

# Therapeutic inhibition of prolyl hydroxylase domain-containing enzymes in surgery: putative applications and challenges

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**Abstract:** Oxygen is essential for metazoans to generate energy. Upon oxygen deprivation adaptive and protective pathways are induced, mediated by hypoxia-inducible factors (HIFs) and prolyl hydroxylase domain-containing enzymes (PHDs). Both play a pivotal role in various conditions associated with prolonged ischemia and inflammation, and are promising targets for therapeutic intervention. This review focuses on aspects of therapeutic PHD modulation in surgically relevant disease conditions such as hepatic and intestinal disorders, wound healing, innate immune responses, and tumorigenesis, and discusses the therapeutic potential and challenges of PHD inhibition in surgical patients.

**Keywords:** hypoxia, HIF, PHD, PHI, surgery

## Introduction

Since oxygen is indispensable for life, insufficient oxygen availability can lead to cellular dysfunction and ultimately cell death. However, metazoans are able to sense and respond to altered oxygen tension in several physiological and pathological settings. Hypoxia-inducible factors (HIFs) play a central role in this adaptation process and have therefore been the subject of intensive and rapidly expanding research in the past two decades.<sup>1,2-4</sup> The HIF-mediated adaptive response is orchestrated by HIF prolyl hydroxylase domain-containing enzymes (PHDs), which fulfill specific functions in multiple physiological and pathophysiological processes. Pharmacological manipulation of PHDs therefore represents an interesting therapeutic opportunity in various disorders with high relevance in surgery.

This article reviews the current evidence on PHD function in various visceral organs and diseases, which has emerged from studies applying preclinical disease models in gene-deficient mice or on the pharmacological inhibition of PHDs (prolyl hydroxylase domain-containing enzyme inhibitors [PHI]), as well as from expression studies in human disease. We do not provide a comprehensive literature survey, but focus on the clinical and translational aspects of PHD modulation by inhibitors (PHI) in visceral surgery and discuss the putative applications and challenges of this novel therapeutic approach.

## Hypoxia-inducible factors and prolyl hydroxylase domain-containing enzymes

Three HIF isoforms are known (HIF1, -2, and -3), but HIF1 and -2 have been the most extensively studied. While HIF1 is ubiquitously expressed, HIF2 is more restricted.<sup>5</sup> HIFs

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are transcriptional regulatory proteins, which are composed as heterodimers consisting of two subunits: an alpha ( $\alpha$ )-subunit, regulated by hypoxia, and an oxygen-independent beta ( $\beta$ )-subunit, which is constitutively expressed. In normoxia the  $\alpha$ -subunit is hydroxylated, which generates a binding site for von Hippel–Lindau tumor-suppressor proteins of the E3 ubiquitin proteasome ligase complex, resulting in polyubiquitination and subsequent proteasomal degradation.<sup>6</sup> An autosomal recessive mutation in the von Hippel–Lindau (*VHL*) gene (Chuvash syndrome) leads to chronically elevated HIF1 $\alpha$  levels and is associated with polycythemia, peripheral thrombosis, vascular hypertension, vascular abnormalities, and premature mortality.<sup>7</sup> Congenital autosomal dominant mutations in the *VHL* gene (von Hippel–Lindau disease) predispose to cerebral and retinal hemangioblastoma and renal cell carcinoma.<sup>8</sup> In hypoxia, the HIF  $\alpha$ -subunits accumulate and dimerize with the  $\beta$ -subunits configuring active HIF. The heterodimers are translocated from the cytoplasm to the nucleus, where they bind to hypoxia response elements in the promoter region of downstream target genes, thus modulating the adaptive cellular response. More than 150 HIF-target genes have been identified, including those regulating angiogenesis, cell proliferation, metabolism, and apoptosis.<sup>5</sup> This multifold response indicates the great potential for therapeutic manipulation of the HIF pathway.

PHDs function as oxygen sensors, because they require oxygen (besides iron, 2-oxoglutarate [2OG] and antioxidants like ascorbate or glutathione [GSH]) as an essential co-substrate for the hydroxylation of the HIF $\alpha$ -subunit.<sup>9</sup> PHDs are non-heme iron containing 2OG-dependent dioxygenases, and belong to the family of prolyl 4-hydroxylases (P4Hs). The P4H enzyme family consists of collagen- and HIF-P4Hs, which are members of a class of over 60 2OG-dependent dioxygenases.<sup>5,10</sup> The group of HIF-PHDs comprises four members: PHD1, PHD2, PHD3, the factor-inhibiting hypoxia-inducible factor (FIH), all of which display a 42%–59% sequence similarity.<sup>11</sup> All PHDs are able to hydroxylate HIF $\alpha$  in vitro, but it remains unclear in what proportional contribution.<sup>5</sup>

In normoxia and mild hypoxia PHD2 is the main regulator of HIF1 $\alpha$  due to its relatively abundant frequency in most cells.<sup>12,13</sup> In severe and prolonged hypoxia PHD3 regulates HIF2 $\alpha$  more efficiently.<sup>12</sup> Knockout of PHD2 leads to stabilization of HIF1 $\alpha$ , not HIF2 $\alpha$ .<sup>14,15</sup> In contrast, PHD1 and PHD3 double knockout results in accumulation of HIF2 $\alpha$ , not HIF1 $\alpha$ .<sup>15</sup>

PHDs are ubiquitously expressed, however, the PHD homologs display particular, partly overlapping tissue- and subcellular-specific RNA and protein-expression

patterns.<sup>11,12,16</sup> PHD1 is highly expressed in the testis and liver. PHD2, the most abundant homolog, is expressed in all organs. PHD3 is mainly expressed in the heart.<sup>12,16</sup> On the subcellular level, PHD1 is present in the cell nucleus, PHD2 mainly in the cytoplasm, and PHD3 equally in both.<sup>12,16,17</sup> Nevertheless, subsequent studies using monoclonal antibodies have indicated that all PHDs are mostly located in the cytoplasm.

