

Research Article

Therapeutic Potential of Peroxisome Proliferator-Activated Receptor Modulation in Non-Alcoholic Fatty Liver Disease and Non-Alcoholic Steatohepatitis

Leonie Gellrich and Daniel Merk

Institute of Pharmaceutical Chemistry, Goethe University Frankfurt, Max-von-Laue-Str. 9, 60438 Frankfurt, Germany

Abstract. A long neglected hepatic manifestation of the metabolic syndrome, non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) arise as serious health burden with alarming global prevalence. The disease complex is currently attracting considerable interest of drug discovery and many experimental approaches are studied in all stages of clinical development. Peroxisome proliferator-activated receptors (PPARs) have a successful history as pharmaceutical targets in the treatment of several aspects of the metabolic syndrome and, therefore, a putative therapeutic value of PPAR modulators in NAFLD/NASH is obvious. However, so far only the PPAR α/δ agonist elafibranor has revealed clear efficacy and reached an advanced stage of development while the far more established PPAR subtypes PPAR α and PPAR γ have disappointed. Still, clinical trial design and population might have obscured beneficial activities and, in addition, synergistic multi-target approaches as well as selective PPAR modulators could generate safer approaches with higher therapeutic efficacy.

Corresponding Author Daniel Merk

merk@pharmchem.unifrankfurt.de

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1. Introduction

Non-alcoholic steatohepatitis (NASH) is the long-term consequence of non-alcoholic fatty liver disease (NAFLD). NAFLD is defined as hepatic lipid accumulation (steatosis) in the absence of pathologies such as viral hepatitis or alcohol abuse [1] with triglyceride levels above 55 mg/g measured by magnetic resonance imaging (MRI) [2] or determined histologically by a grading system [3]. When hepatic inflammation, hepatocellular injury [4] and hepatocyte ballooning occur additionally, the disease progresses to NASH that may finally lead to fibrosis, cirrhosis, or hepatocellular carcinoma (HCC) [5, 6].

Drug discovery for novel NASH therapies is extensive and there are promising approaches in all stages of clinical development. Nuclear receptors have high significance amongst the experimental targets studied for NASH and as well-established targets in the treatment of metabolic diseases. PPARs have been evaluated for the therapeutic potential as well. The available data of PPAR modulation in the disease complex NAFLD and NASH will be focus of this review.

2. Pathophysiology of Non-Alcoholic Steatohepatitis

Related to the pathologies obesity, insulin resistance, hypertension and hyperlipidemia, NAFLD is considered as the hepatic manifestation of the metabolic syndrome [7]. It has an alarming estimated global prevalence of 25% [8]. Furthermore, 75% of patients with obesity-associated type 2 diabetes have some form of fatty liver [9]. According to recent investigations, 7%-30% of NAFLD patients have NASH making the overall prevalence of NASH range between 1.5% and 6.45% [8]. Without countermeasures, patients with NASH will develop cirrhosis within 10 years at 29% [10] and have a 10-fold higher risk of liver-related mortality [11]. During the next decade NASH is estimated to overtake hepatitis C virus infection as the leading cause of liver transplantation in the US [12].

Most NAFLD patients are asymptomatic [13] or have unspecific symptoms such as fatigue, malaise and right upper quadrant discomfort [14]. Thus, an early diagnosis of the disease is difficult. Additionally, there are diagnostic complexities since only few obesity-independent circulating markers for NAFLD and NASH have been reported [15]. Elevated serum transaminase levels are often used in clinical trials for NASH as surrogate marker, especially alanine aminotransferase (ALT) and aspartate transaminase (AST), because they can indicate hepatocellular damage. However, these surrogate parameters have several limitations, because on one hand drugs can influence transaminase levels and on the other hand, fatty liver and fibrosis can occur without affecting ALT and AST levels [14, 16–18]. Studies relying only on transaminase levels as readout should therefore carefully evaluated. Therefore, new surrogate parameters are required, that have better correlation with the histological outcome. One such potential marker could be adiponectin, which will be discussed later.

Imaging techniques such as ultrasound and MRI are often used to diagnose lipid accumulation in the liver but also have their limitations. With these methods steatosis cannot be distinguished from steatohepatitis and the severity of inflammation, the degree of fibrosis and the stage of the disease cannot be measured [14].

Hence, the gold standard for the diagnosis of NASH is an invasive method, namely liver biopsy followed by histological analysis [19]. However, invasive methods cannot be repeated multiple times and the location, where the liver biopsy is taken is crucial, because parenchymal injury and fibrosis vary in different regions of the liver [20, 21]. In a review of Day [22], which recapitulates the current knowledge about NASH, it is postulated, that a liver biopsy is indicated, when some of the following points apply: ALT is greater than twice normal, AST level are higher than ALT, at least moderate central obesity occurs, non-insulin dependent diabetes mellitus (NIDDM) or impaired glucose tolerance arises, hypertension and hypertriglyceridemia develops [23].

To avoid liver biopsy, a NAFLD fibrosis activity score (NAS) was established with 90% prediction accuracy. It predicts the appearance of fibrosis in NAFLD patients considering age, hypertriglyceridemia, body mass index, platelet count, albumin, and AST/ALT ratio [24].

To understand the pathogenesis of the disease a two-hit hypothesis was suggested in 1998 postulating that the first hit is steatosis caused by insulin resistance, which is the reason for enhanced lipolysis and delivery of free fatty acids to the liver. The first hit determines a sensitizing factor for a second hit through metabolic injuries that cause inflammation in the liver. These metabolic injuries include oxidative stress, abnormal cytokine production, lipid peroxidation,

endoplasmatic reticulum stress, elevated intrahepatic ceramide levels, elevated plasma fatty acids, elevated intestine derived endotoxins and pathogens or high plasma cholesterol levels. After this second hit, hepatic inflammation, necrosis and fibrosis develop [25].

Steatosis under physiological conditions is a common reversible state in homoeostasis to maintain energy balance. In the fasting state the liver accumulates triglycerides and fatty acid flux from adipose tissue is increased [26, 27]. Thus, hepatic steatosis is not necessarily pathological.

Approximately 60% of triglycerides localized in the liver derive from plasma albumin-bound fatty acids and 15% from nutrition. About 25% are result from de novo synthesis [28]. This indicates potential causes and targets for steatosis and that the main source of hepatic lipids is not derived from nutrition or de novo synthesis in the liver but steatosis basically arises because of accumulation of free fatty acids from plasma. One reason for enhanced hepatic fatty acid accumulation is indeed insulin resistance and accordingly type 2 diabetes [29, 30]. When carbohydrate levels are increased, insulin levels are enhanced and thus the transcription of sterol regulatory element-binding protein 1c (SREBP-1c) in adipose tissue and liver X receptor (LXR) in hepatic tissue is upregulated leading to induction of lipogenic genes. SREBP-1c and LXR induce L-type pyruvate kinase (L-PK), acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), which are key glycolytic enzymes converting carbohydrates into triglycerides [31, 32]. SREBP-1c is regulated by peroxisome proliferator-activator receptor (PPAR) α and farnesoid X receptor (FXR). FXR decreases SREBP-1c mRNA expression [33] while PPARa enhances the activity of the SREBP-1c promoter [34]. SREBP-1c is a transcription factor, which regulates multiple genes involved in fatty acid and triglyceride synthesis by binding to a promotor sequence of different genes, called sterol regulatory element-1 (SRE1). SRE1 regulated genes include ATP citrate lyase, which generates acetyl-CoA as well as ACC and FAS, which is involved in the conversion of pyruvate into fatty acids. Both produce palmitate. Another SREBP-1c target gene is stearoyl-CoA desaturase (SCD), which catalyses a ratelimiting step in the synthesis of unsaturated fatty acids and plays a key role in hepatic synthesis of triglycerides and very-low-density lipoproteins (VLDL) [35]. Furthermore, SREBP-1c causes massive production of malonyl-Co-A, which inhibits carnitine palmitoyl transferase-1 (CPT-1), a pivotal enzyme involved in the transport of fatty acids through the inner mitochondrial membrane to the mitochondrial matrix, where their metabolism by β -oxidation takes place. Therefore inhibition of CPT-1 decreases fatty acid transport into the mitochondrial matrix, which in turn lowers β -oxidation [36, 37]. Recent studies have reported, that PPARy is also responsible for the upregulation of lipogenic genes beyond SREBP-1c.

Both transcription factors are crucial for the lipogenic effects detected in NAFLD patients and their expression is upregulated in these patients [38, 39]. Due to elevated plasma glucose levels caused by insulin resistance, the transcription factor carbohydrate response element binding protein (ChREBP) is increased [36]. In the liver, ChREBP is responsible for transactivation of L-PK, ACC and FAS, the above mentioned key glycolytic enzymes, converting carbohydrates to triglycerides [40]. Thus ChREBP initiates triglyceride synthesis from carbohydrates in response to glucose. The transcription factors SREBP-1c and LXR in contrast, are activated by insulin signalling and induce the same pathway of carbohydrate conversion to triglycerides. Forkhead box protein O1 (FOXO1) is a transcription factor that is related to lipolysis and insulin resistance and considered as important player in NAFLD and NASH. It induces phosphoenolpyruvate

carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase). Both enzymes are also induced by glucagon during fasting and in the postprandial state, whereas insulin inhibits their expression. Both are involved in hepatic gluconeogenesis, and therefore, insulin resistance causes a loss of gluconeogenesis inhibition, thus elevating plasma glucose levels. FOXO1 additionally suppresses glucose oxidation, recruits the fatty acid translocase (FAT) also referred to as cluster of differentiation (CD)36, induces lipoprotein lipase (LPL) and stimulates pyruvate dehydrogenase kinase 4 (PDK4), an inhibitor of the oxidation of glucose to acetyl-CoA leading to further glucose accumulation [41, 42].

NAFLD patients display low plasma adiponectin levels and the expression of adiponectin receptors in the liver is decreased [43, 45, 46]. Adiponectin is a peptide hormone produced by adipocytes and is involved in fatty acid oxidation, which could improve hepatic insulin sensitivity and reduce steatosis. Furthermore, the hormone has anti-inflammatory properties. It is secreted from adipose tissue into the bloodstream and responsible for decreased gluco-neogenesis and enhanced glucose uptake through improved insulin sensitivity. Furthermore, adiponectin promotes β -oxidation, upregulates uncoupling proteins and reduces tumor necrosis factor- α (TNF- α) [47–49].

Besides insulin resistance, obesity plays a major role in the development of steatosis, because high caloric intake and obesity-driven lipolysis of triglycerides dramatically enhance the levels of free fatty acids. Additionally, excessive intake of carbohydrates induces de novo lipogenesis (DNL) in the liver and from a non-lipid source further free fatty acids are synthesised exacerbating steatosis [50]. Finally, dysregulated gut physiology and microbiome impairment may also increase dietary fat uptake [51, 52].

Extraordinarily high free fatty acid levels promote the rate of mitochondrial β -oxidation but can overburden this metabolic pathway [53, 54] and the lack of (alternative) fatty acid metabolism routes can contribute to steatosis. Imbalance of fatty acid metabolism can arise from mitochondrial dysfunction leading to increased levels of malonyl-CoA [55]. Moreover, mitochondrial dysfunction upregulates compensatory pathways to decrease pathologically high fatty acid levels such as peroxisomal β -oxidation and microsomal omega-oxidation, which cause oxidative stress [56, 57].

Also decreased fatty acid efflux from the liver can significantly contribute to steatosis via impaired VLDL synthesis or transport by apolipoprotein (APO) B expression, which is essential for triglyceride accumulation into VLDL [14, 58]. In this context, insulin resistance plays a role as well, because in postprandial state insulin targets APO B and leads to VLDL degradation and decreased trigyceride efflux [30].

The progression from NAFLD to NASH is mainly induced through hepatic inflammation, caused by excessive cytokine production, e.g. TNF- α , interleukin (IL)-1, IL-6, macrophage disorder, oxidative stress, direct release of TNF- α by adipose tissue and bacterial overgrowth in small intestine [3].

Arising from hepatic steatosis and alterations in gut microbiota, the liver is sensitized for noxious agents such as endotoxins, a component of the lipopolysaccarid cell wall of gram negative bacteria [59] and other proinflammatory stimuli whereby cytokines are released and macrophages as well as Kupffer cells are activated. Thus inflammation and excessive cytokine production, especially of TNF- α , and nuclear factor- κ B (NF- κ B) signalling are triggered [60, 62, 63].

Oxidative stress develops from reactive oxygen species (ROS) released by mitochondria. ROS are produced dependent on the amount of free fatty acids, which are highly increased in hepatocytes from NAFLD patients [64]. ROS lead to lipid peroxidation which in turn alters mitochondrial DNA. Additionally, lipid peroxidation products bind to mitochondrial proteins inhibiting the transfer of electrons along the respiratory chain. In consequence, further ROS are produced leading to a vicious cycle. These findings are confirmed by NASH patients displaying mitochondrial lesions and reduced activity of respiratory chain complexes [54].

Another source of oxidative stress could be the cytochrome P450 enzymes (CYP)2E1 and CYP3A4 [65]. CYP2E1 plays a key role in alcoholic liver disease and is also important in the pathogenesis of oxidative stress in NAFLD patients, because of its ability to stimulate lipid peroxidation. During phase I of xenobiotic metabolism CYPs bind substrates via the formation of an oxy complex which is subsequently reduced to a peroxy-complex. Thus ROS is generated during the intermediate stages of CYP-mediated biotransformation [66]. Elevated hepatic iron concentrations might also play a role in the development of ROS and NAFLD patients display elevated serum iron in form of serum ferritin. Furthermore, increased hepatic iron storage is correlated to hepatic damage and fibrosis by inducing oxidative stress, understood as an increase in the steady state concentration of oxygen radical intermediates [67, 68].

Hepatic inflammation leads to activation of hepatic stellate cells (HSCs) which occurs in all cases of liver damage. In resting state, HSCs contain vitamin A lipid droplets and are important for fat storage [69]. Activated HSC, so-called myofibroblast-like cells, are the major cell type involved in liver fibrosis, where fibrotic scar tissue replaces liver cells when chronic liver injury occurs [70, 71]. Upon activation by inflammatory stimuli, HSCs enter proliferation and induce contractility. Moreover, activated HSCs influence microcirculation and portal hypertension caused by vasoactive substances and induce chemotaxis of granulocytes and macrophages. Additionally, their vitamin A content decreases and they start secreting collagen, which is responsible for the development of scar tissue leading to fibrotic remodelling and, terminally, cirrhosis [3].

Although NAFLD is one of the most common liver diseases there is currently no FDAapproved pharmacological therapy available but pharmacological interventions are urgently required since lifestyle changes are rarely effective to treat NASH [12]. Drug discovery should especially focus on decreasing hepatic inflammation and hepatocyte damage, no worsening of fibrosis and a decrease in the NAFLD fibrosis activity score.

