SUMMARY, DISCUSSION, AND FUTURE PERSPECTIVES
1. Summary and Discussion

Angiogenesis has been perceived as one of the promoters in liver fibrosis since it was discovered that angiogenesis emerges in parallel with liver fibrogenesis\(^1\). Indeed, some *in vivo* studies in mouse and rat models of bile duct ligation (BDL) or carbon tetrachloride (CCl\(_4\))-exposed fibrosis have shown reduction of fibrosis in animals treated with angiogenesis inhibitors\(^5\)\(^-\)\(^9\). Notwithstanding the foregoing, recent findings showed that angiogenesis inhibition eventually aggravates fibrosis in the liver and decelerates fibrosis resolution\(^10\)\(^-\)\(^12\).

The conflicting findings on the role of angiogenesis in liver fibrosis are described in chapter 1. To date, there is still a dialog whether angiogenesis occurs as a defense mechanism to compensate for the shortage of oxygen supply or whether it stimulates and aggravates the fibrotic condition. Angiogenesis is tightly controlled by the balance of pro- and anti-angiogenic factors\(^13\). These factors are produced by different cell types in the liver including hepatocytes and hepatic stellate cells (HSC)\(^6\), which indicates a multicellular crosstalk in fibrosis-associated angiogenesis. Hepatocytes are often regarded as “victims” and “bystanders” in liver fibrogenesis\(^14\), however they express important angiogenic factors, such as vascular endothelial growth factor A (VEGF-A) and thrombospondin 1 (THBS-1)\(^15\)\(^-\)\(^17\). Yet, the contribution of hepatocytes to angiogenesis in fibrosis has not been extensively investigated yet.

With the aim to address the role of hepatocytes in angiogenesis during liver fibrosis, we developed an interferon alpha (IFN\(\alpha\))-construct by conjugating galactose-polyethylene glycol to IFN\(\alpha\) to obtain a targeted delivery to the asialoglycoprotein receptors (ASGPR) expressed at the sinusoidal membrane of the hepatocytes. In chapter 2, the construct (galactose-PEG-IFN\(\alpha\), GPI) was evaluated *in vitro* using HepG2 cells, a human liver cancer cell line that has been widely used as a model for the study of human hepatocytes. The STAT1 phosphorylation by GPI was partly inhibited by lactosylated HSA, which indicates that the GPI activity was partly mediated by the ASGPR and the remaining effect was most likely mediated by the interferon alpha receptor (IFNAR). *In vitro*, the anti-angiogenic activity of both IFN\(\alpha\) and GPI was shown by the decreased secretion of the pro-angiogenic factor VEGF-A and increased expression of the anti-angiogenic factor THBS-1. In addition, both IFN\(\alpha\)- and GPI-treated HepG2 cells inhibited the tubular formation of HUVEC cells indicating release of anti-angiogenic factors. Interestingly, the anti-angiogenic activity was only shown at the lower dose, while the higher dose did not affect the tubular formation by HUVEC. A similar phenomenon was also observed in the *in vivo* evaluation of both IFN\(\alpha\) and GPI in a fibrosis model of CCl\(_4\)-exposed mice as described in chapter 3. A U-shaped dose-response upon IFN\(\alpha\) used as angiogenesis inhibitor was previously observed in human bladder cancer\(^18\). In addition, other angiogenesis inhibitors also displayed different types
of hormesis in the dose-response. For instance, the proteasome inhibitor bortezomib displayed a bell-shaped dose response in HUVEC, in which the lower dose promoted angiogenesis while the higher dose inhibited it\(^\text{19}\). The molecular mechanism of hormesis in angiogenesis has not been understood yet. This phenomenon might be generated by one receptor, but can also be explained by assuming that the anti-angiogenic agent binds to more than one receptor, in which one receptor mediates angiogenesis stimulation and other receptors mediate the inhibition of angiogenesis\(^\text{20}\). Another explanation is that proteins, such as endostatin may contain both pro-angiogenic and anti-angiogenic sequences and the activity balance of these different sequences may mediate the hormesis phenomenon\(^\text{20,21}\). Nevertheless, there is no specific explanation yet for the hormetic dose-response of IFN\(\alpha\).