Genetic deletion of PHD1 in mice does not cause any phenotypical effects in healthy conditions, but induces remarkable tolerance to muscle ischemia and reduced exercise endurance.<sup>18</sup> Prenatal PHD2 deficiency is embryonically lethal due to placentation defects. Postnatal PHD2 deficiency promotes angiogenesis, polycythemia, and congestive heart failure.<sup>14,19</sup> PHD3 deficiency results in a hypofunctional sympathoadrenal system and reduced blood pressure.<sup>20,21</sup>

## Prolyl hydroxylase domain-containing enzyme inhibitors

PHDs are increasingly considered promising therapeutic targets for pharmacological modulation in various clinical settings involving acute or chronic hypoxia. The biochemistry of PHDs and PHI has been previously reviewed.<sup>4</sup> In general, PHI interfere with PHD activity either nonselectively by replacing their essential co-substrates (iron and 2OG) or directly blocking the enzymes' catalytic site. The PHI deferoxamine, an iron chelator, and cobalt chloride (CoCl<sub>2</sub>), a competitive iron inhibitor, compete for endogenous iron, and therefore can have systemic side effects. Pan-inhibitors, such as L-mimosine, dimethylxalylglycine (DMOG), and ethyl-3,4-dihydroxybenzoate (EDHB), inhibit PHD function by mimicking 2OG, an intermediate of the tricarboxylic-acid cycle.<sup>5,22</sup> However, several other tricarboxylic-acid-cycle intermediates such as citrate, isocitrate, succinate, fumarate, malate, oxaloacetate, and pyruvate also compete for binding to the active site and thus function as PHI.<sup>23,24</sup> Moreover, reactive oxygen species (ROS) and nitric oxide (NO) can act as potent inhibitors of PHD activity [ie, by converting Fe(II) to Fe(III) and by chelating Fe(II), respectively] or via nitric oxide (by chelating Fe[II]), emphasizing the crucial effects of oxidative stress on the PHD–HIF axis.<sup>25,26</sup>

More recently developed PHI preferentially target protein–protein interactions, PHDs' amino- or carboxyl terminal ends (eg, FK506-binding protein 38 [FKBP38]) or their active site (eg, TM6008 and TM6089).<sup>27–29</sup> However, the PHDs' catalytic site is highly conserved, thus hampering the development of isoform-specific PHI.<sup>30</sup> Present research increasingly focuses on the development of small-molecule

inhibitors of PHDs like JNJ-42041935, FG-4497, TRC160334, and AKB-4924.<sup>31–34</sup> The use of small interfering ribonucleic acids (siRNAs) as PHI has also been considered.<sup>35,36</sup>

The greatest challenges remain:

- First, the enormous complexity within the PHD-HIF pathway, which regulates multiple genes, while at the same time interacting with multiple other signaling pathways (eg, the nuclear factor kappa-light-chain-enhancer of activated B cells [NF- $\kappa$ B] pathway, which links hypoxia to inflammation).<sup>37</sup>
- Second, the selectivity of PHI regarding HIF-PHDs: in order to prevent considerable adverse effects, PHIs should not only be selective for HIF-PHD (instead of targeting multiple other 2OG-dependent dioxygenases), but also for different HIF-PHD homologs. However, crystallographic and sequence analyses revealed that the active site is highly conserved among PHDs and FIH, thus hampering the development of isoform-specific PHI.<sup>30</sup>
- Third, the identification of the ideal therapeutic niche, which involves not only careful selection of clinical settings, but also the appropriate timing and duration of PHD inhibitor administration. Direct HIF-independent effects of PHI might have a more rapid onset since they occur posttranslationally, whereas downstream effects of HIF stabilization might be delayed.<sup>38</sup> In this context, it will be essential to define the cut-off between alleviation and aggravation of symptoms, and to balance the benefits and systemic side effects of PHD inhibitor therapy. Moreover, application routes to enable organ-specific treatment would be desirable. Given the well-known clinical symptoms of Chuvash polycythemia and von Hippel–Lindau disease, long-term studies are required to elucidate the effects of permanent PHD inhibition associated with continuous HIF activation. In this context, a combination therapy of PHIs with additional drugs should also be considered to fine-tune therapeutic responses.<sup>5</sup>
- Ultimately, there is an urgent need for clinical studies. Clinical studies about PHD inhibition are scarce, albeit that the first Phase II and III clinical trials testing PHI for anemia in chronic kidney disease are ongoing and appear to be encouraging. Peer-reviewed results, however, are pending.<sup>39</sup>

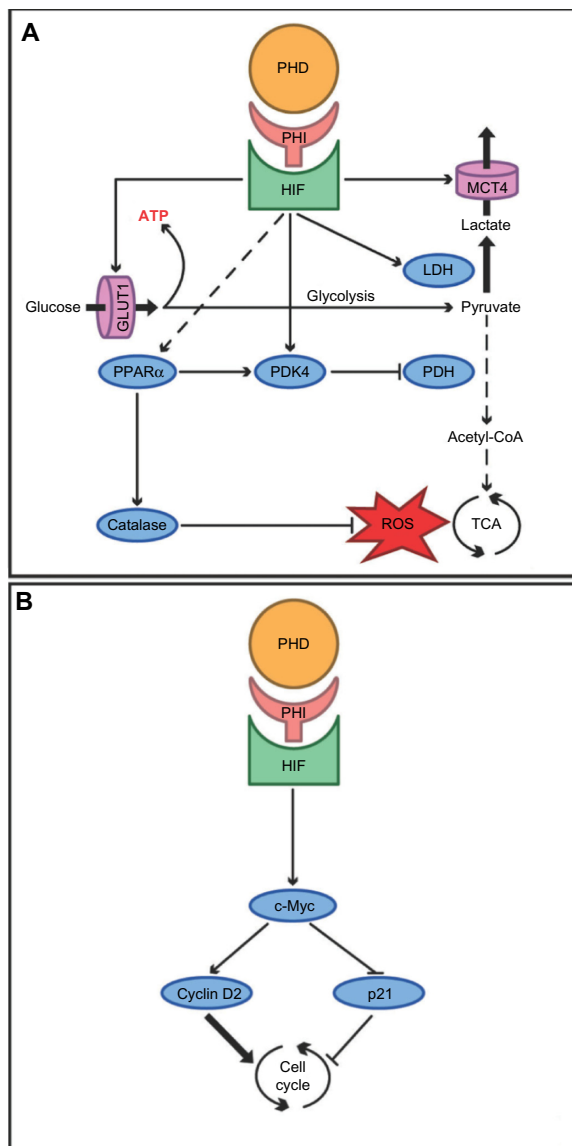
## The putative effects of prolyl hydroxylase domain-containing enzyme inhibitors in the liver

Liver dysfunction following acute or prolonged hepatic ischemia, which might occur after major liver resection or liver

transplantation, represents a major challenge in hepatobiliary surgery.<sup>40</sup> Ischemia/Reperfusion (I/R) injury is considered the major contributor to primary allograft failure secondary to transplantation.<sup>41</sup> Moreover, hepatic I/R injury promotes remote organ inflammation, which may ultimately lead to sepsis and multiorgan failure, emphasizing the key role of organ ischemia in the pathogenesis of severe, systemic complications.<sup>42</sup> Intriguingly, recent insights from preclinical studies suggest that PHD inhibition might exert beneficial effects on liver function in various post-surgical conditions, which are outlined following.

