Chapter

Regulation of Iron Metabolism in NAFLD/NASH

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Abstract

The disturbance of iron metabolism is one of the characteristic features of NAFLD/NASH, and complicated Type2DM, however, as for the mechanisms of the iron deposition observed in the liver of NAFLD/NASH, as well as the correlation between iron metabolism and insulin resistance, the precise pathophysiology and dynamics are still uncertain. In addition, numerous factors might be involved in the pathogenesis of NAFLD/NASH and wide-ranged analysis, as well as multi-targeted treatment, should be considered and challenged for the improvement of the prognosis of NAFLD/NASH. In many NAFLD/NASH cases, a remarkable decline of serum ferritin, as well as the improvement of T2DM, were observed after treatment with Laennec (placenta-derived drug) in accordance with the improvement of the liver dysfunction and histopathological amelioration in the liver. In recent years, it was shown that hepcidin, the principal regulator of iron metabolism exists in human placenta in high concentrations. Then, we examined whether Laennec can restore the pathological background by regulating iron and glucose metabolism in NAFLD/NASH by the action of a "hepcidin inducer".

Keywords: non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), iron metabolism, hepcidin, placenta extract

1. Introduction

Non-alcoholic fatty liver disease (NAFLD), regarded as the 'hepatic manifestation of the metabolic syndrome', is now estimated to affect one billion individuals worldwide [1]. The definition of NAFLD is very simple such as the presence of at least 5% hepatic steatosis but excluding secondary hepatic fat accumulation of general causes, such as congenital hepatic disorders, chronic viral hepatitis, autoimmune hepatitis, excessive alcohol consumption, or long-term use of steatosis-inducing medications [2].

NASH is characterised by the development of histopathological changes in the liver that are nearly identical to those induced by excessive alcohol intake, but in the absence of alcohol abuse, the presence of macrovesicular steatosis and mixed inflammatory infiltrate associated with focal hepatocyte ballooning degeneration and varying amounts of Mallory's hyaline and glycogenated nuclei. However, liver biopsy still remains the "gold standard" for making a definitive diagnosis.

NASH is capable of progressing to cirrhosis and liver failure at the end of clinical course [3, 4]. Actually, NASH is forecasted to become the principal cause of advanced

liver disease in developed countries [5] and the high-ranking indication for liver transplantation [4]. NAFLD has also been gradually acknowledged as an independent risk factor for the development of cardiovascular disease, type 2 diabetes mellitus (T2DM) and hepatocellular carcinoma [6, 7]. The factors that predispose patients to the development of steatohepatitis and fibrosis in NAFLD are not well elucidated, and effective treatment strategies are still lacking [8].

Day and James [9] initially proposed a 'two-hit' model to explain the progression of NAFLD. The 'first hit' constitutes the deposition of triglycerides in the cytoplasm of the hepatocytes. The disease does not progress unless additional cellular events occur (the 'second hit'), which can include oxidative stress, especially that arises from inflammatory cytokines, mitochondrial stress and insulin resistance. Autophagy may also play a notable role in the pathogenesis of NASH.

A new prospect explaining the pathogenesis of NASH was reported by Tilg and Moschen, called the 'multi-parallel hit' hypothesis recently [10]. This hypothesis, based on reports that cytokine-mediated stress and endoplasmic reticulum stress can induce steatosis as well as necroinflammation, suggests that multiple hits take a step together in the development of NASH. The progression of steatosis should, therefore, be regarded as a part of the liver's early 'adaptive' and 'purposive' response to some types of stress rather than as the first hit in the disease development. The close correlation between insulin resistance and iron level has been speculated and examined by many researchers. Even if secondary iron accumulation increases insulin resistance or vice versa, it remains still unclear. Oxidative stress may be the elusive 'second' hit of possibly multiple steps in the progression of steatosis to fibrosing steatohepatitis [11]. This type of response might be originated from and been modified by the activation of hepatic stellate cells (HSCs) [12].

2. Relationship between NASH/NAFLD and iron metabolism

Iron is among the essential trace elements for the existence of all living organisms. It is required for numerous metabolic routines, including energy production, DNA synthesis, oxygen transport and innate immunity, also in the expression of other enzymes involved in the oxidation or reduction of biological substrates and in the activation of iron-containing enzymes, such as the cytochrome system in the mitochondria [13]. Because there is no known physiological mechanism for appropriately and efficiently eliminating the 'too much incorporated' iron, even in severe iron-overloaded conditions, a crucial element in maintaining systemic iron homeostasis is effective and harmonised correspondence among cells that use iron (mainly erythroid precursors), absorb iron from the diet (duodenal enterocytes) and store iron (hepatocytes and tissue macrophages). Therefore, when the iron intake exceeds the cellular provisions and storage capabilities are overflowed, iron toxicity due to overloading may easily develop [14]. Thus, iron balance is maintained through steady and urbane regulatory mechanisms.

Recent studies have established the importance of hepcidin in iron homeostasis as a negative regulator of iron release into the bloodstream by duodenal enterocytes and reticuloendothelial macrophages [15]. Excess iron in the liver promotes steatohepatitis, liver fibrosis, cirrhosis and hepatocellular carcinoma [16]. The discovery of hepcidin and the elucidation of its role in iron metabolism made it possible to develop novel therapies for hemochromatosis, anaemia of inflammation and other iron-related disorders such as NAFLD/NASH [17]. Hepcidin has a distinct and essential

role in controlling the dietary absorption of iron, its storage and its release into the bloodstream. Hepcidin concentrations are strictly controlled, and their pathologic dysregulation leads to numerous human iron-related disorders such as NAFLD/NASH. Our understanding of hepcidin regulation has rapidly increased; however, numerous questions related to hepcidin pathobiology still need to be clarified and addressed [18].

Iron is stringently and elegantly regulated by a mechanism similar to that for glucose control [19]. Like glucose and insulin, the serum iron levels are regulated by a hepatic peptide hormone, hepcidin. Elevated iron levels arouse hepcidin synthesis, which decreases the levels of the iron-exporter ferroportin in macrophages and intestinal cells and reduces serum iron levels, similar to how insulin controls excessive glucose levels [19, 20].

The spectrum of NAFLD ranges from simple steatosis to NASH [21]. Iron is regarded as a putative element that interacts with oxygen radicals, and NASH is associated with high rates of hyperferritinemia together with increased hepatic iron stores [22]. The role of hepatic iron in the progression and pathogenesis of NASH remains unclear and controversial.

It stands to reason that iron is one of the most copious metals on the earth with the potential of high toxicity against living cells. Highly active cells need iron for maintaining their metabolic activity because iron allows optimal and preferable electron transfer, assisting biochemical reactions between different atoms and molecules. The toxicity of iron originated from induction of reactive oxygen species (ROS), which at high levels leads to cellular damage [23, 24]. Progress in understanding the involvement of hepcidin in normal physiology and disease conditions [25], coupled with advances in quantification, make it an increasingly attractive candidate biomarker for assessing iron status and guiding iron intervention strategies [26]. Evidence suggests that a modest degree of iron overload is associated with more advanced liver injury in NAFLD, although the mechanisms by which this might occur remain unclear and vague [27, 28].

Recently, however, it has become increasingly evident that iron in the adipose tissue plays an important role in the pathogenesis of insulin resistance and, therefore, possibly NAFLD [29, 30].

Excessive iron is also a potent cause of cellular injury from oxidative stress due to the generation of reactive oxygen species by the Fenton reaction [31]. Under usual conditions, intracellular protection from iron-induced oxidative stress is facilitated by the sequestration of iron within ferritin [32]. Dysfunctional adipose tissue produces adipokines that promote the development of insulin resistance [29]. The liver, skeletal muscle and adipose tissue are the key sites of insulin action and resistance [33]. In the adipose tissue itself, insulin resistance potentiates lipolysis of triglycerides by the hormone-sensitive lipase [34]. This generates the most free fatty acid (FFA) flux in the liver in NAFLD [35]. Insulin resistance in skeletal muscle as a result leads to reduced uptake of glucose, on the other hand in the liver, insulin resistance enhances gluconeogenesis [36]. Iron and NAFLD-resultant compensatory hyperinsulinemia and relative hyperglycaemia promote hepatic de novo lipogenesis and cholesterol synthesis and reduced catabolism of FFA by oxidation [37]. Oxidative stress is considered an important contributor to the pathogenesis of NASH [38]. Excess hepatic iron can promote oxidative stress via Fenton's reaction and is proposed to be a cofactor in the development of NASH.

The regulatory mechanisms of hepcidin have been investigated in animal models, and only a few studies have investigated the role of hepcidin in human NAFLD patients [39]. Hepcidin is an important regulator of liver inflammation [19], and along with its key role in iron homeostasis, it could play a vital part in NASH

pathogenesis. It was hypothesised that hepcidin and/or its upstream regulatory factors play a key role in the progression of NAFL to NASH [40]. The elevated hepcidin in NASH seems to be either a reflection of hepatocellular inflammation or simply indicating the induced hepcidin in the early stage of NASH. Hepcidin expression actually appears to be directly enhanced by insulin and down-regulated under insulin resistance, suggesting a possible mechanism for iron loading as an early event in the pathogenesis of NAFLD and T2DM. These findings have raised numerous questions and have stimulated exciting clinical research. With that in mind, it is difficult to predict what additional surprises will emerge from the ongoing study of this fascinating viewpoint.

The elevated ferritin and low expression of hepatic inflammatory cytokines (IL-6; 8-fold, NFNB; 5-fold and IL-1E; 4-fold) in patients with NAFLD with hepatic iron deposition could probably be suggestive of the notion that in this cohort, increased hepcidin expression is more likely attributable to hepatic iron deposition rather than inflammation.

Hepatic HAMP gene expression is induced in patients with NASH compared to that in patients with NAFLD, and presumably, in response to excess hepatic iron in NAFLD patients with iron overload. Two possible mechanisms for hepcidin expression in patients with NASH are likely IL-6-mediated stimulation of JAK2/STAT3 pathway, which results in upregulation of HIF1D. Furthermore, increased hepatic *STAT3* gene expression in NASH patients relative to that in NAFLD patients lends support to this putative hypothesis.

The presence of iron deposition in livers of patients with NAFLD can be classified as hepatocellular, reticuloendothelial or both. A study of 849 adult biopsy specimens performed in the United States showed that reticuloendothelial patterns of iron deposition were associated with advanced fibrosis compared with hepatocellular iron patterns. Biopsy specimens with reticuloendothelial iron were also more likely to have definite steatohepatitis [41]. However, an Italian study on 587 patients with NAFLD found that hepatocellular rather than reticuloendothelial iron was associated with an increased likelihood of liver fibrosis [42].

The reason for the discordant results might be explained by the differences in the patient populations; the subjects in the US study were more ethnically diverse and had higher body mass indices and more advanced fibrosis than those in the Italian study. Interactions between iron metabolism and NAFLD are complex and complicated under active investigation by various researchers. In conclusion, they observed that HAMP expression is elevated in NASH patients and in NAFLD patients with hepatic iron deposits. Their data allowed them to study the interdependence of various regulatory signals such as hepatic iron stores, inflammation and hypoxia or oxidative/ER stress on the expression of hepcidin and inflammatory cytokines. Increased hepcidin expression, which attempts to sequester excess iron, thereby reducing oxidative stress, maybe a protective response.

Bekri et al. showed that hepcidin levels are increased in the adipose tissue of severely obese patients compared with those in the liver, suggesting that severe obesity itself causes hypoferremia due to the overproduction of hepcidin in the adipocytes [43].

Asian Indians are neither associated with iron overload nor with *HFE* gene mutations [44].

