1 **Hypoxia-inducible factor prolyl 4-hydroxylases and Metabolism**

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1 **Abstract**

2 Hypoxia-inducible factor prolyl 4-hydroxylases (HIF-P4Hs, also known as PHDs or EglNs) are
3 enzymes that act as cellular oxygen sensors. Inhibition of HIF-P4Hs leads to stabilization of hypoxia-
4 inducible transcription factors (HIFs) which initiate a gene expression program that allows to cope
5 with low oxygen levels and to restore tissue oxygenation. This involves for example upregulation of
6 erythropoiesis and angiogenesis, modulation of inflammatory responses and reprogramming of
7 metabolism. Currently, several pharmacological HIF-P4H inhibitors are in clinical trials mainly for
8 renal anemia. However, recent data suggest that HIF-P4H inhibitors could also be considered to treat
9 metabolic disorders. Here we will discuss the potential of targeting HIF-P4Hs and the HIF pathway
10 for the treatment of obesity, metabolic syndrome, atherosclerosis and fatty liver diseases.

1 **HIF-P4H inhibitors and renal anemia – What about beyond?**

2 After birth the kidney overtakes liver as the major organ responsible for synthesis of erythropoietin
3 (EPO) [1]. Therefore, conditions such as chronic kidney disease (CKD), can cause renal anemia [1].
4 The current anemia treatment in CKD is recombinant EPO, often with iron supplementation.
5 However, due to CKD-associated inflammation involving *e.g.* upregulation of hepcidin, the treatment
6 is often insufficient [1]. Hence, alternative renal anemia treatments are of high demand. Induction of
7 EPO expression and erythropoiesis are also key events of a physiological response to hypoxia, which
8 occurs *e.g.* at high altitude (Fig.1). The genetic program of the hypoxia response is primarily driven
9 by hypoxia-inducible factors (HIFs) (Fig. 1). Under normoxic conditions, the three HIFs known to
10 date, with HIF2 being the primary EPO inducer in kidney, are targeted for VHL-mediated degradation
11 by HIF prolyl 4-hydroxylases (HIF-P4Hs, also known as PHDs or EglNs) (Fig. 1). Three variants
12 have also been identified for HIF-P4Hs [2]. Thus, inhibitors of the HIF-P4H enzymes, which prevent
13 the degradation of HIFs provide a novel, efficient method to treat at least CKD by inducing the
14 endogenous hypoxia response. These inhibitors are chemical analogues of 2-oxoglutarate (2-OG), a
15 reaction cofactor for HIF-P4Hs (Fig. 2), and act as competitive antagonists that stabilize HIFs in the
16 presence of oxygen (Fig. 2). Several such compounds are in clinical trials for treatment of renal
17 anemia (Table 1).

18 Since HIFs regulate many processes beyond erythropoiesis (Fig. 1), HIF-P4H inhibition has
19 been exploited widely in preclinical conditions, and indeed found beneficial in ischemic and
20 inflammatory conditions (for reviews see [3, 4]) and lately in metabolic diseases [4-9]. However,
21 there exists concern about the use of HIF-P4H inhibitors. This arises from findings showing that
22 cancerous lesions typically express high levels of HIF and HIFs are often associated with increased
23 mortality [10]. Hence, close care is required although in tumors HIF stabilization is driven by hypoxia
24 due to uncontrolled proliferation and inadequate vascularization, and HIF itself has not been found to
25 be an oncogene [11]. This review focuses on the potential role of HIF-P4H inhibition in treatment of
26 metabolic disorders.

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28 **Hypoxia and HIFs as metabolic reprogrammers**

29 The hypoxia response consists of an acute phase and a prolonged phase where transcriptional changes
30 orchestrated by HIFs are central (Fig. 1). During extended oxygen deprivation mitochondrial ATP
31 production by oxidative phosphorylation suffers most, because the majority of oxygen is used there
32 as a terminal electron acceptor. As an adjustment, many HIF target genes encode enzymes and factors
33 that decrease oxidative phosphorylation and induce glucose uptake and glycolysis, thereby a minor
34 ATP production can be maintained in the expense of lactate generation (Fig. 3). To cope with the

latter, the HIF response promotes acid-base homeostasis by upregulating lactate export and recycling for gluconeogenesis (Fig. 3).