### Liver ischemia and ischemia/reperfusion damage

Hepatocytes are heavily dependent on mitochondrial adenosine triphosphate (ATP) production. In I/R conditions, however, mitochondrial redox processes promote mitochondrial dysfunction, cellular energy deficiency, oxidative stress, and uncontrolled production of ROS. The resultant oxidative stress is further aggravated by the recruitment of local and circulating inflammatory cells, altogether causing irreversible cell damage and postischemic liver failure.<sup>43,44</sup> Preclinical studies in rodents revealed that PHIs are hepatoprotective in the setting of warm hepatic I/R, an effect that is at least partially mediated by antioxidant effects of the HIF1-target gene for heme oxygenase 1 (HO-1).<sup>45</sup> Interestingly, the combination of I/R injury followed by resection of the nonischemic liver remnant is lethal in up to two-thirds of wild-type mice, but survived by 100% of PHD1-deficient mice. These effects may be attributable to the fact that PHD1 deficiency provides hypoxia tolerance to hepatocytes via stabilization of HIF2 $\alpha$  and reprogramming of basal-cell metabolism (Figure 1A): loss of PHD1 function upregulates basal expression of the pyruvate dehydrogenase kinase isozyme 1 (PDK1), leading to reduced oxidative metabolism and less ROS production in ischemic conditions.<sup>36</sup> These effects seem to be comparable to previously described effects of PHD1 deficiency on the energy metabolism in skeletal myofibers, which are partly mediated by the master regulator of energy metabolism, peroxisome proliferator-activated receptor alpha (PPAR $\alpha$ ), ultimately leading to increased expression of catalase and pyruvate dehydrogenase kinase 4 (PDK4). While catalase contributes to the detoxification of ROS, PDK4 inactivates the enzyme pyruvate dehydrogenase (PDH), causing a shift of glucose metabolism from oxidative to more anaerobic ATP production.<sup>18</sup> Further, direct effects of HIF are increased glucose uptake via glucose transporter 1 (GLUT1), conversion of pyruvate to lactate catalyzed by lactate dehydrogenase, and clearance of lactate



**Figure 1** The putative effects of prolyl hydroxylase domain-containing enzyme inhibitors (PHI) in the liver.

**Notes:** (A) In liver ischemia and reperfusion PHI stabilize hypoxia-inducible factor (HIF), thus inducing a shift of glucose metabolism from oxidative to more anaerobic adenosine triphosphate (ATP) production, and leading to reduced mitochondrial oxidative stress involving the master regulator of energy metabolism, peroxisome proliferator-activated receptor alpha (PPAR $\alpha$ ), as well as catalase, pyruvate dehydrogenase kinase 4 (PDK4), and lactate dehydrogenase (LDH). While catalase detoxifies reactive oxygen species (ROS), PDK4 inactivates pyruvate dehydrogenase (PDH), reducing the conversion of pyruvate to acetyl-CoA. LDH catalyzes the conversion of accumulated pyruvate to lactate. Further, direct effects of HIF comprise increased glucose uptake via glucose transporter 1 (GLUT1) and lactate clearance via monocarboxylate transporter (MCT) 4. (B) PHI may enhance liver regeneration via increased hepatocyte proliferation, which is induced by enhanced expression of the cell-cycle promoter cyclin D2 and decreased expression of the cell-cycle inhibitor p21 in a c-Myc-dependent fashion.

**Abbreviations:** Acetyl-CoA, acetyl coenzyme A; TCA, tricarboxylic acid.

via the monocarboxylate transporter 4 (MCT4). Hence, the reduction of hepatic mitochondrial dysfunction and oxidative stress by inhibition of PHDs (PHD1 in particular) can be considered a promising therapeutic strategy for reduction of liver dysfunction following liver ischemia.

In the clinical setting, these effects could be of particular interest in liver transplantation, which remains the sole treatment option for acute liver failure and chronic end-stage liver disease. PHI treatment might facilitate the use of marginal donor organs to a greater extent, and thus represent an interesting opportunity to face the constant shortage of adequate organs. Indeed, results from a small, randomized, controlled clinical trial including 60 liver donors, suggest that induction of HIF1 $\alpha$  stabilization prior to organ retrieval can attenuate graft injury and improve clinical outcomes.<sup>46</sup> As proof of principle it has been demonstrated that in rodent kidney transplantation one single application of the PHD inhibitor FG-4497 6 hours prior to donor organ retrieval is sufficient to stabilize HIF1 and to significantly improve organ function and long-term outcome.<sup>33</sup>

## Liver regeneration

Liver resection is the only potentially curative therapy for primary hepatic malignancies and metastatic liver disease.<sup>47</sup> The extent of resection is, however, limited by the function and volume of the prospective remnant liver.<sup>48</sup> About 13% of patients undergoing extended liver resection develop liver failure, which is associated with high morbidity and mortality.<sup>49</sup> Current research approaches therefore aim at enhancing the regenerative capacity of the liver after resection. Due to altered hepatic hemodynamics, hallmarked by increased portal venous inflow and decreased arterial blood supply, post-resectional regeneration of the liver occurs in a hypoxic environment.<sup>50</sup> Not surprisingly, therefore, HIFs and their hepatic target genes are over-expressed in hepatocytes during liver regeneration.<sup>51–53</sup> Genetic deficiency of PHD1 enhances liver regeneration in preclinical mouse models via increased hepatocyte proliferation, which is induced by enhanced expression of the cell-cycle promoter cyclin D2 and decreased expression of cell-cycle inhibitor p21 in a c-Myc-dependent fashion (Figure 1B). These effects of PHD1 deficiency in the regenerating liver are likely induced by increased stabilization of HIF2 $\alpha$ .<sup>54</sup> Collectively, these results support a potential therapeutic role of PHI for liver regeneration after surgical liver resection.