So far, fibrates as PPAR α agonists, thiazolidinediones (TZDs) as PPAR γ agonists and different dual agonists of PPAR, e.g. the dual PPAR α/δ agonist elafibranor [72], have been studied in clinical trials. Furthermore, vitamin E [12], the FXR agonist obeticholic acid [73] and succeeding compounds as well as insulin-sensitizing agents, particularly metformin are investigated as therapeutic option for the treatment of NAFLD or NASH. In addition, inhibition of stearoyl-CoA desaturase 1 (SCD1) to enhance fatty acid degradation is evaluated. Earlier stages of clinical development also study the potential of apoptosis signal regulating kinase 1 (ASK1) inhibitors and caspase inhibitors as direct anti-inflammatory and anti-fibrotic approaches.

PPARs play a crucial role in drug development for NAFLD and NASH and it is important to consider this group of nuclear receptors more in detail to assess its opportunities and limitations.

3. Role of PPARs in Metabolic Syndrome and NASH

3.1. Peroxisome proliferator-activator receptors (PPARs)

In 1969 for the first time, peroxisome-like particles were detected and in 1990 the first PPAR was cloned from mouse liver [74, 75]. Since then, research on their function and potential use as drug target was very intensive. PPARs are members of the family of nuclear receptors and exist in the subtypes PPAR α , PPAR β/δ (from now on named PPAR δ) and PPAR γ [76]. All subtypes act as ligand-activated transcription factors and usually form a heterodimer with a retinoid X receptor (RXR). PPARs have five or six structural regions (A–F) in four functional domains. The structural region C represents the DNA-binding domain (DBD), which binds to PPAR response elements (PPRE) that constitute direct repeats interspaced by a single residue (DR-1). The E/F domain mediates ligand-binding. In a comparative study of mammalian and amphibian samples, the amino acid sequence discrepancy in the E domain between both lineages was investigated. In contrast to the thyroid hormone receptor (THR) and retinoic acid receptor (RAR) the E domain of PPARs has evolved three times faster which could be the reason for the increasing specialisation of PPAR subtypes [77]. In addition, PPARs have large ligand binding sites and accommodate a variety of ligands [78].

When an agonist binds to the ligand binding domain (LBD), a conformational change in the transactivation domain (activation function 2, AF2) is induced leading to release of corepressors and recruitment of coactivators [79]. Typical corepressors for PPAR which are bound to the LBD in inactive state include nuclear corepressor (NCoR) and the silencing mediator of retinoid and thyroid hormone receptors (SMRT). Characteristic coactivators that are recruited upon activation are cAMP response element-binding protein (CREB) and p300, which acetylate histones, PPAR binding protein (PBP), which bridges PPAR and the transcription initiation machinery, and the PPAR γ coactivator 1-alpha (PGC-1 α), whose role as coactivator is not fully clarified on molecular level [76, 80, 81]. The DBD of PPAR binds to the PPRE in the promoter region of the corresponding target gene and the transcription of PPAR target genes is upregulated [80, 82, 83]. PPARs can also repress target genes through mechanisms that are less well investigated. This "trans-repression" could be the result of stabilization of corepressor recruitment after posttranslational PPAR modification by for example sumoylation [76, 84].

Despite large differences in signalling pathways and tissue distribution, PPARs mainly regulate metabolic pathways and inflammatory processes [77]. In rodents, PPARs also promote peroxisome proliferation and before it was discovered that this activity lacks in humans, PPAR modulating agents were expected to have carcinogenic potential especially in liver, and therefore the research of PPAR as drug target was discontinued for a while. PPAR expression and function differs between rodents and humans, which makes it difficult to evaluate the function and involvement in diseases of PPARs by using animal models. Table 1 gives an overview over significant species differences concerning PPARs in human and rodents [82].

Known available drugs targeting PPARs are the insulin-sensitizing TZDs e.g. pioglitazone and rosiglitazone which activate PPAR γ and were used to treat diabetes mellitus [85]. PPAR α agonists, named fibrates, play a role in the treatment of dyslipidemia by their triglyceride lowering and high-density lipoprotein (HDL) raising properties but their therapeutic relevance has decreased [86, 87].

PPARs are still in the focus of current drug discovery mainly as targets for inflammatory liver diseases but also in chronic inflammatory disorders such as multiple sclerosis [88]. This review gathers available data on the role of PPARs in NAFLD and NASH and evaluates their therapeutic potential. Each PPAR subtype has individual effects on metabolism in different tissues but all affect the various aspects of the metabolic syndrome including NAFLD and NASH (Figure 1).

3.1.1. PPARα

PPAR α is mainly expressed in the liver and other tissues where metabolic processes, especially lipid metabolism, and gluconeogenesis occur, such as adipose tissue, heart, skeletal muscle, intestine, renal cortex and kidney. PPAR α transcriptionally regulates multiple metabolic processes including β -oxidation, lipid transport and gluconeogenesis and is also involved in inflammatory processes [76, 82].

Activation of PPAR α improves plasma lipids. Plasma triglyceride levels are decreased since the balance between hepatic fatty-acid oxidation and glycerolipid esterification is switched towards the catabolic route and, therefore, less triglycerides are available for VLDL synthesis. The metabolism of triglycerides is triggered by the transport of fatty acids through the inner mitochondrial membrane to the mitochondrial matrix, where their metabolism takes place, catalysed by the pivotal enzyme CPT-1 [112] whose expression is regulated by PPAR α . Furthermore, expression of other enzymes, which are important for fatty acid uptake, intracellular transport and β -oxidation are under control of PPAR α . Besides CPT-1 the most important enzymes are fatty acid transport protein (FATP), CD36 and long-chain fatty acid acetylcoenzyme A synthase (LC-FACS) [113].

Transporter of long-chain fatty-acids (LCFAs) can be graduated in three groups, namely FATP 1-6, CD36 and plasma membrane-associated fatty-acid binding protein (FABP)pm. Two of three groups count to PPAR α target genes. FATP is a transporter for LCFAs with Acyl-CoA synthetase (ACS) activity, which leads to an esterification of the fatty acids and therefore an activation for β -oxidation and a prevention of their efflux. Besides its own ACS activity, FATP forms a complex with a long-chain-fatty-acid-CoA ligase (ACSL) to acetylate fatty acids [114–116]. CD36 belongs to the scavenger receptors which recognize oxidized or acetylated low-density lipoprotein (LDL). Except LDL, CD36 recognizes LCFAs and facilitates their transport through membrane barriers into cell compartments [117]. Acetylation of LCFAs via LC-FACS leads to enhanced β -oxidation and decreased efflux of LCFAs as explained above. By the increased β -oxidation and the reduction of DNL, PPAR α also improves hepatic insulin resistance [118].

PPAR α not only promotes degradation of triglycerides, but also induces the hydrolysis of lipoproteins, since LPL expression is induced by PPAR α and the inhibitor of LPL, APO CIII, is repressed by PPAR α [119, 120]. In addition, the amount of HDL cholesterol is increased, because PPAR α activation induces APO AI [121] and APO AII [122] expression in hepatocytes, which are the major HDL APOs. In contrast, in rats PPAR α activation suppresses APO AI expression and lowers HDL.

Besides its role in lipid homeostasis, PPAR α activation inhibits inflammatory genes induced by NF- κ B and directly inhibits NF- κ B by increasing the transcription of the Inhibitor of κ B

	rodents	humans	
expression	PPARa: liver (PPAR α expression is subject to negative and positive regulation by insulin and glucocorticoids) [89, 90], adipose tissue (highly expressed in comparison to human, high levels of PPAR α mRNA are detected in brown fat), heart, kidney, skeletal muscle, GI tract (mucosa of stomach and duodenum), transient expression in the developing central nervous system and during skin maturation [91, 93]	 PPARa: liver (its levels in the liver appear lower than in the rodent liver) [101], adipose tissue, heart, skeletal muscle, intestine, renal cortex and kidney [102] PPAR\delta: ubiquitously expressed (moderate levels in all tissues), highest expression rates are found in small intestine, colon, heart, adipose tissue, inflammatory cells, skin, brain and placenta [102] 	
	PPAR6: ubiquitously expressed (often at higher levels than PPAR α and PPAR γ), most expressed isotype in the adult nervous system [92, 93, 95], weakly expressed in liver, as compared with other tissues such as lung and kidney [91, 93, 96], skeletal and cardiac muscle, testis (very high in sertoli cells) [93], expression is markedly induced in the uterus at the time of blastocyte implantation and remains abundantly expressed in the decidua at the postimplantation stage [97]	PPARy: adipose tissue and immune cells (in contrast to rodent, no expression in spleen or lymphoid tissues) [103]	
	PPARy: white and brown adipose tissues (major sites of expression) [98], intestinal mucosa (high levels in colon and caecum but less in the small intestine) [99, 100], lymphoid tissues (spleen and Peyer's patches) [93, 94], in retina and skeletal muscle (at low levels)		
function	PPARs promote peroxisome proliferation in contrast to human PPARs leading to carcinogenic potential especially in liver [82]. Species specificity in ligand recognition (may be linked to the speed of evolution of the PPAR genes) [104] PPARa: well conserved across species [91, 105], functions in rodents and human are similar and homology of the DBD and LBD is high [106–108]. Peroxisome proliferation leads to an increase in the density of peroxisomes and peroxisomal fatty acid β -oxidation, but PPAR α agonists not only play a role in induction of genes for fatty acid oxidation, but also are carcinogenic in liver (specific to rats and mice) [109].	PPARa: regulates multiple metabolic processes including β-oxidation, lipid transport and gluconeogenesis and is also involved in inflammatory processes in summary regardless of the species, the expression of PPARα correlates with high mitochondrial and peroxisomal oxidation activities [104], however, there is a marked decrease in PPARα expression of human hepatocytes in the presence of an agonist due to differences in the promoter response elements of target genes [111], An inactive PPARα splice variant was found exclusively in human liver samples [101] PPARδ: regulation of fatty acid β-oxidation in skeletal muscle	
	 PPAR5: PPARδ shows species variations in structure and ligand activation profiles [91, 105] PPARy: well conserved across species [91, 105], but the PPARγ motif in E5 in the mouse genome was located in a small (~100bp) fragment of a rodent-specific LINE/L1 transposon species-specific PPRE [110] 	PPARy: major impact on adipocyte differentiation and lipid metabolism, two putative enhancers/PPREs in hASCs (not in the murine genome) [110] the orthologous CD36 loci revealed multiple species-specific regulatory elements [110]	

 Table 1: Comparison of PPAR expression and function between rodents and humans.

(IkB) [123, 124]. Furthermore, it decreases the expression of acute-phase response genes, IL-6 and C-reactive protein (CRP) [125, 126]. In the endothelial tissue, vascular cell adhesion molecule-1 (VCAM-1) expression is reduced lowering the formation of atherosclerotic plaques [127–129]. Another anti-atherosclerotic effect results from PPAR α activation in macrophages, where the cholesterol efflux protein ATP-binding cassette transporter (ABCA1) is induced, causing an increased cholesterol efflux [130]. Furthermore PPAR α reduces induction of COX-2 [82] and stimulates peroxisomal β -oxidation of leukotriene B4 (LTB4), which is important for the negative feedback control of inflammatory lipid mediators [131].

3.1.2. PPARy

PPAR γ is predominantly expressed in adipose tissue and immune cells and has a major impact on adipocyte differentiation and lipid metabolism [82]. Furthermore, glucose transporters are induced by PPAR γ [132], which rendered PPAR γ an attractive drug target for the therapy of diabetes with insulin sensitizers in form of TZDs. In addition, PPAR γ interferes with inflammatory processes and several proteins, such as uncoupling protein 1 (UCP-1), that have an important role in thermogenesis, are upregulated by PPAR γ [133].

PPAR γ is expressed in white and brown adipose tissue at similar levels and is essential for their growth, because PPAR γ upregulates genes, involved in adipocyte functions, such as fatty acid uptake, transport and esterification, lipogenesis, lipolysis and thermogenesis as well as adipokine synthesis and secretion [134]. Preadipocyte cell lines were used to investigate the molecular background underlying PPAR γ induced adipocyte differentiation. It turned out, that PPAR γ is especially required in the early stage of adipogenesis where it induces the transcription factors CCAAT-enhancer-binding proteins (C/EBP)- α C/EBP β and C/EBP δ [98, 135, 136]. C/EBP proteins control cellular proliferation, growth and differentiation. C/EBP α has a positive feedback on PPAR γ , because its activation induces PPAR γ expression [137].

Adipogenesis is an essential process in lipid homoeostasis, because adipocytes are important energy storages. When energy intake exceeds the current need, adipocyte differentiation is upregulated by insulin. Energy mobilization from adipose tissue can be triggered by epinephrine, glucagon and adrenocorticotropic hormone (ACTH) [138].

PPAR γ activation can effectuate the conversion of white adipose tissue into brown adipose tissue. Brown-like adipocytes are called brite or beige adipocytes, which develop from their white counterparts or from adipocyte precursor cells [139, 140]. White adipose tissue is the storage of energy in form of fat while brown adipose tissue is mainly responsible for thermogenesis and negatively associated with adiposity, insulin resistance and aging [141].

Besides adipogenesis, PPAR γ regulates genes involved in lipid metabolism. For instance, LPL and FABP4 also referred as adipocyte Protein 2 (aP2), a carrier protein for fatty acids that facilitates the transport of fatty acids into the adipocytes, are upregulated via PPAR γ [85, 135, 142, 143]. FATP [144] and oxidized LDL receptor 1 (OLR1) [145] are supplemental target genes of PPAR γ , which arrange fatty acid influx into adipocytes leading to internalization and degradation of oxidatively modified low density lipoprotein (oxLDL) by vascular endothelial cells [145]. Thus, PPAR γ plays a role in adipocyte differentiation and transport of fatty acids from periphery to adipocytes and other tissues, but, furthermore, PPAR γ is

important for fatty acid biosynthesis, where the PPAR γ target genes fatty-acyl-CoA synthase (FACS) [142], glycerol kinase [145] and the glycerol transporter aquaporin 7 (AQP7) [146] are involved.

Moreover PPAR γ induces the transcription of a class B scavenger receptor, called CD36, that leads to binding and internalisation of oxLDL, but as well recognizes LCFAs and supports their transport through membrane barriers into cell compartments, which is important for following β -oxidation in mitochondria [147]. Therefore CD36 is important, when elevated fatty acid plasma level occur such as in NAFLD/NASH.