Hypoxia, HIFs and HIF-P4Hs in obesity, metabolic syndrome and beyond

According to WHO statistics a quarter of World's adults has metabolic syndrome, characterized by visceral obesity, dyslipidemia, hypertonia and hyperglycemia or insulin resistance, and which predisposes to type 2 diabetes (T2D), cardiovascular diseases, stroke and hepatic steatosis. Several epidemiological studies associate hypoxia in people living at high altitude with a lower prevalence of obesity, T2D, ischemic heart disease and mortality from it or from stroke, but with harmful effects on chronic obstructive pulmonary disease [12-15]. In addition, mouse data corroborate this. For example, exposure of C57Bl/6J mice to chronic environmental hypoxia (8% O₂ for 3 weeks) associated with decreased fat mass, adipocyte size and white adipose tissue (WAT) inflammation [16] (Fig. 4). In line with the substrate preference (Fig. 1), mice with systemic HIF-P4H-1 (PHD1/EglN2) deficiency lowered their skeletal muscle O₂ consumption through activation of PPAR α as well as HIF2 α -mediated development of hypoxia tolerance [17] but had no difference in body weight [18]. These mice also displayed reduced LDL-C levels, less circulating immune cells, and an improved glucose tolerance [9] (Fig. 4). When *Hif-p4h-1*^{-/-} mice were crossed into a LDL receptor (LDLR) knockout background and fed a Western-type diet, they showed protection against atherosclerosis [9] (Fig. 4). However, these studies are opposed by another report in which HIF-P4H-1-deficient mice exhibited a higher body weight, hepatic steatosis, glucose intolerance as well as systemic insulin resistance [19] (Fig. 4). While the reasons for the apparent differences are not known, these mice displayed a certain degree of protection as their metabolic aggravations were not altered when fed a high fat diet (HFD) [19]. Haplodeficiency for HIF-P4H-2 (PHD2/EglN1), the most abundant isoenzyme, associates with lower body weight [18] (Fig. 4). Further, systemic reduction of it in HIF-P4H-2 hypomorphic mice, which display differing HIF1 α and HIF2 α stabilization in various tissues [20, 21], reduced body weight, adiposity and WAT inflammation, lowered hepatic cholesterol synthesis, improved the HDL/LDL+VLDL ratio, amended glucose tolerance and insulin sensitivity and protected against hepatic steatosis [6, 8] (Fig. 4). When these mice were crossed with LDLR mutant mice protection against atherosclerosis was reported [8] (Fig. 4). The hepatic HIF-P4H-2/HIF1 α axis has also been reported to contribute to post-exercise metabolic homeostasis [22]. Global HIF-P4H-3 (PHD3/EglN3) deficiency caused systemic hypotension [23] but no difference in body weight [18] (Fig. 4). By contrast, global inactivation of the HIF asparaginyl hydroxylase FIH (Fig. 1) reduced body weight, elevated metabolic rate and improved glucose and lipid homeostasis, and offered resistance to HFD-induced weight gain and hepatic steatosis [24]. Moreover, activation of

both HIF1 α and HIF2 α in adipocytes of mice by deleting a negative regulator, e.g. HIF-P4H-2, VHL or FIH (Fig. 1), reduces fat mass [6, 24-26]. Oppositely, endothelial cell-specific deficiency of HIF-P4H-2 increased body and fat weight, impaired glucose tolerance and induced hepatic steatosis and fibrosis (Fig. 4) [27].

Interestingly, the findings from the genetic studies were partially reproduced by the preclinical pharmacological pan HIF-P4H-1-3 inhibitor FG-4497. Treatment of C57Bl/6J mice with FG-4497 phenocopied protection against obesity and metabolic dysfunction seen in the HIF-P4H-2 hypomorphs [6] (Fig. 4). FG-4497-treatment also reduced adiposity and improved glucose tolerance in mice fed a HFD suggesting that in addition to prevention, it could be used for obesity treatment [6]. Along with the improved metabolism, systemic FG-4497 treatment of *Ldlr*^{-/-} mice also reduced aortic plaques, reduced inflammation in WAT and led to higher levels of autoantibodies targeting oxidized-LDL [8] (Fig. 4).

In clinical trials with renal anemia patients the HIF-P4H-1-3 inhibitor roxadustat not only corrected anemia but also reduced serum cholesterol levels and improved the HDL/LDL ratio [28] (Table 1). Similar effects on serum lipids were reported by daprodustat [29] whereas vadadustat did not alter serum cholesterol levels [30] (Table 1), however, vadadustat and molidustat were associated with lowered blood pressure [31, 32] (Table 1). Altogether, data from preclinical, clinical and epidemiological studies support that activation of the hypoxia response via HIF-P4H inhibition is a powerful tool to regulate metabolism (Fig. 4). So far, majority of the data associate HIF-P4H inhibition to be beneficial for metabolic health (see Clinician's Corner).

Hypoxia, HIFs and HIF-P4Hs in fatty liver disease

Fatty liver disease is characterized by an excessive accumulation of macro- or microvesicular lipid droplets in the cytoplasm of hepatocytes. Commonly, the presence of lipid droplets in more than 5% of hepatocytes is defined as steatosis [33]. The lipids, mainly triglycerides, can arise from diet, *de novo* lipogenesis or fatty acids released and re-esterified from the adipose tissue. In addition, ethanol, which in many Western cultures is consumed at a daily rate of 20-30 g, leads to formation of fatty acids. Hence, any imbalance in triglyceride acquisition and removal in the hepatocytes can lead to steatosis. Clinically fatty liver disease is categorized into non-alcoholic fatty liver disease (NAFLD) and alcoholic fatty liver disease both of which can progress to fibrosis, cirrhosis and hepatocellular carcinoma (HCC) [34].

Hypoxia, HIFs and HIF-P4Hs in nonalcoholic fatty liver disease (NAFLD)

1 NAFLD became a major health problem in adults and children, since it parallels the pandemic spread
2 of obesity, T2D and cardiovascular diseases [35]. Currently, the worldwide prevalence of NAFLD is
3 estimated to be ~24% [36]. NAFLD comprises a range of conditions including simple, still benign
4 steatosis and pathological nonalcoholic steatohepatitis (NASH). About 10-30 % NASH patients will
5 progress into cirrhosis and about 40–60% of the cirrhosis patients will develop HCC [37]. Despite
6 growing prevalence, the factors influencing NAFLD and progression to NASH, fibrosis, cirrhosis and
7 HCC are poorly understood. Apart from continued nutrient excess, insulin resistance, impaired
8 autophagy, endoplasmic reticulum stress and reactive oxygen species (ROS) formation [38, 39],
9 hypoxia may occur and aggravate development and progression of NAFLD [40, 41]. The role of
10 hypoxia for steatosis development was also supported by a recent mathematical model [42].
11 Interestingly, steatosis and later cellular damage observed in NASH are mainly found in the less
12 oxygenated pericentral areas of the liver acinus (Fig. 5). This goes in line with the concept of
13 “metabolic zonation” (Fig. 5). In that concept, the metabolic capacities in the smallest functional unit,
14 the liver acinus, are found distributed in different zones which exist according to the proximity of the
15 portal triad and the O₂ gradient (Fig. 5) [43].