## Hepatic steatosis and fibrosis

Fatty liver disease (FLD) caused by abnormal hepatic triglyceride accumulation has an increasing prevalence in Western industrialized countries, and is associated with alcoholic liver disease or metabolic syndrome.<sup>55</sup> Severe steatohepatitis ultimately causes liver fibrosis and cirrhosis.<sup>55</sup>

Both clinical observations and preclinical studies in gene-deficient mice suggest that HIF pathways are significantly involved in the pathogenesis of FLD. For instance, chronic intermittent hypoxia induced by obstructive sleep apnea syndrome is associated with FLD, promoted by activation of the HIF- and NF- $\kappa$ B pathways.<sup>56,57</sup>

In mice, chronic stabilization of HIF2 $\alpha$  stimulates excessive hepatic lipid accumulation, along with impaired  $\beta$ -oxidation of fatty acids and increased hepatocellular lipid storage capacity, as well as increased inflammation and hepatic fibrosis.<sup>52,58</sup> HIF1 $\alpha$  expression likewise correlates with the severity of fibrosis in rodents, and HIF1 $\alpha$ -deficient mice display reduced fibrosis.<sup>59,60</sup> Consistent with these effects in HIF1 $\alpha$ -deficient mice, animals deficient in both PHD2 and PHD3 display severe hepatomegaly and hepatic steatosis.<sup>61</sup> Conversely, HIF1 may also exert protective effects in alcoholic liver disease: ethanol-induced hepatic lipid accumulation is significantly increased in HIF1 $\alpha$ -deficient mice, and DMOG ameliorates steatosis in wild-type, but not in HIF1 $\alpha$ -null mice.<sup>62</sup>

While beneficial effects of PHI in settings of liver ischemia and resection have been well documented in preclinical studies, less is known about the significance of PHD inhibition in the setting of hepatic steatosis and fibrosis. Given the evidence outlined, putative effects of PHI on hepatic steatosis and fibrosis remain ambiguous and most likely depend on the underlying pathogenesis. Thus, while inhibition of collagen-P4Hs reduces collagen formation and prevents liver fibrosis, specific effects of HIF-P4Hs on steatohepatitis and liver fibrosis are subject to further investigation.<sup>63,64</sup>

## The putative effects of prolyl hydroxylase domain-containing enzyme inhibitors in the intestine

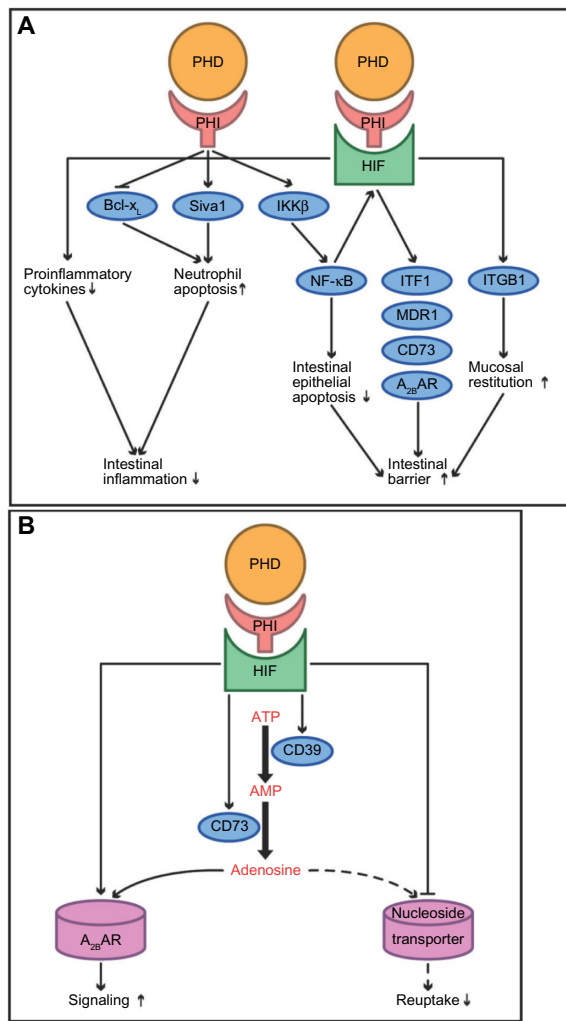
### Inflammatory bowel disease

The two main types of inflammatory bowel disease (IBD), Crohn's disease and ulcerative colitis (UC), are distinct in their pathogenesis, and their patterns of manifestation within the gastrointestinal tract: while Crohn's disease may affect all bowel segments, UC is restricted to the colon. Therefore, surgery can offer a cure for UC: the chronic form, which is resistant to conservative, anti-inflammatory treatment, or fulminant UC are indications for restorative proctocolectomy.<sup>65</sup> Further indications for colectomy comprise bacterial-induced colitis and ischemic colitis.<sup>66,67</sup>

Severe hypoxia of the intestinal mucosa (inflammatory hypoxia) is a hallmark of IBD. At baseline physiological

conditions, the intestinal mucosa already has a hypoxic environment, which is potentiated in IBD through a mismatch of localized vascular damage and increased oxygen demand.<sup>68,69</sup> It has been shown that HIF1 $\alpha$  is significantly overexpressed in human UC.<sup>70</sup> In mice, inactivation of epithelial HIF1 $\alpha$  in the colon aggravates the susceptibility to mucosal inflammation due to downregulation of barrier-protective HIF-target genes (for eg, intestinal trefoil factor 1 [ITF1], multidrug resistance 1 [MDR1], *CD73*, and adenosine A2B receptor [*A<sub>2B</sub>AR*]). Collectively, this results in diminished mucin production, attenuated xenobiotic defense mechanisms, and impaired nucleotide metabolism and signaling (Figure 2A).<sup>71–74</sup> Consistently, the stabilization of HIF1 $\alpha$  by PHI has a protective effect:<sup>68,69</sup> Several PHI, such as DMOG, FG-4497, TRC160334, and AKB-4924, attenuate the severity of colitis in a variety of preclinical mouse models by, first, augmentation of the epithelial barrier via NF- $\kappa$ B and intestinal barrier genes; second, promotion of neutrophil apoptosis; third, lowering the levels of proinflammatory cytokines such as interleukin (IL)-1b, IL-6, and tumor necrosis factor alpha ([TNF $\alpha$ ] a stimulator of NF- $\kappa$ B); and, finally, accelerated initiation of mucosal restitution through fibroblast integrin beta 1 (ITGB1).<sup>31,34,75–77</sup> The iron chelator quercetin promotes similar effects in a rat model of colitis.<sup>78</sup> Among the individual PHDs, PHD1 appears to be a predominant effector of mucosal protection: PHD1 deficiency leads to increased enterocyte density and intestinal barrier function, and decreased apoptosis of colonic mucosal cells.<sup>79</sup> In patients suffering active UC, epithelial expression of PHD1 protein is significantly increased, highlighting these findings' relevance in human IBD.<sup>79,80</sup> Furthermore, loss of PHD3 shortens the lifespan of inflammatory neutrophils by upregulation of the proapoptotic mediator Siva1 and loss of its binding protein, B-cell lymphoma-extra large (Bcl-x<sub>L</sub>), thus limiting the severity of mucosal inflammation.<sup>81</sup> In addition, HIF stabilization supports the immune function of dendritic and mast cells in mice.<sup>82,83</sup>

