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Liver Regeneration

The impact of altered portal hemodynamics on gene expression and growth of the porcine liver

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Contents

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List of papers

1. **Scale-space analysis of time series in circulatory research**. Mortensen KE, Godtliebsen F, Revhaug A. *Am J Physiol Heart Circ Physiol*. 2006. Dec; 291(6): H3012-22. PMID 16877564.

2. **Regenerative response in the pig liver remnant varies with the degree of resection and rise in portal pressure.** Mortensen KE, Conley LN, Hedegaard J, Kalstad T, Sorensen P, Bendixen C, Revhaug A. *Am J Physiol Gastrointest Liver Physiol*. 2008 Mar; 294(3): G819-30. Epub 2008 Jan 10.PMID: 18187521.

3. **Increased sinusoidal flow is not the primary stimulus to liver regeneration.** Mortensen KE, Conley LN, Nygaard I, Sorenesen P, Mortensen E, Bendixen C, Revhaug A. *Comp Hepatol.* 2010 Jan 20; 9:2.PMID: 20148099.

4. **Liver regeneration in surgical animal models – a historical perspective and clinical implications**. Mortensen KE, Revhaug A. Submitted to European Surgical Research.

Abbreviations

ACT	Activated Clotting Time
ALAT	Alanine aminotransferase
ALP	Alkaline phosphatase
ANOVA	Analysis of Variance
ASAT	Aspartate aminotransferase
ATP	Adenosine TriPhosphate
Bcl2	Oncogene B-Cell leukemia 2
Bcl3	B-cell leukemia/lymfoma 3
BCLAF	Bcl2-Associated Transcription Factor 1
Bcl-rambo	B-cell leukemia/lymfoma-rambo
BCLx	B-cell leukemia/lymfomax
BIL	Bilirubin
BTG2	B-cell translocation gene 2
BTG3	B-cell translocation gene 3
Casp7	Caspase 7
CASP-8	Caspase 8
CDK5	Cyclin-dependant kinase 5
CDK6	Cyclin-dependant kinase 6
cDNA	complementary Deoxy Nucleic Acid
CETN2	Centrin, EF-hand Protein 2
CFOS	V-FOS fbj-murine osteosarcoma viral oncogene
CO	Cardiac Output
CVK	Central Venous Catheter
CYCS	Cytochrome C, Somatic
Dapk1	Death associated protein kinase 1
DDAH	Dimethylarginine dimethylaminohydrolase
DDAH2	Dimethylarginine dimethylaminohydrolase 2
ECG	Electro Cardiogram
EGF	Epidermal Growth Factor
ELISA	Enxyme Linked ImmunosorbentAnalysis
eNOS	endothelial Nitrice Oxide Synthase
FAIM2	FAS apoptotic inhibitory molecule 2
GEO	Gene Expression Omnibus
GO	Gene Ontology
GSSG	Glutathione disulfide
GSTP1	Glutathione-transferase, PI
GT	Glutamyl transpeptidase
HE	Hematoxylin-Eosin
HGF	Hepatocyte growth Factor
HNRPK	Heterogenous nuclear ribonuclear protein K
HPLC	High Pressure Liquid Chromatography
HPR	High Pressure Resection
HSF-1	Heat-shock transcription factor 1
HSP	Heat Shock Protein
IGFBP3	Insulin Growth Factor Binding Protein 3
IGFBP5	Insulin Growth Factor Binding Protein 5
IL-1	Interleukin-1
IL-10	Interleukin-10
IL-6	Interleukin-6
JNK	Jun-N terminal Kinase
KIF20-A	Kinesin-like protein 20A
L-NAME	*N*G-nitro-L-arginine methyl ester
LPR	Low Pressure Resection
LPVB	Left Portal Vein Branch
MAP	Mean Arterial Pressure
MAPK13	Mitogen activated protein kinase 13
MAPK2K1	Mitogen-activated protein kinase 1
MAPK6	Mitogen-activated protein kinase 6
MAPK8IP2	Mitogen activated protein kinase –8 interacting protein 2
MCTS1	Malignant T-cell amplified sequence 1
MDM2	Mouse double minute 2 homolog
MIB1	Mindbomb, drosophila, homolog of, 1
MPVT	Main Portal Vein Trunk
MRI	Magnetic Resonance Imaging
mRNA	messenger Ribo Nucleic Acid
NFkappaB	Nuclear Factor kappa B
NME1	Nonmetastatic cells 1, protein expressed in 1
NME2	Nonmetastatic cells 2, protei expressed in 2
NO	Nitric Oxide
NOS3	Nitric Oxide Synthase 3
NOSIP	Nitric Oxide Synthase Interacting Protein
NPM1	Nucleophosmin/Nucleoplasmin family, member 1
OMIM	Online Inheritance in Man
PAI-1	Plasmin Activator Inhibitor-1
PCR	Polymerase Chain Reaction
PHx	Partial Hepatectomy
PKB/AKT	Protein kinase B
PTMA	Prothymosin, alpha
PVP	Portal Venous Pressure
RAF/MEK1	MAPK/ERK kinase 1
RhoB	RAS homolog gene family, member B
RHVP	Right Hepatic Venous Pressure
RPVB	Right Portal Vein Branch
RT-PCR	Reverse Transcriptase - Polymerase Chain Reaction
SBDSP	Shwachman-Bodian-Diamond Syndrome gene
SCAP2	Src-associated protein 2
SCYL1	SCY1-like1
SIN-1	3-morpholinosydnonimine-1

SiNoS	Significant Nonstationarities
SiZer	Significant Zero crossings for derivatives
SMA	Superior Mesenteric Artery
SOD1	Superoxide Dismutase 1
SVR	Systemic Vascular Resistance
TC	Truncus Coeliacus
TGF-alpha	Transforming Growth Factor-alpha
TGF-beta	Transforming Growth Factor-beta
TNF-alpha	Tumor Necrosis Factor-alpha
UBE2C	Ubiquitin-conjugating enzyme E2C
UBE2M	Ubiquitin-conjugating enzyme E2M
VEGF	Vascular Endothelial Growth Factor

Introduction

The fact that the liver regenerates after parenchymal loss has been known for a long time as reflected in the 8th-century BC Greek myth of Prometheus, a Titan and champion of human kind, who stole fire from Zeus and gave it to mortals. For this he was punished by Zeus having him bound to a rock in Caucasus where a great eagle ate his liver every day only to have it grow back to be eaten the next day and so forth for many years until the hero Heracles (Hercules) shot the eagle with an arrow and freed Prometheus from his chains.

The phenomenon of liver regeneration is most likely a natural developmental protective mechanism having prevented liver failure upon mammalian exposure to plant toxins, viral hepatitis and possibly parenchymal loss due to liver trauma. In modern times, liver regeneration after partial hepatectomy (PHx) has been studied extensively partly because the organ affords the possibility to study the molecular and genetic control of the cell cycle as it occurs in a synchronised manner after resection, triggered by the changes imposed on the organ after parenchymal loss. Furthermore, the need to understand the mechanisms behind and find treatment strategies for the acute and chronic failing liver has resulted in a vast amount of research on this topic.

Modern liver surgery has seen the development of split-liver grafting [1] and increasingly more aggressive, multimodal treatment of primary and secondary liver malignancies, progressively expanding the limits of resectability [2]. Despite continuous improvement in surgical technique and perioperative intensive care, patients still occasionally suffer from deficient regeneration and functional failure in the so-called small-for-size syndrome (SFSS) after liver transplantation if the graft is of marginal size (graft weight/body weight ratio, GRWR < 0.8 %) [3] or if the liver remnant is too small after extended hepatectomy (< 25 % of remaining, functionally normal liver)[2]. Post-resectional liver dysfunction is also a potential problem with the increasing practice of neoadjuvant chemotherapy for colorectal metastasis [4]. The vast amount of research to date performed on liver regeneration has had relatively little practical consequence for the patient with a failing liver except the development of liver support systems such as MARS, bridging the patient to transplantation, re-transplantation or as a support during recuperation of the native liver [5] . Contemporary liver surgery therefore calls for a better understanding of the mechanisms controlling liver regeneration in order to design new treatment strategies to support the functionally deficient failing organ and, at the same time, enhance its regenerative capacity be

it a small-for-size graft or a failing remnant after extended hepatectomy or a functionally deficient organ after neoadjuvant chemotherapy followed by liver resection.

Background

Liver anatomy and physiology

In most mammals, the liver weighs an average of 2.5 % of the total body weight and receives approximately 30 % of the cardiac output. It receives dual blood supply – 75 % portal flow from the splanchnic organs (stomach, duodenum, small- and large intestine, pancreas and spleen) and 25 % arterial flow from the aorta. The hepatic blood drains to three major liver veins which join the vena cava inferior draining in turn to the right atrium. The liver is the largest internal organ in the body with vital functions in carbohydrate and lipid metabolism and protein synthesis. It also has important immunological functions in filtering the portal blood via the reticuloendothelial system, removing bacteria and endotoxin translocated from the gut to the portal circulation and contributes to the digestive process by its production of bile. The liver is also plays a major role in drug metabolism. The functional unit of the liver, the liver lobule, consists of rows of hepatocytes lined on one side by fenestrated sinusoidal endothelial cells, and on the other by biliary cells forming bile canaliculi. The blood from the hepatic artery and the portal vein are united in the liver sinusoids perfusing dynamic fenestrated endothelial lining, draining at last into hepatic venules that coalesce into liver veins. The liver accommodates passively increases in blood flow from the portal circulation to a large degree [6], although there is some intrinsic flow regulation: - intrahepatic portal vascular resistance sites are found in post sinusoidal veins [7,8] and pre-sinusoidal sinusoidal flow is regulated by the Hepatic Arterial Buffer Response (HABR) (increased portal flow to the liver results in increased washout of adenosine surrounding pre-capillary sphincters, resulting in hepatic arteriole constriction. Conversely, reduced portal vein flow results in a compensatory increase in the hepatic arterial flow [9]). Portal vein ligation illustrates the compliance of the vascular bed in the liver – upon ligation of one half of the liver, the flow to the contralateral side increases by a factor of four [10]. This is clearly seen in the expanding, distended portal vein tributaries in portal vein angiograms after portal vein embolization [11]. Hepatic flow is further under sympathetic regulation from circulating noradrenaline and local nerve endings [12] and is influenced by systemic nitric oxide [13,14], endothelin-1 and cyclooxygenase-derived prostaglandins [15] and histamine [16].

Models of regeneration

Portal flow

The study of liver regeneration was largely triggered by Eck's seminal paper on complete portocaval shunting (PCS = Eck fistula) in dogs in 1877 [17] which led to the belief that the liver was not dependant upon portal blood perfusion. However, this was later contested by Hahn in 1893 [18] whose dogs deteriorated with the Eck fistula, showing signs of liver atrophy, weight loss and encephalopathy. The changes incurred by PCS were, for many ensuing years, thought to be the result of a lack of portal flow through the liver (as distinguished from the lack of the hepatotrophic substances transported to the liver in the portal blood). This "flow theory" seemed unquestionable after Child's model of portocaval transposition in 1953, where, after a 70 % PHx in dogs, the portal vein and vena cava inferior were switched surgically, resulting in the liver remnant regenerating by 50 % despite its receiving only systemic blood from the caudal stump of the vena cava inferior [19]. Further solid support to this theory came from several canine experiments conducted in the 1950's and early 60's with portal vein arterialisation (after portocaval shunting) showing that this manoeuvre would, not only arrest the changes incurred by the Eck fistula such as "meat intoxication", weight loss of the animals and liver atrophy [20-22], but also allow liver regeneration to occur after a 42 % PHx [23].