16 The occurring physiological oxygen gradient, among other morphogens, is considered to be a
17 major modulator of metabolic zonation [43, 44]. In line, it was shown that the expression of the fatty
18 acid synthase (FASN) can be induced by hypoxia [45]. Thereby, HIFs, which are expressed in a
19 zoned manner [46], appear to have not entirely identical functions. While deletion of the common
20 HIF destruction protein VHL [47] and constitutive activation of hepatic HIF2 α , but not HIF1 α , in
21 mice resulted in increased lipid storage associated with steatosis [48], HIF1 α suppressed expression
22 of the β -oxidation involved medium- and long-chain acyl-CoA dehydrogenase (MCAD and LCAD)
23 genes [49]. Accordingly, transcriptomic profiling revealed overlapping but not entirely identical
24 HIF1 α and HIF2 α target genes [47]. In line, hepatocyte-specific deletion of HIF2 α in mice
25 ameliorated NAFLD after feeding either a choline-deficient L-amino acid-defined or a
26 methionine/choline-deficient diet [50]. The NAFLD reduction appeared to be mediated by down-
27 regulated macrophage M1 polarizing histidine-rich glycoprotein (HRGP) expression [50], which
28 links hepatocyte function with inflammation.

29 HIF2 α appears not only to be a stronger regulator than HIF1 α with respect to lipid storage and
30 insulin sensitivity [51], but also with the lipid profile. Studies with hepatocyte-specific VHL,
31 VHL/HIF1 α and VHL/HIF2 α knockout mice revealed that HIF2 α is the major driver of a selective
32 autophagy pathway, namely pexophagy [52]. Since peroxisomes account for about 20% of O₂
33 consumption and 35% of H₂O₂ production in liver [53], it is conceivable that cells reduce the number
34 of peroxisomes during hypoxia. As a consequence of the HIF2 α -driven pexophagy, the decreased

peroxisomal metabolic activity and the HIF2 α -mediated PPAR α antagonism [52, 54] shift the lipid profile to a pattern which is similar to that seen in patients with NAFLD [55]. Hence, a pattern showing an increase of triglycerides with very long-chain and polyunsaturated fatty acids as well as a decrease in docosahexaenoic and arachidonic acid [52, 55]. Moreover, the effects of HIF2 α on pexophagy were not restricted to normal hepatocytes. In particular a reduced number of peroxisomes was found in clear cell renal cell carcinomas with high HIF2 α levels [52]. Another layer of complexity was added by a study linking stabilization of intestinal HIF2 α with NAFLD. Here, deletion of HIF2 α from intestinal cells or the HIF2 α specific inhibitor PT2385 prevented HFD-induced hepatic steatosis [56].

In line with the substrate preference (Fig. 1), it was demonstrated that liver specific lack of HIF-P4H-2 induced HIF1 α , whereas lack of HIF-P4H-3 primarily activated HIF2 α , and that the apparent severe hepatic steatosis in the HIF-P4H-2/HIF-P4H-3 double knockout mice was mainly the result of HIF2 α activation [57]. In addition to steatosis, the liver specific HIF-P4H triple knock-out mice had hepatic vascular malformations and massive erythrocytosis induced by hepatic EPO expression. Interestingly, the presence of a single HIF-P4H-1 or HIF-P4H-3 allele minimized steatosis and vascular malformations while high hematocrit levels persisted, whereas in HIF-P4H-2/3 double knockouts all these parameters were rather normal [58]. Further, HIF-P4H-2 deficiency in mouse liver was shown to enhance hepatic and renal gluconeogenesis from lactate produced in skeletal muscle (Cori cycle) and hence contribute to reduced circulating lactate [59]. Similar results were obtained upon oral administration of the HIF-P4H inhibitors REC2923 or roxadustat, and these effects could be advantageous *e.g.* in T2D patients suffering from metformin-associated lactic acidosis [60]. In addition, liver-specific HIF-P4H-3 deletion in mice was shown to improve insulin signaling and to counteract diabetes [5]. Thereby, HIF-P4H-3 lack acted mainly through HIF2 α stabilization, which then increased insulin receptor substrate-2 (IRS2) transcription and insulin-dependent PKB/Akt activation. Interestingly, a further increase in HIF2 α (and HIF1 α), obtained by additional HIF-P4H-1 and/or HIF-P4H-2 deletions caused liver steatosis and did not lead to additive metabolic improvements [5].

Another report, although not in liver, links HIF-P4H-2 inactivation with regulation of glycolysis, glycogen storage and inflammation [61], the latter being a feature of NASH. In that study, myeloid-specific loss of HIF-P4H-2 in mice caused a glycolysis-dependent delay in inflammation resolution paralleled by enhanced neutrophil survival and augmented chemotaxis. Application of the HIF-P4H inhibitor molidustat also enhanced neutrophil function and delayed resolution of inflammation [61]. Hence, while the liver and kidney-specific lack of HIF-P4H-2 appear to be

1 beneficial under conditions such as HFD or lactic acidosis, the latter study suggests that in myeloid
2 cells it may aggravate inflammation.