Collectively, these observations indicate that PHIs represent a promising tool for the clinical treatment of IBD. Of note, it was likewise observed that high doses of FG-4497 might cause vascular occlusion within the intestine, most likely secondary to elevated hematocrit levels, thus underscoring the challenges of dose finding for therapeutic HIF stabilization.<sup>84</sup> Hitherto, research has focused on the significance of PHIs in acute colitis, however, their potential in the treatment of chronic colitis and in the maintenance of remission remains to be addressed. Remarkably, it has been recently shown in rodents that treatment with DMOG 24 hours after



**Figure 2** The putative effects of prolyl hydroxylase domain-containing enzyme inhibitors (PHI) in the intestine.

**Notes:** (A) In inflammatory bowel diseases PHI may attenuate the severity of colitis by, first, augmentation of the epithelial barrier via induction of intestinal barrier-protective hypoxia-inducible factor (HIF) target genes for intestinal trefoil factor 1 (ITF1), multidrug resistance 1 (MDR1), cluster of differentiation (CD73), and adenosine A<sub>2B</sub> receptor (A<sub>2B</sub>AR) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB); second, promotion of neutrophil apoptosis (induction of proapoptotic Siva1 and inhibition of its binding protein B-cell lymphoma-extra large [Bcl-x<sub>L</sub>]); third, attenuating levels of proinflammatory cytokines; and, finally, accelerating initiation of mucosal restitution (through integrin beta 1 [ITGB1]). Moreover, PHI can induce inhibitor of nuclear factor kappa-B kinase subunit beta (IKKβ), a promoter of NF-κB, which in turn likewise affects the HIF. (B) In intestinal ischemia and reperfusion, PHI may stimulate adenosine generation via increased HIF stabilization, which is considered gut protective. Adenosine triphosphate (ATP) is converted into adenosine monophosphate (AMP) by CD39. Subsequent conversion of AMP to adenosine is catalyzed by CD73. Furthermore, HIF stabilization amplifies adenosine signaling by upregulation of A<sub>2B</sub>AR, and enhances extracellular adenosine concentrations by repressing equilibrative nucleoside transporters (thus inhibiting its reuptake).

radiation-induced enteritis is still gut protective, emphasizing the imperative to further explore the perfect timing of PHD inhibitor administration in the setting of IBD.<sup>85</sup>

## Intestinal ischemia and reperfusion

Bowel damage caused by intestinal I/R represents a frequent challenge in gastrointestinal surgery. While early symptoms

of intestinal ischemia are mostly nonspecific, the clinical suspicion can suffice as an indication for surgical exploration and either revascularization or resection. Common reasons for intestinal I/R are strangulated hernias, mesenteric artery occlusion (due to thrombosis or embolism), or circulatory collapse (eg, in septic conditions).<sup>86</sup> Current research on novel treatment strategies to alleviate intestinal ischemia injury is therefore highly warranted.

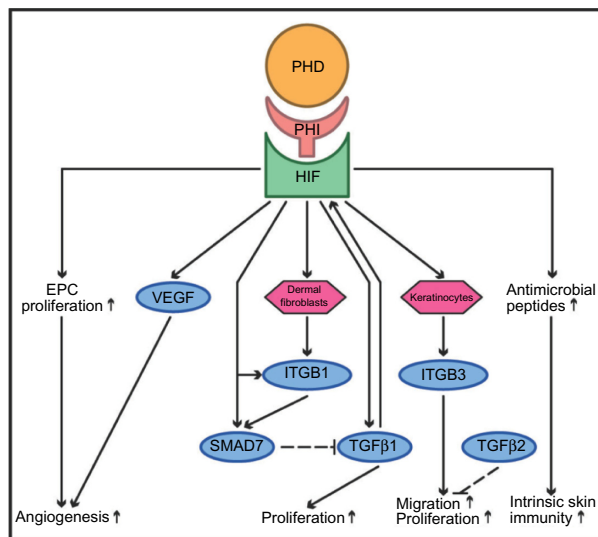
It has been demonstrated that adenosine and its receptors are protective in intestinal I/R injury. Extracellular adenosine is generated from ATP or adenosine diphosphate, which are converted into adenosine monophosphate (AMP) in a process driven by the ectopyrase CD39. The subsequent conversion of AMP to adenosine is catalyzed by CD73. Both CD39 and CD73 are induced by hypoxia (Figure 2B).<sup>72,87</sup> In mice, CD39 deficiency causes increased severity of intestinal inflammation and CD73 deficiency amplifies intestinal injury and organ failure in response to I/R.<sup>88–90</sup> The effects of adenosine in the setting of intestinal ischemia are importantly mediated by A<sub>2B</sub>AR, which is likewise induced in hypoxia via HIF.<sup>91</sup> While conditional deletion of intestinal epithelial HIF1α aggravates intestinal I/R damage in a mouse model of short superior mesenteric artery occlusion (SMAO; 15 minutes), DMOG significantly attenuates I/R injury by increased extracellular adenosine generation involving CD73, and signaling via A<sub>2B</sub>AR.<sup>91,92</sup> Furthermore, HIF amplifies extracellular adenosine concentrations by repressing equilibrative nucleoside transporters (thus inhibiting its reuptake), and by repressing its further metabolism by the adenosine kinase, which reconverts adenosine into AMP.<sup>93,94</sup>

While these insights indicate a putative benefit of PHIs in the setting of intestinal I/R, conflicting data likewise exist: in a mouse model of severe SMAO (90 minutes), partial deficiency of HIF1 seems to be protective against intestinal I/R injury, emphasizing that duration and severity of I/R injury determine the role of HIF in this setting.<sup>95</sup> However, in other rodent models of severe SMAO (60 minutes), induction of the HIF-target gene for HO-1 causes a significant reduction of intestinal I/R injury.<sup>96</sup>

In summary, PHI may represent an interesting novel treatment opportunity in clinical settings of intestinal ischemia, despite these apparently paradoxical preclinical data. However, it remains a major challenge to determine the therapeutic niche for PHD inhibitor treatment in this setting, and more preclinical studies are required to identify the perioperative timing of PHD inhibitor application, in order to balance the beneficial and deleterious effects of HIF stabilization.