The authors suggested that hyperferritinemia in NASH is a non-specific effect of hepatic necroinflammation, reflecting its function as an acute phase protein as a result. Serum ferritin is known to increase because of released from damaged hepatocytes. The authors also previously concluded that serum ferritin levels reflect

oxidative stress as well as hepatic iron concentrations and hepatocyte damage in chronic liver disease [45]. Elevated serum ferritin in NASH may be caused by iron-unrelated oxidative stress, such as that derived from FFA, lipid peroxide, cytokines and induction of cytochrome P450 enzymes (CYP2E1 and CYP4AC-) [46]. Thus, the role of hepatic iron in the pathogenesis of NASH or abnormal iron indices in NASH remains debatable and unsettled.

Under usual conditions, intracellular protection from iron-induced oxidative stress is facilitated by sequestration of iron within ferritin [47]. Pathologic states of iron overload often led to saturation of the serum iron transporter, transferrin, which increases the serum levels of toxic non-transferrin bound iron (NTBI). NTBI is readily absorbed by tissues such as the liver and cardiac muscle [48]. The association between hyperferritinemia, insulin resistance and T2DM is compelling. The odds ratios for developing diabetes in those with elevated serum ferritin levels were high at 3.61 for women and 4.94 for men [49–51]. The connotation between hyperferritinemia and histologic markers of liver injury in NAFLD is reasonably firm. In 2004, Bugianesi et al. [43] showed that serum ferritin concentration is not associated with hepatic iron concentration in NAFLD, but is a marker of severe histologic damage.

In an earlier study by Chitturi et al. [44] of 93 patients with NASH, 33% of whom had advanced fibrosis, the authors found that serum ferritin concentration was not an independent predictor of advanced fibrosis. This implies that the ferritin association with NAFLD is not simply a result of NAFLD itself causing hyperferritinemia. Moreover, the results suggest that the link between hyperferritinemia and NAFLD could be explained by insulin resistance. It also appears that it has a direct role in the activation of hepatic macrophages and HSCs. In humans with NAFLD, reticuloendothelial iron has been shown to be associated with apoptosis, indicated by increased serum cytokeratin-18 (CK-18) fragments and increased hepatic TUNEL staining of liver sections [52]. Iron may also contribute to liver injury in NAFLD by generating endoplasmic reticulum stress [53]. Additionally, hepatic iron loading in mice upregulates cholesterol biosynthesis pathways; this has been proposed as an additional mechanism of iron-induced liver injury in NASH [54].

3. Deteriorative effects of iron against NASH/NAFLD

Iron overload is one of the important risk factors for diabetes. The relationship between iron and diabetes was first recognised in pathologic conditions – namely hereditary hemochromatosis and thalassemia – but high levels of dietary iron also enhance the risk of diabetes. It is generally recognised that iron plays a direct and causal role in diabetes pathogenesis, mediated both by β-cell failure and insulin resistance.

Iron is capable of generating hydroxyl radicals from peroxide and can also inhibit antioxidant defences such as SOD2 [55]. Highly elevated iron levels have been linked to oxidative damage to DNA, lipids and proteins that, in turn, have been implicated in cardiovascular disease, diabetes, atherosclerosis and neurological degeneration, as seen in Alzheimer's disease [56].

Iron homeostatic pathways are tightly associated with inflammatory stressors. Inflammation causes significant upregulation of hepcidin, largely through interleukin-6 (IL-6), and results in large increases in serum ferritin levels.

There is a greater prevalence of iron deficiency in obese (39%) and overweight (12%) children and adolescents than in normal-weight children, the prevalence of

iron deficiency in whom is only 4% [28]. The association of iron deficiency with obesity has been confirmed in other populations, which include children and adults of both sexes [57]. The conceivable cases for causality, in turn, can be made in both directions: normal or high iron stores might be required to support higher rates of fatty acid oxidation so that iron-deficient individuals are less able to mobilise and use high fat, or, conversely, the inflammatory nature of obesity might trigger increased hepcidin levels, which limit the absorption of dietary iron.

In the progression of diabetes, ROS can cause both β-cell failure and insulin resistance. β-cells are particularly sensitive to ROS because of low expression of antioxidants such as catalase and SOD2, overexpression of which has been associated with increased β-cell viability [58]. ROS can cause β-cell dysfunction by multiple mechanisms including decreased insulin gene expression secondary to decreased expression of transcription factors necessary for β-cell differentiation, maintenance and insulin gene transcription.

ROS have also been reported to directly affect circulating human insulin by hydroxylation of phenylalanine residues that result in lower affinity binding to the insulin receptor [59]. Finally, ROS can induce insulin resistance through multiple mechanisms; for example, through the activation of FOXO1, which prevents downregulation of gluconeogenesis even in the presence of insulin signalling [60]. Hypoxia-inducible factors 1 and 2 (HIF-1 and HIF-2) regulate cellular responses against low oxygen by upregulating transcription of a diverse set of proteins involved in angiogenesis, erythropoiesis and glycolytic flux [61]. HIFs also regulate iron metabolism, and under conditions of low iron levels, HIF-2 upregulates DMT-1 and DCYTB, whereas HIF-1 upregulates DMT-1 and decreases ferritin [62]. Conversely, cellular iron levels regulate HIF protein levels through the control of prolyl hydroxylase (PHD) activity [63].

Emerging data demonstrate that iron plays an important role in metabolic regulation and the pathophysiology of diabetes. Iron overload is common in T2DM [64, 65]. On the contrary, iron depletion seems to be protective for the development of diabetes. Rats with iron-deficiency anaemia are more insulin sensitive than controls [66], and phlebotomy improves the insulin sensitivity and glycemia, both in nondiabetic subjects [67] and T2DM subjects with high ferritin [68]. These studies suggest that iron plays an important role both in the development and improvement of diabetes. However, the precise molecular mechanisms of iron-associated diabetes are not well understood at present [69].

4. Ameliorating effects of 'hepcidin inducer Laennec and Porcine' for the progression of NASH/NAFLD

NASH is a severe form of fatty liver disease that is defined by the presence of inflammation and fibrosis, ultimately leading to cirrhosis and hepatocellular carcinoma. Shindo et al. [62] evaluated the effect of human placenta extract (HPE) and Laennec treatment in a mouse model of NASH. In the methionine- and choline-deficient (MCD) diet-induced liver injury model, fibrosis started in the regions around the sinusoids.

They dispensed the MCD diet with high-salt loading (add 8% NaCl in the drinking water) to mice deficient in the vasoprotective molecule RAMP2 for 5 weeks, with or without HPE. In both the HPE and control groups, fibrosis was observed in regions adjacent to the sinusoids, but fibrosis was not so pronounced in the HPE-treated mice. Levels of TNF-α and MMP9 expression were also significantly reduced in

HPE-treated mice, and oxidative stress was suppressed in the perivascular region. These observations indicate that HPE ameliorates NASH-associated pathologies by suppressing inflammation, oxidative stress and liver fibrosis.

HPE has been prescribed clinically to treat chronic hepatitis, liver cirrhosis and other hepatic diseases for more than 40 years in Japan. In an experimental animal model of hepatitis, HPE reportedly ameliorated hepatic injury through liver regeneration and inhibition of inflammatory reactions and hepatocyte apoptosis [70, 71]. Moreover, Shimokobe et al. recently reported that HPE is effective in NASH patients who were unresponsive to lifestyle intervention [72].

As for histopathological changes in the liver, silver staining histological sample revealed fibrotic areas adjacent to the sinusoids in both groups; however, the fibrosis was not so severe in HPE-treated mice. By using immunofluorescent staining, the authors observed high expression levels of p67 phox, a cytosolic component of NADPH oxidase, in the perivascular regions of all mice; however, the expression levels were less marked in HPE-treated mice. Levels of 4HNE, a lipid peroxidation product, were also decreased in HPE-treated mice. Judging from these observations, it is indicated that HPE treatment for ameliorated liver injury is possible by reducing inflammation, oxidative stress and fibrosis.

In an earlier study, HPE was shown to suppress inflammation in a chronic arthritis rat model using complete Freund's adjuvant [73]. Direct effects of HPE on the production of pro-inflammatory cytokines and mediators have also been reported. For example, HPE reportedly inhibits the production of nitric oxide, TNF-α and cyclooxygenase-2 in lipopolysaccharide-stimulated RAW264.7 macrophages [74]. In the present study, the authors found that HPE significantly suppressed TNF-α expression in an MCD fed-diet model. This suggests that HPE may suppress the progression of chronic inflammation initiated by lipid accumulation within hepatocytes of the NASH patients.

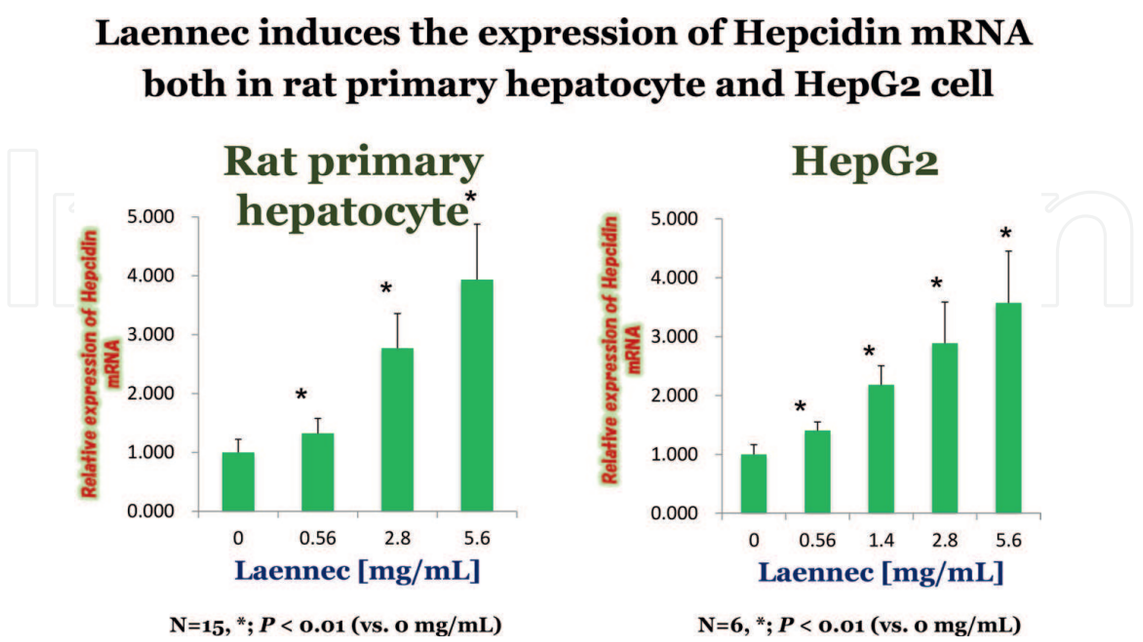

Figure 1.
The most important basic data is expressed. Laennec induces the expression of hepcidin mRNA both in rat primary hepatocyte and HepG2 cells in a dose dependent manner; this means that in the human body, the possibility of hepcidin mediated action of Laennec might be the main route or at least one of the important mechanism of its efficacy in regulating iron metabolism.

Besides inflammation, oxidative stress seems to also contribute to chronic liver injury. In that regard, HPE showed both anti-oxidative and anti-inflammatory activities in rats exposed to benzopyrene (BaP) [75]. Application of H_2O_2 to cultured cells is performed to evaluate the cellular damage caused by oxidative stress. It is also observed that serum hepcidin levels are typically elevated in individuals with NASH [76]. As this in itself fails to explain iron loading in NASH, one might consider that dysregulated iron metabolism occurs in NASH independently of hepcidin.