3 In summary, the role of HIF-P4Hs in NAFLD appears to be complex and not yet fully
4 understood. While the data mainly from liver/hepatocyte-specific settings with inhibition of all HIF-
5 P4Hs or stabilization of HIF2 α indicate a NAFLD promoting role, the data also suggest that
6 restricting the inhibition to selected HIF-P4H isoenzymes may have beneficial effects.

8 *Hypoxia and HIFs in alcoholic liver disease (ALD)*

9 ALD has several features in common with NAFLD. Steatosis is seen in ~90% of the heavy drinkers.
10 About 30% of them will develop cirrhosis and ~10% of those will progress to HCC [37]. While
11 gender and ethnic specific differences exist, *e.g.* women are more susceptible to ALD than men,
12 obesity has evolved as a common and central risk factor that accelerates ALD [62]. Moreover, other
13 liver diseases such as hepatitis B or C virus infections promote the disease and progression into HCC
14 [37].

15 Acute or chronic ethanol ingestion is supposed to act directly on hepatocytes by finally inducing
16 apoptosis via organelle dysfunction, mediator release and oxidative stress [63]. The latter, together
17 with hormones (*e.g.* catecholamines), contributes to an increase in oxygen consumption [64]. As a
18 result, this leads to hypoxia, which is best visible in the perivenous area of animal models (Fig. 5)
19 and in patients with ALD [63, 65]. Accordingly, HIFs may play a role in the pathogenesis of ALD.
20 Given the common aspects between NAFLD and ALD, such as steatosis as result of HIF2 α activation,
21 one could expect a similar role in ALD. However, studies with HIF2 α in ALD are, to the best of our
22 knowledge, not yet available and only a few studies with respect to HIF1 α in ALD are published.

23 In one study hepatocyte-specific deletion of HIF1 α protected mice from liver damage and lipid
24 accumulation when fed an ethanol containing diet (habituation to a Lieber-DeCarli liquid diet with
25 5% ethanol (v/v) over a period of 2 weeks, then maintained on the 5% diet for 4 weeks). *Vice versa*,
26 hepatocyte-specific overexpression of degradation-resistant HIF1 α resulted in hepatomegaly and
27 steatosis upon ethanol feeding [66]. By contrast, another study using the same animal model and
28 almost identical study protocol (Lieber-DeCarli liquid diet with increasing concentrations of ethanol
29 from 1%-4% within the first week, followed by 6% for a further 4 weeks) showed that hepatocyte-
30 specific knockout of HIF1 α aggravated steatosis [67]. Mechanistically, this appeared to be an indirect
31 regulatory effect involving regulation of lipogenic genes such as FASN or acyl-CoA carboxylase
32 (ACC) via the transcription factor SREBP-1c and its repressor differentiated embryo chondrocyte 1
33 (DEC1) [67]. All in all, more studies are needed to understand the role of HIFs and HIF-P4Hs in
34 ALD.

Hypoxia, HIFs and HIF-P4H in liver fibrosis/cirrhosis/HCC

The pathogenic process from NAFLD/ALD to fibrosis, cirrhosis and HCC is rather complex and involves beside the hepatocytes non-parenchymal cells. For example, hepatocellular damage results in activation of Kupffer and hepatic stellate cells. Drinking alcohol also increases gut permeability and links the Kupffer cells to the intestinal microbiome that can trigger inflammatory responses via toll-like receptor 4 in Kupffer cells and hepatocytes [68]. Consequently, this leads to further recruitment of macrophages, cytokine release and mainly stellate cell-mediated extracellular matrix deposition [69].

Upon fibrosis, the changed tissue architecture causes resistance to blood flow and hypoxia [70]. In fact, this coincides with expression of HIF targets in human liver [71] and HIF1 α stabilization in mouse models [72, 73]. While to date the participation of HIF2 α in liver fibrosis was not really investigated, some reports show that hypoxia mainly via HIF1 α can regulate genes important for collagen synthesis and angiogenesis in stellate cells [74]. With respect to the associated inflammation, the chemokine receptor CXCR4 and its ligand, stromal cell-derived factor-1 (SDF-1), which both are required for infiltration of inflammatory cells, are HIF-target genes [75]. Indeed, deletion of HIF1 α or HIF1 β in cells of the myeloid lineage reduced bile duct ligation-induced fibrosis in mice [76].

Another mechanism contributing to fibrosis is epithelial-mesenchymal transition (EMT). HIF1 α and HIF2 α were shown to regulate EMT in HCC cells [77] via the Snail family [78, 79]. Reports from the kidney show that HIF1-dependent mechanisms contribute to fibrogenesis by inducing expression of Snail1 [80] and another HIF target Twist [81, 82], hence implying that a similar mechanism could also work in liver, however, direct evidence needs to be obtained.

Although the HIF-dependent regulation of Snail appears to be a driver of EMT and to promote fibrosis and HCC, it may also represent an important feedback mechanism for NAFLD-associated insulin resistance. A recent report showed that hepatocyte-specific deletion of Snail1 enhanced insulin-stimulated lipogenesis in hepatocytes and aggravated NAFLD in mice, which was opposed by liver-specific Snail1 overexpression. Specifically, Snail repressed FASN promoter activity epigenetically via recruitment of HDAC1/2 and deacetylation of H3K9 and H3K27. Thus, this raises in contrast to the rather lipogenic effects of HIFs, the possibility that Snail1 induction by HIFs could partially counteract NAFLD in obesity [83].