## The putative effects of prolyl hydroxylase domain-containing enzyme inhibitors in wound healing

Wound healing is of greatest interest to the surgeon, especially if this complex process – in which inflammation, (neo-)angiogenesis, and reepithelialization play pivotal roles – is impaired.<sup>97</sup> Like the intestinal mucosa, the skin is constitutively hypoxic even under baseline conditions, resulting in continuous HIF1 $\alpha$  stabilization within the basal epidermal layer.<sup>98,99</sup> Skin injury further enhances hypoxia and epidermal HIF1 $\alpha$  stabilization, which represents a major stimulus for wound healing. Subsequently, several angiogenic factors are released, and circulating endothelial precursor cells are recruited to initiate revascularization (Figure 3).<sup>100,101</sup> Accordingly, heterozygous loss of HIF1 $\alpha$  impairs wound healing in mice, and dermal suppression of HIF1 $\alpha$  and its target genes negatively affects the wound-healing process in diabetic mice.<sup>102,103</sup> HIF1 $\alpha$  stabilization by DMOG crucially improves wound closure via enhanced angiogenesis, which is stimulated by increased levels of vascular endothelial growth factor and enhanced proliferation



**Figure 3** The putative effects of prolyl hydroxylase domain-containing enzyme inhibitors (PHI) in wound healing.

**Notes:** Hypoxia-inducible factor (HIF) stabilization by PHI may improve wound healing, first, via enhanced angiogenesis (upregulation of vascular endothelial growth factor [VEGF] and endothelial progenitor cell [EPC] proliferation); second, via keratinocyte proliferation in the basal skin layer (reduced activity of the canonical transforming growth factor beta (TGF $\beta$ ) 2 pathway and increased transcription of integrin beta [ITGB] 3); third, via increased proliferation of dermal fibroblasts directly through HIF-dependent stimulation of TGF $\beta$ 1 and indirectly through induction of ITGB1, which in turn reduces the activity of mothers against decapentaplegic homolog 7 (SMAD7), a strong inhibitor of TGF $\beta$ 1. In a positive feedback loop, TGF $\beta$ 1 can further stabilize HIF by selectively inhibiting prolyl hydroxylase domain-containing enzyme (PHD) 2. Finally, PHI may improve wound healing through production of antimicrobial peptides, and thus strengthen the intrinsic immunity of the skin layer.

of endothelial precursor cells, a process that appears to be specifically mediated by inhibition of PHD2.<sup>103–106</sup> Specific depletion of PHD2 in epidermal keratinocytes accelerates reepithelialization in a murine model of skin wound healing: this effect is promoted by reduced activity of the canonical transforming growth factor beta (TGF $\beta$ ) 2-pathway and increased HIF1 $\alpha$ -dependent expression of  $\beta$ 3-integrin, thus enhancing the migration and proliferation of keratinocytes.<sup>107</sup> Consistently, HIF1 $\alpha$  deficiency in keratinocytes delays wound healing in aged mice.<sup>108</sup> In contrast, in dermal fibroblasts HIF stabilization by DMOG and FG-4497 induces the transcription of ITGB1.<sup>76</sup> Integrin signaling through  $\alpha$ 3 $\beta$ 1-integrin inhibits SMAD7, likewise a HIF-responsive gene and potent inhibitor of TGF $\beta$ 1, and thus enhances cutaneous reepithelialization in a murine model of skin wound healing.<sup>109,110</sup>

While hypoxia increases TGF $\beta$ 1 in human dermal fibroblasts in vitro, TGF $\beta$ 1 likewise stabilizes HIF1 $\alpha$  through selective downregulation of PHD2, thus underscoring the extensive crosstalk between the HIF- and TGF $\beta$ -pathways in wound healing.<sup>111,112</sup> Furthermore, in mice HIF1 $\alpha$  directly stimulates the production of antimicrobial peptides in keratinocytes, including cathelicidin, thus strengthening the intrinsic immunity of the skin layer.<sup>113</sup> In a mouse model of skin abscess, topical treatment of keratinocytes with the HIF1-stabilizing PHD inhibitor AKB-4924 leads to increased bactericidal capacity against skin pathogens.<sup>114</sup> In contrast to these reported effects of HIF1 $\alpha$ , keratinocyte-specific deletion of HIF2 $\alpha$  accelerates wound closure by increasing keratinocyte migration, and reducing the bacterial load in the acute inflammatory phase.<sup>115</sup>

Despite these adverse effects caused by HIF2 $\alpha$ , these pre-clinical data collectively indicate that PHIs might represent an interesting therapeutic tool in skin wound healing, especially in the restoration of complicated wounds such as in diabetic patients or in ischemic flaps, or as an adjunctive therapy in antibiotic-resistant bacterial wound infections.<sup>103–106</sup> A major advantage of PHI application in this setting might be the opportunity for topical administration, which would reduce systemic side effects.

