MECHANISMS OF LIVER REGENERATION IN A CLINICAL MODEL FOR PREOPERATIVE INDUCTION OF LIVER HYPERTROPHY PRIOR TO MAJOR LIVER RESECTION

A thesis submitted to Imperial College London

for the degree of Doctor of Philosophy

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Final submission May 2021

Dedicated to all those who fight against cancer:

Patients,

Doctors,

Researchers

and

My beloved family.

Abstract

The liver is a central organ for homeostasis and has the unusual and remarkable property of regeneration with rapid restoration of its volume and function.

Liver regeneration (LR) is a very enigmatic and complex process involving numerous intra and extrahepatic signals and pathways. Interestingly, genetic knockdown studies have often demonstrated delays in the course of regeneration, but no single signal has been identified to be both sufficient and necessary for LR. Furthermore, LR can be modified by multiple patient, liver or trauma-related factors.

As a consequence, diverse clinical applications have been developed and used extensively including hepatic resections to remove liver tumours, split liver transplantation from donors, portal vein occlusion, artificial support in acute liver insufficiency or cellular therapy.

Recently, a surgical technique named associated liver partition with portal vein ligation for staged hepatectomy (ALPPS) has shown an unprecedented property of accelerating LR. This procedure results in a greater speed of cell hypertrophy in the future liver remnant when compared with the gold standard portal vein embolization (PVE). These techniques are used for preoperative optimization of a small future liver remnant, avoid postoperative liver failure and, in turn, improve morbidity and mortality. Unfortunately, tumoral progression has been observed after PVE precluding the potential of

curative hepatectomy. The ALPPS procedure may speed this hepatic resection and increase resectability rates. However, due to its associated high morbidity and mortality rates, diverse groups have developed modifications of the ALPPS technique (ALPPS variants). This includes radiofrequency assisted liver partition ablation with portal vein ligation for staged hepatectomy (RALPPS). By associating a line of necrosis with radiofrequency ablation following portal vein ligation, this two-stage technique allows a safe and rapid LR without an increased morbidity or mortality associated with the procedure.

Herein, a general review of the process of LR is presented as well as further research of the intrinsic mechanisms of LR induced by PVE and the ALPPS variant, RALPPS.

Statement of originality

I, Ana Belen Fajardo Puerta, hereby declare that the work presented in this thesis is my own aside from the following assistance and help:

- Experiment in chapter 4 was performed with the assistance and supervision of Dr Ben Jones, Dr George Tharakan and Professor Tricia Tan in the Department of Metabolism, Digestion and Reproduction (Hammersmith Campus, Imperial College London, London, UK).
- Normal liver tissue and metastatic liver tissue samples were acquired from surgery undertaken by Professor Long R Jiao at Hammersmith Hospital, Imperial College NHS Trust, London, UK.
- Portal vein embolization was performed by senior radiologists Dr Robert Thomas and Dr Paul Tait at Hammersmith Hospital, Imperial College NHS Trust, London, UK.
- Experiments and analysis in chapter 5 were performed with the assistance and supervision of Dr Maria Elena Lopez Jimenez in the Department of Surgery and Cancer (Hammersmith Campus. Imperial College London, London, UK).
- RNA-sequencing was carried out by Genomic Laboratories, MRC Clinical Sciences Centre (London, UK).
- RNA-sequencing and relative bioinformatic analyses were conducted by Dr Leandro Castellano (Imperial College London, London, UK).

I am very appreciative of the help from the aforementioned.

Copyright Declaration

Acknowledgements

First and foremost, I would like to express my most sincere gratitude to Professor Long R Jiao for his guidance and constant support on the direction of this thesis. Secondly, I am very grateful to the co-supervisors Dr Elena Lopez-Jiménez, Mr Adam Frampton and Mr Mikael Sodergren for their help during this period.

I am extremely grateful to all those patients who gave consent for using their clinical data, blood and tissue biopsy samples making this research possible.

I am very thankful to all the staff and colleagues at Sainsbury's wing private patients and the Department of HPB Surgery (Hammersmith Hospital) as well as the Emergency Surgery team at Chelsea & Westminster Hospital.

A very special and well-deserved mention goes to my very good friends. Thank you very much, Anna Lewandowska because your friendship is the most valuable treasure I have had in many years and hopefully many more to come. Thank you, Dr Elena Lopez for your constant support not only as a co-supervisor and enthusiastic scientist, but as a very close friend. You have played a key role during this period and a big part of this PhD has been possible because of you. To my dearest friend from uni Dr Juani Cordero who despite of the distance I have felt very close. I am also very thankful to a great female surgeon, friend and mentor, Dr Beatriz Perez Cabrera. You are such an inspiration! Thank you all for believing in me.

Como no puede ser de otra forma, mi más sincero agradecimiento a mis padres, hermanos y sobrinos, por su inmenso cariño, paciencia y cercanía a pesar de la distancia. Este trayecto ha sido todo un reto repleto de dificultades a sortear que, sin duda, ha merecido la pena. Gracias por apoyarme y estar siempre conmigo.

Agradecida pues por esta increíble oportunidad que la vida me ha brindado, haciendo posible uno más de mis muchos sueños: estudiar en una universidad de gran prestigio internacional como es Imperial College London.

Papers and presentations

1. Fajardo Puerta AB, Lopez-Jimenez E, Jiao LR. Expression profiling and genetic characterisation of liver regeneration following PVE and RALPPS prior to major liver resection. In preparation.
2. Fajardo Puerta AB, Lopez-Jimenez E, Jiao LR. Impact of cytokines and circulating hormones responsible for liver regeneration following induction of liver hypertrophy with PVE and RALPPS prior to major liver resection. In preparation.
3. Fajardo-Puerta AB, Mato Prado M, Frampton AE, Jiao LR. Gene of the month: HGF. J Clin Pathol. 2016 Jul;69(7):575-579.
4. Jiao LR, Hakim DN, Gall TM, Fajardo A, Pencavel TD, Fan R, et al. A totally laparoscopic associating liver partition and portal vein ligation for staged hepatectomy assisted with radiofrequency (radiofrequency assisted liver partition with portal vein ligation) for staged liver resection. Hepatobiliary Surgery and Nutrition. 2016 Aug;5(4):382-387.
5. Jiao LR, Fajardo Puerta AB, Gall TM, Sodergren MH, Frampton AE, Pencavel T, Nagendran M, Habib NA, Darzi A, Pai M, Thomas R, Tait P. Rapid Induction of Liver Regeneration for Major Hepatectomy (REBIRTH): A Randomized Controlled Trial of Portal Vein Embolisation versus ALPPS Assisted with Radiofrequency. Cancers. 2019; 11(3):302.

6. Laparoscopic RALPP: a totally laparoscopic radio-frequency-assisted liver partition with portal vein regeneration. Oral presentation. 15th World Congress of Endoscopic Surgery. 9-12/11/16. Shanghai-Suzhou. China.

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Abbreviations

°C: Celsius degrees

ACE: angiotensin-converting enzyme

ACSM1: Acyl-CoA synthetase medium chain family member 1

Alb: albumin

ALN: first stage, left liver, normal liver tissue

ALP: alkaline phosphatase

ALPPS: associated liver partition with portal vein ligation for staged hepatectomy

ALT: alanine aminotransferase

ALT: first stage, left liver, tumour

ARN: first stage, right liver, normal liver tissue

ART: first stage, right liver, tumour

ATDC: Ataxia-Telangiectasia Group D-Associated Protein

Bi: bilirubin

BLN: second stage, left liver, normal liver tissue

BLT: second stage, left liver, tumour

BRAK: breast and kidney-expressed chemokine

BRN: second stage, right liver, normal liver tissue

BRT: second stage, right liver, tumour

BSA: body surface area

BW: body weight

c-MET: mesenchymal epithelial transition receptor

C3P1: complement component 3 precursor pseudogene

CASH: chemotherapy-associated steatohepatitis

CCL3: C-C motif chemokine ligand 3

CDK4: cyclin-dependent kinase 4

CLDN2: Claudin 2

CLRM: colorectal liver metastasis

COX-2: cyclooxygenase-2

CRP: C reactive protein

CT: computed tomography

CTV: computed tomography volumetry

CXCL14: C-X-C motif chemokine ligand 14

DAMPs: Damage-associated molecular patterns

DFS: disease free survival

DNA: deoxyribonucleic acid

e.g.: example given

ECM: extracellular matrix

EDTA: ethylenediaminetetraacetic acid

EGF: epithelial growth factor

EGFR: epithelial growth factor receptor

eLVD: extended liver venous deprivation

ERα: estrogen receptor alpha

F: French

FGF: fibroblast growth factor

FGFR: fibroblast growth factor receptor

FLR: functional liver volume

FLR: future liver remnant

FLRV: future liver remnant volume

FXR: Farnesoid X receptor

GPC3: glypican-3

GPI linked protein: glycosylphosphatidylinositol linked protein

GRBWR: graft-to-recipient body weight ratio

h: hours

HB-EGF: heparin binding epidermal growth factor

HBS: hepatobiliary scintigraphy

HBV: hepatitis B virus

HCC: hepatocellular carcinoma

HCV: hepatitis C virus

HGF: hepatic growth factor

HIP1R: huntingtin interacting protein 1

HLA-H: human leukocyte antigen class 1 gene

HPB: hepatopancreaticobiliary

I/R injury: ischemia reperfusion injury

ICG: indocyanine green

IFNγ: interferon-gamma

IKB: inhibitory KB protein

IL6: interleukin 6

ILK: integrin linked kinase

INR: international normalized ratio

Kg: kilogram

KGR: kinetic growth ratio

L: liter

LBWI: liver-to-body weight index

LDLT: living donor liver transplantation

LFTs: liver function tests

LINC00319: long intergenic non-protein coding RNA 319

Log: logarithm

LOS: length of hospital stay

LPS: lipopolysaccharide

LR: liver regeneration

LVD: liver venous deprivation

MDT: multidisciplinary team

MET: mesenchymal to epithelial transition

mg: milligram

Min: minutes

MIP1A: macrophage inflammatory protein 1 alpha

miRNA: microRNA, micro ribonucleic acid

MIS: minimally invasive surgery

ml: milliliter

MRI: magnetic resonance imaging

mRNA: messenger ribonucleic acid

MT-CO2: Mitochondrially Encoded Cytochrome C Oxidase II

NAFLD: non-alcoholic fatty liver disease

NASH: non-alcoholic steatohepatitis

NECAB2: Neuronal calcium-binding protein 2

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

NGS: next generation sequencing

NK cells: natural killer cells

OGC: oesophago-gastric cancer

OS: overall survival

p: probability value

PAMP: pathogen-associated molecular pattern

PCDHB19P: protocadherin beta 19 pseudogene

PDGF: platelet-derived growth factor

PH: partial hepatectomy

PHLF: post-hepatectomy liver failure

PLIN5: Perilipin 5

POD: postoperative day

PT: prothrombin time

PTEN: phosphatase and tensin homolog

PVE: portal vein embolization

PVL: portal vein ligation

RALPPS: radiofrequency assisted liver partition with portal vein ligation for staged hepatectomy

RCT: randomized clinical trial

RFA: radiofrequency ablation

RIN: RNA Integrity Number

RNA-seq: RNA-sequencing

RNA: ribonucleic acid

RNU1-27P: RNA U1 small nuclear 27 pseudogene

RPL12: ribosomal protein L12

RPL23AP2: ribosomal protein L23a pseudogene 2

RPL41P1: ribosomal protein L41 pseudogene 1

RT: room temperature

RT-qPCR: reverse transcription quantitative real time polymerase chain reaction

RU: relative units

S: survival

Secs: seconds

sFLR: standardized future liver remnant

SFSS: small-for-size syndrome

SIRT: selective internal radiation therapy

SLC7A9: solute carrier family 7 member 9

SMAD: gene with genetic homology of Caenorhabditis elegans SMA ("small" worm phenotype) and Drosophila MAD ("Mothers Against Decapentaplegic")

SNORD3D: small nucleolar RNA C/D box 3D

SOCS1: suppressor of cytokine signaling 1

SOS: sinusoidal obstruction syndrome

SSPO: subcommisural Organ Spondin

STAT3: signal transducer and activator of transcription 3

TACC2: transforming acidic coiled coil containing protein 2

TGFβ: transforming growth factor beta

TGFRβ: transforming growth factor receptor beta

TGFα: transforming growth factor alpha

TIPS: trans jugular intrahepatic porto-systemic shunt

TLTV: total liver tumour volume

TLV: total liver volume

TNF: tumor necrosis factor

TNFR: tumour necrosis factor receptor

TNFα: tumor necrosis factor alpha

TRIM29: Tripartite Motif Containing 29

TV: tumour volume

VEGF: vascular endothelial growth factor

VEGFR: vascular endothelial growth factor receptor

vs: versus

Y: years

Yap: Yap-associated protein

µg: microgram

µl: microliter

CHAPTER 1. INTRODUCTION

1.1 Background

Liver cancer is the 6th most frequent cancer in the world and causes 8.2% of all cancer related deaths (Globocan, 2018). Currently, hepatic resection provides the best chance of long-term survival and cure. Postoperative liver failure is the main cause of death after hepatic resection. This liver failure can be due to a massive resection, underlying liver disease such as non-alcoholic steatohepatitis (NASH) or chemotherapy associated steatohepatitis (CASH) or even advancing age (1). To avoid this complication, the liver has the intrinsic ability of regeneration to maintain its homeostasis and functions. Other surgical procedures such as living donor liver transplantation also relies on the liver regeneration process, both for the donor and the recipient. The size of the initial liver-graft volume will converge to the standard liver volume over time, regardless whether it was smaller or larger than the standard liver volume (2). It is known that the liver requires a precise and accurate size to provide for the needs of the body (3).

The need for research on the process of liver regeneration is evident considering its relevant clinical applications both in medicine and surgery. Being the only internal mammalian organ that can fully regenerate, liver regeneration has become an important tool for understanding pathways and mechanisms of organogenesis and tissue repair.

Over decades, many researchers have conducted investigations to enlighten the understanding of the pathophysiological molecular and cellular mechanisms hidden behind this marvel. Much of this knowledge have been elucidated with the introduction of the two-thirds partial hepatectomy model in rodents by Higgins *et al.* (4). The process of liver regeneration after partial hepatectomy is a highly complicated process involving several types of signaling pathways and different organs in a well-orchestrated manner. The replacement of the liver mass is achieved from a rapid priming and activation of the quiescent hepatocytes, entering into the cell cycle to proliferate, and followed by a termination phase renewing its quiescence (5).

Studies on liver regeneration will contribute to our understanding of pathways governing liver regeneration.

1.2 General anatomical and physiological aspects of the liver

The liver is a multifunctional solid organ present in all vertebrates. Being considered as the heaviest and largest solid organ, the liver is located in the right upper quadrant of the abdomen in the human with an average weight of 1.5 Kg in the adult (2.5% of the total body weight). Numerous factors such as age, gender or body size have been described to modify its size and weight.

From the anatomical point of view, the falciform ligament divides the liver externally into a left and a right hepatic lobe. A further functional division can be made taking into consideration its portal blood supply and biliary drainage. Thus, the two main right and left portal veins split the liver into two lobes, left

and right, division which can be made by an imaginary line that runs from the inferior vena cava to the gall bladder fossa, the Cantlie's line. The eight segmental portal branches separate further the liver into eight functional segments as described by Couinaud *et al.* (6, 7).

The blood supply of the liver, the blood inflow, is dual: 75% from the portal vein and 25% from the proper hepatic artery. The portal vein supplies deoxygenated blood along with nutrients and toxins from the gastrointestinal track, pancreas and spleen. On the contrary, oxygenated blood is provided by the hepatic artery. The hepatic blood drainage, the blood outflow, is ensured via three hepatic veins (left, median and right) which drain directly into the inferior vena cava (8).

The main cell that populates the liver is the hepatocyte (60-80% of the cell population), but other non-hepatocyte cells such as Kupffer, stellae, endothelial and bile ductular cells (also named cholangiocytes) similarly compose and play important roles within the hepatic parenchyma.

The functional microscopic unit is the hepatic lobule. This hexagonal columnar structure has a portal triad on each of its angles (comprised of branches of the hepatic artery, portal vein and bile duct), cords of hepatocytes, hepatic sinusoids, bile canaliculi and a central vein in the centre. The sinusoid blood contains a mixture of oxygenated and partially deoxygenated blood (one third from the hepatic artery and two thirds from the portal vein, respectively) and flows from the portal triads to the central vein which in turns drains to one of the three hepatic veins (right, median and left hepatic vein) and these, to the inferior vena cava. The bile runs in the bile

canaliculi in the opposite direction of the blood, towards the portal duct for drainage. The space of Disse separates the hepatocytes from the sinusoidal endothelial cells, which lack of intercellular junctions allowing contact between the blood and hepatocytes. The Kupffer cells are resident macrophages lined along the hepatic sinusoids. This perisinusoidal space contains the extracellular matrix (ECM) produced by the hepatic stellae cells. Each functional segment contains nearly 10.000 lobules.

The liver is known to be of vital importance for maintaining the metabolic homeostasis of the body. This is achieved via synthesis, degradation and storage of metabolites. This organ is responsible for multiple substantial functions including carbohydrate, lipid and alcohol metabolism; storage of diverse substances such as glycogen, vitamin A, D, K, B12, folic acid, iron and copper; synthesis, secretion and metabolism of bile; production of diverse proteins (e.g. blood clotting factors, albumin, apolipoproteins, angiotensinogen, caeruloplasmin, C reactive protein, transferrin, globulins…); blood reservoir; conversion of ammonia to urea; destruction of old red blood cells and immunological clearance of pathogens by Kupffer cells. Apart from the homeostasis and metabolic functions, the liver is the first natural filter of micro-organisms and toxins (xenobiotics) from the bowel through the entero-hepatic circulation.

During homeostasis and pathological conditions, the hepatic functions are undertaken mainly by the hepatocytes which are influenced by several organ specific factors (e.g. chemokines, growth factors and extracellular matrix) and the non-hepatocyte cells (e.g. Kupffer, endothelial, stellate and biliary duct

cells) (9). Furthermore, depending on their position in the liver lobule, the hepatocytes can undertake different functions. Hepatocytes located around the portal triad receive blood rich in nutrients and oxygen from the portal vein and hepatic artery, respectively. These hepatocytes are specialized in gluconeogenesis, oxidation of fatty acids and synthesis of cholesterol. On the other hand, those hepatocytes located around central veins receive blood with lower levels of nutrients and oxygen and undertake processes such as glycolysis, lipogenesis, bile acid synthesis and drug detoxification by cytochrome P450 enzymes. This phenomenon is called as metabolic zonation and explains the observed zonal damage depending on the type of injury (10).

One of the most fascinating aspects of the liver is that although this organ remains quiescent with no basal hepatocyte proliferation, in response to an injury, it has the intrinsic ability to not only maintain the body homeostasis, but to rapidly recover by a compensatory growth mechanism known for centuries: liver regeneration (LR) (5, 11-13).

1.3 Liver regeneration

1.3.1 History and models of liver regeneration

Liver regeneration (LR) is a very complex, well-orchestrated and strictly regulated process present in all vertebrates, from the zebrafish to the human. Although this remarkable capacity was firstly described by the Greek myth of Prometheus, where after being punished by Zeus for stealing the fire, this titan was chained to a rock and an eagle would eat his liver daily to have it

successfully regenerated at night (Eschilo: 525 BC) (14), its underlying molecular and cellular mechanisms still remain unclear. Given its therapeutic significance and clinical applications, LR has been a great focus of interest by many researchers and clinicians in the last decades (12, 15-18).

Until the 1950s, LR was mainly analyzed at a cellular level with microscopic observations. In the 1960s, with the advent of electron microscopy, the analysis of the hepatocyte ultrastructure was one of the main focuses. It was with the development of gene targeting technology from 1989, where the effort has been also directed to identify the main genes and pathways involved in different scenarios and models of LR (19).

During the years, numerous experimental models have been used to achieve a deeper understanding of the process and multiple pathways involved in LR. From using liver cells cultures to in vivo animal models including the zebrafish, rodents or pigs; the regenerative process has been triggered and studied by administrating hepatotoxic substances (e.g. carbon-tetrachloride, D-galactosamine, paracetamol, ethanol or thioacetamide), bacterial particles, virus, paracetamol or by performing in-vivo procedures such as portal vein occlusion, liver resections or in the setting of liver transplantation. Genetic modifications and sequencing descriptions have also been investigated as well as cell therapy that triggers the process (17, 20).

One of the most widely accepted models to study LR is the two-thirds partial hepatectomy in rodents firstly described in rats by Higgins and Anderson in 1931 (4). This easily reproducible technique involves resection of the left lateral, left medial and right medial lobes achieving a 66% hepatic loss.

It can be performed in about fifteen minutes and given the fact that it is not affected by the acute inflammatory response or necrosis, which typically occurs after administration of toxic chemicals, it provides a more precise chronology for the genetic, histologic and biochemical changes along the regenerative phenomenon (5, 21). As a result of adaptations and discrepancy in outcomes along the decades, a similar surgical model was described in depth by Mitchell and Willenbring for partial hepatectomy in mice in 2008 (22). Also genetically modified animal models with knockout genes have been used in this setting to have a better understanding of the liver pathophysiology (23).

In addition to the aforementioned partial hepatectomy models, pharmacological models using diverse hepatotoxic substances in different animals have been described as mentioned above. Although the latter are easy to perform and can simulate liver diseases by inducing necrosis with great clinical relevance, they are difficult to reproduce given the different types or doses of toxins used, inter-species variability or possible underlying hepatic diseases (13).

In the human, the inherent ability of the liver to regenerate has not only been studied, but applied widely in different scenarios: staged liver resection with hepatic resection to achieve removal of primary or secondary liver tumours in the future liver remnant followed by blood flow modulation with portal vein ligation or embolization, splenic artery ligation prior to stage 2 liver resection, living donor transplantation, and prolonged chemical exposure or viral injury to liver (16, 17, 24-26).

Unfortunately, limitations in terms of the applicability of results from experimental rat studies to humans are evident. The rat liver differs not only in size and anatomy, but in its metabolism as it is higher than in the human. With this regard, in the setting of a partial hepatectomy (PH), the regenerative process in the rat is almost complete after 5 days in comparison with the human where the liver mass is not fully restored until 2-3 months later. Additionally, studies have shown that the upper limit of parenchymal removal to avoid impairment of the liver functions in PH is 90-95% in rats (27, 28), but decreases in humans to 70-80% of the total liver volume (29, 30). Nonetheless, in spite of the differences noted, the principles of the underlying regulation in rat liver regeneration have been widely applied to the complex hepatic regeneration process in humans (31).

In 2012, a novel surgical procedure was described by Schnitzbauer *et al.* showing an impressive acceleration of the liver regeneration mechanism: the associated liver partition with portal vein ligation for staged hepatectomy (ALPPS) (25). This two-staged procedure revealed a greater hypertrophy of the non-deportalized liver in a much shorter period of time when compared to the gold standard, portal vein embolization (PVE). To unravel the mechanistic insights behind this process, a few groups have investigated this further even by generating animal models which can mimic the process (32, 33). At present, many questions remain unclear with regards of liver regeneration and further research needs to be undertaken.

1.3.2 Mechanisms and phases of liver regeneration

The cell population within the liver can be divided into two main groups: the parenchymal cells (hepatocytes and cholangiocytes) and the non-parenchymal cells (sinusoidal endothelial cells, Kupffer cells and stellae cells) of endodermic and mesodermal origin, respectively. Approximately 80% of the liver weight is populated by the hepatocytes, which carry out most of the metabolic and synthetic functions of the liver (34). In normal conditions, majority of the hepatocytes remain in a quiescent status (G0 phase) but can enter into cell cycle after hepatic loss or injury (11).

In contrast to other organs where a removal of a portion is followed by a limited regeneration, the liver regenerates until it achieves the appropriate size and weight, also called the liver-to-body-weight ratio, to provide the needs of the body and maintain the internal homeostasis (3). This proportionated relationship between the liver weight and the rest of the body has been also described as the “hepatostat” where LR can be seen as one aspect of it. After a partial hepatectomy, the remnant liver increases to a 100% of its original liver size but with a different shape. On the contrary, in situations where the body metabolism changes such as cachexia, responses to chemotherapy or chronic inflammatory conditions, the liver decreases in size (5, 12, 16). In clinical and experimental liver transplantation, it has been observed how the donor liver size adapts to the recipient body surface. This phenomenon has even been reported in the baboon xenograft to the human (17). Moreover, it has been demonstrated in rat models that the regeneration process is dependent on the extent of the hepatectomy. The process increases significantly after 90% of a partial hepatectomy in comparison with 30% and

70% hepatectomies. On the contrary, liver function is compromised inversely to the degree of partial hepatectomy, where the early postoperative period after 90% partial hepatectomy is critical with regard to liver failure (29).

It has been suggested that similar mechanisms participate in LR after performing a partial hepatectomy than by administration of the above mentioned hepatotoxic substances although some differences have been found (16). It seems that this mechanism of regeneration involves different pathways depending on the type of injury, acute (after hepatic resection or administration of chemicals) or chronic loss of hepatocytes. In chronic injury scenarios, a continuous proliferation of hepatocytes takes place with an effort to restore the liver mass which can lead to cirrhosis or cancer (3).

Although it has been suggested that LR appears to recapitulate what is observed during development (35, 36), it is yet to be demonstrated whether LR is due to several cell types or a single cell of origin (35, 37). Two types of replacement of tissue loss during liver regeneration have been observed: a proliferation of cells of each cellular compartment and a trans differentiation of facultative stem cells (16, 38). During the acute liver injury, no “stem cells” are involved, and new hepatocytes and cholangiocytes are generated from proliferation of old hepatocytes and cholangiocytes, respectively. On the other hand, when the proliferative ability of either cell compartment is impaired, e.g. experimental manipulations (39), the other type will transdifferentiate to fully restore the hepatic histology (40, 41). Trans differentiation of each cell type can occur in situations of fulminant hepatic failure where up to 25% can unexpectedly recuperate (38).

The liver is the only visceral organ able to regenerate after partial resection and chemical injury (13). It does not follow the steps of blastema formation but a compensatory hypertrophy (increase in cell volume) followed by hyperplasia (increase in cell number) (13).

In severely damaged liver with impaired hepatocyte proliferation, the facultative stem cells proliferate and have the potential to transdifferentiate into both hepatocytes and biliary epithelial cells. On the contrary, LR after a partial hepatectomy does not require such progenitor cells, but proliferation of the quiescent hepatocytes. Interestingly, whereas lost tissue or removed limbs in salamander or amphibians is a true epimorphic regrowth from a blastoma formed by trans-differentiation of adult cells to mesenchymal cells; in hepatic resections, the remnant tissue undergoes a “compensatory hyperplasia” to recover the original liver mass within about two weeks in rodents. Hence, the liver does not recover its original lobular structure or shape, but increases in size (12, 16, 17, 19, 35).

The volume of the liver resected seems to influence on the type of regeneration induced. A 30% removal of liver mass in mice will lead into hypertrophy, whereas a 70% hepatectomy provokes an hypertrophy as a precedent of cell proliferation, hyperplasia (42).

During acute liver injury, stem cells are not involved in the restoration of the hepatic loss. In this scenario, proliferation of both compartments, hepatocytes and cholangiocytes, generates new hepatocytes and cholangiocytes, respectively. Interestingly, it has been demonstrated with experimental manipulations that if one of these compartments is impaired,

the other compartment can work as a facultative stem cells and transdifferentiate into the other. This phenomenon of trans differentiation between hepatocytes and cholangiocytes has been controversial but recently clarified and may play an important role in the scarce spontaneous recovery of fulminant hepatic failure (38, 40, 41).

Multiple and complex signaling pathways mediated by growth factors, cytokines and hormones have been identified to take part of this process. Interestingly, not a single disrupted pathway will completely stop it. On the contrary, alternate pathways will come into play to achieve the adequate regeneration and provide for the needs of the body and survival (12, 15-17, 35, 43, 44).

After partial hepatectomy, mature hepatocytes undergo oscillating cell divisions encompassing the first wave of proliferation 60% of the hepatocytes, continued by waves of less division (45). By integrating the cellular and molecular mechanisms, Fausto *et al.* described a three-phase model for LR after a partial hepatectomy which corresponds to the phases that hepatocytes pass through: initiation, induction or priming (entering from quiescent G0 to G1), progression or proliferation (G1 to proliferation or M) and termination (M to G0). This model has been assessed in different species (15-17, 35, 46).

1.3.2.1 Induction or priming phase

During this phase, the liver cells are prepared for replication by entering in the cell cycle. In partial hepatectomies, one of the first modifications is the rise of blood flow up to the sinusoids leading to stimulation of endothelial cells and

initiation of LR (47). The liver has a dual blood supply: 75% from the portal vein and 25% from the proper hepatic artery. The portal vein supplies deoxygenated blood along with nutrients and toxins from the gastrointestinal track, pancreas and spleen. On the contrary, the oxygenated blood is provided by the hepatic artery. Haemodynamic changes are observed promptly after PH e.g. increase of portal blood flow without affecting the arterial supply resulting in an increase availability per hepatocyte of growth factors and cytokines (11). As the portal venous pressure increases in the remnant liver, this provokes a mechanical stress on the endothelial cells which in turn express an increased activity of urokinase plasminogen activation (uPA) (48).

The earliest documented biochemical change is the increase of urokinase activity produced by hepatocytes although it is not clear whether other cells also contribute with this activation. Urokinase activates plasminogen into plasmin and the latter activates metalloproteinases triggering the remodeling of the extracellular membrane (ECM) as well as activation of the hepatic growth factor (HGF), a potent mitogenic signal for hepatocytes (49). A nuclear migration in hepatocytes of beta-catenin and Notch is also observed and enhanced by proteins of the Wnt family, the HGF receptor MET and EGFR. As a result, there is a rapid and profound change in gene expression patterns of hepatocytes and hundreds of genes normally not expressed in quiescent G0 hepatocytes are rapidly activated (16). Within the first 4 hours after partial hepatectomy in mice, 95% of the hepatocytes change their gene expression, from quiescent state of G0 to G1 phase of the cell cycle (35). Whether the cell will divide or not depends on a critical control point denominated restriction

point R. The activation of Cyclin D1 has been considered this event of no return. Until then, this process is reversible, allowing the hepatocytes to return to its G0 previous state (12, 15-17, 35).

1.3.2.2 Progression phase

This phase corresponds with the transition of hepatocytes from G1 to completion of mitosis or phase S. As mentioned before, many extracellular signals contribute in a meaningful way in selective aspects of the regenerative process. During the progression phase, the complete mitogens hepatic growth factor (HGF) and endothelial growth factor receptor (EGFR) will induce the proliferation of the cells and other auxiliary mitogens such as norepinephrine, IL6, tumour necrosis factor (TNF), bile acids or leptin will enhance this effect. It has been suggested that deprivation of the auxiliary mitogens can cause delay but does not abolish LR. Both categories of mitogens, complete and auxiliary, rise shortly after partial hepatectomies in mice and several transcription factors are activated: notch, beta-catenin, integrins and NF-κB. The latter plays a very important role in advancing hepatocytes into the cell cycle and it is influenced largely by TNF. If TNF is absent, other signaling pathways such as endothelial growth factor (EGF), HGF or norepinephrine may activate NF-κB signaling pathway to complete the regenerative process. The activation of STAT3 (signal transducer and activator of transcription-3) occurs within 1h after partial hepatectomy by IL6 and norepinephrine (12, 16).

Although, many studies of LR focus mainly on cellular kinetics of hepatocytes, other cells that populate the liver are also actively involved during all this process to achieve a final normal size. After partial hepatectomy,

hepatocytes and biliary cells enter into proliferation quicker than endothelial and stellae cells. On the contrary, in situations where either hepatocytes or biliary epithelial cells cannot proliferate and cannot participate in regeneration, alternate regenerative cellular pathways can be activated. Here facultative stem cells, oval cells or recruitment of bone marrow mesenchymal cells may play an important role (12).

After partial hepatectomy, the proliferation of hepatocytes proceeds as a wave from periportal to centrilobular areas, reaching the latter in 48h in rats, and synthesize effective growth factors toward the adjacent cells. Hepatocytes produce vascular endothelial growth factor (VEGF) which stimulates production of HGF by endothelial cells. The penetration of endothelial cells between the early proliferating hepatocytes forms vascular channels and these eventually acquire the structure of endothelial sinusoids. During LR, bone marrow mesenchymal cells are a source of endothelial cells and most of HGF produced by endothelial cells derives from those who were generated from bone marrow mesenchymal precursors (13).

In human, the peak of hepatocyte proliferation varies depending on underlying disease, nutritional status and extent of hepatectomy. Although the rate of hepatocyte proliferation has been shown to decrease with age, the liver does not lose the capacity to restore its full mass with aging (3).

Regarding the hepatic stem cells, several studies argued that there is a small population of committed tissue specific hepatic stem cells which become activated and results in the generation of progenitor cells when hepatocyte proliferation is blocked, however, they haven’t been identified in a

reproducible manner as they are not evident in simple histological examination of the liver. Other studies suggest the possibility that they come from other sources, such as from haematopoietic stem cells from the bone marrow, they enter in the liver and function as hepatic stem cells to generate progenitor cells. On the other hand, it seems that these cells are derived from fusion between bone marrow and haematopoietic stem cells and hepatocytes (50).

The oval cells also known as progenitor cells have phenotypic markers of both hepatocytes and biliary cells and can differentiate in either direction. They have been considered as the equivalent of facultative hepatic stem cells. Strong evidence supports that biliary cells are the origin of oval cells and progenitor cells. Therefore, it has been postulated that the biliary compartment is associated with continual renewal of hepatocytes throughout the life (16).

The innate immune system, particularly Kupffer cells and natural killer cells (NK), also participate in the response to liver injury. On one hand, Kupffer cells secrete tumour necrosis factor alpha (TNFα) and interleukin-6 (IL6) facilitating the activation of transcription 3 (STAT3) in hepatocytes and initiate the proliferation of liver progenitor/stem cells. On the other hand, NK cells are the most important source of interferon-gamma (IFNγ). By activating STAT1 and antagonizing STAT3 activation in hepatocytes, NK cells modulate LR in a negative manner (51).

1.3.2.3 Terminal or apoptotic phase

In general, the termination process has been much less investigated than the priming and proliferation phases (13). During the termination phase, a remodellation of the tissue with a return of the hepatocytes to the initial G0 phase takes place. In keeping with the hepatostat concept, there is evidence of a wave of apoptosis which corrects the extra number of hepatocytes and restores the original liver size. If this restrain fails, the aberrant regenerative response can lead to carcinogenesis in severe cases. Remodellation of the ECM along with several proliferative inhibiting factors such as integrins, TGFβ, decorin, glypican-3, activins and Yes-associated protein have demonstrated to play an important role (12, 13, 16). Hence an important balance between mitogens and proliferative inhibiting factors is needed along the process.

The mentioned remodellation of the ECM consists of a new synthesis of components including glycosaminoglycans and different types of collagen. Besides, a complex signaling pathway is activated by integrins. Integrin linked kinase (ILK) suppresses proliferation of hepatocytes and biliary epithelial cells. In ILK (hepatocyte targeted)-deficient mice, a prolonged regeneration process is observed after a partial hepatectomy and the final liver weight is higher than the original before surgery (12).

At the beginning of the liver regeneration, TGFβ is eliminated from the liver and its receptors in proliferating hepatocytes are dramatically decreased. At the termination of the process, TGFβ is re-synthesized by stellae cells and stored by binding decorin in the ECM. Decorin, a GPI linked protein to hepatocytes, also plays a role in termination of regeneration by both storing

TGFβ and inhibiting the HGF receptor MET and EGFR. Activins A and B are members of the TGFβ superfamily and its receptors have similar structure to TGFβ receptors, sharing signaling pathways. Activin A has an identical structure than TGFβ. Produced by the hepatocytes in an autocrine manner, this protein suppresses the hepatocyte growth by inhibiting DNA synthesis through SMAD pathway. Furthermore, activin induces cell death in hepatocytes in rats causing a reduction in liver mass. Furthermore, its apoptotic effects are 90% less potent than TGFβ (52).

Glypican-3 (GPC3) expression occurs at the end of liver regeneration. Although it is one of the most intensely expressed proteins in HCC, it is not a growth stimulator, but part of a growth suppressor. Deficit of GPC3 in humans, Sympson-Golabi-Behmel syndrome, is associated with organ enlargement. In transgenic mice with expression of GPC3, hepatostat is downregulated as the hepatocytes have suppressed regeneration and the liver weight at the end of the regenerative process is decreased (50).

The Yap-associated protein (Yap) could be the endpoint of the signals determining the functions of hepatostat. It is a nuclear transcription factor which levels are regulated by a system of kinases, the Hippo pathway. A targeted overexpression of Yap in hepatocytes results in a massive hepatic enlargement. When the hepatostat is downregulated by overexpression of GPC3 or upregulated by targeted elimination of ILK, there is a concomitant decrease or increase of Yap protein in the hepatocyte nuclei (5, 11, 16, 50).

1.3.3 Factors influencing liver regeneration

As mentioned earlier, liver regeneration is a very complex process in which many intra hepatic and extrahepatic signals and pathways are involved to complete a successful restoration according to the body needs and maintain its internal homeostasis. In addition to these convoluted interactions, the liver regeneration machinery can be modified depending on multiple factors related to the patient, the liver itself or trauma/injury related.

1.3.3.1 Patient-related factors

1.3.3.1.1 Age

Despite of many physiological changes in the elderly, the main hepatic functions are preserved (53).

From the macroscopic point of view, the liver shrinks up to 40% in volume; acquires a brownish appearance due to accumulation of lipofuscin and damaged proteins from lysosomal dysfunction (54); and its blood and bile flow decreases (55). This process where damaged proteins are degraded by the lysosomes, named autophagy, is reduced with aging.

From the metabolic point of view, gluconeogenesis decays enabling a lipid accumulation which can contribute to the steatosis observed in the elderly (56, 57). The dk4-C/EBPalpha-p300 axis plays a critical role in regulating age-associated disorders as higher levels of cyclin-dependent Kinase 4 (CDK4) have been associated to the process of liver steatosis or non-alcoholic fatty liver disease (NAFLD) in the elderly (58). There is a decreased activity of liver

cytochromes and gradual rise in of bilirubin in plasma (59). Furthermore, aged stellae cells become more active causing a susceptibility for fibrosis (60).

Although it has been described that ageing impairs liver regeneration severely, with poor recovery after massive hepatic resections or ischaemia/reperfusion injury induced in liver transplantation, the aged hepatocytes maintain their proliferative capability when exposed to particular stimuli (61, 62). After partial hepatectomy in rats, DNA synthesizing capacity of nuclei from hepatocytes was found to be higher in younger rats that of aged rats (63). In humans, a recent study has demonstrated a higher risk of post hepatectomy liver failure after right hepatectomy in elderly patients with a cut off at 75 years old (35% *vs* 7%, p <0.001) (64). This could be probably secondary to either a delay or impairment in the restoration of the liver mass associated with the phenomenon of senescence. Intricated changes in circadian rhythms as well as epigenetics alterations have been deemed as responsible for this impairment of the liver regeneration process in the elderly. Curiously, this specific variation in circadian rhythm can be reverted by autophagy in the aged liver (65).

Overall, with an immune response dysfunction and impaired liver regenerative capacity, elderly people are susceptible to acute and chronic liver diseases, such as fibrosis, viral infections and non-alcoholic fatty liver disease (NAFLD) with potential progression towards cirrhosis and hepatocellular carcinoma (HCC). Moreover, the risk of non-alcoholic steatohepatitis (NASH) and progression to HCC is increased with metabolic risk factors such as hypertension, diabetes, hyperlipidaemia and obesity associated in the elderly

(66). Interestingly, senolytic drugs have shown to eliminate senescent cells diminishing the steatosis in the aged liver (67).

1.3.3.1.2 Gender

Currently, there is no evidence of differences in liver regeneration after partial hepatectomy between men and women. On the other hand, estrogen receptors have been found on hepatocytes and an increased production of oestradiol via ovary and testes has been demonstrated after partial hepatectomy promoting liver regeneration in mice. Oestradiol induces oestrogen receptors (ER) mainly in periportal hepatocytes facilitating its proliferation (68). On the contrary, a delayed liver regeneration with decreased production of oestradiol was observed in ovariectomized and orchiectomized mice (69). After PH in *ERα* Knockout mice, LR was delayed with a lower rate of hepatocyte proliferation, suppressed production of TNG-α and IL6, and activation of NF-κB and STAT3 resulting in a fatty liver (70).