The findings that EMT can be regulated by HIFs goes in line with higher HIF1 α and HIF2 α levels in HCC than in non-tumor-surrounding tissues [84]. These findings are important since HCC is the second leading cause of cancer-related death worldwide [85] and HIF1 α was positively correlated with higher HCC grade and reduced survival [86, 87].

1 NAFLD/NASH, ALD, T2D, abuse of drugs and congenital disorders contribute to cirrhosis and
2 HCC in only about 20% of the cases. About 80% HCC cases are based on chronic hepatitis B and C
3 infections [88]. Interestingly, the hepatitis virus B X-protein was able to enhance HIF1 α levels in cell
4 cultures and correlated positively with the expression of HIF1 α in HCC material from hepatitis B
5 virus infected patients [89]. Moreover, hepatitis C virus glycoproteins promoted EMT and hepatoma
6 cell migration via HIF1 α -dependent upregulation of Snail and Twist [90].

7 The possible involvement of the HIF system in HCC is also indirectly reflected by the currently
8 used therapies or phase III clinical trials against HCC. Drugs, such as sorafenib, regorafenib,
9 lenvatinib, cabozantinib and ramucirumab, target tyrosine kinase receptor family members binding
10 vascular endothelial growth factor, placental-derived growth factor, stem cell factor and stem cell-
11 derived factor, which are virtually directly or indirectly regulated by hypoxia/HIFs [91, 92]. Although
12 this picture is not entirely complete since various other important players feed into the pathogenesis
13 of HCC, these findings suggest a supportive role of hypoxia and HIF1 α in disease progression,
14 whereas the role of HIF2 α appears to be conflicting. Two studies indicated that HIF2 α promotes HCC
15 [86, 93] and another study reported that HIF2 α inhibits HCC progression [94].

16 When HCC induced with diethylnitrosamine was studied in HIF-P4H-2 haplodeficient (*Hif-*
17 *p4h-2*^{+/-}) mice, these mice had more aggravated HCC growth potentially due to Notch activation [95].
18 Importantly, the effects of HIF-P4H-2 haplodeficiency appeared to be a rather late event and
19 manifested during tumor nodule formation and not early on during neoplastic transformation [96].
20 Interestingly, the *Hif-p4h-2*^{+/-} mice appeared to be less prone for metastasis. Although not shown for
21 HCC, those mice generate less metastasis than wild-type upon transplantation of *Hif-p4h-2*^{+/+} tumor
22 cells. These effects are due to an improved vessel architecture within the tumor mediated mainly via
23 HIF2 α [97]. Moreover, metastasis was also reduced when the *Hif-p4h-2* haplodeficiency was
24 introduced to a genetic breast cancer model (PyMT mice). Mechanistically, metastasis reduction was
25 the result of a decreased activation of cancer associated fibroblasts, and a HIF-independent reduction
26 of TGF- β 1 release and matrix production [98]. In line, when testing the systemically administered
27 HIF-P4H pan-inhibitor ethyl-3,4-dihydroxybenzoate in a preclinical setting of extended partial
28 hepatectomy to remove colorectal liver metastases, it was found to augment liver regeneration [99].

29 Conflicting data with respect to HIF-P4H-2 expression and HCC in human samples were
30 reported. One study associated high HIF-P4H-2 expression with larger and higher stage tumors and
31 poorer prognosis [100], whereas another one reported an association of lower hepatic HIF-P4H-2
32 levels with cancerous tissue and a longer patient survival upon elevated HIF-P4H-2 levels [101].
33 Interestingly, HIF-P4H-3 expression seems to be a favorable prognostic marker for patients with
34 HCC [102] since its downregulation in HCC was associated with more aggressive tumor behavior

1 and poor prognosis [103]. Altogether, the mechanisms involved in the progression of fatty liver
2 diseases to fibrosis/cirrhosis/HCC are many and involve not only the HIF system but also the cross-
3 talk with various other signaling pathways. This likely represents an underlying cause for
4 contradictory appearing data on the roles of HIFs and HIF-P4Hs and call for more studies to draw
5 conclusions.
6

Concluding remarks

Various lines of evidence indicate that hypoxia, inhibition of HIF-P4Hs and stabilization of HIFs are causally linked to various aspects of metabolism (Fig. 4). Hence, disrupting that causal chain via HIF-P4H inhibition could be an efficacious therapeutic option to treat the global epidemic of obesity, metabolic syndrome, T2D and fatty liver diseases. However, at the moment it is uncertain whether the current HIF-P4H inhibitors, which have been optimized to induce erythropoiesis, can be of ultimate benefit due to the pleiotropic and context dependent activities of the different HIFs in particular, as well as lacking information on specific diseases, *e.g.* ALD. From the data reviewed here, it appears that HIF-P4H inhibition could be beneficial with respect to obesity, metabolic syndrome and hyperlipidemia and under conditions similar to T2D (Fig. 4). Regarding NAFLD, a dose-dependent HIF-P4H inhibitory window, primarily targeting HIF2 α , and avoiding aggravation of steatosis and inflammation could mediate advantage. Thus, additional molecular studies are necessary to address whether the observed steatosis upon combined genetic HIF-P4H-1-3 deficiency is solely dependent on the dosage of HIF2 α , and to which extent other, HIF-independent effects contribute to metabolism regulation. On the other hand, HIF2 α inhibition, for example with the novel specific inhibitor PT2385 in clinical trials for renal carcinoma [56], may offer potential in fatty liver diseases. The knowledge gained will substantiate the common and different involvement of HIF-P4Hs and HIFs in the regulation of metabolism. At the end, the one or the other inhibitor, dependent on the dose, duration and features, like HIF-P4H isoenzyme, HIF α paralogue or cell type selectivity, could be a therapeutic option to treat *e.g.* NAFLD.