So far, the only approved therapeutic use of PPAR γ agonists is their insulin sensitizing effect. Insulin resistance occurs often in combination with obesity, in particular visceral obesity. High glucose levels, caused by insulin resistance, induce DNL and, therefore, arrange further adipose tissue and enhance free fatty acid concentrations in plasma leading to accumulation of fat in the liver. In addition, when insulin resistance occurs, insulin levels in plasma are elevated as compensatory effect. Under physiological conditions, insulin has anti-lipolytic effects and prevents hydrolysis of triglycerides into glycerol and free fatty acids. Since free fatty acids play a crucial role in progression of NAFLD to NASH, insulin has positive effects on fatty liver, which could be utilised as therapeutic option by the insulin sensitizing effect of PPAR γ agonists.

NAFLD patients are frequently obese. In obesity, dysfunctional fat cells develop that release cytokines, such as TNF- α , IL-6 and resistin supporting the development of insulin resistance and decreasing insulin sensitizing cytokines like adiponectin [148, 149]. PPAR γ activation on one hand leads to elevated adiponectin levels and on the other hand can resolve insulin resistance by increasing the expression and translocation of the glucose transporters 1 (GLUT)-1 and -4, which improves glucose uptake into liver and skeletal muscle cells [150]. Furthermore PPAR γ agonists decrease IL-1 β , IL-6 and TNF- α levels [151, 152].

Additionally, it was found out that PPAR γ increases nitric oxide bioavailability in cultured endothelial cells potentially due to a repression of the NADPH oxidase enzyme complex. Therefore, a decreased superoxide anion production and less oxidative stress occur, which is another beneficial effect preventing steatohepatitis in NAFLD patients [153, 154].

PPAR γ activation has positive and negative impact on NAFLD an NASH. Adipogenesis and differentiation of adipocytes is not desirable for the treatment, but the weight gain induced by PPAR γ agonists mainly tended to be peripheral fat rather than central, thus no exacerbation of NAFLD and NASH is expected by PPAR γ activation. The positive aspect of PPAR γ as drug target are the insulin sensitizing and anti-inflammatory effects as well as its adiponectin increasing property and its beneficial influence on oxidative stress [155].

3.1.3. PPARδ

PPAR δ is ubiquitously expressed throughout the body, but the highest expression rates are found in small intestine, colon, heart, adipose tissue, inflammatory cells, skin and brain [113, 156]. Its main function is regulation of fatty acid β -oxidation in skeletal muscle. In contrast to PPAR α , PPAR δ has little influence on β -oxidation in the liver. The receptor can influence lipid metabolism and reduce inflammation and insulin resistance [157–160] but, so far, PPAR δ is the least investigated receptor amongst PPARs. The PPAR δ agonist GW501516 was studied as promising drug candidate for the treatment of dyslipidaemia but it was withdrawn due to safety concerns in clinical trials [161]. One reason was its potential carcinogenicity. It was detected that in colon cancer cells PPAR δ is upregulated by the adenomatous polyposis coli (APC) tumour suppressor pathway [162]. Potentially PPAR δ could then be activated by products of cyclooxygenase-2 (COX-2). This could also explain the beneficial effects of non-steroidal anti-inflammatory drugs against colorectal tumorigenesis. The carcinogenic potential of GW501516 was the main reason for its failure during development. Of note, the dual PPAR α and PPAR δ ligand elafibranor showed none of these effects [162].

Apart from GW501516 and elafibranor, which will be discussed later, most experiments to study PPAR δ were conducted in cell culture models and some animal studies. Since there are major differences between animal and human PPAR functions, their results must be evaluated carefully.

To identify target genes of PPAR δ subcutaneous white adipose tissue isolated from obese patients was incubated with selective agonists of all three PPARs (GW7647 for PPAR α , GW0742 for PPAR δ and BRL49653 (rosiglitazone) for PPAR γ). All subtypes decreased leptin and IL-6 secretion, whereas only PPAR α and PPAR δ agonists increased hepatocyte growth factor secretion. The PPAR δ agonist down-regulated angiogenin and induced TIMP metallopeptidase inhibitor 1(TIMP-1) release [163].

Other studies have reported that PPAR\delta activation induces SCD1. Although SCD1 has lipogenic properties by stimulating hepatic synthesis of triglycerides and VLDL, it has beneficial effects on oxidative stress. Endoplasmic reticulum stress is induced by saturated fatty acids and SCD1 catalyses the formation of monounsaturated fatty acids from unsaturated fatty acids [164, 165].

In brown fat, Twist-related protein 1 (Twist-1) is expressed and inhibits PGC-1 α . PGC-1 α mediates mitochondrial oxidative metabolism and uncoupling. PPAR δ seems to act down-stream of Twist-1 signal transduction but also increases Twist-1 expression indicating that Twist-1 serves as a negative-feedback to modulate the PGC-1 α /PPAR β / δ -regulated brown fat metabolism [166].

In a mouse myoblast cell line (C2C12), PPAR δ induced the expression of FOXO1, which suppresses glucose oxidation, recruits CD36, induces LPL and stimulates PDK4, an inhibitor of the oxidation of glucose to acetyl-CoA [167]. Furthermore, many genes involved in lipid utilisation, cholesterol efflux, energy uncoupling, and β -oxidation were upregulated [168] including UCP-1, UCP-2, UCP-3, FABP3, LPL, M-CPT-1 and adipose differentiation-related protein (ADRP). UCP carrier proteins are expressed in the mitochondria of brown fat and involved in thermogenesis [169]. FABP3 supports the transport of LCFAs from cytoplasm to the nucleus and M-CPT-1, the isoform of carnitine palmitoyl transferase 1 in muscles, catalyses the transport of LCFAs from cytosol into mitochondria by the transfer of an acyl group [168]. ADRP is involved in the formation of lipid droplets [170].

In C2C12 cells and in human myotubes angiopoietin-like protein 4 (ANGPTL4) was identified as target gene of PPAR β/δ [171]. ANGPTL4 inhibits LPL and increases lipolysis and fatty acid oxidation and decreases blood glucose levels. Furthermore, it is an inhibitor of angiogenesis and apoptose [172]. However, PPAR δ has as well pro-angiogenic properties in heart via calcineurin and in skeletal muscle via vascular endothelial growth factor A (VEGF-A) and platelet endothelial cell adhesion molecule (PECAM-1) [173, 174].

PPARδ selective agonists elevated HDL levels in diabetic mice [175] and GW501516 increased HDL while decreasing triglyceride and insulin levels in obese rhesus monkeys [176]. A first study in healthy volunteers displayed increased circulating HDL levels [177]. In contrast, in overweight volunteers, GW501516 treatment did not affect HDL levels. In the same trial, circulating triglycerides, APO B, LDL, insulin levels, liver fat content and urinary isoprostanes were decreased [161]. Further mouse studies with GW501516 also showed attenuated weight gain and insulin resistance by increasing β-oxidation of fatty acids in skeletal muscle [178]. Additionally, PPARδ exhibits anti-inflammatory activity by preventing the activation of macrophages and Kupffer cells [179]. Accordingly, GW501516 reduced NF- κ B activity and stimulated I κ B α expression [180]. In adipocytes, PPARδ activation decreased IL-6 levels [181].

4. In Vivo Effects of PPAR Modulation in NASH

4.1. PPARa

PPAR α agonists have been studied to evaluate their potential efficacy in the treatment of NAFLD and NASH in animal models and in clinical trials. Most results are derived from rodent models using a variety of high fat diets to induce fatty liver disease. However, several animal studies were conducted with Wy-14,643, which is defined as selective for PPAR α by many authors, but has considerable potency on PPAR γ , as well [182, 183]. Thus, interpretation of these studies should consider this concern and evaluate the results as arising from activation of PPAR α and PPAR γ .

4.1.1. PPARα animal studies

There are different methods to induce NASH in animal models. Mainly different diets were conducted, that result in liver injury similar to NASH. One method is to apply a methionine and choline deficient (MCD) diet to rodents which in consequence develop progressive fibrosing steatohepatitis. In one study, after 5 weeks MCD diet-fed wild-type and PPAR α knockout mice displayed moderate steatohepatitis and elevated ALT levels. Afterwards the mice were treated with Wy-14,643 which prevented intrahepatic triglyceride accumulation and resolved steatohepatitis in wild-type mice. Wy-14,643 increased FABP and enzymes essential for β -oxidation. The knockout mice were unaffected by Wy-14,643 [184]. In another trial, MCD diet-fed mice were treated with Wy-14,643 and examined after five and twelve days to detect, whether Wy-14,643 has additional antifibrotic effects, beyond the already observed effect on steatohepatitis. The study revealed significantly reduced ALT levels and hepatic lipoperoxides and less severe steatohepatitis. After twelve days, hepatic triglycerides and histology were normal. To identify the fibrosis reducing mode of action, expression of relevant genes including matrix metalloproteinases (MMPs), its inhibitors TIMP-1 and TIMP-2 and transforming growth factor beta 1 (TGF- β 1) were studied but only TIMP-2 and MMP-13 were decreased [185]. Thus, beyond the benefit on steatohepatitis no additional effect on fibrosis could be confirmed.



Figure 1: Contributing factors and molecular targets in NAFLD & NASH. Insulin resistance is an important factor in the pathology of non-alcoholic fatty liver disease (NAFLD) and elevated insulin levels play a crucial role in disease manifestation and progression. Insulin enhances the transcription of sterol regulatory element-binding protein 1c (SREBP-1c) and liver X receptor (LXR), which upregulate the three key glycolytic enzymes L-type pyruvate kinase (L-PK), acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). These enzymes catalyse conversion of carbohydrates to triglycerides. SREBP-1c additionally causes malonyl-Co-A production, which inhibits carnitine palmitovl transferase-1 (CPT-1), the pivotal enzyme for the transport of fatty acids through the inner mitochondrial membrane to the mitochondrial matrix, where their metabolism by β -oxidation takes place. When high glucose levels occur, carbohydrate response element binding protein (ChREBP) is upregulated, which further promotes conversion of carbohydrates to triglycerides. The transcription factor forkhead box protein O1 (FOXO1) induces phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) which are usually induced by glucagon and repressed by insulin. Both enzymes are involved in hepatic gluconeogenesis, and therefore, insulin resistance leads to a loss of gluconeogenesis inhibition, thus elevating plasma glucose levels. FOXO 1 additionally induces cluster of differentiation (CD)-36, which recognizes long-chain fatty-acid (LCFAs) and mediates their transport through membrane barriers into cellular compartments. Lipoprotein lipase (LPL) which hydrolyses triglycerides to free fatty acids and glycerol is also upregulated by FOXO1 further increasing the amount of free fatty acids. Another FOXO1 target gene is pyruvate dehydrogenase kinase 4 (PDK4), an inhibitor of glucose oxidation to acetyl-CoA leading to further glucose accumulation. The peptide hormone adiponectin also seems to be crucially involved in the pathogenesis of NAFLD and is downregulated in affected patients. It improves insulin sensitivity and promotes β -oxidation, reduces gluconeogenesis and has anti-inflammatory properties by inhibiting tumor necrosis factor- α (TNF- α). When free fatty acids are elevated, mitochondrial β -oxidation can be overburdened leading to further hepatic accumulation and steatosis. As compensatory effect peroxisomal β-oxidation and microsomal omegaoxidation are activated and cause oxidative stress. Impaired very low density lipoprotein (VLDL) synthesis and transport by apolipoprotein (APO)-B expression, which is essential for triglyceride accumulation into VLDL, may also contribute to hepatic fat accumulation. Insulin is also involved in this context, because in postprandial state insulin targets APO-B and leads to VLDL degradation. The progression of NAFLD to non-alcoholic steatohepatitis (NASH) is mainly caused by hepatic inflammation. Inflammation is triggered by alterations in gut microbiota leading to release of endotoxins by gram negative bacteria and reactive oxygen species (ROS). ROS are produced as a result of dysfunctional mitochondria, by CYP enzymes in lipid peroxidation, or by iron, which increases the steady state concentration of oxygen radical intermediates. Hepatic inflammation activates hepatic stellate cells (HSCs) leading to fibrotic remodelling and, terminally, cirrhosis.

Figure 1: (preceding page). PPAR α can counteract the disease by inducing CPT-1 and CD36, thus improving the transport of fatty acids into mitochondrial matrix. Furthermore, PPAR α upregulates enzymes that mediate β oxidation, decreases de novo lipogenesis and inhibits NF- κ B signalling by increasing the transcription of the Inhibitor of κ B (I κ B). However, PPAR α also promotes hydrolysis of lipoproteins by inducing LPL expression and LPL inhibitor APO CIII repression which increases free fatty acid levels. PPAR γ also induces CD36 promoting fatty acid uptake into mitochondria and increases adiponectin levels that improve insulin sensitivity. Furthermore, PPAR γ can reduce ROS formation and oxidative stress by repression of the NADPH oxidase enzyme and TNF- α . On the other hand, PPAR γ activation promotes adipocyte differentiation, fatty acid biosynthesis and LPL expression, which enhance fatty acid formation. PPAR δ can influence lipid metabolism, but mainly in skeletal muscle (not depicted). The receptor induces the expression of FOXO1, which has negative and positive effects on NAFLD. On one hand, flux of fatty acids into mitochondria is improved by CD36, but on the other hand glucose levels are enhanced by PDK4, PEPCK and G6Pase. In addition, LPL is induced by FOXO1 and directly by PPAR δ . APO B levels are decreased when PPAR δ is activated. A positive impact of PPAR δ is the reduction of NF- κ B activity and stimulation of I κ B α expression.

Fibrates which are less potent PPAR agonists compared to Wy-14,643 were also studied in NASH animal models. The single fibrates have a different pattern of selectivity. Clofibric and fenofibric acid activating both PPAR α and PPAR γ , with a 10-fold selectivity for PPAR α . Bezafibrate has no specificity for any of the three PPAR subtypes [186]. Again this lack of selectivity must be considered for the interpretation of the following studies.

APO E2 knockout mice who were fed a western-type high fat diet under fenofibrate treatment revealed a decrease in hepatic macrophage accumulation and reduced steatosis. Expression of inflammatory genes was diminished and genes involved in β -oxidation were increased [187]. Another mouse model, using a high-caloric and high-cholesterol diet (HCD) to induce NAFLD, reported promising data for fenofibrate which caused a significant improvement of NAFLD symptoms, reduced apoptosis and repressed genes related to oxidative stress in endoplasmic reticulum such as inositol-requiring enzyme 1 α (IRE1 α) and X-box binding protein 1 (XBP1). Moreover, phosphorylation of c-Jun N-terminal kinase (JNK) was decreased [188]. In a very recent animal study with mice on fructose-enriched diet (FED) to induce hepatic dyslipidemia and insulin resistance, fenofibrate positively affected the expression of several genes involved in hepatic metabolism, inflammation and fibrosis such as suppressor of cytokine signaling 3 (SOCS-3), apoptosis antigen 1 (CD95), mast cell degranulating peptide, TGF- β 1 and leptin. Additionally, reductions in body weight, insulin resistance estimated by Homeostatic Model Assessment (HOMA), liver triglycerides, AST/ALT ratio and TNF- α were observed [189].