## The putative effects of prolyl hydroxylase domain-containing enzyme inhibitors in the innate immune response

From a surgical perspective, abdominal sepsis is highly relevant since it is associated with a lethality of 25–30%,

and is one of the leading causes for mortality on surgical intensive care units.<sup>116</sup> Sepsis represents a severe systemic inflammatory response syndrome due to bacterial pathogens, which can ultimately lead to multiorgan failure. Clinical sepsis symptoms and septic multiorgan failure are induced by an overwhelming innate immune response, which is importantly stimulated by hypoxia in a HIF-dependent manner (Figure 4).<sup>117,118</sup> The intertwined relationships between hypoxia, inflammation, and innate immunity have been well-documented:<sup>68,119</sup> tissue hypoxia causes accumulation of inflammatory cells, and leakage across epithelial and vascular barriers.<sup>120,121</sup> Remarkably, inflammation potentiates tissue hypoxia due to an imbalance of decreased oxygen supply (caused by vascular thrombosis) and increased oxygen demand (due to enhanced consumption by both resident and inflammatory cells).<sup>84,92,122</sup> At the cellular and subcellular level, HIF1 $\alpha$  enhances the adhesion and migration of hypoxic myeloid cells through  $\beta$ 2-integrin transcription, and is indispensable for anaerobic ATP-generation and myeloid cell survival in inflamed tissues, as well as for their ability to aggregate and invade.<sup>117,123</sup> HIF1 $\alpha$  likewise

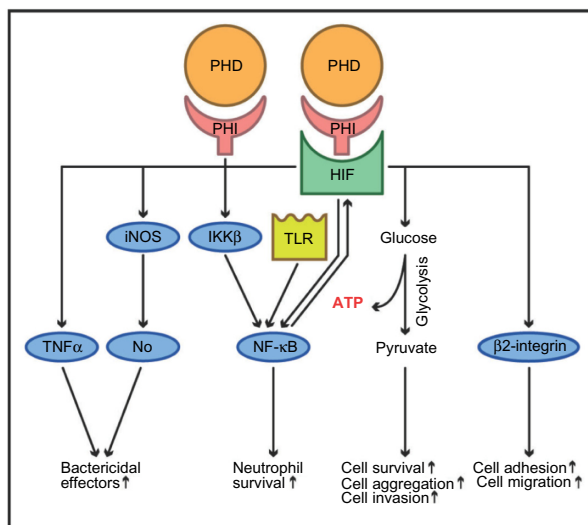
stimulates bactericidal effectors of the innate immune response such as TNF $\alpha$ , inducible nitric oxide synthase and subsequently nitric oxide, and promotes neutrophil survival via NF- $\kappa$ B.<sup>124–127</sup>

From a clinical perspective, the significance of HIF in the innate immune response appears to be double-edged. Depending on the type and extent of the underlying inflammatory disease, both adverse and beneficial effects have been described: while HIF-dependent effects in myeloid cells importantly support the ability of the immune system to resolve localized bacterial invasion and tissue inflammation, HIF1 $\alpha$  can play an adverse role in sepsis. In murine sepsis models, macrophage-specific deletion of HIF1 $\alpha$  is protective against lipopolysaccharide-induced mortality by reducing proinflammatory cytokines such as IL-1 $\beta$ , IL-6, or TNF $\alpha$ .<sup>128,129</sup> Furthermore, HIF1 $\alpha$  amplifies the proinflammatory NF- $\kappa$ B pathway directly by upregulation of NF- $\kappa$ B, as well as indirectly, through induction of toll-like receptors (TLR).<sup>127,130</sup> In a reciprocal self-reinforcing process, NF- $\kappa$ B likewise stimulates HIF1 $\alpha$  transcription.<sup>131</sup>

Of note, PHDs regulate both pathways and are, therefore, important mediators of the innate immune response.<sup>84</sup> PHD1 represses NF- $\kappa$ B in normoxia by hydroxylation and consecutive inactivation of I $\kappa$ B kinase beta (IKK $\beta$ ), a promoter of NF- $\kappa$ B.<sup>132</sup> PHD3 interacts with IKK $\beta$  independently of its hydroxylase function by blocking the interaction of IKK $\beta$  with its chaperone heat shock protein 90 (Hsp90), which is required for IKK $\beta$  phosphorylation and release of NF- $\kappa$ B.<sup>133</sup> Accordingly, global or myeloid cell-specific deficiency of PHD3 aggravates clinical symptoms and the lethality of abdominal sepsis in preclinical mouse models via stabilization of HIF1 $\alpha$  and increased activity of NF- $\kappa$ B.<sup>134</sup>

Finally, apart from their multifold effects on innate immune functions, HIF and PHDs likewise affect adaptive immunity. For instance, specific stabilization of HIF1 $\alpha$  in T-cells impairs the survival of septic mice due to reduced T-cell proliferation and proinflammatory cytokine secretion.<sup>135</sup>

From a surgical, as well as from an anesthesiologic standpoint, these insights, which were mostly generated in preclinical mouse models, are highly relevant concerning putative applications of PHI in critically ill or septic patients. For instance, boosted activation of the innate immune response via PHI could be an interesting therapeutic option in bacterial infections of immunodeprived patients, but it may likewise have severe side effects. More experimental evidence is required to evaluate the therapeutic implications



**Figure 4** The putative effects of prolyl hydroxylase domain-containing enzyme inhibitors (PHI) in innate immune response.

**Notes:** Hypoxia, inflammation, and innate immunity have intertwined relationships. Hypoxia-inducible factor (HIF) stabilization by PHI may enhance the adhesion and migration of hypoxic myeloid cells (through beta 2-integrin transcription), and is indispensable for myeloid-cell adenosine triphosphate (ATP) generation, survival, aggregation, and invasion in inflamed tissues. Hypoxia-inducible factor 1 alpha (HIF1 $\alpha$ ) stabilization likewise stimulates bactericidal effectors of the innate immune response such as tumor necrosis factor alpha (TNF $\alpha$ ) and nitric oxide synthase (iNOS) (producing nitric oxide [NO]), and promotes neutrophil survival via nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B). The NF- $\kappa$ B pathway is directly amplified by HIF1 $\alpha$ , as well as indirectly through the induction of toll-like receptors (TLR). PHIs may likewise induce inhibitor of nuclear factor kappa-B kinase subunit beta (IKK $\beta$ ), a promoter of proinflammatory NF- $\kappa$ B. In a reciprocal self-reinforcing process, NF- $\kappa$ B directly stimulates HIF1 $\alpha$  transcription.

**Abbreviation:**  $\beta$ 2-integrin, beta 2-integrin.



of PHIs for the modulation of the innate immune response in specific inflammatory disease settings.