Considering this, a prerequisite for the use of HIF-P4H inhibitors in general, and in particular in liver disease, should be that it would not aggravate pre-existing liver diseases. Although in majority of reports HIF-P4H inhibition is associated with protection against inflammation [3, 4], the recent findings from mice where the HIF-P4H inhibitor molidustat aggravated inflammation [61] suggests that long-term use of currently available HIF-P4H inhibitors in situations such as NASH requires caution and may be contraindicated. On the other hand, all HIF-P4H inhibitors currently in clinical trials appear to be well tolerated, but only one has been tested in an open-label study with eight subjects having moderate hepatic impairment (liver cirrhosis Child-Pugh score 7-9) and eight subjects with normal hepatic function. When these patients received a single oral dose of 100 mg roxadustat under fasting conditions they did not show differences of clinical significance in pharmacokinetics and pharmacodynamics [104].

Moreover, the recent identification of non-HIF α HIF-P4H substrates (Fig. 2) suggest existence of several cross-talk options which may be of special interest in HCC given the potential opposing

1 actions of HIF1 α and HIF2 α in cancer progression [105]. Because of its poor prognosis, limited
2 treatment options and high risks of tumor recurrence and metastases [88], it is important to better
3 understand the contribution of HIFs and the HIF-P4H system in the pathogenesis of HCC and its
4 preceeding entities fibrosis and cirrhosis. In particular, it would be extremely important to understand
5 at which stage and under which conditions either HIF or HIF-P4H inhibitory effects with or without
6 HIF stabilization would be of value for the treatment of fibrosis/cirrhosis or even HCC (see
7 Outstanding Questions). Considering this, HIF-P4H inhibitors that exploit differences in binding of
8 HIFs and non-HIF substrates would be of interest, but at current, it is unknown how difficult it will
9 be to develop those in a clinically acceptable manner.

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2 We apologize to all researchers who excellently contributed to the field and whose work could not be
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1 **Highlights**

2 Inhibition of HIF-P4Hs, which act as molecular oxygen sensors, is the so far best understood
3 mechanism to regulate the oxygen sensing pathway.

4

5 Several HIF-P4H inhibitors are currently in phase 3 randomized clinical trials for treatment of renal
6 anemia with no reported serious side effects until now.

7

8 Recent data from preclinical, clinical and epidemiological studies support that besides anemia
9 activation of the hypoxia response via HIF-P4H inhibition is a powerful tool to regulate metabolism
10 and may be beneficial for conditions such as obesity, metabolic syndrome, T2D, NAFLD/NASH or
11 liver fibrosis.

1 **Outstanding Questions**

- 2 Can HIF-P4H inhibitors be used in treatment of diseases beyond renal anemia in metabolic disorders,
3 such as obesity, metabolic syndrome, NAFLD, ALD, T2D or atherosclerosis?
- 4 HIF stabilization is associated with cancers. Is chronic HIF-P4H inhibition that results in long-term
5 HIF stabilization safe?
- 6 Which HIF-P4H isoenzyme and/or tissue should be targeted to treat metabolic diseases?
- 7 Are there HIF-independent HIF-P4H substrates that mediate effects on metabolism and which could
8 be targeted by even more selective HIF-P4H inhibitors?
- 9 Can HIF α isoform agonists be used instead of HIF-P4H inhibitors to receive the same or more
10 selective effects?
- 11 Can HIF-P4H inhibition be achieved by other means than chemical antagonists, such as exposure to
12 high altitude or lowering of tissue oxygenation for example by manipulation of hemoglobin levels?

1 **Clinician's Corner box**

- 2 • The cellular oxygen sensors HIF-P4Hs can be inhibited by oral administration of small molecules
3 which can stabilize HIF and initiate the hypoxia response under normoxia
- 4 • HIF-P4H inhibitors (such as roxadustat, vadadustat, molidustat and daprodustat (Table 1)) have
5 advanced up to phase III clinical trials for treatment of renal anemia because they upregulate
6 erythropoietin expression
- 7 • Preclinical data suggest that HIF-P4H inhibition may have beneficial effects in treatment of
8 obesity and metabolic dysfunction
- 9 • In clinical trials for anemia HIF-P4H inhibitors have been reported to reduce serum total
10 cholesterol levels and improve HDL/LDL ratio
- 11 • More clinical trials assessing the potential of HIF-P4H inhibitors in metabolic disorders are
12 required

1 **Glossary**

2 **Alcoholic liver disease (ALD):** develops based on alcohol overconsumption, and is characterized by
3 steatosis, alcoholic and chronic hepatitis as well as in chronic cases often with liver fibrosis or
4 cirrhosis.

5 **Angiogenesis:** the formation of new blood vessels from pre-existing vessels.

6 **Autophagy:** an intracellular degradation process that takes place via the delivery of cytoplasmic
7 entities to the lysosomes, where macromolecules are lysed and their components recycled.

8 **Chronic kidney disease (CKD):** impairs gradually kidney function and erythropoietin production
9 and results in renal anemia . Caused by type 1 or type 2 diabetes (T2D), high blood pressure, infection
10 or polycystic kidney disease.

11 **Epithelial-mesenchymal transition (EMT):** a process by which epithelial cells lose their adhesion
12 and cell polarity and gain migratory and invasive properties.