1.3.3.1.3 Nutritional status

The process of liver regeneration after hepatectomy requires a great amount of energy mirrored by a depletion of the ATP levels and an increment of its degradation product, inorganic phosphate (71). It is known from protein-calorie starvation rat models that malnutrition is linked to delayed LR and higher mortality rates after partial hepatectomy (72). Regarding the effects of specific nutrients in LR there is very scarce information in the literature (73). From one hand, administration of glutamine-supplemented parenteral nutrition, a source of protein and DNA synthesis, significantly promoted LR in

70% hepatectomized dogs (74). On the other hand, dextrose supplementation has shown controversial results with inhibitory effect in PH mice (75), but improvement of the nutritional status and quicker normalisation of nitrogen balance was observed in a randomised clinical trial comparing peripheral dextrose *vs* hypertonic glucose following hepatectomy (76). Sarcopenia has been associated with a poorer kinetic growth rate and degree of hypertrophy in patients with colorectal liver metastasis following portal vein embolization (77, 78).

1.3.3.1.4 Diabetes mellitus

Several facts have demonstrated that insulin is an important hepatotrophic factor delivered via portal blood during liver regeneration (79). Although insulin *per se* does not cause liver regeneration and is not mitogenic in cultured hepatocytes, portocaval shunts have been associated to liver atrophy and injecting Insulin into a portal vein above the shunt (Eck's fistula) successfully restores the liver volume in dogs (3, 80).

In diabetic patients with reduced insulin secretion, the synthesis of RNA and DNA is decreased on day one preventing the restoration process after hepatectomy (81). Additionally, a multiple regression analysis in patients who underwent PVE found diabetes mellitus to be a risk factor of poor regeneration in the future liver remnant (82). Therefore, from the practical point of view, in situations where liver regeneration is encouraged, such as hepatectomy or PVE, strict control of glucose levels should be aimed by the clinicians (83).

1.3.3.2 Liver-related factors

1.3.3.2.1 Biliary obstruction

Different molecular mechanisms have been associated to impair LR when there is an accumulation of toxic bile salts. Cholestasis has been demonstrated to trigger the Fas pathway leading to apoptosis. Fas death receptors are activated by TNFα and Fas ligand, releasing mitochondrial cytochrome-c and initiating apoptosis mediated by caspases (84). Altered expression of MYC, C/EBP, cyclin E, HGF, EGF or IL6 has been also observed (85-89).

The impairment of the enterohepatic circulation that occurs during biliary obstruction also affects the regenerative competence of the liver after partial hepatectomy. In the clinical practice, the use of an external biliary drainage decreases the LR process in comparison with the use of an internal biliary drainage after partial hepatectomy (90, 91).

1.3.3.2.2 Intrinsic liver disease: steatosis, steatohepatitis, fibrosis and cirrhosis

As mentioned earlier, post hepatectomy liver failure is one of the main risks for morbidity and mortality after extended hepatectomy. This risk is even higher in the presence of underlying liver diseases which drives a poor regenerative response such as steatosis, alcoholic or non-alcoholic steatohepatitis, fibrosis or cirrhosis.

In liver surgery, the steatotic liver shows worse tolerance to injury in comparison with healthy hepatic parenchyma. Postoperative complications

after hepatectomy in the presence of marked steatosis (≥30%) are significantly higher, and even more in the presence of steatohepatitis. Moreover, liver grafts with severe steatosis (≥60%) have worse survival rates with a very high rate of primary disfunction probably to a failure in the regeneration mechanisms (92).

The pathogenesis of non-alcoholic fatty liver disease (NAFDL) has been associated with adipokines such as leptin and adiponectin. Furthermore, leptin has been found to have a profibrogenic role in vitro studies being associated with hepatic fibrosis. Lipid accumulation causes free-radical injury from fatty acid oxidation and this provokes mitochondrial damage. Several studies have studied the influence of steatosis in liver regeneration after partial hepatectomy and have characterised the involvement of different adipokines (93). In a meta-analysis performed in 2010, patients with steatosis who underwent a major hepatic resection had up to two fold increased risk of postoperative complications (94).

A recent model of ALPPS in cirrhotic rats has shown that although ALPPS promoted liver regeneration, there was a significant impaired liver function with a delayed peak of hepatocyte proliferation in cirrhotic rats (33).

1.3.3.3 Drugs and chemotherapy

Several drugs have shown some beneficial effects on liver regeneration following partial hepatectomy (e.g. recombinant human erythropoietin, iron supplements and its combination) (95).

On the other hand, many frequently prescribed drugs can impair the regenerative capacity by inducing histological changes such as hepatic steatosis (e.g. statins, antibiotics, steroids, antivirals, calcium channel blockers, antidiabetic agents or non-steroidal anti-inflammatory drugs) (96, 97). Widely used chemotherapy agents such as oxaliplatin and irinotecan have been deemed as hepatotoxic with the described sinusoidal obstruction syndrome (SOS) (98) and chemotherapy-associated steatohepatitis (CASH) (99), respectively. A protective effect of bevacizumab, a monoclonal antibody targeting VEGF, has been found against oxaliplatin-associated liver toxicity (100) and it also appears to improve survival in colorectal liver metastases (101). Impairment of liver regeneration secondary to sinusoidal obstruction may also be prevented with the administration of aspirin (102).

In the clinical practice, clinicians should be aware of all medication before preoptimizing liver induction for regeneration which must be taken into consideration.

1.3.3.4 Surgical factors

During hepatic surgery, technical aspects intrinsic to the hepatectomy *per se* can modulate the response of the remnant liver regeneration.

Firstly, the surgical trauma and manipulation associated to liver surgery cause a profound cell damage. Experimental and human studies have demonstrated that the manipulation of the liver during its mobilization is associated with a profound hepatocellular damage as a result of an infiltration

of inflammatory cells and upregulated genes linked to acute inflammation (103).

Secondly, and as a consequence of the hepatoduodenal ligament occlusion (Pringle maneuver), haemodynamic changes, ischaemia/reperfusion injury and interruption of the entero-hepatic circulation take place. Once the liver is mobilized, clamping the hepatic pedicle is used to minimize bleeding during the hepatic transection. However, this vascular occlusion can imply ischaemic/reperfusion injury if the clamping is prolonged in time with important cell damage (93, 104).

The acute oxidative and nitrosative stress causes hepatocellular death. Injured cells die and expose their intracellular content to the extracellular matrix, including self-antigens known as damage-associated molecular patterns (DAMPs), triggering a sterile immune response (103, 105). Kupffer cells release TNFα and IL6 (106, 107) which in turn activate the proliferation of hepatocytes and subsequently the rest of pathways and cascades that contribute to liver regeneration (16). Decreased tolerance to I/R injury with a poorer liver restoration has been observed in partial hepatectomy using vascular occlusion in steatotic than non-steatotic livers (108, 109).

Haemodynamic changes also occur during a partial hepatectomy with a redirection of the portal and arterial blood flow to the remaining parenchyma (5). Reduction of hepatic liver mass results in portal hypertension and hyperperfusion. This increase in blood pressure promotes an interaction between platelets and endothelial cells (110), making the sinusoidal fenestrations wider (111) and facilitates the entry of molecules and platelets

into the space of Disse (112). Increased portal hypertension can lead to decreased arterial perfusion due to the hepatic arterial buffer response and impair the microcirculation causing post hepatectomy liver failure (113). Looking for a clinical use by lowering the portal pressure and minimising liver injury, vasoactive agents in the splanchnic area such as propranolol, somatostatin and terlipressin have been used after 90% hepatectomy in rats. All treatment groups showed increased hepatocyte proliferation but terlipressin significantly increased median survival compared to control group (114). On the contrary, earlier reports have demonstrated that betablockers may impair liver regeneration by lowering the portal flow and blocking effects of adrenalin (115).

Another physiological mechanism that takes place after partial hepatectomy is the interruption of the liver-gut axis when part of the portal vein system is ligated (116). This increases further the immune response by altering the intestinal mucosal barrier and appearing in blood a pathogen-associated molecular pattern (PAMP), the lipopolysaccharide (LPS). LPS not only generates a pathogen-mediated inflammation but enhances liver regeneration by activating Kupffer cells which release TNFα and IL6 (117, 118). This phenomenon called endotoxemia also promotes an accumulation of platelets in the regenerating liver which in turn and via degranulation sequestrate more sinusoidal cells and hepatocytes for further proliferation (119).

1.3.3.5 Liver transplantation

As well as in partial hepatectomies, the regeneration process is key during liver transplantation. Several factors may influence the regenerative ability of the transplanted liver graft: size of the graft, donor's age, concomitant viral hepatitis and bacterial infections, portal hypertension, poor venous drainage and immunosuppression.

A very important variable in liver transplantation is the size of the graft. In the same manner that liver to body weight ratio becomes restored after partial hepatectomy, the ratio between graft-size and recipient, named graft-to-recipient body weight ratio (GRBWR), adjusts in order to achieve a similar volume to the original native liver and maintain internal homeostasis (120). Although controversial, the minimal accepted standard volumes is 0.6%-0.8% GRBWR (121, 122) or 40% of the standard liver volume (SLV) (123, 124), though other donor and recipient characteristics need to be taken in consideration (125). Otherwise, recipients may develop small-for-size syndrome and poor graft survival (126).

In the small for size syndrome, small grafts may regenerate up to the appropriate size, but prolonged cholestasis and significant functional impairment has been observed secondary to ischemic injury (127).

The age of the donor is important as aged grafts do not regenerate as quick as younger grafts showing poorer survival (128).

Hepatitis B and C virus infections (HCV, HBV) inhibit liver regeneration with unknown mechanisms (129, 130). The effect on the kinetics of viral replication

in HCV-positive patients has also been investigated. In proliferating hepatocytes, HCV RNA synthesis is increased, suggesting that viral replication is regulated by cell-cycle-dependent factors and progressive disease has been observed (131).

In liver transplant, a balance of the portal blood flow needs to be achieved. From one side, an adequate flow has been associated with a more rapid regeneration, but on the contrary, when the graft is hyperperfused, LR is impaired significantly with increased mortality (132). To avoid this situation splenic artery ligation, with or without splenectomy or portocaval shunting has been used (133). The venous outflow of the liver has shown to be relevant as well in this regenerative process. With time, atrophy of poorly drained segments has been observed (134).

To prevent allograft rejection, different immunosuppressors are routinely used (e.g. corticoids, cyclosporine, sirolimus or tacrolimus). These can interfere in the restoration of the graft even on a dose-dependent fashion (135, 136).

As mentioned earlier, portal pressure can interfere with the process of regeneration. To minimize liver injury with portal hypertension vasoagents such as propranolol somatostatin or terlipressin has been investigated, some of them with contradictory results like the betablockers (114, 115).

1.3.3.6 Bile acid metabolism

Bile acids and its interaction with Farnesoid X receptor (FXR), a nuclear receptor of ligand-activated transcription factor, have been demonstrated to contribute to normal liver regeneration (116, 137, 138). After partial

hepatectomy, there is a transient bile acid overload that lasts for 48 to 72 hours. The bile acids help in promoting the first wave of hepatocyte proliferation though biding several nuclear receptors or by indirect activation. At the same time, and to protect themselves, the hepatocytes start a bile acid detoxification and elimination resulting in the excretion of bio-transformed bile acids into the bile canaliculi. Once in the intestine, the excreted bile acids are reabsorbed by the enterocytes, the Farnesoid X receptor (FXR) is activated and FGF19/FGF15 is produced and released into the enterohepatic circulation. FGF19/FGF15 binds its cognate receptor, fibroblast growth factor receptor 4 (FGFR4) promoting a secondary wave of hepatocyte proliferation (116). Partial hepatectomy in Knockout *FXR* mice has shown a significantly impaired liver regeneration (139).

Earlier reports have demonstrated that biliary external drainage decreases the capacity of liver regeneration, whereas internal biliary drainage does not suppress this ability (90, 140).

1.3.3.7 Redox status

During hepatectomy, phenomena like ischemia/reperfusion, transient bile acid stasis or concomitant liver disease, create a pro-oxidative state with an over production of reactives such as hydrogen peroxide (H2O2)(116). Some authors have suggested a possible induction of hepatocyte proliferation depending on the intracellular concentration of H2O2 during the first 24 hours after partial hepatectomy in rat and mice (141, 142). This has been described

as regulated by the ERK-cyclin/d1-pRB pathway (141) as well as Notch signaling (143).

1.3.3.8 Inflammation, cell damage and growth factors

Many growth factors, cytokines and other molecules are involved in liver regeneration with diverse roles and its modification can influence the biology of the process. The complete hepatic mitogens include those molecules able to stimulate hepatocyte DNA synthesis causing liver hypertrophy *in vivo* and mitosis in hepatocytes cultures. These molecules include: HGF, EGF, TGFα, HB-EGF and amphiregulin. Many other molecules which do not induce DNA synthesis, liver growth or hepatocyte mitosis, can cause delays LR without abrogating it if inhibited or not present: TNFα, IL6, FGF, VEGF, complement proteins, insulin, serotonin, norepinephrine and cyclooxygenase 2 (COX-2) (144). A review of the most relevant molecules follows.

1.3.3.8.1 Complete hepatic mitogens

1.3.3.8.1.1 Hepatic growth factor (HGF)

HGF is one of the first circulating factors found to promote liver regeneration (145, 146). Also known as Scatter factor, it is involved in a number of cellular processes, such as proliferation, growth, survival, and metabolism (147, 148). Along with the ligands to the EGF receptor, it has been described as a complete mitogen for hepatocytes by inducing both DNA synthesis in hepatocytes *in vitro* and liver enlargement when administered *in vivo* (50). HGF binds and activate the multifunctional tyrosine kinase receptor c-Met (149). This receptor dimers with the insulin receptor contributing to

different metabolic activities such as glucose regulation (16). Its mitogenic activity of HGF initiated signaling requires the function of the transcription factor C/EBPbeta (150).

Many studies have demonstrated its crucial role in liver regeneration after partial hepatectomy in experimental models. During partial hepatectomy, HGF is utilized in the liver in a biphasic manner. Firstly, a rapid increase of peripheral blood levels of HGF is observed 1 h after hepatectomy (151). As a consequence, a first wave of MET activation occurs within 30 minutes and peaks at 60 minutes (152). In a similar manner, there is a biphasic increase of its activated receptor by tyrosine phosphorylation only 1 to 5 and 60 mins in PH rats (152). Within the first three hours after hepatectomy, the endogenous reservoir of HGF in the extracellular matrix, especially important around the portal triads (153, 154), becomes depleted probably secondary to the increased urokinase activity which degrades ECM (155). After these three hours and with a peak at 24 hours post hepatectomy (155, 156), an endogenous synthesis of HGF is undertaken in the liver by stellate cells and endothelial cells (157, 158). Extra-hepatic sources of HGF from other organs (e.g. spleen, lung, kidney) (159, 160) or platelets (161) have been also described but its relevance is not known. *HGF* mRNA transcription in different organs after partial hepatectomy has been shown as a response of increases in circulating norepinephrine (162) and insulin-like growth factor (163).

The important function of c-MET receptor in liver regeneration has been observed in different experimental studies revealing that its impairment cannot be compensated by other factors. From a delayed regeneration with

defective exit of hepatocytes of quiescence G1 phase and decreased S phase entry to severe liver necrosis and death within 48 hours was observed in partial hepatectomies of mice with knockout or mutant *c-MET* (164, 165). Besides, injection of short harpin RNAs against HGF and c-MET in rats inhibits mitosis and increases apoptosis within 24 hours post hepatectomy (166).

1.3.3.8.1.2 Epidermal growth factors receptor (EGFR) ligands: epidermal growth factor (EGF) and transforming growth factor (TGFα)

Epidermal growth factor (EGF) and transforming growth factor α (TGFα) belong to the seven members of the EGF family and share a common receptor, the epidermal growth factor receptor (EGFR).

EGFR, also known as ErbB1, is one of the four high-affinity receptors for the EGF family (EGFR/ErbB1, HER2/ErbB2, HER3/ErbB3 and HER4/ErbB4) (167). Epidermal growth factor (EGF), transforming growth factor-alpha (TGFα), amphiregulin, heparin binding-EGF-like growth factor (HB-EGF) are the ligands to EGFR described in the context of liver regeneration after partial hepatectomy and comprise the only other complete mitogens besides HGF (11)(47)(50). A peak of activation by phosphorylation of the EGFR is observed one hour after PH in rats, following similar kinetics as that of c-MET (152).

After partial hepatectomy in hepatocyte-targeted *EGFR* knockout mice, mortality was observed in one third. In the surviving mice, the regeneration process was delayed but finally completed (168). Another study with administration of shRNAs against EGFR in rats demonstrated decreased

hepatocyte proliferation after partial hepatectomy but a compensatory activation of other receptors such as MET, ErbBw and ErbBe was identified (169). Once again liver mass restoration did occur, concluding that the EGFR pathway is important but not essential for hepatocyte proliferation.

The earliest studied EGFR ligand and first growth factor ever isolated was EGF (16). EGF has been shown to be mitogenic to hepatocytes both in cell cultures (170) and *in vivo* when administered to rats (171). Although produced in several tissues, the main source of EGF during liver regeneration are the Brunner's glands in the duodenum increased by circulating norepinephrine (172). EGF is taken almost entirely by the liver through the portal circulation and it concentrates around the portal triads (173). EGF is also produced by salivary glands (173, 174) and it seems to play an important role in liver regeneration as sialectomy in mice have shown delayed hepatocyte proliferation after PH but restored by exogenous administration of EGF (175, 176).

TGFα is produced by the hepatocytes during liver regeneration (177) probably functioning in an autocrine or paracrine manner (178). It is also a ligand of EGFR but more potent hepatocyte mitogen than EGF probably due to a different ligand-receptor processing (179). *TGFα* is expressed at 20 h after initiation of cell cycle in hepatocytes. *TGFα* knockout mice have normal liver regeneration after partial hepatectomy (180).

Other EGFR ligands involved in liver regeneration after partial hepatectomy are amphiregulin and HB-EGF. Amphiregulin is produced by hepatocytes and its expression is induced in cultures by interleukin-1 beta and prostaglandin E2

(181, 182). Its expression has been related to the protein Yap and Hippo kinase pathway (183). *Amphiregulin* Knockout mice have impaired liver regeneration (181, 182).

HB-EGF is a potent mitogen for cultured hepatocytes (184) and in contrast to EGF and TGFα it is produced by Kupffer cells and endothelial cells during liver regeneration (16, 185). Interestingly, *HB-EGF* is expressed in 2/3 hepatectomy, but not on 1/3 partial hepatectomy in mice. Furthermore, a similar effect in DNA replication is found when HB-EGF is administered in a 1/3 partial hepatectomy as to a 2/3 partial hepatectomy. *HB-EGF* Knockout mice have delayed regeneration response (186).

EGFR is expressed in biliary cells, Kupffer cells and endothelial cells and its modifications are reflected in the liver regeneration much later in comparison with alterations of *c-MET* (187).

Both c-MET and EGFR are crucial for liver regeneration and its baseline homeostasis. Recently, its combined disruption in mice after partial hepatectomy has shown not only to abolish regeneration causing liver failure due to reduced hepatocyte proliferation or apoptosis of non-parenchymal cells, but a further impairment in other liver functions such as lipid synthesis, urea cycle and mitochondrial functions and activation of inflammation pathways (188).

1.3.3.8.2 Incomplete or auxiliary hepatic mitogens

1.3.3.8.2.1 Interleukin 6 (IL6)

Interleukin-6 (IL6) is an extensively investigated multifunctional cytokine involved in the acute phase response of the innate immunity as well as in the development of cancer (189-191). Classically, IL6 has been found to play an important role in triggering the process after partial hepatectomy (192). Its plasma levels increase rapidly after a partial hepatectomy (16) and mice lacking of IL6 have a delayed liver regeneration secondary to a loss of STAT3 activation and decreased cell cycle progression (193). Spontaneous recovery of the process with completion of regeneration could be due to a later activation of STAT3 by EGF and HGF (194-196). This delay could also be rescued by administration of IL6 or stem cell factor (SCF) (197). Exogenous administration of IL6 *per se* in animals of cultures does not induce hepatocyte proliferation, but it has been observed to be mitogenic in biliary epithelial cells cultures (198). IL6 is secreted mainly by Kupffer cells under the stimulation of TNFα (16) and by hepatocytes under certain circumstances(190). Its expression is regulated by LPS/MyD88 pathway, by complement components and by ICAM-1 activation (199, 200). When IL6 binds to its transmembrane receptor IL-6R present in hepatocytes and some leukocytes, protein gp130 dimerizes and signaling pathway is activated by the IL6R-gp130 complex (201, 202). The importance of TNFα and its receptor TNFR1 in liver regeneration resides in the upregulation of IL6. In *TNF receptor 1* knockout mice, there is a lower expression of IL6 which is rescued by a preoperative administration of IL6 (203).

An important role of IL6 is the regulation of HGF during liver regeneration. It has been demonstrated that HGF is produced by non-parenchymal cells in

response of IL-6 (204, 205). Besides, HGF decreases the production of IL6 by in bone-marrow derived macrophages by preventing NF-KB accumulation (206). This fact suggests an extra anti-inflammatory role of HGF apart from its mitogenic and apoptotic roles during liver regeneration (207).

Regarding the pro-regenerative role of IL6 during the liver repair, there is published data with conflicting results (208). Although majority of studies support a positive effect in hepatocyte proliferation (209, 210), some experimental studies have revealed IL6 to impair liver regeneration. In primary hepatocyte cultures stimulated by EGF and TNF, IL6 suppresses DNA synthesis and proliferation (211-213).

Furthermore, high levels of IL6 were associated with defective liver regeneration in a model of hepatic failure by inducing the expression of protein inhibitor of activated STAT3 and the suppressor of cytokine signaling (SOCS) (214). Also overexpression of STAT3, a downstream component of IL6 signaling pathway, was found to impair liver regeneration of fatty livers (215).

Besides, this discrepancy has also been observed in patients with chronic liver diseases who present elevations of IL6 (216, 217).

It has been hypothesized that the contradictory results regarding the role of IL6 in liver regeneration may result from use of mice with different genetic background, from differences in the surgical procedure, or from the use of different anaesthesia or analgesia (44).

1.3.3.8.2.2 Fibroblast growth factor (FGF)

Fibroblast growth factors family (FGFs) is a large family of proteins comprising more than 20 members where acidic FGF (aFGF or FGF-1) and basic FGF (bFGF or FGF-2) are best characterised. FGFs contribute to development, wound repair, hematopoiesis or angiogenesis by activating four transmembrane tyrosine kinase receptors (FGFR 1-4) (218). All these receptors are expressed in every cell of the liver including the endothelial cells, but FGFR4 is exclusively expressed in hepatocytes (219) and its ligand in human is FGF19 (FGF15 in mouse). The production of FGF15/19 is stimulated by bile acids in enterocytes via Farnesoid X receptor. The ileal FGF15/19 to hepatic FGFR4 axis has been demonstrated to have an endocrine effect in the hepatocytes regulating liver lipid metabolism, but its role in liver regeneration has been controversial (16, 220). Initially, it was demonstrated that FGF15/19 has no role in liver regeneration as loss of its receptor, FGFR4, does not alter regeneration after partial hepatectomy in mice (221). This suggested that other FGFRs may be required in the repair process.

A more recent experiment has shown upregulation of ileal and serum FGF15/19 after partial hepatectomy in mice which promotes cell cycle progression, proliferation and reduction in lipid accumulation in hepatocytes both in vitro and in vivo (222).

In studies after partial hepatectomy in zebrafish expressing a dominant-negative *FGFR* mutant (223) and in mice expressing a dominant-negative mutant of *FGFR2* (224), impaired liver regeneration was observed. Furthermore, increased mortality due to liver failure was demonstrated after partial hepatectomy in FGFR1/FGFR-2-deficient, suggesting a cytoprotective

role for FGFR1 and FGFR2 as FGFR signaling by controlling the upregulation of detoxifying enzymes of the cytochrome P450 (44).

The ligands FGF-1, FGF-2 and keratinocyte growth factor (KGF) have been shown to increase DNA synthesis in cultured hepatocyte (225, 226) and during S phase, hepatocytes have an increased expression of FGF-1 and FGF-2 (227, 228).

FGF-2 is a potent angiogenetic factor that can stimulate endothelial cell proliferation, differentiation and migration (229). In Knockout *FGF-2* mice after partial hepatectomy, a reduction in cell proliferation with likely delayed proliferation of endothelial cells is observed at day 4. Interestingly, an upregulation of vascular endothelial growth factor (VEGF) compensates the phenomena and liver regeneration takes place normally (230).

1.3.3.8.2.3 Tumour necrosis factor alpha (TNFα)

Tumour necrosis factor (TNF) is a pro-inflammatory cytokine which has shown pleiotropic effects during liver regeneration. Along with IL6, TNF is an important regulator of the priming phase of the quiescent hepatocytes becoming competent to enter the cell cycle. The production of TNF by Kupffer cells occurs within 30-120 mins after partial hepatectomy in mice. Via portal circulation, lipopolysaccharide (LPS) and complements C3a and C5a activate the nuclear factor kB (NF-κB) pathway (231) by degrading the inhibitory KB protein (IKB) which normally blocks NF-κB in the cytoplasm. NF-κB then migrates into the nucleus activating the transcription of TNF, IL6 and cyclin D1 (13). TNF binding with its receptor TNFR1 accelerates in a vicious loop the

activation of NF-κB. After partial hepatectomy in mice lacking TNFR1, there is a decreased activity of NF-κB and delayed regeneration is observed (232). By contrast, liver regeneration is not impaired after PH in mice with knockout of *TNFR2* suggesting that TNFR1 has a more important role in activation of liver regeneration (233).

From one hand, TNF activates death pathways in hepatocytes causing liver failure in mice models (234-236). On the other hand, TNF has also been associated with enhancement of proliferation pathways in cells that have been previously primed by growth factors to enter into the cell cycle (237). The lack of these factors will lead to activate death pathways TNF (237). Experimental studies have revealed delayed regeneration when using TNF neutralizing antibodies in PH mice and in *TNFR1*-deficient mice where NF-κB activation is decreased (233, 238). NF-κB can be also activated by c-MET and EGFR alone (239). In addition to enhancing a better response to the mitogenic effect of HGF and EGF in primed hepatocytes, TNF enhances activation of TGFα and thus EGFR. TNF also has a role in the activation of MMP9 and regulation of expression of stem cell factor (*SCF*) (240, 241).

1.3.3.8.2.4 Vascular endothelial growth factor (VEGF)

The vascular endothelial growth factor (VEGF) family is involved in angiogenesis and lymphangiogenesis by activating the tyrosine kinase receptors VEGFR1-3 (242). It has been demonstrated that VEGF is crucial for vasculogenesis and hematopoiesis in mice, as heterozygous *VEGF* knockout causes death during embryonic development. Among the five types of the

VEGF family in mammals, *VEGF-A* is strongly upregulated by hepatocytes during liver regeneration promoting proliferation of sinusoidal endothelial cells 48 hours after hepatectomy (243). Besides, activation of VEGFR1 releases HGF from the endothelial cells and this in due increases hepatocyte proliferation (244). Treatment of rats with neutralizing antibodies against VEGF-A diminishes proliferation of both hepatocytes and sinusoidal endothelial cells after PH impairing the liver regeneration process (243, 245, 246). After partial hepatectomy in rats, the complex process of angiogenesis occurs from preexisting vessels at 2-3 days and lasts for another 2-3 days. This new vessel formation occurs in different steps Firstly, proliferating hepatocytes release angiogenic growth factors such as VEGF, PDGF-A, FGF and angiopoietins. VEGF stimulates the production of the protease MMP by the endothelial cells. The released MMP enzyme breaks the collagen structure allowing migration of endothelial cells (247). Proliferation of endothelial cells, smooth muscle cells and fibroblasts occurs within the new avascular clusters of newly replicated hepatocytes (11). Subsequent tubulogenesis, stabilization and maturation of the vessels are allowed by FGF, PDGF and angiopoietin allowing the formation of sinusoids (13).

1.3.3.8.2.5 COX-2

COX-2 is an enzyme that regulates prostaglandin synthesis. In the context of liver regeneration, the observed endotoxemia post hepatectomy activates COX-2 in Kupffer cells in mice and drives a higher expression in hepatocytes in rats (248). COX-2 promotes hepatocyte proliferation in the early regeneration though NF-kb, ERD1/2 and MAPK pathways (249-251).

1.3.3.8.2.6 Complement signalling

Complement activation and its signaling have shown to be essential for a normal regenerative response after partial hepatectomy or toxic injury. The proinflammatory proteins C3a and C5a have shown to be relevant during the priming phase. These activate Kupffer cells and neutrophils amplifying the sterile immune response (107, 252). Inhibition of this system abrogates the regenerative response after partial hepatectomy (107, 253).

1.3.3.8.3 Mito-inhibitors

1.3.3.8.3.1 Transforming growth factor beta (TGFβ)

The TGFβ family includes three ligands, TGFβ1-3, which interact with three transmembrane receptors (TGFβ type I-III) expressed mainly in hepatocytes (254). Among these ligands, TGFβ-1 is the most studied in liver regeneration (16). TGFβ plays a very important role in termination of liver regeneration (3). Its mito-inhibitory effect has been demonstrated in hepatocyte cultures and other epithelial cells. Furthermore, exogenous administration of TFGβ delays the process in two-thirds partial hepatectomy rat model (208). Secreted by non-parenchymal cells (Kupffer, stellae, endothelial cells and platelets), the inactive pro-TGFβ1 remains latent in the ECM bound to decorin along with the pro-HGF (255). Shortly after partial hepatectomy, the increased activity of urokinase degrades the ECM via an integrin signaling pathway and activates the HGF. The now active HGF and TGFβ are released to the blood stream following a similar kinetic and trigger entry of hepatocytes from G0 to G1. Both HGF and TGFβ levels elevate at 3 hours and reach a peak at 24-72 hours

following a similar kinetic (256). TGFβ in plasma is bound and hence blocked by alpha-2 macroglobulin (257) enabling hepatocyte proliferation. Interestingly, immunochemistry studies have shown a removal of TGFβ in the same direction as the wave of hepatocyte proliferation, from periportal to pericentral (258). Later in the middle of the proliferation phase, pro-TGFβ becomes active with the induction of the gene of cation-independent mannose 6-phosphate receptor (*CIMPR*)(208). Studies suggests that early after partial hepatectomy, TFGβ is eliminated from the surroundings of the hepatocytes but re-synthesized to regulate other necessary processes relevant at later stages of liver regeneration like promoting neovascularization (259, 260) or production of connective tissue proteins by fibroblasts and mesenchymal cells (261). *TGFβ1* RNA levels increase at 4 hours after partial hepatectomy reaching a maximum at 72 hours (262). *TGF*β expression is upregulated upon PH but a transient resistance is observed which could be due to upregulation of its inhibitors*, SnoN* and *Ski*, or downregulations of its receptors (263). Mito-inhibitory effects after 24-72 hours of partial hepatectomy have been observed in cultures of isolated hepatocytes despite increasing TGFβ activation (264). Delay of hepatocyte proliferation has been observed after administration of TGFβ and in mice with overexpressed *TGF*β in hepatocytes (265, 266). Elimination of TGFβ receptor type II conduced to hyperproliferation of hepatocytes and a higher liver to body weight ratio at one week after PH in mice but this effect was transient and did not prolong liver regeneration (267). A similar experiment revealed enhanced hepatocyte proliferation, but this effect was not found at 120 h after PH (268).

1.3.3.9 Other extrahepatic factors

During liver regeneration after partial hepatectomy, other extrahepatic cells compartments are involved in the process such as the lung, kidney, duodenum, bone marrow or circulating platelets.

HGF is not only produced by the stellae cells in the liver, but also in distant organs such lung and kidney (11). EGF is continually produced in the duodenum and secreted to the lumen where it is absorbed intact entering in the portal circulation via the gut-liver axis (116).

The bone marrow has been reported to mobilize cells after partial hepatectomy in rodents and humans After 3-6 days of the partial hepatectomy, the bone marrow replaces sinusoidal endothelial cells by mobilising endothelial progenitor cells which colonize the hepatic sinusoids, acquire fenestrations and produce HGF (269, 270).

In liver transplantation, bone marrow cells migrate to the graft modulating the process of regeneration by multiple pathways, although its mechanisms are not clear. Experimental studies of transplantation of bone marrow cells in mice with fibrotic livers revealed an improvement in the impaired hepatic cells proliferation (271).

Platelets have been suggested to play part in the liver regeneration process. After partial hepatectomy in mice, there is a rapid but transient platelet accumulation in the liver remnant mediated by release of von Willebrand factor by sinusoidal cells. Platelets adhere to the endothelium with a consequent activation and release of platelet granules which stimulate hepatic

proliferation via Akt and ERK1/2 pathways. Secretion of growth factors such as IL6 by the sinusoidal endothelial cells is also promoted by platelets (119). Thrombosis and platelet transfusions have shown to improve liver regeneration. On the other hand, in thrombocytopenic mice, liver regeneration is reduced compared to mice with normal platelet count. Besides, administration of antibody against Von Willebrand factor has shown low influx of platelets in the liver and poor regenerative response (272).

1.3.3.10 Bacterial infections

Some studies in rats have reported that bacterial infections can modify the process of liver regeneration either by enhancing it with the upregulation of proinflammatory cytokines (HGF, IL6 or TNFα) (273, 274) or, in the case of hyperinflammation and intraperitoneal sepsis, delaying it (275).

1.3.3.11 Genetic factors: miRNA and lncRNA

The regulatory RNAs known as microRNA (miRNA) have been attributed among others an auxiliary role in liver regeneration (276). After partial hepatectomy, some of these transcriptomic regulators are upregulated (*miR-21, miR-33, miR-153, miR-743b*) and others, downregulated *(let-7b, let-7f, let-7g, miR-22a, miR-23b, miR-26a, miR-30b* and *miR-122a*) (277-280).

Overall, studies have suggested that miRNAs are essentially inhibitors of liver regeneration. From one side, the lack of Dicer 1 in mice, enzyme responsible for producing miRNA, has revealed a proliferative liver phenotype (281, 282). From the other side, some miRNA are able to deter the translation

of mRNA to functional proteins, such as mediators of proliferation or cell cycle regulators (283-286).

Recently, *miR-155* has been found to facilitate cell cycle and accelerate hepatocyte proliferation by targeting the suppressor of cytokine signaling 1 (*SOCS1*) (287). This suppressor attenuates *c-MET* signaling and is normally downregulated during liver regeneration (288).

Long noncoding RNAs (lncRNAs) play important roles in diverse biological processes but its role in liver regeneration is not well understood. Recently, *LncHand2* has been identified as a critical mediator of liver repopulation by inducing c-MET pathway (289).

1.4 Clinical relevance and applications of induction of LR in humans

The relevance of LR lies in those situations where the multiple hepatic functions are compromised and, therefore, need to be restored in order to maintain the body homeostasis. In a similar manner than in animal models, the LR process has been thoroughly investigated and applied in different clinical scenarios in humans: after partial or extended hepatectomy to remove primary or secondary tumours of the liver; combined or not with in-flow modulation techniques such as portal vein occlusion (ligation or embolization) or splenic artery ligation; after split liver transplantation in both donor and recipient; during cellular therapies and, more often, in those situations where a prolonged chemical or viral exposure has provoked focal necrosis of the

hepatocytes such as the artificial liver support in acute hepatic failure (26, 42, 290-294).

Currently, hepatic resection remains the main chance for long-term survival in primary and secondary liver malignancies (295-297). Large liver tumour resections can be extremely challenging for hepatobiliary surgeons (298). The concept of major or extended liver resections includes the removal of more than 3 segments of the liver. These major resections are associated with higher postoperative complications and mortality in comparison to minor hepatic resections (299-302). One of the most feared complication after a major hepatectomy is liver failure. The latter has been mainly associated to the future liver remnant volume (FLRV) (303-306). Although the minimum or acceptable FLRV to minimize this risk remains controversial, studies have shown that the upper limit for a partial hepatectomy is around 70-80% in healthy livers (1, 307) and above that limit, a greater resection may lead to impairment of the liver functions and mortality (303, 308-310). Taking into consideration that LR is influenced by several factors, further studies suggested that the cut-off value depends on each individual and more precisely, the underlying hepatic disease. In case of cholestasis, cirrhosis or steatosis the FLRV between 30-40% is needed to prevent liver failure and mortality (94, 309, 311, 312).

From the surgical point of view, there is no other choice but to rely on this intrinsic phenomena to prevent postoperative liver failure and prolong survival in patients whose liver tumours clearance requires an extended hepatectomy to achieve a clear resection margin, a major determinant factor for long-term

survival (313). The latter can occur in up to 45% of the resectable cases (314). Therefore, in liver surgery, it has been established that a limiting factor for major liver resections is the size of the future liver remnant (FLR). A small size can cause postoperative liver failure and have deleterious consequences. To maintain a normal postoperative liver function, around 25% of the total liver volume is considered to be sufficient in a patient without any pre-existing liver disease (315).

Different strategies have evolved along the years with the aim to increase resectability rates and achieve curative resections in patients initially diagnosed with irresectable liver tumors. From one hand, neoadjuvant chemotherapy achieves a reduction of the tumoral bulk (downsizing) allowing rescue liver surgeries (316). From the other hand, in patients with insufficient preoperative FLRV or to prevent small for size syndrome, several procedures have been used to preoptimize the liver remnant using similar principles.

By relying on the intrinsic phenomenon of liver regeneration, these procedures include: portal vein occlusion either by ligation (PVL) or radiological embolization (PVE) (317-319), two stage hepatectomy (TSH) combined or not with PVE (320, 321), associated liver partition with portal vein ligation for stage hepatectomy and its variants (25, 322) or, the most recently described, the liver venous deprivation with its extended modification (323, 324).

Surgical planning and preoperative assessment of individual risk for postoperative liver failure is crucial in patients who undergo a hepatic resection. Not only the surgeon needs to determine the best surgical strategy

to achieve a radial oncological outcome depending on size, aetiology and localization of the tumour, but to prevent postoperative complications such as liver failure secondary to a potential excessive removal of liver volume. Technical aspects to reduce the damage of the remnant liver include a careful mobilization, minimise hepatic pedicle occlusion, ensuring adequate portal/arterial inflow and venous drainage during liver resection preventing congestion.

Volumetric analysis has been described as a tool to predict postoperative liver failure and death in patients with liver tumours (290, 325). In an attempt to expand resectability criteria and safety for patients with inadequate FLR volume, interventional and surgical techniques have been used to induce liver regeneration on the future liver remnant prior surgical resection (26).

The observation of an ipsilateral atrophy and a contralateral hypertrophy of the liver lobes after portal vein ligation was the key concept for the first clinical report of a portal vein embolization in 1984 (24). Since then, this radiological interventional procedure has become a very important preoperative tool to prevent liver failure and increase the safety of extensive liver resections (292, 326). A preoperative portal vein embolization or portal vein ligation in two-stage hepatectomies can achieve sufficient hypertrophy with an interval of 6-8 weeks. A more rapid parenchymal hypertrophy has been observed in patients who undergo a new surgical technique: the associating liver partition and portal vein ligation procedure (ALPPS) (25). This novel two staged technique has been criticized given its high postoperative morbidity and mortality rates in comparison with the current gold standard, the portal vein embolization.

Although the reported volume increase of FLRV after ALPPS has been greater than the achieved with PVE (74% *vs* 12%), a high postoperative morbidity rate is associated with it ranging from 33% to 58% (294, 327, 328).

Several modifications of the novel ALPPS have been described in order to diminish the postoperative complications: partial ALPPS (partial split of 50-80%; hypertrophy of FLR 80-90%); laparoscopic microwave ablation and portal vein ligation (FLR hypertrophy 78-90%); associating liver tourniquet and portal ligation for staged hepatectomy (median FLR hypertrophy 61%); and sequential associating liver tourniquet and portal ligation for staged hepatectomy (FLR hypertrophy 77%) with a potential decrease in morbidity particularly after stage one. Given the variability in techniques of ALPPS meaningful statistical comparisons of outcomes is limited. Not physically splitting the liver at the first stage may decrease morbidity; however, randomized controlled trials are needed to determine benefits in technical variations (329, 330). In our center, a promising technique has been described to achieve a rapid increase in FLR volume (mean FLR hypertrophy 62%) within a short period of time as seen in ALPPS but without its unacceptable increased morbidity and mortality rates. This technique uses in-line radiofrequency to create a virtual liver partition with portal vein ligation - Radiofrequency Assisted Liver Partition with Portal Vein Ligation (RALPP) (331, 332). Experimental models with ALPPS in rats or porcine models have been investigated to understand this quick induction of liver regeneration (333, 334).