Portal blood constituents

With the advent of auxiliary liver grafting in the early 60's unveiling the phenomenon of graft atrophy due to the portal steal effect of the native liver, came the realization that there must be certain hepatotrophic substances delivered to the liver in the portal blood only, which the organ is dependant upon to regenerate and / or maintain its volume and function [24]. One could no longer regard the liver's homeostasis as a result of mechanical portal flow stimulus. Numerous experiments with canine models of split portocaval transposition (one portal branch perfused with blood from vena cava inferior and the other portal branch perfused with portal blood draining splanchnic organs, with similar flow rates and oxygen tension) in the period 1965 to 1978 revealed the importance of hormonal and nutritional influence of portal blood on liver regeneration, in particular insulin. After three months of this vascular rearrangement in dogs, one observed hypertrophy with glycogen deposition, increased DNA synthesis and mitosis on the side receiving splanchnic blood and atrophy on the side receiving systemic blood [25-28]. Substituting the blood flow in one of the portal vein branches with

arterial flow (and increased oxygen delivery) over three months could not compensate for the qualitative loss of the portal blood stimulus [29].

Given that the trophic substances seemed to be found in the portal blood, subsequent investigations were designed to disclose their origin. Surgical models with separation of the portal inflow coming from the upper GI-tract (distal stomach, duodenum, pancreas and spleen) from that originating from the small intestine (model of splanchnic flow division), and various degrees of splanchnic evisceration confirmed that the major trophic substances emanated from the upper GI-tract and consisted of insulin and possibly glucagon [30-36].

With the importance of portal blood constituents for liver maintenance and regeneration firmly established, research evolved to screen for other potential hepatotrophic substances in the portal blood. Canine models of Eck fistula in the early 90's, revealed that Insulin and partly T3, IGF-II, HSS, TGF-alpha, and HGF inhibited liver atrophy [37]. TGF-beta increased the atrophy but this effect was reversed upon concomitant insulin infusion.

Despite the numerous surgical models with splanchnic vascular manipulation and liver transplantation over a period of approximately 100 years implying the dominant role of humoral regulative mechanisms initiating liver regeneration and maintaining liver homeostasis, new studies in the late 90's appeared suggesting that increased sinusoidal flow and sinusoidal shear stress after PHx could play a role after all [38,39]. Increased endothelial shear stress was shown to trigger NO production [40-42] and later, liver regeneration after PHx in rats was shown to be inhibited by the administration of the NO antagonist N^G –nitro-L-arginine methyl ester, and restored by the NO donor 3-morpholinosydnonimine-1 (SIN-1) [43,44]. These studies therefore implicated that the increase in sinusoidal flow per gram remaining liver parenchyma after PHx initiates liver regeneration through de novo synthesis of NO. So the question of whether liver flow per se is detrimental to liver regeneration seems unsettled.

The role of liver oxygen status & energy load

In a canine Eck fistula model in 1952, it was observed that arterialisation of the portal vein stump over 4 months prevented liver atrophy [45]. Consequent long term canine experiments performed the next ten years in animals with Eck fistula and portal vein stump arterialisation showed that this altered hepatic vascularity was compatible with life, although many reported the development of vascular damage, and liver fibrosis progressing to cirrhosis [46-50]. In 1954, one observed superior liver regeneration in dogs after a 42 % PHx model with Eck fistula and arterialisation of the portal vein stump (controls regenerated to 80 % of original

volume versus 103 % in the arterialised group) and hypothesized that the increased oxygen delivery contributed to this difference [51]. Later, liver grafts were shown to survive on arterial blood only [52] and arterializing of the portal vein stump could reverse the atrophy caused by an Eck fistula [53]. Furthermore, recent porcine models of liver PHx with portal vein arterialisation have even shown enhanced regeneration compared to pigs with portal perfusion of the liver remnant also suggesting beneficial effects of increased oxygen delivery [54]. In the clinical setting, portal vein arterialisation has been found useful in counteracting the portoprival state of the liver and hepatic encephalopathy in cirrhotic patients with portocaval shunting [55,56] and beneficial in humans after extended hepatectomy [57].

Increased energy status in the remnant may explain beneficial effect of arterialisation. Rodent models of PHx from the 1970's and canine models from the 1990's have shown that the capacity of the liver remnant to regenerate after PHx is dependant upon an increased supply of energy [58-61] and arterialisation of the liver remnant leads to improved survival in rats after extended hepatectomy [62-64].

The liver as a source of growth factors

The liver itself is also a source of growth factors and cytokines which play a vital role in regeneration. In 1952, Glinos and Gey found the serum of partially hepatectomized rats to exert a growth-promoting action on fibroblasts in tissue culture [65]. Around the same time, other investigators reported an increased number of mitoses in the non-hepatectomized partner in parabiotic rats with cross circulation indicating the presence of growth stimulating factors in the effluent from the liver remnant [66,67]. This hypothesis was corroborated by later by observations of increased liver cell mitosis in intact rodent livers injected with serum from hepatectomized counterparts [68,69]. To circumvent the changes in portal hemodynamics caused by PHx, canine experiments in the early1960's with autotransplantation of small liver grafts to the jejunal mesentery, followed by randomization to 70 % PHx of the native liver or control, revealed that the autografts in the animals with 70 % PHx did not undergo atrophy, indicating again a growth stimulus from the resected liver to the autografts via the systemic circulation [70]. In later canine models of heterotopic allografting in the 1960's, the grafts did not suffer from atrophy when the native liver, receiving all the portal blood, was resected, again indicating a growth stimulating effect from the liver effluent after PHx [71]. Later, Starzl extracted cytosol from hepatectomized canine livers (48 and 72 hours after PHx) injecting it into the portal vein stump of dogs with Eck fistula, observing a proliferative response [72]. A year later it was observed that the growth-

stimulating factor in the cytosol extract from regenerating canine livers (termed Hepatic Stimulatory Substance, SS) was organ specific in that it did not stimulate any glomerular proliferative activity when injected into the renal artery. Investigating how factors in the recipient liver influenced the action of SS, Terblanche injected regenerative liver extract into the portal vein perfusing normal canine livers without any response. However, an augmented proliferative response was seen upon injecting the extract into the portal vein of resected livers 48 and 72 hours after PHx [73]. Further investigations of a possible growth stimulatory substance in the liver effluent from partially hepatectomized pigs was performed by van Hoorn-Hickman in 1981 by cross circulation with recipient animals or exchange perfusion. Increased thymidine kinase activity and mitotic indices in biopsies from portocaval shunted (recipient) pigs corroborated Starzl's previous observations in dogs [74]. Kahn also showed in 1982 that a stimulatory substance was transferred from a transplanted partially hepatectomized liver to the host liver (which had a portocaval shunt), stimulating a proliferative response in the latter (as judged by increased thymidine kinase activity and mitotic indices)[75].

Contemporary research – has it helped?

The focus of research on liver regeneration after PHx has expanded greatly during the last three decades since the pioneering surgical models described above. Focus has turned from examining "causative" factors such as portal- and hepatic arterial blood flow and its content towards the intrinsic consequences these changes have in the extra cellular matrix, the intracellular signal transduction mechanisms and genetic response in the liver during regeneration. Studies in various cell culture models, rodent knockout- and knockdown models, stem cell transplantation, microarray analysis, the impact of the immune system, blood platelets and serotonin, the complement system, cytokines, and the interaction between the many different cell types now known to regulate the regenerative process has unquestionably added much knowledge to the research on liver regeneration. However, the picture has become quite complex and seemingly increasingly intangible when it comes to the clinical application of the knowledge gained. This is partly expressed in recent reviews by authorities on liver regeneration, summarizing the vast amount of published literature on the molecular control of liver regeneration in the past 20-30 years. Concluding her review in 2004, Rebecca Taub writes, "what remains unclear is how the size of the liver is determined", "how the known molecular pathways necessary for liver regeneration are altered in human disease", and that "greater insight will be required to develop improved pharmacological therapeutics and surgical approaches" [76]. In Nelson Fausto's review from 2006, the author concludes that what is needed is a more "rigorous effort to apply the knowledge gained in experimental work to solve clinical problems"[77]. And in his recently published Rous-Whipple Award Lecture (2010) Michalapoulos concludes that "liver failure, essentially a failure of regeneration, should be subject to mechanistic analysis based on knowledge already gained on regeneration, and perhaps therapeutic interventions may be designed with impact on human liver disease" [78].

How then has research on liver regeneration in the past 133 years benefited the patient with liver cirrhosis, acute liver failure or liver metastasis? The recognition of the importance of portal blood to liver homeostasis and regeneration was obviously detrimental to the pioneers of liver transplantation as they observed how the auxiliary graft would undergo atrophy without portal blood constituent stimulus [79]. The earlier canine and porcine experiments of portal vein arterialisation [80-87] also illustrated to the transplantation surgeon, that the auxiliary graft could be supplied perfused by PVA as an option to leave the hilus of the native liver untouched and in cases of portal vein thrombosis [88-91].

Furthermore, as part of the emerging multimodal three stage treatment of colorectal metastases [2], surgeons now often embolize the portal vein before performing large resections in order to stimulate liver hyperplasia in the remnant to be, thereby avoiding postoperative liver failure, having appreciated that diverting portal flow away from one side to the other resulting in hypertrophy the future remnant (described as the “parenchymal shift” by Rous and Larimore in 1920 [92]).

Aims

Faced with the prospect of an increasing incidence of primary and secondary liver malignancies due to an ageing population and the rising use of multimodal treatment with neoadjuvant chemotherapy to downstage colorectal metastasis, contemporary liver surgery necessitate a better understanding of the mechanisms controlling liver regeneration in order to design new treatment strategies to support the functionally deficient failing organ be it due to a minimal remnant or cytotoxic side-effects. On this basis, the present thesis has the following aims:

Paper 1

To illustrate how analysis of real time data in time series (derived from research on liver hemodynamics) using novel statistical analysis may complement traditional statistical methods as the technique enables the observer to utilize all data points from real time sampling and explore the data at multiple time scales.

Paper 2

To investigate if the pressure and flow differences in the liver remnant after a 62% and a 75% resection generate different gene activation patterns corroborating the theory of shear stress induced initiation of the regenerative response.

Paper 3

To distinguish the effects two different stimuli potentially initiating liver regeneration after partial hepatectomy: - increased sinusoidal flow/shear-stress in the liver remnant vs. increased delivery of hepatotrophic factors in the blood perfusing the liver remnant.

Paper 4

To review the published English literature on large animal research on liver regeneration in order to learn from the past century of research and depict the future challenges.