13 **Erythropoietin (EPO):** hormone secreted by the kidney promoting erythropoiesis in bone marrow.

14 **End stage renal disease (ESRD):** advanced state of CKD.

15 **Hepatocellular carcinoma (HCC):** the most common type of primary liver cancer in adults and the
16 most common cause of death in people with cirrhosis.

17 **Hepcidin:** Peptide hormone produced by liver that inhibits iron transport. Upregulated in
18 inflammatory conditions.

19 **Hypoxia:** a condition in which the body or a region of the body is deprived of adequate oxygen supply
20 at the tissue level. Can result *e.g.* from anemia or ischemia.

21 **Hypoxia-response element (HRE):** genomic sequence 5'-RCGTG-3' which HIF binds to in the
22 regulatory region of target genes.

23 **Metabolic syndrome:** clustering of conditions including obesity, hyperglycemia or insulin
24 resistance, hypertriglyceridemia, hypertension and low HDL levels. Increases risk for cardiovascular
25 diseases, T2D and NAFLD.

26 **Myelodysplastic syndrome (MDS):** group of disorders in which immature blood cells in the bone
27 marrow do not mature properly.

28 **Non-alcoholic fatty liver disease (NAFLD):** includes a range of conditions from benign steatosis
29 to NASH. Although mainly affecting hepatocytes, other cells such as stellate cells, CD4+ and CD8+
30 T cells or NK-T cells, contribute to NAFLD.

31 **Non-alcoholic steatohepatitis (NASH):** characterized by steatosis with concomitant infiltration of
32 liver tissue with inflammatory leukocytes as well as fibrosis. One third of NASH patients will
33 progress into cirrhosis, which constitutes a basis for development of HCC.

34 **Type 2 Diabetes (T2D):** chronic and progressive condition caused by impaired insulin response
35 resulting in hyperglycemia.

36 **White adipose tissue (WAT):** major energy storing tissue in human body composed of adipocytes.

1 **Figures and figure legends**

2

A)

B)

3

4 **Figure 1. Oxygen sensing via acute responses and altered gene expression**

5 (A) Lack of oxygen initiates adaptive responses involving short-term and long-term modifications
6 without and with changes in gene expression, respectively. Structural changes driven by modified
7 gene expression allow long-term adaptation to altered oxygenation. The processes can be interlinked
8 when short-term responses include changed channel or enzyme activities which regulate transcription
9 factors. (B) Long-term responses to hypoxia in metazoan cells are primarily regulated by HIFs. HIFs
10 are αβ-heterodimeric transcription factors which bind to hypoxia response elements (HREs) in
11 regulatory regions of genes. The HIF system regulates >300 genes to balance oxygen supply and
12 demand (for HIF target genes see [106]). The three α-subunits, HIF1α-3α (green), are labile under

1 normoxia, whereas the β -subunit (HIF1 β /ARNT) (rose) is stable. When oxygen is available, three
2 HIF-P4H isoenzymes (PHDs/EglNs) (orange) hydroxylate one or two proline residues (4-Hyp) in
3 HIF α subunits, which marks them for ubiquitinylation by the von Hippel-Lindau (pVHL) protein
4 (yellow) and proteasomal degradation. The catalytic activity of HIF-P4Hs is highly dependent on
5 oxygen and hypoxia destabilizes HIF α [107]. HIF-P4H-2 (PHD2/EglN1) is the most abundant
6 isoenzyme and the major one regulating HIF α stability. In kidney, inactivation of HIF-P4H-2 was
7 sufficient to induce EPO expression while in brain pericytes and hepatocytes it required simultaneous
8 inactivation of HIF-P4Hs 2 and 3 or all HIF-P4Hs, respectively [57, 108]. FIH (magenta), which
9 shares the same reaction mechanism and cofactors with HIF-P4Hs but is less dependent on oxygen,
10 hydroxylates an asparagine residue in HIF1 α /2 α . The hydroxyasparagine (Asn-OH) prevents binding
11 of transcriptional coactivators, like CBP/p300 (petrol), and therefore inhibits HIF target genes
12 expression.

13

1

2 **Figure 2. Regulators of HIF-P4H catalytic activity**

3 HIF-P4Hs 1-3 belong to 2-oxoglutarate-dependent dioxygenases (2-OGDDs) with ~70 members in
 4 humans that share the same reaction mechanism but act on different substrates (proteins, DNA, RNA,
 5 and fatty acids). They require Fe^{2+} , 2-oxoglutarate (2-OG) and O_2 as cofactors and hydroxylate
 6 proline residues to 4-hydroxyproline (4-Hyp). One atom of oxygen becomes incorporated into the
 7 substrate while 2-OG decarboxylates to succinate and CO_2 . HIF-P4Hs also require a reducing factor,
 8 such as vitamin C, L-cysteine, reduced glutathione (GSH) or DTT [109]. Several naturally or
 9 pathologically occurring 2-OG analogues, such as fumarate, succinate or S-2-hydroxyglutarate (S-
 10 2HG), have been shown to inhibit HIF-P4H catalytic activity competitively. The oncometabolite R-
 11 2HG, generated by cancer-associated isocitrate dehydrogenase variants, did not inhibit HIF-P4Hs but
 12 could instead act as an alternative cofactor for 2-OG to promote catalysis [110]. Chemical 2-OG
 13 analogue inhibitors, such as the preclinical pan 2-OGDD inhibitor dimethyl oxalylglycine (DMOG)
 14 and the renal anemia therapeutic roxadustat, have been developed. In effect, it is the ratio of the 2-
 15 OG analogue to 2-OG which appears to determine the effect on catalytic activity. Other divalent
 16 metals (*e.g.* Zn^{2+}) and iron chelators (such as desferrioxamine (DFO)) can also inhibit HIF-P4H
 17 catalysis as can reactive oxygen species (ROS). In addition to HIFαs, other non-HIFα substrates have
 18 emerged for HIF-P4Hs. Among those are the HIF-P4H-1 substrates FOXO3a [111], I-kappaB kinase
 19 [112], the HIF-P4H-2 substrates N-myc downstream-regulated gene 3 (NDRG3) [113], AKT1/2
 20 [114] and the HIF-P4H-3 substrates β 2-adrenergic receptor [115], pyruvate kinase M2 [116] and
 21 acetyl-CoA carboxylase [117]. Chemical analogues are indicated by italics.