The contribution of adipose tissue-derived hepcidin to the serum hepcidin pool is uncertain, however, this is another potential factor that may explain the increased serum hepcidin levels in NASH. Further complexity in these relationships arises when one considers that iron deficiency has been shown to be associated with obesity, and in women with obesity and NAFLD [77, 78]. Together, these findings suggest that the interaction between iron and lipid metabolism is multi-faceted. It seems that 'just enough' but 'not too much' iron may be critical for preventing dysfunctional lipid metabolism.

Previous studies [73–75] have revealed that the administration of Laennec significantly improved T2DM complicated with NASH and other chronic liver diseases, suggesting the importance of iron regulation on insulin-resistant T2DM showing hyperferritinemia.

Thus, more experimental and clinical studies are required to confirm or refute the claim that hepcidin has a role in T2DM. To shed light on the factors that alter hepcidin expression, the authors performed experiments with HepG2 and HuH7, human hepatoma cell lines that are widely used for this purpose. Despite the considerable advances made recently, further explorations are required to investigate the cellular mechanisms and functions of peripheral hepcidin, as well as its regulation in different organs (**Figures 1** and 2).

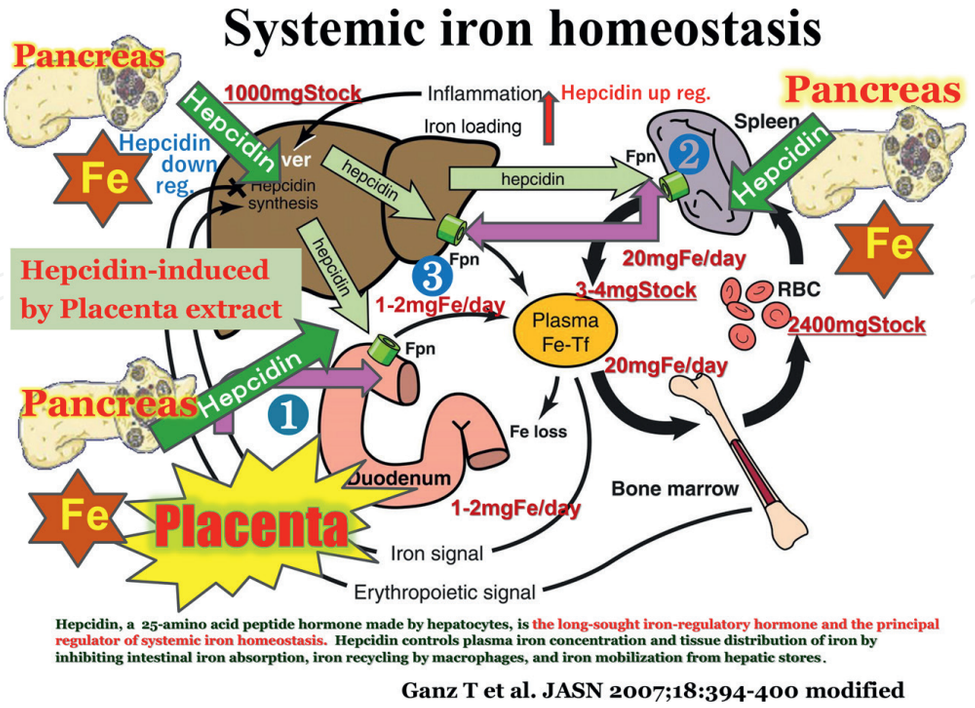

Figure 2.
Systemic iron homeostasis is regulated by hepcidin. Judging from the clinical data of Laennec, formerly obtained through the treatment of chronic liver diseases such as NASH and chronic hepatitis type C, the sites of action of Laennec resemble those of hepcidin. Hepcidin is also expressed in the pancreas of rats and humans, which means that pancreas is an iron-regulating organ beyond their proper glucose–regulatory function. According to basic data, the regulation of hepcidin expression is similar in the liver and in the endocrine pancreas.

5. Placenta-derived drugs Laennec (i.v) and Porcine (oral) are capable of improving T2DM complicating with NASH/NAFLD through the action of 'hepcidin inducer'

The discovery of hepcidin and its role in iron metabolism could lead to novel therapies for hereditary hemochromatosis and other iron-loading diseases. Laennec (parenteral) and Porcine (oral), which are hepcidin inducers, actually improved iron overload in a hereditary hemochromatosis patient, without performing sequential phlebotomy. This suggests the possibility of not only improving the prognosis of hereditary hemochromatosis but also ameliorating complications, such as type 2 diabetes, liver fibrosis and hypogonadism. Laennec and Porcine can completely replace continuous venesection in patients with hereditary hemochromatosis and may improve other iron-overloading disorders caused by hepcidin deficiency and/or insufficiency [73, 75] (**Figures 1–4**).

The association with hepcidin is supposed in many kinds of human diseases, furthermore, most of these diseases are influenced by alterations in hepcidin concentrations [73, 76]. Hepcidin-targeted therapies may improve the manifestations and biochemical abnormalities of patients with iron disorders. Although no specific hepcidin therapies are currently available, several compounds are under development as hepcidin agonists or antagonists [77]. Moreover, hepcidin either has a primary or a secondary role in insulin resistance, which is a characteristic of T2DM. However, it remains inconclusive whether serum hepcidin levels are an independent risk factor in the etiopathogenesis of T2DM. By inducing preferable and appropriate amounts of hepcidin, placenta-derived drugs could improve the clinical course of NASH complicated with T2DM and hyperferritinemia by attenuating

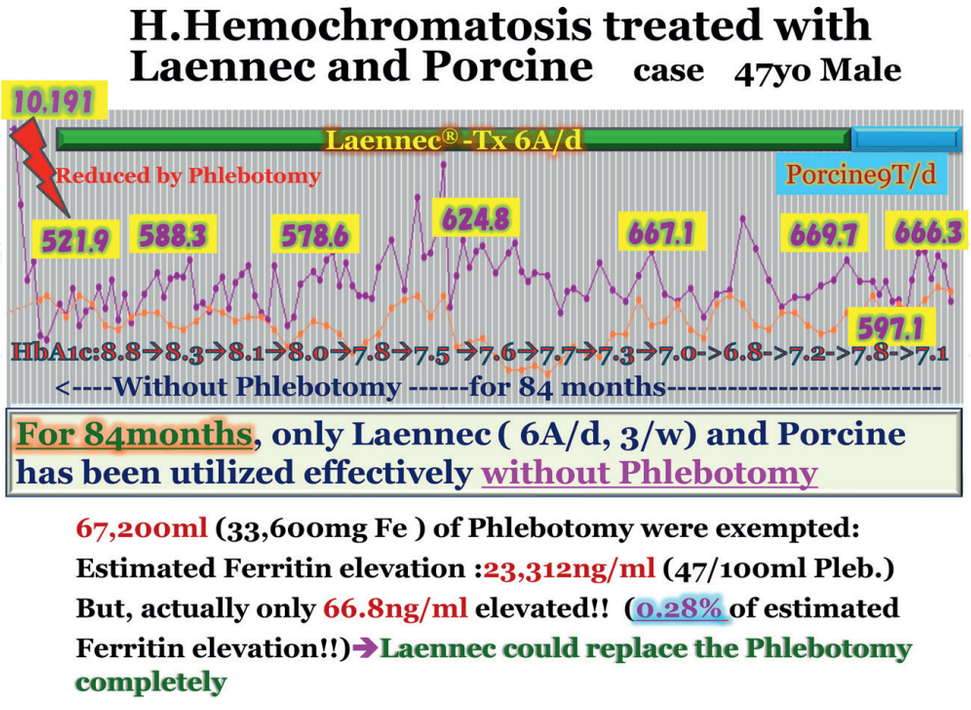

Figure 3.
H. hemochromatosis treated with Laennec and porcine. Case, 47-year-old male. For 74 months, only Laennec (6A/d, 3/w) and porcine have been utilised effectively without phlebotomy. 67,200 ml (33,600 mg Fe) of phlebotomy were exempted: Estimated ferritin elevation 23,312 ng/ml (47/100 ml phlebotomy). Actually only 66.8 ng/ml elevated (0.28% of estimated ferritin elevation).

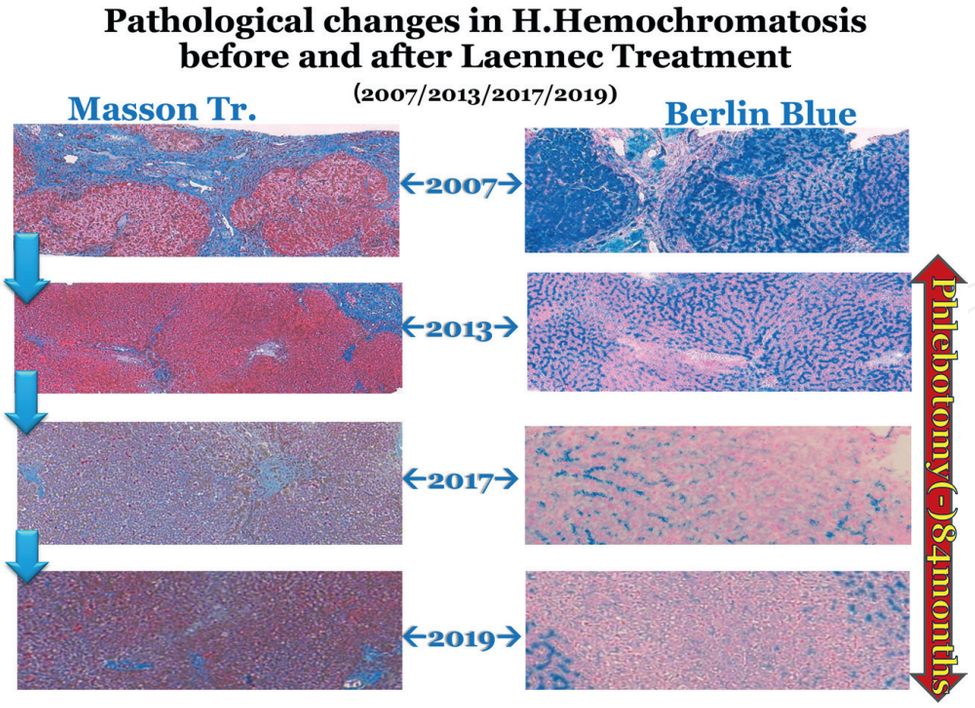

Figure 4.
After treatment with Laennec without phlebotomy for 84 months, the histological evaluation revealed a remarkable reduction in iron deposition and fibrosis. At the same time, remarkable improvements in the quality of life (QOL) and erectile dysfunction (ED) were observed. During these periods, oral administration with porcine was replaced with Laennec for 8 months; however, the efficacy remained the same.

the iron-induced oxidative stress and iron accumulation in both hepatocytes and pancreatic β-cells [78].

It is generally confirmed that iron overload causes insulin deficiency by promoting pancreatic β-cell apoptosis. Because of their stringent dependence on mitochondrial glucose metabolism and their limited antioxidant capacity [79], β-cells are extremely susceptible to oxidative stress. Through their divalent metal transporter, pancreatic β-cells can avariciously take up non-Tf-bound iron [80], which can promote oxidative stress by catalysing the Fenton reaction. Elevated iron levels oxidise various biomolecules, such as nucleic acids, proteins, and lipids, which may contribute to the development of T2DM by decreasing the insulin secretion from pancreatic β-cells, with a concomitant increase in insulin resistance [14, 76].