Overall, PPAR α agonists showed promising effects on steatosis and steatohepatitis in the few animal models. Their impact on fibrosis is not consistent. Wy-14,643 revealed no effect on fibrosis, whereas fenofibrate might possess some therapeutic potential. But it is possible, that its activity on PPAR γ was responsible for this outcome. While the trials in rodents indicate promising outcomes, studies in humans showed more variation (Table 2).

4.1.2. PPARa clinical trials

In 1996 a pilot study with clofibrate in 16 patients with liver biopsy proven NASH and hypertriglyceridemia was conducted. Besides 2 g per day clofibrate treatment, 24 patients were treated with 13-15 mg/kg/d ursodeoxycholic acid and both treatments were compared to each other, but no placebo control group was recruited. No changes in ALT, AST, γ -glutamyltransferase (GGT), bilirubin, triglycerides or cholesterol levels were observed. Only alkaline phosphatase (ALP) levels were significantly reduced under clofibrate. Furthermore, no improvement in the histological grade of steatosis, inflammation or fibrosis could be confirmed after 12 months of treatment. In contrast, ursodeoxycholic acid improved values of ALP, ALT, GGT and the histological grade of steatosis [190]. Another trial enrolled 46 patients with histologically proven NASH that received 600 mg gemfibrozil per day or placebo. After 4 weeks, mean serum ALT, AST and GGT levels were significantly decreased contradicting the former trial, but the higher number of patients might have enabled a more robust result [191]. Collectively 74% of treated patients in comparison to 30% in the placebo group had a significant reduction of ALT levels. No changes in triglyceride values or mean body weight could be detected. Neither a biopsy to examine a histological effect nor an analysis of side effects were conducted. A third pilot trial investigated not only surrogate markers, but also analysed histological parameters. It enrolled 16 patients with biopsy-confirmed NAFLD that received 200 mg fenofibrate per day for 48 weeks. Unfortunately, a placebo control was missing. At the end of treatment, histological outcomes were determined from liver biopsy revealing a reduced grade of hepatocellular ballooning and degeneration, but no effects on steatosis, lobular inflammation, fibrosis or NAFLD activity score could be observed. Surrogate parameters showed a significant decrease in triglyceride, glucose, ALP, GGT, ALT and AST levels as well as increased APO A1 levels. Furthermore, an improvement of insulin sensitivity and decreased insulin levels were observed [192]. A rather short trial reported by Riserus et al. for only 2 weeks with 12 healthy moderately overweight participants receiving 20 µg of the selective PPARa agonist GW590735 or placebo per day, revealed triglyceride lowering activity but no effects on APO B, LDL, insulin or HDL were observed. A histological examination to determine the efficacy of GW590735 was not conducted [161].

In summary, animal models suggest significant beneficial effects of PPAR α or PPAR α/γ activation in NAFLD and NASH. The few and rather small clinical trials on humans fail to confirm the promising in vivo activity. Their outcomes are inconsistent and not very promising. This might in part be due to different functions and distribution patterns of PPAR α or PPARs in general in human and rodents. Additionally, the lack of clinical efficacy could also arise from underpowered studies.

4.2. PPARδ

Similar to PPAR α , most data for PPAR δ modulation in NAFLD/NASH has been reported from animal studies while there are only few clinical trials (Table 2).

4.2.1. PPARδ animal studies

The PPARδ agonist GW501516 was evaluated in rat L6 myotubes and a mouse model with high fat diet to induce NAFLD/NASH. In vitro data from a microarray indicated that enzymes of mitochondrial fatty acid-oxidation, electron transport, and ketogenesis were induced. Especially PDK4, which is involved in glucose oxidation, and genes important for fatty acid transport and activation, e.g. FATP, LC-FACS and CPT-1 were upregulated. Accordingly, GW501516 ameliorated diet-induced obesity and insulin resistance in mice. Furthermore, PPARδ activation

led to an improved metabolic rate by enhanced β -oxidation. In addition, the proliferation of mitochondria was improved and lipid droplets were reduced in the skeletal muscles of GW501516treated mice [178].

Another animal study compared GW501516 as selective PPAR δ agonist and bezafibrate, a PPAR pan-agonist, activating PPAR α and PPAR δ and with lower potency PPAR γ [193], in a MCD diet model to induce NASH in mice. Both agents, administered over 5 weeks, normalized hepatic triglyceride levels and the amount of fat droplets within hepatocytes. In addition, inflammation and the number of activated HSC, that cause fibrosis, were decreased. Specific target genes were monitored in hepatic tissue after isolation of the liver, indicating increased fatty acid β -oxidation after bezafibrate and GW501516 treatment by enhanced acyl-CoA oxidase (ACO), CPT-1, FABP and peroxisomal ketothiolase gene expression. Furthermore, inflammatory cytokines, such as TGF- β 1, IL-6, IL-1 β , monocyte chemoattractant protein (MCP)-1, TNF- α and NF- κ B were decreased. Bezafibrate but not GW501516 reduced plasma ALT levels and enhanced plasma adiponectin. This could be due to its dual agonism on PPAR α and PPAR δ [194].

An alternative PPAR δ agonist that has been used in NAFLD animal models is GW0742. It was studied in Otsuka Long Evans Tokushima Fatty (OLETF) rats which are often used as model of type II diabetes with obesity or to evaluate fatty liver and hepatic inflammation. To induce NASH, mice were fed a MCD diet. Upon treatment with GW0742 over 5 weeks, intrahepatic triglyceride content, expression of inflammatory cytokines such as TNF- α and MCP-1 as well as PGC-1 α gene expression were significantly decreased [195].

4.2.2. PPARδ clinical trials

Beyond these preclinical animal data, there are just two small clinical trials examining potential effects on fatty liver of selective PPAR^δ agonists. A small study reported by Risérus et al. investigated the effect of 10 mg GW501516 in 18 healthy but moderately overweight participants over 2 weeks versus placebo and 20 μ g of the PPAR α agonist GW590735. In comparison to the PPAR α agonist, GW501516 showed much more promising results with significant reductions in fasting plasma triglycerides, APO B, LDL, insulin, liver fat content and urinary isoprostanes. Urinary isoprostanes are prostaglandin-like substances, which develop when fatty acids react with free radicals in a peroxidation. They serve as inflammatory mediators and markers for lipid peroxidation, respectively oxidative stress. No effect was found on HDL levels. Furthermore, an improvement of β -oxidation was indicated by a higher skeletal muscle expression of CPT-1b [161]. The second PPARδ agonist used in a clinical trial was MBX-8025 which was evaluated in 181 dyslipidaemic and overweight patients. 50 or 100 mg MBX-8025 were compared to 20 mg atorvastatin or combined with atorvastatin for 8 weeks. Both, MBX-8025 alone and the combination with atorvastatin showed promising metabolic effects. APO B, non-HDL-cholesterol, especially LDL, triglycerides, free fatty acids, high-sensitivity CRP and the liver enzymes GGT and ALP were reduced while HDL levels were increased. Unfortunately, specific parameters concerning NAFLD or NASH or biopsies were not studied [196].

In summary, available data on PPAR δ in NAFLD/NASH is exiguous, similar to PPAR α . Based on data from rodent models, PPAR δ seems very promising to treat NASH and clinical data is less disappointing. Still, more experiments with selective PPAR δ agonists are required to reveal the receptor's exact molecular signalling pathways and to confirm the therapeutic value of PPAR δ modulators in clinical trials.

4.3. PPARγ

PPAR γ has long been successfully used as therapeutic target for the treatment of type 2 diabetes since its activation has insulin sensitizing activity. Additionally, anti-inflammatory effects have been reported, that could be very beneficial in the therapy of NAFLD and NASH as well [85]. Since TZDs were approved drugs, many clinical data from large patient collectives are available (Table 2). Most of them have been withdrawn because of safety concerns. Pioglitazone was associated with bladder tumors [197], rosiglitazone revealed an increased risk of cardiac events (although the FDA in 2013 revised the assessment) [198] and troglitazone, an early member of the TZDs, caused drug-induced hepatitis and was already withdrawn in 2000. Moreover, there are numerous animal experiments for TZDs as experimental NAFLD/NASH therapeutics.

4.3.1. PPARy animal studies

Pioglitazone was studied in a rat model of liver fibrosis induced by a choline-deficient L-amino acid-defined (CDAA) diet. First assessment after 2 weeks indicated that pioglitazone improved hepatic steatosis, because liver weight was reduced and triacylglycerol content was decreased. Furthermore, pioglitazone prevented liver fibrosis, and reduced pre-neoplastic lesions in the liver. After another 10 weeks pioglitazone prevented the activation of HSCs resulting in reduced expression of type I procollagen, MMP-2, that is increased when HSCs are activated and may be involved in liver carcinogenesis, TIMP-1 and TIMP-2. MMP-13 was not affected, which mainly hydrolyses extracellular matrix and can degrade fibrous collagen, e.g. types I and III collagens, two major factors in the formation of hepatic fibrosis. In addition, pioglitazone reduced serum ALP and bile acid levels in a dose dependent manner, but had no influence on ALT levels. It was observed that activated HSC also referred to as myofibroblast-like cells, who express alpha smooth muscle actin (aSMA), showed proliferation in the livers of rats fed CDAA diet for 10 weeks and pioglitazone was able to reduce the area of aSMA-positive cells. Moreover the area of placental glutathione S-transferase (GST-P), a marker for hepatocarcinogenesis, was reduced by pioglitazone [199].

Another animal study using various methods (carbon tetrachloride (CCl_4) injection, cholinedeficient diet, bile duct ligation(BDL)) of inducing fibrosis showed less beneficial effects. Mice were analysed for severity of hepatic fibrosis in pioglitazone treated animals versus untreated controls. In the CCl_4 and in choline-deficient diet induced fibrosis, pioglitazone revealed beneficial effects in reducing hepatic fibrosis, hydroxyproline content, a major component of the protein collagen which plays a key role for collagen stability, hepatic mRNA expression of collagen type I, and profibrotic genes, as well as the amount of aSMA. However, antifibrotic effects were lost when pioglitazone was administered after 5 weeks instead of 2 weeks after fibrosis induction. Moreover, pioglitazone failed to reduce fibrosis in the BDL model, independent from the time of administration. Thus, the study indicates a time and severity dependent effect of pioglitazone on fibrosis [200]. Pioglitazone was also tested in a murine MSD diet model. Besides steatohepatitis, effects on systemic insulin sensitivity were studied after pioglitazone treatment. However, no effect on insulin sensitivity was observed, although pioglitazone is known to have insulin sensitizing effects [85]. Still, pioglitazone was able to reduce hepatic fat accumulation and steatohepatitis. Adiponectin levels were enhanced, but ACO and ACC, which are important for β -oxidation, were unchanged [201].

Hsiao et al. evaluated the hepatoprotective effect of pioglitazone in male C57BL/6 mice treated with a 30% fat diet and 100 mg/kg/day pioglitazone for 8 weeks. Tissue oxidative stress was measured by malondialdehyde concentration, which arises when ROS degrades polyunsaturated lipids. Pioglitazone normalized malondialdehyde levels. Additionally, oxidative DNA damage was studied by immunohistochemical 8-oxoguanine (8-oxoG) staining. 8-oxoG develops as most common DNA lesions resulting from ROS. In this experiment, pioglitazone reduced the number of DNA lesions. Furthermore, the expression of antioxidative genes and the potential to repair oxidative DNA damage was evaluated. Under high fat diet, the expression of superoxide dismutase (Sod)1, Sod2, 8-Oxoguanine glycosylase (Ogg1) repairing DNA by base excision and MutY, another DNA glycosylase involved in oxidative DNA damage repair, were significantly decreased but this reduction could be reversed by pioglitazone treatment [202].

4.3.2. PPARy clinical trials

In 2001, the first clinical trial investigating the efficacy of a TZD in the treatment of NASH was reported. 10 female patients with histologically proven NASH were treated with 400 mg troglitazone per day for 6 months. During the study, seven out of ten patients were responder and only this group of patients was included in data evaluation. ALT and AST levels fell significantly, but biopsy comparisons before and after therapy showed persistent steatohepatitis in all cases. Four of these seven patients experienced a one-point improvement in necroinflammatory grade. Via electron microscopy, an elongation of the mitochondria after therapy was detected, but the number of mitochondria did not change significantly. Additionally, no data concerning hepatic dysfunction has been reported [203]. It was a rather short and small study, so it is difficult to assess its outcome. Furthermore, using ALT and AST levels to evaluate the efficacy on steatohepatitis is rather unspecific and histological examinations revealed no efficacy. Still, a longer trial might be able to confirm the positive outcome of the surrogate parameters [203].

Neuschwander-Tetri et al. studied rosiglitazone during a longer and larger trial. Within the class of TZDs, rosiglitazone is the most potent derivative [204]. 30 patients, including both men and women underwent liver biopsies before and after treatment. All displayed biopsy proven NASH, obesity and elevated ALT levels. The participants received 4 mg rosiglitazone twice daily for 48 weeks, which was the highest recommended dose for the treatment of diabetes. No placebo group was recruited, so the outcome cannot be evaluated without concerns. It was observed that the global necroinflammatory score was significantly improved and biopsies of 10 patients proved that they did not meet the criteria for NASH anymore. Furthermore, hepatocellular ballooning and zone 3 perisinusoidal fibrosis were improved, but the global fibrosis score remained unchanged. Serum levels of ALT, AST, ALP and GGT were decreased, which implicates less cholestatic injury [205]. However, no correlation between baseline liver fat and baseline GGT level or changes in liver fat and changes in GGT level could be demonstrated.

Furthermore, no significant correlation was found between changes in ALT level and changes in ballooning or changes in the global necroinflammatory score. Cholesterol and triglyceride levels remained stable throughout the trial. Histological examinations showed a shift in parenchymal localization of steatosis and changes in the relative grade of portal inflammation. Moreover, a shift of inflammation from predominantly lobular to more portal based, was observed. After 48 weeks the treatment with rosiglitazone was discontinued but patients were observed for another 6 months. After this follow-up, enzyme levels had increased to near pre-treatment levels, and the insulin-sensitizing effects of the therapy was not sustained. As side effects, haemoglobin levels were decreased and typically for TZDs patients gained weight. This weight gain mainly tended to be peripheral rather than central fat [155] and therefore it might not increase risks associated with the metabolic syndrome since central or abdominal obesity is strongly correlated with cardiovascular diseases, diabetes and dyslipidaemia. The risk of the appearance of heart diseases, hypertension, insulin resistance and type 2 diabetes is much higher in patients with central obesity than peripheral obesity [206]. In conclusion, there was no correlation between changes in ALT level and changes in ballooning or changes in the global necroinflammatory score. The authors state this could be due to the relatively small cohort size [149] but eventually liver enzyme levels are not perfect surrogate parameters to estimate the severity of the disease. Nonetheless rosiglitazone was able to resolve NASH in nearly half of the patients without improving fibrosis. Additionally, all benefits lasted only as long as the drug intervention.