Hypothesis

Paper 1

With the advent of increasing computational power and real-time recordings from hemodynamic studies, one is increasingly dealing with vast amounts of data in time series the analysis of which with traditional methods of statistical analysis is inadequate.

Paper 2

Increase in the flow per gram remaining liver tissue, as reflected in the increase in portal vein pressure, determines the regenerative response in the liver remnant after liver resection.

Paper 3

According to the “flow theory” of liver regeneration, it is the increased sinusoidal flow in itself, and not the increased delivery of potential hepatotrophic substances in the portal blood which is the primary stimulus to liver regeneration.

Paper 4

Reviewing the published literature on large animal research on liver regeneration will enable the scientist to formulate relevant hypotheses for future experiments.

Materials and methods

Animals and instrumentation

Castrate sus scrofa domesticus were used for all experiments which were conducted in compliance with the institutional animal care guidelines and the National Institute of Health's Guide for the Care and Use of Laboratory Animals [DHHS Publication No. (NIH) 85-23, Revised 1985]. The animals were an average of three months old, weighing on average 32 kg (SD 2 kg). All animals were operated upon between 0800 and 1200 hours. Anesthesia was induced with intramuscular Ketamine (10 mg/kg) and atropine (0.05 mg/kg) and Fentanyl (0.2mg/kg), and maintained with 1.5-2% Isoflurane mixed with 55% oxygen. Analgesia was maintained with Fentanyl 0.01 mg/kg. Respiratory rate was adjusted to achieve an end tidal CO_2 between 3.5 and 6 KPa. Mean alveolar concentration of Isoflurane was maintained at 1.3 using a Capnomac (Nycomed Jean Mette). Before surgery, all animals received a single i.m. shot of antibiotic prophylaxis (Enrofloxacin, 2.5 mg/kg). For hemodynamic monitoring (in all acute experiments), a 16G central venous catheter (CVK, Secalon® T) was placed in the left femoral artery for continuous arterial blood pressure recording (MAP). A 7 French 110 cm angiographic catheter (Cordis®, Johnson&Johnson) was placed in the hepatic vein draining segments V and VIII via the right internal jugular vein for right hepatic venous pressure monitoring. A paediatric CVK (Arrow® International) was placed in the portal vein with the tip approximately 5 cm from the liver hilus for portal pressure monitoring. A 5 French Swan-Ganz catheter (Edwards Life sciences™) was floated via the right external jugular vein to the pulmonary artery for cardiac output (CO) measurements.
Calibrated transducers (Transpac 3™, Abbott Critical Care Systems, Chicago, IL, USA) were used for real time pressure registration. The transducers were connected to an amplifier (Gould, 2800S, Ohio, USA). Pulsatile signals were displayed on a monitor, digitalized and stored electronically (Advantech, Industrial Computer). All recordings were logged every fourth second. Perivascular ultrasonic flow probes (CardioMed Systems, Medistim A/S, Oslo, Norway) were placed around the portal vein (12mm probe). Signals were displayed on a monitor and stored electronically (Advantech, Industrial Computer). Blood extraction was performed prior to biopsy sampling. Samples were taken from the portal vein, femoral artery and hepatic vein. IL-1, IL-6, TNFα, TGF-α, TGF-β, and EGF were analyzed using ELISA (Quantikine®, R&D systems, and Searchlight® Pierce Biotechnology, MA, USA) and ASAT,

ALAT, GT, pyruvate, glucose, lactate, ALP levels were quantified by calorimetric, UV-photometric and HPLC analysis (Roche®, PerkinElmer®).

Paper 1

All calculations in paper 1 are based on hemodynamic recordings from experiments reported in paper 2 and 3. (Figure 1)

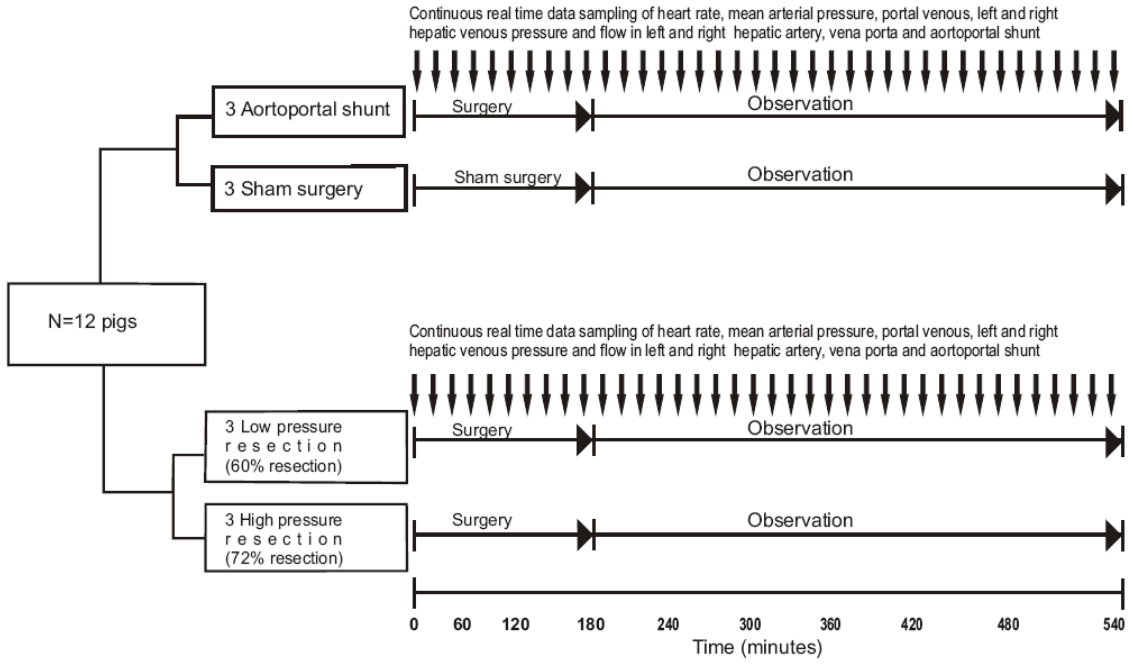

Figure 1. Illustrates below illustrates the four experimental groups from which the data are extracted.

Five sets of hemodynamic data were analyzed: Mean Arterial Pressure (MAP), Heart Rate (HR), Right Hepatic Venous Pressure (Vp), Portal Venous Pressure (Pp), and Portal Venous Flow (Pf). Each set was analyzed with three different statistical methods: 1) Repeated Measures ANOVA, 2) Linear Mixed Models and 3) Time series analysis with the Significant Nonstationarities (SiNoS) method. For analysis with the two first methods, data was extracted from the real time data material with exact 10-minute intervals and analyzed using SPSS 13 for Windows statistical package. P values ≤ 0.05 were considered statistically significant. When analyzing with Repeated Measures ANOVA, we studied within-group trends with within-subjects effects for time and group*time. Overall group difference was analyzed with between-subjects effects. When analyzing with Linear Mixed Models we performed multiple local ANOVAs for consecutive one-hour time periods starting at t = 0 minutes (0-1hr, 1-2hrs,

etc) and t = 30 minutes (30-90', 90-150' etc) and for two-hour time periods starting at t = 0 minutes (0-2hrs, 2-4hrs etc). We defined time as a fixed factor and subject as a random effect. As recommended by Norusis [93], an autoregressive AR1 covariance matrix was used. Time, group and group*time interaction were tested.

To analyze all data points from the real time data, we used time series analysis with the Significant Nonstationarities (SiNoS) method. Briefly, in SiNoS, the data is analyzed simultaneously for several time horizons by basing the inference on repeated tests along the time series, comparing the estimated parameters on consecutive segments of the series. The lengths of the segments represent the time scales for the analysis and different lengths are used to detect changes on different time scales, making it possible to judge which scales that seem to have the most meaningful interpretation. For a chosen test point, t1, we perform two-sampled hypothesis tests for the mean (μ), the variance ($\sigma 2$) and the first lag autocorrelation coefficient (ρ), comparing estimated values in the two windows W1 and W2 on each side of t1. The total length of the two windows W1 and W2 represents the scale for the analyses. For each scale the hypothesis testing is repeated along the time series by sliding the two consecutive windows to the next chosen test point t2. By applying different window lengths, potential changes are explored on different time scales**.** To avoid a large number of false detections, we adjust for multiple testing with the method of False Discovery Rate (FDR) introduced by Benjamini and Hochberg [94].

Paper 2

Six castrate sus scrofa domesticus were used for all experiments. Three pigs underwent a 62 % liver resection removing segments II, III, IV, V and VIII (low-portal pressure resection, LPR) and three underwent a 75 % resection removing in addition segments VI and VII (high-portal pressure resection, HPR).

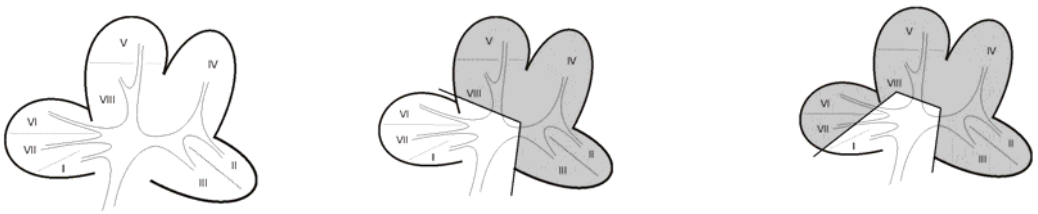

Figure 2. Illustrates the segmental anatomy of the porcine liver and two different grades of resection (PHx).

All hemodynamic recordings were made with sampling points every 4th second for six hours after completion of liver resections. Biopsies for microarray analysis were taken from the remaining segments VI, VII in the LPR series and from segment I in the HPR series and placed immediately in RNALater (Ambion®). Sampling time points were 1, 30, 90, minutes and 3, 4 and 6 hours after resection.

The microarray experiments were conducted as a common reference design using liver total-RNA purified from an unrelated animal as the reference allowing comparison of all the different samples to each other (the acute phase response due to the operation in itself could in theory affect gene expression in both groups of liver remnants, however, as the perioperative conditions were almost identical, we considered this effect to be equal for the two resection groups and chose not to use a sham as the reference). Total-RNA was purified and aminoallyl-cDNA (aa-cDNA) was synthesized from 20 µg of total-RNA. The reference sample was labeled with Alexa 488, each individual sample was labeled with Alexa 594 and the samples were combined pair wise and hybridized to the pig array DIAS_PIG_55K2,

which consists of 26879 PCR products amplified from unique cDNA clones. Following hybridization, washing and drying, the slides were scanned and the median intensities were computed. Statistical analysis was carried out in the R computing environment using the Bioconductor package Linear Models for Microarray Analysis (Limma) [95,96]. The $\log_2$-transformed ratios of Alexa-594 to Alexa-488 were normalized within-slide using print tip-loess and analyzed to identify genes being significantly differentially expressed by time within treatment as well as between treatments. Time contrasts were formed referring to the sample taken at time point 1 min. The genes found significantly expressed between at least two time points were further analyzed referring to Online Mendelian Inheritance in Man (OMIM®, [97]) and Gene Ontology® (GO®) to group the genes by function. More detailed descriptions of the microarray experiments are available at the NCBIs Gene Expression Omnibus [98-100] through the GEO series accession number GSE6860.