Our results in \textit{chapter 3} show that the aim to reveal the role of hepatocytes in fibrosis-associated angiogenesis cannot be achieved by using GPI, since we did not observe a notable difference in the cellular distribution of GPI and native IFN\(\alpha\). Both molecules were present on hepatocytes, endothelial cells, and (myo)fibroblasts, apparently at similar relative concentrations. We hypothesize that this localization is mainly due to binding to the IFNAR present on the aforementioned cell types in the liver. IFNAR is a common receptor for type I interferons including IFN\(\alpha\) and consists of two subunits, a low- (IFNAR1) and a high-affinity (IFNAR2) receptor component\(^\text{22,23}\). It has been previously shown that IFNAR is expressed on mouse hepatocytes\(^\text{24}\). However, there has not been any study yet showing the distribution of IFNAR expression in the liver and whether it is present on other cell types than hepatocytes. Nevertheless, the reported IFNAR expression in human umbilical vein endothelial cells\(^\text{25}\) and cardiac fibroblasts\(^\text{26}\) suggests that IFNAR might also be expressed by endothelial cells and fibroblasts in the liver. An immunohistochemical staining of IFNAR is required to confirm this finding. Expression of this receptor on endothelial cells and fibroblasts would explain the uptake of both compounds in these cells, as found in our studies.

We have shown in \textit{chapter 3} that IFN\(\alpha\) and GPI inhibited angiogenesis, yet there was no effect observed on fibrogenesis. IFN\(\alpha\) was previously shown to have antifibrotic effects in BDL and CCl\(_4\) models of liver fibrosis in rats\(^\text{27,28}\). Our \textit{in vivo} study did not show a similar result and this might be due to the difference in species as we used mice, and the dose of IFN\(\alpha\). In the rat studies 100,000 IU of IFN\(\alpha\) was dosed, while the highest dose that we used was 200 ng IFN\(\alpha\)/day which equals to ca. 50,000 IU of IFN\(\alpha\). Nevertheless, the lower dose that we used was sufficient to inhibit angiogenesis in the CCl\(_4\)-exposed mice without any significant effect on liver fibrogenesis. Therefore, we conclude that angiogenesis has no direct role in fibrogenesis but may occur as the consequence of increased profibrotic factors that also have angiogenic effects. This angiogenesis
inhibition without any effect on liver fibrogenesis was also observed by Kantari-Mimoun et al. using genetic ablation of VEGF in scar-infiltrating myeloid cells. The absence of VEGF in those myeloid cells inhibited angiogenesis in 12-week CCl4-exposed mice but did not affect liver fibrogenesis.12

VEGF-A is a potent angiogenic factor that enhances endothelial cell proliferation, promotes vessel sprouting and branching and increases microvessel permeability.29 Since VEGF-A is a pro-angiogenic factor, an increased VEGF-A expression in the fibrotic liver is expected. Interestingly, our result in chapter 3 showed a decreased VEGF-A expression at mRNA and protein level in the CCl4-exposed mice. Other recent studies also showed a similar phenomenon, in which VEGF-A is decreased in fibrogenesis and re-augmented during fibrosis resolution.9,12 To further study the role of VEGF-A in fibrosis, in chapter 4 we studied the correlation between VEGF-A expression and fibrosis markers in mice with a low and a high level of liver fibrosis. We discovered a negative correlation between VEGF-A and procollagen 1α1 expression suggesting that VEGF-A has no effect on fibrogenesis in CCl4-exposed mice. Thus, we sought to investigate the direct role of VEGF-A in a fibrosis model of precision-cut liver slices (PCLS). PCLS incubated for 48 h has been well validated as a model to study liver fibrosis.30-33 However, this model has never been previously used to investigate fibrosis-associated angiogenesis. Similar to the in vivo situation, we observed an increased CD31 expression and decreased vascular endothelial growth factor receptor 1 and 2 (VEGFR1 and VEGFR2) expression along with an increased procollagen 1α1 expression in untreated mouse PCLS cultured for 48 h. One of the advantages of using PCLS is the possibility to study these processes in the human liver, which provides a better clinical translation of the result. In human PCLS, we also observed an increased CD31 expression after 48 h of PCLS incubation. Interestingly, the expressions of VEGFR1 and VEGFR2 were increased in human PCLS during 48 h of incubation. The increased expression of both receptors is consistent with previous reports on cirrhotic patients.12,34 The different response in mouse and human PCLS indicates that the intertwined processes of angiogenesis and fibrosis are not identical in mouse and human.