Promrat et al. reported a trial for NASH that excluded diabetic patients. Only adult nondiabetic patients with biopsy-proven NASH and elevated serum ALT or AST were enrolled. 3 months before treatment, participants were instructed to lose weight, follow a healthy diet and avoid over-the-counter vitamin, mineral, or herbal supplements. After this, 18 out of 20 included patients entered 48-week therapy with 30 mg pioglitazone per day. According to liver biopsy, two thirds of the patients achieved improvements in histological features including steatosis, cellular injury, parenchymal inflammation, mallory bodies, which are damaged intermediate filaments in hepatocytes and fibrosis. The NASH activity index was reduced by at least one point in all patients. For the defined histological response, a reduction in the NASH activity index by at least 3 points with improvements of at least 1 point each in steatosis, parenchymal inflammation, and hepatocellular injury was required which was matched by 67% of patients. Liver fat and volume examination by MRI revealed significant reductions in both parameters. Secondary outcomes included a reduction of serum ALT and AST levels. ALT values were normalized in 72% of patients. This decrease was gradual with an onset after 4 weeks of treatment and lowest levels between weeks 40 and 48. In addition, AST and ALP levels were reduced, insulin sensitivity was improved while serum glucose levels remained constant, hepatic fat content and size decreased and free fatty acid levels were decreased to some extent. There were no significant changes in total cholesterol, triglycerides, LDL and HDL cholesterol levels. Again, the degree of histological improvements was not reliably correlated with changes in serum aminotransferase levels. 83% of patients with a histological response had normal ALT levels, but 50% of patients who did not achieve a histological response had normal ALT values as well. As major side effect, weight gain of 3.5 kg in average occurred in 72% of patients. Unfortunately, the study enrolled no placebo treated control group. Moreover, no follow-up evaluating long-term effects was conducted [207].

Another clinical trial (NCT00227110) in 2006 enrolled patients with impaired glucose tolerance or type 2 diabetes and liver biopsy–confirmed NASH. They were treated with 45 mg pioglitazone per day in combination with a hypocaloric diet over 6 months. This study included a control group, treated with a hypocaloric diet plus placebo. During the trial, participants were assessed by MRI to diagnose hepatic histologic features and hepatic fat content. Additionally, oral glucose tolerance tests were conducted. Diet in combination with pioglitazone improved glycaemic control and glucose tolerance. Furthermore, hepatic insulin sensitivity and glucose clearance were improved, which resulted in reduced free fatty acids, glucose and insulin in plasma. ALT and AST values were reduced, as well as hepatic fat content. Histological examination showed an improvement in steatosis, ballooning, necrosis and inflammation. TNF- α and TGF- β levels were reduced while adiponectin levels were significantly increased. However, also this trial failed to confirm a significant reduction in fibrosis. As side effects fatigue and mild lower-extremity edema occurred and potentially due to dietary intervention. Pioglitazone intake caused only a modest weight gain (2.5±0.5 kg) and increase in body fat of 1.5±0.5% [208].

Lutchman et al. conducted a small study in 18 patients with NASH treated with 30 mg pioglitazone daily for 48 weeks to investigate the serum levels of selected adipokines and proinflammatory cytokines. The aim was to correlate changes to improvements in liver histology and identify a better surrogate parameter to estimate the efficacy of TZD treatment. As result, they only detected increased adiponectin levels while the levels of other studied cytokines, including leptin, IL-1a, IL-6, and TNF- α , remained unchanged. Hepatic steatosis and the NASH activity index score could be correlated to the adiponectin level indicating on one hand, that adiponectin might be a major target of TZDs, rather than proinflammatory cytokines. On the other hand, it could be a better surrogate parameter for estimation of histological outcomes in NAFLD/NASH treatment [209].

The long-term efficacy of 30 mg pioglitazone per day was assessed in another trial lasting 12 months. Only non-diabetic patients with histologically proven NASH were included. Overall 74 patients were recruited and received 30 mg pioglitazone daily or placebo. Both groups were instructed to reduce their calorie intake by 500 Kcal/day, and to perform modest exercise, starting 3-months before drug intervention. 61 patients of 74 had a liver biopsy both at the beginning and the end of the study. After 12 months a reduction in glucose levels (-0.1 mmol/L vs. +0.4 mmol/L), haemoglobin A1c (HbA_{1c}; -0.18% vs. +0.16%), insulin C peptide level (-78 pmol/L vs. +42 pmol/L), ALT (-36.2 U/L vs. -10.9 U/L), GGT (-41.2 U/L vs. -9.4 U/L) and ferritin ($-11.3 \mu \text{g/L}$ vs. $-90.53 \mu \text{g/L}$), a storage for iron, were observed. Histological examination detected reduced degree of steatosis, hepatocellular injury, lobular inflammation, mallory bodies and fibrosis. In addition, there was a modest increase in serum adiponectin upon pioglitazone treatment. Hepatic steatosis was improved by dietary intervention and physical exercise alone, too. Improvements in fibrosis could be observed as well but failed to reach statistical significance upon treatment with pioglitazone. Only hepatocellular injury and the amount of Mallory bodies were significantly improved by pioglitazone [210].

Ratziu et al. studied the efficacy of rosiglitazone in two trials whereby FLIRT2 was an enlargement of the former FLIRT1 trial. 63 Patients with a histological diagnosis of NASH and elevated ALT levels were enrolled in the placebo-controlled trial FLIRT1. The verum group was treated with 4 mg/day rosiglitazone for the first month and 8 mg/day thereafter for 1 year

in total. 4 months after the end of treatment, patients were examined again. Both groups, verum and placebo, were instructed to lose weight if they were obese or overweight. In addition, they had to follow a healthy diet and to exercise at least twice a week. Until then, this was the longest placebo-controlled study of a TZD derivative in NASH. Primary end points for the assessment of the trial were improvements in histologic score of steatosis, normalization of serum transaminase levels and improvements in necroinflammation and fibrosis. Steatosis was reduced in 47% verum patients vs 16% in the placebo group. 38% vs 7% had a reduction in transaminase levels. This effect occurred rapidly during the first 4 months and was stable during the whole treatment period. No significant improvement in liver necroinflammation or liver fibrosis could be detected. Some secondary end points were also not matched, as there was no improvement in other histologic lesions, including hepatocyte ballooning. Still, progression of hepatocyte ballooning, portal inflammation, perisinusoidal fibrosis and overall fibrosis was significantly lower with rosiglitazone. Furthermore, significant reductions in fasting glucose level, HbA_{1c}, and surrogate serum markers of insulin resistance (hyperinsulinemia, HOMA index) were observed. Another aspect of the study was the detection of responder and non-responder. It turned out, that only half of the patients responded and the authors assume that a predictor for response is the absence of diabetes and in the presence of strong steatosis. Decreases in insulin levels, serum glucose and HOMA levels were significantly more pronounced in responders. The loss of liver fat was associated with an improvement of these parameters. In addition, it was observed that serum adiponectin levels correlated negatively with reduction in steatosis and responders had higher baseline adiponectin levels. Moreover, responders had lower median-GGT values, less frequently diabetes and a higher grade of steatosis than non-responders. An improvement of steatosis correlated with reduction of transaminase levels, improvement in insulin sensitivity and increase in adiponectin levels but not with weight changes. 4 months after end of treatment, the long-term efficacy of rosiglitazone was evaluated. ALT, glucose, HbA_{1c}, cholesterol, LDL and haemoglobin levels were normalized to a healthy level and there was still an improvement in insulin levels and HOMA values. Therefore, the authors assume that long term use of TZDs is required and a safety evaluation of long-term TZD intake is required. Side effects detected in this trial beyond weight gain were painfully swollen legs, muscular cramps and reduced serum haemoglobin levels. No hepatotoxicity or cardiovascular events occurred [211].

To study, whether a longer treatment had beneficial effects on the prognosis of the disease, the extensional trial FLIRT2 was conducted. 40 patients that completed FLIRT1 including the final examination after 4 months without treatment were enrolled. 22 received placebo during FLIRT1 and 18 were treated with rosiglitazone. In FLIRT2, all 40 were treated with 8 mg rosiglitazone daily for another 2 years. Both groups demonstrated a reduction in serum insulin by 26%, in HOMA by 30%, and ALT level by 24% but no significant change in the mean NAS, ballooning score, fibrosis stage, or area of fibrosis, was achieved. In the group receiving placebo in FLIRT1, steatosis significantly decreased after 2 years but in the group, that had already received verum previously, no additional improvement could be detected. Thus, the promising first trial, indicating a longer treatment might improve the symptoms of NAFLD an NASH, was not confirmed [212].

In 2010, a phase 3 trial (NCT00063622, PIVENS) was reported that lasted 96 weeks and enrolled 247 adults without diabetes who had biopsy-confirmed NASH. Three groups received

either 30 mg pioglitazone daily, 800 IU vitamin E daily or placebo. 90% of all patients underwent a liver biopsy after the end of treatment. The primary outcome was resolution of steatohepatitis, which was only achieved in the vitamin E group. Pioglitazone displayed no benefit compared to placebo. As secondary outcomes, histological features were evaluated and both vitamin E and pioglitazone showed an improvement. Liver biopsies indicated that both treatments lead to a significant reduction in steatosis, lobular inflammation and the activity score for NAFLD but fibrosis scores were not significantly reduced. Vitamin E improved scores for hepatocellular ballooning while pioglitazone was not effective in this scoring system. The serum levels of ALT and AST were reduced by both interventions. Additionally, pioglitazone reduced insulin resistance. Pioglitazone treated patients gained more weight than those who received vitamin E or placebo. The mean increase was about 4.7 kg at week 96. Also this long study enrolling nearly 250 participants failed to demonstrate beneficial effects of a PPAR γ agonist on fibrosis in NASH. Treatment with vitamin E seemed superior to pioglitazone, although patients with diabetes or with cirrhosis were excluded from the trial and thus the results cannot be generalized [213].

Another trial evaluated, whether a drug combination could be more beneficial for NAFLD and NASH treatment and ameliorate weight-gain of TZDs. Therefore, 4 mg rosiglitazone and 500 mg metformin twice daily were combined and compared to 4 mg rosiglitazone twice daily and 50 mg losartan once daily and both treatments were matched with 4 mg rosiglitazone twice daily treatment alone. The trial lasted 48 weeks, enrolled 137 patients with biopsy-proven NASH and assessed differences in the improvement of steatosis, hepatocellular inflammation, and fibrosis. Histological examinations revealed no significant difference between the 3 groups but in all groups, an improvement in steatosis, hepatocellular inflammation, ballooning degeneration and especially fibrosis was detectable. Serum aminotransferase levels were reduced in all three groups, as well. No reduced weight gain was achieved by the combination of rosiglitazone with metformin or losartan. Thus, use of combination therapy with metformin or losartan had no additional beneficial effects in this study. Still, this trial was able to detect a significant fibrosis improvement in contrast to all former studies with glitazones in NASH. Unfortunately there was no comparison to a placebo group, which lowers the impact of this study [214].

An Indian study compared 1200 mg pentoxifylline divided in 3 doses per day and 30 mg pioglitazone per day in 60 biopsy proven NASH patients with elevated ALT levels for 6 months. In addition to drug intake, patients were instructed to reduce their caloric intake by 500 kcal/day and perform exercise. Pioglitazone was superior to pentoxifylline but both agents were reported to improve transaminase levels, insulin resistance (HOMA), adiponectin levels and steatosis. TNF- α was not significantly reduced with either intervention. Pioglitazone improved lobular inflammation, portal inflammation and Brunts grade which is a staging score developed to reflect localization and extent of fibrosis [215].

Another study (NCT00994682) was conducted to assess the efficacy and safety of longterm pioglitazone treatment in patients with NASH and prediabetes or diabetes. 101 biopsy proven NASH patients received 45 mg pioglitazone per day or placebo over 18 months and were instructed to follow a hypocaloric diet. A reduction of at least 2 points in the NAFLD disease activity score in 2 histologic categories without worsening of fibrosis was defined as primary outcome and occurred in 58% of pioglitazone treated patients. Additionally, 51% had resolution of NASH. As secondary outcomes, histological improvements, hepatic triglyceride content and metabolic parameters were evaluated. Some individual histologic scores, including the fibrosis score could be improved. Although the fibrosis score was reduced in the verum group, the effect on fibrosis was rather modest with a reduction by -0.5 points. Placebo treatment had no effect on fibrosis. Furthermore, hepatic triglyceride content was reduced and insulin sensitivity was improved. Consistent with previous trials pioglitazone caused weight gain by about 2.5 kg compared to placebo. Although the study aimed at evaluating the efficacy of pioglitazone treatment in patients with NASH and diabetes, a subgroup analysis of the population which is actually suffering from diabetes and NASH is missing [216].

Since the outcome of fibrosis improvement by TZD treatment is not obvious from the above discussed studies, a meta-analysis evaluated the available four high quality randomized, placebo-controlled trials (NCT00063622 [213], Aithal et al. [210], NCT00227110 [208], FLIRT1 [211]). Three of the trial used pioglitazone and one study rosiglitazone. The meta-analysis concludes, that TZDs are able to improve ballooning degeneration, lobular inflammation and steatosis. Additionally, necroinflammation could be significantly reduced, calculated by statistical analysis over the 4 studies. However, including all 4 studies no significance regarding fibrosis improvement could be detected. Without involving the FLIRT1 trial, the improvement in fibrosis was statistically significant. However, evaluating each of the single trials revealed no significant fibrosis but more clinical data would be required to confirm this activity [217].

Another systematic review supports the thesis that pioglitazone is superior to rosiglitazone. But the key aim of this systematic analysis was to evaluate, whether adiponectin is a main target of TZD treatment. Again 4 studies were analysed, including the study of Belfort et al. [208], Lutchman et al. [209], Ratziu et al. (FLIRT1) [211] and Sharma et al. [215]. In total, 187 patients with liver biopsy proven NASH were investigated, which were treated 6-12 months with a TZD. In all studies, a significant elevation of adiponectin was observed, as well as a significant improvement of steatosis. Lobular inflammation was only significantly improved during pioglitazone treatment, not with rosiglitazone, supporting the former thesis that pioglitazone might be superior to the more potent rosiglitazone. The best efficacy in ballooning and fibrosis was found in trials with pioglitazone in the highest dose or the longest duration of therapy. Insulin resistance and liver function were improved as well. Moreover, it could be verified, that an increase in circulating adiponectin levels correlates with histological improvement. These findings might support the development of selective PPAR γ modulators (SPPARMs), which are discussed below [218].