Genetic therapy has also been investigated in the field of liver regeneration. There are several experimental studies and clinical trials where genetic transference has been used to enhance LR. Natural genes, gene segments, chimeric genes, oligodeoxynucleotids or siRNAs can be transferred using diverse methods with either viral or non-viral vectors. Viral vectors such as retrovirus, adenovirus, Adeno-associated, HSV, lentivirus o bacilovirus have been used but they have a risk of toxicity. Another way of triggering this regenerative process is to target specific receptors of liver cells promoting its replication. Some recombinant growth factors which promote liver regeneration have shown a short half live needing continuous administration. Therefore to overcome this problem, gene transfer technology has been used in experimental animal models after partial hepatectomies or chemical injuries to transfer genes that encode these factors (335).

With respect of cellular therapy in liver regeneration, several kinds of rat models with transplantation via the portal vein of bone marrow cells, hepatocytes or adipocyte-derived mesenchymal cells, in the setting of acute or chronic liver damage have been investigated with promising results. Advances in stem cell research suggest that the use of individualized and minimally invasive cell therapy is a potential alternative to liver transplantation. While allo-hepatocyte transplantation has been performed for metabolic hepatic disease, auto-bone marrow transplantation has shifted toward mesenchymal stem cells transplantation for liver cirrhosis (20).

1.5 Available methods for preoperative induction/modulation of liver regeneration

During the last decades, different surgical and radiological methods have been described and performed to extend the frontiers of resectability of both large liver tumours and bilobar tumours by preoptimizing the future liver remnant before hepatectomy (336). Among others methods, portal vein ligation or portal vein embolization have been widely used enabling to reduce complications such as post-hepatectomy liver failure and small-for-size syndrome in patients with insufficient future liver remnant (319, 337, 338). A brief overview of the different techniques currently used to induce liver regeneration in the clinical practice follows.

1.5.1 Portal vein ligation (PVL)

It is well stablished that ligation of a branch of the portal vein, either right or left, generates haemodynamic changes that can lead without any parenchymal loss to hypertrophy of the contralateral liver lobe and atrophy of the ipsilateral liver lobe (339, 340). This observation was already made in 1920, when Rous and Larimore ligated a major branch of the portal vein in a rabbit (341).

The first use of PVL in a two-stage hepatectomy in humans was performed by Honjo *et al.* in 1965 (342). At that time, these authors detected a marked atrophy of the ligated liver lobe in an attempt to suppress tumour growth by ipsilateral portal venous ligation of patients with HCC (343). These

observations were the base of attempting a less invasive procedure, the portal vein embolization, as preoperative induction of liver regeneration for major hepatectomy.

Currently, PVL is part of the first step in a two-stage hepatectomy (344). PVL is feasible with the laparoscopic approach (345) which has been shown to reduce the presence of adhesions during a two-stage hepatectomy facilitating the performance of the second step (346).

1.5.2 Portal vein embolization (PVE)

Based in the hypertrophy-atrophy observation made after unilateral PVL by Honjo *et al.* (342), the first portal vein embolization (PVE) was performed in a patient with hilar bile duct carcinoma by Makuuchi *et al.* in 1984 (24). Given its success, PVE was also applied to patients with other types of liver tumours such as colorectal liver metastases and hepatocellular carcinoma (317, 347).

Nowadays, PVE is considered the gold standard procedure to induce liver regeneration prior hepatectomy in both primary and secondary liver tumours for patients with inadequate future liver remnant volume (FLRV) (348, 349).

From the technical point of view, the embolization of the branches of the portal vein can be performed percutaneously (either transhepatic or trans-jugular) (350, 351) or intraoperatively with a trans-ileocolic approach (317).

Several embolic agents are used for PVE alone or in combination (fibrin glue, cyanoacrylato, gelatin sponge, thrombin, metallic coils, microparticles and absolute ethanol), but due to the diverse PVE techniques, different

imaging times or volumetric methodology used, the comparison between the different materials is difficult and the choice is at the hand of the radiologist who performs the procedure (352).

From the safety point of view, PVE is a technique with minimal complications. Patients can have pain, nausea, vomiting of mild fever in the first 72 hours. Generally, the changes in the liver function tests (e.g. increased bilirubin and prolonged prothrombin time) are transient and return to normal in 2-3 days suggesting that the inflammatory reaction and necrosis are minimal after PVE. On the other hand, a more severe side effects have been also described such as haemoperitoneum, haemobilia, portal thrombosis or migration of the embolic agent to the portal system of the FLRV (353, 354).

The meta-analysis performed by Abulkir *et al.* in 2008 included 37 publications with 1088 patients who underwent PVE prior to hepatic resection. This publication showed a mean hypertrophy rate of the FLRV after PVE of 11.9% after an average of 29 days. A transient liver failure following hepatic resection was observed in 2.5% and 0.8% of patients developed acute renal failure and died. Major morbidity from pulmonary embolism was seen in 2.2% with no mortality. After hepatectomy the morbidity rate was 16.0% with a 1.7% mortality rate (355). A further case series of PVE have demonstrated an increase in the FLRV from 8.0% to 49.9 % (356, 357).

Initially, some disadvantages were reported with the PVL technique *per se* such as the need for an operation with general anaesthesia or increased risk of adhesions resulting in a more difficult resection in the second stage. Furthermore, PVL was suggested to be less effective at increasing the FLRV

than PVE (358, 359). On the contrary, other studies showed right portal vein ligation as effective as embolization in inducing hypertrophy of the remnant liver volume before right hepatectomy (319, 360). More recently, two systematic reviews and meta-analysis have demonstrated no significant differences in safety or rate of FLR hypertrophy between PVL and PVE for elective liver resection (349, 361).

An advantage of PVL is that its decision to proceed with PVL can be made intraoperatively during a two-stage hepatectomy obviating an additional PVE procedure (349).

One of the shortcomings described with PVL and PVE is that the tumour may continue growing and progressing over the 4-8 week waiting period of the regenerating process (338, 362).

Some factors have been described to influence the magnitude of the liver growth after PVE including the volume of the FLR, embolic material used, pre-existing conditions or the extension of the embolized parenchyma. The greater the FLRV before PVE, the less ability to increase in volume (363). Different rates of regeneration have been observed when using different materials (364). Modest effect is seen with biological products such as gelfoam or fibrin glue, in contrary to the higher effect observed with absolute ethanol. Pre-existing liver diseases such as hepatitis, cirrhosis or diabetes have shown to influence the liver regeneration after PVE (82, 365). Besides, the addition of including embolization of segment 4 portal branches in the right portal vein embolization before extended right hepatectomy has been questionable despite of producing a hypertrophy up to 56% in experienced hands (366). In a

recent Scandinavian multicenter cohort study, Bjornsson *et al.* demonstrated that the additional S4 embolization to the standard right PVE prompted a growth of the FLR of 48% *versus* the 38% achieved with PVE alone (*p*= 0.01) (367).

Sequential PVE followed by trans-arterial embolization (TAE) for complete portal and hepatic artery occlusion has been described as a safe and effective technique to treat hepatocellular carcinomas (HCC). It minimizes the time between the induction of liver regeneration and completion hepatectomy with a lesser possibility of the tumour to become unresectable (368). Furthermore, limited clinical experience has been reported with sequential hepatic vein embolization in patients with insufficient FLR following PVE with a good tolerance and effectiveness (369).

1.5.3 Associating liver partition with portal vein ligation for staged hepatectomy (ALPPS)

In 2012, Schnitzbauer *et al.* proposed an alternative method to the gold standard PVE, the ALPPS procedure. Firstly performed in 2007 in Germany, this novel two-step technique comprises a right portal vein ligation combined with an *in-situ* liver splitting along the falciform ligament in small-for-size settings followed by resection of the deportalized liver in a second step within a few days (25). The median volume of hypertrophy in the left lateral liver lobe was 74% after a median waiting period of 9 days (range= 5-28) in a series of 25 patients with diverse liver tumours. Despite of this rapid liver regeneration,

morbidity was reported in 68% of the patients, with a post-operative bile leak of 24% and a high mortality rate (12%). To avoid postoperative bile leaks from the raw surface of the liver, de Santibañes *et al.* described a modification of the ALPPS technique consisting in wrapping the whole diseased ischaemic liver in a hermetic plastic bag, which was subsequently reported in a letter to editor (370, 371). However, the morbidity rate remained high at 58% (327).

ALPPS was received by the HPB community with both enthusiasm and preoccupation and its first international expert meeting was held in February 2015 (372). An international online registry (http://www.alpps.net) was then set up. In 2015, data from this registry showed a liver failure rate (by ISGLS criteria) and a mortality rate of 30% and 8.8%, respectively (373). In the meantime, some authors suggested that portal vein embolization should remain the gold standard (348).

The need of systematic reviews and meta-analysis was then evident to address the controversy whether ALPPS or traditional staged hepatectomy such as portal vein embolization (PVE) and two-staged hepatectomy (TSH) is better. Several groups performed this research with similar results.

The systematic review and meta-analysis by the group of Clavien in 2016 revealed a greater increase of the FLR (76% *vs* 37%; $p< 0.001$) and higher rate of completion of stage 2 (100% *vs* 77%; $p< 0.001$) at the price of superior morbidity (73% *vs* 59%; $p= 0{\cdot}16$) and mortality (14% *vs* 7%; $p= 0.19$) after stage 2 ALPPS in comparison with PVE (374).

One of the first systematic reviews in 2015 stablished that ALPPS achieves a hypertrophy rate of 61-93% after a median interval between two stages of 9-14 days with resectability rates of 95-100%. The authors stablished that ALPPS leads to a less drop out due to progressive disease than PVE but a cost of a higher morbidity and mortality (375).

Interestingly, in a systematic review and meta-analysis (2018) looking into operative results and oncological outcomes between ALPPS and traditional staged hepatectomy (TSH) in patients with extensive colorectal liver metastasis, no significant differences in the final FLR were found (mean difference: 31.72, 95% CI: -27.33 to 90.77, p= 0.29) although kinetic growth seemed to be faster with ALPPS (mean difference 19.07 ml/day, 95% CI 8.12-30.02, p= 0.0006). In keeping with previous published data, TSH showed a lower rate of postoperative complications (overall morbidity: RR: 1.39, 95% CI: 1.07-1.8, p= 0.01; I^2: 58%, p= 0.01; major morbidity: RR: 1.57, 95% CI: 1.18-2.08, p= 0.002; I^2: 0%, p= 0.44). Overall survival was comparable following ALPPS *vs* THS (376).

In a systematic review and meta-analysis of ALPPS and TSH with or without preoperative PVE, Liu *et al.* found again that ALPPS was associated with greater future liver remnant (FLR) (RR: 4.87; 95% CI, 3.41-6.33), a higher rate of completion of stage 2 resection (RR: 1.32; 95% CI, 1.21-1.44), but again a higher morbidity (RR: 1.19, 95% CI, 0.96-1.47) and mortality (RR: 2.11, 95% CI, 1.02-4.33) after the second stage compared with TSH (377).

A recent review of the literature after ten years of ALPPS, suggested a bias in the information from diverse retrospective studies with positive and

negative conclusions on ALPPS (322). Similar conclusions were obtained from an international HPB meeting celebrating the 10th anniversary of ALPPS held in 2017 where this innovative procedure was recognized as a two-stage hepatectomy but with a clear need of defining further its indications, technical aspects as well as long-term oncological outcomes (378).

A recent meta-analysis including 1200 patients from 19 studies has shown no difference in mortality among ALPPS, two-staged hepatectomy, and portal vein embolization/ligation (379).

The intrinsic mechanisms underlying the accelerated regeneration observed in a much shorter period of time than PVL or PVE (338, 340, 375, 380), including patients with cirrhosis or end stage liver tumours (381, 382), are not well understood. Many clinical and experimental research has emerged with the great interest to elucidate the effect of adding a transection line between the right and left lobes to a portal vein ligation without any liver mass removal can fast-track the process of liver regeneration in comparison with portal vein occlusion on its own (32, 33, 333, 340, 383-388).

Parenchymal congestion has been suggested as a possible mechanism of rapid FLR hypertrophy during ALPPS. Kawaguchi *et al*. found in a small series of 8 patients, an extrapolated kinetic reduction in volume of the ventral aspect of the right paramedian sector which became 11 times more congested after the first stage in ALPPS than in classical two-stage hepatectomy (9.8±11.6 ml/day *vs* 1.8±1.3 ml/day, p= 0.001) (389). For further characterisation, these authors designed a rat model of ALPPS with venous congestion demonstrating a greater regeneration rate with a higher hepatocyte density and smaller

hepatocyte size at 48 h and 7 days in the ALPPS model with congestion than the ALPPS alone or sham (390).

Not only venous congestion has been evaluated in the ALPPS technique, but also the effect of a prolonged cholestasis has been suggested as a reflection of the poorer and immature regeneration of bile canalicular-ductule networks observed in the FLR of ALPPS patients in comparison with PVE ($p<$ 0.001) (391). The likely prolonged cholestasis before the second stage in ALPPS patients, may be the cause of the observed poorer regeneration of bile canalicular-ductule networks in the FLR than in PVE patients.

The main pathogenic mechanisms for small-for-size syndrome (SFSS) and post hepatectomy liver failure are portal hyper perfusion and a secondary constriction of hepatic artery (hepatic artery buffer response) with subsequent hypoxia of the liver remnant. In fact, to prevent portal hyper perfusion of the transplanted liver and minimize the risk of a small for size graft, diverse inflow modulation techniques has been sought as crucial such as splenic artery ligation, splenectomy, or portocaval shunting (293). Conversely, Dili *et al.* demonstrated with the aid of a rat model of ALPPS that the actual effect of hypoxia of the FLR protects its liver function and facilitates an adequate early neoangiogenesis response with synchronicity between hepatocytes and liver sinusoidal endothelial cells proliferation. Although portal hyper perfusion was similar in ALPPS and SFSS, arterial perfusion of the FLR was significantly lower in ALPPS' FLR causing a higher hypoxia which in turn would upregulate neo-angiogenic genes with larger liver sinusoids (392).

Another suggested mechanism of stimulating proliferation from a rat model is the massive liver necrosis and inflammatory response driving an upregulation of cytokine expression on the regenerated liver (383). Kambakamba *et al.* found higher levels of IL6 and PCNA in ALPPS group than in controls with a FLV growth rate double than the PVL group (393).

Interestingly, another experiment revealed a similar increase of the FLR when injecting plasma after radiofrequency ablation of an organ (spleen, kidney or lung) in the PVL group than in the ALPPS group. The authors suggested that localized trauma or inflammatory response may accelerate the induction of hepatocyte proliferation (32).

Genetic expression has also been studied in ALPPS. The activation of AMPK/mTOR pathway and its molecules expression in 11 patients undergoing ALPPS has been investigated. At the second stage, AMPK and Akt was increased only in the occluded liver lobe, but *mTOR, S6K1, 4E-BP1, TSC1* and *TSC2* expression were observed in the regenerated liver lobe (394).

Despite of its potential to enhance liver regeneration and increase resectability rates, ALPPS and its variants are still considered controversial procedures. From the preclinical point of view, its underlying mechanisms of liver repair remain not very well understood given the difficulty of interpreting results from many different models (395). From the clinical point of view, not only its high morbidity but also its indications have been criticized in a recent review of the international registry (396).

1.5.4 ALPPS variants

In order to minimize the associated complications initially reported with the ALPPS technique various alternative methods which mimic the accelerated LR associated to ALPPS have been proposed by different liver surgeons worldwide. Among these novel techniques named as “variant ALPPS” are Tourniquet-ALPPS, partial ALPPS, hybrid ALPPS, mini-ALPPS, MIS-ALPPS, LAPS and RALPPS (329, 331, 397-402).

One of the first ALPPS variants was described by Robles *et al.*, the Tourniquet-ALPPS or ALTPS (associating liver tourniquet and portal ligation for staged hepatectomy). In this procedure, instead of splitting of the liver parenchyma during the first stage, a tourniquet is positioned on Cantlie’s line or the umbilical ligament if a right hepatectomy or right tri-sectionectomy is planned (397). ALTPS has been recently evaluated and compared to two-stage hepatectomy (THS) for colorectal liver metastases in terms of long-term outcome in a propensity score matching analysis. After showing an increased volume in Tourniquet-ALPPS (68% *vs* 39%; *p*= 0.018) both techniques revealed no differences in terms of postoperative complications, disease free survival or overall survival (403).

To preserve middle hepatic vein, Petrowsky *et al.* described the partial ALPPS (p-ALPPS) where the goal was to achieve 50% of liver parenchyma transection in comparison with the “complete” ALPPS. Later on, due to location of tumours or hepatic veins p-ALPPS transection ranged from 50-80% (398). This procedure which can be performed with a minimally invasive approach, laparoscopic partial ALPPS, seems to be less aggressive than the

original ALPPS, facilitating an earlier second stage without inflammatory adhesions (404). On the other hand, in hepatocellular carcinoma patients with underlying chronic liver disease such as chronic hepatitis or cirrhosis, associating a full split down to the inferior vena cava to portal vein ligation for staged hepatectomy induced greater and quicker increase of FLRV than partial ALPPS (405).

The hybrid partial ALPPS was described by Li *et al.* to avoid "all-touch" and achieve oncological efficacy to treat tumor infiltrating the right portal vein or biliary bifurcation. This technique accounts of three stages: a parenchymal transection in the first stage, PVE one day later and a second stage to complete hepatectomy (406).

In the Mini-ALPPS, de Santibañes *et al* intended to invert the ALPPS paradigm by minimizing the first stage impact with a combination of a partial ALPPS with an intraoperative portal vein embolization during the first stage. This approach achieved a mean FLR hypertrophy of 62.2% (49-79%) (399). A totally laparoscopic Mini-ALPPS was successfully achieved combining a partial liver transection with laparoscopic percutaneous cannulation of the inferior mesenteric vein for intraoperative trans-mesenteric PVE (407).

Minimally invasive surgery (MIS) to perform complete ALPPS has been described both laparoscopically and robotically. The first successful ALPPS procedure using a total robotic approach was reported by Vicente *et al.* in 2016 (408). In a recent systematic review of the literature, minimally invasive ALPPS (MIS-ALPPS) appears to be safe, with potentially lower morbidities and mortalities relative to open patients but numbers are scarce and registry

studies are needed (409). A further review comparing open *vs* MIS ALPPS concludes that although minimally invasive approach would seem better in terms of morbidity and mortality than the open approach, more research is needed (410).

In the LAPS procedure, associating laparoscopic microwave ablation with portal vein ablation for staged hepatectomy, Gringeri *et al.* reported an increase of the FLR doubling its size in 10 days (390 to 693 cm3), by creating a necrotic groove between the right and left liver lobes with microwave ablation in a minimally invasive stage one approach (402).

Furthermore, a percutaneous microwave ablation along the future resection line in combination with PVE after 3 days in HCC and cholangiocarcinoma has been described (411) with concerns of needle track seeding in resectable liver tumours (412).

1.5.5 Radiofrequency assisted liver partition with portal vein ligation for staged hepatectomy (RALPPS)

As the other ALPPS variants, the radiofrequency assisted liver partition with portal vein ligation for staged hepatectomy (RALPPS) emerged as a technical modification to improve safety on the ALPPS procedure. The group of Professor LR Jiao proposed in 2015, replacing the transection of the parenchyma between the right and left sides of the liver in the first stage of ALPPS with a 1cm wide line of necrosis caused by a radiofrequency ablation probe. In this first description, 5 patients had a rapid hypertrophy comparable

with the standard ALPPS approach. This new technique increased FLR by a median of 62.3% (range, 53.1%-95.4%) in 21.8 ± 9.4 days. When comparing a historical cohort of 5 matched PVE patients where FLR increased by a median of 24.6% (range, 8.4%-35.4%) after 55.4 ± 15.6 days, RALPPS revealed a significant gain of FLR by 38% (p= 0.079) accomplished with a reduction of 34 days (p= 0.003) (331).

One of the advantages of this ALPPS variation is the feasibility of completing both stages laparoscopically (or robotically), including clearance on the FLR on the first stage for patients with bilobar liver disease. In 2016, a successful case of entirely laparoscopic RALPPS along with a detailed description of the technique was reported in a patient with bilobar liver metastasis and small FLR. A 57.9% increase of volume in the FLR was observed in 14 days where both laparoscopic stages were completed with no major morbidity or mortality (332).

A randomised controlled trial comparing the efficacy and safety of RALPPS with PVE in is presented in this PhD (413).

1.5.6 Liver venous deprivation (LVD)

A more innovative option for inducing liver hypertrophy is the novel interventional procedure named “liver venous deprivation” (LVD). By simultaneously embolising both right portal and right hepatic veins, LVD achieves a comparable increase of volume of the FLR to ALPPS (323) and similar morbidity and mortality rates when comparing patients undergoing

PVE prior major hepatectomy (414). Moreover, in a recent study comparing LVD and PVE alone before major hepatectomy with patients with small FLR, completion hepatectomy rate was similar in the two techniques, but a greater FLRV was achieved in the LVD group (135% *vs* 124%; *p*= 0.034) (415).

From the mechanistic point of view, the randomized experimental study in a porcine model conducted comparing PVE and LVD by Schadde *et al.* demonstrated that simultaneous portal and hepatic vein occlusion accelerates liver hypertrophy secondary to abrogating porto-portal collateral formation without necrosis of the deportalized liver compared to PV occlusion alone (416).

As mentioned in the PVE section, series of either sequential or combined PVE with hepatic vein embolization (HVE) in preoperative FLRV optimization have been reported. A review of this combination showed a FLR increase of 33-63.3%, morbidity of 10.3% with no PHLF and a mortality rate of 5.1% (417).

A further step with this technique is the extended liver venous deprivation (eLVD) which includes the embolization of the right portal vein along with the embolization of the right and middle hepatic veins (324). This novel method may challenge the ALPPS procedure and the current gold standard PVE, but more studies are necessary (418, 419).

Currently, two randomized controlled trials comparing PVE to LVD, DRAGON1 and HYPER-LIV01, are being held (420).

1.5.7 Selective internal radiation therapy (SIRT)

Selective internal radiation therapy (SIRT), an unilobar injection of radioactive microspheres of Ytrium-90 (Y90) into the arterial supply of liver tumours used as a locoregional treatment for patients with HCC, has been reported to cause hypertrophy of the contralateral liver. This has led to an interest of using SIRT with a doble advantage: as a strategy for volume optimization before hepatic resection by inducing FLR hypertrophy and simultaneously instigating tumour control (421). A matched-pair analysis which compared SIRTS to PVE revealed a significant hypertrophy in the non-embolised lobe, but with a lower speed than PVE (SIRT 29% after 46 days *vs* PVE 61.5% after 33 days, $p< 0.001$) (422, 423).

Moreover, some authors have reported a successful combination of SIRTs and PVE encouraging this strategy as a future treatment option to increase the FLR before hepatectomy (424, 425).

1.6 Quantification / measurement of liver regeneration

In the clinical practice, measuring liver regeneration has been performed as a reflection of modifications of both liver volume and liver functions. In order to assess this process, it is important to stablish that volume may not equal or reflect function.

1.6.1 Liver function assessment

Along the years, many tests have been developed to predict liver function, but none of them have shown sufficient sensitivity and specificity to demonstrate its full multifunctional capacity. As shown in Table 1.1, diverse

investigations indicate different functions or events within the liver parenchyma or biliary tree. These tests can be classified into conventional blood parameters, metabolic clearance tests and molecular nuclear imaging techniques (426).

Function or event measured	***Test***
Cholestasis	Bilirubin, alkaline phosphatase, gamma-glutamyl transpeptidase
Necrosis	Transaminases
Synthesis	Coagulation factors, prothrombin time, albumin, urea
Hepatic perfusion	Indocyanine green clearance, galactose clearance, sorbitol clearance
Microsomal function	Aminopyrine breath test, antipyrine clearance, caffeine clearance, lidocaine clearance, methacetin breath test
Alcohol abuse	γ-glutamyl transpeptidase
Functional hepatocyte mass	^{99m}Tc-GSA

Table 1.1. Available tests for assessment of different liver functions.

- Conventional blood tests and clinical grading systems

In the daily practice, the liver function is assessed by a clinical examination along with blood tests mainly the liver function test (LFTs), clotting screen and albumin levels in plasma (427).

The so called LFTs is a blood chemistry analyses which involves serum levels of bilirubin, transaminases, alkaline phosphatase and γ-glutamyl transpeptidase. It is widely use in the daily clinical practice not as a truly measure of the function of the liver but as a reflection of different processes that can happen within the liver. For instance, transaminases (aspartate aminotransferase and alanine aminotransferase) are released by damaged hepatocytes in conditions such as ischaemia, necrosis, hepatitis, severe sepsis or cancer. The liver-specific alkaline phosphatase, expressed in biliary epithelium, increases during cholangitis or processes involving biliary obstruction. γ-glutamyl transpeptidase is elevated by high alcohol intake and it is expressed by both hepatocytes and biliary cells. Serum bilirubin reveals liver uptake, conjugation and excretion though the biliary tree.

Overall, clotting screen and albumin levels give an estimation of liver synthetic function as albumin and other proteins involved in hemostasis and fibrinolysis (vitamin K dependent coagulation proteins, factor V, XIII, fibrinogen, antithrombin, α2-plasmin inhibitor, and plasminogen) are exclusively synthesized by the liver.

Apart from the LFTs, other blood tests such as albumin, prothrombin time, lactate, glucose and ammonia along with bilirubin can help to differentiate whether an hepatic damage is acute or chronic.

These widely available and passive liver function test have been used to develop useful clinical grading systems. As an example, assessment of chronic liver disease and suitability for hepatic resection and transplant is performed with the Child-Pugh score (also known as the Child-Pugh-Turcotte score). This classification, initially designed to predict mortality in patients with cirrhosis in 1964 by Child and Turcotte (428), provides a scoring system based in five clinical and laboratory features (Table 1.2). The Child-Pugh score stablishes three categories of liver disease severity and associated expected survival rates (Table 1.3).

Another system used in transplantation for end-stage liver disease is the Model for End-Stage liver disease (MELD) (429). This score is derived from a linear regression model based on three laboratory results: international normalized ratio (INR), serum bilirubin and creatinine levels. Its formula is as follows:

MELD = 3.78× ln [serum bilirubin (mg/dL)] + 11.2× ln [INR] + 9.57× ln [serum creatinine (mg/dL)] + 6.43

	1 point	***2 points***	***3 points***
Total bilirubin, μmol/L	< 34	34-50	> 50
Serum albumin, g/dL	> 3.5	2.8-3.5	< 2.8
Prothrombin time, seconds (INR)	< 4 (< 1.7)	4-6 (1.7-2.2)	> 6 (> 2.2)
Ascites	None	Mild (or suppressed with medication)	Moderate to severe (or refractory)
Hepatic encephalopathy	None	Grade I-II (or suppressed with medication)	Grade III-IV (or refractory)

Table 1.2. Child-Pugh score system for chronic liver disease.

Points	***Class***	***One-year survival***	***Two-year survival***
5-6	A	100%	85%
7-9	B	80%	60%
10-15	C	45%	35%

Table 1.3. Child-Pugh score scoring system reflecting associated one or two-year survival depending on points from clinical and laboratory test features.

The MELD scoring system is a prognostic tool for assessing the severity of chronic liver disease in general. Although initially derived from patients undergoing TIPS (transjugular intrahepatic portosystemic shunt) procedures, this score has substituted the older Child-Pugh score in the priorization of the lists for liver transplantation. The higher the score, the less ill the patient is (430). Alternatives to MELD score have been developed subsequently such as: MELD-Na, MELD-sarcopenia, UKELD, D-MELD, iMELD, and the newest MELD-Plus (431).

In the acute phase after liver resection and during regeneration, liver functions will depend on alterations of metabolism, perfusion, extension of resection and quality of the liver parenchyma. Of all complications after major hepatic resection, post hepatectomy liver failure remains the most feared with mortality rates around 80% often associated with sepsis and ischemia-reperfusion injury (432). As mentioned earlier, being the liver a multifunctional organ with no accurate single test determining its whole diversity of functions, defining liver failure post hepatectomy after exceeding a critical cut-off remains a challenge.

A widely used definition for liver failure after hepatic resection is the “50-50 criteria” described by Balzan *et al.* in 2005. Here liver failure is defined as prothrombin time (PT) <50% (or INR> 1.7) and serum bilirubin >2.92 mg/dl on day 5 after liver resection. The authors claimed a 59% mortality risk when these criteria were met in comparison to 1.2% when they were not met (sensitivity 70%; specificity 98%) (432).

More simplistically, liver failure has also been defined with a “peak bilirubin criterion” when bilirubin level is higher than 7 mg/dl (433).

Additionally, and with some controversies, postoperative peak of transaminases has been correlated with postoperative morbidity and mortality rates after liver resection, including postoperative liver failure (434).

Given the lack of consensus in the definition of liver failure after hepatic resections, in 2011 the International Study Group of Liver Surgery (ISGLS) defined post hepatectomy liver failure (PHLF) as “the impaired ability of the liver to maintain its synthetic, excretory, and detoxifying functions, which are characterized by an increased international normalized ratio and concomitant hyperbilirubinemia (according to the normal limits of the local laboratory) on or after postoperative day 5”. The severity of post-hepatectomy liver failure was also classified depending on its clinical management: grade A, requiring no change in clinical management; grade B, requiring change in clinical management but not invasive therapy; and grade C, where invasive therapy is needed. The risk of perioperative mortality with grades B and C were 12% and 54%, respectively (435).

In a review correlating morbidity and mortality with hypertrophy and liver function in ALPPS published in 2017, Kang *et al.* revealed that the most common criteria used for PHLF definition was the ISGLS group definition followed by the “50-50-criteria”. In this review, PHLF rate was around 30% (range, 8-36%) (436).

PHLF shares pathophysiological mechanisms with the transplant term short-for-size syndrome (SFSS). In SFSS, acute liver failure occurs in the recipient when the transplanted liver donor is too small. Both conditions present a hepatic dysfunction secondary to a reduction of liver mass and hemodynamic changes including portal hyper flow and venous congestion (127).

-Metabolic clearance tests

The metabolite clearance tests are dynamic quantitative tests measuring the elimination of a substance in time by the liver. Some of them reflect the cytochrome P450 activity such as the lidocaine-monoethylglycinexylidide (MEGX test), caffeine elimination or C-aminopyrine breath test. Others reflect the hepatic cell mass like the galactose elimination test, or the protein synthetic function like amino acid clearance test.

Unfortunately, due to their complexity, poor availability and high cost, many of these qualitative tests are rarely applied (307, 437).

Among all the metabolic clearance test, the most widely used is the indocyanine green (ICG) clearance test (438). Indocyanine green (ICG) is a trycarbocyanine dye rapidly cleared from the blood by the hepatocytes and excreted into the bile which can be determined by blood sampling or pulsed spectrophotometry. After ICG injection, serum ICG level falls exponentially and, in approximately 20 mins, 97% of the dye is excreted into bile. Therefore, it has been used as a marker of biliary excretion, reflecting a global function of the liver e.g. when the overall liver function is poor, the rate of clearance in

the blood in ICG will be slower. Results of the test can be expressed as percentage of retention at 5 or 15 minutes post injection (ICG-R5 or ICG-R15) or, more commonly, as ICG plasma disappearance rate (ICG-PDR) with normal ranges between 16-25%/min. An ICG-R15 higher than 20% indicate impaired hepatic functional reserve indicating the need of preoperative liver optimization techniques (437). Disadvantages of this test is that ICG clearance is influenced by hepatic blood flow, cellular uptake and biliary excretion. Therefore, it may be misrepresentative in cases of underlying liver disease or when variations of hepatic blood flow like shunting or thrombosis are present (426). Furthermore, ICG does not reflect the function of each liver segment separately. Some of the liver segments may be more functional than others or even have different vascularization (439). Although ICG clearance is one of the most widely used quantitative test, disagreements with clinical outcomes have been reported. In the setting of ALPPS, where portal flow is affected by definition, ICG clearance test has not been validated as means to assess liver function (440).

- Molecular nuclear imaging techniques: hepatobiliary scintigraphy (HBS)

The use of molecular nuclear imaging techniques in the field of liver surgery offers very specific imaging methods known as hepatobiliary scintigraphy (HBS). These techniques provide simultaneous morphological and physiological information revealing regional functional differences in the liver in terms of quality of biliary drainage. HBS has been used to assess the risk of postoperative liver failure after major hepatic resection and liver transplantation by either using ^{99m}Technetium-galactosyl human serum

albumin (GSA) or ^{99m}Technetium-mebrofenin (^{99m}Tc-N-(3-bromo-2,4,6-trimethyacetanilide) iminodiacetic acid) (IDA) (441).

After injecting ^{99m}Tc-galactosyl human serum albumin (GSA), this radio compound binds asialoglycoprotein receptors on the hepatocyte cell membrane selectively. Hence, its uptake is related to hepatocyte cell mass and likely influenced by hepatic blood flow. On the other hand, this substance is not excreted by bile, so it is not suitable to evaluate the biliary tree morphology. The most commonly used parameters in planar dynamic ^{99m}Tc-GSA scintigraphy is the hepatic uptake ratio of ^{99m}Tc-GSA (LHL15) and the blood clearance ratio (HH15) (441, 442).

^{99m}Tc-mebrofenin HBS uses an iminodiacetic acid (IDA) analogue which also uptakes exclusively in the liver. In contrast with ^{99m}Tc-GSA, 99mTc-mebrofenin is excreted in the bile canaliculi. This excretional phase enables to define the quality of biliary drainage.

In the clinical practice, pre-operative hepatobiliary scintigraphy (HBS) can predict post-operative liver function and guide when it is safe to proceed with major hepatectomy after PVE and ALPPS. HBS has been suggested to be useful in timing the second stage after PVE and ALPPS, after 3 weeks and 8-10 days, respectively (443).

Dynamic ^{99m}Tc-mebrofenin HBS (hepatobiliary scintigraphy) have also been used allowing measurement of functional volume of the liver during the hepatic uptake and excretion phase.

Moreover, the combined modality of ^{99m}Tc-mebrofenin scintigraphy with single-proton-emission CT, can provide a better idea of the differential hepatocyte function within the different liver segments than the routine scintigraphy.

With the premise that FLR volume may not necessarily equals function, the group of van Gulik *et al.* described the use of ^{99m}Tc-mebrofenin scintigraphy with SPECT-CT to monitor FLR volume and function in patients who underwent the first stage of ALPPS procedure. Provided the greater importance of FLR function over its volume, they proposed this strategy as an useful tool to predict the safest timing of resection in the second stage of ALPPS (443).

1.6.2 Liver volume assessment

The average adult liver weighs about 1.2-1.5 kg with an inter-individual variable volume and shape. Different factors such as age, gender or body size affect the liver weight and volume. In keeping with the concept of the "hepatostat", a proportionated relationship between the liver weight and the rest of the body, the liver requires a precise and accurate size to provide for the needs of the body. After hepatectomy, the remnant liver increases to a 100% of its original liver size although with a different shape. On the contrary, in situations where the body metabolism changes such as cachexia, responses to chemotherapy or chronic inflammatory conditions, the liver decreases in size (5, 12, 16). Besides, in the setting of liver transplantation, it has been observed how the donor liver size adapts to the recipient body surface (17).

Postoperative liver failure is the most common cause of mortality post hepatic resection (444). A thorough preoperative volumetric analysis of the liver has been described as a crucial tool to predict and minimize the risk of postoperative liver failure and death in patients undergoing either liver resection or liver transplantation (290, 325). Unfortunately, the safe limits for hepatic resection in terms of future liver remnant volume (FLRV), percentage of remaining functional liver volume compared with pre-operative functional liver volume, remains under debate. The minimal and functional FLRV varies on each patient depending on its liver function and underlying liver disease (299, 300, 433). Furthermore, different studies have shown that the incidence of PHLF, post hepatectomy liver failure, increases proportionally with the numbers of segments resected. Schindl *et al.* demonstrated an PHLF incidence of 1% and 30% when resecting 1-2 segments or 5 or more, respectively (304). Others have demonstrated that removing more than half of the total liver volume is responsible of 80% deaths secondary to PHLF (310, 432).

As mentioned before, studies have shown that the upper limit of partial hepatectomy to avoid the most feared complication, post hepatectomy liver failure, is 70-80% in humans (1, 307) and 90-95% in rats (21, 28). As a general rule, to prevent liver failure post hepatectomy different liver volumes for the future liver remnant have become acceptable: >20% if normal parenchyma; 30-35% when mild steatosis, cholestasis or early cirrhosis (Child's-Pugh A) is present; and 40% in severe steatosis and cholestasis (445, 446). In patients with stablished cirrhosis, resection up to 50% of the liver volume can be safe if there is no functional impairment or portal hypertension (447). In patients

with more advanced chronic liver disease, Child's-Pugh B or C, even small resections can result in PHLF (448).

A preoperative volumetric assessment of the future liver remnant (FLR) may indicate whether its size is insufficient or preoptimization of its volume with either interventional or surgical techniques is needed to achieve a safe resection. Along the years, a number of different imaging modalities have been used to calculate the liver volumetry such as computer tomography, magnetic resonance imaging (MRI) and ultrasound (US). Currently, estimations of total liver volume, tumoral tissue volume and/or future remnant liver volume can be calculated preoperatively either by planimetry of single CT or MRI slides or, more recently, with the aid of a virtual surgical planning software tools, here additional 3-dimensional reconstruction provides invaluable information on vascular and biliary anatomy, tumor extent and relationship with important structures (26, 437).

Nowadays, CT is the most common first-line imaging modality in liver surgery. Not only it is highly available and low-cost, but it enables tumour staging, monitoring of the disease, preoperative volumetric evaluation and location of tumour in relation to main biliary and vascular structures (449).

CT volumetry (CTV) is the imaging modality most widely used in the assessments of liver volumes. The volumes of interest are total liver volume (TLV), standardized total liver volume (sTLV), functional liver volume (FLV), future liver remnant (FLR) and tumour volume (TV).

Different formulas based on biometric data have been used to estimate total liver volume. Unfortunately, it remains unclear which formula is the most accurate. Body surface area (BSA) and body weight (BW), predict total liver volume (standardized total liver volume, sTLV) using the following formulas:

TLV = -794.41 + 1,267.28 x body surface area (m2)

TLV = 191.80 + 18.51 x body weight (kilograms)

Standardized total liver volume (sTLV) is an estimation of the total liver volume calculated from a linear regression formula which correlates body surface area or weight with total liver volume (308, 450). Functional liver volume (FLV) is provided by the subtraction of TV from TLV. Enhanced CT scan with contrast can calculate the future liver remnant volume (FLV) and tumour volume. By using the FLRV measured by CT volumetry and the TLV based on BSA or body weight (FLRV/sTLV ratio), a useful parameter to assess whether preoptimization of liver volume is needed before liver resection and the degree of FLR hypertrophy as a response: future liver remnant volume/total liver volume ratio. Future liver remnant (FLR) is dependent of the predicted volume liver remnant either as a percentage of the total liver volume (TLV) or as a percentage of an ideal total liver volume, sTLV (*sFLR = FLR/sTLV x100*) (451).

Graft weight-to-recipient body weight ratio (GRBWR) has been also used to predict minimum adequate graft volume in liver transplantation. Although controversial, the minimal accepted GRBWR is 0.6-0.8% (450).

Kinetic growth ratio is calculated as percentage of growth per day (*percentage-point difference between the sFRL volume before and after the intervention/ time elapsed*) or CC growth per day (*FLR after intervention – FLR prior to intervention/ time elapsed*). The increase of FLR in patients with CRLM is highest the first week after portal vein occlusion (452). In the setting of ALPPS, the liver kinetic growth rate has shown to predict the risk of postoperative liver failure after the second stage (453, 454). A recent study including a multicenter cohort of patients who underwent ALPPS procedures with volumes calculated with 16 different formulas showed that the Vauthey formula (*estimate total liver volume = 18.51 × body weight + 191.8*) provides the most accurate prediction of the actual future remnant liver volume (455).

As mentioned earlier, liver ultrasound (US) and magnetic resonance imaging (MRI) have also been used to calculate volumes, but more often to assess treatment response. When MRI is compared to CT scan, there is a good correlation of the liver volume measurement using both techniques. The main benefit of MRI is the superior tissue contrast allowing a better delineation of hepatic tumours and better segmentation of liver margins. On the contrary, MRI is more expensive, unsuitable for claustrophobic patients and motion artefacts can affect the quality of the images. Regarding the ultrasound which is operator-dependent, 3D US has shown good concordance with CTV, but poor correlation with conventional 2D US (449).