In our clinical data: Laennec has been administered [73] (**Figures 3** and **4**) to a patient with hereditary hemochromatosis without phlebotomy. HbA1c levels have further improved by Laennec treatment (more than 2% declined) for 84 months without changing the medications for diabetes treatment. These results are probably due to the additive efficacy of Laennec in reducing iron-originated ROS, enhancing the anti-inflammatory action with concomitant improvement in liver fibrosis, and diminishing the iron deposition in hepatocytes. Laennec was also administered to patients with NASH with T2DM (**Figures 5–8**); treatment with Laennec significantly improved the T2DM, reduced the serum ferritin level, and decreased the iron deposition in the hepatocytes [73, 74]. The regulation of iron and glucose metabolism is possibly due to the pancreatic β-cells' ability to co-release insulin and hepcidin.

The data published by Kulaksiz et al. [81] demonstrated that hepcidin is expressed in the pancreas of rats and humans. Further analysis showed that it was localised in the β-cells of the islets of Langerhans. In addition, in vitro experiments performed

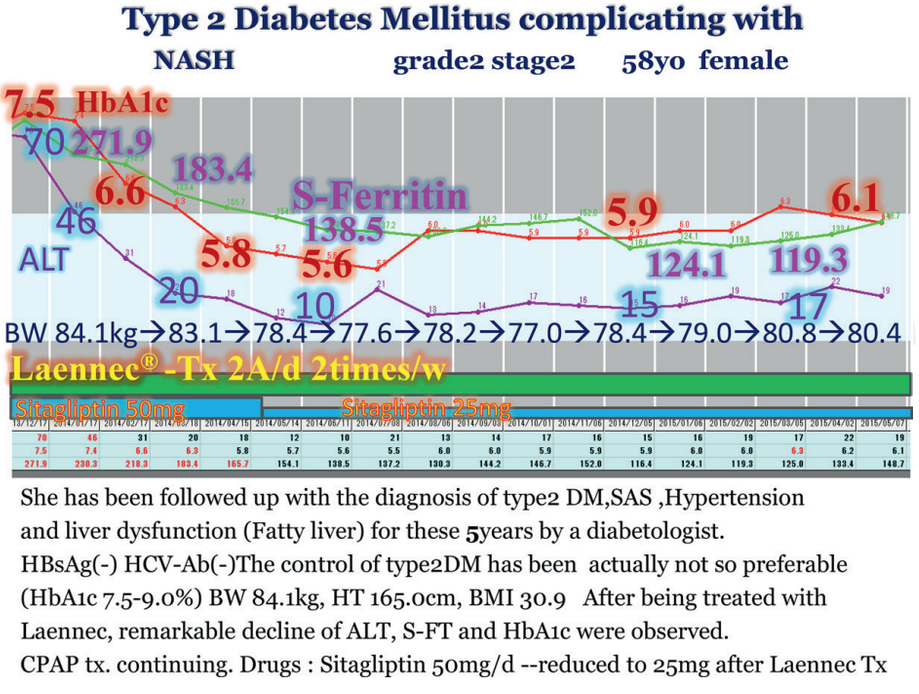

Figure 5.
Type 2 diabetes mellitus complicating with NASH grade 2, stage 2, 58 years old female. H.P.I: she has been followed up with the diagnosis of type 2 DM, SAS, hypertension and liver dysfunction (fatty liver) for these 5 years by a diabetologist. HBsAg (–) HCV (–) the control type 2 DM has been actually not so preferable (HbA1c 7.5–9.0%) BW 84.1 kg, HT 84.1 kg, HT 165.0 cm, BMI 30.9. As for SAS: CPAP tx continuing drugs for DM: sitagliptin 50 mg/d reduced to 25 mg after Laennec Tx.

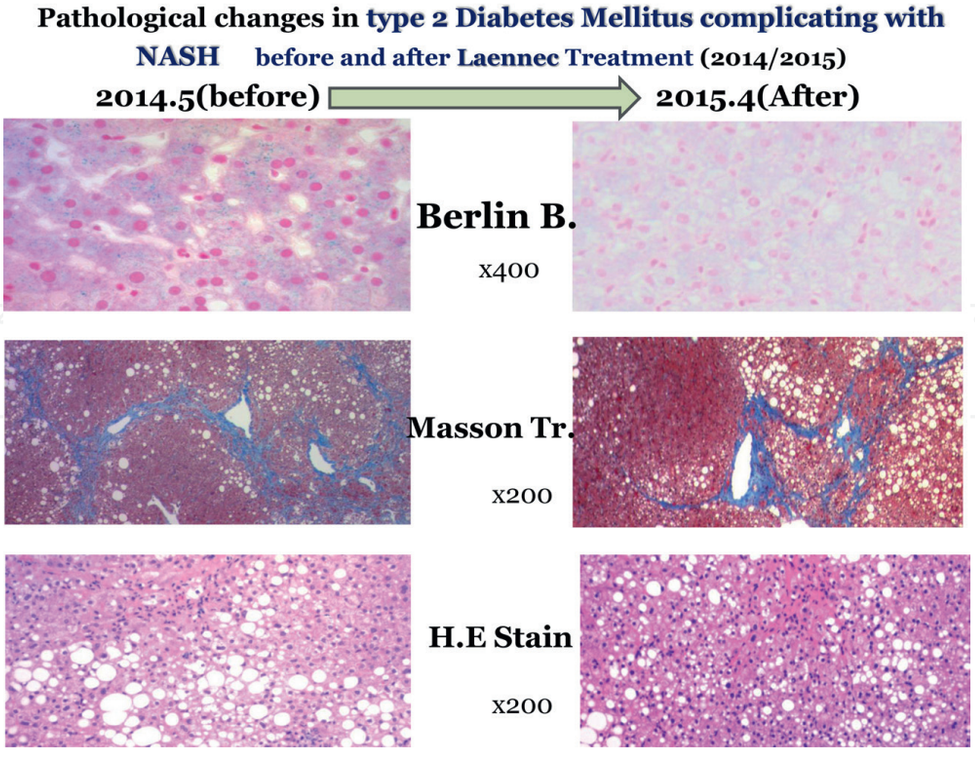

Figure 6.
The histological evaluation revealed the presence of fatty metamorphosis, inflammation, pericellular fibrosis, ballooning of hepatocyte, Mallory body and iron deposition mainly in the hepatocyte. After treating with Laennec, the histological evaluation revealed remarkable improvement in iron deposition and fibrosis, as well as amelioration of inflammation and fatty metamorphosis.

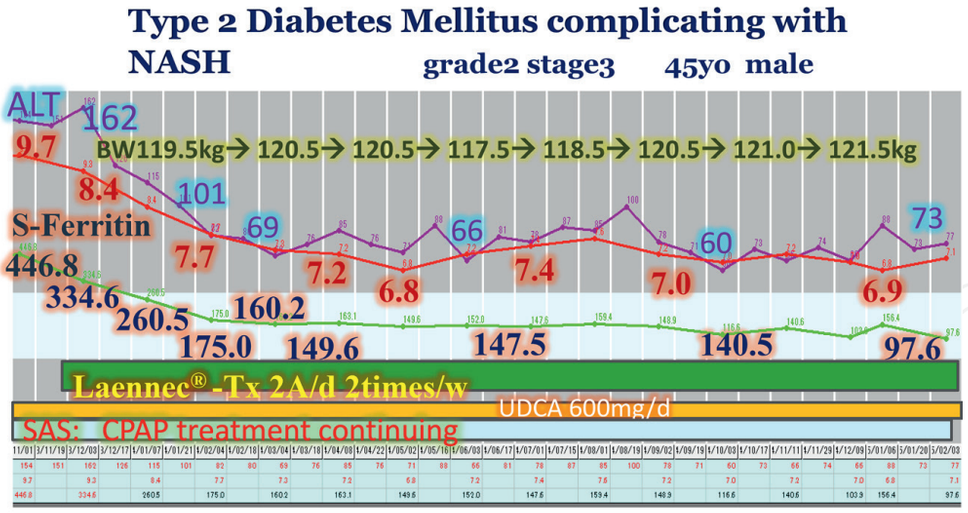

Figure 7.
Type 2 diabetes mellitus complicating with NASH grade 2, stage 3, 45 years old male. H.P.I: he has been followed up with the diagnosis of type 2 DM, SAS and liver dysfunction (fatty liver) for these 8 years by a diabetologist. The control of type 2 DM has been actually poor (HbA1c 9–10%) HBsAg (−) HCV-ab (−) BW 119.5 kg, HT 176.6 cm, BMI 38.3. As for SAS: CPAP tx continuing drugs for DM: [1] metformin 2250 mg/d [2] sitagliptin 100 mg/d [3] gliclazide 40 mg/d.

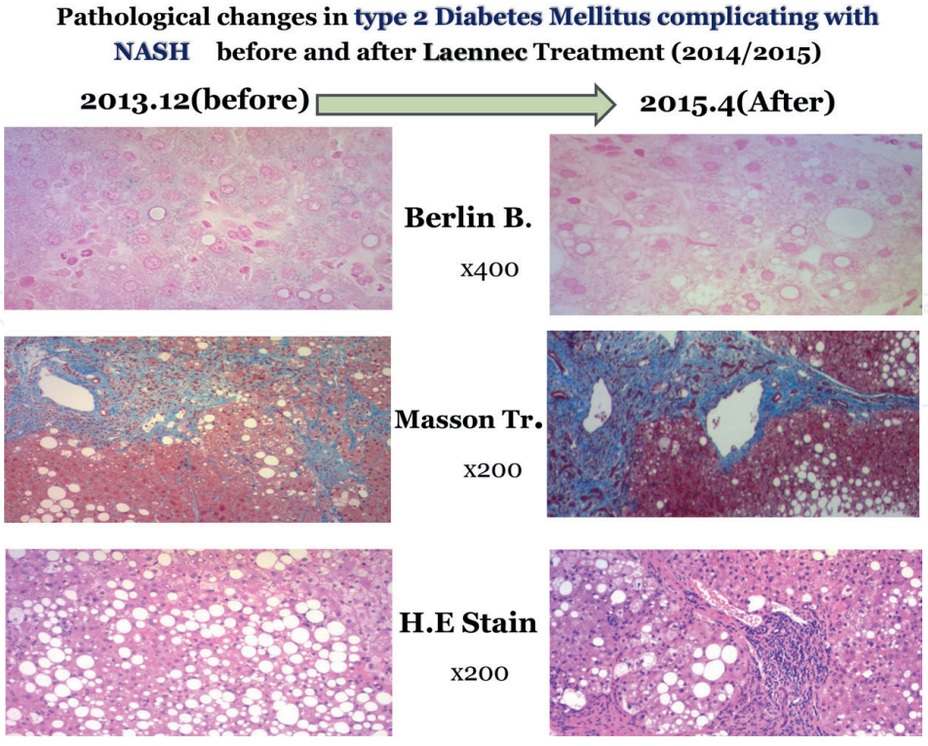

Figure 8.
Histological evaluation revealed the presence of fatty metamorphosis, inflammation, pericellular fibrosis and iron deposition in the hepatocyte. After treating with Laennec, the histological evaluation revealed remarkable improvement in iron deposition and fibrosis as well as amelioration of inflammation and fatty metamorphosis. In addition to these pathological changes, remarkable improvements in biochemical data and QOL were observed.

in this study demonstrated that hepcidin expression in β-cells is directly regulated by iron. Iron is important for normal insulin secretion. However, excessive amounts of iron can affect β-cell function in hemochromatosis models [82–84], causing iron accumulation in the islets, a reduction in insulin secretion and an increase in the apoptosis of β-cells. In contrast, a reduction in the iron pool was shown to protect against diabetes and loss of β-cell function in an obese (ob/ob) mouse model [85]. These observations suggest that hepcidin produced by β-cells may be involved in the intrinsic regulation of pancreatic iron and glucose homeostasis [22].