In summary, the variety of trials with TZD in NAFLD/NASH fail to confirm the promising data from animal models. Still, significant improvements in histological parameters, especially hepatic steatosis and inflammation could be observed during TZD treatment. Whether the agents can reduce or reverse fibrosis remains to be confirmed. Potentially, a less efficacious and potent PPAR γ agonist is superior since the more potent agents might promote adipocyte differentiation and fat storage. This assumption and the problem of significant weight gain during TZD treatment further highlight the promising role SPPARMs could have in NASH to bypass side effects and improve for example adiponectin levels.

4.4. Dual PPAR agonists

4.4.1. Dual PPAR agonists animal studies

As mentioned above, bezafibrate constitutes a pan agonist of PPAR α , PPAR δ with lower potency on PPARy [193]. A few rodent models respective NAFLD and NASH demonstrated potential efficacy of bezafibrate. In addition to the examinations of Nagasawa et al. who compared bezafibrate with the PPAR\delta agonist GW501516 [194], Nakano et al. studied bezafibrate and pioglitazone in male KK-Ay/TaJcl mice fed a MCD diet for 7 weeks as type 2 diabetes mouse model. Moreover, an in vitro HSC model with an immortalized rat HSC (RI-T) was conducted. The cells were stimulated with TGF- β 1 to induce fibrosis. In vivo, bezafibrate was able to reduce hepatic triglyceride content and the accumulation of fat droplets within hepatocytes. Pioglitazone in comparison did not affect triglyceride content of the liver. Furthermore, in contrast to pioglitazone, bezafibrate decreased ALT levels and the concentration of thiobarbituric acid-reactive substances (TBARS). TBARS develop during lipid peroxidation as side-products and can be used to estimate the extend of ROS creation. Both, bezafibrate and pioglitazone increased adiponectin levels. The effect of bezafibrate on inflammation was determined by the number of foamy macrophage clusters and neutrophil infiltration, which were significant reduced. Moreover, bezafibrate and pioglitazone prevented HSC activation, measured by the number of α SMA-positive HSC. Via Masson trichrome stain, a three-colour staining to distinguish keratin or muscle fibres from collagen or bone, and to distinguish cytoplasm from cell nuclei was conducted. Thereby, collagen can be detected as fibrosis marker. Bezafibrate, but not pioglitazone, seemed to prevent hepatic fibrosis in the mouse model. Moreover, bezafibrate increased the mRNA levels of ACO and CPT-1, indicating an enhanced β -oxidation. Also TNF- α , IL-1 β , IL-6 and MCP-1 were reduced during bezafibrate treatment and anti-oxidative enzymes such as SOD-1 were elevated. Pioglitazone only affected Il-6 and SOD-1. In the in vitro HSC model, the fibrogenic response of several different PPAR modulators was studied by investigating α SMA, α_1 (I)-collagen and fibronectin-1 levels. All were decreased with bezafibrate superior to pioglitazone. In addition, fenofibrate as specific PPAR α agonist had the strongest decreasing effect on fibrogenic target genes. The PPARδ agonist GW501516 had no significant effect. The authors assume that an anti-fibrotic effect could be result of mainly PPAR α and additionally PPAR γ activation but to verify this hypothesis, more target genes need to be considered [219].

Although these animal models indicate a superior efficacy of the pan agonist bezafibrate, no clinical studies for the treatment of NASH with humans have been reported that would be required to confirm the promising in vivo data.

With the glitazars acting as dual agonists of PPAR α and PPAR γ there is another class of PPAR modulators. Glitazars were developed with the aim to treat diabetic patients, which develop dyslipidemia as comorbidity resulting in atherosclerotic coronary heart disease [220]. However, as consequence of several cardiovascular [221, 222] and renal [223] side effects, most glitazars have been withdrawn. Only saroglitazar is marketed in India.

Ji-Ming et al. reported promising in vivo data from rats on high fat diet treated with ragaglitazar, Wy-14,643 or rosiglitazone. Insulin sensitivity and lipid metabolism were assessed and indeed, hepatic triglyceride accumulation as well as visceral adiposity were stronger improved upon glitazar treatment than with rosiglitazone or Wy-14,643. Furthermore, in comparison with rosiglitazone and Wy-14,643 insulin sensitivity improvement was more pronounced and adiponectin values were elevated, significantly correlating with lipid content and insulin activity in liver. Hence, the dually active glitazars might be superior to selective PPAR modulators, but due to safety concerns their future is unknown. In India, saroglitazar is currently investigated in a phase 3 study (NCT02265276, GLAZED), evaluating its efficacy in NAFLD in comparison with pioglitazone. Though, according to the clinical trial database of United States National Library of Medicine, the completion date has passed and the status has not been verified in more than two years [224].

Another dual PPAR agonist, in particular the dual PPAR α and PPAR δ agonist elafibranor (GFT505), is currently amongst the farthest developed pharmacological NASH treatments. It has proven therapeutically effective during several animal studies and a clinical trial (GOLDEN-505). Elafibranor is currently the most promising PPAR targeting approach in NASH treatment (Table 2).

Staels et al. have extensively studied elafibranor in NASH models with human APO E2 transgenic mice fed with a western diet as well as MCD fed mice and rats, where fibrosis was induced by CCl4. All the rodent models showed that elafibranor decreased steatosis and inflammation with lower levels of IL-1 β , TNF- α and EGF-like module-containing mucin-like hormone receptor-like 1 (F4/80), a member of adhesion G protein-coupled receptors and a specific marker for eosinophils. Furthermore, profibrotic genes such as TGF- β , TIMP-2 and collagens were downregulated. Human APO E2 transgenic mice where used for PPAR α knockout experiments to assess the effects of elafibranor in absence PPAR α revealing that western diet induced steatosis and inflammation were resolved, despite of PPAR α knockdown, but plasma triglyceride levels and the concentration of free fatty acids were not affected, suggesting involvement of PPAR α . In rats, GFT505 underwent extensive enterohepatic cycling.

4.4.2. Dual PPAR agonists clinical trials

Two clinical studies of elafibranor reported by Cariou et al. evaluated effects and safety of elafibranor in abdominally obese patients with either combined dyslipidemia or prediabetes. The trials were not designed to study the treatment of fatty liver, but already indicate a potential use regarding fatty liver diseases. The first trial enrolled 94 patients with obesity and dyslipidemia while the second study was conducted in 47 obese patients with prediabetes. Patients were treated with 80 mg elafibranor per day for 28 days in trial one and 35 days in trial two. Elafibranor was able to significantly reduce fasting plasma triglycerides and increased HDL cholesterol in both studies. However, LDL only decreased in obese prediabetic patients. Additionally, a significant decrease of HOMA-index and fasting plasma glucose was detected in the same population. A reduction of GGT levels was observed in both studies. No verum specific side effects could be detected. These two trials both indicate a beneficial effect of the dual PPAR α and PPAR δ agonist for the treatment of NAFLD and NASH, because insulin sensitivity is a major contributor of fatty liver disease and decreases in triglycerides as well as circulating non-HDL-cholesterol levels seem very beneficial for a therapy of NAFLD and NASH [225].

Elafibranor was then specifically studied as potential therapy of NAFLD and NASH in the GOLDEN-505 trial (NCT01694849). Selection criterion to be included in this phase IIb trial

was a histologic diagnosis of non-cirrhotic NASH. Patients were excluded if they consumed alcohol daily, if steatohepatitis had a secondary cause, or if any other chronic liver disease was detected. In total, 276 patients were enrolled and separated in three treatment groups receiving 80 mg or 120 mg elafibranor once daily or placebo over 52 weeks. The primary outcome was the resolution of NASH without fibrosis worsening. Resolution of NASH was defined as the absence of at least one of the following symptoms: steatosis, ballooning or inflammation. Worsening of fibrosis was defined as no progression to bridging fibrosis or when bridging fibrosis was the initial diagnosis, no progression to cirrhosis. The endpoint was not matched, because there was no statistically significant difference between elafibranor and placebo but NASH resolution occurred in a higher number of patients in the 120-mg elafibranor group. Using a post-hoc analysis with a modified definition, the results of the response rate changed. The modified definition describes resolution of NASH as disappearance of ballooning in combination with either disappearance of lobular inflammation or the persistence of mild lobular inflammation only. Furthermore, any increase in fibrosis is regarded as fibrosis worsening. With this definition, the response rate was significantly higher for the 120-mg arm than for placebo while the 80-mg group revealed no superiority to placebo for both definitions of response. As secondary outcomes, changes in NASH activity score from baseline biopsy at the end of treatment as well as changes and improvements in individual histologic scores of steatosis, ballooning, inflammation, and fibrosis were defined. Again no significant difference between elafibranor and placebo could be detected. The efficacy of the 120-mg dose to reduce the NASH activity score by 2 points and to improve steatosis, ballooning and lobular inflammation was slightly improved, but did not reach a level of statistical significance. Patients receiving elafibranor had improved ALT, GGT and ALP levels. Furthermore, lipid parameters, including triglycerides, LDL and HDL were improved and in diabetic patients, elafibranor improved fasting serum glucose and HbA_{1c}, as well as markers of insulin resistance such as fasting insulin, HOMA-index and circulating free fatty acids. Additionally, there was a reduction in systemic inflammatory markers, mainly high-sensitivity CRP and fibrinogen at both doses. Histologic changes were estimated by a panel of serum biomarkers of steatosis and fibrosis (SteatoTest, Fatty Liver Index, Fibrotest/FibroSure, and the NAFLD Fibrosis score) and showed significant reductions in patients treated with elafibranor 120 mg only. Overall, high placebo induced NASH improvements affected the outcome of the study. Patients with mild steatohepatitis, had a high placebo response rate leading to a lack of significance in the verum groups. Therefore, the authors suggest that elafibranor might have higher efficacy in more severe disease. Further studies to confirm this assumption are required, however. Concerning side effects, elafibranor was well tolerated and did not cause weight gain or cardiac events. Only a mild, reversible increase in serum creatinine was observed, indicating renal side effects [72].

Currently the phase 3 study RESOLVE-IT (NCT02704403) to evaluate the efficacy and safety of elafibranor in NASH patients is ongoing. Inclusion criteria for participants are liver biopsy confirmed diagnosis of NASH, and in contrast to GOLDEN-505, at least 1 point in each component of the NAS score (steatosis scored 0-3, ballooning degeneration scored 0-2, and lobular inflammation scored 0-3) with a total score of at least 4. In addition, a fibrosis stage between 1 and 4 according to the NASH CRN fibrosis staging system is required [226].

Reference	PPAR subtype, intervention	Population, duration	NAFLD/NASH related outcomes	comments
Laurin et al. (1996) [190]	- PPARα: 2 g/d clofibrate - 13-15 mg/kg/d ursodeoxycholic acid	40 patients with biopsy-confirmed NASH 12 months	 no changes in ALT, AST, GGT, bilirubin, triglycerides and cholesterol no improvement in the histological grade of steatosis, inflammation or fibrosis ALP↓ 	- no placebo control
Basarangoglu et al. (1999) [191]	- PPARα: 600 mg/d gemfibrozil - placebo	46 patients with biopsy-confirmed NASH and persistent elevated ALT and AST levels 4 weeks	 ALT↓, AST↓, GGT↓ no change in mean triglyceride levels and mean body weight 	- no histology
Fernandez- Miranda et al. (2007) [192]	- PPARα: 200 mg/d fenofibrate + diet and physical exercise	16 patients with biopsy-confirmed NASH 48 weeks	 triglycerides↓ glucose↓, tendency to improved insulin sensitivity ALT↓, AST↓, GGT↓, ALP↓ Apo-A1↑ hepatocellular ballooning↓ no change in grade of steatosis, lobular inflammation, fibrosis or NAFLD activity score 	- no placebo control
Riserus et al. (2008) [161]	- PPARα: 20 μg/d GW590735 - PPARδ: 10 mg/d GW501516 - placebo	18 healthy but moderately over-weight participants (6 per group) 2 weeks	GW590735: no effect except triglyceride lowering activity GW501516: - fasting plasma triglycerides↓ (-30%), Apo- B↓ (-26%), LDL↓ (-23%), insulin↓ (-11%), liver fat content↓ (-20%), urinary isoprostanes↓ (-30%), CPT-1b↓	- no histology
Bays et al. (2011) [196]	- PPARδ: 50 mg/d or 100 mg/d MBX-8025 +/- 20 mg/d atorvastatin	181 over-weight participants with mixed dyslipidemia 8 weeks	APO-B↓, non-HDL-cholesterol↓, LDL↓, HDL↑, triglycerides↓, GGT↓, ALP↓ combination with atorvastatin showed more efficacy	 no NAFLD/NASH specific parameters no histology
Caldwell et al. (2001) [203]	- PPARγ: 400 mg/d troglitazone	10 female patients with biopsy-confirmed NASH 6 months	 7/10 responded (with normal ALT) outcome for responders: ALT↓, AST↓ persistent steatohepatitis in all cases biopsy revealed one-point improvement in necroinflammatory grade in 4/7 elongation of mitochondria 	- no placebo control - very short duration

Table 2: Clinical trials of PPAR modulators for NAFLD/NAS	SH.
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Reference	PPAR subtype, intervention	Population, duration	NAFLD/NASH related outcomes	comments
Neuschwander- Tetri et al. (2003) [149]	- PPARγ: 4 mg rosiglitazone twice daily	30 overweight or severely obese patients with increased ALT levels and biopsy-confirmed NASH (50% with impaired glucose tolerance or diabetes) 48 weeks	 mean global necroinflammatory score improved biopsies of 10 patients (45%) no longer met criteria for NASH after treatment hepatocellular ballooning↓ no statistically significant improvement in global fibrosis score ALT↓, AST↓, GGT↓, ALP↓ no significant changes in cholesterol and triglyceride levels liver enzyme levels had increased to near pre-treatment levels 6 months after treatment 	- no placebo control
Promrat et al. (2004) [207]	- PPARγ: 30 mg/d pioglitazone + healthy diet + pre-treatment weight-loss + no vitamin/mineral/ herbal supplement	18 nondiabetic patients with biopsy-confirmed NASH and elevated serum ALT or AST 48 weeks	 steatosis, cellular injury, parenchymal inflammation, Mallory bodies, hepatocellular injury and fibrosis significantly improved in 67% NASH activity score decreased by at least one point in all patients ALT, AST, ALP normalized improved insulin sensitivity hepatic fat content↓ liver size↓ no significant changes in total cholesterol, triglycerides, LDL and HDL cholesterol levels 	- no placebo control
Luyckx et al. (2006) (NCT00227110) [208]	- PPARγ: pioglitazone) - placebo + hypochaloric diet	55 patients with biopsy-confirmed NASH and impaired glucose tolerance or diabetes 6 months	 improved glycaemic control and glucose tolerance plasma free fatty acids↓ insulin levels↓ AST↓, ALT↓ hepatic fat content↓ improvement in steatosis, ballooning necrosis and inflammation TNF-α level decreased by 11% TGF-β level decreased by 18% adiponectin↑ no significant difference in fibrosis vs placebo 	- variations in fibrosis assessment by percutaneous biopsy [227]