Volume estimations by virtual surgical planning systems have shown to be comparable or even superior to the radiologist's CT or MRI planimetry (456). These softwares offer a precise visualization of the liver structures, its

anatomical relation with the tumoral tissue and some of them have a volume-safety margin function allowing a preoperative surgical planning with a safe tumour-free resection (449).

1.6.3 Liver function *vs* liver volume in liver regeneration

In order to assess LR in the context of surgical resections or transplant, it is important to stablish that volume may not equal or reflect function. While volume has been widely studied as a strong predictor of postoperative outcome, it is basically a surrogate measure for function.

Firstly, there is no single test that can predict all liver functions at once (e.g. uptake, synthesis, biotransformation or excretion). Secondly, despite of its wide availability, one of the disadvantages of the CT volumetry (CTV) is that as a morphological method, it does not reflect the liver function, but its anatomical structure and size. In healthy patients with normal liver parenchyma, the functional liver volume may be proportional to the total liver volume measured. On the contrary, in patients with compromised liver function, there is a discordance between liver volume and function. In the latter, CTV would not be enough for the preoperative assessment of the FLR. In such situations, there is a need for utilization of combined functional and morphological assessment in the evaluation of FLR for more accurate prediction of postoperative liver failure (427). Therefore, in patients with normal liver parenchyma, CTV is useful. On the contrary, in those patients with

suspicious of diseased parenchyma measurement of FLRV should preferably be combined with a quantitative liver function test such as Child-Pugh or ICG test.

Overall, it is agreed that the preoperative evaluation of the FLR should be a multidisciplinary approach where surgeons and radiologists assess resectability, establish the most appropriate surgical plane, determine tumour burden and the presence of a diseased parenchyma (steatosis or cirrhosis) (427).

The current methods for assessing the growth of the FLR depends mainly on CT volume reconstructions. Here either cellular hypertrophy, secondary to parenchyma oedema, or true liver regeneration involving hyperplasia of the liver cell population is not accurately differentiated (373, 457).

The question whether liver volume parallels liver function was raised on the evidence of the spectacular enhanced liver regeneration induced by ALPPS associated on the other hand with significant rates of liver failure (373).

Controversy remains in the field of PVE. From one hand, after portal vein embolization in rabbits, it has been demonstrated that hepatic transection increased liver volume but not function (458). On the other hand, liver function preceded the volume increase in PVE in other study (459). In the case of ALPPS, volume does not appear to reflect functional growth (460) but similar histological features in ALPPS and PVE have been demonstrated (391, 461).

From the histological point of view, it has been observed that regeneration after 30% removal of liver tissue was mediated only by hypertrophy and no

hyperplasia whilst after 70% hepatectomy, hypertrophy anteceded cell proliferation in mice (42).

After 70% partial resection in humans, 80-90% recovery of the liver mass occurs within 6-12 months. On the contrary, recovery of the liver function is highly variable and depends on the parameter investigated. As an example, bilirubin and international normalized ratio normalize in 10 days, but albumin and galactose clearance test take longer time recovering within 90 days (462).

Some authors have suggested that the estimation of the future liver remnant with a combination of future liver remnant volume ratio (FLRV%) by CT volumetry and liver function by ^{99m}Tc-mebrofenin HBS before hepatectomy can predict PHLF and mortality and guide the indication of portal vein occlusion (337, 459).

In the setting of ALPPS and its variants, PHLF has been associated to the discrepancy between liver volume and function. A recent study investigated how HBS, hepatobiliary scintigraphy, and daily gain in volume of the FLR (KGRFLR) can be used to predict PHLF after ALPPS. A high risk of PHLF was revealed in patients with a KGRFLR≤ 4.1% per day and a HBSFLR≤ 2.7%/min/m2 where completion hepatectomy in the second stage should be reconsidered (463). In ALPPS, scintigraphy has shown to over-estimate liver volume versus liver function (460). In a dynamic evaluation of liver volume and function before and after the two stages of ALPPS in 9 patients, Sparrelid *et al.* showed that the increase in function was half of the increase in volume after stage one. These authors advocate functional assessment before both ALPPS

stages and further delay the second stage if functional evaluation is no adequate (464).

1.7 Impact of liver regeneration in liver tumour progression

Current evidence suggests that induction of liver regeneration after major resection associated or not to portal vein occlusion might facilitate tumour growth by increasing the size of established metastasis or the appearance of new liver lesions (465-467). In fact, already in 2001, Kokudo *et al.* stablished a 20% rate of failure to proceed with completion hepatectomy due to insufficient FLRV or disease progression after PVE (468). Tumour progression after preoperative PVE has been observed and investigated in rabbit models (465, 469).

The interaction between liver regeneration and tumour progression has been predicted to be secondary to four possible mechanisms. Firstly, an up-regulation of growth factors and cytokines can be responsible for both hepatocyte and tumour cell proliferation. Secondly, an interruption of portal blood flow might be associated with an increase of the hepatic arterial flow (known as the hepatic arterial buffer response) being the latter the main route of vascularization of liver metastasis. Thirdly, the growth of dormant micrometastasis could be triggered by angiogenesis of liver regeneration and finally, the remodelling of the extracellular matrix could favour detachment of tumour cells and subsequently engrafting to other hepatic sites. Unfortunately, a wide variety of experimental models in mouse and rats have

been used to study this effect in colorectal liver metastases with conflicting results (470).

From the clinical point of view, this relationship has tremendous relevance in patients with bilateral liver metastasis. After portal vein embolization or ligation, tumour progression could occur either by an increase of tumour volume in the occluded liver or the induction of new metastases in the future liver remnant. Therefore, a two-staged hepatectomy with clearance of the future liver remnant can be justified before portal vein occlusion. To expedite the second stage and theoretically prevent further tumour proliferation by inducing a quicker liver regeneration response, the novel technique ALPPS with its variations may play an important role. Many studies comparing the efficacy as well as short and long-term outcomes of these new approaches with the gold standard portal vein embolization are being conducted at the present time.

The effect of the first stage during ALPPS on tumour proliferation, in terms of recurrence or early tumour progression, remains a concern not only for intrahepatic tumoral tissue *per se* but also for distant metastatic disease elsewhere (471, 472). This has been recently investigated in mice and humans with initially unresectable colorectal liver metastasis who underwent ALPPS procedures, failing to identify any accelerated tumour growth or progressive disease (393).

1.8 Hypothesis

1.8.1 General aims

To examine the liver regeneration process at a genetic, molecular and macroscopic level after inducing liver hypertrophy with PVE or RALPPS techniques in patients with liver tumours.

1.8.2 Specific aims

- **Literature review**

To perform an updated literature review about the liver regeneration process including its clinical applications and novel models of accelerated liver regeneration.

- **Experimental work**

To understand the intrinsic changes occurred during the liver regeneration process when PVE or RALPPS techniques are performed through:

- Detection of circulating cytokines and growth factors plasma levels involved in liver regeneration (HGF, EGF, FGF, IL6, TNFα, TGFα, VEGF, and TGFβ).
- Analysis of differential expression of the transcriptome in liver tissue and colorectal liver metastasis during liver

regeneration. Characterise expression profiles associated to different areas of the liver.

- **Randomised clinical trial - REBIRTH:**

  To understand and compare the impact of PVE and RALPPS on liver regeneration in terms of volumetric changes, safety and postoperative complications rates.

CHAPTER 2. MATERIALS AND METHODS

All research work during this PhD took place in the Department of Surgery and Cancer at Hammersmith Hospital where access to clinical data and patients' samples from Imperial College NHS Trust were available.

The experimental work was undertaken in separate laboratories at Hammersmith Campus (Imperial College London, London, UK):

- **Circulating liver regeneration biomarkers assay.** 6th floor Commonwealth building in collaboration with Professor Tricia Tan's group. Department of Metabolism, Digestion and Reproduction.
- **Next generation sequencing assay: transcriptional characterization of liver tissue and colorectal liver metastasis during liver regeneration.** 4th floor IRDB building in collaboration with Dr Johnathan Krell's group (Department of Surgery and Cancer), 2nd floor ICTEM building (Agilent® 2100 Bioanalyzer®) and Imperial BRC Genomics Facility.

2.1 Circulating liver regeneration biomarkers' assay

A literature review of the following biomarkers involved in liver regeneration was performed in a well-known international publications' repository, PubMed. The exploratory process began with the following terms: liver regeneration, hepatectomy, PVE, ALPPS, HGF, EGF, FGF, IL6, TNFα, TGFα, VEGF, and TGFβ.

In order to identify modifications of peripheral plasma levels in the aforementioned analytes in patients who underwent PVE or RALPPS at different time points, a simultaneous analysis was performed with the Milliplex MAP Assay Kit (EMD Millipore, MA, USA). The process is explained in detail in the following sections.

2.1.1 Patient plasma samples: collection and storage

The samples collected consisted of peripheral blood prior or after induction of LR in patients with liver tumours who underwent either RALPPS (n= 10) or PVE (n= 13) in the Department of Surgery and Cancer at Hammersmith Hospital between November 2015 and June 2019.

Peripheral blood in three EDTA-coated tubes (20 ml) were taken at the following defined time points: 2 hours prior to induction of liver regeneration (RALPPS n= 9; PVE n= 11); 24 - 48 hours post induction of liver hypertrophy (RALPPS n= 6; PVE n= 5), and 2 hours before second stage liver resection (RALPPS n= 6; PVE n= 9). The samples were kept on ice and taken to the

laboratory. After 10 mins of centrifugation at 1000xg at 4°C, plasma was obtained and subsequently stored in 1ml Eppendorfs at -80°C until analysis.

2.1.2 Multiplex liver regeneration assay

The analysis of the circulating factors (HGF, EGF, FGF, IL6, TNFα, TGFα, VEGF, TGFβ) in the plasma samples was performed using the immunoassay Milliplex® MAP Assay Kit (EMD Millipore, MA, USA) according to the manufacturer instructions.

A concise explanation follows. Based on xMAP® technology (Luminex Corporation, Austin, TX, USA), the Milliplex®immunoassay is a quantitative method which can analyse multiple biomarkers simultaneously on a 96 well plate from human serum, plasma or cell culture samples. Briefly, duplicates or triplicates of plasma samples, standard and control were distributed in a 96-well flat bottom plate where magnetic beads coated with monoclonal antibodies were added. These monoclonal antibodies are specific for each biomarker. An incubation overnight permits the analyte to be captured on the surface of the bead. After washing by using a hand-held magnetic separator block which allows the magnetic beads to be securely held to each well, further incubation with specific detector antibodies with a reporting fluorescent mark is performed for one hour. Once the 96 well plate is ready to be analysed, it is inserted in the MAGPIX® platform (Luminex Corporation, Austin, TX, USA) which contains an xPOTENT 4.2 software (Luminex Corporation, Austin, TX, USA) with a fluorescent imager hardware. The results

of each analyte are automatically calculated by the software and given in pg/ml.

In the present thesis, a total of 46 plasma samples were analysed in duplicates with a volume of plasma of 25 µl per well. Three types of customized kits were used as there were some differences in the protocols and the selected analytes could not be plexed together.

- HCYTOMAG-60K (Human Cytokine/Chemokine Magnetic Bead Panel) (EMD Millipore, MA, USA).
- HAGP1MAG-12K (Human Angiogenesis/Growth Factor Magnetic Bead Panel 1) (EMD Millipore, MA, USA).
- TGFBMAG-64K-01 (TGFß1 Single Plex Magnetic Bead Kit) (EMD Millipore, MA, USA).

2.1.3 Data analysis and groups for comparison

After following slightly different protocols depending on the Kit manufacturer's instructions, the 96 well plates were inserted independently in the MAGPIX® platform. The software xPOTENT 4.2 provided the results of each analyte automatically in pg/ml which were then further analysed with the software PRISM 8 using the appropriate standard curves.

The following groups were taken into consideration for the analyses:

- Pre liver induction in RALPPS patients (n= 9).

- Post liver induction in RALPPS patients <48 h (n= 6).
- Pre second stage in RALPPS patients (n= 6).
- Pre liver induction in PVE patients (n= 11).
- Post liver induction in PVE patients <48 h (n= 5).
- Pre second stage in PVE patients (n= 9)

Different comparisons were performed between the stablished groups:

- Pre stage one and post stage one in RALPPS patients.
- Pre stage one and post stage one in PVE patients.
- Pre stage one and pre stage two in RALPPS patients.
- Pre stage one and pre stage two in PVE patients
- Pre stage one in RALPPS *versus* PVE patients.
- Post stage one in RALPPS *versus* PVE patients.
- Pre stage two in RALPPS *versus* PVE patients.

2.1.4 Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS, version 25, IBM, Chicago, IL, USA). Distribution of continuous variables was analyzed using the Shapiro-Wilk test and expressed as mean ±standard deviation. Non-parametric Mann-Whitney U test was used to

compare two means of independent samples within the different groups. Differences were considered significant if $p \leq 0.05$.

2.2 Next generation sequencing assay: transcriptional characterisation of liver tissue and colorectal liver metastasis during liver regeneration

In this experiment, RNA extraction and purification from patients' tissue samples were performed and their subsequent analysis by NGS to identify genetic dysregulation after induction of liver regeneration either with RALPPS or PVE was undertaken in:

a) Normal parenchyma of the future liver remnant and the embolized or ligated hepatic lobe.
b) Colorectal liver metastasis.

2.2.1 Patient liver tissue samples: types, collection and storage

Sixty-nine normal liver and colorectal liver metastases tissue samples were harvested from patients with liver tumours who underwent RALPPS (n= 10) or PVE (n= 8) procedures in the Department of Surgery and Cancer at Hammersmith Hospital between October 2016 to July 2017.

The tissue samples were retrieved at two different time points. Firstly, at the beginning of the first stage operation in both RALPPS and PVE patients, normal looking liver parenchyma and colorectal liver metastases (CRLM) tissue biopsies (<0.5 cm thickness) from both liver lobes when possible were harvested with scalpel. Tissue samples were immediately immersed in

Allprotect Tissue Reagent (Qiagen, Hilden, Germany) to stabilize the RNA at room temperature and stored at –80°C freezer until further analysis.

Similar harvesting and storage were performed at the beginning of the second stage operation, once liver regeneration had occurred either by PVE or RALPPS procedures previously.

The samples were classified according to:

- Time of the extraction: A or B, for first and second stage operation, respectively.
- Site: L or R, for left or right liver lobe, respectively.
- Nature of the tissue: N or T, for macroscopic normal liver parenchyma or tumoral tissue, respectively.

Therefore, tissue samples from the first resection were named as first stage left-sided normal liver parenchyma (ALN), first stage right-sided normal liver parenchyma (ARN), first stage left-sided tumour (ALT), and first stage right-sided tumour (ART). After the induction of liver regeneration either with PVE or RALPPS, samples were labelled as second stage left-sided normal liver parenchyma (BLN), second stage right-sided normal liver parenchyma (BRN), second stage left-sided tumour (BLT), and second stage right-sided tumour (BRT) (Table 2.1).

	Patient	ARN	ALN	ART	ALT	BRN	BLN	BRT	BLT
RALPPS	**1**	1	1		1	1	1	1	
RALPPS	**2**	1	1				1	1	
RALPPS	**3**	1	1			1	1	1	
RALPPS	**4**	1	1	1	1	1	1		
RALPPS	**5**	1	1		1	1			1
RALPPS	**6**	1	1	1	1	1	1	1	
RALPPS	**7**	1	1			1	1	1	
RALPPS	**8**		1		1				
RALPPS	9	1							
RALPPS*	10	1	1			1	1		
TOTAL		**9**	**9**	**2**	**5**	**7**	**7**	**5**	**1**
PVE	1					1	1		
PVE*	2					1	1		
PVE	3	1	1	1	1				
PVE*	4					1	1		
PVE	5						1		
PVE	6					1	1		
PVE	**7**	1	1	1		1	1	1	1
PVE	**8**	1	1	1	1				
TOTAL		**3**	**3**	**3**	**2**	**5**	**6**	**1**	**1**

Table 2.1. Distribution of tissue sample retrieved for RNA extraction. Any empty cell means there is no sample collected.

ALN: first stage, left normal liver tissue; ALT: first stage, left tumoral liver tissue; ARN: first stage, right normal liver tissue; ART: first stage, right tumoral liver tissue; BLN: second

stage, left normal liver tissue; BLT: second stage, left tumoural liver tissue; BRN: second stage, right normal liver tissue; BRT: second stage, right tumoural liver tissue.

* Patients with non-colorectal liver metastasis (one ovarian; two cholangiocarcinoma).

2.2.2 Total RNA isolation and purification

The total RNA extraction from tissue was performed with the RNeasy® Mini Kit (Qiagen, Hilden, Germany) as per manufacturer's instructions. Briefly, this method can purify up to 100 μg of RNA longer than 200 nucleotides by combining the selective binding properties of a silica-based membrane with the speed of micro spin technology. RNAs with less than 200 nucleotides such as 5.8S rRNA, 5S rRNA, and tRNAs are selectively excluded.

This technique requires the following materials:

- RNeasy® Mini Kit (Qiagen, Hilden, Germany)
- RNeasy Mini Spin Columns (pink)
- Collection Tubes (1.5 ml)
- Collection Tubes (2 ml)
- Buffer RLT contains guanidine thiocyanate
- Buffer RW1 contains a small amount of guanidine thiocyanate
- Buffer RPE
- Buffer RLC contains guanidine hydrochloride
- RNase-Free Water
- Cole-Parmer LabGEN 125 tissue homogeniser kit (speed motor 30,000 rpm) (Cole-Parmer, UK)

The tissue samples (<2 mm in diameter) are lysed and homogenised in a few seconds in the presence of a buffer which contains guanidine thiocyanate and immediately inactivates RNAses (600 µl buffer RLT), with the Cole-Parmer LabGEN 125 tissue homogeniser. The lysate then is centrifugated at full speed for 3min and the supernatant is transferred to another tube. To provide adequate binding conditions of the RNA to the RNeasy membrane, ethanol 70% is added to the sample, and the mix is applied to a RNeasy mini spin column and centrifugated at 8000 xg 15 secs. Contaminants are washed away after 15 secs of centrifugation with 350 µl RW1 buffer. A mix of 10 µl DNase I stock solution to 70 µl Buffer RDD is added and incubated for 15 mins at room temperature (RT). Further washing contaminants is then performed by adding 350 µl RW1 and centrifuge 15 secs; adding 500 µl RPE, centrifuge 15 secs and discard and adding 500 µl RPE, centrifuge 2 min and discard. The RNeasy spin column is then centrifuged at full speed 1 min and placed in a new collection tube. Finally, the membrane-bound total RNA is eluted in RNase-free water by adding 50 µl RNase-free water to the column, waiting for 1 min at RT and centrifuge 8000 xg 1 min.

2.2.3 Nanodrop quantification

Immediately after total RNA was purified, RNA concentration was quantified by absorbance at 260 nm and 280 nm using the Nanodrop spectrophotometer (Nanodrop, Wilmington, Delaware, USA). Spectrophotometric quantification allows the calculation of RNA concentration by using the formula A260 x dilution x 40= µg RNA/ml. Under neutral pH, an

absorbance of 1 unit at 260 nm equals to 44 µg of RNA per ml (A260=1→ 44 µg/ml). A260 readings should be greater than 0.15 to ensure significance and a ratio A260/280 around 2 is considered pure (without contaminants).

2.2.4 Quality check

2.2.4.1 RNA integrity analysis by Bioanalyzer

As small amounts of RNA may be difficult to determine photometrically, further integrity and quantification of the RNA samples were evaluated by using an Agilent® 2100 Bioanalyzer® 6000 Nano Assay (Agilent Technologies, Palo Alto, CA, USA) as per manufacturer's protocol.

The Bioanalyzer® estimates the RNA integrity based in an algorithm denominated RNA Integrity Number (RIN) which corresponds to the quantified value given by the Agilent software. RIN can be between 1-10, being 10 the maximum quality, 7 could be considered as optimal and between 1-3 degraded. The Agilent® 2100 Bioanalyzer® 6000 Nano Assay is able to detect total RNA concentrations ranging from 25 to 500 ng/µL. In the graphic representation, ribosomal RNAs should appear as sharp peaks and ratio of 28S rRNA to 18S rRNA should be around 2:1. Samples that show a lack of a ribosomal RNA peaks or small increment towards smaller sized RNAs reflects major degradation either before or during purification of RNA.

2.2.4.2 Selected tissue samples for NGS and feasible groups for comparisons

Thirty-two total RNA samples with appropriate quality related with the mentioned techniques in the quality check were sent to the BRC Genomics

Facilities at Imperial College for next generation sequencing. Hence, 8 and 6 patients who underwent RALPPS or PVE procedures, respectively, were finally included in the experiment.

The RNA samples were distributed as (Tables 2.2 and 2.3):

- Normal tissue (ALN): n= 9 (6 RALPPS; 3 PVE)
- Left normal tissue post RALPPS (BLN): n= 5
- Left normal tissue post PVE (BLN): n= 4
- Right normal tissue post RALPPS (BRN): n= 5
- Tumoral tissue pre RALPPS (ART or ALT): n= 6
- Tumoral tissue post RALPPS (BRT): n= 3

	Patient	ARN	ALN	ART	ALT	BRN	BLN	BRT	BLT
RALPPS	**1**	1	1		1	1	1	1	
RALPPS	**2**	1	1				1	1	
RALPPS	**3**	1	1			1	1	1	
RALPPS	**4**	1	1	1	1	1	1		
RALPPS	**5**	1	1		1	1			1
RALPPS	**6**	1	1	1	1	1	1	1	
RALPPS	**7**	1	1			1	1	1	
RALPPS	**8**		1		1				
RALPPS	9	1							
RALPPS*	10	1	1			1	1		
TOTAL		**9**	**9**	**2**	**5**	**7**	**7**	**5**	**1**
PVE	1					1	1		
PVE*	2					1	1		
PVE	3	1	1	1	1				
PVE*	4					1	1		
PVE	5						1		
PVE	6					1	1		
PVE	**7**	1	1	1		1	1	1	1
PVE	**8**	1	1	1	1				
TOTAL		**3**	**3**	**3**	**2**	**5**	**6**	**1**	**1**

Table 2.2. Distribution of tissue sample sent for mRNA sequencing after quality check (marked in yellow). Any empty cell means there is no sample collected. Samples written in red were not amenable for NGS due to inadequate quality/quantity.

ALN: first stage, left normal liver tissue; ALT: first stage, left tumoral liver tissue; ARN: first stage, right normal liver tissue; ART: first stage, right tumoral liver tissue; BLN: second stage, left normal liver tissue; BLT: second stage, left tumoural liver tissue; BRN: second stage, right normal liver tissue; BRT: second stage, right tumoural liver tissue.

* Patients with non-colorectal liver metastasis (one ovarian; two cholangiocarcinoma).

	ARN	ALN	ART	ALT	BRN	BLN	BRT	BLT
RALPPS	0	6	2	4	5	5	3	0
PVE	0	3	0	0	0	4	0	0

Table 2.3. Summary of the final distribution of tissue samples sent for RNA sequencing after quality check.

ALN: first stage, left normal liver tissue; ALT: first stage, left tumoral liver tissue; ARN: first stage, right normal liver tissue; ART: first stage, right tumoral liver tissue; BLN: second stage, left normal liver tissue; BLT: second stage, left tumoural liver tissue; BRN: second stage, right normal liver tissue; BRT: second stage, right tumoural liver tissue.

The feasible groups for comparison after the obtention of the RNA sequencing quality check were:

a. RALPPS samples:

   - Pre normal liver (ALN or ARN; n= 6) *vs* left normal liver post regeneration (BLN; n= 6).
   - Pre normal liver (ALN or ARN; n= 6) *vs* right normal liver post regeneration (BRN; n= 3).
   - Pre tumoural tissue (ART or ALT; n= 5) *vs* right tumoural tissue post regeneration (BRT; n= 5).

b. PVE samples:

- Pre normal liver (ALN or ARN; n= 3) *vs* left normal liver post regeneration (BLN; n= 4).

c. PVE *vs* RALPPS samples:

- Left normal liver post regeneration by PVE (BLN; n= 4) *vs* left normal liver post regeneration by RALPPS (BLN; n= 6).

2.2.5 Library preparation

2.2.5.1 Ribosomal RNA depletion

In order to purify the extracted total RNA due to its high degradation, ribosomic RNA depletion was undertaken with the Globin-Zero Gold rNA Removal Kit (illumina, San Diego, CA, USA) as per manufacturer's instructions. This kit allows rRNA depletion from 1-5 μg total RNA samples and increase the accuracy for the library preparation, avoiding the noise that the ribosomic RNA could generate.

2.2.5.2 Library preparation

Library preparation for the rRNA-depleted total RNA samples was performed using:

- NEBNext Ultra II Directional RNA Library Prep Kit (NEB E7760) (illumina, San Diego, CA, USA). This kit is compatible with rRNA depletion reagents and generates high yield, high quality strand-specific libraries from amounts of total RNA as low as 5 ng.

- NEBNext Multiplex Oligos for Illumina (Dual Index Primers Set 1) (illumina, San Diego, CA, USA). This kit enables to analyse up to 96 different samples by a PCR amplification.
- 100 ng of RNA input per sample (rRNA depleted previously).

2.2.6 Next generation sequencing

Next generation sequencing was performed by Imperial BRC Genomics Facility (Imperial College London) by using the ultra-high throughput platform HiSeq4000 (Illumina, San Diego, CA, USA). The libraries were all pooled into 1 pool and sequenced on 6 lanes (2x75bp = paired-end with segments of 75 base pairs). An automated cluster was generated then for data analysis.

2.2.7 Bioinformatic analyses of RNA-seq data

2.2.7.1 Expression profiling

RNA-seq data analysis was performed as previously described by Ottaviani *et al.* (473) by Dr Leandro Castellano, Imperial College London, with some modifications. Specifically, quality reads from fastq files were assessed using FasQC version 0.10.1 (http://bioinformatics.babraham.ac.uk/projects/fastqc/).

The reads for each sample were then mapped on the reference human genome, version hg19, obtained from the University of California Santa Cruz (UCSC) genome browser (https://genome.ucsc.edu/) by using HISAT2 (https://ccb.jhu.edu/software/hisat2/index.shtml). Next, raw reads were

counted on genes by using HTSeq (https://htseq.readthedocs.io/en/release 011.1/) and the Gencode annotation v19. Differential gene expression analysis and normalization of the data was performed by using DESeq2 method (https://bioconductor.org/packages/release/bioc/html/DESeq2.html) from Bioconductor (https://bioconductor.org/).

Genetic expression for each group of comparisons was given in Excel spread sheets with resultant *p-adjusted* values and Log fold changes (Log2FC). In each comparison, genes with Log2FC>0 and Log2FC<0 were selected as up-regulated and down-regulated, respectively. Further, by using differentially expressed genes data, hierarchical clustering was created with its corresponding heatmaps and volcano plots for the different comparisons.

2.2.7.2 Pathways analysis

A standard gene pathways analysis was undertaken following two different reference databases:

- KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways (http://www.genome.jp/kegg/pathway.html): a collection of manually written pathway maps representing the knowledge about molecular interactions, reactions and relations for metabolism, genetic and environmental information processing, cellular processes, organismal systems, human diseases and drug development.

- WikiPathways (http://www.wikipathways.org): a twelve-year-old database of biological pathways maintained by and for the scientific community.

Given that the pathway enrichment software automatically normalizes the enrichment for variation in gene set sizes, normalization is not accurate for extremely large or extremely small gene sets. Gene sets fewer than 25 genes or more than 500 genes, could not generate significant results. Hence, software ignores gene sets that are fewer than 25 or more than 500 genes for performing the pathways analysis.

In this research, functional analysis was built on significant differential expressed genes obtained by adjusting *p* values. However, this was only possible for two comparisons:

- PVE ALN *vs* BLN when $p \leq 0.05$ and $p \leq 0.15$.
- BLN PVE *vs* BLN RALPPS when $p \leq 0.15$.

2.2.8 Validation of results by quantitative real-time RT-Polymerase Chain Reaction (RT-qPCR)

To confirm the results obtained by the NGS analysis, a quantitative real time RT-qPCR was performed with the Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, EEU) compatible with TaqMan® Low Density Array (Applied Biosystems, Foster City, CA, EEU).

For the validation, total RNA samples were selected from two different comparisons: ALN *vs* BLN in PVE (n= 3 *vs* n= 4) and BLN in PVE *vs* BLN in RALPPS (n= 4 *vs* n= 5), the same ones that could be included for the pathways enrichment analyses.

Firstly, 1 µg of total RNA was reverse transcribed to complementary DNA (cDNA) in a 20 µl reaction mixture using the High-capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, EEUU) according to manufacturer's protocol.

Secondly, cDNA was amplified in 20 µl of 1× TaqMan Universal Master Mix II, no UNG (Applied Biosystems, Foster City, CA, EEUU), with thirteen predeveloped TaqMan assay probes (Applied Biosystems, Foster City, CA, EEUU) (Table 2.4), according to manufacturer's instructions. The TaqMan probe sets were selected from the gene probes catalogue available in Applied Biosystems. *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* was stablished as a housekeeping gene and nuclease free water was used as negative control.

RT-qPCR reactions were developed by using the sequence detector system ABI Prism 7900HT (Applied Biosystems, Foster City, CA, EEUU). The experiment was performed in three technical replicates and placed in 384 well plates where cDNA fragments were amplified. The amplification process is based on repeated thermal cycles involving three consecutive steps: denaturation (95°C), annealing (50°C to 56°C) and extension (72°C). These three stages are repeated 40 times, exponentially increasing the number of copies of DNA each time.

Quantification was performed by using the comparative $2^{-\Delta\Delta Ct}$ method (474) and the analysed results were expressed as relative units (RU).

All analyses were performed in triplicate, and relative RNA levels were determined using *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* as internal control. The Mann-Whitney test was used to compare the two genetic classes for each comparison.

Gene name	**TaqManGene Expression Assay ID**
EGR1	Hs00152928_m1
FADS2	Hs00188654_m1
CCL3	Hs00234142_m1
PER2	Hs00256143_m1
KRT5	Hs00361185_m1
MAPK4	Hs00969401_m1
CDH3	Hs00999915_m1
C3P1	Hs01032950_g1
ACSM1	Hs01048215_m1
ENO3	Hs01093275_m1
LRP2	Hs01118981_m1
SCD	Hs01682761_m1
GAPDH	Hs99999905_m1

Table 2.4. List of target genes with corresponding TaqMan probes used for RT-qPCR.

2.3 Randomized clinical trial

2.3.1 Study design, protocol and participants

This is a two-arm prospective single center randomized clinical trial. The first step was to prepare a detailed protocol which included all the relevant information for the trial. Specific consent forms were also generated as well as information leaflets for patients.

2.3.2 Ethical approval

This RCT was approved by the National Research Ethics Service (IRAS: 148741) and registered online.

(https://clinicaltrials.gov/ct2/show/NCT02216773)

2.3.3 Selection criteria

Inclusion criteria:

- Age ≥ 18 years.
- Any patient requiring right or extended right hepatectomy with preoperative FLRV/TLV:
    - ≤ 25% in patients without preoperative chemotherapy.
    - ≤ 35% in patients with preoperative chemotherapy.
- WHO performance status 0, 1 or 2.

- Patient able to comply with protocol requirements and deemed fit for surgical resection.
- Written informed consent.

Exclusion criteria:

- Inability to give informed consent.
- Pregnancy.
- WHO performance status 3 or 4.
- New York Heart Association Classification Grade III or IV.

2.3.4 Randomization and masking

Within two weeks of randomization using the website http://www.sealedenvelope.com, the patients underwent either RALPPS or PVE to increase their insufficient FLRV for staged hepatectomy preoperatively. There was no masking during the intervention, data collection, data analysis or data interpretation.

2.3.5 Procedures

2.3.5.1 Portal vein embolization (PVE)

Two senior radiologists (R.T., P.T.) performed the PVE as standard. In case of bilobar disease, PVE was performed during the same admission after the

stage-1 operation where clearance of the FLR was achieved. Under local anaesthesia and with fluoroscopy, percutaneous puncture of the portal vein was performed. Each segmental portal branch of the right lobe was identified and injected with a mixture of polyvinyl alcohol and Iohexol (Omnipaque, GE Healthcare, Bucks, UK). To further increase the FLRV, segment 4 was embolized when necessary. The track was sealed with a combination of polyvinyl alcohol and coils. After six weeks, volumetric changes were assessed with a re-staging triple-phase CT scan.

2.3.5.2 Radiofrequency assisted liver partition with portal vein ligation for staged hepatectomy (RALPPS)

The RALPPS technique has been described previously by Gall *et al.* (329, 331, 332). First stage procedure was performed laparoscopically or robotically when possible using a five-port technique (2x10 mm working ports on each side of the abdomen) (Figure 2.1). Briefly, following resection of tumour from the left lobe for those requiring staged liver resection with bilobar disease, cholecystectomy was performed to use the cystic duct as a reference and by being retracted medially to expose the hilus for dissection. The right hepatic artery was isolated and slung with a non-absorbable suture of 2/0 Prolene for later identification at the second stage. The portal vein was carefully separated from the common hepatic duct behind the right hepatic artery and ligated with two Hem-o-loks (Teleflex, NC, USA). Then, ultrasound-guided radiofrequency ablation with either cool tip RFA (Covidien, Hampshire, UK) or laparoscopic Habib Sealer (Laparoscopic Habib TM 4X, Rita, USA) was performed along the now visible demarcation line between left and right

lobes. For the open approach, the abdominal cavity was accessed through a right subcostal incision with upper midline extension. procedure was performed in a similar manner. In this group, re-staging triple-phase CT scan was performed two weeks after the first stage operation.

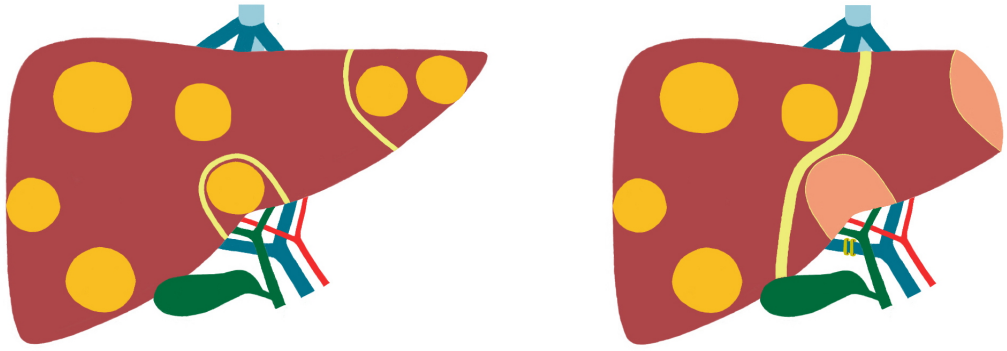

Figure 2.1. First stage RALPPS: wedge resection in segment 2 and 3. Right hepatic artery identification. Ligation of right portal vein with two Hem-o-loks (Teleflex, NC, USA). Radiofrequency ablation with either cool tip RFA (Covidien, Hampshire, UK) or laparoscopic Habib Sealer (Laparoscopic Habib TM 4X, Rita, USA) along demarcation line.

Adapted from *Jiao LR et al., Hepatobiliary Surg Nutr. 2016 Aug; 5(4): 382–387.*

2.3.5.3 Hepatic resection

Patients with adequate FLRV (>25% and >35% if prolonged course of chemotherapy) after first stage RALPPS or PVE, underwent open, laparoscopic or robotic right or extended right hepatectomy named second stage (Figure 2.2). In the minimally invasive procedures, a five-port technique was used, and the specimen was retrieved with a 15 mm Endocatch Bag (Medtronic, Watford, UK) and retrieved via Pfannenstiel incision. For the open liver

resection, a right subcostal incision with upper midline extension was performed. Haemostasis was achieved with Harmonic scalpel (Ethicon Endo-Surgery, Cincinnati, OH, USA). A 20 F Robinsons drain was placed at the resection margin and removed postoperatively when minimal output.

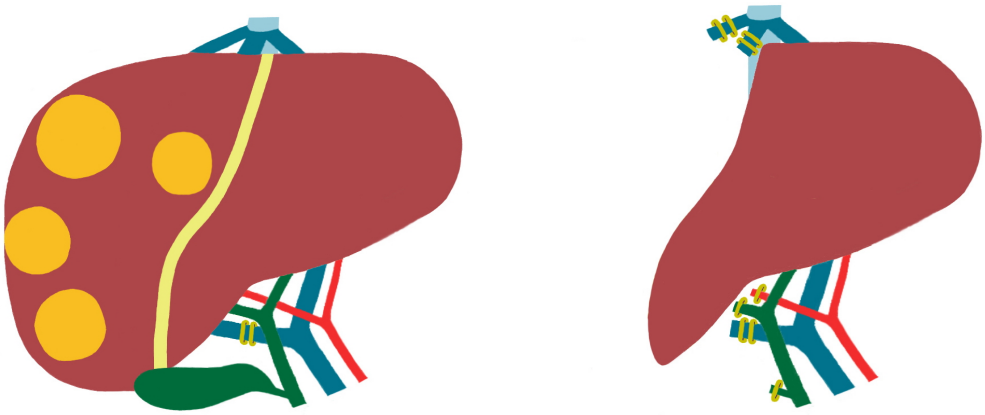

Figure 2.2. Hepatectomy during second stage RALPPS: right hepatic artery identified and ligated. Right Glissonian pedicle divided with Endo GIA 60. Liver parenchyma division through necrotic line with Harmonic scalpel (Ethicon Endo-Surgery, Cincinnati, OH, USA). Right and middle hepatic veins divided Endo GIA 60 (Medtronic, Watford, UK).

Adapted from *Jiao LR et al., Hepatobiliary Surg Nutr. 2016 Aug; 5(4): 382–387.*

2.3.6 Volumetric study of the liver

Serial transverse CT scans before first (PVE or RALPPS) and second stages (hepatectomy) were performed (Figure 2.3). Measurements of total liver volume (TLV), future liver remnant volume (FLRV) and total liver tumour volume (TLTV) were calculated with the software package ImageJ (Image Processing and Analysis in Java, National Institute of Health) as previously described by Dello *et al.* (475). The FLRV which is the liver parenchyma

situated to the left of Cantlie's line was also defined as the proportion of FLRV to TLV minus TLTV.

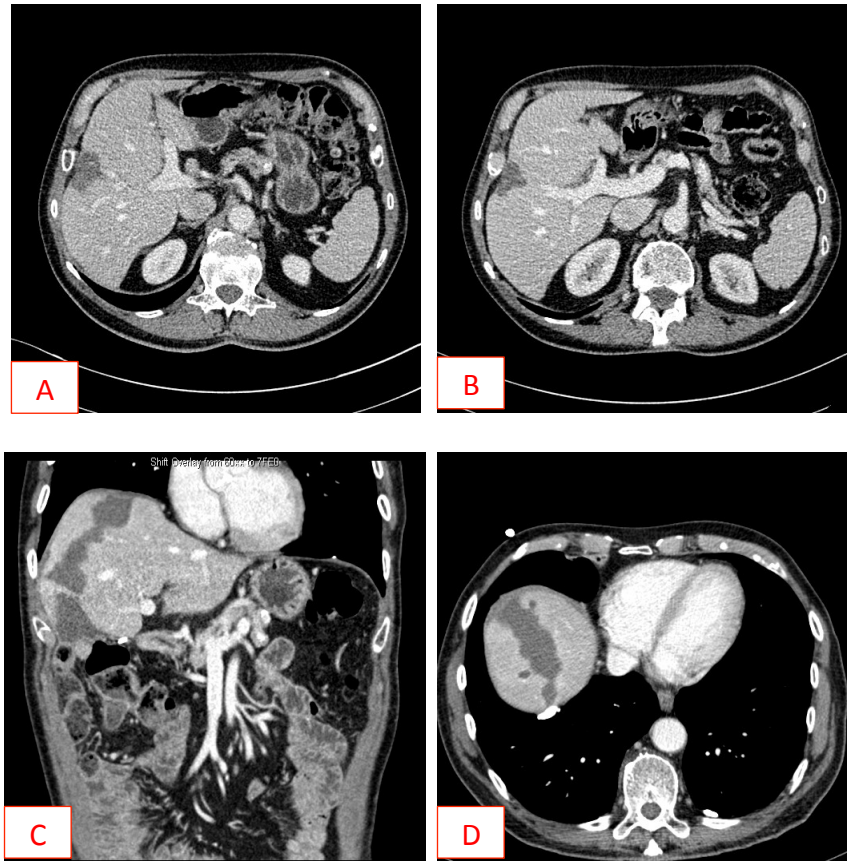

Figure 2.3. CT scan images showing an example of RFA demarcation line post stage one.