The data was analyzed within each resection group (LPR and HPR) in two ways: Firstly, using top-tables for each of the five time intervals (1'to 30', 1'-90', 1'-3h, 1'-4h, 1'-6h post PHx) we classified all genes into 14 functional groups by molecular function and biological process according to the GO® and OMIM®. Secondly, using K-means clustering we defined 20 clusters in each group and selected from these 8 clusters of special interest on the basis of their non-uniform profiles for closer analysis with GO® and OMIM®. For direct comparison of the two resection groups we investigated the genes which were expressed in both groups at each time point, selecting those which had a statistically different expression level in the two groups (herein termed "within time point contrasts"). We avoided using fold change cutoff values because this approach fails to take the uncertainty of variability into account (i.e. a gene may exhibit a tenfold change and yet not be significant because of its variability), and has the potential of excluding genes of biological importance whose expression values are below the cutoff value.

For RT-PCR validation, sets of two primers and a probe for 8 chosen target genes and 1 control gene (18S) were designed using the Primer Express software package (version 2.0; Applied Biosystems). To avoid genomic DNA contamination the primers were designed to span exon boundaries. The probes for the target genes were labelled with either the fluorescent reporter VIC or SYBR Green. 5 μg total RNA of each sample was reverse transcribed using random hexamer primers and SuperScript II Reverse Transcriptase (Invitrogen). Real-time quantitative RT-PCR was performed on the ABI Prism 7900HT sequence detection system (Applied Biosystems) by monitoring the increase in fluorescence due to the binding of SYBR green or VIC to double-stranded DNA. The amplification

conditions were as follows: 50°C for 2 minutes, 95°C for 10 minutes and 40 cycles each of 95°C for 15 seconds and 60°C for 1 minute. Each cDNA sample was run as technical triplicates. The standard curve method was used to calculate the relative mRNA levels. The quantity mean of the triplicate measurements were normalized against the 18S gene and mean of the biological replicates were calculated as well, yielding a single value for each time point. The profiles of these values were compared to the profiles of the microarray data.

Paper 3

This paper is a combination of an acute- and chronic series of partial portal vein arterialisation (Figure 3).

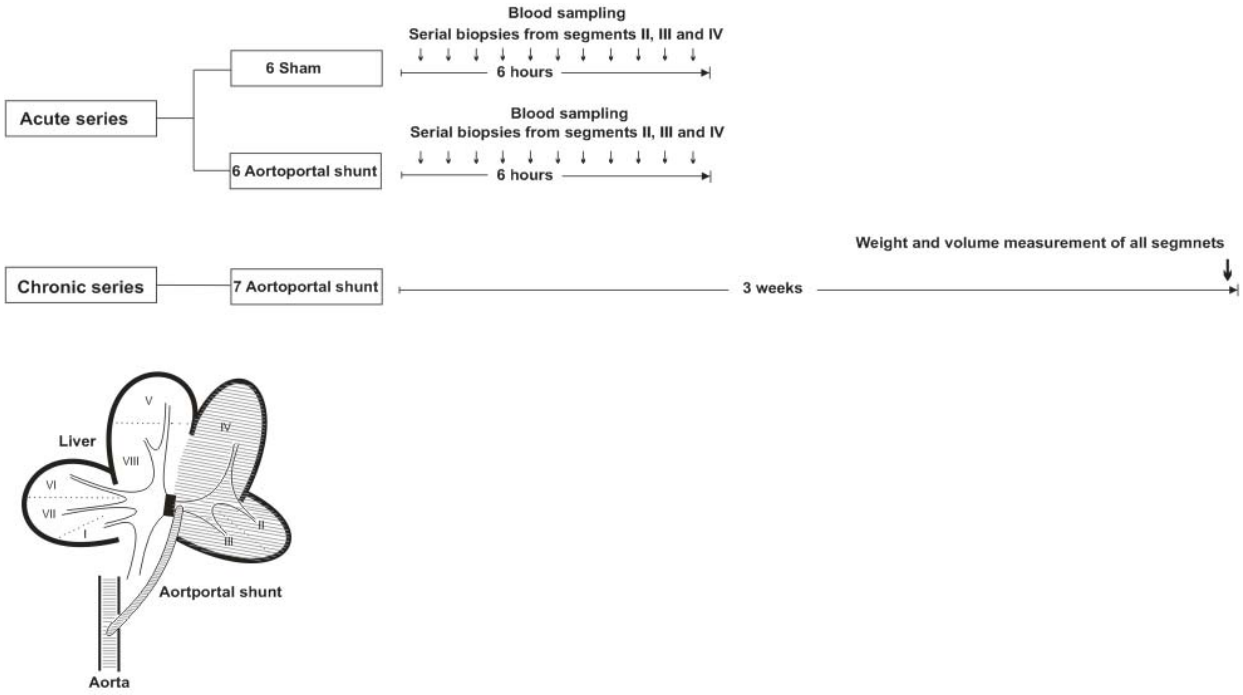

Figure 3. Illustrates the three experimental groups and the arterialization of segments II, III and IV.

Acute series: After a midline laparotomy and placement of all catheters and flow probes as described above, we isolated and recorded the flow in the left portal vein branch (LPVB). When the activated clotting time (ACT) was above 250 seconds, a 5mm Propaten Gore-Tex™

graft was anastomosed end-to-side from the aorta (between truncus coeliacus (TC) and the superior mesenteric artery (SMA)) to the LPVB. The LPVB was then ligated proximal to the bifurcation to prevent backflow to the main portal vein trunk (MPVT). The opening of the shunt was regarded as time = 0 and noted. Flow in the shunt was standardized in each experiment to 1000 mL/minute by gradual shunt constriction using a ligature and a perivascular flow probe. Sham surgery consisted of all the steps above except for the establishment of the aortoportal shunt.

Chronic series: After a midline laparotomy, a similar shunt was placed from the aorta to the LPVB once the animal had received 5000 IE Heparin i.v. We used an interposed aorta graft from a donor pig in the chronic series as the Gore-Tex grafts™ tended to become occluded. The LPVB was ligated proximal to the portal bifurcation to prevent backflow to the MPVT. Flow was standardized (by concentric constriction with a ligature) to 1000mL/minute. Upon relaparatomy three weeks later, the shunt was isolated and flow measured. The flow in the MPVT (now supplying the right liver only) was recorded.

In the acute series, sequential biopsies were taken from the shunted segments II, III and IV at time points 1, 5, 10, 30, 60, 90 minutes and 2, 3, 4 and 6 hours after shunt opening (t = 0). The sampling time points were the same as in a previous study of liver regeneration after PHx [101] using the same microarray platform allowing the direct comparison of gene expression profiles found in the present experiments with the former. Biopsies were placed immediately in RNAlater (Ambion®).

Blood extraction was performed before biopsy sampling. Samples were taken from the portal vein, femoral artery, and hepatic vein draining both sides of the liver. Aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), glutamyl transpeptidase (GT), glucose, bilirubin (Bil) and alkaline phosphatase (ALP) levels were quantified by calorimetric, ultraviolet-photometric, and HPLC analysis (Roche, PerkinElmer).

For cytokine analysis, a multiplex kit was developed including four different cytokines; TNF-α, IL-1α, IL-6 and IL-10. Serum samples was analyzed in duplicates using the Luminex 200™ with the Bioplex manager software (BioRad, Hercules, CA) [102].

In the sham series, liver biopsies were taken from segments II, III and IV and blood was sampled from the same locations at the same time points as in the shunted animals.

In the chronic series, only peroperative arterial blood gas samples were taken (directly from the aorta) to monitor respiratory status.

To evaluate the long-term (3 weeks) effects of arterial hyperperfusion on the liver parenchyma we took biopsies from both the shunted and the portally perfused sides of the liver before and after shunting. Specimens were fixed in buffered formalin, paraffin embedded, and stained with Hematoxylin-Eosin (HE) to evaluate tissue architecture. To evaluate proliferative activity, sections were stained with Ki67 and Phosphohistone H3. The proliferative index was estimated by counting the number of Ki67 positive cells relative to the number of non-stained hepatocytes per liver lobuli. Connective tissue distribution was studied using Reticulin staining. An independent pathologist (EM) reviewed the sections in a blinded manner.

Paper 4

A literature search was performed in Pubmed with the following terms: liver regeneration, animal, canine, porcine, and pig. Further peer reviewed research papers were obtained from the reference lists of the articles retrieved.

Summary of results

Paper 1

We found SiNoS analysis more comprehensive when compared to traditional statistical analysis in four ways: One, the method allows better signal to noise detection; two, including all data points from real time recordings in a statistical analysis permits better detection of significant features in the data; three, analysis with multiple scales of resolution facilitates a more differentiated observation of the material; and four, the method affords excellent visual presentation by combining group differences, time trends and multiscale statistical analysis allowing the observer to quickly view and evaluate the material. It is our opinion that SiNoS analysis of time series is a very powerful statistical tool that may be used to complement conventional statistical methods. Figure 4 below will be used as an illustration of this.

Figure 4.a illustrates curves representing a measurement of MAP every fourth second over a period of nine hours. The curves in both sham and aortoportal shunt groups are almost superimposed until shunt opening at 180 minutes (corresponding to measurement nr. 2800) where we observe an immediate and sustained fall in MAP in the shunt group. For the sake of illustration and comparison, a vertical line representing the time of aortoportal shunt opening is drawn through all figures. Figure 4.b illustrates the Plot of Smooths derived from the Gaussian Kernel smoothing with different bandwidths and the Mean SiNoS plot (figure 4.c) displays the corresponding significance map. Significant changes in the difference in MAP between the shunt and sham group are illustrated for the whole period of observation. The y-axis in figure 4.c represents the window width of analysis and the x-axis the time points throughout the experiment. White areas depict time windows in which there is a statistically significant decrease in the MAP difference between the shunt and sham groups, and black areas time windows in which there is a statistically significant increase in the MAP difference. Dark grey areas represent time frames of no statistical significant change and light grey areas time frames with too few data points for statistical inference to be made at the respective scale of analysis.

At window width 800 (on the Y-axis of figure 4.c) in the time period of measurement nr.1000 to 1500, the MAP in the shunt group rises transiently above the MAP in the sham group (which is seen on closer inspection of figure 4.a, in the time period of approximately 58 to 90 minutes). This pressure increase is flagged as significant as there is a white area in the Mean SiNos map corresponding to this time period (figure 4.c). Despite the statistical significance, the change probably only represents random pressure fluctuation. However, further on, in the time period measurement nr. 2500 to nr. 3200, the MAP difference changes again significantly in the opposite direction. By referring to figure 4.a we see that this corresponds to the fall in MAP in the shunt group when the shunt is opened. SiNoS analysis detects instantly the fall in MAP upon shunt opening. When the time frame is increased the change in difference is also significant over a longer time period. For example, taking the window width 2520 we see a significant change in MAP difference from shunt opening to measurement nr. 5000 – that is, if we take any time point from measurement nr. 2500 to nr. 5000 and compare the MAP of the preceding 168 minutes with the following 168 minutes, there will be a significant change in MAP difference from the first time period to the next. It follows from this that the steeper the changes in MAP difference, the smaller time frame needed to detect it.

