Agonists and the Risk of Nonalcoholic Fatty Liver Disease: A Cohort Study. *Hepatology (Baltimore, Md)* 2021;**74**:2467–77. doi:10.1002/HEP.32012

87 Bril F, Kalavalapalli S, Clark VC, *et al.* Response to Pioglitazone in Patients With Nonalcoholic Steatohepatitis With vs Without Type 2 Diabetes. *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association* 2018;**16**:558-566.e2. doi:10.1016/J.CGH.2017.12.001

88 Barr J, Vázquez-Chantada M, Alonso C, *et al.* Liquid chromatography-mass spectrometry-based parallel metabolic profiling of human and mouse model

serum reveals putative biomarkers associated with the progression of nonalcoholic fatty liver disease. *Journal of proteome research* 2010;**9**:4501–12. doi:10.1021/PR1002593

89 Ooi GJ, Meikle PJ, Huynh K, *et al.* Hepatic lipidomic remodeling in severe obesity manifests with steatosis and does not evolve with non-alcoholic steatohepatitis. *Journal of hepatology* 2021;**75**:524–35. doi:10.1016/J.JHEP.2021.04.013

90 Gorden DL, Myers DS, Ivanova PT, *et al.* Biomarkers of NAFLD progression: a lipidomics approach to an epidemic. *Journal of lipid research* 2015;**56**:722–36. doi:10.1194/JLR.P056002

91 Warshauer JT, Lopez X, Gordillo R, *et al.* Effect of pioglitazone on plasma ceramides in adults with metabolic syndrome. *Diabetes/metabolism research and reviews* 2015;**31**:734–44. doi:10.1002/DMRR.2662

92 Gastaldelli A, Miyazaki Y, Mahankali A, *et al.* The effect of pioglitazone on the liver: role of adiponectin. *Diabetes care* 2006;**29**:2275–81. doi:10.2337/DC05-2445

93 Miyazaki Y, Glass L, Triplitt C, *et al.* Effect of rosiglitazone on glucose and non-esterified fatty acid metabolism in Type II diabetic patients. *Diabetologia* 2001;**44**:2210–9. doi:10.1007/S001250100031

94 Gastaldelli A, Stefan N, Häring HU. Liver-targeting drugs and their effect on blood glucose and hepatic lipids. *Diabetologia* 2021;**64**:1461–79. doi:10.1007/S00125-021-05442-2

95 Rosso C, Kazankov K, Younes R, *et al.* Crosstalk between adipose tissue insulin resistance and liver macrophages in non-alcoholic fatty liver disease. *Journal of hepatology* 2019;**71**:1012–21. doi:10.1016/J.JHEP.2019.06.031

96 Klein S, Gastaldelli A, Yki-Järvinen H, *et al.* Why does obesity cause diabetes? *Cell metabolism* 2022;**34**:11–20. doi:10.1016/J.CMET.2021.12.012

97 Miyazaki Y, Mahankali A, Matsuda M, *et al.* Effect of Pioglitazone on Abdominal Fat Distribution and Insulin Sensitivity in Type 2 Diabetic Patients. *The Journal of Clinical Endocrinology & Metabolism* 2002;**87**:2784–91. doi:10.1210/JCEM.87.6.8567

98 Basu A, Jensen MD, McCann F, *et al.* Effects of Pioglitazone Versus Glipizide on Body Fat Distribution, Body Water Content, and Hemodynamics in Type 2 Diabetes. *Diabetes Care* 2006;**29**:510–4. doi:10.2337/DIACARE.29.03.06.DC05-2004

99 Shadid S, Jensen MD. Effects of Pioglitazone Versus Diet and Exercise on Metabolic Health and Fat Distribution in Upper Body Obesity. *Diabetes Care* 2003;**26**:3148–52. doi:10.2337/DIACARE.26.11.3148

100 Martins FO, Delgado TC, Viegas J, *et al.* Mechanisms by which the thiazolidinedione troglitazone protects against sucrose-induced hepatic fat accumulation and hyperinsulinaemia. *British Journal of Pharmacology* 2016;**173**:267. doi:10.1111/BPH.13362

101 Silva JCP, Marques C, Martins FO, *et al.* Determining contributions of exogenous glucose and fructose to de novo fatty acid and glycerol synthesis in liver and adipose tissue. *Metabolic Engineering* 2019;**56**:69–76. doi:10.1016/J.YMBEN.2019.08.018

102 Bril F, Kalavalapalli S, Clark VC, *et al.* Response to Pioglitazone in Patients With Nonalcoholic Steatohepatitis With vs Without Type 2 Diabetes. *Clinical Gastroenterology and Hepatology* 2018;**16**:558-566.e2. doi:10.1016/J.CGH.2017.12.001

103 Apostolopoulou M, Gordillo R, Koliaki C, *et al.* Specific Hepatic Sphingolipids Relate to Insulin Resistance, Oxidative Stress, and Inflammation in Nonalcoholic Steatohepatitis. *Diabetes care* 2018;**41**:1235–43. doi:10.2337/DC17-1318

104 Luukkonen PK, Zhou Y, Sädevirta S, *et al.* Hepatic ceramides dissociate steatosis and insulin resistance in patients with non-alcoholic fatty liver disease. *Journal of hepatology* 2016;**64**:1167–75. doi:10.1016/J.JHEP.2016.01.002