Adapted from *Jiao LR et al., Hepatobiliary Surg Nutr. 2016 Aug; 5(4): 382–387.*

2.3.7 Blood tests

Blood samples were taken before stage one and two, as well on post-operatively day one, three and five after completion hepatectomy. Samples were analyzed for bilirubin (umol/L), alanine transaminase (ALT, in IU/L),

alkaline phosphatase (ALP, in IU/L); albumin (g/dL), haemoglobin (Hb, g/dL), c-reactive protein (CRP, in mg/L) levels as well as prothrombin time (PT, in seconds) and activated partial thromboplastin time (APTT, in seconds).

2.3.8 Endpoints/Outcomes

2.3.8.1 Primary endpoint

- Percentage of increase in FLRV

2.3.8.2 Secondary endpoints

- 30-day morbidity
- 30 and 90-day mortality
- Time to second operation

2.3.9 Postoperative complications

Post procedural complications after both stages were graded as per the Clavien-Dindo classification (476). Serious complications defined as ≥3b.

The definition of postoperative liver failure as “The impaired ability of the liver to maintain its synthetic, excretory, and detoxifying functions, which are characterized by an increased international normalized ratio and concomitant hyperbilirubinemia on or after postoperative day 5” was taken as the one given by the International Study Group of Liver Surgery (435).

Any patient who did not proceed to second stage hepatic resection due to disease progression (local, regional or systemic) or insufficient FLRV (≤25% in chemo naïve patients and ≤35% in those who had preoperative chemotherapy) was stablished as failure of treatment.

2.3.10 Data analysis

2.3.10.1 Power calculation and sample size

The sample size was based on the pilot data previously published where 5 patients who had RALPPS procedure were matched by age, gender, preoperative liver function and pathology to 5 historical patients who had PVE (331). By assuming a two-sided testing, the sample size of each arm was calculated using the equation designed for two proportions; α was set at 0.05 to control for type 1 error (false-positive result) and β at 0.10 to control for type 2 error (false-negative result).

After power calculation, a total sample of 16 patients was estimated. However, given the small size of the pilot data, it was decided to aim to recruit 25 patients to the trial with an interim analysis after 16 patients had achieved adequate follow-up.

2.3.10.2 Statistical analysis

Statistical analyses were carried out using the SPSS v22 (Statistical Package for Social Sciences, IBM, Chicago, IL, USA). A descriptive analysis was

performed reporting quantitative data as median and range or mean with standard deviation. Differences between groups were analysed using the unpaired Student's t-test and Mann-Whitney U test for parametric and non-parametric data, respectively. For categorical variables, X^2-test was used. All statistical tests were two-sided and differences were considered significant when $p < 0.05$.

CHAPTER 3. ROLE OF HEPATOCYTE GROWTH FACTOR (HGF) IN LIVER REGENERATION

3.1 Background

Hepatocyte growth factor (HGF) is a multifunctional cytokine produced by cells of mesenchymal origin. This molecule binds specifically the tyrosine-kinase receptor c-MET (cellular mesenchymal-epithelial transition), also named MET which is present in epithelial, endothelial and hematopoietic progenitor cells. The HGF/MET axis is known to be involved in several biological processes, such as cell proliferation, metabolism, motility, survival, morphogenesis and carcinogenesis (147, 148, 477, 478). Hence, this growth factor and its interaction with the c-MET receptor are widely investigated and remain the focus of numerous research works (479-484).

3.2 HGF structure, functions and c-MET receptor

The HGF molecule was purified for the first time in rat platelets in 1984, and four years later in humans. It was described as a potent mitogenic factor for mature rat hepatocytes in vitro (485-488). Its primary structure was determined in 1989. Since then, different transcript variants encoding several isoforms have been recognized by alternative splicing of the gene (489).

HGF gene is located on the long arm of the chromosome 7 (q21.1) and spans 71,433 bases of genomic DNA formed by 18 exons and 17 introns. It

encodes the inactive pre-pro-HGF protein, a single chain of 728 amino acids (83134 Da), which includes a signal sequence (1-31), a heavy alpha-chain (32-494; 69kDa) and a light beta-chain (495-728; 34kDa). The first exon contains the signal peptide and a 5'-untranslated region. The following ten, twelve and six remaining exons encode the alpha-chain with four Kringle structures, the short spacer region between the alpha- and beta-chains and the beta-chain, respectively (490-492).

A two-cleavage process is necessary to activate the inactive pre-pro-HGF into pro-HGF. Firstly, the signal peptide of the pre-pro-HGF is degraded, generating the pro-HGF, and secondly, a further cleavage between Arg494 and Val495 will result in the pro-HGF molecule. Multiple serum or cell-membrane proteases have been described to be involved in this activation process such as HGF activator (HGF-A), urokinase-type plasminogen activator, plasma kallikrein, coagulation factors XII and XI, matriptase and hepsin (493). Among them, HGF-A is the principal protease responsible for the activation of pro-HGF in serum. The activation process of HGF plays an important role in the regulation of tissue regeneration and susceptibility to pathological conditions. As an example, impaired restoration of epithelia after mucosal injury was observed in *HGF-A* knock-out mice although normal development was achieved (494). Also, a lower capacity to activate HGF has been observed in fibroblasts from patients with idiopathic pulmonary fibrosis compared with control fibroblasts (495). A final disulphide bond between the alpha and beta chains achieves the finally active heterodimeric HGF molecule.

HGF belongs to the plasminogen subfamily of S1 peptidases, but no proteolytic activity has been identified, probably due to the substitution of two out of three amino acids required in the catalytic triad (496). Besides, it has been suggested that HGF could be evolutionally derived from proteases implicated in the coagulation cascade and fibrinolysis, due to a very similar organization. Its alpha-chain has 38% homology with plasmin, and the beta-chain is structurally similar to the catalytic domains of serine proteases, although with some differences in the amino acids in the active site (477).

HGF gene expression is upregulated by several growth factors, cytokines and prostaglandins: b-FGF (basic fibroblast growth factor), OSM (oncostatin M), HIF-1α (hypoxia-inducible factor 1 alfa) and NF-κB (nuclear factor kappa B); and downregulated by tumour growth factor beta (TGFβ) (497).

A very distinctive feature of the HGF molecule is that In contrast to other growth factors, HGF binds exclusively to the product of the *c-MET* proto-oncogene, also mapped on chromosome 7, the multifunctional tyrosine kinase receptor c-MET (149, 498-500). The c-MET receptor is a 190kDa protein, comprising a ligand-binding extracellular domain, a transmembrane region and a cytoplasmic domain with tyrosine kinase activity. Although HGF alfa-chain has a higher affinity for MET, it is the beta-chain which activates the receptor (501, 502). Upon MET dimerization, kinase activation results in auto-phosphorylation of tyrosines Y1349 and Y1356, and recruitment of several substrates, including growth factor receptor-bound protein 2, Shc, p85 subunit of phosphatidylinositol 3’ kinase, phospholipase C γ, signal transducer and activator of transcription 3 (STAT3) and Grb2-associated binding protein 1. The

activation of c-MET causes a diversity of downstream signaling pathways within the cell involved in diverse processes such as proliferation, survival, motility, invasion and angiogenesis (Figure 3.1) (503-515). Furthermore, this receptor dimers with the insulin receptor contributing to different metabolic activities such as glucose regulation (16). Conditional knockouts of *c-MET* in mice have revealed the implication and roles of the HGF/MET axis in multiple biological and physiological e.g. organogenesis, morphogenesis, tissue regeneration and carcinogenesis (516).

In 1991, two proteins were found to be identical to HGF: the human lung fibroblast-derived mitogen and the Scatter Factor (SF), a fibroblast-derived factor for epithelial cells, involved in cell migration during embryogenesis and tumour progression. Encoded by the same chromosome bands as HGF, these molecules are ligands for c-MET receptor (517-521). A further protein, a tumour cytotoxic factor derived from fibroblasts, which leads into cell death in several cancer types, was also discovered to be the same molecule as the HGF protein (522). These research works had an important influence for further investigations on the role of HGF in cell growth and motility during embryogenesis, tissue regeneration including liver regeneration, carcinogenesis and tumour progression. Targeted knockouts of *HGF* or c-*MET* have resulted in lethal embryogenesis secondary to impaired development of the placenta and liver (523, 524).

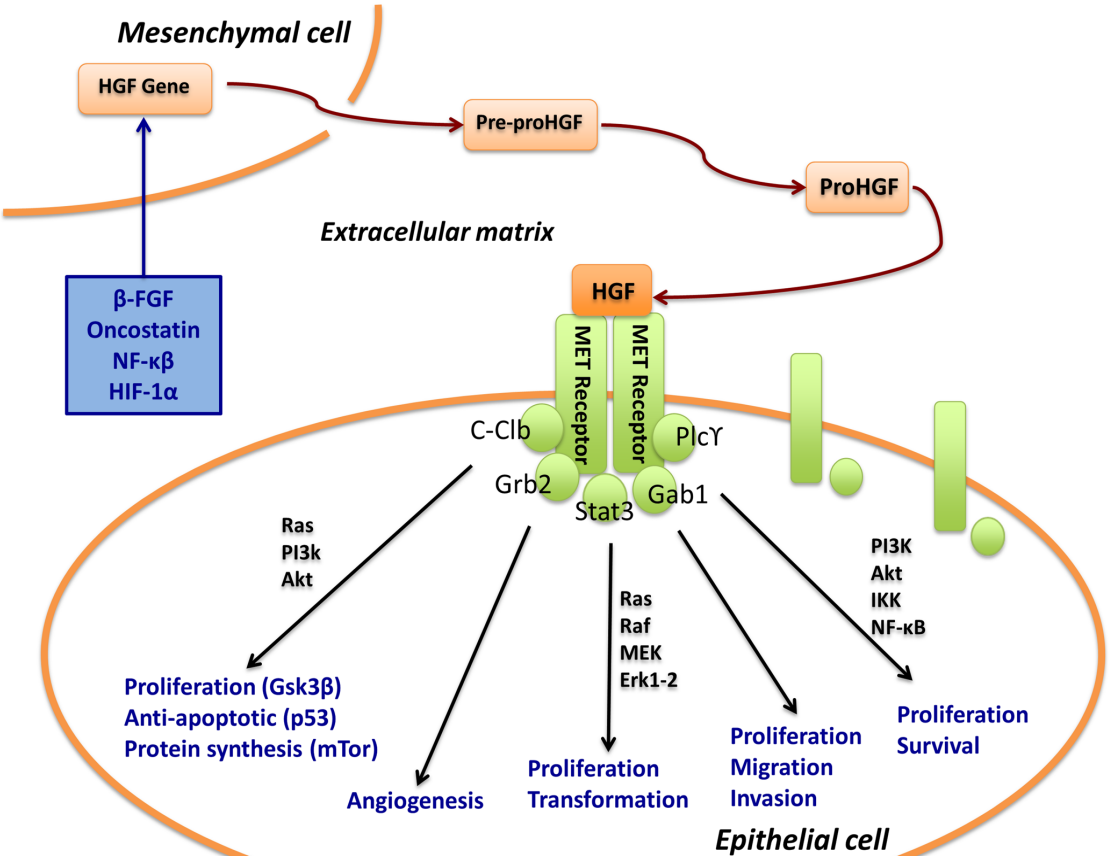

Figure 3.1. The HGF/MET axis. Once HGF is activated by a two-cleavage process in the extracellular matrix, it binds to the MET receptor on epithelial cells, promoting its dimerization and auto-phosphorylation of tyrosine residues. The recruitment of adaptor proteins generates different downstream signaling pathways which evoke diverse cellular responses as shown.

Key: C-Cbl: Casitas B-lineage Lymphoma; Erk1-2: extracellular-signal-regulated kinases; FAK: focal adhesion kinase; GAB1: Grb2-associated binding protein 1; Grb2: growth factor receptor bound protein 2; Gsk3β: glycogen synthetase kinase 3β; IKK: inhibitor of nuclear factor kappa-B kinase; MEK, mitogen-activated protein kinase/ERK kinase; mTOR: mammalian target of rapamycin; NF-kβ: nuclear factor kappa-B;; PI3K: phosphatidylinositol 3' kinase; PLCγ: phospholipase C-γ;Stat3: signal transducer and activator of transcription 3. Adapted from (497).

Reproduced from *Fajardo-Puerta et al., et al Gene of the month: HGF Journal of Clinical Pathology 2016;69:575-579.*

From the practical and clinical point of view, the one-to-one ligand-receptor relationship between HGF and c-MET receptor, makes the HGF/MET pathway a very interesting target for drug development, either by activation or inhibition of the axis. Currently, three pharmacologic inhibitors of HGF-MET axis are being developed with promising results: anti-HGF antibodies, anti-MET antibodies and MET kinase inhibitors (525).

3.3 HGF and liver regeneration

As mentioned earlier, the liver has the capacity to restore to almost its optimal volume after liver resection (12). HGF is one of the first circulating factors found to promote liver regeneration (145, 146). Along with the ligands to the EGF receptor, HGF has been described as a complete mitogen for hepatocytes by inducing DNA synthesis in hepatocytes in vitro and liver enlargement when administered in vivo (50). Activation of the HGF/MET axis generates a cascade of intracellular signaling for the G1-S progression of hepatocytes. Numerous research works have demonstrated its crucial role in liver regeneration after different forms of liver injury, such as partial hepatectomy, ischaemia or hepatitis. Immediately after the liver injury, there is an increased activity of proteinases and intense intracellular signaling provoking an intense remodeling of the extracellular matrix (526). The levels of active HGF rise rapidly in the liver due to its increased production by Kupffer, stellate and sinusoidal endothelial cells, and subsequently activation by urokinases (11, 151, 527). In the scenario of partial hepatectomy, HGF has been observed to be utilized in the liver in a biphasic manner. Firstly, a rapid

increase of peripheral blood levels of HGF is observed 1 h after hepatectomy (151). As a consequence, a first wave of MET activation occurs within 30 minutes and peaks at 60 minutes (152). In a similar manner, there is a biphasic increase of its activated receptor by tyrosine phosphorylation only 1 to 5 and 60 mins in PH rats (152). Within the first three hours after hepatectomy, the endogenous reservoir of HGF in the extracellular matrix, especially important around the portal triads (153, 154), becomes depleted probably secondary to the increased urokinase activity which degrades ECM (155). After these three hours and with a peak at 24 hours post hepatectomy (155, 156), an endogenous synthesis of HGF is undertaken in the liver by stellate cells and endothelial cells (157, 158). Production of HGF from other intact organs (e.g. spleen, lung, kidney) (159, 160) or platelets (161) have been described but its relevance is not known. It has been suggested that the increase of *HGF* mRNA transcription from different organs after partial hepatectomy may be a response of increases in circulating norepinephrine (162) and insulin-like growth factor (163). Overall, this results in a well-adjusted liver restoration and growth.

Available evidence from different experimental studies has revealed the relevance of c-MET receptor in liver regeneration. It has been observed that c-MET impairment cannot be compensated by other factors. Delayed regeneration with defective exit of hepatocytes of quiescence G1 phase and decreased S phase entry severe liver necrosis and death within 48 hours was observed in partial hepatectomies of mice with knockout or mutant *c-MET* (164, 165). Besides, injection of short harpin RNAs against *HGF* and *c-MET* in

rats inhibits mitosis and increases apoptosis within 24 hours post hepatectomy (166).

3.4 HGF/MET axis in cancer

HGF/MET axis has been described to play a crucial role in some cancers such as hepatocellular carcinoma (HCC), oesophagogastric or colorectal cancers. Due to the already mentioned proximity between *HGF* and *MET* on chromosome 7, the polysomy of this chromosome has been suggested to lead to malignancy due to an over production of both molecules (528). In general, over-expression, mutations, amplification of the MET receptor and/or changes in its kinase activity have been linked to different types of cancer and poor outcomes (529).

Colorectal cancer (CRC) is the 2nd and 3rd most common cancer worldwide in female and male, respectively. Around one third of the patients even after curative surgery will develop distant metastasis (530). In this scenario, by regulating the expression of cadherins and extracellular membrane proteases, the HGF/MET axis has been suggested to contribute in the metastatic progression and invasiveness of the tumoral cells (531). Besides, MET amplification seems to be a late event in CRC progression being more common in advanced tumour stages (532) and with higher expression in metastatic than in primary tumour tissue (533). Cetuximab and other agents targeting the epidermal growth factor receptor (EGFR), have been used in patients with unresectable metastatic CRC with a 10-20% response rate (534, 535). A

mechanism described for this resistance is the activation of the HGF/MET axis. It has been demonstrated that a dual activation of both EGFR and MET receptors in CRC cells increases cell proliferation synergistically (536). Cetuximab could inhibit this cell growth by 60-80%. However, adding HGF to cetuximab-treated cells phosphorylated MET, but not EGFR, reestablishing cell proliferation. As a consequence, a blockade of the HGF/MET axis may therefore increase response to EGFR inhibitors in CRC, and dual target therapy should be examined further (536). A recent study has suggested that serum HGF and epiregulin levels may be associated with resistance to anti-EGFR treatment. This study showed that high levels of serum HGF and epiregulin before treatment with anti-EGFR antibodies were associated with poor survival in KRAS wild-type patients with metastatic CRC. Therefore, HGF might be a potential biomarker to predict response and prognosis factor in combination therapy with anti-EGFR antibodies like cetuximab and HGF/MET inhibitors (537).

Hepatocellular carcinoma (HCC) is the 3rd most common cause of cancer-related mortality worldwide with a multifactorial aetiology and extensive molecular and phenotypic heterogeneity (538). Among other signaling pathways, the HGF/MET axis may play a crucial role during the carcinogenesis process (539-541). In comparison to healthy controls, patients who suffer from HCC have a significantly higher serum concentration of HGF (526, 542). Furthermore, over-expression of *HGF* and c-*MET* has been detected in 33% and 20-48% of HCC tissues, respectively (543-547). Poor prognosis and aggressive phenotype have been described when up-regulation of *MET* is

present in patients with HCC. This upregulation has been associated with tumour migration, vascular invasion, neo-angiogenesis and, therefore, poor survival (548). Although disbalance of the HGF/MRET axis seems to impact on survival in HCC patients, other studies have shown contradictory results (549-554). Nonetheless, targeted therapies with pharmacological inhibitors of the HGF/MET axis are being applied in clinical trials in HCC and other types of malignancies. These inhibitors could be a promising second line treatment for patients with advanced HCC (541, 555, 556). A recent study has demonstrated that the interaction of mixed-lineage leukaemia (MLL) protein with the HGF/MET axis, promotes cell invasion and metastasis in HCC. Theoretically, the inhibition of this interaction could potentially decrease the incidence of distant metastasis but not its proliferative capacity (557).

Oesophago-gastric cancer (OGC) is the 5th most common malignancy worldwide. Interestingly, a clear geographical difference has been observed in overall survival with 70% and 25% surviving 5 years in Japan and Europe, respectively. This suggests the need of screening for early detection and treatment. Different alterations of oncogenes and pathways have been linked to OGC. Among them, tumorigenesis and metastasis in gastric cancer has been described secondary to *MET* over-expression and/or amplification. These alterations have been observed in 75–90% and 1.5–20% of the cases, respectively (558-560). However, mutations of *HGF* or *c-MET* are extremely rare in OGC (561, 562). Trials with several drugs targeting the axis include tyrosine kinase inhibitors (crizotinib, a dual c-MET and ALK inhibitor), and

monoclonal antibodies that neutralize HGF (Rilotumumab) or c-MET (Onartuzumab)(563-567).

CHAPTER 4. CIRCULATING ANALYTES INFLUENCING LIVER REGENERATION

4.1 Background

To elicit an effective regeneration after liver injury, precise communication between the liver cell population is guaranteed by multiple signals among which growth factors and cytokines are included. After being secreted by the cells, these molecules bind their target cells via a specific cell-surface receptor inducing changes in function, synthesis of additional cell products or even prompting proliferation or apoptosis. Many of these signals rise in blood circulation at different time points during the liver regeneration process either due to aid the regeneration *per se* or to maintain the body needs and its homeostasis (16).

From one side, growth factors are polypeptides involved in diverse activities such as promotion of cell survival, locomotion, contractility, differentiation or angiogenesis. By binding receptors of restricted or multiple target cells, these factors stimulate the transcription of genes that may be silent in quiescent cells. Some of these factors are involved in tissue regeneration and repair.

From the other side, cytokines are soluble proteins or glycoproteins produced by a large spectrum of cells which can have a wide variety of different biologic activities depending on the target cell to which it binds. Majority are secreted, but they can also be expressed on the cell membrane or

held in reservoirs in the extracellular matrix. Intracellular signal cascades and pathways are activated when they bind to specific receptors on the target cell surface. Certain key features are shared among these molecular signaling: most cytokines have more than one action (pleiotropy); their biological effects can also be observed in another cytokines (redundancy); majority act in a nanomolar to femtomolar range (potency) and most are part of a cascade of cytokines released in order acting in synergy and inhibited by other cytokines or soluble receptors (part of network or cascade).

Originally, cytokines were not labelled the same as growth factors as couldn't activate pathways associated to mitogenesis. Nowadays, cytokines are stablished as important mediators of inflammation and immune response and some of them can be considered as growth factors as they can have growth-promoting activities and participate in wound healing or regeneration such as TNF and IL6 in liver regeneration.

In the context of liver regeneration, promoting and proliferating factors stimulate quiescent hepatocytes into mitosis during the initiation and proliferation stages. Subsequently, anti-proliferation factors will stop cell proliferation when the remnant liver reaches a suitable volume and function ensuring a safe and stable liver regeneration (208).

Depending on the mode of action, these signals have been categorized in complete mitogens and auxiliary mitogens. Complete mitogens are those who have direct hepatotrophic effect causing hepatocyte DNA synthesis in vitro and liver enlargement in vivo. These include hepatic growth factor (HGF) and epidermal growth factor receptor (EGFR) ligands: EGF, TGFα, amphiregulin and

HB-EGF. The hepatotrophic effect is mainly through the Ras-MAPK signaling and PI3K/AKT signaling pathway by binding to the corresponding receptors, c-MET and EGFR. Auxiliary mitogens include very diverse molecules such as bile acids, norepinephrine, endothelial growth factor, insulin-like growth factors, estrogen and serotonin. Although not mitogenic *per se*, its lack may delay liver regeneration. The combination of mitogenic and auxiliary mitogens factors induce the entry of the hepatocytes into the cell cycle triggering its proliferation, production of further growth signals and a stimulation of growth signals production by other cells. In order to avoid failure of the regenerative process, there is not a single signaling pathway whose complete elimination had resulted with complete failure of liver regeneration (16).

External signaling molecules involved in liver regeneration after 2/3 partial hepatectomy include HGF, EGF, FGF, IL6, TNFα, TGFα, VEGF, TGFβ. This combination of mitogenic growth factors and auxiliary mitogens converge upon hepatocytes to induce their entry into the cell cycle, its proliferation and further production by other cells of growth signals directed back to hepatocytes (16).

The knowledge about circulating analytes influencing liver regeneration after partial hepatectomy in human remains limited and it has mainly been extrapolated from animal models (11, 46). Only a few studies have looked into the serial changes of these biomarkers in the context of partial hepatectomy for resection of liver tumours or living-donor liver transplantation in humans (568-579). Additionally, there is even more limited research focused on the modifications of growth factors and cytokines in plasma during liver

regeneration induced by other techniques such as portal vein occlusion or the more novel technique ALPPS and its variants (580, 581).

4.2 Aims

To analyse the changes in different time points of peripheral plasma of relevant liver regeneration analytes (HGF, EGF, FGF, IL6, TNFα, TGFα, VEGF, TGFβ), before and after induction of liver regeneration in patients with inoperable metastatic liver disease with two different techniques, portal vein embolization (PVE) and radiofrequency assisted liver partition with portal vein ligation (RALPPS).

4.3 Results

4.3.1 Samples, multiplex assay results and groups for comparison

A total of 23 patients with metastatic liver disease who needed preoperative induction of liver regeneration for an insufficient FLR in the Department of Surgery and Cancer at Hammersmith Hospital between November 2015 and June 2019 were included in this experiment. Ten patients underwent RALPPS procedure and 13 patients had PVE.

Peripheral plasma samples were taken when possible before inducing liver regeneration (pre stage one; RALPPS n= 9; PVE n= 11), within 48 hours after stage one (post <48 h stage one; RALPPS= 6; PVE n= 5) and 2 hours before second staged hepatectomy (pre stage two; RALPPS n= 6; PVE n= 9) and kept at ≤-80°C (Tables 4.1 and 4.2):

- Pre stage one in RALPPS patients (n= 9).
- Post <48 h stage one in RALPPS patients (n= 6).
- Pre stage two in RALPPS (n= 6).
- Pre stage one in PVE patients (n= 11).
- Post <48 h stage one in PVE patients <48h (n= 5).
- Pre stage two in PVE (n= 9).

	Pre stage one	**Post stage one (<48h)**	**Pre second stage**
RALPPS	9	6	6
PVE	11	5	9

Table 4.1. Number of samples taken from RALPPS and PVE at different time points: before stage one, <48 h after stage one and before stage two.

	Pre stage one	D1 post stage one	D2 post stage one	Pre second stage
1	1			
2	1			1
3	1			
4	1			1
5	1			
6	1			1
7				1
8	1	1	1	1
9	1	1	1	1
10	1	1	1	
11				1
12	1			1
13	1			
14	1			1
15	1			1
16				1
17	1			1
18	1			
19	1			1
20	1	1		1
21	1	1		1
22	1	1		1
23	1	1	1	

Table 4.2. Distribution of samples taken from RALPPS (n= 10) and PVE(n= 13) patients at different time points: before stage one, day one after stage one (D1), day two post stage one (D2) and before stage two.

The plasma samples were analysed in duplicates with the Multiplex assay kit (EMD Millipore, MA, USA) as per manufacturer's instructions, and results were provided by the MAGPIX® software in pg/ml. Biomarkers concentrations at the different time points (pre stage one, post stage one and pre stage two) are expressed in Tables 4.3 and 4.4 for RALPPS and PVE, respectively.

RALPPS	HGF	EGF	FGF	TGFα	IL6	TNFα	VEGF	TGFβ
PRE S1	79.79	22.97	116.50	3.94	68.07	15.99	130.64	68.22
PRE S1	59.44	0	0	0	0	10.80	0	44.07
PRE S1	96.49	0	0	0	0	10.85	0	69.36
PRE S1	35.19	21.69	236.84	0	0	43.41	0	175.72
PRE S1	73.03	19.29	120.42	3.46	5.28	13.36	94.67	245.95
PRE S1	83.19	0	0	0	0	10.29	0	85.19
PRE S1	79.00	0	47.95	0	0	11.19	0	212.78
PRE S1	46.71	6.32	54.99	0	0	9.48	0	312.35
PRE S1	86.99	47.95	58.25	0	10.18	13.79	0	408.47
<48H POST S1	148.25	0	39.99	0	75.41	11.76	0	205.28
<48H POST S1	142.36	0	68.69	0	132.75	17.13	0	263.81
<48H POST S1	1132.50	13.35	47.95	0	0	8.67	0	310.61
<48H POST S1	74.99	14.23	49.78	0	5.28	10.21	0	166.26
<48H POST S1	79.00	39.99	51.56	0	22.41	15.50	0	370.77
<48H POST S1	91.97	68.69	72.79	0	35.84	14.37	0	419.26
PRE S2	73.03	0	0	0	0	7.13	0	108.66
PRE S2	13.88	11.35	142.68	0	0	28.02	15.06	153.54
PRE S2	105.84	0	35.63	0	0	11.82	10.89	209.36
PRE S2	206.74	3.66	108.59	0	0	20.34	39.17	443.21
PRE S2	81.00	0	51.56	0	0	11.76	0	200.13
PRE 2S	48.75	12.39	78.01	0	0	9.97	0	139.58

Table 4.3. Values of peripheral circulating biomarkers expressed in pg/ml before stage one (pre S1), in less than 48 h after stage one (<48 h post S1) and before stage two (pre S2) in RALPPS patients.

PVE	HGF	EGF	FGF	TGFα	IL6	TNFα	VEGF	TGFβ
PRE S1	119.21	0	0	0	1.60	23.94	0	133.68
PRE S1	198.67	0	22.15	0	0	15.94	8.59	128.67
PRE S1	233.86	0	0	0	0	13.49	0	80.43
PRE S1	194.39	129.23	935.39	29.23	173.42	223.98	598.07	282.97
PRE S1	116.65	3.94	122.62	0	0	17.01	93.73	511.78
PRE S1	245.97	0	0	0	76.65	2.24	0	312.04
PRE S1	127.89	0	102.82	0	0	17.00	0	108.95
PRE S1	120.70	13.36	35.49	0	58.67	18.84	0	445.20
PRE S1	60.92	21.45	109.86	0	0	27.53	58.24	245.00
PRE S1	90.98	0	0	0	0	8.18	0	222.42
PRE S1	66.96	59.24	257.39	0	0	15.99	327.69	200.13
<48H POST S1	115.77	0	17.65	6.69	77.86	16.81	0	283.37
<48H POST S1	160.01	6.32	39.99	5.80	71.44	15.67	11.05	577.33
<48H POST S1	120.70	22.94	130.70	0	11.08	50.59	80.85	707.05
<48H POST S1	88.99	44.46	231.30	0	0	17.78	308.08	205.28
<48H POST S1	84.99	0	0	0	0	11.27	0	257.68
PRE S2	204.61	0	0	0	32.36	15.59	76.81	523.21
PRE S2	168.87	0	0	0	0	13.95	0	78.43
PRE S2	719.29	49.53	350.87	12.43	10771.51	112898.3	208.07	142.58
PRE S2	188.60	0	0	0	0	14.44	0	103.07
PRE S2	66.24	0	41.08	0	0	11.41	15.06	76.61
PRE S2	204.29	0	103.02	0	0	10.25	0	130.31
PRE S2	77.00	16.54	89.98	0	0	27.05	50.07	978.84
PRE S2	64.95	44.29	102.89	0	0	12.74	182.75	231.69
PRE S2	68.98	0	0	0	0	7.85	0	178.21

Table 4.4. Values of peripheral circulating biomarkers expressed in pg/ml before stage one (pre S1), in less than 48h after stage one (<48 h post S1) and before stage two (pre S2) in PVE patients.

Liver regeneration analytes were then compared between the following different groups:

- Pre stage one and post stage one in RALPPS patients.
- Pre stage one and post stage one in PVE patients.
- Pre stage one and pre stage two in RALPPS patients.
- Pre stage one and pre stage two in PVE patients
- Pre stage one in RALPPS *versus* PVE patients.
- Post stage one in RALPPS *versus* PVE patients.
- Pre stage two in RALPPS *versus* PVE patients.

Shapiro-Wilk normality test failed to prove a normal distribution within the values of the quantitative variables. Hence, non-parametric tests were used to analyze the difference of plasma concentrations of the analytes between the aforementioned groups. The statistical non-parametric Mann-Whitney U test was used for comparison of means of the biomarker's concentrations at the different times of RALPPS and PVE patients (Tables 4.5-4.9).

4.3.2 Pre stage one *vs* post stage one in RALPPS and PVE

Comparisons of biomarkers before and after stage one in RALPPS and PVE (Table 4.5) demonstrated only within the RALPPS group an increment after 48 hours of IL6 levels significantly higher within the RALPPS group in comparison with the baseline (p= 0.047).

	RALPPS		*p*-value	PVE		*p*-value
	Pre stage one (n= 9)	**Post stage one (n= 6)**		**Pre stage one (n= 11)**	**Post stage one (n= 5)**	
HGF	71.09 ± 20.02	278.18 ± 419.72	0.052	143.29 ± 64.66	114.09 ± 30.13	0.364
EGF	13.14 ± 16.42	22.71 ± 26.84	0.626	20.67 ± 40.21	14.74 ± 19.08	0.672
FGF	70.55 ± 77.53	55.13 ± 12.79	0.953	144.16 ± 274.24	83.93 ± 93.53	0.774
TGFα	0.82 ± 1.64	0	0.232	2.65 ± 8.81	2.46 ± 3.44	0.213
IL6	9.28 ± 22.33	45.28 ± 50.64	0.047	28.21 ± 55.35	32.08 ± 39.19	0.417
TNFα	15.46 ± 10.68	12.94 ± 3.26	0.906	34.92 ± 63.07	22.42 ± 15.94	0.955
VEGF	25.03 ± 50.48	0	0.232	98.76 ± 192.36	79.99 ± 131.88	0.762
TGFβ	180.23 ± 126.17	289.33 ± 96.78	0.126	242.84 ± 138.27	406.14 ± 222.10	0.100

Table 4.5. Concentrations expressed in mean ± SD of peripheral circulating analytes before and after stage one in RALPPS and PVE patients.

4.3.3 Pre stage one *vs* pre stage two in RALPPS and PVE

No statistically significant differences were found when comparing pre stage one and pre stage two levels in both techniques RALPPS and PVE (Table 4.6).

	RALPPS		*p*-value	PVE		*p*-value
	Pre stage one (n= 9)	**Pre stage two (n= 6)**		**Pre stage one (n= 11)**	**Pre stage two (n= 9)**	
HGF	71.09 ± 20.02	88.21 ± 65.92	0.768	143.29 ± 64.66	195.87 ± 205.87	0.970
EGF	13.14 ± 16.42	4.57 ± 5.84	0.385	20.67 ± 40.21	12.26 ± 20.42	0.637
FGF	70.55 ± 77.53	69.41 ± 51.49	0.858	144.16 ± 274.24	76.43 ± 112.65	0.666
TGFα	0.82 ± 1.64	0	0.232	2.65 ± 8.81	1.38 ± 4.14	0.942
IL6	9.28 ± 22.33	0	0.129	28.21 ± 55.35	1200.42 ± 3589.17	0.574
TNFα	15.46 ± 10.68	14.84 ± 7.82	1	34.92 ± 63.07	12556.84 ±37628.05	0.305
VEGF	25.03 ± 50.48	10.85 ± 15.32	0.528	98.76 ± 192.36	59.19 ± 81.99	0.839
TGFβ	180.23 ± 126.17	209.08 ± 120.74	0.637	242.84 ± 138.27	271.44 ± 298.78	0.470

Table 4.6. Concentrations expressed in mean ± SD of peripheral circulating analytes before stage one and before stage two in RALPPS and PVE patients.

4.3.4 Pre stage one in RALPPS *vs* PVE

When comparing pre stage one between RALPPS and PVE, a higher concentration of HGF was observed in the PVE group (p= 0.006) (Table 4.7).

	Pre stage one		*p-value*
	RALPPS (n= 9)	**PVE (n= 11)**	
HGF	71.09 ± 20.02	143.29 ± 64.66	0.006
EGF	13.14 ± 16.42	20.67 ± 40.21	0.715
FGF	70.55 ± 77.53	144.16 ± 274.24	0.969
TGFα	0.82 ± 1.64	2.65 ± 8.81	0.501
IL6	9.28 ± 22.33	28.21 ± 55.35	0.755
TNFα	15.46 ± 10.68	34.92 ± 63.07	0.119
VEGF	25.03 ± 50.48	98.76 ± 192.36	0.349
TGFβ	180.23 ± 126.17	242.84 ± 138.27	0.271

Table 4.7. Concentrations expressed in mean ± SD of peripheral circulating analytes before stage one in RALPPS and PVE patients.

4.3.5 Post stage one in RALPPS *vs* PVE

Comparisons between RALPPS and PVE after 48 hours of inducing liver regeneration revealed no statistically significant differences among all the

cytokines or growth factors except for VEGF. Interestingly, this growth factor was present in the PVE group and not detected in the RALPPS group. The most potent mitogens, HGF and EGF, had higher peripheral plasma concentrations in the RALPPS procedure but this did not achieve statistical significance. Levels of TGFβ, an inhibitor of liver regeneration, were higher in PVE than RALPPS but did not reach statistical significance (Table 4.8).

	Post stage one		***p-value***
	RALPPS (n= 6)	**PVE (n= 5)**	
HGF	278.18 ± 419.72	114.09 ± 30.13	1
EGF	22.71 ± 26.84	14.74 ± 19.08	0.709
FGF	55.13 ± 12.79	83.93 ± 93.53	0.647
TGFα	0	2.46 ± 3.44	0.104
IL6	45.28 ± 50.64	32.08 ± 39.19	0.580
TNFα	12.94 ± 3.26	22.42 ± 15.94	0.100
VEGF	0	79.99 ± 131.88	0.037
TGFβ	289.33 ± 96.78	406.14 ± 222.10	0.522

Table 4.8. Concentrations expressed in mean ± SD of peripheral circulating analytes after stage one in RALPPS and PVE patients.

4.3.6 Pre stage two in RALPPS *vs* PVE

Before the second stage, biomarkers did not show any significant changes within the two different techniques although higher levels of IL6 and TNFα were observed in the PVE group in comparison with the RALPPS group (Table 4.9).

	Pre stage two		***p-value***
	RALPPS (n= 6)	**PVE (n= 9)**	
HGF	88.21 ± 65.92	195.87 ± 205.87	0.346
EGF	4.57 ± 5.84	12.26 ± 20.42	1
FGF	69.41 ± 51.49	76.43 ± 112.65	0.548
TGFα	0	1.38 ± 4.14	0.414
IL6	0	1200.42 ± 3589.17	0.232
TNFα	14.84 ± 7.82	12556.84 ±37628.05	0.556
VEGF	10.85 ± 15.32	59.19 ± 81.99	0.351
TGFβ	209.08 ± 120.74	271.44 ± 298.78	0.637

Table 4.9. Concentrations expressed in mean ± SD of peripheral circulating analytes before stage two in RALPPS and PVE patients.

4.4 Discussion

It is well established that after a partial hepatectomy, rapid orchestrated biochemical changes, including the release of different cytokines and growth factors, occur to promote liver regeneration and maintain the liver functions. Among others, these molecules include HGF, EGF, FGF, IL6, TNFα, TGFα, VEGF and TGFβ.

During the priming phase, macrophages will produce IL6 and TNFα to prepare quiescent hepatocytes to move into mitosis. This progression within the cell cycle will be triggered by the release of the mitogens HGF, EGF and TGFα, and enhanced by other auxiliary mitogens. Proliferating hepatocytes will then secrete many other growth factors such as TGFα, FGFs, TNFα and VEGF targeting non-parenchymal cells. On the contrary, antiproliferative factors like TGFβ will stop cell proliferation once the remnant liver reaches a suitable volume ensuring an adequate liver function and internal homeostasis (16, 208).

The knowledge of serial changes of growth factors and cytokines in peripheral blood after partial hepatectomy in humans is scarce and mainly extrapolated from animal models (46). A few groups have investigated these variations post hepatectomy either after resections of liver tumours or after living liver donations (569, 574-579). Furthermore, there is even more limited literature focused on the serial changes of circulating growth factors and cytokines during induction of liver regeneration by either portal vein occlusion or with the more novel technique ALPPS and its variants (580).

With the advent of the unprecedented rapid regeneration observed at the first stage of the ALPPS procedure and trying to unveil its underlying mechanisms, the group of Clavien *et al.* generated a rat model and hypothesized the presence of circulatory growth factors responsible for a much faster liver regeneration than PVL on its own. These authors established that future work should focus on the identification of these specific molecules, leading to discovery of novel pathways of liver regeneration (32). Only a recent comparison has been made for the assessment in peripheral plasma of growth factors associated with liver regeneration in patients who underwent ALPPS and PVE procedures by Sparrelid *et al.* (580). Furthermore, Dhar *et al.* looked at the expression of multiple cytokines (IL2, IL6, IL13, GM-CSF, VEGF, INFγ, MIP1α, CINC-1) within liver tissue samples during ALPPS, PVL or sham procedures in rats (581).

The present research is a prospective observational study on patients who underwent a preoperative induction of liver regeneration with the gold standard PVE and the more novel ALPPS variant, RALPPS procedure. Peripheral plasma levels of relevant circulating factors for liver regeneration including HGF, EGF, FGF, IL6, TNFα, TGFα, VEGF and TGFβ were analysed at different time points in order to find modifications between two techniques, RALPPS and PVE.