Sampling MAP with 10-minute intervals yields a somewhat similar picture graphically (figure 4.d). In contrast to the approximately 8000 data points used in figure 4.a, b and c, we are now dealing with 54 data points. As in the real time raw data, several noise spikes appear, though fewer, and a fall in MAP in the shunt group at time point 180 minutes is seen corresponding to shunt opening.

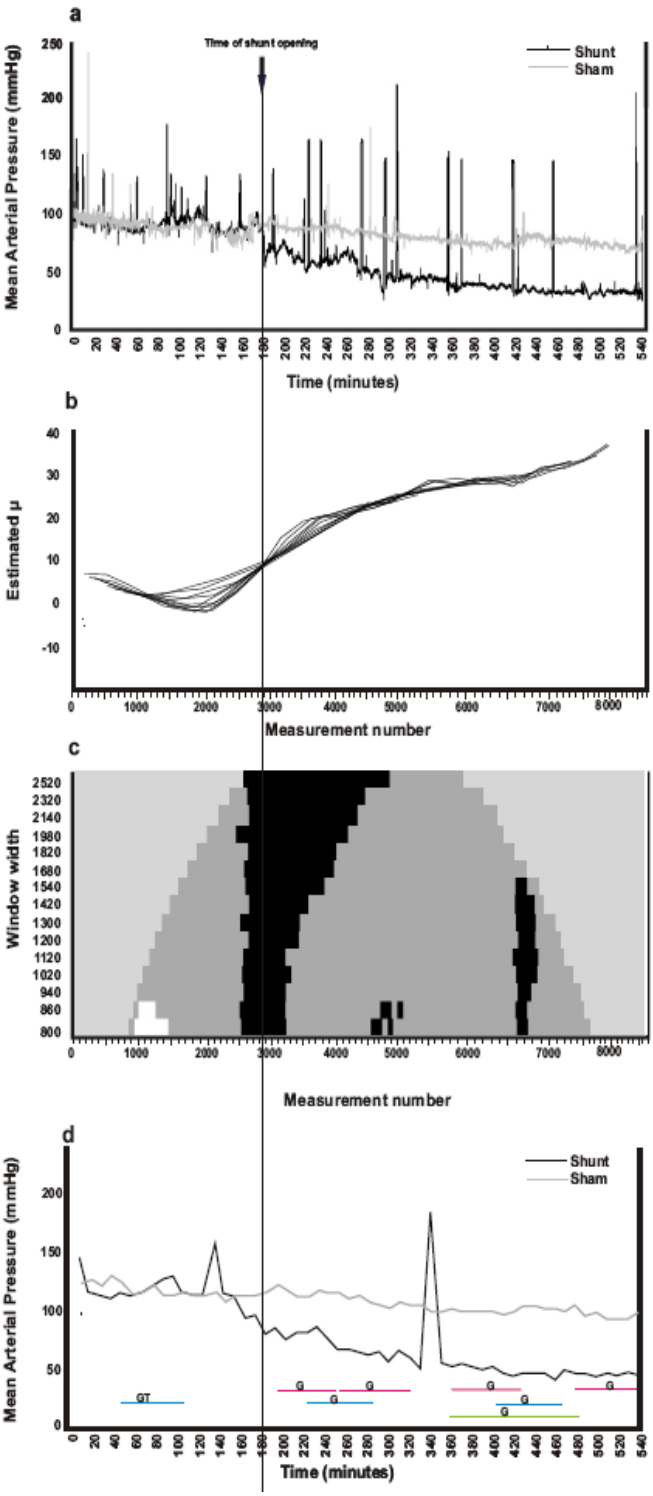

Figure 4. Mean Arterial Pressure (MAP) in shunt vs. sham series.
a) MAP curves for the shunt (black) and sham (grey) groups based on real-time recordings over 9 hours.
b) Plot of Smooths.
c) Mean SiNoS plot for the difference in MAP between shunt and sham groups.
d) MAP curves based on sampling every 10 minutes.

Analysis of this dataset with Repeated Measures ANOVA (figure 5) indicates significant within subject effects of time (p=0.000) and time*group interaction (p=0.008). However, it is not until 260 minutes after shunt opening (at time point 440 minutes) that a significant difference in MAP trends in the two groups is detected (within subjects contrasts, p=0.042). This is followed by a period of significant and non-significant within subject contrasts until experiment termination with significance values varying from p=0.029 to p=0.044. Analyzing between-group effects reveals no group difference. In other words, contrary to SiNoS analysis, repeated measures ANOVA does not detect the fall in MAP upon shunt opening until 4 hours has passed and only at a few and sporadic time points does ANOVA find significant group differences

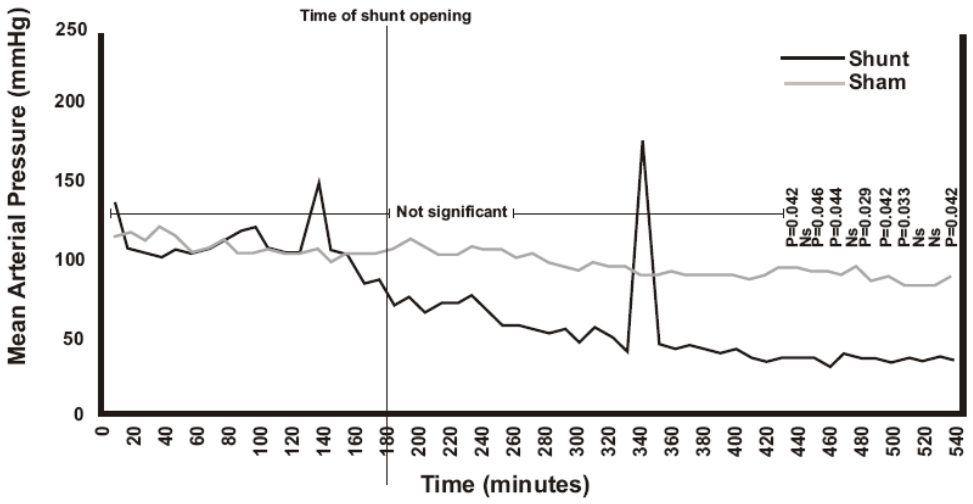

Figure 5. MAP curves based on sampling every 10 minutes. Ns; non-significant within-subjects contrasts when analyzed with repeated measures ANOVA..

Performing local ANOVAs with a Linear Mixed Model along the MAP dataset reveals significant group differences in the mean MAP in several time periods after shunt opening (marked with “G” for the respective time periods in figure 4.d). Only from 40 to 100 minutes do we find a significant group*time_interaction (marked with “GT” in figure 4.d). This corresponds to the time period where SiNos also detected a group difference (marked by a white area in figure 4.c). Interestingly, Mixed Models ANOVA performed over a one-, and two hour time period immediately after shunt opening does not find a significant group*time interaction upon shunt opening, although the two curves split and stay so at this point (figures 4.a and 4.d). We observe, however, that Mixed Model ANOVA does find significant group differences over almost the entire period after shunt opening (figure 4.d). We do, of course, believe that the group difference occurring after shunt opening found by both SiNos, mixed models ANOVA, and partly repeated Measures ANOVA is biologically significant, as the MAP would be expected to fall upon opening a large central arteriovenous shunt.

Paper 2

Six pig livers were resected with 62% (Low Portal Pressure Resection, LPR) and 75% (High Portal Pressure Resection, HPR) resulting in a portal venous pressure increase from a baseline of 6.1 mmHg to 8.2 and 12 mmHg respectively (figure 6). By sampling consecutive biopsies from the liver remnants we found differentially expressed genes in the HPR group to have functions related primarily to apoptosis, nitric oxide metabolism and oxidative stress, whereas differentially expressed genes in the LPR group potentially regulate the cell cycle (figure 7). Common to both groups was the upregulation of genes regulating inflammation, transport, cell proliferation and development and protein metabolism. Also common to both groups was both up- and downregulation of genes regulating cell-cell signaling, signal transduction, cell adhesion and translation. Genes regulating the metabolism of lipids, hormones, amines, and alcohol were downregulated in both groups (figure 8). The genetic regenerative response in the liver remnant to varies according to the level of resection.

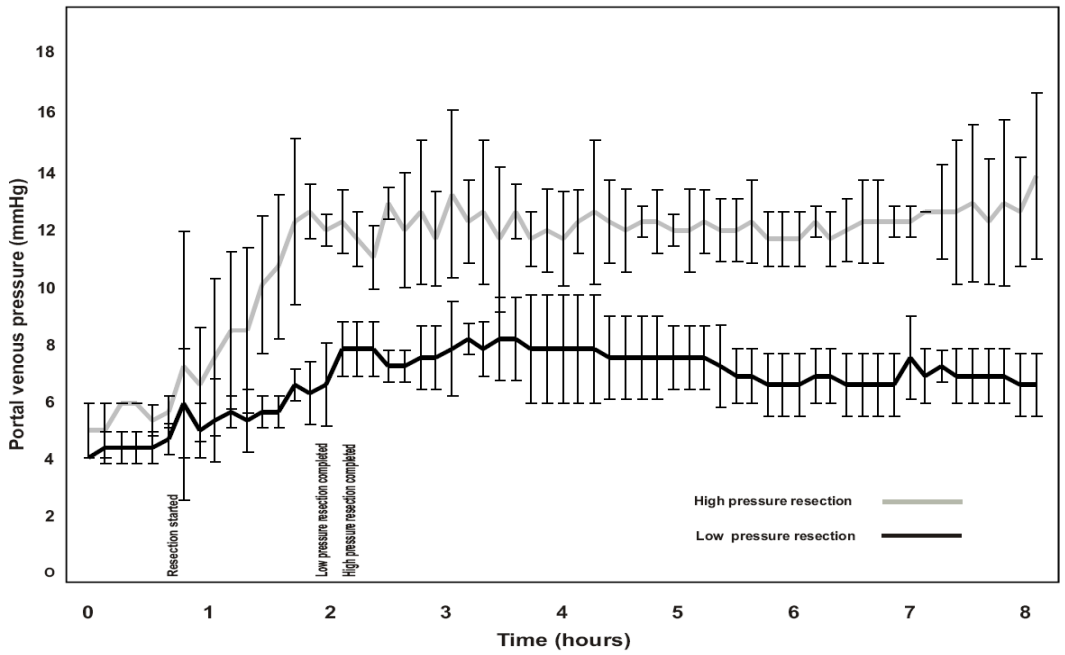

Figure 6. Portal venous pressure in the High Portal venous Pressure Resection (HPR) and Low Portal venous Pressure Resection (LPR) series (mean pressure ± SD).