Reference	PPAR subtype, intervention	Population, duration	NAFLD/NASH related outcomes	comments
Lutchman et al. (2006) [209]	- PPARγ: 30 mg/d pioglitazone	18 patients with biopsy-confirmed NASH 48 weeks	- adiponectin \uparrow - no change in cytokines levels (IL-1a, IL-6, and TNF- α) - histological improvements assessed by semi-quantitative scoring revealed reduced steatosis, parenchymal inflammation, cell injury and fibrosis - correlations between change in adiponectin level and improvement in steatosis (P = 0.03) as well as in a summary NASH activity index score (P = 0.01)	- no placebo control
Aithal et al. (2008) [210]	- PPARγ: 30 mg/d pioglitazone - placebo + diet and exercise	74 non-diabetic patients with biopsy-confirmed NASH (61 completed study) 12 months	reduction in: - plasma glucose \downarrow , HbA _{1c} \downarrow , insulin C peptide \downarrow - ALT \downarrow , GGT \downarrow - ferritin \downarrow - hepatocellular injury \downarrow (P = 0.005) - mallory bodies \downarrow (P = 0.004) - fibrosis \downarrow (P = 0.05)	
Ratziu et al. (2008) (FLIRT1) [211]	- PPARγ: 4 mg/d in the first month then 8 mg/d rosiglitazone + diet and exercise + pre-treatment weight-loss	63 patients with biopsy-confirmed NASH and elevated ALT level 1 year	- improvement in steatosis - ALT normalized - no improvement in hepatic necroinflammation, fibrosis, hepatocyte ballooning or lobular inflammation/necrosis - lower progression of hepatocyte ballooning (P = 0.026), portal inflammation (P = 0.02), and overall fibrosis (P = 0.05) with rosiglitazone -fasting glucose level \downarrow , HbA _{1c} \downarrow , HOMA index \downarrow - only 50% of patients responded - predictors of response were: absence of diabetes, severe steatosis, lower GGT levels	
Ratziu et al. (2010) (FLIRT2, extension of FLIRT1) [212]	- PPARγ: 8 mg/d rosiglitazone	40 patients that had completed FLIRT1 2 years (extension)	 no significant change in mean NASH activity score, ballooning score, fibrosis stage or area of fibrosis steatosis significantly decreased only in patients that had received placebo in FLIRT1 -> no benefit of extended treatment 	

Reference	PPAR subtype, intervention	Population, duration	NAFLD/NASH related outcomes	comments
Sanyal et al. (2010) (PIVENS) [213]	- PPARγ: 30 mg/d pioglitazone - vitamin E (800 IU daily) - placebo	247 patients with biopsy-confirmed NASH 96 weeks	 pioglitazone had no benefit over placebo in resolving steatosis (34% and 19%) total NAFLD activity score↓ (-1.9 vs0.5), steatosis score↓ (-0.8 vs0.1), lobular inflammation↓ (-0.7 vs0.2), hepatocellular ballooning↓ (-0.4 vs0.2), fibrosis stage↓ (-0.4 vs0.1) ALT↓, AST↓, GGT↓, ALP↓, bilirubin↓ superior efficacy with vitamin E 	- very variable cohort (diabetes, cirrhosis, etc.)
Torres et al. (2011) [214]	- PPARγ: 4 mg/d rosiglitazone +/- losartan (50 mg/d) or metformin (2x 500 mg/d)	137 patients with biopsy-confirmed NASH 48 weeks	 no difference in efficacy between groups significant improvement in fibrosis improved steatosis score (-0.85, -0.82, -0.74) and hepatocellular inflammation (-0.58, -0.32, -0.34) in all groups ALT↓, AST↓, ALP↓ (all groups) no benefit of metformin concerning weight-gain 	- no placebo control
Sharma et al. (2012) [215]	- PPARγ: 30 mg/d pioglitazone - pentoxifylline (1200 mg/d) + reduced caloric intake + exercise	60 patients with biopsy-confirmed NASH and elevated ALT 6 months	 pioglitazone: AST↓, ALT↓, insulin resistance↓ (HOMA), adiponectin levels↑ steatosis↓, lobular inflammation↓, portal inflammation↓, Brunts grade↓ TNF-α was not significantly reduced neither with pioglitazone nor with pentoxifylline no significant change of fibrosis 	- no placebo control
Cusi et al. (2016) [216]	- PPARγ: 45 mg/d pioglitazone - placebo + hypocaloric diet	101 patients with biopsy-confirmed NASH 18 months	 reduction of at least 2 points in NAFLD disease activity score in 2 histologic categories without worsening of fibrosis in 58% resolution of NASH in 51% steatosis↓, inflammation↓, ballooning↓, fibrosis↓, liver fat content↓ ALT↓, AST↓, triglyceride levels↓, free fatty acids↓ 	

Reference	PPAR subtype, intervention	Population, duration	NAFLD/NASH related outcomes	comments
Cariou et al. (2011) [225]	- PPARα/δ: 80 mg/d elafibranor - placebo	S1: 94 abdominally obese patients with dyslipidemia, 28 days S2: 47 abdominally obese patients with pre-diabetes, 35 days	 significant reduction in fasting plasma triglycerides HDL cholesterol↑ LDL cholesterol↓ only in S2 S2 study: significant decrease of HOMA-index, fasting plasma glucose and fructosamine 	
Ratziu et al. (2016) (GOLDEN- 505) [72]	- PPARα/δ: 80 or 120 mg/d elafibranor - placebo	276 patients with biopsy-confirmed non-cirrhotic NASH	 no significant difference between elafibranor and placebo in resolution of NASH without fibrosis worsening NASH resolved in more patients without fibrosis worsening in the 120-mg arm (protocol definition: 21% vs. 17%, modified definition: 19% vs 12%) liver enzymes, lipids, glucose profiles, and markers of systemic inflammation were significantly reduced in the elafibranor 120-mg arm no changes in NAS between end of treatment and baseline biopsy no changes and improvements in individual histologic scores of steatosis, ballooning, inflammation, and fibrosis 120 mg elafibranor reduced the NAS by 2 points (48%) and improved steatosis, ballooning, and lobular inflammation with increasing baseline severity, both elafibranor doses improved liver function tests (ALT, GGT and ALP) and lipid parameters (triglycerides, LDL cholesterol, HDL cholesterol) 	 high placebo response in patients with mild steatohepatitis many non-responders

5. Synergistic Multi-Target Modulation as Superior NASH Treatment?

As discussed above, NASH is a very multi-factorial disease that arises from a variety of causes and results in several pathological conditions leading to liver damage and promoting further disease progression. It might, therefore, be rather effective to address more than one molecular target in order to generate an effective pharmacological response in NASH treatment. In theory, modulation of each PPAR subtype provides numerous beneficial effects for NASH treatment but so far, only the dual PPAR α /PPAR δ agonist elafibranor could translate PPAR modulation into clinical efficacy supporting the assumption that modulation of a single target might not be sufficient to treat NASH. Combined activation of PPAR α and PPAR δ by elafibranor might generate a synergistic effect by producing beneficial effects in different tissues. While PPAR α activation amongst others promotes hepatic degradation of lipids by β oxidation, PPAR δ agonism predominantly exhibits extrahepatic activity improving metabolic balance which in turn improves liver health. Similar potential synergies with PPAR modulation are also plausible for several other targets that have revealed beneficial effects in NASH treatment.

Although anti-inflammatory effects of PPAR activation have repeatedly been reported and elafibranor caused improvement of hepatitis in clinical trials, PPARs predominantly regulate metabolic pathways and many of their anti-inflammatory effects in liver are a consequence of improved metabolic balance than a direct activity. PPARs promote β -oxidation and lipolysis and enhance clearance of lipids from the liver leading to reduced steatosis. To support these metabolic improvements with anti-inflammatory, liver cell protective or anti-fibrotic activity, combining PPAR activation with an anti-inflammatory approach holds much promise as potentially synergistic therapeutic strategy. In NASH, metabolic stress in the liver leads to oxidative stress and a permanent inflammatory process causing cell damage and ultimately cell death through apoptosis. Several anti-inflammatory targets have already proven efficacy against this aspect of NASH in animal models or even clinical trials. Except for the anti-oxidative activity of vitamin E, two anti-inflammatory strategies evolve as experimental approaches in NASH treatment [12, 228, 229]. The chemokine receptor antagonist cenicriviroc blocking the chemokine receptors (CCR)2 and CCR5 is currently the most advanced anti-inflammatory agent in the NASH pipeline. Chemokine ligand (CCL)2 and CCL5 amongst others have been found upregulated in NASH patient biopsies [230] and might significantly contribute to hepatic inflammation. Cenicriviroc currently undergoes a prospective phase IIb trial [231] to assess its anti-inflammatory and anti-fibrotic potential in NASH. Inhibitors of the ASK1 such as selonsertib (GS-4997) also have great anti-inflammatory and cell-protective potential and three studies for the ASK1 inhibitor selonsertib as NASH treatment are recruiting (NCT02781584, NCT03053050, NCT03053063). These anti-inflammatory approaches could support the beneficial metabolic effects of PPAR activation by directly reducing hepatic inflammation and thereby providing liver cell protective activity. Under reduced inflammatory conditions, liver cell apoptosis and fibrosis would also be expected to decrease.

A direct reversal of fibrosis is very desirable in NASH treatment but promising anti-fibrotic effects from animal models have not translated to a comparable activity in clinical trials so far. Two strategies directly aiming at preventing and reducing fibrosis in NASH are in clinical development with the caspase inhibitor emricasan in phase IIa and galectin 3 inhibitors in phase I [228, 229, 232–234]. Although there is also some limited evidence that PPAR γ activation itself might have anti-fibrotic activity [235], combination of anti-fibrotic approaches with PPAR agonists holds therapeutic promise. By preventing apoptosis of hepatocytes and reducing the formation of fibrotic tissue, caspase inhibitors or galectin 3 inhibitors could protect the liver architecture and retain hepatic function which might also result in synergistic efficacy

against NASH when combined with PPAR activation that predominantly ameliorates hepatic metabolism and reverses steatosis.

Beyond combining agents to improve metabolic balance with direct anti-inflammatory or anti-fibrotic mechanisms also targeting metabolic anti-NASH effects in different tissues holds promise for synergistic activity. In addition to the dual activity of elafibranor, this might also be achievable by simultaneously activating FXR and PPAR δ . FXR exhibits its effects mainly in liver and intestine. Via PPAR α and the transcription factor sterol regulatory element binding protein 1c (SREBP1c), the nuclear receptor promotes β -oxidation and lipid clearance in liver. Additionally, FXR enhances cholesterol excretion from liver into bile and prevents biosynthesis of hepatotoxic bile acids by repressing cholesterol 7α hydroxylase (CYP7A1). Finally, FXRmediated repression of NF- κ B signaling in hepatocytes and hepatic macrophages/Kupffer cells can reduce hepatic inflammation and fibrogenesis [229]. PPAR δ activation in contrast, only moderately affects hepatic β -oxidation and lipid metabolism but improves metabolic balance by promoting utilization of lipids for energy generation in muscles. Additionally, PPAR δ activation has beneficial effects on cholesterol levels and the ratio of HDL/LDL which might ameliorate the cholesterol accumulation caused by FXR activation. Finally, dual modulation of FXR and PPAR α might provide synergistic effects by activating hepatic lipid clearance via two essential regulators. As discussed above, both receptors promote anti-steatotic effects in the liver, PPAR α mainly by enhancing β -oxidation and FXR by inducing the expression of PPAR α as well as by SREBP1c mediated effects on hepatic lipid and glucose metabolism. Clinical trials and practice will have to reveal the most favorable synergistic combinations of pharmacological approaches in NASH treatment but the multifactorial nature of the disease especially in later stages will most likely require more than one pharmacodynamic mechanism for sufficient therapeutic efficacy.

6. Selective PPARy Modulators

Selective modulation of nuclear receptors is getting increasingly into focus of drug discovery as an improved strategy to exploit their pharmacological potential as targets with reduced side effects. Such selective strategies include tissue selective modulation, gene selective modulation and partial activation with reduced transactivation efficacy.

Also some SPPARMs have been reported (Table 3) that might have value in NASH treatment. Amongst SPPARMs, partial agonists gained most attention. Several potent examples have been developed and studied in vivo. PAM-1616 [236], KR62980 [237], SPPAR γ M5 [238], INT131 [239, 240], L312 [241], MK-0533 [242] and MBX-102 [243] constitute partial PPAR γ agonists that have revealed anti-diabetic activities with lower typical PPAR γ mediated side-effects such as weight gain, cardiac hypertrophy and edema. Clinical trials of INT131 [244] have already confirmed that such partial agonism can translate into robust effects on glycemic control in human since INT131 was not inferior to pioglitazone but did not cause weight gain and fluid retention in phase 2.

name	structure	type
PAM-1616 [236]	S S S S S S S S S S S S S S S S S S S	- partial agonist - fully dissociates co-repressors
KR62980 [237]		- partial agonist
SPPARγM5 [238]	F ₃ C ₀ COOH	- partial agonist
INT131 [239, 240, 244]		- partial agonist - 40% transactivation compared to rosiglitazone
L312 [241]		- partial agonist - blocks Cdk-5 mediated PPARγ-phosphorylation
МК-0533 [242]	F ₃ CO COOH	- partial agonist
MBX-102 [243]	CI CF3	 partial agonist fully dissociates co-repressors full efficacy in transrepression
S26948 [245]		- only recruits GRIP1 in vitro - does not promote adipocyte differentiation and weight-gain

Table 3: Selective PPAR γ modulators (SPPAR γ Ms).

name	structure	type
FK614 [246, 247]		 fully dissociates co-repressors specific profile of co-activator recruitment promotes adipogenesis
LG100641 [248]	о соон	- antagonist - tissue/cell-type specific?
SR1664 [249]	O ₂ N H H H	- non-agonist - blocks phosphorylation at Ser-273
GQ16 [252]	Br S OCH ₃	- partial agonist - blocks phosphorylation at Ser-273

Structural data from an INT131-PPAR γ co-crystal structure indicates that partial activation of the receptor is explained by incomplete stabilization of the transactivation helix (H12). INT131 forms only lipophilic contacts to this helix whereas full agonistic TZDs participate in several H-bonds with H12 [240]. Partial PPAR γ activation seems superior to full agonists concerning side effect profiles in vivo but retain beneficial metabolic effects. However, the effects on metabolism did not exceed the activity of classical TZDs. As discussed above, pioglitazone or rosiglitazone alone were not effective in NASH treatment and, therefore, it is unlikely that partial PPAR γ agonists alone will perform significantly better.