In accordance with this study, the possibility of replacing phlebotomy with placenta-derived drugs, Laennec and Porcine, was evidenced through the pharmacological mechanism of inducing hepcidin production and suppressing iron-related oxidative stress [86]. Furthermore, these results strongly suggest that Laennec and Porcine are considerably effective not only for H.H but also for other iron loading diseases, such as β-thalassemia, MDS, NASH complicated with T2DM, and autoimmune liver disease [primary biliary cirrhosis (PBC) and autoimmune hepatitis (AIH)] [73–75]. The efficacy of removing iron from the liver will improve the prognosis of patients with these types of iron-loading hepatic disorders. Thus, more experimental and clinical studies are required to confirm the claim that hepcidin plays an important role in chronic liver diseases and complicating T2DM [87]. These are promising drugs that can suppress iron-induced oxidative injury as well as iron deposition in multiple organs, which will improve the prognosis of patients who developed iron-overloading disorders (**Figures 5–8**) [73–75].

In addition, in some types of β-thalassemia (especially intermediate), inducing hepcidin by administering Laennec and Porcine can improve the iron-overloading conditions in these patients without affecting the underlying cause of their haemolytic anaemia.

In general, serum hepcidin levels are typically elevated in individuals with NASH [68]. As this in itself fails to explain the cause of iron loading in NASH, one might consider that dysregulated iron metabolism occurs in NASH independently of hepcidin. One of the possible mechanisms by which hepcidin inducer Laennec is capable of improving iron metabolism in NAFLD/NASH might be the induction of the alternative route of hepcidin, which might be relevant in the progression of NAFLD/NASH. Furthermore, there might be some kind of 'hepcidin resistance' in NAFLD/NASH patients, which is observed in T2DM patients as 'insulin resistance'.

In conclusion, the present study suggests that s-ferritin elevation in our patients is a marker of metabolic syndrome with hepatic steatosis and insulin resistance and not of iron overload. The direct pathogenic mechanism, however, remains unknown. In the absence of in vivo data, any iron-independent role of hepcidin in the host defence remains speculative.

6. Laennec and Porcine also have the effects of anti-inflammation and immune modulation in the treatment of chronic liver diseases

For the clinician, the serum elevation of ferritin in chronic liver diseases (CLD), with the exception of hemochromatosis, is always assumed to be a non-specific marker of hepatic inflammation and not of iron overload [88]. It is generally considered that iron is a putative component that interacts with oxygen radicals, and high rates of hyperferritinemia together with increased hepatic iron stores have been demonstrated in NASH [22].

In the progression and pathogenesis of NASH, the role of hepatic iron still remains debatable. It is empirically accepted that iron-restricted diets or phlebotomy reduce hepatic damage as well as insulin resistance (IR) in patients with NAFLD/NASH [89]. However, the exact mechanisms involved in iron accumulation in NASH remain unresolved. Several mechanisms such as dysregulation of iron-regulatory molecules, genetic factors linked to IR and erythrophagocytosis by Kupffer cells might be responsible for hepatic iron overload in NASH [90].

A simple clinical study of phlebotomy treatment was reported by Chakraberti and Adams, performed on a series of 56 patients with histology-proven NAFLD [91]. Liver biopsy and liver iron concentration (LIC) were evaluated at entry and 6 months after phlebotomy. They did not find any significant correlation between hepatic inflammation as measured by NAS score, LIC and the level of serum ferritin and other genetic markers of inflammation, such as ESR and CRP. The authors had the conclusion that elevated serum ferritin is associated with hepatic iron accumulation, but that liver inflammation is not the cause of increased serum ferritin in patients with NAFLD.

Recently, other authors [92] demonstrated that elevated ferritin levels reflect iron stores, and not hepatic inflammation, being predictors of vascular damage in NAFLD. Irrespective of the underlying mechanisms, the only certainty is that an increased serum ferritin level in NAFLD is not a marker or a cause of inflammation, but a consequence of iron accumulation within the hepatocyte.

The resident macrophages in the sinusoids of the liver, Kupffer cells, have been widely implicated in hepatic injury such as endotoxin–mediated liver injury. Kupffer cells are known to express the death ligands, tumour necrosis factor α (TNF α), TNF-related apoptosis-inducing ligand (TRAIL), and Fas ligand [93, 94].

As a consequence of chronic tissue damage, HSCs, as well as other extracellular matrices – producing cells such as fibroblasts and myofibroblasts – undergo a process of activation towards a phenotype characterised by increased proliferation, motility, contractility and synthesis of extracellular matrix components. HSC activation is regulated by several soluble factors, including cytokines, chemokines, growth factors and products of oxidative stress as well as by extensive changes in composition and organisation of extracellular matrix components. Controlled cell death (apoptosis) could also be a mechanism underlying the termination of HSC proliferation. Spontaneous apoptosis is detected in parallel with HSC activation. Hepatic fibrosis is a complex dynamic process mediated by the death of hepatocytes and the activation of HSCs. Lipid peroxidation including the generation of ROS, TGF-β and TNF-α can be implicated as a cause of hepatic fibrosis.

The major source of ROS production in hepatocytes is NADH and NADPH oxidases localised in mitochondria. NADH and NADPH oxidases leak ROS as part of their operation. Hepatic fibrosis itself causes no symptoms but can lead to end-stage cirrhosis. In cirrhosis, the failure to properly replaced destroyed hepatocytes and the excessive collagen deposition to distort blood flow through the liver (portal hypertension) results in severe liver dysfunction.

Under physiological conditions, hydrogen peroxide plays an important role in intracellular signalling. In terms of pathological actions, ROS participate in the development of liver diseases. In this situation, hydrogen peroxide is converted into the hydroxyl radical, which is a harmful and highly reactive ROS, in the presence of transition metals such as iron. At the cellular levels, origin of hepatic fibrosis is initiated by the damage of hepatocytes, followed by the accumulation of neutrophils and macrophages including Kupffer cells on the sites of injury and inflammation in the liver. When hepatocytes are continuously damaged, leading to cell death, the

production of extracellular matrix proteins such as collagens predominates over hepatocellular regeneration. Overproduced collagens are deposited in injured areas instead of destroyed hepatocytes.

Judging from our clinical data presented before [73–75], Laennec and Porcine could have improved chr. liver diseases (CHC, NASH, PBC, AIH etc.) through the mechanisms of anti-inflammatory and immune modulative actions, which were evidenced through the fundamental examinations performed by Shindo et al. [62].

7. Heterogeneity of NASH/NAFLD from the viewpoints of etiopathogenic backgrounds and their sensitivity to each treatment procedure.

NAFLD, the most common chronic liver disease in the United States, European and Asian countries is an extremely heterogeneous disorder in its etiopathogenic backgrounds and clinical manifestations [95]. The pathogenesis of NAFLD is multifactorial and complicated; thus, several systemic alterations and individual variations have been implicated and discussed [96].

The primary insult of lipid excess is followed by variable contributions from pathogenic drivers, such as lipotoxicity and immune system response with activation; and modifiers, such as genetic susceptibilities, high calory diet, added small amount of alcohol, and dysbiosis. Although there are considerable heterogeneities in NAFLD progression and the development of NASH, only a subset of NAFLD develops into NASH, which is the most unsolved problem and mysterious issue.

Potential explanations for this variability include differences in etiopathogenic drivers [2], dynamic multiphasic progression, constitutional/genetic backgrounds, complicated diseases, biological reactions, metabolic responses, etc. [97].

7.1 With or without 'T2DM and insulin resistance'

Diabetes is highly prevalent in patients with NASH/NAFLD and vice versa.

Three studies using Fibroscan showed that 12–18% of diabetic patients are estimated to have significant liver fibrosis by different cut-offs [98–100]. Decreased levels of HbA1c [101] were more strongly associated with fibrosis improvement in 39 Japanese patients with diabetes and NAFLD who underwent sequential liver biopsies. Thus, these three clinical parameters, including ALT, body weight and HbA1c (ABC), can become the milestones for the treatment of NASH, although the appropriate goal of each parameter to ameliorate hepatic fibrosis will be established in the near future.

In a cross-sectional multicentre study conducted by JSGNAFLD, the presence of diabetes was found to be associated with advanced fibrosis in 1365 biopsy-proven NAFLD patients [102]. 'With or without T2DM' is a crucial problem for treating NAFLD/NASH patients because complicated T2DM itself modulates the clinical manifestations of NAFLD/NASH and affects the sensitivities to treatment procedures and the prognosis of each disease. If some effective treatments are developed and preferable improvement of NAFLD/NASH can be achieved, the control of T2DM will improve unexpectedly. Mounting evidence suggests that more experimental and clinical studies are needed to confirm or refute the claim that hepcidin has a role or relevance with T2DM complicated with NAFLD/NASH.

Recently, attention has been shifting towards the iron regulatory hormone hepcidin and its possible role in the etiopathogenesis of T2DM. Interpreting this critically, notably, hepcidin in the pancreas is expressed exclusively in the islets of Langerhans,

which constitute merely a small compartment of the total pancreatic parenchyma. Very likely, the regulation of iron and glucose metabolism is distinctly coupled at least at the pancreatic level by the co-release of insulin and hepcidin [81].

7.2 With or without 'hyperferritinemia and iron overloading, related with T2DM'

A strong correlation between iron overload and several manifestations of the metabolic syndrome including NAFLD and T2DM has been demonstrated recently. It has clashed that increased ferritin levels observed in most patients with NASH are due to the underlying necro-inflammatory condition, which assists the release of tissue iron and ferritin into the blood [103].

The corelation between NASH and IR has recently been weaved as a 'new iron overload syndrome' characterised by hyperferritinemia. The dysmetabolic iron overload syndrome has now been established as a frequent finding in the general population, occurring in about one-third of patients with NAFLD and metabolic syndrome. Altered regulation of iron transport genetic factors is considered to be the main contributor to iron overload [26]. The exact mechanisms underlying the deposition of hepatic iron remain unknown and unsettled. One of the clinical factors associated with steatosis, IR and subclinical inflammation, often in the presence of predisposing features of NASH, is the build-up of iron in the liver accompanied by increased levels of serum ferritin, which is highly suggestive of the central role of iron in disease progression [104].

Iron is known to generate highly reactive hydroxyl radicals through the Fenton reaction, and the resultant ROS may contribute to liver damage. Significant increases in hepatic 8-OHdG generated by OH radicals have been reported in patients with NASH, particularly in correlation with iron overload, IR and severity of hepatic steatosis [26]. The mechanisms involved in iron accumulation in NAFLD, and in inducing IR, metabolic, hepatic and vascular damage by iron accumulation are not yet well understood and should be further investigated [92].

The association between hyperferritinemia, insulin resistance and T2DM has been discussed among hepatologists, diabetologists and endocrinologists recently. There is an increased prevalence of T2DM associated with two common iron overload conditions, HFE hereditary hemochromatosis (HH) and β-thalassemia major [28]. The persistence of the association between serum ferritin concentration and T2DM after correction for hsCRP implies that inflammation alone does not entirely explain the association between hyperferritinemia and diabetes.

Bugianesi et al. [43] found that the serum ferritin concentration is not associated with hepatic iron concentration in NAFLD but is a marker of severe histologic damage in 2004. In the large NASH Clinical Research Network (CRN) cohort of 628 patients, Kowdley et al. [105] demonstrated that a serum ferritin concentration greater than 1.5 times the upper limit of normal was independently associated with advanced fibrosis and increased NAFLD activity score. However, other studies have not found such a clear association [39, 106].