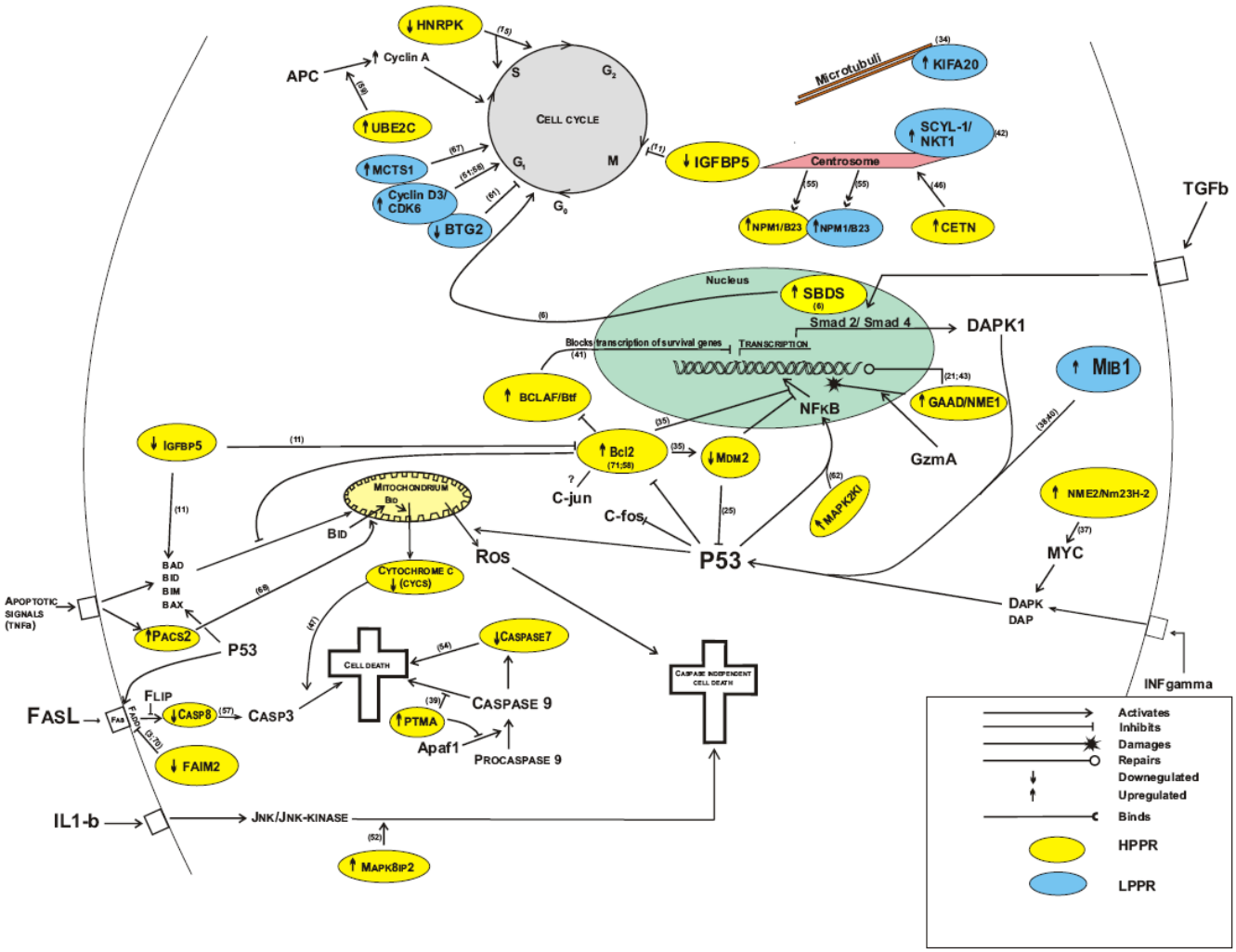

Figure 7. Schematic presentation and suggested interaction of differentially expressed genes regulating cell cycle and apoptosis in both resection series. (Yellow markings - HPR – high pressure resection, 75 % PHx. Blue markings - LPR – low pressure resection, 62 % PHx).

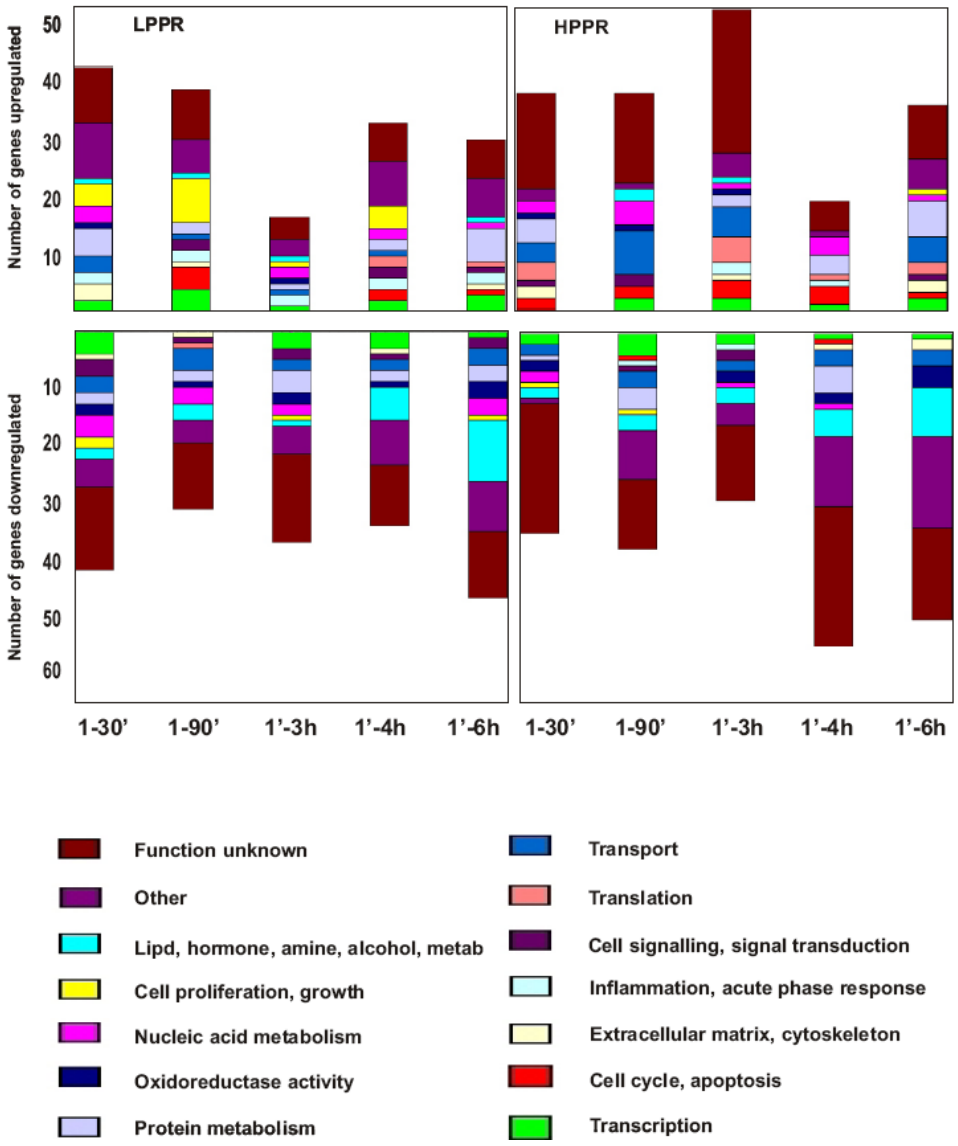

Figure 8. Functional classification of differentially expressed genes in the low pressure group (LPR) and high pressure group (HPR) in top-tables according to the functional classification methods of Gene Ontology and Online Mendelian Inheritance in Man (OMIM).

Paper 3

We constructed an aorto-portal shunt to the left portal vein branch creating a standardized four-fold increase in flow to segments II, III and IV. The impact of this manipulation was studied in both an acute model (6 animals, 9 hours) using a global porcine cDNA microarray chip and in a chronic model observing weight and histological changes (7 animals, 3 weeks). Gene expression profiling from the shunted segments does not suggest that increased sinusoidal flow per se results in activation of genes promoting mitosis. Hyperperfusion over three weeks results in the whole liver gaining a supranormal weight of 3.9 % of the total body weight (versus the normal 2.5 %). Contrary to our hypothesis, the weight gain was observed on the non-shunted, portally perfused side, without an increase in sinusoidal flow (figure 9).

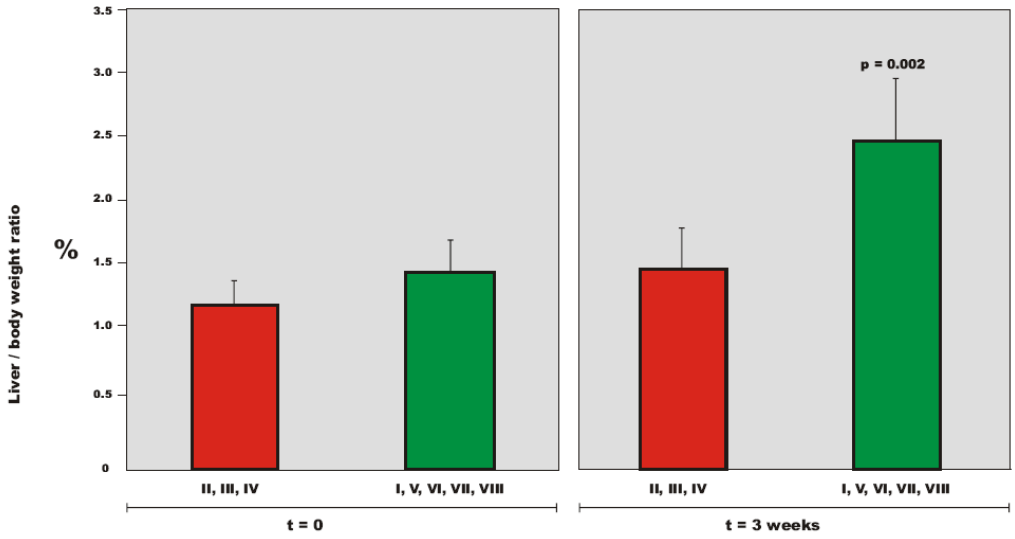

Figure 9. Liver / body weight ratio (%) by segments before and after 3 weeks of aortoportal shunting of segments II, III and IV. The total liver weight increases over three weeks, the increase occurring in the non-shunted segments (I, V, VI, VII and VIII).

The portally perfused and shunted sides revealed marked microscopic and macroscopic differences after three weeks (figure 10). In this study, an isolated increase in sinusoidal flow did not have the same genetic, microscopic or macroscopic impact on the liver as that seen in the liver remnant after partial hepatectomy, indicating that increased sinusoidal flow may not be a sufficient stimulus in itself for the initiation of liver regeneration.