This experiment aimed to find differences in the aforementioned biomarkers before and after liver regeneration with either PVE or RALPPS in a total of 23 patients. Within the RALPPS group, patients' samples were analysed at pre stage one (n= 9), <48 h post stage one (n= 6) and pre second stage (n=

6). A slightly higher number of samples was used for the PVE groups: pre stage one (n= 11), <48 h post stage one (n=5) and pre second stage (n= 9).

As mentioned earlier, the pro-inflammatory cytokine IL6 produced by activated macrophages primes hepatocytes to enter in the cell cycle and stimulates the production of HGF in the early stages of liver regeneration along with TNFα (193, 582). Levels of HGF and IL6 in serum have been investigated in patients after partial hepatectomy as early postoperative liver failure markers. Their levels increased postoperatively and correlated with the magnitude of the resection (572). In 1995, Nishizaki *et al.* divided a series 62 patients who underwent a partial hepatectomy in two different groups for comparing: those whose HGF increased postoperatively and peaked on postoperative day 1 or 3 (group 1, 79%) *versus* those were HGF decreased (group 2, 21%). Interestingly, regeneration of the FLR one month after hepatectomy was significantly higher in group 1 than in group 2 ($p< 0.01$). The incidence of postoperative hepatic failure was significantly higher in group 2 (15%) than in group 1 (0%) ($p< 0.05$). They conclude that HGF could be an indicator of liver regeneration and may serve as a predictor factor of PHLF (569).

The small series of Nakashima *et al.* of 4 healthy liver donors who underwent partial hepatectomy compared to 3 patients who underwent hysterectomy revealed higher circulating levels of HGF and TGFβ after hepatectomy. Serum levels of IL6 with IL10 peaked on postoperative day 1 (POD1). Blood samples were obtained before surgery and on POD1, 3, and 7. These authors also looked into the immune response after partial hepatectomy, more specifically lymphocyte subsets. Whereas white blood

cells counts increased, lymphocytes decreased on POD1 and 3 with lymphocyte subsets changes (CD4/CD8) and T-helper cell (Th)1/Th2 ratios still reduced on POD7. Authors suggested that the immune suppression observed after partial hepatectomy includes reductions in CD4(+) helper T cells, particularly Th1 cells. (577).

In line with the observations made by Sparrelid *et al.* where plasma levels of IL6 increased on postoperative day one after ALPPS in comparison to baseline (p= 0.004) (580), similar findings were observed in the RALPPS group in the present research. Concentrations of IL6 were significantly higher post stage one in the RALPPS procedure than preoperatively in the RALPPS group (p=0.047). A raise of IL6 levels were also detected post stage one in the PVE group, although this change did not reach statistical significance (p= 0.417) (Table 4.5). This modification of IL6 was also found post right lobe hepatectomy in healthy liver donors by Sasturkar *et al.* (575).

In a rat model for ALPPS where Yang *et al*. compared cytokine levels in normal and cirrhotic livers, serum levels of IL6 and TNFα were higher in the cirrhotic group the first and second day after surgery than in the normal group (33).

Unfortunately, in this research no significant differences were found in the comparisons of the peripheral plasma concentration levels before stage one and before stage two in both groups PVE and RALPPS (Table 4.6). This lack of statistic difference could be explained for several reasons. Firstly, a small sample size of patients could have been not enough to reveal subtle differences of concentrations in the peripheral plasma. Secondly, peripheral

blood levels may not represent a more accurate intrahepatic or portal blood levels. In 1999, Higaki *et al.* showed in 17 patients that HGF levels were higher in portal than peripheral serum after partial hepatectomy in humans throughout the study (570). Thirdly, and likely the most plausible explanation, could be that the process of liver regeneration with its associated modifications in plasma levels occurred between the two time points, returning to similar baseline levels prior second stage.

HGF levels are higher in different liver diseases such as cirrhosis, HCC and hepatitis (571). Unexpectedly, a higher baseline level of HGF in the PVE group than the RALPPS group was detected (p=0.006) (Table 4.14). The nature of this finding is difficult to explain as none of the patients had underlying hepatic disease. Looking into the raw data, all RALPPS patients had HGF concentrations below 100 pg/ml in comparison with the majority of the PVE patients where the minimum and maximum quantities detected were 60.92 pg/ml and 245.97 pg/ml, respectively (Tables 4.3 and 4.4). Whether plasma levels before stage one are different between RALPPS and PVE groups within this mitogenic factor, it is arduous to establish. As a consequence, the differences of HGF levels between pre stage one and other time points (less than 48 hours and pre second stage) could be a bias.

Comparisons within 48 hours after inducing liver regeneration with RALPPS or PVE, revealed no statistically significant differences among all the cytokines or growth factors plasma concentrations, except for VEGF. Interestingly, levels of VEGF, a factor produced by proliferating hepatocytes and involved mainly in neoangiogenesis during later stages of regeneration (16, 47), were higher post

stage one in PVE in comparison with the undetectable levels of VEGF at the same time point in RALPPS (p= 0.037). Sparrelid *et al.* found significantly diminished VEGF levels after both stages of ALPPS to then increase after four weeks post stage two (580). In the present experiment, although without statistically significant difference, VEGF levels dropped after first stage of RALPPS group to then increase before the second stage.

The most potent mitogens, HGF and EGF, had higher peripheral plasma concentrations after stage one of RALPPS procedure in comparison with the PVE group, but this did not reach statistical significance. Regarding HGF levels, the group of Sparrelid *et al.* detected an increase on day one post ALPPS with a peak before the second operation without statistical significance (580). In contrast, in the RALPPS group, we observed an increase of HGF after stage one with a decline before the second operation. In the PVE group, levels of HGF decreased after occluding the portal vein and increased before the second operation. None of these changes were statistically significant. The other potent mitogen, EGF, decreased on day one post op and was also lower before the second operation in the Sparrelid's ALPPS cohort. In the present research, the PVE group showed a similar trend regarding the plasma EGF levels, but not the RALPPS group as EGF increased after first stage and decreased before second stage.

Of interest, TGFβ, an inhibitor of hepatocyte proliferation, has been reported to be upregulated not only during the late phases of liver regeneration but also during the early regeneration after partial hepatectomy in rat models (262). This singularity is suggested to be a protective mechanism

against unrestrained cellular growth, as well as acting to terminate the regeneration process. In this research, plasma levels of TGFβ increased after induction of liver regeneration with both techniques with higher levels after stage one but without statistically significant differences (Table 4.5 and 4.6). When comparing both techniques, levels of TGFβ were higher in PVE than RALPPS at all time points, but none of these comparisons reached statistical significance $p \leq 0.05$ (Table 4.8).

Prior the second stage, none of the biomarkers showed any significant changes within the two different techniques. High levels of IL6 and TNFα were observed in the PVE group in comparison with the RALPPS group (Table 4.9).

In 2013, Matsumoto *et al.* investigated the postoperative serial changes of serum levels of nine growth factors including HGF and VEGF (before, postoperative day 1,3,5 and 7) and CT volumetry (before and postoperative day 14) in 16 healthy liver donors who underwent a partial hepatectomy. FLR before the operation tended to be inversely correlated with serum HGF on POD1. The ratio of liver volume on POD14 to liver volume on POD0 was significantly correlated with HGF levels on POD1. Furthermore, HGF levels on POD1 seemed to be higher in those with higher liver regeneration (when ratio of liver volume on POD14 to liver volume on POD0 >150%), although this not reached significant statistical difference. VEGF levels increased during liver regeneration with statistical difference on POD7 but no differences were found depending on the volumetric growth rate. The authors suggested that HGF may be associated with the early phase of LR after partial hepatectomy (PH) in humans.

In patients treated with PH or cryosurgery for colorectal liver metastasis, de Jong *et al.* found comparable responses in both groups of HGF, IGF-I, and IL6 with IL6 peak levels at the end of the operation followed by peak levels of HGF and CRP on postoperative day one. Upregulation of acute phase protein production was higher in patients after cryosurgery than in patients after partial hepatectomy (574).

In 2005, Efimova *et al*. looked into the kinetics of postoperative changes in serum levels of HGF, EGF, VEGF, TGFα of 18 healthy donors who underwent a right hepatectomy at 2 hours after resection, and daily for 5 days postoperatively with enzyme-linked immunosorbent assay kits. At all-time points, postoperative HGF was found to be significantly higher than preoperative levels of HGF ($p< 0.01$), with a highest peak within 2 hours after operation and decreasing progressively until day 5. In contrast, no significant differences in EGF, VEGF or TGFα levels were found before and after the operation. ELISA did not detect TGFα levels suggesting very low levels of TGFα post liver resection (576).

In a series of 25 donors who underwent a right hepatectomy for living donor liver transplantation, Sasturkar *et al.* concluded that HGF, IL6, TNFα, TPO (thrombopoietin) are involved in early phase of regeneration and TGFβ and IFN in the termination phase. More specifically, HGF and IL6 levels increased significantly on day 1 post hepatectomy in comparison to postoperative day 42 ($p= 0.001$; $p< 0.001$, respectively). Levels of TGFβ were significantly higher on POD14 and 42 ($p= 0.008$) (575).

In summary, comparisons of plasma concentrations of relevant biomarkers for liver regeneration at three different time points (pre stage one, <48 h after stage one and before stage two) in RALPPS and PVE patients revealed the following findings.

From one hand, no significant statistical differences were identified within the PVE group between the three different time points. On the other hand, within the RALPPS group, higher levels of IL6 were found to be significantly higher <48h after stage one (p= 0.047) and the rest of the analised biomarkers did not reach statistically significant differences.

When comparing the concentration levels of these biomarkers between the two techniques, RALPPS and PVE, only two events revealed statistically significant differences ($p\leq$ 0.05) in the three time points. Firstly, HGF was found to have a much higher baseline level in the PVE group than in RALPPS before inducing liver regeneration (p= 0.006). This is of unknown significance. Secondly, VEGF, levels were undetectable in the RALPPS group after 48 hours in comparison to the PVE group (p= 0.037), which is a similar finding than the ALPPS group studied by Sparrelid *et al.* (580).

Further research with a larger sample size and extra time points such as 6h and 12 hours post stage one after inducing liver regeneration with PVE and RALPPS techniques may reveal possible differences in the presence in plasma of the aforementioned biomarkers between these two liver regeneration techniques.

CHAPTER 5. GENETIC PROFILING OF LIVER REGENERATION

5.1 Background

From maintaining its homeostasis to unveiling the pathobiology of liver diseases, the fascinating process of liver regeneration has become a great focus of interest. Due to the recent description of the three billion bases sequence that compile the human genome and the development of new molecular techniques such as next generation sequencing (NGS), there is an increasing interest in deciphering at a genetic level the multiple biological processes that the liver undertakes, including liver regeneration.

The concept of "transcriptome" can be defined as the entire mRNA transcript pool within a cell, organ or tissue (583). Understanding the expression and regulation of transcriptomes in a specific context is crucial to comprehend human biology and disease.

Along the years, diverse methods of transcriptome analysis have been used. From the most commonly used, the gene microarray which requires a pre-existing knowledge of the gene sequence, to the open methods were novel sequences can be recognized. Amongst the latter, both Sanger sequencing and NGS have the most sensitive read-out (584).

The transcriptome of the liver has been described as one of the most complex among all the organs. The liver is *per se* a quiescent organ with no basal hepatocyte proliferation and it is populated by a great variety of different cells. In response to a hepatic injury, the regeneration process is

triggered and diverse cellular processes, such as acute-phase response, inflammation, cell division, apoptosis, angiogenesis, cell senescence and cell adhesion take place in an orderly manner to maintain its homeostasis and restore its multiple functions. This complex mechanism is possible with a combination and interaction of growth factors, cytokines, hormones and transcription factors between a heterogeneous cell population not only within but also outside the liver (584).

Genetic profiling of liver regeneration has been investigated mainly in animal models after partial hepatectomy, and less frequently, in human (585).

In animals, the most frequently used model for the study of liver regeneration is the 70% partial hepatectomy (PH) described in rats by Higgins in 1931 (4). In this setting, genetic profiling has been performed not only on a tissue-level but on individual cell types, either on isolated hepatocytes or non-parenchymal cells. Research has pointed out that despite the heterogeneous cell population of the liver, many patterns of expression after partial hepatectomy are similar in hepatocytes and non-parenchymal cells (586). Comparisons at a genetic level of the regenerating liver tissue and isolated hepatocytes have shown similar changes of biological activities, but with different strengths and timing (585).

One of the pioneering groups researching liver regeneration at a genetic level lead by R Taub, investigated induction patterns of genes including both human and rat liver tissue in 1992. Their work consisted on a simultaneous analysis of a panel of genes in different cell types and clinical settings: after perfusion in a donor liver, hepatic ischaemia (after 2 hours of tying of right

lobe in right hepatectomy for haemangioma), fulminant hepatic failure (child about to be transplanted), and regenerating liver rat tissue after 70% partial hepatectomy (586). These authors defined several patterns of expression of immediate-early, delayed-early and liver-specific genes during 9 days after PH in rats.

Further dynamic analyses of gene expression during liver regeneration at different time points have allowed to describe by other research groups up to six distinct temporal gene expression patterns in rats subjected to two-thirds partial hepatectomy: an activation of the hepatocytes and progression from G0 to G1 during the early phase (2-6 hours after PH) followed by a progression into the cell cycle in the early (8-16 hours after PH), intermediate (16-24 hours after PH), early-late (24-36 hours after PH) and late (36-72 hours after PH) phases and finally, the less understood and investigated terminal phase (more than 72 hours after PH) (587-589). In the terminal phase, differences of gene expression in a 2/3 PH rat model have shown upregulation in five pathways: *PPAR* signaling pathway; lipid metabolism; complement, coagulation and fibrinolytic cascades; extra cellular matrix (ECM) remodeling and xenobiotic biotransformation (590).

Therefore, genetic changes within liver regeneration after hepatectomy in the rodents are well known, but not many investigations have been undertaken to unveil this process in humans. Unfortunately, species differences in liver regeneration exists. For instance, the patterns and sequences of liver regeneration in non-human primate models are significantly different compared with other non-primate species. As an example, a peak of

expression of a marker of liver regeneration, *Ki-67*, appears within hours, 72h and 2-3 weeks in rat, dogs or Rhesus macaques' models respectively, after PH (591). This implies, along with different anatomical aspects (size or lobe architecture) or clinical situation (preoperative chemotherapy with subsequent NASH or CASH or even pre-existing steatosis) between the human and the animal models, some limitations for the translation of the results from these animal models to humans.

The first whole genetic profile of liver regeneration immediately after a partial hepatectomy in humans was achieved in 2007. The sampling of standard donor right lobectomy was taken from the edge of the future liver remnant at the beginning of the resection and five hours later (591). A more specific expression profiling has also been investigated in the setting of liver transplant in humans. Furthermore, differences in adult living donor liver grafts in comparison with deceased donor grafts have been studied.

With the advent of innovative procedures such as ALPPS and its modifications, revealing a much faster induction of liver regeneration than PH *per se*, the underlying mechanisms of this rapid regeneration have become a new focus of interest.

A few groups have investigated in more details the genetic changes in the future liver remnant after ALPPS in animal models (32, 581) or in the clinical settings (394, 592) to improve the understanding of this accelerated liver regeneration and to help in refinement of the procedure for clinical benefits.

During the development of the first animal model of ALPPS in mice in 2014, the group of PA Clavien found an upregulation of the gene expression of the auxiliary mitogens IL6 and TNFα in mouse FLR after first stage ALPPS and compared it to patients who underwent either PVL or ALPPS. By comparing the same gene expression of IL6 and TNFα, and plasma levels of IL6 one hour after first step, in PVL or ALPPS patients, this upregulation of the *IL6-TNFα-STAT3* pathway was significantly higher after ALPPS first step than PVL alone (32). Another study showed the expression of multiple cytokines in liver tissue samples during ALPPS, PVL and sham procedures in rats (581).

In a prospective study of patients who underwent ALPPS procedure, the activation of mTOR pathway was associated with the induction of proliferation observed in hepatocytes of the future liver remnant (394). A recent genetic observational study based in 21 patients with CRLM from the LIGRO RCT who underwent either ALPPS or PVE and had tumour recurrence within one year, looked into the characterization of the biology of the rapid recurrences after performing a mutational analysis of *KRAS, NRAS, BRAF, PIC3CA* and *TP53* genes of the metastatic liver tissue and correlated to early tumour recurrence (593).

Nowadays, there are still many unknowns about the underlying process that allows the liver regeneration and the molecular mechanisms orchestrating this complex scenario.

5.2 Aims

To identify transcriptional dysregulation in liver tissue and colorectal liver metastasis after induction of liver regeneration in RALPPS and PVE patients.

In the current PhD, genetic modifications in liver tissue after PVE and RALPPS are investigated. Samples of macroscopically normal liver tissue during first stage of patients who posteriorly underwent PVE were compared at a genetic level with the regenerated left liver tissue secondary to PVE. Within the RALPPS patients, genetic differences are assessed between macroscopically normal liver parenchyma tissue, taken during the first stage, and liver tissue taken during the second stage from the deportalized right liver lobe and the regenerated left lobe. Moreover, right-sided metastatic colorectal liver tissue samples were taken during first and second stage to look for the influence of the liver regeneration process in the tumour.

5.3 Results

5.3.1 Samples and groups for comparison

In this experiment, liver tissue samples of 18 patients with unresectable liver cancers who underwent either RALPPS (n= 10) or PVE (n= 8) to induce liver regeneration from October 2016 to July 2017 at Hammersmith Hospital (Imperial College London NHS Trust) were included. All patients had colorectal liver metastasis apart from one case diagnosed of ovarian liver metastasis and two with cholangiocarcinoma requiring liver resection for tumour clearance.

Total RNA (RNA) was purified in a total of 69 tissue samples (RALPPS, n= 45; PVE, n= 24). The table below shows the sample distribution amongst the different groups of RALPPS and PVE before and after liver regeneration induction; the site of the liver, right or left; and whether the tissue was macroscopically normal parenchyma or metastatic tissue (Table 5.1).

	ARN	ALN	ART	ALT	BRN	BLN	BRT	BLT
RALPPS	9	9	2	5	7	7	5	1
PVE	3	3	3	2	5	6	1	1

Table 5.1. Total number of tissue samples for total RNA extraction and purification (A= first stage; B=second stage; R=right; L=left; N=normal liver tissue; T=tumoral tissue).

After mRNA quality check, 32 samples from 8 RALPPS patients and 6 PVE patients were sent for NGS (Table 5.2). These mRNA samples were distributed as:

- Normal tissue (pre intervention): n= 9 (6 RALPPS; 3 PVE)
- Left normal post RALPPS (BLN): n= 5
- Left normal post PVE (BLN): n= 4
- Right normal post RALPPS (BRN): n= 5
- Tumoral tissue pre RALPPS (ART or ALT): n= 6
- Tumoral tissue post RALPPS (BRT or BLT): n= 3

	ARN	ALN	ART	ALT	BRN	BLN	BRT	BLT
RALPPS	**0**	**6**	**2**	**4**	**5**	**5**	**3**	**0**
PVE	**0**	**3**	**0**	**0**	**0**	**4**	**0**	**0**

Table 5.2. Total number of RNA samples analysed by NGS (A= first stage; B=second stage; R=right; L=left; N=normal liver tissue; T=tumoral tissue).

The conditions for comparisons were:

- RALPPS group
    - Left normal liver tissue prior RALPPS (ALN n= 6)
    - Tumoral liver tissue (colorectal liver metastasis) prior RALPPS (ART n= 2; ALT n= 4)
    - Right normal liver tissue post RALPPS (BRN n= 5)
    - Left normal liver tissue post RALPPS (BLN n= 5)
    - Right tumoral (colorectal liver metastasis) liver tissue post RALPPS (BRT n= 3)
- PVE group
    - Left normal liver tissue prior PVE (ALN n= 3)
    - Left normal liver tissue post PVE (BLN n= 4)

The feasible groups for comparison after mRNA extraction were:

- Normal liver tissue (n= 9) *versus* colorectal liver metastasis (n= 6)
- In RALPPS procedure
    - Pre Normal liver (ALN; n= 6) *vs* left post regeneration (BLN; n= 5)
    - Pre Normal liver (ALN; n= 6) *vs* right post regeneration (BRN; n= 5)
    - Pre tumoral tissue (ART or ALT; n= 6) *vs* right tumour post regeneration (BRT; n= 3)
- In PVE group
    - Left or Right Pre Normal liver (ALN or ARN; n= 3) *vs* left post regeneration (BLN; n= 4)
- Left post regeneration in PVE (BLN; n= 4) *vs* left post regeneration in RALPPS (BLN; n= 5)

5.3.2 Total mRNA samples unsupervised hierarchical clustering

Unsupervised hierarchical clustering method was used to evaluate the expression profiles and identify similarities and differences between global transcriptional profiling of all tissue samples (Figure 5.1). The distribution of genetic variation across the samples is reflected in the principal component analysis (PCA) (Figure 5.2). In this figure, the PC1 (X axis) reveals a 76% of variance between the samples. This is in keeping with two different types of samples which correspond to normal parenchyma and tumour tissues.

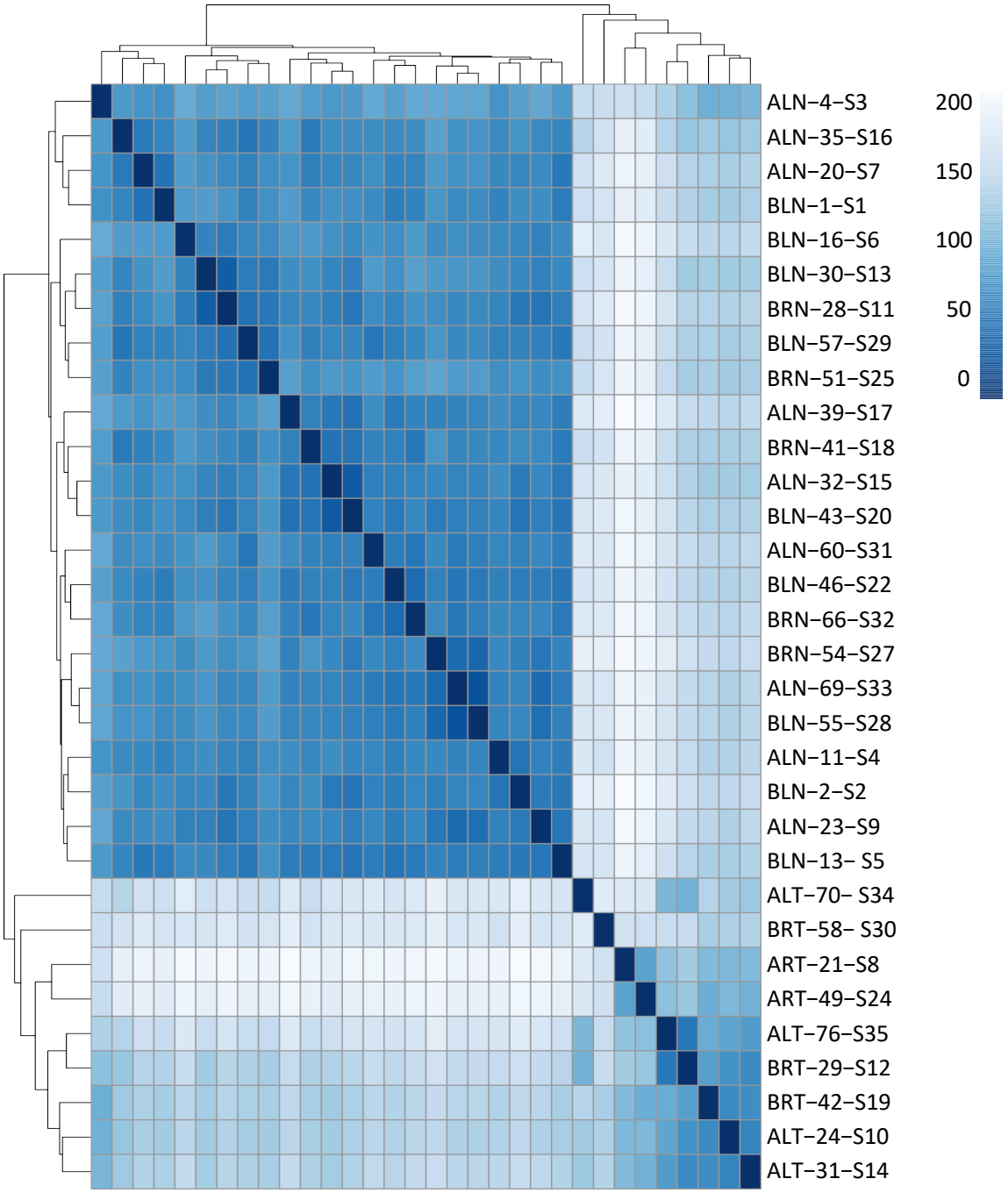

Figure 5.1. Unsupervised hierarchical clustering representation of the total number of samples (n= 32) included for transcriptional profiling by RNAseq. The different groups included were ALN, ARN, ALT, BLN, BRN and BRT.

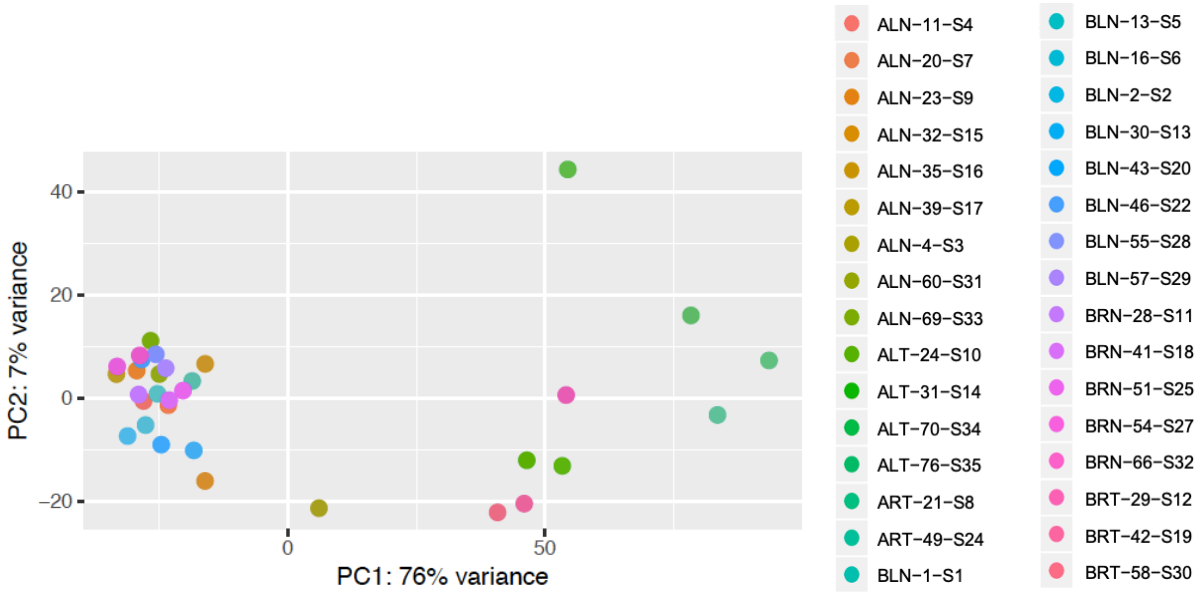

Figure 5.2. Principal component analysis (PCA) of the samples obtained from gene expression data analysis.

5.3.3 Normal liver tissue *versus* colorectal liver metastasis

A total of 9210 genes, both up- and down-regulated, were identified to show significantly differentially expression when normal macroscopic hepatic tissue (n= 9) was compared to colorectal liver metastases (n= 6) (*adjusted p-value*≤ 0.05). When *adjusted p-value*≤ 0.15 was considered, a total of 10801 genes were significantly differentially expressed, increasing the number of deregulated genes.

Unsupervised clustering of these total RNA samples is shown in Figure 5.3. The correspondent volcano plot can be found in Appendix section (Supplementary figure A.1).

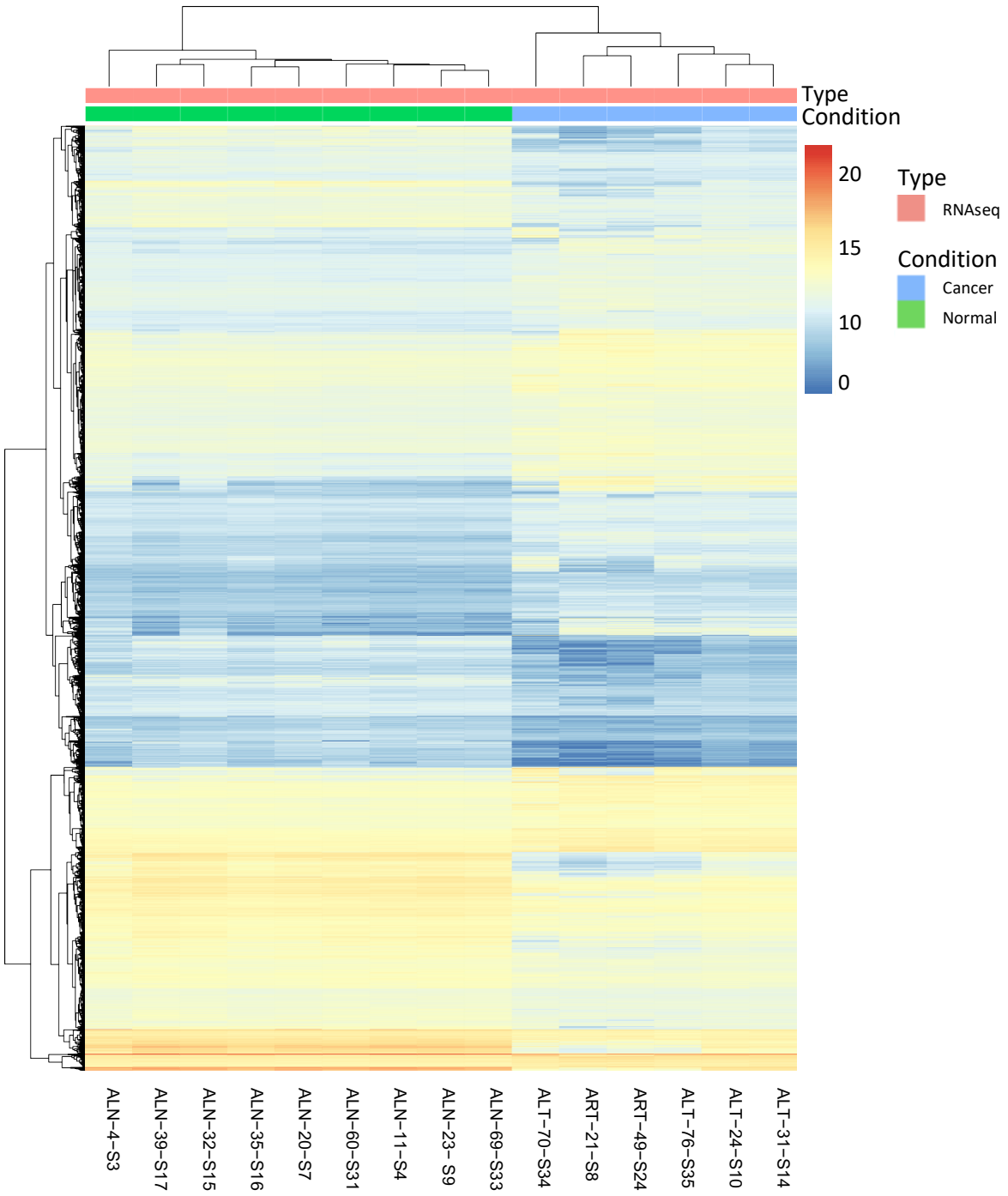

Figure 5.3. Unsupervised clustering of mRNA in normal *vs* colorectal liver metastatic tissue samples (ALN *vs* ALT/ART comparison).

5.3.4 RALPPS group

5.3.4.1 Normal liver *vs* left liver post regeneration

In this comparison, when normal liver tissue (ALN; n= 6) was compared to the regenerating liver tissue after RALPPS procedure (BLN; n= 5), two genes were found to be significantly expressed *RPL23AP2* (*adjusted p-value*= 0.038) and *SNORD3D* (*adjusted p-value*= 0.038). Both genes were found to be down regulated in the regenerated liver tissue after RALPPS in comparison with normal liver tissue (Tables 5.3 and 5.4).

Gene Name	log2FoldChange	*p-adj*
RPL23AP2	4.181	0.038
SNORD3D	1.259	0.038

Table 5.3. Normal liver tissue *vs* left post regeneration liver tissue in RALPPS patients. Two significantly differentially expressed genes were found in this comparison when an adjusted p-value≤ 0.05 was applied.

Gene (Alias)	***Location***	***Description***	***Functions and Associated diseases/conditions (references in the literature)***
RPL23AP2 Ribosomal Protein L23a Pseudogene 2	19p13.12	***Pseudogene***	***Unknown*** ***2 publications (594, 595)*** NGS experiment revealed this gene as a candidate biomarker for three different human cancers (prostate, small-cell lung cancer and lung adenocarcinoma). Suggested among other transcripts as best candidate reference genes to differentiate normal lung and small-cell lung cancer tissues (595).
SNORD3D Small nucleolar RNA C/D box 3D U3-4 RNU3-4 U3 snoRNA D U3 small nucleolar RNA D	17p11.2	***Non-coding RNA***	***Unknown*** ***12 publications (596-607)*** Downregulated in liver tissue of patients with non-alcoholic fatty liver disease (NAFLD) and vitamin D deficiency (596). Upregulated in neonatal human articular cartilage (600); monocytes in response to pneumococci (603); blood of patients with complex regional pain syndrome (607); ACTH-secreting pituitary adenomas (598); after δ-tocotrienol treatment in plasma of chronic hepatitis C patients; hyperthermic response of breast cancer cells (601); after garlic extract treatment in bladder cancer EJ cells (604); after MPP treatment in alpha-synuclein triplication dopaminergic cells of Parkinson's disease (605); after strigolactone treatment in prostate cancer cells (606).

Table 5.4. Location, description, function and references of significantly differentially expressed genes found in normal liver tissue vs left post regeneration liver tissue in RALPPS patients (adjusted p-value≤ 0.05).

The related supervised clustering representing the results for this comparison is shown in Figure 5.4. Its correspondent volcano plot is shown in the Appendix section (Supplementary figure A.2).

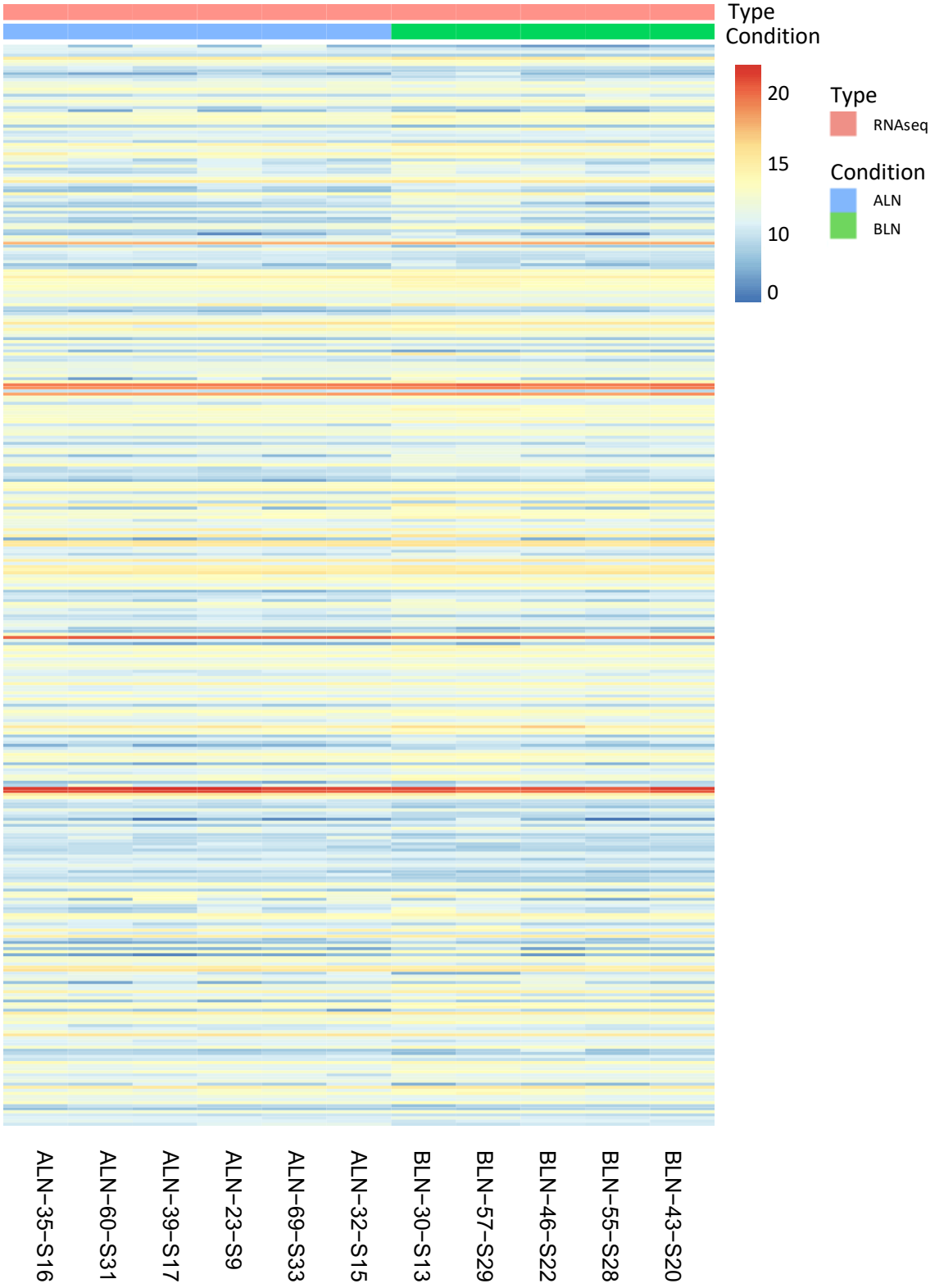

Figure 5.4. Supervised clustering of RALPPS ALN (n= 6) *vs* BLN (n= 5) comparison.

5.3.4.2 Normal liver *vs* right liver post regeneration

No significant statistical difference between the genetic profile of the normal liver parenchyma (ALN; n= 6) *versus* the ligated right-sided liver lobe (BRN; n= 5) after RALPPS procedure was found.

When reducing the level of significance to an *adjusted p-value*≤ 0.15, three genes were found to be differentially expressed within this group: *MT-CO2* (*adjusted p-value*= 0.094); *RNU1-27P* (*adjusted p-value*= 0.124); and *RP11-79H23.3* (*adjusted p-value*= 0.124). Log2FC values revealed that *MT-CO2* was downregulated in the occluded right-sided liver tissue after liver regeneration and the other two genes, *RNU1-27P* and *RP11-79H23.3*, were upregulated in comparison with the normal liver tissue pre-intervention (Tables 5.5 and 5.6).

Gene Name	log2FoldChange	*p-adj*
MT-CO2	0.628	0.094
RNU1-27P	-2.539	0.124
RP11-79H23.3	-1.238	0.124

Table 5.5. Normal liver tissue *vs* right post regeneration liver tissue in RALPPS patients. Three significantly differentially expressed genes were found in this comparison when an *adjusted p-value*≤ 0.15 was applied.

Gene ***(Alias)***	***Location***	***Description***	***Functions and Associated diseases/conditions (references in the literature)***
MT-CO2 Mitochondrially Encoded Cytochrome C Oxidase II COII MTCO2 COX2 PTGS2	Ch1	***Protein coding RNA***	***13 publications (608-623)*** Catalyzes the conversion of arachidonic acid to prostaglandin H2. Promoting cell growth, enhancing angiogenesis, inhibiting cell apoptosis, carcinogenesis and cancer progression. Upregulated in maternally inherited diabetes, hearing loss and mitochondrial myopathy (610-612) and some cancers (colorectal, gall bladder, cervical, thyroid and hepatocellular) (613-616). Poor prognosis in colorectal, breast, nasopharyngeal or tongue cancers (617-620). COX2 inhibition with aspirin or celecoxib may have benefit in the prevention, treatment or survival of some cancers like colorectal carcinoma (608, 613) (621-623).
RNU1-27P RNA, U1 small nuclear 27, pseudogene RNU1-7 RNU1-7P	14q13.1	***Pseudogene***	***Unknown*** ***No publications***
RP11-79H23.3	8q21.13	***Long non-coding RNA***	***Unknown*** ***1 publication (624)*** Down regulated in bladder cancer tissues with potential tumor suppressor role in progression of bladder cancer suggesting role of

			inhibition of cell proliferation, migration, angiogenesis and tumorigenesis (624).