Notably, in an Italian cohort of 587 patients with NAFLD, Valenti et al. [26] showed that serum ferritin concentration did not predict fibrosis stage >1. As would be expected, the serum ferritin concentration was higher in the patients who had hepatic iron staining than those who did not, but those with non-parenchymal iron had much higher ferritin values (606 μg/L) than those with hepatocellular iron (serum ferritin 354 μg/L) $P < 0.0001$. This suggests that macrophage iron can cause hyperferritinemia either by direct release of ferritin or cytokine-mediated stimulation of ferritin released by other cells.

Moreover, these results tend to suggest that the link between hyperferritinemia and NAFLD could be explained by insulin resistance. In NAFLD pathogenesis, the role of hepatic iron has largely focused on the generation of oxidative stress by iron. Considering oxidative stress is an established key component of NASH pathogenesis [107], the role of iron mediating liver injury in NAFLD via this mechanism has been well studied. Oxidative stress leads to cell death via depletion of ATP, NAD and glutathione, and by direct damage to DNA, lipids and proteins within hepatocytes in NASH. Furthermore, oxidative stress leads to an increase in the production of pro-inflammatory cytokines and fibrogenic responses. Not only does oxidative stress potentiate steatohepatitis, characterised by inflammation and cell death, but it can also increase steatosis by preventing the secretion of very low-density lipoprotein (VLDL) through increased degradation of apolipoprotein B100 (ApoB100) [108].

In conclusion, iron has been gradually recognised as a regulator of adipose tissue function. There are definite pieces of evidence which support the role of iron in the regulation of adipose tissue inflammation, adipokine regulation and adipose tissue lipolysis. At present, most pieces of evidence support the role of adipose tissue iron in the pathogenesis of insulin resistance and T2DM, although clearly, these mechanisms may be highly relevant in NAFLD.

It has been suggested that elevated serum ferritin is associated with several metabolic disorders. However, no reported study has assessed the association between serum ferritin and sarcopenia despite the close relationship between sarcopenia and metabolic disorders.

The question then arises whether the concentration of hepcidin in T2DM subjects is primary or secondary to elevated body iron stores. This might be an important assignment to find a key to the settlement of the dispute.

7.3 With or without 'iron deposition in hepatocyte and/or Kupffer cell'

Although iron is indispensable for normal physiology and biochemical reactions, excess iron is toxic and harmful because it can accelerate the Fenton reaction that generates noxious reactive oxygen species (ROS) and severely damages cells and tissues in the human body. Thus, maintenance of body iron homeostasis is pivotal, particularly because there is no physiological pathway for removal of excessive iron from the body [109]. Systemic iron regulation is mediated via the liver-secreted iron regulating hormone hepcidin under normal physiological conditions [110]. Several studies have reported the fibrosis-enhancing effects of iron. For instance, induced collagen deposition in gerbil [111], iron elevated collagen gene expression in HSCs and increased TGF-β expression in rats [112], and promoted cirrhosis in mice [113]. Ramm et al. [114] demonstrated the correlation between LIC and HSC-activation in humans, resulting in increased expression of α-SMA and collagen deposition in patients with hemochromatosis for the first time. Similar results were observed in rat HSCs, wherein iron increased HSC-cell proliferation and selectively increased collagen synthesis without affecting non-collagen proteins [115].

In the pathogenesis of NAFLD, iron has been widely implicated, therefore represents a potential target for treatment. Correlations between the serum ferritin concentration and NAFLD are noted in most studies, although serum ferritin is an indistinct measure of iron loading. A large number of mechanisms underlying the pathogenic role of hepatic iron in NAFLD have been demonstrated in animal and cell culture models. However, the human data linking hepatic iron to liver injury in NAFLD is not so clear, with seemingly conflicting evidence, supporting either an

effect of iron in hepatocytes or within reticuloendothelial cells. Adipose tissue has emerged as a key site where iron may have a pathogenic role in NAFLD [116].

An investigation of the serum ferritin level and histological findings including iron deposition in 628 patients with NAFLD was performed by Kowdley et al. [105]. This large cross-sectional study revealed that elevated serum ferritin (>1.5 × UNL) was associated with advanced hepatic fibrosis (odds ratio [OR], 1.66; 95% confidence interval [CI], 1.05–2.62; P = 0.028) and a higher NAS (OR, 1.99; 95% CI, 1.06–3.75; P = 0.033). Elevated serum ferritin levels (seen in approximately 20% of the subjects) were associated with greater iron accumulation in the body (i.e., a high serum iron and transferrin-iron saturation) and greater hepatic iron deposition in both the reticuloendothelial system and hepatocytes.

It was also elucidated that the patients with increased serum ferritin levels also had higher serum transaminases and gamma-glutamyl transferase and a lower platelet count. Interestingly, even in patients without a hepatic iron overload on histology, higher serum ferritin was correlated with advanced stage of the disease.

7.4 With or without 'inflammation and elevation of cytokines'

One of modifying factors in NAFLD/NASH is hepatic iron content (HIC) [117]. Iron accumulation exacerbates hepatic oxidative stress and can, therefore, affect susceptibility to oxidant stress induced by fatty acid oxidation [118]. HIC is susceptive to factors that differ among individuals (polymorphisms in genes such as HFE) and factors that might change during the lifetime of individuals, including sex-related factors (menstruation or pregnancy) and diet (consumption of greasy meal, roughage or red meat) [119]. The lipotoxic outcomes of identical fatty acid exposures can, therefore, differ based on complicated factors that modulate hepatic iron content. Inflammation is required to clear damage-related debris and stimulate local accumulation of other wound-healing cells, such as liver progenitors and myofibroblasts. However, excessive inflammation can compromise the viability of residual hepatocytes and promote over-growth of progenitors and myofibroblasts, laying the groundwork for progressive fibrosis to liver cirrhosis and carcinogenesis. Therefore, the liver is variably repopulated with relatively immature or dysfunctional hepatocytes as long as wound-healing responses are active and continuing. This potentiates metabolic stress and increases the risk of liver cancer [120].

Recent studies have suggested several possibilities in progressing liver fibrosis, involving inflammation caused by OS associated with lipid peroxidation, endogenous toxins of fructose metabolites, cytokine activation and NO [9]. Mitochondrial dysfunction not only facilitated the production of ROS but also contributed to the progression of NAFLD by inducing hepatic inflammatory cytokines. The network of obesity, IR and adipokine/cytokine has been hypothesised to induce both liver fat accumulation and NASH development [121]. ROS along with products of lipid peroxidation leads to increased release of several cytokines (tumour necrosis factor-alpha (TNF-α), Fas ligand), which play a key role in cell death, inflammation and fibrosis [107]. Lipid peroxidation, release of inflammatory cytokines and cell death are the consequences of ROS-mediated mechanisms. Biologically active lipid peroxidation products and cytokines take action together by inducing hepatic inflammation, leading to the development of diverse hepatic lesions associated with NASH. The inflammatory response is induced because of the upregulation of pro-inflammatory cytokines including TNF-α, interleukin (IL) 1 and IL-6 [122], which play an essential role in directing polymorpho-and mono-nuclear leukocytes into flamed tissue. The

effects of TNF-α in NASH are enhanced through an abnormal cytokine profile and increased expression of the TNF-α-receptor in the liver [123]. This contributes to additional lipid peroxidation of the mitochondrial membranes, thereby worsening their function and further inducing OS [94]. Adipose tissue shows prominent deregulation of genes related to inflammation in patients with NASH. Induction of NADPH oxidase by TNF-α also leads to inflammation through the expression of TNF receptor-1 and activation of nuclear factor kappa B (NF-κB).

In the pathogenesis of steatohepatitis, hepatic inflammation and fibrogenic progression are pivotal features. Although hepatocyte damage and ROS are regarded to be the initial triggers of inflammation, additional factors such as mitochondrial dysfunction and ER stress have also been implicated as contributory factors in the progression of NAFLD to NASH, by promoting generation of signals and mediators of inflammation.

The association of elevated serum iron values and increased hepatic tissue ferritin deposition with hepatic inflammation and IR in patients with NASH have been well established [94]. In contrast, ROS and lipid peroxidation cause direct damage to hepatocytes by affecting membranes, proteins and DNA [124]. The ensuing damage to nuclear and mtDNA results in necro-inflammation, particularly in the nuclei/cytoplasm of hepatocytes and sinusoidal cells.

The results of current research suggest that hepcidin may dampen inflammatory cytokines through a mechanism that is not well understood. Because excessive inflammation is damaging in many infections, the potential role of hepcidin as a mediator of the innate immune response is a new and unexpected area of study.

7.5 With or without 'sleeping apnoea syndrome (SAS), CPAP-Tx (+) or (−)'

Another serious clinical condition in understanding NAFLD/NASH is the presence of obstructive sleep apnoea (OSA), characterised by upper airway obstruction (causing intermittent hypoxia and ROS), and interrupted sleep [125]. Both conditions have been associated together as a cause/result/modifying factor or potential co-occurring complications of obesity and NAFLD/NASH [126, 127].

The two-hits hypothesis is one of the prevalent theories for the development of NASH. This theory indicates that benign hepatic steatosis may be the first hit, and then, another precipitating factor (second hit) may load and progress the pathogenesis of NAFLD/NASH [128]. The involvement of OSA as a second hit in NASH development is evidenced by both experimental and epidemiological reports.

In a mice experiment, Zamora-Valdés and Méndez-Sánchez evidenced that exposure to a high-fat diet along with chronic intermittent hypoxia was associated with lobular inflammation and fibrosis and with significant increases in the hepatic levels of pro-inflammatory cytokines (interleukin 1β and 6 and tumour necrosis factor α), as well as collagen-1α mRNA. Other in vivo experiments showed concordant results [129]. Oxidative stress and the release of hypoxia-inducible factor-1 are hypothesised to be the main players in this association [130].

Concurrently, epidemiological studies have showna higher prevalence of NASH in OSA patients, as well as a higher prevalence of OSA in NASH patients and vice versa. However, the evidence remains largely inconclusive, i.e., some studies have reported significant elevation in serum liver enzymes in OSA patients [131], whereas other studies failed to record such observations [132]. In a sample of 54,169 participants, significant association between NASH and OSA was observed. At the same time,

significant associations between NASH and obesity, DM and metabolic syndrome were also observed, indicating the possible involvement of these conditions in the pathogenesis of NASH.

Some convincing mechanisms were speculated to explain this association lately. Oxidative stress remains the predominant hypothesis. This occurs through repetitive cycles of hypoxia/reoxygenation every night, which disturb mitochondrial respiration along with bouts of catecholamine release, inducing metabolic changes [133]. Moreover, hypoxia stimulates fibrosis and angiogenesis by enhancing the expression of hypoxia-inducible factor-1α, vascular endothelial growth factor, angiotensin-I-converting enzyme and transforming growth factor β1 [134].

Furthermore, hypoxia is an established risk factor for inflammation [135]. This study documented a significant association between NASH and other complicating factors such as obesity, DM and metabolic syndrome. The association between NASH and hypertension, obesity and DM shows the full picture of metabolic syndrome. If the NASH patients complicating with SAS could be treated by CPAP appropriately, the more enthusiastically they continue the procedure, the more will the grade of liver fibrosis and hyperferritinemia as well as T2DM ameliorate by degrees. This modification by treating SAS with CPAP will complicate the clinical data, manifestations and susceptibility to newly developed drugs for NAFLD/NASH patients.

7.6 With or without 'reactive oxygen species (ROS) and Antioxidant dynamics'

A 'two-hit' theory has been postulated to help explain the mechanisms underlying the development of advanced NAFLD. The 'second hit' has yet to be completely described; extensive research has identified several possible mechanisms, including oxidative stress (OS)-induced inflammation with lipid peroxidation, cytokine activation and excess production of reactive oxygen and nitrogen species (ROS/RNS) [124].