Upon treatment with VEGF-A, we observed an increased CD31 expression in the mouse PCLS indicating the responsiveness of the model to VEGF-A, but no effect was observed in the fibrosis marker procollagen 1α1. In addition, an enhanced CD31 expression was observed in five out of eight human PCLS. Interestingly, the responsive PCLS also showed an increased VEGFR1 expression upon VEGF-A treatment, which was not observed in the non-responsive PCLS. We discovered that the non-responsive PCLS had a higher basal expression of VEGFR1 compared to the responsive ones. Thus, we speculate that in these PCLS the stimulation with VEGF-A could not further increase the VEGFR1 and CD31 expression.
Another interesting finding in chapter 4, which is related to species-specific response, is the effect of VEGF-A treatment on procollagen 1α1 expression. In mouse PCLS, we found that VEGF-A treatment has no direct effect on liver fibrogenesis. In contrast, treatment of human PCLS with low dose of VEGF-A increased the procollagen 1α1 expression. Previous in vitro studies have shown a direct effect of VEGF-A on proliferation, migration, and transformation of human HSC but not on the procollagen 1α1 expression. The effect of VEGF-A on procollagen 1α1 expression in human PCLS was not observed at the higher dose. We hypothesize that this might be related to the increased VEGFR1 expression. The exact role of VEGFR1 is not yet understood, but some studies indicate that VEGFR1 acts as decoy receptor. So, if VEGF-A activates HSC and VEGFR1 acts as decoy receptor, the effects of VEGF-A on HSC are transient. LeCouter et al. suggest that VEGFR1 protects the liver from CCl4-exposed injury in mice without affecting angiogenesis. Overall, we conclude that the potent pro-angiogenic factor VEGF-A does not have a direct effect on fibrogenesis, even though HSC express both VEGFR1 and VEGFR2. Our results support the study of Kantari Mimoun et al. and Yang et al. who concluded that VEGF-A affects fibrogenesis indirectly through the effects on the infiltration of pro-fibrotic immune cells. We could not observe this indirect effect because blood cells are not present in our PCLS model. Thus, the effect of immune cells infiltration is not included in our studies.

The results in chapter 4 not only showed the effect of VEGF-A but also highlighted a new model to study fibrosis-associated angiogenesis that needs to be further validated. Therefore, in chapter 5 we aimed to develop an alternative model using PCLS as a liver-specific approach to investigate fibrosis-associated angiogenesis. The advantages and disadvantages of the currently available angiogenesis assays have been described in chapter 1. The main limitation of these assays is that the distinctive features of hepatic angiogenesis are not well reflected in the models. Our results in chapter 5 showed that the rat PCLS respond positively towards treatment with the pro-angiogenic factor VEGF-A and the anti-angiogenic sunitinib. The response of PCLS towards VEGF-A treatment was in line with our finding in mouse PCLS, which was described in chapter 4. In rat PCLS, however, we did not observe the parallel increase of CD31 with procollagen 1α1 expression in the untreated PCLS. Interestingly, after VEGF-A treatment the increased CD31 expression was highly correlated with the increased procollagen 1α1 expression in rat PCLS. This indicates that VEGF-A might play role in rat liver fibrogenesis. Yet, we did not observe this in mouse and human PCLS. Again, we highlight the species-specific response in the intertwined processes of angiogenesis and fibrosis.

The CD31 mRNA expression is not sufficient to discriminate between formation of new endothelial cells or increased expression in the existing endothelial cells. For this reason, we
evaluated the CD31 expression at the protein level in slices after five days of incubation where fibrosis was more developed. An increased protein expression of collagen I and III was observed in the five-day incubated PCLS. Our results showed a significant increased area of CD31 positive staining and increased CD31 staining intensity in the slices incubated in an enriched culture medium developed for long term culture of liver cells, Regenemed (Reg), but not in the commonly used Williams' Medium E medium (WME). Both media caused a similar increase in collagen expression, but the collagen deposition was located in different areas of the slices. In addition, it was demonstrated that Reg-incubated slices developed hypoxic regions inside the slices due to the thickening of the slices that might block the oxygen penetration to the inner cell layers. Hypoxia is an important stimulus in both angiogenesis and fibrosis. Structural and anatomical changes in liver fibrogenesis have been proposed as factors that cause a reduced oxygen supply. To compensate for the lack of oxygen supply, angiogenesis is initiated.