Gene-selective PPAR γ modulation might hold even more therapeutic potential. But in contrast to the large number of partial PPAR γ agonists, only few compounds have been found to selectively modulate PPAR γ activation in a gene-selective manner which is much more difficult to achieve. So far, the most specific modulation of PPAR γ has been achieved with SPPARMs displaying significantly distinct co-activator recruitment profiles.

S26948 is a PPAR γ ligand with comparable affinity and transactivation efficacy as rosiglitazone in reporter gene assays. However, its co-activator recruitment profile was significantly different from rosiglitazone as it only recruited glutamate receptor interacting protein 1 (GRIP1) while rosiglitazone also recruited DRIP205 and PGC-1 α . The compound did not cause adipocyte differentiation or lipid accumulation in vitro but in vivo exhibited comparable antidiabetic and lipid-lowering activity as rosiglitazone. In contrast to rosiglitazone, S26948 caused no weight gain in vivo. Additionally, the PPAR γ modulator reduced liver weight and liver fat content while promoting hepatic lipid oxidation. In a mouse model of atherosclerosis, S26948 reduced plasma cholesterol and lipid levels to a less atherogenic profile. These results indicate that specific PPAR γ modulation might also be effective in improving liver health in NASH [245].

The SPPARM FK614 [246, 247] revealed a clearly different pharmacodynamic profile than S26948. It robustly dissociated co-repressors SMRT and NCor from PPAR γ and recruited PGC-1 α with comparable efficacy as thiazolidindiones while C/EBP and steroid receptor coactivator-1 (SRC-1) were significantly less recruited upon FK614 binding. In contrast to S26948, FK614 promoted adipocyte differentiation and lipid accumulation with similar efficacy as thiazolidindiones. However, the long-term effect of FK614 on mature adipocytes differed from thiazolidindiones as treatment with the SPPARM resulted in a more favorable expression profile of genes involved in insulin resistance. This activity might as well improve liver health in NASH by redistributing fat from the liver to the periphery but clinical trials with thiazolidindiones in NASH have revealed that this effect alone is not sufficient and the resulting promotion of adipose tissue formation and weight gain is hardly desirable.

Beyond its partial PPAR γ transactivation potency, MBX-102 has been discussed as more effective in PPAR γ transrepression activity. While it only moderately induced PPAR γ target gene expression, repression of pro-inflammatory genes was markedly more pronounced. In vitro, MBX-102 displayed nearly full efficacy in inducing the release of co-repressors NCOR and SMRT from PPAR γ but only moderately recruited the co-activators C/EBP, SRC-1, PGC-1 α , DRIP205 and transcriptional mediators/intermediary factor 2 (TIF2) with efficacies below 30% which gives a hint for the mechanism of the compound's interesting pharmacological profile [243]. According to the limited available data, the partial PPAR γ agonist PAM-1616 might also have gene-selective modulatory properties as it induced the glucose transporter GLUT-4 with higher efficacy compared to rosiglitazone than other PPAR γ target genes [236].

LG100641 was identified as PPAR γ antagonist with specific modulatory profile. It inhibited co-activator recruitment to PPAR γ induced by rosiglitazone, prevented adipocyte differentiation upon rosiglitazone treatment and blocked TZD induced PPAR γ target gene expression. However, LG100641 did not reduce basal glucose uptake by adipocytes but a clear molecular mechanism to explain the compound's activity has not been demonstrated and other targets might be involved [248].

Recently, inhibition of cyclin-dependent kinase 5 (CdK-5) mediated phosphorylation of PPAR γ has been proposed as another way of modulating the nuclear receptor [249–251]. Under high fat diet, PPAR γ phosphorylation at Ser-273 was found significantly upregulated in adipose tissue changing the expression of adiponectin and other genes involved in insulin sensitivity. However, the phosphorylation only affected a subset of PPAR γ target genes which might be due to differential recruitment of co-regulatory proteins. PPAR γ phosphorylation is blocked by PPAR γ agonists such as rosiglitazone but independent from their activating properties since PPAR γ ligands lacking agonistic potency could also be identified. Such nonagonist ligand SR1664 revealed robust antidiabetic activity in vivo suggesting that specific modulation of PPAR γ that prevents phosphorylation at Ser-273 might be sufficient to retain anti-diabetic activity. The expression profile of PPAR γ target genes under treatment with the non-agonist ligand thereby significantly differed from a typical PPAR γ agonist expression profile. As major advantage, the non-agonist modulator did not induce fluid retention in the

animals or promote body fat mass whereas TZDs increased body fat and caused weight gain. Since insulin resistance and type 2 diabetes are closely linked to NAFLD and NASH, these preliminary observations indicate that blocking PPAR γ phosphorylation at Ser-273 might also be effective in NASH treatment. Several studies have developed small-molecule blockers of PPAR γ phosphorylation lacking agonistic potency and have shown their anti-diabetic activity [241, 252, 253]. However, their effect on hepatic fat content and liver health has not been studied, so far. PPAR γ agonists tend to reduce liver fat by redistributing and disposing fat into peripheral adipose tissue causing weight gain. Since increased body fat mass and weight gain have not been observed with the non-agonist PPAR γ modulators that block phosphorylation at Ser-273, their effect on liver steatosis and hepatic lipid metabolism might be significantly different and the value of this mode of PPAR γ modulation in NASH needs to be further studied.

7. Conclusion & Outlook

PPARs have a changeful history as drug targets. PPAR γ agonistic thiazolidinediones markedly improved the therapy of type 2 diabetes mellitus as insulin sensitizers but few years ago their therapeutic significance ended with market withdrawals and warnings due to severe side effects. PPAR α agonistic fibrates are still marketed and can be used to treat hyperlipidemia but have also significantly lost in significance since alternative therapeutic approaches turned out superior. And finally, PPAR δ was considered as very promising target to treat metabolic disorders until the first selective PPAR δ agonist GW501516 failed in clinical trials due to unresolved safety concerns. However, the growing interest in therapeutic options for non-alcoholic steatohepatitis might help PPARs to a new rise as drug targets.

As discussed above, all three PPAR subtypes are crucially involved in the various aspects of the metabolic syndrome. PPAR α regulates transport and distribution as well as synthesis and degradation of lipids. Its activation promotes the utilization of lipids as energy source in β oxidation leading to decreased lipid concentrations especially in liver. Thus, PPAR α activation seems a very reasonable strategy to treat NASH which is highly linked to increased hepatic lipid levels. Accordingly, promising efficacy was observed in rodent models but most clinical trials failed to translate this into robust therapeutic benefit. A significant issue of almost all studies on PPAR α in NASH might be the used PPAR α agonists since hardly any selective compound was studied. Therefore, the results of isolated PPAR α activation in NASH are hardly known and further studies with potent and selective agents will be necessary to clearly analyze its therapeutic value.

PPAR γ mainly modulates glucose homeostasis and promotes insulin sensitivity. Thus, its relation to NASH becomes clear only at second glance because insulin resistance, diabetes and NAFLD/NASH are connected as manifestations of the metabolic syndrome. Notably, type 2 diabetes is amongst the strongest risk factors of NAFLD/NASH and some trials with thiazolidinediones have indeed revealed some efficacy in NASH treatment. Still, PPAR γ activation is well-known for causing weight gain which constitutes a major issue in NAFLD/NASH patients that often already are obese. Although PPAR γ activation was shown to decrease hepatic fat content, this effect was mainly due to redistribution of fat from liver to visceral fat. PPAR γ modulators have been most extensively studied in clinical trials for NASH but with merely disappointing results. However, PPAR γ drug discovery remains very intensive and there are many ligands of newer generations that either partially activate the nuclear receptor, cause a specific interaction profile with different co-activators and repressors or modulate the receptor's phosphorylation state. These new approaches have shown retained anti-diabetic activity in vivo but caused significantly less side effects such as weight gain. Thus, selective PPAR γ modulators also hold considerable potential in NASH but much more research will be necessary to confirm this hypothesis.

PPAR δ remains the least studied PPAR subtype also with the great success of elafibranor in clinical trials. It holds a lot of promise in NASH treatment by improving lipid utilization in peripheral organs, especially in skeletal muscle, combined with anti-inflammatory activity. Elafibranor is very likely to become the first approved PPAR targeting agent to treat NASH and revealed efficacy in reducing steatosis, hepatitis and fibrosis in clinical trials, at least in severe cases. However, the great efficacy of elafibranor might arise from its dual activity profile since it also activates PPAR α and thereby combines peripheral and hepatic anti-NASH activity. On one hand, this makes the interpretation of PPAR δ 's isolated role in NASH treatment rather difficult but on the other hand indicates that dual or multi-target agents might possess superior efficacy in this multifactorial disease complex.

Multi-target modulation as innovative, additive and potentially synergistic strategy holds promise especially in a multifactorial context as it is present in the metabolic syndrome in general and in NASH in specific. In this context, PPARs seem very promising targets that can be combined with supportive modulation of other signaling pathways or enzymatic cascades. Since PPARs mainly cause metabolic improvements that positively affect liver health and can reduce hepatic steatosis by promoting metabolic lipid elimination, combination of PPAR activation with anti-inflammatory and anti-apoptotic strategies has obvious potential. Thereby, the beneficial metabolic consequences of PPAR modulation could be markedly supported potentially leading to superior efficacy.

Despite of intensive research, several putative therapeutic effects of PPAR modulation in NASH have not been successfully translated into clinical efficacy, yet. Potential reasons are the considerable species differences in PPAR functions and expression pattern. Therefore, animal models and trials in human are not comparable in various aspects (Table 1). Additionally this might also be due to the design, population and biomarkers of the respective clinical studies. Improved NASH research requires better biomarkers with more predictive potential as well as more innovative study design. In addition, a more individualized therapeutic approach might be required in NASH. The great number of patients suffering from the disease, of course, have individual genetic background and individually suffer from different co-morbidities. Accordingly, several of the above discussed trials have identified specific populations of responders and non-responders that must be considered in future drug development for NASH. Then, not only elafibranor but also other PPAR targeting drugs might reveal great therapeutic efficacy in NASH treatment.

8. Abbreviations Used

8-oxoG: 8-oxoguanine ABCA1: ATP-binding cassette transporter ACC: acetyl-CoA carboxylase ACO: acyl-CoA oxidase ACS: acyl-CoA synthetase ACSL: long-chain-fatty-acid-CoA ligase ACTH: adrenocorticotropic hormone ADRP: adipose differentiation-related protein AF2: activation function 2 ALP: alkaline phosphatase ALT: alanine aminotransferase ANGPTL4: angiopoietin-like protein 4 aP2: adipocyte Protein 2 APC: adenomatous polyposis coli APO: apolipoprotein AQP7: aquaporin 7 ASK1: apoptosis signal-regulating kinase 1 aSMA: alpha smooth muscle actin AST: aspartate transaminase BDL: bile duct ligation C/EBP: CCAAT-enhancer-binding proteins C2C12: mouse myoblast cell line CCL: chemokine ligand CCl₄: carbon tetrachloride CCR: chemokine receptors CD: cluster of differentiation CD95: apoptosis antigen 1 CDAA: choline-deficient L-amino acid-defined CdK-5: cyclin-dependent kinase 5 ChREBP: carbohydrate response element binding protein COX-2: cyclooxygenase-2 CPT-1: carnitine palmitoyl transferase-1 CREB: cAMP response element-binding protein CRP: C-reactive protein CYP: cytochrome P450 enzyme CYP7A1: cholesterol 7α hydroxylase DBD: DNA-binding domain DNL: de novo lipogenesis DR-1: direct repeat 1 DRIP205: vitamin D-interacting protein 205 F4/80: EGF-like module-containing mucin-like hormone receptor-like 1 FABP: fatty acid binding protein FACS: fatty-acyl-CoA synthase FAS: fatty acid synthase FAT/CD36: fatty acid translocase FATP: fatty acid transport protein

FED: fructose-enriched diet FOXO1: forkhead box protein O1 FXR: farnesoid X receptor G6Pase: glucose-6-phosphatase GGT: γ-glutamyl transpeptidase GLUT: glucose transporter GRIP1: glutamate receptor interacting protein 1 GST-P: glutathione S-transferase HbA_{1c}: haemoglobin A1c HCC: hepatocellular carcinoma HCD: high-caloric and high-cholesterol diet HDL: high-density lipoprotein HOMA: Homeostatic Model Assessment HSC: hepatic stellate cell IL: interleukin IRE1 α : inositol-requiring enzyme 1 α IκB: Inhibitor of κB JNK: c-Jun N-terminal kinase LBD: ligand-binding domain LCFA: long-chain fatty acid LC-FACS: long-chain fatty acid acetyl-coenzyme A synthase LDL: low-density lipoprotein L-PK: L-type pyruvate kinase LPL: lipoprotein lipase LTB4: leukotriene B4 LXR: liver X receptor MCD: methionine and choline deficient diet MCP: monocyte chemoattractant protein MMP: matrix metalloproteinase MRI: magnetic resonance imaging NAFLD: non-alcoholic fatty liver disease NAS: NAFLD fibrosis activity score NASH: non-alcoholic steatohepatitis NCoR: nuclear corepressor NF-κB: nuclear factor-κB NIDDM: non insulin dependent diabetes mellitus Ogg1: 8-Oxoguanine glycosylase OLETF: Otsuka Long Evans Tokushima Fatty OLR1: oxidized LDL receptor 1 oxLDL: oxidatively modified low density lipoprotein PBP: PPAR binding protein PDK4: pyruvate dehydrogenase kinase 4 PECAM-1: platelet endothelial cell adhesion molecule PEPCK: phosphoenolpyruvate carboxykinase PGC-1α: PPARγ coactivator 1-alpha

PPAR: peroxisome proliferator-activator receptor PPRE: PPAR response element RAR: retinoic acid receptor ROS: reactive oxygen species RXR: retinoid X receptor SCD1: stearoyl-CoA desaturase 1 SMRT: silencing mediator of retinoid and thyroid hormone receptors SOCS-3: suppressor of cytokine signaling 3 Sod: superoxide dismutase SPPARM: selective PPARy modulators SRC-1: steroid receptor coactivator-1 SRE1: sterol regulatory element-1 SREBP-1c: sterol regulatory element-binding protein 1c TBARS: thiobarbituric acid-reactive substance TGF- β 1: transforming growth factor beta 1 THR: thyroid hormone receptor TIF2: transcriptional mediators/intermediary factor 2 TIMP: tissue inhibitor of metalloproteinase TNF- α : tumor necrosis factor- α Twist-1: Twist-related protein 1 TZD: thiazolidinedione UCP: uncoupling protein VCAM-1: vascular cell adhesion molecule-1 VEGF-A: vascular endothelial growth factor A VLDL: very low density lipoprotein XBP1: X-box binding protein 1

Competing Interests

The authors declare no competing interests.

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