The main source of radicals in biological systems is molecular oxygen, which readily accepts electrons, the most important of which being the hydroxyl radical (•OH), the superoxide anion ($O_2^{\bullet -}$) and nitric oxide radical (NO•). These unstable and reactive radicals are natural by-products of the intracellular metabolism and from exogenous substances, which have the ability to react with biological compounds including proteins, FFA and DNA [136, 137]. On the other hand, the main endogenous intracellular sources of ROS are mitochondria, the endoplasmic reticulum (ER) and peroxisomes, superoxide anion radicals ($O_2^{\bullet -}$) are produced because of enzymatic activity, such as with xanthine oxidase (XO) and cytochrome P450 metabolism [107, 138].

In a normal situation, a fine balance exists between prooxidant and antioxidant mechanisms, and OS, which has been long recognised as a key mechanism responsible for liver damage and disease progression in NAFLD, is believed to occur due to an imbalance in favour of prooxidation [139]. Numerous pieces of evidence accumulated over the past decade suggest that mitochondrial dysfunction plays a significant role in steatosis and steatohepatitis. ROS overproduction is induced by mitochondrial dysfunction, and the ensuing increase in the lipid peroxidation and protein oxidation has a detrimental effect on fat homeostasis in the liver. Mitochondria remain the main source of ROS in hepatocytes, although other subcellular organelles have also been shown to participate in the process [140, 141].

As a matter of fact, peroxisomes can oxidise long-chain FFA more rapidly than mitochondria, thereby increasing the cell's capacity to metabolise FFA. However, H_2O_2, which is an end-product of peroxisomal β-oxidation, is converted into the highly reactive OH radical with ease. By promoting toxic accumulation of ROS, which

triggers other signalling pathways within the cell, chronic ER stress may also contribute to OS. The relationship between ER stress and OS works both ways because ROS generated through inflammation or damage to organelles (e.g., mitochondria) may also accelerate ER dysfunction [140, 141].

Because of either excessive production of ROS within the hepatocyte or reduced antioxidant defences, oxidative stress occurs and accumulates within the hepatocytes. Most antioxidant enzymes, copper/zinc superoxide dismutase (Cu/Zn SOD) and manganese-superoxide dismutase (MnSOD), which are mainly present in the cytoplasm and mitochondria, promote the reduction of $O_2^{\bullet-}$ to H_2O_2. Another antioxidant enzyme, glutathione peroxidase (GPx), facilitates the subsequent conversion of H_2O_2 to H_2O [137]. In correlation with disease severity, a breakdown in the antioxidant defences plays a significant role in OS associated with NASH, as evidenced by decreased hepatic glutathione (GSH) and diminished SOD, GPx, catalase and glutathione transferase activities [107].

Lipid peroxidation to release more reactive aldehydes is augmented by the resultant increase in mitochondrial ROS, which further damages the mitochondrial DNA (mtDNA) and respiratory chain polypeptides [142].

In summary, mitochondrial dysfunction not only impairs fat homeostasis in the liver but also leads to an overproduction of ROS, which is deliberated to be an important factor in producing lethal hepatocyte injury associated with NAFLD [107].

7.7 With or without 'liver fibrosis and promoting signals'

It is interesting that iron-loading is frequently observed in chronic liver diseases regardless of the aetiology. The Fenton reaction is induced by excessive iron. At the same time, it generates unquenchable amounts of free radicals that cause grave cellular and tissue damage and thereby contribute to fibrosis. In addition, excess iron can induce fibrosis-promoting signals in the parenchymal and non-parenchymal cells, which accelerate disease progression and exacerbate liver pathology. Liver fibrogenesis is the normal process of tissue repair. It is mediated via a complex network of interrelated and regulated signalling interactions between the resident parenchymal cells (hepatocytes), non-parenchymal cells, Kupffer cells, hepatic stellate cells (HSCs), liver sinusoidal endothelial cells, biliary epithelial cells, liver associated lymphocytes and non-resident infiltrating immune cells. HSCs located in the space of Disse between the hepatocytes and liver sinusoids play a pivotal role in liver development and regeneration via fibrogenesis [143].

The fruitless regenerative response perpetuates variable repair-related expansion of immature liver cells, inflammation, vascular remodelling and fibrogenesis, which results in more advanced or severe NASH. By degrees, functional hepatic parenchyma is progressively replaced by scar, and the liver becomes enriched with neoplastic immature hepatocytes; this can account for the increased risk of cirrhosis and liver cancer in patients with severe NASH [144].

The histologic features of NASH indicate the ongoing repair responses to chronic hepatocyte lipotoxicity and vary with the severity of lipotoxicity and success of the wound-healing process. The liver can usually undergo repair and regeneration after acute injury or when chronic injury causes a minor increase in the rate of hepatocyte death. Therefore, there is no progressive replacement of hepatic parenchyma with scar, and the risk for liver cancer remains low in numerous patients with minimal hepatic lipotoxicity and mild NASH [120].

In conclusion, the findings indicate that HSCs, during fibrogenesis in vivo, may not be directly subjected to oxidant stress, and when exposed to various oxidant stressors in vitro, do not turn on the fibrogenic machinery.

7.8 With or without 'amenorrhea or menopause complicated with Mets'.

In menopausal women, oestrogen is one of important hormones for the regulation of glucose metabolism, because it has capacity in exerting a protective effect on pancreatic beta cells and plays an important role in regulating appetite and improving insulin resistance in insulin target organs. It is one of the crucial problems that oestrogen may also play an important role in the progression of NAFLD and NASH. It has been empirically considered that postmenopausal women are at an increased risk of NAFLD and might show metabolic features of insulin resistance. For example, increased total and visceral adiposity in peri- and postmenopausal women is associated with an increased risk of insulin resistance, dyslipidaemia, hypertension, diabetes and cardiovascular disease. Furthermore, postmenopausal women with NAFLD are at an increased risk of portal inflammation, ballooning and fibrosis due to their inability to suppress oxidative stress and fibrosis by lowering their oestrogen levels [145].

It is really recognised that oestrogen replacement therapy has some beneficial effects in patients with liver fibrosis. The risk of NAFLD is greater among postmenopausal women than among premenopausal women [146].

It is possible that the loss of protection conferred by oestrogens', combined with other factors, underlies the increased NAFLD risk in postmenopausal women. NAFLD can easily progress to a more dangerous condition called NASH, which indicates there is both inflammation and liver cell damage, along with fat in the liver [147].

In addition, menopause or amenorrhea actually means 'relative iron overload' for women who develop obesity at the same time. These women have a high risk of deteriorating NAFLD/NASH. In such situations, Laennec as a 'hepcidin inducer' might be a preferable and effective treatment. In my experiences, 13 biopsy-proven NASH cases were extremely sensitive to placenta-derived Laennec treatment. In five cases, second liver biopsy revealed a diminishing liver fibrosis and inflammation, as well as a decrease in iron deposition. In these NASH cases, iron deposition was mainly observed in the Kupffer cells [73, 74, 78].

7.9 With or without 'lipolysis and lipotoxicity'

In discussing the prognosis of liver steatosis, NAFLD does not necessarily lead to NASH because NAFLD is an extremely heterogeneous condition. This heterogeneity exists in part because different types of lipids with different cytotoxic potentials accumulate in the NAFLD, and individuals with NAFLD differ in their ability to defend against lipotoxicity. Differences in these wound-healing responses among individuals determine whether the lipotoxic livers regenerate, leading to stabilisation or resolution of NASH, or develop progressive scarring, cirrhosis and possibly liver cancer.

The perception that the lipotoxic potential of various types of lipids differs can help explain why the outcomes of hepatic steatosis vary as a matter of fact. Interventions that block the accumulation of lipotoxic lipids might, therefore, be used to prevent or treat NASH. Multiplying of fatty acids within the mitochondria could also dissipate the protonmotive force that typically occurs during mitochondrial respiration [148]. This makes mitochondria more vulnerable to other insults that collapse the mitochondrial membrane potential, such as tumour necrosis factor alpha

(TNFα), and could lead to the release of mitochondrial factors that promote apoptosis [149]. Complete cessation of the mitochondrial electron transport and ATP synthesis is caused by extreme depolarisation of mitochondrial membranes, resulting in cellular necrosis [150]. Because damaged mitochondria cannot efficiently metabolise fatty acids, fatty acids accumulate [151], leading to further hepatic lipid accumulation [152], and promoting inflammatory [153] and fibrogenic responses as well as mitogenic responses that could be carcinogenic [154].

Lipotoxicity induces several different types of cellular stresses, including ER stress [154] and impaired autophagy [155]. In addition, it promotes a sterile inflammatory response that can potentiate liver cell injury and death. At the adipocyte level, metabolic dysregulation because of impaired insulin post-receptor signalling leads to excess lipolysis of triglycerides (TGs) and NEFA release into the circulation. At the molecular level, lipotoxicity leads to endoplasmic reticulum (ER) stress, lysosomal dysfunction, inflammasome activation, cell death and activation of inflammatory responses due to lethal and sublethal hepatocellular injury [156].

NASH occurs because lipotoxic hepatocytes release factors that initiate wound-healing responses to replace dying hepatocytes [157]. Wound healing is a complex multifaceted process that can restore the liver structure and function to a healthy state [158].

7.10 With or without 'the intestinal microbiome and enterohepatic circulation'

While the causal links between the microbiota and NAFLD have not been fully elucidated, disruption in intestinal permeability [159] and bacterial-derived ligands (e.g., LPS) and metabolites (e.g., secondary bile acids, short chain fatty acids) are putative mediators of this association.

It was elucidated recently that the presence of bacterial strain (*Klebsiella pneumoniae*), which produces high levels of endogenous alcohol was associated with NAFLD in a human cohort [160]. Bile acids are synthesised and secreted by hepatocytes and are involved in the absorption of dietary lipids. They are transported back to the liver by enterohepatic circulation and act on the nuclear farnesoid X receptor (FXR), which is also expressed on hepatocytes, thereby affecting glucose [161] and lipid metabolism. Further, the release of FGF after ileal FXR activation is a feedback mechanism that reduces bile acid synthesis, hepatic steatosis and IR [162]. Through their antimicrobial effects, bile acids also modulate the relationship between gut microbiota and chronic liver disease [163] and improve glucose metabolism by activation of G-protein coupled bile acid receptor (GPBAR1) in enterocytes. Therefore, targeting these mechanisms, for example, with an FXR agonist, is an attractive strategy for NAFLD therapy [164]. Gut-derived hormones, such as GLP-1, play a crucial role in controlling nutrient intake, absorption and metabolism and are attractive targets for metabolic disease in general, as well as in the liver [96].

7.11 With or without small amount of alcohol, so-called 'NASH + ASH'

NAFLD and alcohol-related fatty liver disease (AFLD) [165] are already undoubtedly, and will continue to be, leading drivers of progressive liver disease and hepatocellular carcinoma (HCC) worldwide. Severe alcohol abuse leads to accelerated disease progression with higher rates of HCC, liver-related deaths and poor prognosis. In contrast, NAFLD is most frequently related to metabolic dysfunction (MAFLD: metabolic dysfunction-associated fatty liver disease) and is associated with an increased risk of cardiometabolic disease and cancer.

Although the main environmental triggers of fat accumulation differ between AFLD and NAFLD, they are frequently superimposed, and the pathogenesis of inflammation and progressive liver damage share numerous mechanisms [166].

The progression of liver damage is accelerated when, especially at times of acute insults during the natural history of the disease, excess fat and lipotoxicity lead to inflammation, hepatocellular damage and fibrogenesis, in a condition referred to as 'steatohepatitis' (NASH and acute alcohol-related steatohepatitis (ASH)) [167].