Although culturing the PCLS for five days was sufficient to observe intrahepatic angiogenesis, the technique is relatively time consuming including sample preparation, staining, imaging and image analysis. Therefore, we developed another assay which was modified from the well-known aortic ring assay, where outgrowth of endothelial cells is used as marker for angiogenesis. Instead of using aorta rings, we used rat PCLS embedded in collagen matrix. An outgrowth of cells was observed in these collagen-embedded PCLS incubated for five days in WME. Immunofluorescence staining revealed that in WME the outgrowths were CD31 positive, identifying them as endothelial cells. Treatment with sunitinib prevented the outgrowth, but there was no response to VEGF-A treatment. This might be due to the endogenous VEGF production, which was already sufficient to induce maximum sprouting. However, we cannot exclude that the additional VEGF-A was not sufficient to stimulate a notable increase of the angiogenic sprouts. We also evaluated the influence of medium on the sprouting since we discovered that Reg-incubated slices showed a significant increase in the number of blood vessels in the liver tissue. In collagen-embedded PCLS incubated for five days in Reg, we observed massive outgrowths in the collagen matrix. However, none of the outgrowths was CD31 positive. We discovered that the cells were alpha smooth muscle actin positive indicating that they may be fibroblasts. The result was not consistent with our finding in Reg-incubated slices, in which an increased number of blood vessels was observed. Thus, we conclude that this medium is not suitable for the collagen-embedded PCLS assay.

2. Concluding Remarks and Future Perspectives
We applied different approaches to understand angiogenesis in liver fibrogenesis, started from designing the GPI construct from the anti-angiogenic IFNα which was recognized by the ASGPR
expressed by hepatocytes. This targeting strategy was aimed to reveal the role of hepatocyte in liver fibrosis. Since we discovered that the construct was also associated with other liver cell types after administration in mice \textit{in vivo}, we cannot draw any conclusion on whether the anti-angiogenic effect of GPI was due to an effect on the hepatocytes. However, we discovered a consistent phenomenon of both IFN\textalpha and GPI showing a U-shaped dose response in inhibiting HUVEC tubular formation and decreasing CD31 expression in the \textit{in vivo} fibrosis model of CCl\textsubscript{4}-exposed mice. In addition, we did not observe any difference in the expression of procollagen 1\alpha\textsubscript{1} although angiogenesis was inhibited. Thus, we concluded that angiogenesis did not have a significant role in liver fibrogenesis.

This finding was also supported in our study on VEGF-A, a potent angiogenic activator, which had no direct effect on liver fibrogenesis in the fibrosis model of PCLS treated with VEGF-A. However, in human PCLS we observed that the lower dose of VEGF-A increased procollagen 1\alpha\textsubscript{1} expression. Thus, the results of our studies highlighted the importance of using human tissue to have a more clinically relevant model that reflects pathogenesis in liver fibrosis. For future work, a wide range of VEGF-A concentration may give a useful information on the effect of VEGF-A in the human PCLS. Besides, evaluation at protein level may provide additional information on the translational regulation of VEGF-A in liver fibrogenesis.

Another issue in the study of fibrosis-associated angiogenesis is the lack of specific assays that represent the physiological condition in liver fibrogenesis. We tried to solve this issue by developing three different methods using PCLS as fibrosis model. The response of the model towards pro- and anti-angiogenic treatment, the parallel increase of CD31 expression with procollagen 1\alpha\textsubscript{1} expression and the evidence of CD31 positive outgrowths in the collagen-embedded PCLS indicate that we have a promising model that can be used to study fibrosis-associated angiogenesis. In the future, this model will be further validated by using molecules that have been known to have profibrotic effects and human PCLS to investigate human-specific responses.

Since there is no effective treatment yet for liver fibrosis, different approaches need to be done to understand the complexity of the processes involved in liver fibrosis. One of them is using knock-out mice to have a better understanding on the specific effect of angiogenic factors. This is because of the available anti-angiogenic inhibitors are multi tyrosine kinase inhibitors which are not specific for angiogenesis but also affect the fibrosis. These inhibitors might lead to a false conclusion on the correlation or causal relation between angiogenesis and fibrosis.

Ultimately, PCLS offer a promising model to investigate fibrosis-associated angiogenesis with the opportunity of using human tissue for translation of the results to patients. The collagen-embedded PCLS is a novel approach in investigating liver-specific angiogenesis with real-time evaluation, which may accelerate the work in this relevant field and will contribute to the reduction,
replacement and refinement of animal experiments by avoiding *in vivo* animal experiments with serious discomfort.

In conclusion, our approaches are not sufficient to resolve the puzzling relation between angiogenesis and fibrosis and the role of hepatocytes in liver fibrosis. However, our studies do bring further insights and introduce a new *ex vivo* model using PCLS to study angiogenesis. Recent findings on the involvement of macrophages in bridging both processes can be included in the PCLS model of fibrosis as a new approach to unravel the link between fibrosis and angiogenesis.

It is clear that although important steps have been taken in this area, much is needed to be done.

**REFERENCES**

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