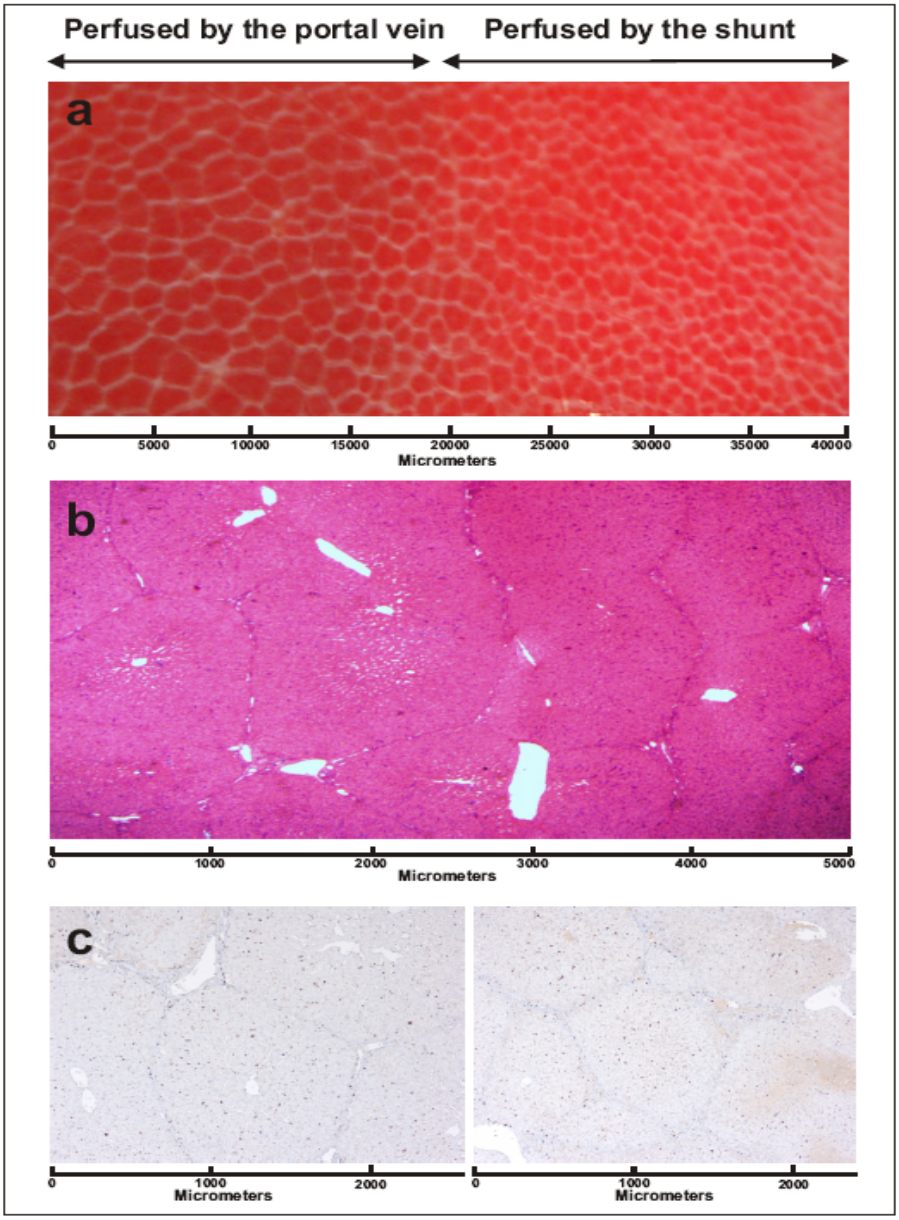

Figure 10. Macro-and microscopic changes after three weeks of shunting: **a)** Close-up photograph of the transition zone between shunted and portally perfused in-vivo liver after three weeks. The shunted side exhibits smaller condensed lobuli and a brighter (hyperoxygenized) color, while the portally perfused side exhibits larger lobuli, **b)** HE stained section of the transition zone showing more condensed lobuli on the shunted side and larger lobuli with dilated portal venules and central veins on the portally perfused side, **c)** sections from areas perfused by the portal vein and by the shunt showing an even distribution of Ki67 positive cells (control sections of sham and baseline livers all show a lower density of Ki67 positive cells).

Paper 4

Apart from the surgical principles and practice of preoperative portal vein embolization to induce hyperplasia of the remnant to be after PHx, and portal vein decompression by portosystemic shunting to reduce sinusoidal congestion in the case of SFSS after liver transplantation, there is still no novel patient therapy available to aid and augment the process of liver regeneration be it after extended liver resections, after a toxic liver insult or in the cirrhotic patient (MARS substitutes the failing liver, but does not stimulate regeneration) - this in spite of all the modern technological advancements and the knowledge gained on the microscopic and molecular aspects of liver regeneration in the past 20-30 years. We suggest that it is time to turn back to the systemic large animal surgical research on liver regeneration as it offers a more integrated, systemic biological understanding of this complex process, and that a more clinically relevant progression could possibly be made with a closer collaboration between the hepatologist, liver surgeon/transplant surgeon and the laboratory scientist.

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Discussion

Paper 1

Large amounts of research has previously been done on the hepatic vascular bed and the regulation of liver flow [6-10,12,16,103-119] in particular by Wayne W. Lautt in Canada. Therefore, it was not our intention to describe well-known phenomena such the hepatic arterial buffer response (HABR) or analyze in detail the systemic changes incurred by liver resection and or aortoportal shunting. However, when studying the changes in mean arterial pressure, pulse, portal venous pressure and hepatic venous pressures, changes in flow in the liver arteries, portal vein and in the established shunt occurring during different grades of liver resection and arterial shunting of the left portal vein, we soon realized that using repeated measures ANOVA and Linear Mixed Models ANOVA was inadequate when sampling every fourth second for the nine hours that the acute experiments presented in paper 2 and 3 lasted. We speculated that the scale of resolution with which the hemodynamic changes were analyzed and the method with which true signals were selected from (instrumentation) noise would influence what we saw / found and, accordingly, which conclusions we could infer. We therefore searched for an alternative manner to analyze our repeated measures and eventually applied the Significant Nonstationarities (SiNoS) method.

Paper 1 illustrated that the novel SiNoS analysis of our hemodynamic data is potentially a very useful adjunct to Repeated Measures ANOVA and Linear Mixed Models ANOVA. We advocate the use of this method of statistical analysis of repeated measures of real time data sampled in hemodynamic research because:

1. The method's plot of smooths gives good resolution of signal from noise, a great advantage when interpreting hemodynamic data from experimental surgery.
2. The method allows for analysis of all data points from real time recordings that seems to increase the ability to detect significant features in the material.
3. Analysis with multiple scales of resolution facilitates a more differentiated observation of the material, and

4. The graphical display afforded by this method, enables the researcher to appreciate the data in a more varied manner than is likely with more traditional graphics.

The statistical method developed in paper does not employ the often-used Bonferroni correction but the method of False Discovery Rate (FDR) introduced by Benjamini and Hochberg [94]. When examining the data after statistical analysis one must therefore bear in mind the various biological and technical explanations for the trends detected. When this is done, many trends falsely labeled statistically significant by SiNoS may be explained by viewing them in light of the experimental or technical intervention.

Paper 2

In this paper we aimed to investigate whether the grade of resection and hence the portal venous pressure and sinusoidal shear stress increase, would influence the regenerative response, as suggested by the "flow theory" described above.

The study showed that the regenerative response (as quantified by gene expression in the liver remnant) in the first six hours immediately after completion of the resection, is indeed influenced by the grade of resection and increase in portal pressure:- A "low pressure" resection (LPR) (62% liver resection) stimulated primarily the progression through the G1-phase of the cell cycle whereas a " high pressure" (HPR) (75%) resection, primarily inhibited the apoptotic apparatus, regulated NO metabolism and oxidative stress.

Of all the 26 cell-cycle genes differentially expressed in this experiment, a major part (18 genes) was found in the HPR group (figure 7). Qualitatively, the genetic response after a high grade resection seems to center around the regulation of apoptosis, inhibiting death-promoting pathways, particularly the caspase system, whereas, after a lower grade resection the differential regulation is primarily of genes regulating the cell cycle and cytoskeletal framework. Specifically, we observed in the LPR group, that genes promoting progression through the G_1 phase were upregulated and genes inhibiting progression were downregulated. In addition, genes associated with the microtubuli apparatus and centrosomes were found upregulated. Over time, it seems that apoptosis is downregulated during the earlier time points (within 90 minutes) whereas cell cycle progression and microtubuli/centrosome regulation is regulated somewhat later (90 minutes to 4 hours post PHx). From these results it would seem that a higher grade of resection primarily results in an inhibition of the apoptotic apparatus whereas a lower grade resection stimulates primarily G1- phase cell cycle progression.

As the "flow theory" mentioned above potentially implies the activation of eNOS by increased sinusoidal shear stress after PHx, it was of interest to look for any differential expression of this gene (or iNOS and cNOS) in our experiments. We observed several genes regulating the activity of eNOS to be differentially expressed in the HPR group: - NOSIP competes with caveolin-1, recently found essential for liver regeneration [120], in the binding of eNOS. Upon binding eNOS, NOSIP (upregulated in the HPR group) translocates the enzyme from the plasmalemma to the Golgi apparatus and possibly the mitochondria,

reflecting functional regulation by cellular compartmentalization after PHx. DDAH2 (upregulated in the HPR group) regulates eNOS activity indirectly by its degradation of ADMA, which in turn converts eNOS to methylamine and citrulline. This could possibly reflect a very early physiological response in NO regulation and neovascularization in the liver remnant as angiogenesis is central to neovascularization of regenerated hepatocyte islands in the regenerating liver [121]. Genes encoding Calsium-calmodulin (essential for eNOS activity) were upregulated in both resection series and HSF-1 (activating eNOS via PKB/AKT) was upregulated in the HPR series, reflecting activation of eNOS in the liver remnants.

Interestingly, several genes, previously found activated in response to various cellular stresses were found to be downregulated in our liver remnants (SOD1, catalase, and GSTP1). Taken together, this suggests that the cells in the liver remnants were under reduced oxidative stress over time. This is in contrast to Fausto's metabolic theory of reactive oxygen species (ROS) triggering the regenerative response after PHx [122]. Dimmeler et al showed that laminar flow shear stress protects against oxidative stress by the upregulation of SOD [123] and Hojo et al [124] found that laminar fluid shear stress inhibited H_2O_2 induced JNK activation and increased GSH/GSSG ratio. Of the genes associated with redox cycling, all were differentially expressed in the HPR, whereas only one (catalase) was found in the LPR group. Taken together, this may suggest that the increased shear stress in the HPR group decreases the level of oxidative stress within the endothelial- and juxtaposed hepatocytes.

A microarray experiment cannot specifically falsify or confirm a null hypothesis, as it is, in essence, a screening technique. Yet, we applied the method in paper 2 to shed light on regeneration theories of portal pressure and sinusoidal shear stress and ventured to hypothesize that the immediate regenerative response in the liver remnant after a liver resection would vary according to the level of resection because of the differences in portal pressure incurred by varying levels of resection. We believe that the results in this paper indicate that there are qualitative and quantitative differences in the regenerative response depending on the level of resection and that these differences may be caused by differences in portal, and hence sinusoidal pressure and shear stress in the respective liver remnants. We concluded that further refined in vivo models of shear stress in the liver needed to be explored in order to investigate increased sinusoidal flow per se (without prior resection), in order to isolate the phenomenon of increased flow.