Just as all heavy drinkers do not progress to cirrhosis and HCC, nor do all patients with non-alcoholic steatosis progress. However, if NASH patients drink small amounts (EtOH: male <210 g/w, female <140 g/w) of alcohol, in my clinical experience of following more than 200 biopsy-proven NASH patients, the progression of liver fibrosis and deposition of iron at the hepatocytes seem to be more conspicuous compared to those who do not drink any alcohol.

A small amount of alcohol seems to modify and accelerate clinical manifestation and the progression of NASH.

8. How to find a breakthrough for NAFLD/NASH treatment?

As mentioned before, NAFLD/NASH is in general a heterogeneous group of chronic liver diseases characterised by the accumulation of fat in the liver. The heterogeneity and variation of NAFLD/NASH as well as the sensitivity to many kinds of treatment procedures are reflected in a clinical and histologic spectrum, where some patients develop isolated steatosis of the liver, termed non-alcoholic fatty liver, whereas others develop hepatocyte injury, ballooning, inflammation and consequent fibrosis termed as NASH and progress to liver cirrhosis/hepatocellular carcinoma.

Further research is required to determine why progressive scarring develops in only some patients with NASH, define the mechanisms that shift effective regeneration to pathologic scarring [168] and determine how wound-healing responses might be modulated to heal lipotoxicity without scarring [169].

Based on these findings, the risk for NASH is determined by the susceptibility of hepatocytes to toxic lipids and potential for repair of lipotoxic liver damage. Therapies for NASH might, therefore, include those that prevent hepatic lipotoxicity by alleviating systemic metabolic stress [170].

A nascent understanding of this heterogeneity would also suggest that 'combination therapy' might be one of the options for preventing the progression of NASH; however, considering the remarkably wide-ranged heterogeneity of the disease, it may be extremely expensive and sometimes futile. One possible treatment procedure might be the trial with 'bioactive drugs', which have multiple sites of action such as 'antioxidant', 'metabolic regulator' and 'anti-inflammatory effects' at the same time. The sites of action brought about by Laennec on NASH treatment might be so to speak "multicentric" and "covering a wide area" comparing with so called newly developing "monotherapy drugs".

The most important mechanism of Laennec/Porcine might be conducted by the regulation of iron metabolism, which is needed in many kinds of biochemical and biophysical reactions (**Figure 9**). Laennec/Porcine might act on multiple targets, affecting diverse pathological processes and leading to an increased ability to adapt.

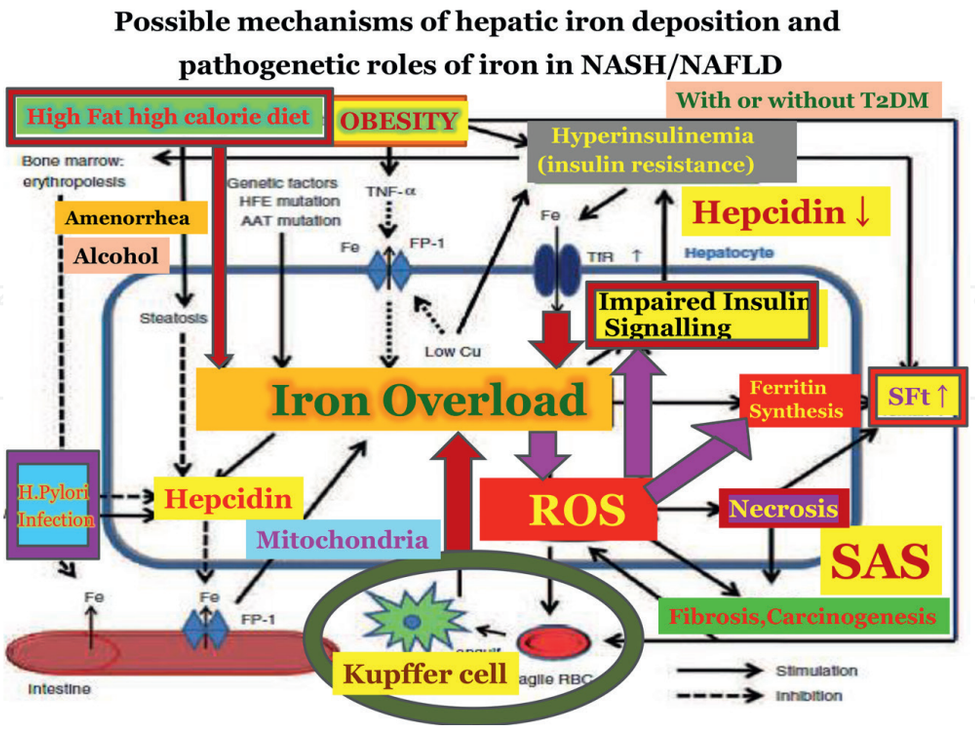

Figure 9.
Possible mechanisms of hepatic iron deposition and pathogenetic roles of iron in NASH/NAFLD. 'Iron reduction therapy' such as phlebotomy or dietary iron restriction may be promising for patients with NASH/NAFLD to reduce insulin resistance as well as serum transaminase activities. Iron is a potent catalyst of oxidative stress and may act synergistically with other promoters of lipid peroxidation by catalysing these reactions. Iron overload can also directly cause lipid peroxidation, and one of the subsequent products, malondialdehyde, has been shown to activate HSCs in vitro, the major source of fibrogenesis in liver injury. Excessive triglyceride accumulation is the most likely first step. The second step may be related to an increase in oxidative stress, which in turn, triggers liver cell necrosis and activation of HSCs, both leading to fibrosis and ultimately to the development of cirrhosis. One of the potential cofactors suspected to enhance this oxidative stress is excessive hepatic iron accumulation (by the courtesy of Ref. [171], partially modified by the author).

This fits seamlessly in the pathophysiologic model of NAFLD/NASH since diverse pathological processes are involved. This concept is really compatible with the site of action induced by Laennec/Porcine. So that, Laennec/Porcine is capable of covering wide range of pathological abnormalities in NAFLD/NASH (**Figures 10** and **11**). If the drug is safe, has no apparent side effects, is cost beneficial (250–300 USD/m), has multiple mechanisms, which will ameliorate NAFLD/NASH spectrum according to the individual pathogenic background, and its pharmacodynamics are clarified, attempts should be made to use such a drug for treating the patients with NAFLD/NASH (**Figures 10** and **11**).

In conclusion, considering numerous factors being involved in the pathogenesis of NAFLD/NASH, one of the most preferable and reliable drugs for the control of these diseases might be the "Bioactives" such as Laennec/Porcine, which have multi-ranged sites of action and the potential to modulate iron metabolism appropriately through the action of "hepcidin inducer".

Further studies should confirm the role of iron overload and the meaning of hyperferritinemia in patients with chronic liver diseases, including NAFLD/NASH. 'Hepcidin inducing therapy' using Laennec/Porcine might be one of the preferable treatment options for controlling wide-ranged NAFLD/NASH along with complicating T2DM (**Figures 10** and **11**).

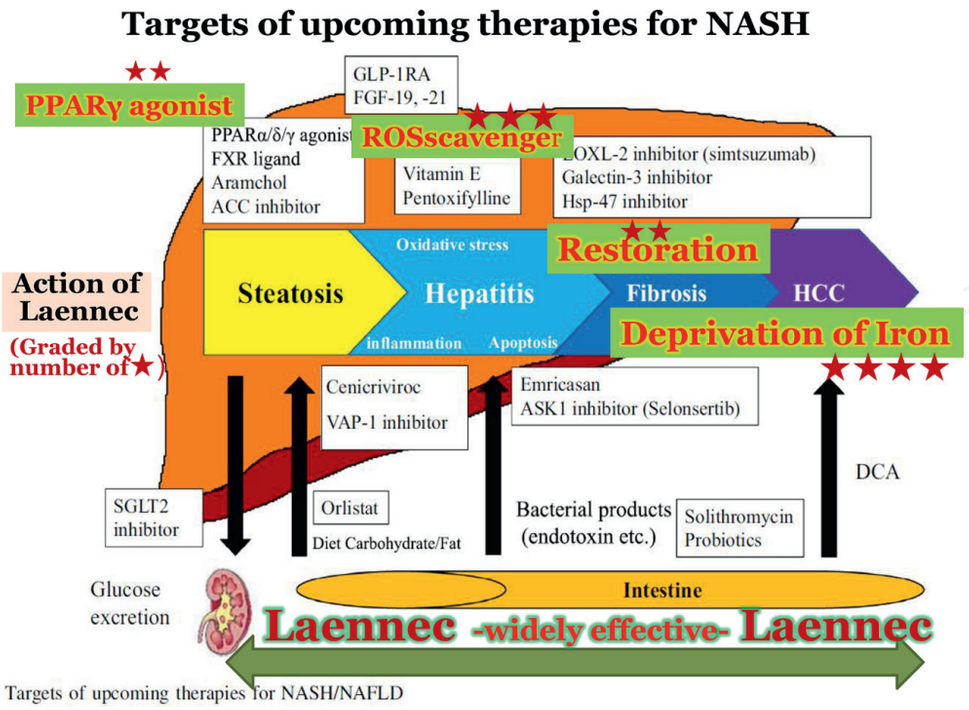

Figure 10.
Targets of upcoming therapies for NASH are shown in this figure. The supposed sites of action of Laennec seem to be widespread compared with those of other newly developed 'single targeted drugs'. The expected sites of action of Laennec are graded ★★—★★★★ tentatively judging from formerly obtained data and clinical observations (by the courtesy of Ref. [172], partially modified by the author).

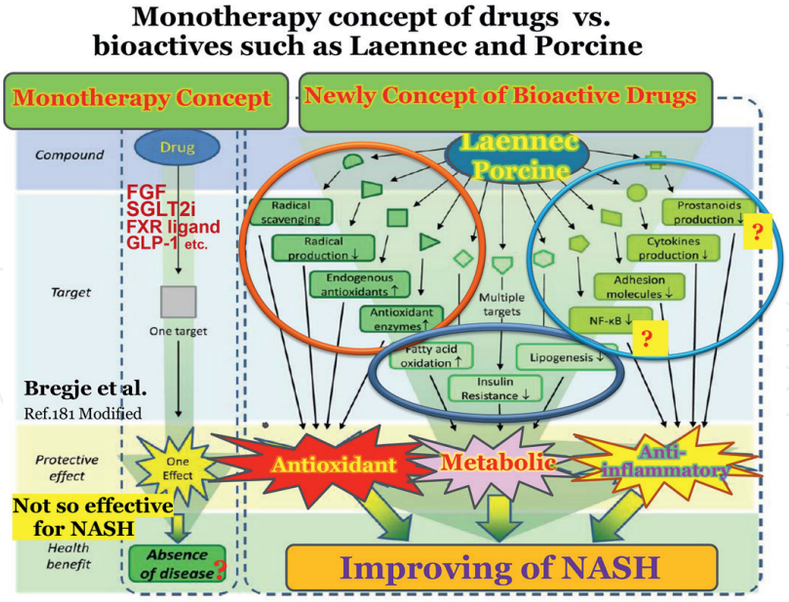

Figure 11.
Traditional concept of drugs vs. the mode of actions (Laennec and Porcine), 'traditional' concept of action of drugs versus the contemporary concept of mode of actions such as Laennec and Porcine. Traditional drugs are developed to act on one target, leading to the absence of disease; however, the target diseases are complicated and consist of many kinds of factors. Laennec and Porcine act on multiple targets, affecting diverse pathological processes and leading to increased ability to adapt. This fits seamlessly in the pathophysiologic model of NAFLD since it involves diverse pathological processes. (Dr. Bregje Van De Wier, Ref. [173], partially modified by the author)

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