Table 5.6. Location, description, function and references of significantly differentially expressed genes found in normal liver tissue *vs* right post regeneration liver tissue in RALPPS patients (*adjusted p-value*≤ 0.15).

The related supervised clustering showing the results for this comparison is shown in Figure 5.5. Its correspondent volcano plot is shown in the Appendix section (Supplementary figure A.3).

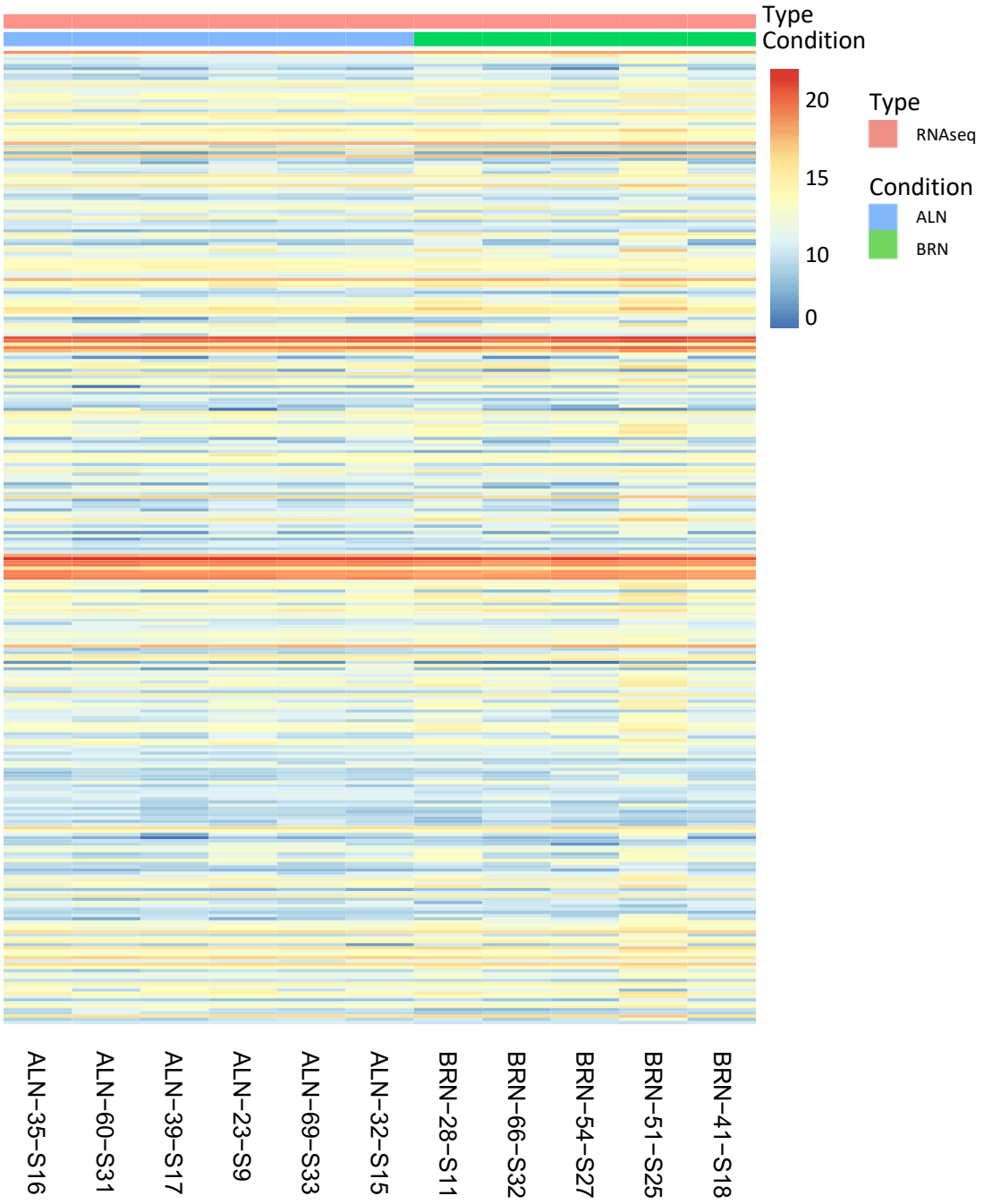

Figure 5.5. Supervised clustering of mRNA RALPPS ALN (n= 6) *vs* BRN (n= 5).

5.3.4.3 Tumoral tissue *vs* right-sided tumour post regeneration

Three genes were found to be significantly expressed in tumoral tissue before (ALT or ART; n= 6) and after the process of regeneration (BRT; n=3) when *adjusted p-value*≤ 0.05. *RPL41P1* (*adjusted p-value*= 1.62E-05) and *CLDN2* (*adjusted p-value*= 8.60E-05) were upregulated whereas *AC159540.1* (*adjusted p-value*= 0.006) was downregulated in the colorectal liver metastasis of the right liver after liver regeneration with RALPPS (Tables 5.7 and 5.8).

When reducing the level of significance *adjusted p-value*≤ 0.15, other eight genes were found to be differentially expressed within this group. Most of the genes appeared to be downregulated after RALPSS procedure (Tables 5.23 and 5.24).

In the following Table 5.7, the eleven deregulated genes for this comparison are shown.

Gene Name	log2FoldChange	*p-adj*
RPL41P1	-4.231	1.62E-05
CLDN2	-4.753	8.60E-05
AC159540.1	3.127	0.006
SBSPON	-3.867	0.064
CTD-3080P12.3	-3.127	0.069
CLEC4G	-3.414	0.099
GSTA2	-2.808	0.099
SLC7A9	-1.815	0.099
CLEC4M	-2.417	0.103
RP11-582J16.4	3.167	0.121
PROM2	-3.170	0.129

Table 5.7. Tumoral tissue post-regeneration (BRT) *vs* tumour pre-regeneration (ART/ALT) post regeneration in RALPPS patients. Eleven significantly differentially expressed genes were found in this comparison when an *adjusted p-value*≤ 0.15 was applied.

Gene (Alias)	***Location***	***Description***	***Functions and Associated diseases/conditions (references in the literature)***
RPL41P1 Ribosomal protein L41 pseudogene 1 RPL41L2 dJ1065O2.1	20p11.22	***Pseudogene affiliated to lncRNA class***	***Unknown*** ***No publications***
CLDN2 Claudin 2	Xq22.3	***Protein coding RNA***	***106 publications*** Intercellular permeability at the tight junctions. Proliferation, migration, invasion and cell survival. Overexpressed in proximal tubules of kidneys and gastrointestinal tract, mainly in the liver, gall bladder, pancreas and small bowel. Dysregulated in inflammatory bowel diseases and some cancers (gastric, colorectal, lung, breast, renal and osteosarcoma). Higher expression in breast liver metastases than in primary cancer cells (625).
AC159540.1	2q11.2	***Long non-coding RNA***	***Unknown*** ***No publications***

Table 5.8. Location, description, function and references of significantly differentially expressed genes found in tumoral tissue post-regeneration (BRT) *vs* tumour pre-regeneration (ART/ALT) post regeneration in RALPPS patients (*adjusted p-value*≤ 0.15).

The related supervised clustering showing the results for this comparison is shown in Figure 5.6. Its correspondent volcano plot is shown in the Appendix section (Supplementary figure A.4).

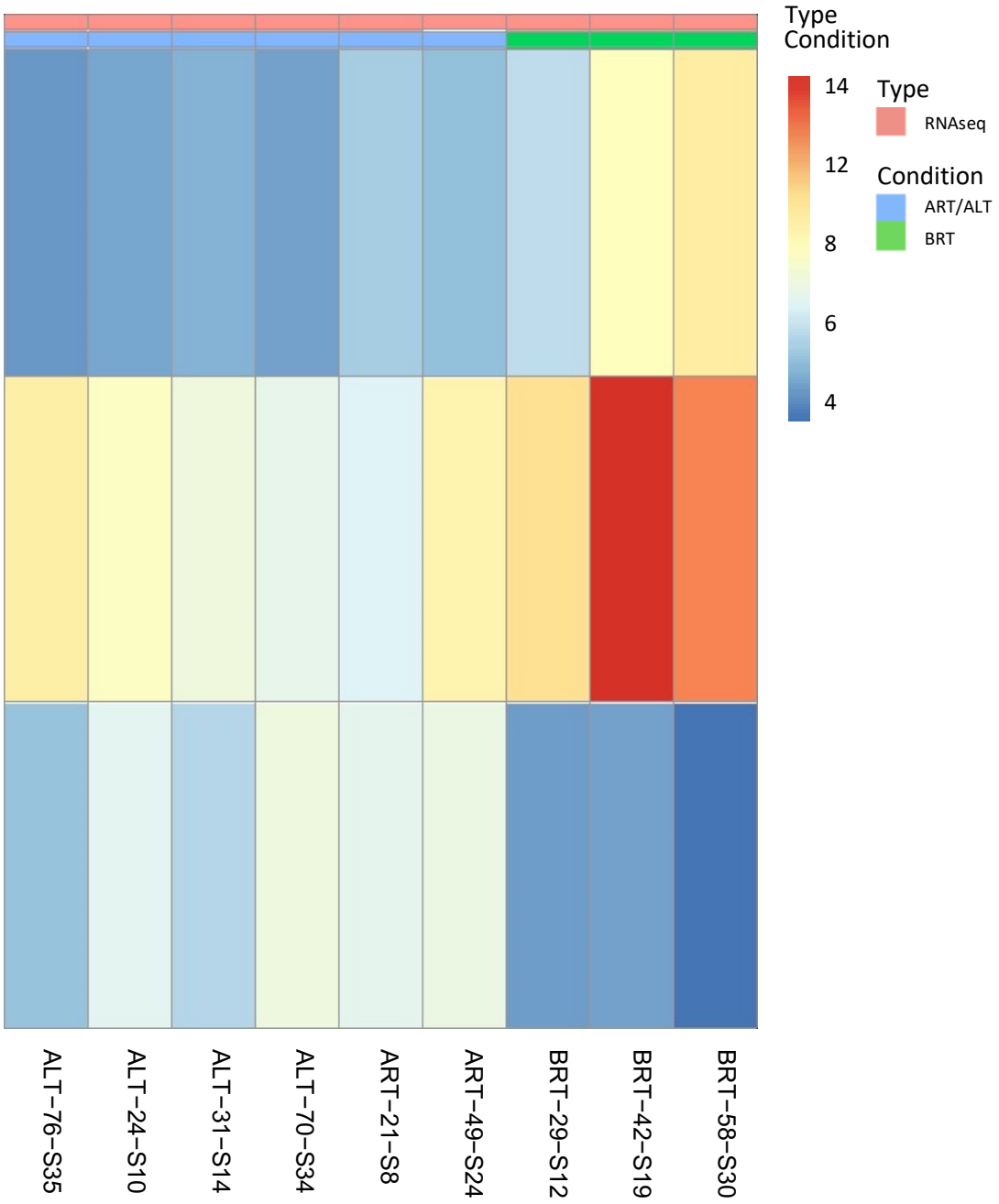

Figure 5.6. Supervised clustering representing the comparison ALT/ART (n= 6) *vs* BRT (n= 3) in RALPPS patients. Three significantly differentially expressed genes were found in this comparison when an *adjusted p-value*≤ 0.05 was applied.

5.3.5 PVE

5.3.5.1 Normal liver *vs* left liver post regeneration

In the comparison of mRNA of 3 macroscopically normal liver parenchyma samples and 4 tissue samples of the left-sided liver lobe after portal vein embolization, 32 genes were found to be statistically significantly expressed (*adjusted p-value*≤ 0.05). Among them, 15 genes were significantly upregulated in the regenerated liver post embolization and the rest were downregulated (Table 5.9). Considering a significance of *adjusted p-value*≤ 0.15, 66 genes were significantly expressed.

The related supervised clustering showing the results for this comparison is shown in Figure 5.7. Its correspondent volcano plot is shown in the Appendix section (Supplementary figure A.5).

Gene Name	log2FoldChange	*p-adj*
KRT5	5.458	2.12E-05
CDH3	3.860	4.87E-05
RP11-278L15.6	-2.283	4.87E-05
LRP2	2.316	0.000
UPK3B	3.443	0.000
NOL4	-2.921	0.000
BNC1	4.082	0.003
HSFX2	6.115	0.003
LRG1	1.674	0.003
MAPK4	2.790	0.004
RP11-64D22.2	-2.087	0.005
EGR1	-2.587	0.007
VIPR2	2.712	0.007
HIST2H2BF	1.680	0.008
DSC3	3.609	0.009
ENO3	-1.497	0.010
FADS2	-2.336	0.011
RARRES1	2.540	0.011
PTPRQ	3.820	0.019
CHAD	-1.863	0.019
SLC12A8	1.206	0.023
SYN1	2.319	0.023
PER2	-1.596	0.023
AMZ1	-3.414	0.026
OR10J6P	-1.466	0.026
PKHD1L1	3.025	0.026
SCD	-1.674	0.026
SKAP1	-1.058	0.026
TCEA2	-1.015	0.026
RP11-538D16.2	-2.495	0.031
AC159540.1	2.365	0.037
RN7SL600P	-2.104	0.048

Table 5.10. Normal tissue pre regeneration ALN (n= 3) *vs* post regeneration process BLN (n= 4) in PVE patients. Thirty-two significantly differentially expressed genes were found in this comparison when an *adjusted p-value*≤ 0.05 was applied.

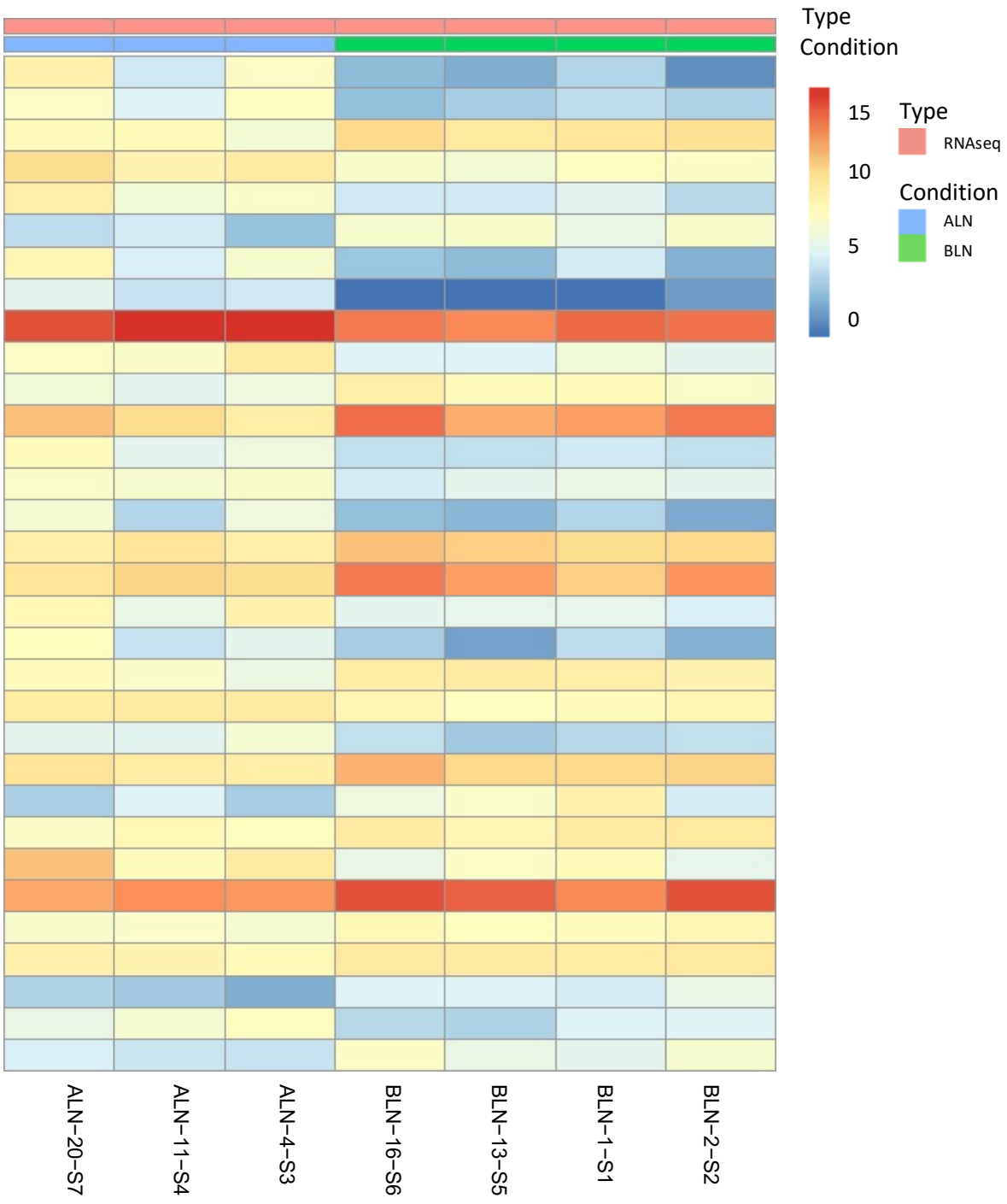

Figure 5.7. Supervised clustering representing the comparison ALN (n= 3) *vs* BLN (n= 4) in PVE patients. Thirty-two significantly differentially expressed genes were found in this comparison when an *adjusted p-value*≤ 0.05 was applied.

5.3.6 PVE *vs* RALPPS post regeneration

When comparing the transcriptional profile of regenerated left-sided liver tissue (PVE, n= 4; RALPPS, n= 5), significant differences in genetic expression after liver regeneration were found between PVE and RALPPS techniques in 14 genes (*adjusted p-value*≤ 0.05). Furthermore, 29 genes were highly expressed when significance was *adjusted p-value*≤ 0.15 (Tables 5.11 and 5.12). A volcano plot of this comparison is shown in the Appendix section (Supplementary figure A.6).

Gene Name	log2FoldChange	*p-adj*
C3P1	0.831	1.17E-07
ACSM1	2.522	0.001
RPL41P1	-3.711	0.001
RP11-108K14.4	1.208	0.002
TACC2	0.569	0.004
HIP1R	0.516	0.009
CCL3	-2.053	0.011
HLA-H	-6.315	0.011
LINC00319	2.179	0.011
RPL12	-0.523	0.015
SSPO	1.175	0.016
TRIM29	1.600	0.018
PLIN5	0.916	0.021
NECAB2	1.356	0.028

Table 5.11. Normal tissue post regeneration in PVE (BLN, n= 4) *vs* normal tissue post regeneration in RALPPS (BLN, n= 5) in PVE patients. Fourteen significantly differentially

expressed genes were found in this comparison when an *adjusted p-value*≤ 0.05 was applied.

Gene (Alias)	***Location***	***Description***	***Functions and Associated diseases/conditions (references in the literature)***
C3P1 Complement component 3 precursor pseudogene	19p13.2	***LncRNA pseudogene***	***Unknown*** ***6 publications*** Downregulated in malignant cholangiocytes (626) and kidney cancer cells (627).
ACSM1 BUCS1 MACS1	16p12.3	***Protein coding RNA*** *Acyl-CoA synthetase medium chain family member 1*	***25 publications* (628-637)** Fatty acid beta-oxidation and cytochrome P450 and valproic acid pathways (630, 631). Deregulated in squizophrenia and major depression (628, 629) and in some tumours like breast (632-635) and prostate cancers (636, 637).
RPL41P1 Ribosomal protein L41 pseudogene 1 RPL41L2 dJ1065O2.1	20p11.22	***Pseudogene***	***Unknown*** ***No publications***
RP11-108K14.4	10q26.3	***Pseudogene***	***Unknown*** ***No publications***
TACC2	10q26.13	***Protein***	***60 publications***

AZU-1 ECTACC		***coding RNA*** *Transforming acidic coiled-coil containing protein 2*	Tumorigenesis. Dispensable for normal development and its deficiency does not lead to cancer (638). Prognostic marker in HCC (639), breast (640), prostate (641) and infant acute lymphoblastic leukaemia (642). Successful smoking cessation (643). Strong association with late onset of Alzheimer disease (644).
HIP1R HIP3 HIP12 ILWEQ	12q24.31	***Protein coding RNA*** *Huntingtin interacting protein 1*	***62 publications*** Mental retardation, expressive language disorder, congenital cataract 8, gastritis and Parkinson disease. Receptor trafficking by facilitating the formation of clathrin-coated vesicles, carriers of proteins or surface receptors such as tyrosine kinases receptors. Low levels of its transcript and low expression of its protein are associated with worse survival in patients treated with RCHOP for diffuse B-cell lymphoma (645).
CCL3 C-C motif chemokine ligand 3 MIP1A SCYA3 G0S19-1 LD78ALPHA MIP-1α	17q12	***Protein coding RNA*** *Macrophage inflammatory protein 1 alpha protein (MIP1A)*	***1365 publications*** Inflammatory response. Both resistance and susceptibility to infection by human immunodeficiency virus type 1. Antitumour or protumour behaviours. Prognostic biomarker in multiple cancers including haematological malignancies. Immune response in sepsis, Toll-like receptor signaling, and senescence and autophagy in cancer pathways (646).
HLA-H	6p22.1	***Protein***	***26 publications***

HLAHP		***coding RNA*** *Human leukocyte antigen class 1*	Immunity. Major histocompatibility complex gene which presents foreign antigens to the immune system.
LINC00319 Long intergenic non-protein coding RNA 319 PRED49 C21orf125 NCRNA00319	21q22.3	***Non-coding RNA***	***10 publications*** Oncogenic. Tumorigenesis, cell proliferation and invasion in lung cancer (647). Carcinogenesis and poor prognosis in nasopharyngeal carcinoma (648). Accelerate tumour growth and metastasis in gastric cancer (649).
RPL12 Large ribosomal subunit protein UL11	9q33.3	***Protein coding RNA*** *Ribosomal protein L12 (part of 60S)*	***154 publications*** Involved in the "rRNA processing in the nucleus and cytosol" and "Viral mRNA translation" pathways. Neuropathy, distal hereditary motor type VIII and Diamond-Blackfan anaemia.
SSPO Subcommissural organ spondin SCO-spondin	7q36.1	***Pseudogene***	***19 publications*** Associated to metabolism of proteins and O-glycosylation of TSR domain-containing proteins pathways. Peptidase inhibitor activity. Cell adhesion, cell differentiation and central nervous system development (650).
TRIM29 ATDC Ataxia-Telangiectasia Group D-Associated	11q23.3	***Protein coding RNA*** *Tripartite Motif Containing 29 protein*	***87 publications*** Regulation of macrophage activation in response to viral or bacterial infections within the respiratory tract. Transcriptional regulatory factor in carcinogenesis and/or differentiation via

Protein			inhibition of p53 nuclear activities (651, 652). Tumourigenesis, tumour progression and poor prognosis in many types of cancers: pancreas (653), prostate (654), *oesophageal* (655), lung (656), breast (657, 658), colorectal (659, 660), gastric (661), thyroid (662), cervical (663), bladder (664), osteosarcoma (665) or nasophagingeal (666).
PLIN5 MLDP LSDA5 LSDP5 OXPAT	19p13.3	***Protein coding RNA*** *Perilipin 5 protein*	***26 publications*** Prevents intracellular lipolytic degradation by coating the storage of lipid droplets. It is involved PPAR signaling pathway. Controversial roles in lipotoxicity and insulin resistance (667-669). Protective for ischaemic heart disease due to its role in stabilization of the cardiac lipid metabolism following ischaemia in mice (670). Upregulated in liver biopsies from non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) patients along with glucose-6-phosphatase gene and down regulation of Notch1 pathway (671).
NECAB2 EFCBP2 stip-2	16q23.3	***Protein coding RNA*** *Neuronal calcium-binding protein 2*	***25 publications*** Binds and modulates the function of the adenosine A2 and metabotropic glutamate type 5 receptors. Investigated in human *versus* rat spinal cord expression (672). Inherited ataxias and disorders of Purkinje cell degeneration (673). Molecular genetics of successful smoking cessation (674).

			Brain genetic factors for autism (675).

Table 5.12. Location, description, function and references of significantly differentially expressed genes found in normal tissue post regeneration in PVE (BLN, n= 4) *vs* normal tissue post regeneration in RALPPS (BLN, n= 5) in PVE patients (*adjusted p-value*≤ 0.05).

LncRNA: Long non-coding RNA. TSR: thrombosponding type 1 repeats.

The related supervised clustering showing the results for this comparison is presented in Figure 5.8. Its correspondent volcano plot is represented n in the Appendix section (Supplementary figure A.6).

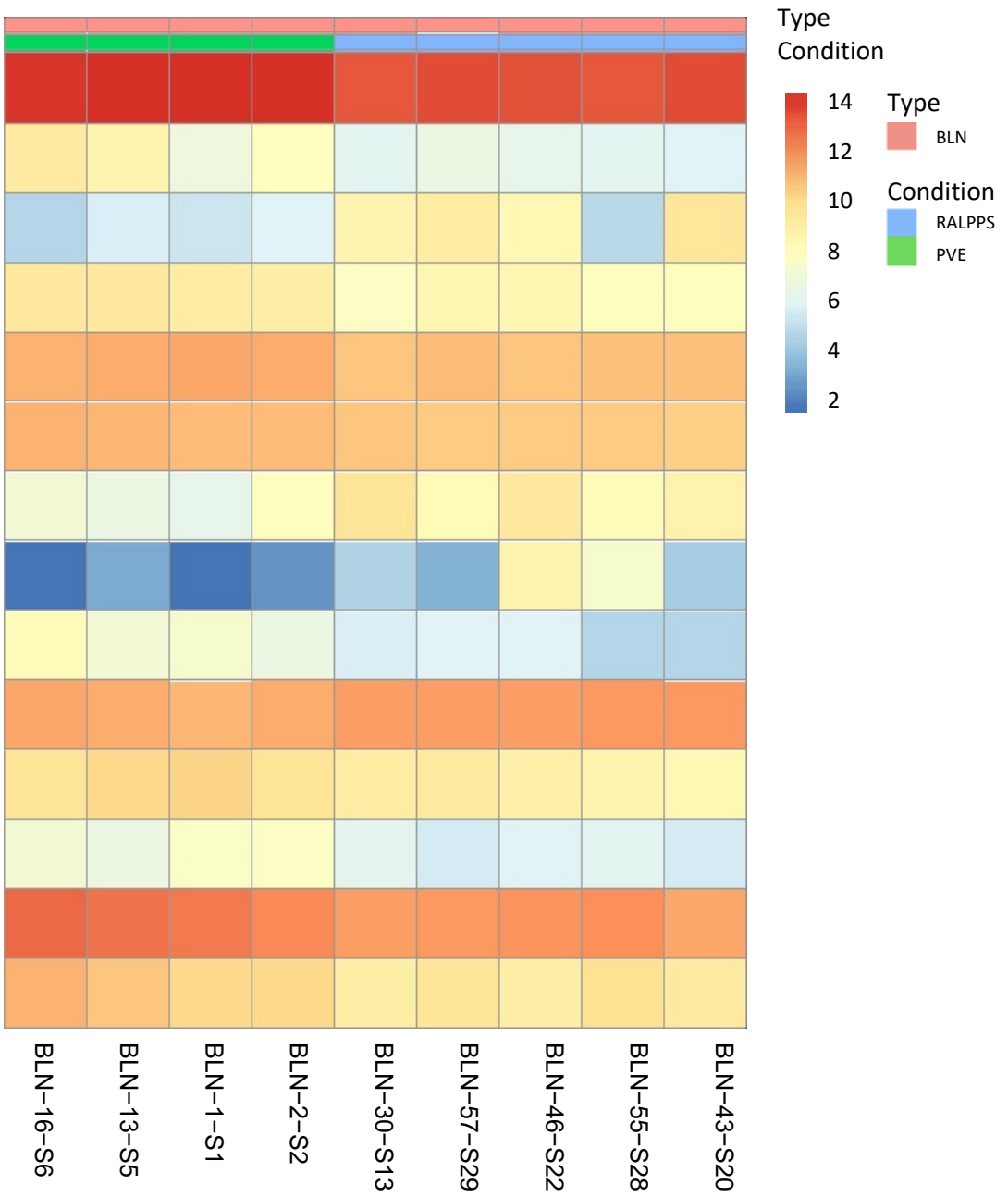

Figure 5.8. Supervised clustering representing the comparison BLN PVE (n= 4) *vs* BLN RALPPS (n= 5) patients. Fourteen significantly differentially expressed genes were found in this comparison when an a*djusted p-value*≤ 0.05 was applied.

5.3.7 Gene expression validation by real time RT-qPCR

To validate the RNA sequencing data, expression levels of selected significantly differentially expressed genes were assessed by RT-qPCR for the two following comparisons:

- ALN *vs* BLN in PVE patients
- BLN PVE *vs* BLN RALPPS patients

5.3.7.1 ALN *vs* BLN in PVE patients

A total of 9 significantly differentially expressed genes were analysed by RT-qPCR in 7 patients' tissue samples, distributed between ALN PVE condition (n= 3) and BLN PVE condition (n= 4) were used for the validation process. Some of the analysed genes were overexpressed in ALN PVE condition (*CDH3, KRT5, MAPK4* and *LRP2*) and some others (*EGR1, ENO3, PER2, FADS2* and *SCD*) were under-expressed, in comparison with BLN PVE condition. Results expressed in Mean and SD of relative units for each sample group (ALN and BLN) in PVE patients are reflected in Table 5.13, Figures 5.9 and 5.10.

	ALN		BLN	
	Mean	**SD**	**Mean**	**SD**
CDH3	5.2	4.4	0.2	0.1
EGR1	129.9	111.2	1001.4	479.1
ENO3	23.4	10.2	77.2	12.6
FADS2	35.3	9.2	207.1	112.1
KRT5	1.55	1.34	0.07	0.11
LRP2	5.4	4.8	1.3	0.8
MAPK4	2.9	1.6	0.8	0.8
PER2	4.1	2.1	12.0	6.6
SCD	176.8	44.3	584.8	187.4

Table 5.13. Results obtained by RT-qPCR for ALN *vs* BLN in PVE patients' comparison. Validation of 9 significantly expressed genes selected from RNA-seq data analysis. The data shows the mean and the standard deviation for each sample group in the comparison.

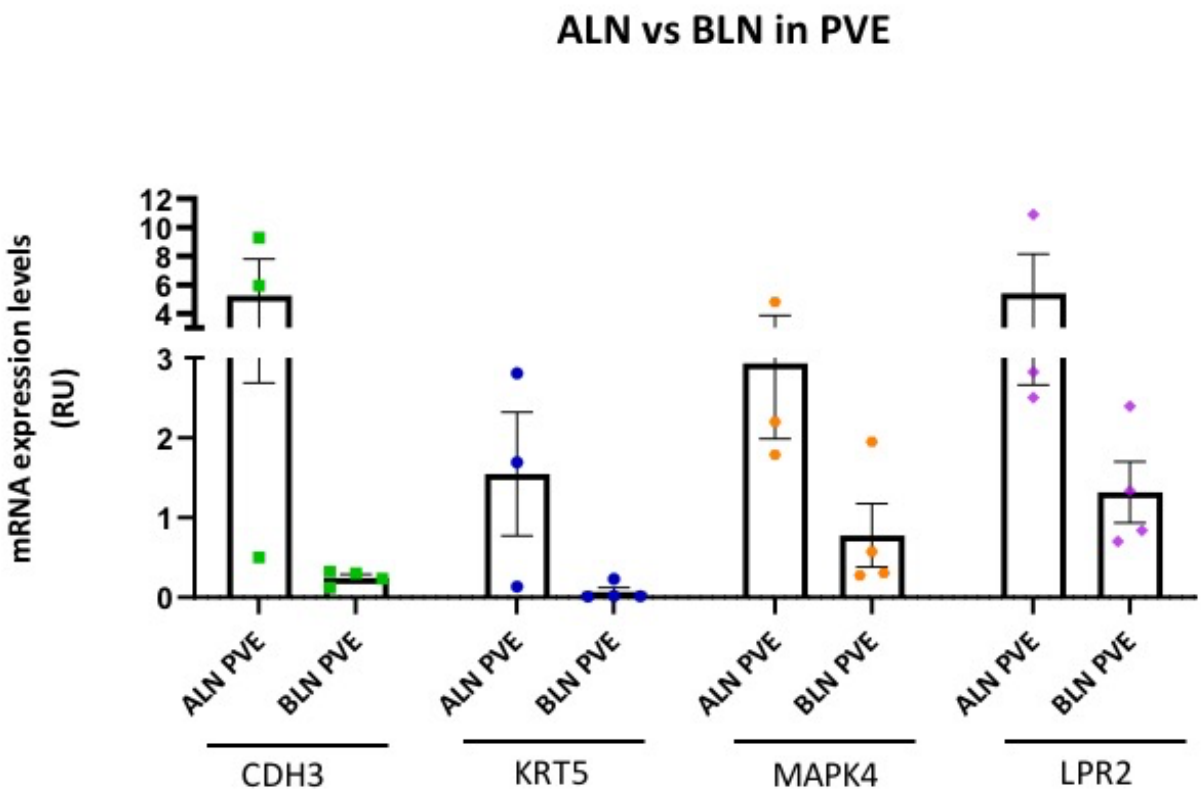

Figure 5.9. Quantitative real-time RT-PCR analysis of four genes significantly differentially expressed between ALN PVE patients tissue samples (n= 3) and BLN PVE patient tissue samples (n= 4). The analysed genes (*CDH3, KRT5, MAPK4* and *LRP2*) were overexpressed in ALN PVE patients' samples. The relative mRNA levels (RU = relative units) were determined using *GAPDH* as internal control. The black bar represents the mean of each condition and the different symbols (square, circle, hexagon and rhomboid) represent each biological replicate per condition.

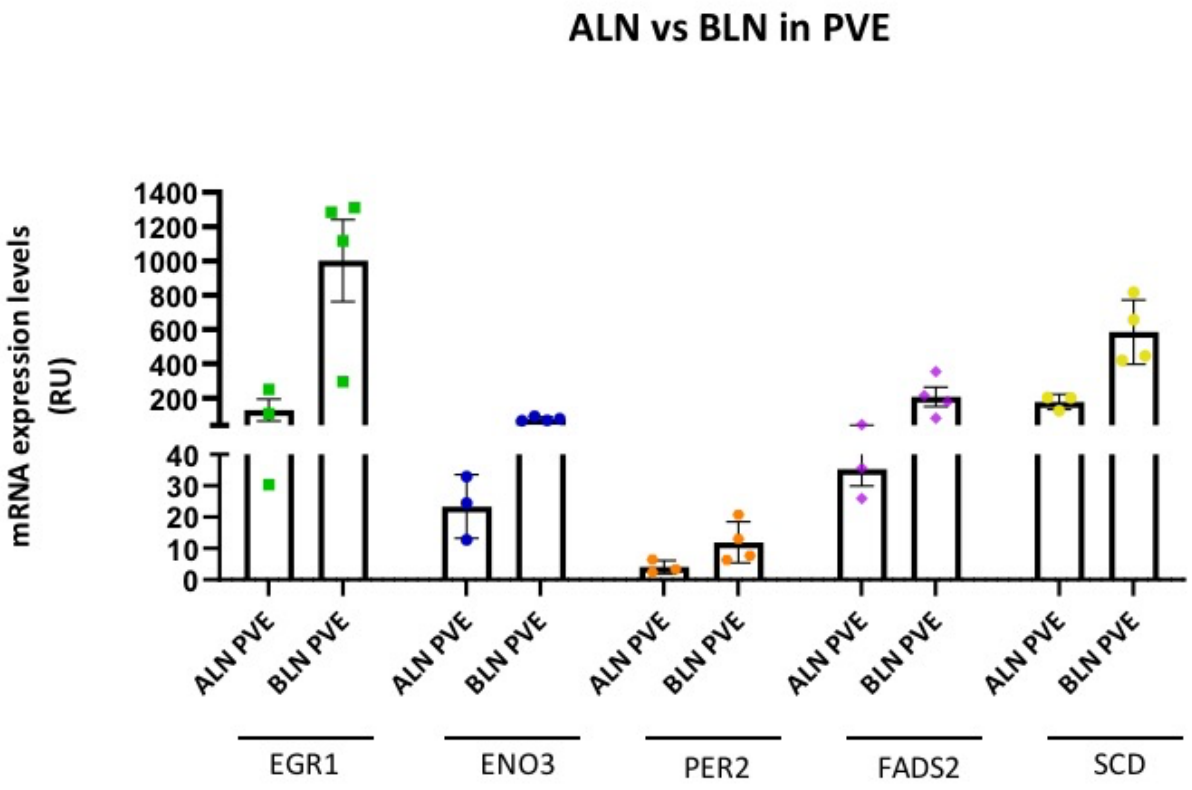

Figure 5.10. Quantitative real-time RT-PCR analysis of five genes significantly differentially expressed between ALN PVE patients' tissue samples (n= 3) and BLN PVE patients' tissue samples (n= 4). The analysed genes (*EGR1, ENO3, PER2, FADS2* and *SCD*) were under expressed in ALN PVE patients' samples. The relative mRNA levels (RU = relative units) were determined using *GAPDH* as internal control. The black bar represents the mean of each condition and the different symbols (square, circle, hexagon and rhomboid) represent each biological replicate per condition.

Even when these results perfectly correlate with the information obtained by RNA-seq, when we applied the Mann-Whitney test to compare these two genetic classes, all the analysed genes showed a strong trend to the significance but didn't reach a significant *p-value*≤ 0.05, so for this comparison no significantly results were obtained. Six of the analysed genes obtained a *p-value* very close to 0.05, *p-value*= 0.057: *CDH3, LRP2, EGR1, ENO3, FADS2, SCD.* The three remaining genes (*KRT5, MAPK4, PER2)* obtained a *p-value*= 0.114, also in line with the RNAseq results for this comparison.

These results could be due to the variability between human patients' samples for the analysed conditions that increases the standard deviation between them and the limited number of samples for each condition within the comparison.

5.3.7.2 BLN PVE *vs* BLN RALPPS patients

A total of 9 patients' tissue samples distributed between BLN PVE condition (n= 4) and BLN RALPPS condition (n= 5) were used for the validation by RT-qPCR. Two of the analysed genes were overexpressed in BLN PVE condition (*ASCM1* and *C3P1)* and one (*CCL3*) was under-expressed, in comparison with the BLN RALPPS condition (Table 5.14).

	PVE		RALPPS	
	Mean	**SD**	**Mean**	**SD**
ACSM1	8.6	5.4	0.9	0.5
C3P1	21.8	168.3	46.1	23.1
CCL3	8.6	5.8	15.8	5.6

Table 5.14. Results obtained by RT-qPCR for BLN in PVE *vs* BLN RALPPS patients' comparison. Validation of 3 significantly expressed genes selected from RNA-seq data analysis. The data shows the mean and the standard deviation for each sample group in the comparison.

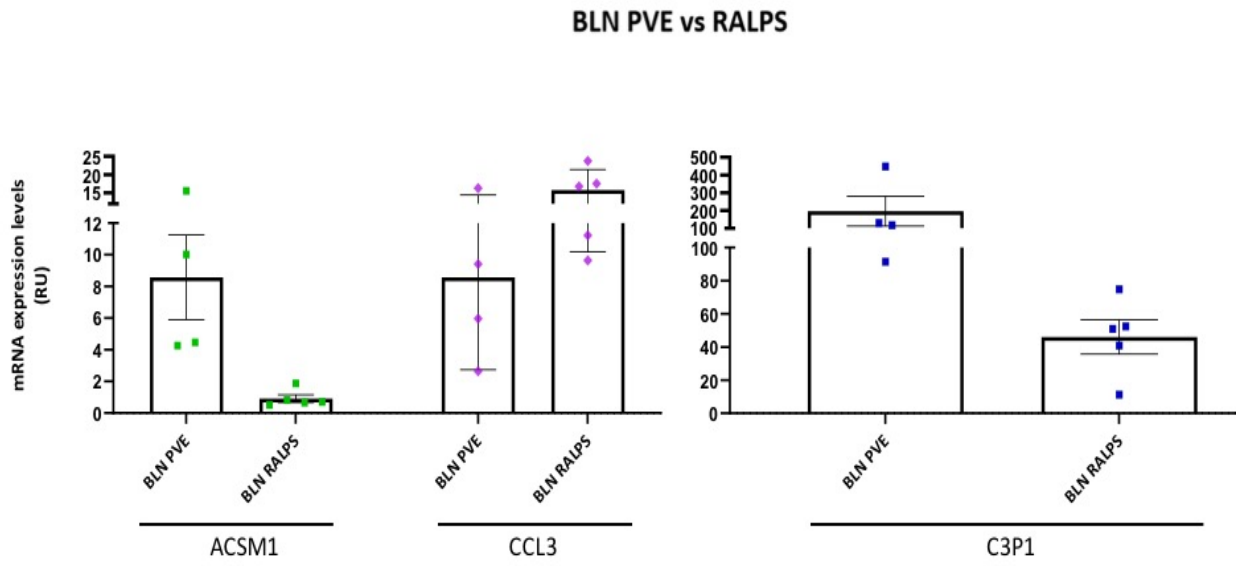

Figure 5.11. Quantitative real-time RT-PCR analysis of three genes significantly differentially expressed between BLN PVE patients' tissue samples (n= 4) and BLN RALPS patients' tissue samples (n= 5). The analysed genes (*ACSM1, CCL3* and *C3P1)* were deregulated between both conditions. The relative mRNA levels (RU= relative units) were determined using *GAPDH* as internal control. The black bar represents the mean of each condition and the different symbols (square and rhomboid) represent each biological replicate per condition.