Paper 3

The hemodynamic changes in the liver remnant resulting from PHx results not only in increased flow and shear stress in the remaining sinusoids, but also increased delivery of hepatotrophic factors to the replicating hepatocytes. Therefore, to distinguish the effects of these two potentially different stimuli (increased sinusoidal flow/shear-stress vs. increased delivery of hepatotrophic factors), and further scrutinize the potential effects of increased sinusoidal flow, we hypothesized in paper 3 that, according to the "flow theory" of liver regeneration, it is the increased sinusoidal flow in itself, which is the primary stimulus to liver regeneration (and not the increased delivery per gram remaining liver of hepatotrophic factors in the portal blood). Consequently, selectively increasing the flow to segments II, III and IV (by creating an aortoportal shunt) should lead to similar gene expression profiles as those seen shortly after PHx, and over time, lead to hyperplasia / hypertrophy of these segments.

Gene expression profiling from the shunted segments does not suggest that increased sinusoidal flow per se results in activation of genes promoting mitosis. Hyperperfusion over three weeks results in the whole liver gaining a supranormal weight of 3.9 % of the total body weight (versus the normal 2.5 %). Contrary to our hypothesis, the weight gain was observed on the non-shunted side without an increase in sinusoidal flow. These results indicate that the isolated increase in sinusoidal flow does not have the impact on the liver as that seen in the liver remnant after partial hepatectomy, demonstrating that increased sinusoidal flow per se is not a sufficient stimulus for the initiation of liver regeneration.

How can we explain our observation that the non-shunted, portally perfused side of the liver grows after three weeks while the weight percentage of the shunted side does not change in the same period? Firstly, the shunted blood was arterial in origin. It may be that this increase in oxygenation of the liver parenchyma may have been unphysiological to such an extent that any potential growth stimulating flow stimulus on the endothelial surface was suppressed. However, a high oxygen tension in portal venous blood has been shown to be beneficial for regeneration after extended PHx in rats and for the outcome of acute liver failure in swine [125,126]. Furthermore, analysis of the flux of liver enzymes, GT, ALP and

bilirubin flux across the liver bed and cytokine analysis of blood draining the shunted segments in the acute series, and histological analysis of HE stained sections, does not suggest any immediate deleterious effect on the liver parenchyma as a result of the shunting. Secondly, ligating the left portal vein branch proximal to the anastomosed aortoportal shunt resulted in a portal pressure increased from 6.22 mmHg to 8.55 mmHg ($P<0.05$) however, the flow per gram liver in these portally perfused (not shunted) segments remained unchanged (1.57 to 1.53 mL/gram/minute, not significant) whereas the flow in the shunted segments increased significantly from an average of 0.61 to 2.89 mL/gram/minute after shunt opening giving a 4.75 fold increase in flow which is similar to the flow increase seen after a 75% PHx [101]. Thus, it may be that it is not the quantity of blood perfusing the liver sinusoids in the remnant which is detrimental to liver regeneration, but rather the quality of the blood (with hepatotrophic factors) as previously suggested by Michalapoulos [127]. Supportive of this theory is the findings of Ladurner et al. where extended hepatic resection with or without decompressive portocaval shunting (and thus significant differences in flow in the liver remnant) did not reveal differences in liver regeneration [128]. Conceivably equally important, are the increased metabolic tasks per gram remaining liver imposed on the liver remnant which may lead to its growth.

Finally, is it justifiable to study the process of liver regeneration without performing a resection? In our opinion, yes, because the moment one performs a liver resection, the relative increase in growth factors supplied, and the increase in metabolic demand on the liver remnant confounds the study of an isolated increase in flow per gram remaining liver parenchyma. It is therefore necessary to create an “unphysiological “state to study an isolated phenomenon in vivo.

Paper 4

The purpose of paper 4 was to summarise previous experimental in-vivo research on liver regeneration in animals, beginning with the Eck fistula model in 1877 and up to present day investigations, focusing on how this field has developed as a result of the interplay between clinical challenges and preclinical surgical research.

The focus of research on liver regeneration after PHx during the last three decades has turned from examining extrinsic hepatic factors such as portal- and hepatic arterial blood flow and its content, to the intrinsic consequences these changes have in the extracellular matrix, the intracellular signal transduction mechanisms and genetic response in the liver. Newer studies in various cell culture models, rodent knockout- and knockdown models, stem cell transplantation, microarray analysis in rodent and porcine models, the impact of the immune system, blood platelets and serotonin, the complement system, cytokines, and the interaction between the many different cell types now known to regulate the regenerative process has unquestionably added much knowledge to the research on liver regeneration but at the same time, made the picture complex and seemingly increasingly intangible when it comes to the clinical application of the knowledge gained.

The surgical principles and practice of preoperative portal vein embolization to induce hyperplasia of the remnant to be after PHx, and portal vein decompression by portosystemic shunting to reduce sinusoidal congestion in the case of SFSS after liver transplantation are well established. Apart from these, there are no novel patient therapies available to aid and augment the process of liver regeneration after extended liver resections, toxic liver insults or in the cirrhotic patient. This is in spite of all the modern technological advancements and the knowledge gained on the microscopic and molecular aspects of liver regeneration in the past 20-30 years. We suggest that it is time to turn back to the systemic large animal surgical research on liver regeneration as it offers a more integrated, systemic biological understanding of this complex process, and that a more clinically relevant progression could possibly be made with a closer collaboration between the hepatologist, liver surgeon/transplant surgeon and the laboratory scientist.

General conclusions

1. SiNoS analysis of time series is a very powerful statistical tool that may be used to complement conventional statistical methods in the analysis of time series in circulatory research.
2. The genetic regenerative response in the liver remnant to varies according to the level of resection and rise in portal pressure.
3. An isolated increase in sinusoidal flow does not have the same genetic, microscopic or macroscopic impact on the liver as that seen in the liver remnant after partial hepatectomy, indicating that increased sinusoidal flow in itself is not be a sufficient stimulus in itself for the initiation of liver
4. The vast amount of research to date performed on liver regeneration has had relatively little practical consequence for the patient with a failing liver with a few exceptions. Contemporary liver surgery therefore calls for a better understanding of the mechanisms controlling liver regeneration in order to design new treatment strategies to support the functionally deficient failing organ and, at the same time, enhance its regenerative capacity.

Models, topics and hypotheses for future research

Many aspects of liver regeneration are unanswered. The work on the present thesis has resulted in some ideas for future experiments:

1. **Can increased energy reserves in the liver remnant aid regeneration?** Rodent models of PHx from the 1970's and canine models from the 1990's have shown that the capacity of the liver remnant to regenerate after PHx is dependant upon an increased supply of energy [130-132]. After PHx in rats, Yoshioka et al showed that oxygen supply to the liver increases by increased hepatic artery flow. Simultaneously, the hepatic oxygen extraction rate increases, while the total energy load decreases along with increased DNA synthesis [133]. Arterialization of the liver remnant leads to improved survival in rats after extended hepatectomy [134-136], and this has also been shown to be beneficial in humans after extended hepatectomy [137]. In investigating the mechanisms behind SFSS, Smyrniotis et al studied the hemodynamic changes in different sized liver grafts in pigs finding that while the portal pressure and flow per gram liver increased inversely with graft size, the hepatic artery flow decreased. However, the hepatic arterial buffer response was preserved, even showing an increased response with decreasing graft size [138]. One could therefore hypothesize that a graded portal vein arterialization could prove beneficial for the function and regeneration of the marginal liver remnant and the small-for-size liver graft, as arterialization potentially leads to an optimal oxidative status and energy charge in the hepatocytes. Accordingly, a surgical model of extended hepatectomy with arterialization of the functionally small and deficient remnant with observations of energy charge and histological signs of regeneration could cast light on this aspect and potentially be used as a bridge to complete regeneration in patients with small-for-size grafts.

2. **The resected liver is itself a source of mitotic stimulus – can we utilize this fact?** In 1952, Glinos and Gey found the serum of partially hepatectomized rats to exert a growth-promoting action on fibroblasts in tissue culture [139]. Bucher and Wenneker reported an increased number of mitoses in the non-hepatectomized partner in parabiotic rats with cross circulation indicating the presence of growth stimulating factors in the effluent from the liver remnant [140,141]. This

hypothesis was corroborated in *in-vivo* rodent models by several investigators who observed increased liver cell mitosis in intact animals injected with serum from hepatectomized counterparts [142,143]. To circumvent the changes in portal hemodynamics caused by PHx, Siegel conducted canine experiments in the early1960's with autotransplanting small liver grafts to the jejunal mesentery, later randomizing the animals to a 70 % PHx of the native liver. In contrast to control groups, the autografts in the animals with 70 % PHx did not undergo atrophy, indicating again a growth stimulus from the resected liver to the autografts via the systemic circulation [144]. Similarly, another experiment showed that autografts transplanted to the neck, did not undergo atrophy, tentatively stimulated by the native liver manipulated with an Eck fistula (in contrast to animals without an Eck fistula) [145]. Thomford showed similar results in 1965 in dogs with heterotop allografts, where the grafts did not suffer from atrophy when the native liver, receiving all the portal blood, was resected; again indicating a growth stimulating effect from the liver effluent after PHx [146]. 14 years later Starzl extracted cytosol from hepatectomized canine livers (48 and 72 hours after PHx) injecting it into the portal vein stump of Eck fistula dogs observing a proliferative response [147]. Further investigations of possible growth stimulatory substances in the liver effluent from partially hepatectomized pigs were performed by van Hoorn-Hickman in 1981 by cross circulation with recipient animals or exchange perfusion. Increased thymidine kinase activity and mitotic indices in biopsies from portocaval shunted (recipient) pigs corroborated Starzl's previous observations in dogs [148]. Kahn also showed in 1982 that a stimulatory substance was transferred from a transplanted partially hepatectomized liver to the host liver (which had a portocaval shunt), stimulating a proliferative response in the latter (as judged by increased thymidine kinase activity and mitotic indices). The authors speculated whether this phenomenon could be clinically useful in aiding liver regeneration in the host liver in patients with liver failure treated by auxiliary liver grafting [149]. This theory leads to the speculation of whether the development of an acute or acute-upon-chronic liver failure large animal model could be used to test the benefit of injecting serum extracts of liver hepatotrophic substances from resected livers in aiding the regeneration of the damaged liver? Is it possible that the remnant liver after an extensive liver resection could be aided in regeneration by infusion of a concentrate of the patient's own serum in the portal vein and could a

small-for-size graft procured from living donor split-liver-grafting profit or in some way be supported by the stimulus that the serum of the donor could offer?

3. **What stops the regeneration process? What is the hepatostat? And how ca we utilize it to aid the liver with insufficient regeneration?**

   An area that has received relatively little attention to date has been the research on the metabolic control of liver size and the mechanisms controlling the termination of regeneration. As the liver to body weight ratio is relatively well conserved across species (i.e. 2.5 %) there seems to be a "hepatostat" regulating liver size. Is there a systemic metabolic or hormonal feedback mechanism involved, or is it primarily intracellular? We suggest a model of liver transplantation of a large graft into a small recipient with a longitudinal metabolic, hormonal and genetic study parallel with volumetric measurements of the liver undergoing functional atrophy. The aim being to detect substances both within the liver itself acting in some paracrine manner and substances in the systemic circulation possibly acting through a negative-feedback axis.

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