22

Figure 3. Reprogramming of energy metabolism by HIF target genes.

While HIF2 α seems to have a dominant role in lipid metabolism, HIF1 α appears to have a stronger impact on structural maintenance, mitochondrial function and carbohydrate metabolism [118]. Indeed, HIF1 α regulates numerous genes involved in glucose uptake (GLUT1, 3) and glycolysis (HK1, 2, GAPDH, PFKL, ENO1, PGK1) [91, 119]. This seems to be even more evident under challenging conditions, such as liver regeneration [120]. Among the HIF1 α target genes, lactate dehydrogenase A (LDHA) directs glycolytic flux to lactate production and pyruvate dehydrogenase kinases 1 and 3 (PDK1, 3) block pyruvate entry into the mitochondrial tricarboxylic acid (TCA) cycle [121, 122]. Monocarboxylate transporter 4 (MCT4) and carbonic anhydrase IX (CA9), both additional HIF1 α target genes, export lactate for reuse in gluconeogenesis and contribute to balancing acid-base homeostasis, respectively [123]. With respect to mitochondrial function it is important to note that HIF1 α induces expression of the electron transport chain (ETC) complex I protein NADH dehydrogenase [ubiquinone] 1 alpha subcomplex, 4-like 2 (NDUFA4L2) [124] and promotes a switch from the cytochrome c oxidase COX4-1 subunit to COX4-2 in electron transport complex IV by upregulating expression of LON, a mitochondrial protease that is required for COX4-1 degradation [125]. Consequently, this inhibits complex I activity and increases efficiency of complex IV leading to reduced oxygen consumption and decreased production of detrimental ROS. Similar to HIF2 α , HIF1 α also contributes to selective autophagy via enhanced expression of BNIP3 [126] and miR210 [127]. HIF1 α promotes mitophagy and blocks Fe/S cluster assembly which additionally prevents ROS production and cell death. Red, HIF target genes.

1

2 **Figure 4. Functions and processes reported to be mediated by HIF-P4H inhibition in key**
 3 **metabolic tissues.** Inhibition of HIF-P4H isoenzymes has been reported to influence body weight,
 4 glucose and lipid metabolism, inflammation, immunology and cellular differentiation and
 5 regeneration in skeletal muscle, liver and white adipose tissue (WAT), as indicated. The variables
 6 measurable from blood are indicated under circulation.

Figure 5. The oxygen gradient in liver and metabolic zonation. (A) Classic liver lobule with a central vein (CV), portal triads (PT) containing branches from the portal vein (blue dot), hepatic artery (red dot) and bile duct (green dot). The blood coming from the portal vein and hepatic artery flows as a mixture through the sinusoids to the central vein. Due to metabolism and elimination the composition of blood changes, and among others an oxygen gradient ranging from about 60 to 65 mmHg (84-91 $\mu\text{mol/l}$) (dark blue) in the periportal (pp) blood to about 30 to 35 mmHg (42-49 $\mu\text{mol/l}$) (light blue) in the perivenous (pv) blood is formed. The intracellular pO_2 is again ~ 15 mmHg lower, *i.e.* 45-50 mmHg in the periportal and 15-20 mmHg in the perivenous cells, respectively (for review see [43]). Although being subdivided, three principal zones can be distinguished: the periportal zone (1), the intermediary zone (2) and the perivenous/pericentral/ centrilobular zone (3). (B) Distribution of major metabolic pathways. pp, periportal; pv, perivenous; AA, amino acids; Cho, cholesterol synthesis; Ggn, glycogen; Lac, lactate; GS, glutamine synthesis. For more details see [43] and references therein.

1 **Table 1. HIF-P4H inhibitors in clinical trials**

Substance (other names)	Company	Clinical trial (most advanced phase completed)	Indications
Roxadustat (FG-4592, ASP1517)	FibroGen/Astellas Pharma/Astra Zeneca	III	CKD, ESRD, anemia, MDS
Daprodustat (GSK1278863, GSK1278863A)	GlaxoSmithKline	III	Anemia, tendon injuries, surgical procedures, peripheral vascular disease, wound healing, diabetic foot, kidney disease
Vadadustat (AKB-6548, MT6548)	Akebia Therapeutics, Mitsubishi Tanabe Pharma Corporation	II	CKD, anemia
Molidustat (BAY85-3934)	Bayer	II	Anemia, chronic renal insufficiency, kidney diseases
FG-2216	FibroGen	II	Renal anemia
Desidustat (ZYAN1)	Cadila Healthcare	I	CKD
DS-1093a	Daiichi Sankyo	I	CKD, anemia, renal anemia
JTZ-951	Akros Pharma	I	CKD

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