## The putative effects of prolyl hydroxylase domain-containing enzyme inhibitors in cancer

Hypoxia occurs in virtually all human tumors because expanding cancer cells rapidly outgrow the development of nourishing blood vessels, and because tumor vessels are poorly functional and chaotic, altogether leading to insufficient oxygen supply.<sup>136,137</sup> Accordingly, HIF is upregulated in a variety of tumor entities, and has been demonstrated to enhance tumor aggressiveness, associated with poor prognosis and resistance against anticancer therapy.<sup>24,138–140</sup> HIFs importantly influence all major aspects of cancer biology, including cell survival, resistance to apoptosis, angiogenesis, invasion, and metastasis.<sup>141</sup> On one hand, clinical disorders such as the hereditary von Hippel–Lindau disease underscore the tumor-promoting role of HIF2 $\alpha$ .<sup>8</sup> On the other hand, tumor-suppressive functions of HIF have likewise been demonstrated, and the effects of HIF1 and HIF2 on tumor growth can be intriguingly different:<sup>142</sup> for instance, HIF1 $\alpha$  (but not HIF2 $\alpha$ ) decreases the activity of the cell-cycle regulator Myc and may have antiproliferative functions. Overexpression of HIF2 $\alpha$  in rat glioma tumors enhances angiogenesis, but reduces tumor growth by increasing tumor-cell apoptosis.<sup>143–145</sup>

The impact of PHI on occult or dormant tumor disease may lead to significant side effects in patients undergoing treatment with PHI, and putative applications of PHI in gastrointestinal surgery (such as protection of liver function following major resections) might specifically apply for cancer patients. From a clinical standpoint, it will therefore become crucial to sort out the specific effects of PHI on tumor growth and metastasis. Although immunohistochemical analyses of human cancers have predominantly revealed increased expression of PHDs, conflicting evidence exists about how they specifically influence tumorigenesis.<sup>11,146</sup> In fact, the effects of the individual PHDs on tumor growth appear to be heterogeneous, and the impact of pharmacological pan-inhibition of PHDs on tumor growth is poorly defined. In the following, we summarize hitherto available evidence regarding specific effects of PHD1, PHD2, and PHD3 on tumor growth, which has mostly been generated by functional genomic studies.

PHD1 overexpression in tumor cells can inhibit tumor growth in mice.<sup>147</sup> Conversely, downregulation of PHD1 *in vitro* impairs cyclin D1, thus suppressing the proliferation of various cancer cell lines.<sup>148</sup>

Broad, but partly conflicting, evidence has been generated concerning the tumor-specific effects of PHD2. While overexpression of PHD2 in pancreatic cancer cells appears to impair tumor growth, downregulation of PHD2 in pancreatic-, colorectal-, and breast-cancer cells stimulates tumor growth in immunodeficient mice, altogether suggesting tumor-suppressive functions of PHD2.<sup>149–151</sup> Moreover, PHD2 haplodeficiency stimulates hepatocarcinogenesis in mice.<sup>152</sup> In human patients, PHD2 expression is positively correlated with gastric-cancer survival, and may serve as a prognostic marker.<sup>152,153</sup> PHD2 deficiency is likewise associated with the immortality of human endometrial cancer cells, while the reintroduction of PHD2 induces senescence.<sup>154</sup> In the literature one case is reported describing a patient with a PHD2 mutation, which was associated with multiple tumors.<sup>155</sup> Conversely, in osteosarcoma cells PHD2 deficiency significantly alleviates tumor growth by the matrix metalloproteinase-induced transformation of TGF $\beta$  into a tumor suppressor.<sup>156,157</sup> This is remarkable in the context of the mentioned potential positive feedback loop between TGF $\beta$  and HIF1 $\alpha$ .<sup>111</sup> However, in breast-cancer cells, TGF $\beta$  seems to be a tumor promoter.<sup>158</sup> In rodents, haplodeficiency of PHD2 reduces tumor-cell invasion, intravasation, and metastasis without affecting primary tumor size, and improves the delivery of chemotherapy and accompanied tumor response to chemotherapy by normalizing the tumor vasculature.<sup>159,160</sup> At the same time, stabilization of HIF1 $\alpha$  may counteract the efficiency of chemotherapy via increased expression of MDR1.<sup>74</sup> PHD2 can also contribute to tumorigenesis independently of HIF: inactivation of PHD2 in colon carcinoma cells stimulates tumor growth, mediated by NF- $\kappa$ B-dependent expression of IL-8 and angiogenin, thereby stimulating angiogenesis and vasculogenesis through the recruitment of bone-marrow-derived vascular modulatory cells.<sup>150</sup>

PHD3 expression is decreased in human colorectal-cancer tissue and associated with higher tumor grade and metastasis. In this context, PHD3 seems to be a HIF-independent tumor suppressor acting via NF- $\kappa$ B.<sup>133</sup> Moreover, PHD3 is essential for the apoptosis of sympathetic neuron precursor cells *in vitro*. Thus, loss of PHD3 contributes to the development of pheochromocytomas.<sup>21</sup>

In summary, the reviewed conflicting data on HIF and PHD in tumorigenesis might be in part due to the following: first, different experimental setups *in vivo* or *in vitro* with inactivation of PHDs only in tumor cells, in tumor micro-environments (fibroblasts, endothelial, epithelial and immune cells), or in both; and, second, the HIF-dependent or -independent functions of PHDs, such

as on NF- $\kappa$ B or TGF $\beta$ .<sup>120,138,157</sup> Obviously, modulation of PHDs might enhance tumor growth, which may limit the clinical use of PHIs in any therapeutic approach. More preclinical studies elucidating the precise cancer- and stromal-cell-specific functions of PHDs in tumorigenesis are warranted.

## Conclusion

The possible applications of PHI in various diseases are multifold, and open a broad perspective for clinical implications in surgery. Although several ongoing clinical trials are encouraging,<sup>39</sup> translation to the patient's bedside mandates more preclinical and clinical studies, which need to settle several problems: first, PHI with certain selectivity for the individual PHDs, as well as for different tissues, are required; second, the timeframe of PHD inhibitor application in preconditioning and treatment has to be defined to balance the benefits and potential side effects of constant HIF stabilization; third, alternative PHD inhibitor application pathways such as local application in wound healing or enteral application in IBD are desirable to minimize systemic side effects; and, finally, the significance of PHI in tumorigenesis (either tumor suppressive as anticancer therapeutics, or cancer promoting as a severe systemic side effect) remains elusive and needs to be clarified.

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## Disclosure

The authors declare no conflicts of interest in this work.

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