The results obtained correlate with the information previously delivered by RNA-seq. The Mann-Whitney test was applied to compare these two genetic classes. Two out of three analysed genes were significantly differentially expressed (*ASCM1* and *C3P1*, *p-value*= 0.016) and the remaining gene showed a strong trend to the significance (*CCL3*, *p-value*= 0.063) (Figure 5.11).

For this comparison, an increase in the significance was observed. This could be due to a higher number of samples analysed in these two conditions.

5.3.8 Pathway analysis

Pathway analysis was performed using Wikipathways and KEGG annotations in two different comparisons: pre and post regeneration stages using PVE (ALN *vs* BLN in PVE) and post regeneration samples comparing the two different surgical strategies (PVE *vs* RALPPS in BLN samples). This analysis could not be done in the rest of the comparisons due to the low number of differential expressed genes does not allow to detect any altered pathway. Those gene sets showing an *adjusted p-value*≤ 0.25 were considered enriched between classes under comparison.

5.3.8.1 Pre and post regeneration in PVE

In order to avoid the loss of relevant information for this study, different thresholds were applied for the selection of the differentially expressed genes in this pathway analysis (n= 32 for *adjusted p-value*≤ 0.05 and n= 66 for *adjusted p-value*≤ 0.15) in ALN *vs* BLN in PVE patients' comparison.

In the PVE cohort, before and after liver regeneration, pathways analysis for all significantly differentially expressed genes (n= 32; *adjusted p-value*≤ 0.15) revealed 10 and 3 enriched pathways with Wikipathways and KEGG repositories, respectively (Tables 5.15-5.16). This result was obtained using the most restrictive threshold for the analysis.

WIKIPATHWAYS ANALYSIS		
Name	***P-value***	***Adjusted p-value***
PPAR signalling pathway WP3942	0.005	0.137
let-7 inhibition of ES cell reprogramming WP3299	0.009	0.137
Liver X Receptor Pathway WP2874	0.016	0.137
SREBF and miR33 in cholesterol and lipid homeostasis WP2011	0.025	0.137
Overview of nanoparticle effects WP3287	0.029	0.137
Serotonin Receptor 4/6/7 and NR3C Signalling WP734	0.029	0.137
Fatty Acid Biosynthesis WP357	0.035	0.137
GPCRs, Class B Secretin-like WP334	0.038	0.137
Signal Transduction of S1P Receptor WP26	0.039	0.137
Monoamine Transport WP727	0.049	0.147

Table 5.15. Pathways enrichment analysis for ALN *vs* BLN in PVE using Wikipathways. The analysis was performed considering as significantly enriched those pathways with an *adjusted p-value*≤ 0.15.

KEGG PATHWAYS ANALYSIS		
Name	***P-value***	***Adjusted p-value***
Biosynthesis of unsaturated fatty acids_Homo sapiens_hsa01040	0.001	0.018
Fatty acid metabolism_Homo sapiens_hsa01212	0.003	0.040
PPAR signalling pathway_Homo sapiens_hsa03320	0.005	0.054

Table 5.16. Pathways enrichment analysis for ALN *vs* BLN in PVE using Wikipathways. The analysis was performed considering as significantly enriched those pathways with an *adjusted p-value*≤ 0.15.

In the same PVE comparison, using a more permissive threshold for allowing to unveil possible deregulated functions, pathways analysis for significantly differentially expressed genes Pathway analysis for genes (n= 66; *adjusted p-value*≤ 0.25) revealed 15 enriched pathways using Wikipathways and only 3 enriched pathways with KEGG repository (Tables 5.17-5.18).

WIKIPATHWAYS ANALYSIS		
Name	***P-value***	***Adjusted p-value***
Brain-Derived Neurotrophic Factor (BDNF) signalling pathway WP2380	0.012	0.223
let-7 inhibition of ES cell reprogramming WP3299	0.019	0.223
PPAR signalling pathway WP3942	0.021	0.222
Hfe effect on hepcidin production WP3924	0.023	0.223
Methylation Pathways WP704	0.029	0.223
Steroid Biosynthesis WP496	0.033	0.223
Liver X Receptor Pathway WP2874	0.033	0.223
Mammary gland development pathway - Involution (Stage 4 of 4) WP2815	0.033	0.223
Hair Follicle Development: Cytodifferentiation (Part 3 of 3) WP2840	0.034	0.223
Iron metabolism in placenta WP2007	0.039	0.223
Human Complement System WP2806	0.041	0.223
Transcriptional cascade regulating adipogenesis WP4211	0.042	0.223
Valproic acid pathway WP3871	0.042	0.223
Osteopontin Signalling WP1434	0.042	0.223
SREBF and miR33 in cholesterol and lipid homeostasis WP2011	0.052	0.223

Table 5.17. Pathways enrichment analysis for ALN *vs* BLN in PVE using Wikipathways. The analysis was performed considering as significantly enriched those pathways with an *adjusted p-value*≤ 0.25.

KEGG PATHWAYS ANALYSIS		
Name	***P-value***	***Adjusted p-value***
Biosynthesis of unsaturated fatty acids_Homo sapiens_hsa01040	0.003	0.067
Transcriptional misregulation in cancer_Homo sapiens_hsa05202	0.003	0.067
Fatty acid metabolism_Homo sapiens_hsa01212	0.011	0.165

Table 5.18. Pathways enrichment analysis for ALN *vs* BLN in PVE using KEGG. The analysis was performed considering as significantly enriched those pathways with an *adjusted p-value*≤ 0.25.

5.3.8.2 PVE and RALPPS after regeneration

When comparing specimens of regenerated liver from PVE and RALPPS patients, the pathway enrichment analysis of the 29 significantly differentially expressed genes (*adjusted p-value*≤ 0.25) showed 4 enriched pathways using Wikipathways and other 4 pathways were enriched using KEGG databases in the RALPPS group (Tables 5.19 and 9.20).

WIKIPATHWAYS ANALYSIS		
Name	***P-value***	***Adjusted p-value***
Gastric acid production WP2596	0.010	0.104
Alanine and aspartate metabolism WP106	0.017	0.104
Valproic acid pathway WP3871	0.019	0.104
Sulfation Biotransformation Reaction WP692	0.024	0.104

Table 5.19. Pathways enrichment analysis for BLN PVE *vs* BLN RALPPS using Wikipathways. The analysis was performed considering as significantly enriched those pathways with an *adjusted p-value*≤ 0.25.

KEGG PATHWAYS ANALYSIS		
Name	***P-value***	***Adjusted p-value***
Butanoate metabolism_Homo sapiens_hsa00650	0.040	0.212
Glyoxylate and dicarboxylate metabolism_Homo sapiens_hsa00630	0.040	0.212
Fructose and mannose metabolism_Homo sapiens_hsa00051	0.045	0.212
Alanine, aspartate and glutamate metabolism_Homo sapiens_hsa00250	0.049	0.212

Table 5.20. Pathways enrichment analysis for BLN PVE *vs* BLN RALPPS using KEGG annotations. The analysis was performed considering as significantly enriched those pathways with an *adjusted p-value*≤ 0.25.

5.4 Discussion

Genetic expression profiles of liver regeneration after partial hepatectomy have been explored intensively in animal models. However, there is scarce information about the changes in liver transcriptome during liver regeneration in humans where this process has been investigated mainly after hepatic resection in the setting of liver transplant or tumour removal.

The underlying genetic mechanisms behind the accelerated liver regeneration induced by ALPPS or its modifications remain unclear. In order to improve the understanding of this enhanced liver regeneration and to help in refinement of the procedure for clinical benefit, a few groups have investigated in more detail the genetic changes observed induced by ALPPS in animal models (32, 581) and humans (394, 592). To date no full genetic characterisation of liver regeneration in ALPPS or its modifications has been performed in humans. In a series of 11 patients who underwent ALPPS, Uribe *et al.* suggested a possible association between liver remnant volume increase and a molecular upregulation of mTOR pathway although there was no control group for comparison. This author hypothesized that the regenerative process after ALPPS may be secondary to molecules entering the remnant liver tissue through portal flow, which, in turn, modulate the mTOR/AMPK pathway. In addition, a recent observational study with a cohort of ALPPS patients included in the Ligro trial, correlated a set of mutations in colorectal liver metastasis with relapses within 12 months from the hepatectomy, but no genetic characterisation was performed regarding the liver regeneration process (593).

In this research, genetic modifications in macroscopically normal liver tissue after liver regeneration induced by RALPPS (n= 10) and PVE (n= 8) in patients with unresectable liver cancers were investigated. Furthermore, the influence of stimulation of liver regeneration by RALPPS at a genetic level was also investigated in metastatic colorectal liver tissue in the deportalized liver lobe. Once quality checks were performed in the purified total RNA of the 69 tissue samples from RALPPS (n= 45) and PVE (n= 24) patients, 32 samples were sent for NGS. The representation of the total number of samples with an unsupervised hierarchical clustering and a principal component analysis (PCA) confirmed the different nature of the non-tumoral and tumoral tissue analysed with two different transcriptional profiling groups (Figures 5.1 and 5.2). This finding was also evident in the unsupervised hierarchical clustering when comparing normal liver tissue (n= 9) *versus* colorectal liver metastasis (n= 6) (Figure 5.3).

NGS results of the different comparisons demonstrated genetic dysregulations in not only protein-coding RNA, but also in non-coding RNA including pseudogenes and long non-coding RNA. Non-coding RNA has been historically thought to be of no purpose or non-functional. Nevertheless, recent studies indicate that they have some regulatory roles. Long non-coding RNAs are a class of RNA transcripts greater than 200 nucleotides in length which do not encode proteins. Accumulating evidence suggest its overexpression may play essential roles in tumorigenesis, cancer progression and thus be potential biomarkers and therapeutic targets (676, 677).

In the present work, genetic modifications secondary to RALPPS technique (n= 8) in tissue from the regenerated liver lobe, the occluded liver lobe and from colorectal liver metastasis located in the deportalized liver lobe were investigated.

When comparing the regenerated liver after RALPPS procedure to normal liver parenchyma, *RPL23AP2* (*adjusted p-value*= 0.038) and *SNORD3D* (*adjusted p-value=* 0.038) were found to be significantly downregulated in the regenerated liver (Tables 5.3 and 5.4). Of note, none of these genes have been associated to the process of liver regeneration in the current literature. The pseudogene *RPL23AP2* has been mentioned only in two papers related to a comparative analysis of processed ribosomal protein pseudogenes in mammals and in the differentiation of normal lung tissue from cancer as a good reference candidate (585, 586). From the limited literature where the non-coding RNA *SNORD3D* is described, one paper established to be down regulated in the liver tissue of patients with non-alcoholic fatty liver disease (NAFDL) (587).

Although no statistically significant difference was found when comparing normal liver tissue to the occluded liver in RALPPS, a lower level of significance with an *adjusted p-value*≤ 0.15 showed three differentially expressed genes (Tables 5.5 and 5.6). In the occluded liver, *MT-CO2* (*adjusted p-value*= 0.094) was found downregulated, *RNU1-27P* (*adjusted p-value*= 0.124) and *RP11-79H23.3* (*adjusted p-value*= 0.124) were upregulated. Interestingly, none of these genes have been associated to the process of liver regeneration in the current literature. The protein-coding RNA *MT-CO2,* also named *COX-2*,

promotes cell growth, angiogenesis, inhibits cell death, carcinogenesis and cancer progression. In the context of liver regeneration, the observed endotoxemia post hepatectomy activates COX-2 in Kupffer cells and drives a higher expression in hepatocytes in rats (248). COX-2 promotes hepatocyte proliferation in the early regeneration though NF-kb, ERD1/2 and MAPK pathways (249-251). Its downregulation in the deportalized liver after RALPPS indicates that processes in favour of tissue restoration such as cell growth and angiogenesis are compromised whereas cell death may be enhanced. The upregulation of *RNU1-27P,* a pseudogene which function is yet to be determined and the long non-coding RNA *RP11-79H23.3* with a possible role as tumour suppressor in bladder cancer (615), suggests a role in suppressing cell restoration.

The occluding effect of RALPPS in the colorectal liver metastasis showed three differentially expressed genes (Tables 5.7 and 5.8). *RPL41P1* (*adjusted p-value*= 1.62E-05) and *CLDN2* (*adjusted p-value*= 8.60E-05) were upregulated in the colorectal liver tissue of the occluded liver after induction of liver regeneration with RALPPS. Whereas the pseudogene *RPL41P1* has not been described before, the protein coding *CLDN2* has been extensively investigated. *CLDN2* encoding protein, Claudin 2, is a paracellular channel protein localised at tight junctions and overexpressed in liver tissue with roles in proliferation and migration. This is an interesting finding in line of how liver regeneration can induce or trigger tumour progression after major resection associated or not to portal vein occlusion (465-467). On the other hand, some groups have reported that tumor progression induced by ALLPS procedure is unlikely given

the short treatment period in between the two stages (371). Supporting this oncological benefit from ALPPS, the immuno-histochemical analysis of *Ki67*, a marker of tumour proliferation, performed by Tanaka *et al.* in CRLM tissue of patients treated with ALPPS and two-stage hepatectomy, demonstrated a greater *Ki67* expression in resected CRLM tissue at second stage in the classical two-stage hepatectomy than in the ALPPS group (p= 0.01) (592). Similarly, there was no significant difference in the genetic expression of the mRNA that encodes Ki67, *MKI67*, in the colorectal liver metastasis tissue after RALPPS (*adjusted p-value*= 0.982). The downregulation in the metastatic tissue located in the occluded liver lobe after RALPPS of the long non-coding gene *AC159540.1* (*adjusted p-value*= 0.006) whose function remains unknown suggests a possible role or implication in tumour suppression or favoring apoptosis.

Genetic modifications after the liver regeneration induced by PVE (n= 6) were also investigated. In this group, when comparing the regenerated liver tissue to normal liver parenchyma, 32 genes were found to be significantly expressed (*adjusted p-value*≤ 0.05) (Table 5.10). From all these genes, 15 were upregulated in the regenerated liver. Given the quantity of genes significantly deregulated after PVE, a pathways analysis was feasible. In this scenario, the most striking result was the enrichment in lipid metabolism pathways such as transcriptional cascade regulating adipogenesis, biosynthesis of unsaturated fatty acids and fatty acid metabolism (Tables 5.17 and 5.18) (*adjusted p-value*≤ 0.25). During hepatic regeneration post liver resection, the liver accumulates lipids from adipose tissue lipolysis in order to provide energy substrate for the

cell proliferation. Although this physiological post-resectional steatosis may be helpful, an excess of lipid accumulation can cause impaired liver regeneration and cell death. This is important during extended hepatic resections where a small FLR can have a lower lipid storage capacity, and a higher risk of lipid overload provoking liver failure. Moreover, a pathway involved in the tumourigenic process, the transcriptional misregulation in cancer pathway was also enriched using KEGG database in PVE group (*adjusted p-value*≤ 0.25). This finding is consistent with the already known evidence about the tumour progression observed and investigated after induction of liver regeneration, with portal vein occlusion, either with embolization or ligation at staged hepatectomies (338, 362, 465-469).

One of the aims of this PhD was to elucidate the differences, at a genetic level, of the faster liver regeneration observed in RALPPS in comparison to the gold standard PVE in patients with unresectable liver tumours and a small future liver remnant. With this regard, when comparing genetic expression profiles in the regenerated liver tissue after PVE and RALPPS, four genes were found to be upregulated in RALPPS in comparison with PVE when *adjusted p-value*≤ 0.05 (*RPL41P1; CCL3; HLA-H; RPL12*). Of interest, *RPL41P1* is a ribosomal protein pseudogene which has not been described yet in the literature nor its function. Upregulation of *RPL41P1* may suggest a possible role in the process of the enhanced liver regeneration observed with the RALPPS technique in comparison with the conventional preoperative liver induction by PVE. Another interesting finding is the upregulation of two genes which play an important role in immune response, the protein coding gene *CCL3* and the

major histocompatibility complex gene, *HLA-H*. This may suggest a greater involvement of the immune system in the increased regeneration process triggered by the RALPPS technique than with PVE. A similar suggestion have been made by several groups where the presence of liver necrosis and subsequent inflammatory response with an upregulation of cytokine expression may accelerate the induction of hepatocyte proliferation after the first stage in the ALPPS procedure (32, 383, 393). Kambakamba *et al.* found higher levels of IL6 with a future liver volume (FLV) growth rate double in ALPPS group than the PVL group (393). Interestingly, an experiment in rats by Schlegel *et al.* revealed a similar increase of the FLR when injecting plasma after radiofrequency ablation of an organ (spleen, kidney or lung) in the PVL group than in the ALPPS group (32).

Regarding the upregulation of *RPL12* in RALPPS, this gene encodes a ribosomal protein, part of the ribosomal subunit 60s, and plays an important role in ribosomal RNA (rRNA) processing. The eukaryotic ribosomes are macromolecular structures formed by two subunits (60S and 40S) containing several rRNA and ribosomal proteins. These particles convert a genetic code (messenger RNA, mRNA) into an amino acid sequence in order to facilitate protein biosynthesis. The process of "ribosomal RNA processing" involves a conversion of a primary rRNA transcript into more mature ribosomal RNA and is essential for the ribosome formation. As mentioned, in contrast with regenerated tissue after PVE, *RPL12* gene was found upregulated in RALPPS suggesting a potential higher need of protein production to cover multiple cellular functions during the boosted process of liver regeneration.

In the literature, tumour progression has been found to be a shortcoming in the 4-8 week waiting period for the regenerating process after PVE precluding second stage resections and decreasing resection rates (338, 362, 465-469). To expedite the second stage and hypothetically prevent further tumour growth, the novel technique ALPPS and its variants may play an important role by inducing a quicker liver regeneration response and increment resection rates (393, 471, 472). In line with this clinical evidence, interesting results have been discovered in the present research. Three genes involved in tumorigenesis were found to be upregulated in the regenerated liver tissue after PVE in comparison to the RALPPS group: the non-coding RNA *LINC00319* and the protein-coding genes, *TRIM29* and *TACC2* (Table 5.12). Furthermore, upregulation of *SSPO* was also found to be upregulated in PVE. This pseudogene has a role in cell adhesion, cell differentiation, central nervous system development as well as in protein metabolism (650). Besides, *SSPO* belongs to the O-glycosylation of TSR (thrombospondin type 1 repeat) domain-related to pathways regulating many biological processes such as Notch signaling, inflammation, wound healing, angiogenesis and neoplasia (678). Moreover, another upregulated gene in the PVE regenerated tissue was *NECAB2*. By encoding the neuronal calcium-binding protein 2, *NECAB2* binds and modulates the activity of the adenosine A2 and metabotropic glutamate type 5 receptors. Both receptors have been described to regulate signaling activity such as promoting MAPK1/3 (ERK1/2) activation. Mitogen-activated protein kinases (MAPK) are extracellular signal-regulated kinases (ERKs). MAPK1 (ERK2) and MAPK3 (ERK1) are involved in a broad spectrum of cellular processes such as proliferation, differentiation, regulation of inflammatory

responses, cytoskeletal remodeling, transcription regulation, development, cell motility, invasion through the increase of matrix metalloproteinase production and apoptosis (679, 680). Hence this kinase pathway is a central signaling module that participates in numerous physiological and pathological processes (681) and its persistent activation has been described to contribute to oncogenic transformation with a high proportion of human cancers containing mutations in different components of the pathway (682). Additionally, MAPK has a role in the innate inflammatory response. After a pathogen infection or tissue damage, MAPK pathway activation induces the expression of multiple genes in the innate immune cells that together regulate the inflammatory response. Pharmacological targeting of MAPK pathways has a potential role in the treatment of autoimmune and inflammatory diseases (683). Although upregulated *NECAB2* in the PVE group in comparison to RALPPS could suggest a major activation of the MAPK pathways, *MAPK4* was found significantly downregulated in the liver tissue regenerated from PVE in comparison to normal parenchyma (*p= 0.004).*

PLIN5 encodes the protein perpilin 5, which prevents intracellular lipolytic degradation and its impairment has been associated to play a role in the pathogenesis of non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) (671). In the RALPPS regenerated tissue *PLIN5* was downregulated along with *SNORD3D,* another gene which was found to be downregulated in the liver tissue of patients with NAFDL (587). In the present study and similarly than in liver biopsies from NASH or NAFLD patients, *PLIN5* was found upregulated in the PVE regenerated tissue. Besides, high levels of

its encoded protein have been found to protect against lipotoxicity in endurance trained muscle (667). This finding emphasizes the already known importance of lipid metabolism in the process of liver regeneration and, more specifically, in later phases of the liver restoration suggesting a more mature regeneration response induced by PVE than by RALPPS. Moreover, *PLIN5* gene is a part of the peroxisome proliferator-activated receptor (*PPAR)* signaling pathway and was upregulated after liver regeneration secondary to PVE. This finding is consistent with the fact that *PPAR* pathway was found to be enriched in the Wikipathways analysis in the regenerated liver tissue after PVE (Table 5.17). Interestingly, both increased lipid metabolism and activated PPAR signaling pathways have shown to play important roles in late-phase LR in 70% partial hepatectomy rats (684). This may suggest that the LR provoked by PVE is in a later stage than RALPPS. As mentioned in the Introduction of this PhD, LR proceeds along the sequence of three phases: initiation, proliferation and termination. The latter is less investigated. In a microarray experiment in rats after 70% PH, the genes expressing at a higher level in the early phase were mainly involved in the response to stress, proliferation, and resistance to apoptosis, while those expressing at a lower level at the early phase than at the late phase were mainly engaged in lipid metabolism (684).

In this study, considering the pathway analysis of the 29 significantly differentially expressed genes (*adjusted p-value*≤ 0.15) in the comparison of regenerated liver tissue from PVE *versus* RALPPS, two similar pathways in the Wikipathways and KEGG databases were enriched in the RALPPS group: “Alanine and aspartate metabolism” and “Alanine, aspartate and glutamate

metabolism", respectively. In the setting of liver regeneration after PH in rats, metabolism of glutamate, alanine and other amino acids has been found enhanced during almost the whole process suggesting that the metabolism and transport of aminoacids and their derivates are necessary in rat liver regeneration (685). In cancer cell metabolism it is known that some aminoacids, most notably glutamine, are consumed at higher rates that what is required for protein synthesis and at the same time others aminoacids are excreted like alanine, glutamate or proline. In a recent quantitative analysis of aminoacid metabolism in liver cancer, Nilsson *et al.* demonstrated that glutamate was formed in excess in the cytoplasm to rapidly help the cell to increase nucleotide synthesis rate and in turn, sustain growth in liver cancer cells (686). Hence, the enrichment of this aminoacids pathway in the RALPPS regenerated liver tissue may indicate, in its inherent enhanced liver regeneration, a greater nucleotide synthesis for larger cell proliferation.

Overall, the data validation of the RNA sequencing results by quantitative real time RT-qPCR demonstrated a good correlation with the RNA-seq data. Nevertheless, no significant *p-value*≤ 0.05 were reached in PVE ALN *vs* BLN (n= 3 *vs* n= 4) but a strong trend to the significance. With a higher number of patients, an increment of the significance in the BLN in PVE *vs* BLN in RALPPS (n= 4 *vs* n= 5) was observed. These outcomes could be explained due to the variability between human tissue samples and a small sample size for each condition included in the study.

Some limitations of this experiment include the following:

An analysis of dynamic gene expression could not be performed due to the nature of this experiment unlike in animal models. Full thickness liver tissue samples were harvested in two time points: before and after induction of liver regeneration. No cell separation techniques such as short collagenase liver perfusion followed by centrifugation which separates hepatocytes from non-parenchymal cells were used. Given that around 60-70% of the liver cells are hepatocytes and the rest are non-parenchymal cells, it is possible that some of the reported modifications may have occurred in non-parenchymal cells rather than in hepatocytes. On the other hand, similar induction of gene expression in both cells has been reported after partial hepatectomy in rats (586).

Due to the nature of this research, this study only evaluated transcriptional changes and did not evaluate protein translation nor reflected their functionality in hepatocytes. Addition of a western blot could have been useful to determine protein translation.

Other limitations were encountered during the performance of this research. From the total 69 harvested samples, a lack of integrity in 37 RNA samples was revealed. Hence, 32 RNA samples were sent for next generation sequencing. Different scenarios can cause RNA degradation affecting a reliable gene expression analysis. Firstly, after tissue harvesting, an RNA stabilization includes an immediate submersion in an appropriate RNAlater RNA stabilization Reagent; too much or too little tissue can also affect the stabilization. Secondly, RNA later stabilized tissue can be stored up to one day at 37°C, up to a week if exceeded 15–25°C, up to 4 weeks at 2–8°C and can be archived much longer at –20°C or –80°C. Thirdly, inappropriate handling of the

samples or components of the kit can cause RNAse contamination during the experiment.

The pathway analysis with Wikipathways and KEGG annotations could not be performed in all comparisons due to the small number of differentially expressed genes. Furthermore, threshold used for selecting the significantly differentially expressed genes in PVE and RALPPS liver tissue after regeneration was an *adjusted p-value*≤ 0.15, due to the low number of input genes to perform the analysis.

Further research into mechanisms regulating transcription during LR in PVE, ALPPS and its variants is needed to develop new approaches in treatments of liver disease.

CHAPTER 6. VOLUMETRIC AND FUNCTIONAL CHANGES IN LIVER REGENERATION: A RANDOMISED CLINICAL TRIAL COMPARING PVE *VERSUS* RALPPS

6.1 Background

Liver resection remains the only chance for cure and long-term survival of patients with primary or secondary liver tumours. In order to achieve this, a clear resection margin (R0) after major or extended hepatectomy should be pursued (314, 687, 688). Unfortunately, when the preoperative future liver remnant volume is <25% in patients with healthy livers, <35% in patients who have had a prolonged course of chemotherapy or <40% in cirrhotic patients, there is an increased risk of postoperative liver failure. The latter has been stablished as the main cause of death after extensive hepatic resections. Volumetric studies should then be routinely performed in planned extended hepatic resections to calculate the future liver remnant volume (FLRV) and avoid small liver remnants. In the last three decades, several techniques have been developed to induce hypertrophy and regeneration of the small future liver remnant preoperatively, and finally increase resectability rates and minimize postoperative complications. Nowadays, the gold standard technique for induction of liver regeneration preoperatively is the portal vein embolization (PVE). By this technique, interventional radiologists can achieve an increase in FLRV of 11.9-39% (326, 355). More recently in 2012, Schnitzbauer *et al.* described a surgical technique which achieves a faster increase of the FLRV in comparison with PVE (25). By associating liver partition

to portal vein ligation in a staged hepatectomy (ALPPS) a 74% increase of the FLRV was observed in a much shorter period of time. However, the reported morbidity rates for this novel procedure were much higher than those reported for PVE: 33-58% and 16%, respectively (322, 355, 380, 689). Since its description, the ALPPS procedure has been criticized in terms of safety by different groups and whether it truly achieves is a functional gain in this short space of time or it is just a simple hypertrophy or oedema of the FLR (436). Inspired by the speed of liver volume restoration observed and aiming to increase its safety, several variations of the ALPPS technique have been described (329, 331, 397-399, 401, 402).

The radiofrequency associated liver partition with portal vein ligation for staged hepatectomy (RALPPS) was firstly described by Gall *et al.* in 2015 (331). The main difference between the original ALPPS and RALPPS lies in creating a virtual splitting of the liver with radiofrequency ablation instead of a pure transection line during the first stage of the staged hepatectomy. In this first publication a pilot study of five patients who underwent RALPPS procedure were compared with a historical cohort of 5 patients matched for age, sex, initial liver function and pathology who underwent PVE. RALPPS demonstrated to be superior in efficacy to PVE. There was no difference in liver function between the two groups on day 15 post hepatectomy. No patients developed a postoperative bile leak (a common source of morbidity in ALPPS) and there was no mortality at 90 days.

During the first consensus meeting in ALPPS held in 2015, international experts concluded that further evidence was needed before ALPPS could

become a routine tool for preoptimization of small FLRV in staged hepatectomies (372, 690). Two randomized clinical trials emerged from this conference: the LIGRO and REBIRTH trials. The multicenter LIGRO trial led by Sandstrom *et al.* compared differences between PVE and ALPPS and its results were published in 2018 (380). In our group, although RALPPS was demonstrated to be as superior to PVE in a small pilot study, the full comparative efficacy profile in the medium and long term between RALPPS and PVE remained unknown. A randomized controlled trial was required to assess this aspect in a more controlled and unbiased manner. In the present chapter, the single center randomized clinical trial "rapid induction of liver regeneration for major hepatectomy (REBIRTH): portal vein embolization versus radiofrequency assisted ligation for liver hypertrophy" is presented (413).

6.2 Aims

The aim of this trial is to see whether RALPPS can be safely performed to increase the volume of the FLR faster than the gold standard PVE.

Hypothesis:

- Null hypothesis: there is no differences in the induced liver regeneration process nor the clinical outcomes between RALPPS and PVE groups.
- Alternative hypothesis: the induction of liver regeneration and clinical outcomes are more significant in the RALPPS patient group when compared to the PVE group.

6.3 Results

Between 1 July 2015 and October 2017, a total of 59 patients presented in the HPB Multidisciplinary meeting at Hammersmith Hospital required preoperative induction of FLRV for primary and secondary liver tumours. Two patients were not fit for surgery and excluded from the study. From the remaining 57 patients, 28 were randomized to PVE and 29 to RALPPS (Figure 6.1). The median follow-up was 24 months (3-33). There were no lost in follow up.

6.3.1 Patient and tumour characteristics

Regarding patients and tumour characteristics, no differences were found between the two groups (Table 6.1). The median age was similar in the two groups as well as the gender distribution. Colorectal liver metastasis was the most frequent tumour (CRLM, 79.2% in PVE arm *vs* 76.9% in RALPPS arm). A median of 2 and 3 metastases were found in PVE and RALPPS, respectively. These metastases had an average size of 45.2 ± 30.1 mm in the PVE group versus 52.2 ± 37.7 mm in RALPPS (p= 0.53). All patients with CRLM had at least 6 cycles of standard systemic chemotherapy. Furthermore, 18 patients on each group had ten or more cycles before preoperative induction of liver regeneration.

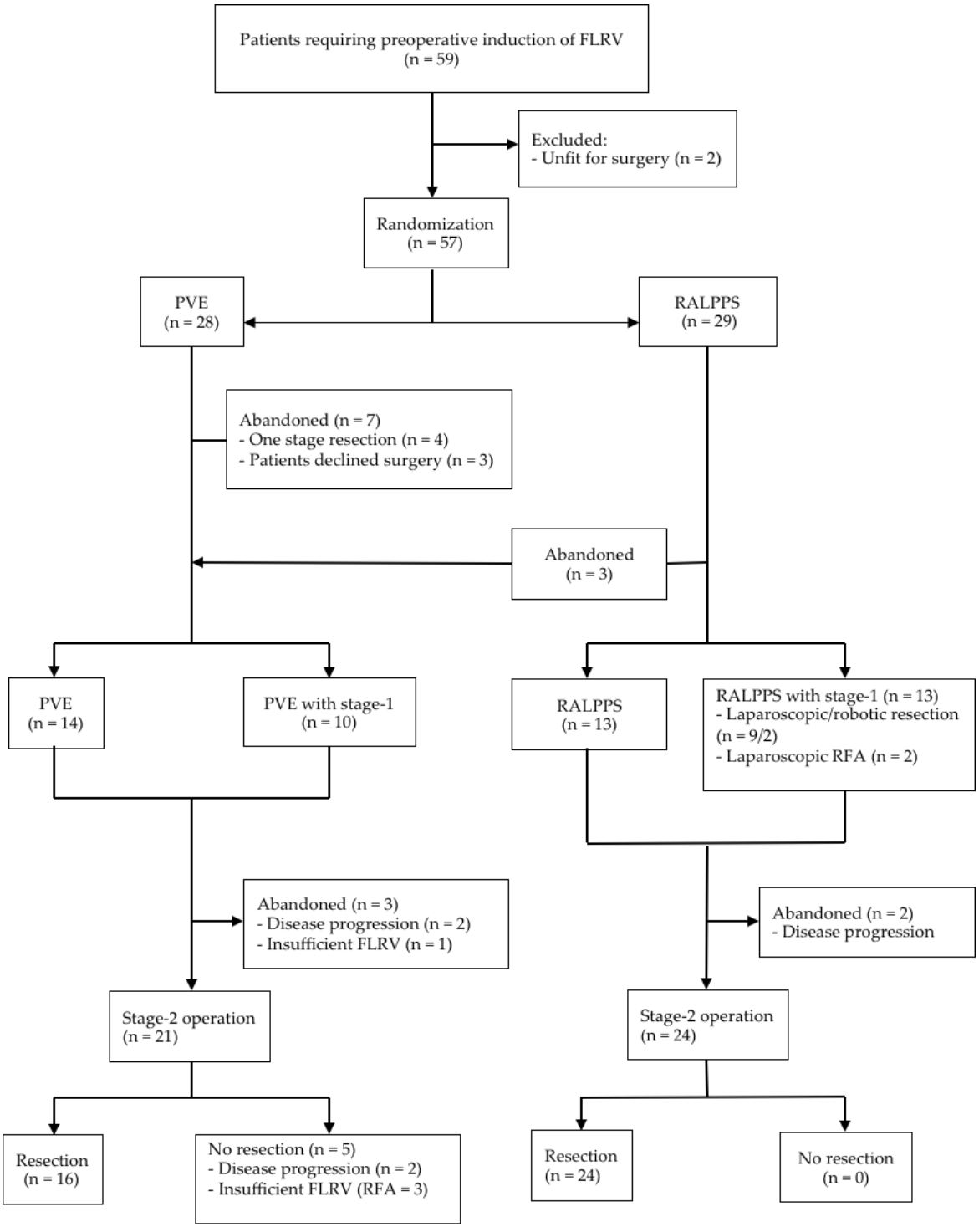

Figure 6.1. Trial profile. FLRV: future liver remnant volume; PVE: portal vein embolization; RALPPS: liver partition with portal vein ligation for staged hepatectomy; RFA: radiofrequency ablation.

	PVE (n= 24)	**RALPPS** (n= 26)	*P value*
Age (mean±SD, in years)	64.3 ± 8.9	62.4 ± 10.2	0.49
Male (%)	12 (50.0)	15 (57.7)	0.78
Type of tumor (%)			0.06
CRLM	19 (79.2)	20 (76.9)	
ICC	4 (16.7)	0 (0)	
HCC	0 (0)	1 (3.8)	
Others*	1 (4.2)	5 (19.2)	
Bilobar liver disease (%)	9 (37.5)	13 (50.0)	0.06
Synchronous metastases (%)	11 (45.8)	9 (34.6)	0.57
Number of metastases (median, range)	2 (0-11)	3 (1-10)	0.18
Size of largest metastasis (median, range in mm)	43(15-108)	39(12-150)	0.53
Extrahepatic disease (%)	1 (4.2)	3 (11.5)	0.61
Primary tumour in situ (%)	1 (4.2)	3 (11.5)	0.61
Neoadjuvant chemotherapy (%)	20 (83.3)	22 (84.6)	0.99
Number of cycles ≥10 (%)	18 (75)	18 (69.2)	0.99

Table 6.1. Patient and tumour characteristics.

* Others: PVE: duodenal adenocarcinoma (n=1); RALPPS: pancreatic NET (n = 1), germ cell ovarian tumor (n=1), endometrial carcinoma (n=1), breast cancer (n=1) and leiomyosarcoma (n=1). Keys: CRLM: colorectal liver metastasis; HCC: hepatocellular carcinoma; ICC: intrahepatic cholangiocarcinoma.

6.3.2 First intervention

6.3.2.1 PVE arm

From the 28 patients randomized to the PVE arm, 7 were excluded as one stage resection could be safely achieved during the first operation in 4 cases and 3 patients declined surgery and underwent adjuvant chemotherapy (Figure 6.1). On the contrary, from the 29 patients who underwent first stage RALPPS, 3 cases were crossed over the PVE arm due to intraoperative technical difficulties. In total, 24 patients had PVE from which 10 of them had undergo a previous stage one minimally invasive tumorectomy for bilobar disease in segment 3 (n= 7) and segment 4 (n= 3). In these cases, liver resection took a median and range of 90 (60-180) minutes. Segment 4 branch was embolized in 18 patients to increase further FLRV. Only five patients suffered from minor complications (Dindo I to IIIa). The median length of stay was 2 days (Table 6.1).

6.3.2.2 RALPPS arm

A total of 29 patients were randomized to the RALPPS arm. Unfortunately, three of these patients had only a diagnostic laparoscopy due to extensive adhesions from previous laparotomy (n= 2) and a large tumour occupying segments 4 and 5 (n= 1) making the dissection of the liver hilum practically

impossible unless conversion to open surgery. Hence, the procedure was abandoned, and these three patients were crossed over to the PVE arm having a successful FLR optimization before completion hepatectomy.

From the other 26 patients who did accomplish the RALPPS procedure, 13 had bilobar disease and clearance of the FLR was achieved in this first operation by RFA (n= 2) and tumorectomy of segment 2 (n= 3), segment 3 (n= 4) and segment 4 (n= 4) (Figure 6.1). The median length of operation was 90 minutes ranging from 60 to 225. There was a median intraoperative blood loss of 310 mL with no perioperative blood transfusion. Minor complications (Dindo I to IIIa) occurred in 6 patients (23%). One patient with metastatic endometrial liver metastases suffered from an acute compartment syndrome in her right lower limb 4 hours postoperatively. This was likely secondary to a vascular injury from a femoral puncture for vascular access during the anaesthetic induction (Dindo IIIb) (Table 6.1).

	PVE (n= 24)	**RALPPS** (n= 26)	*P value*
Details of PVE and RALPPS			
PVE/RALPPS without stage 1	20 (83.3)	13 (50.0)	0.01
PVE/RALPPS with stage 1			
Tumorectomy (lap†/robotic)	4/0	9/2	
RFA (lap/robotic)	0/0	2/0	
Length of operation (median, range in mins)	90 (60-180)	115 (60-225)	0.88
Blood loss (median, range in mins)	300 (10-450)	310 (20-480)	0.88
Perioperative blood transfusion[&] (%)	0	1 (3.8)	0.33
Post procedural complications (%)	5 (20.1)	6 (23.0)	0.2
Dindo I	3	3	
Dindo II	2	2	
Dindo IIIb	0	1	
Length of stay (median, range in days)	2 (1-13)	3 (2-17)	0.06
Details of RALPPS (n=29, %)			
Laparoscopic	n/a	24 (82.8)	
Robotic	n/a	2 (6.9)	
Abandoned	n/a	3 (10.3)	

Table 6.2. Details of stage 1 operation.

†Laparoscopic. [&] Number of patients transfused.

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6.3.3 Volumetric analysis

All patients included in the RCT had serial CT scans before and after liver induction either with PVE or RALPPS. Pre-intervention FLRV among the patients in both groups was similar. As expected by the protocol, the time from the first stage operation to the second CT scan to assess the induction of liver regeneration on the FLR was significantly higher in PVE than in RALPPS (median of 35 *vs* 20 days, respectively). On the other hand, looking into the post-intervention FLRV, the RALPPS technique achieved a significant increase of the FLRV in a much shorter length of time when compared to PVE (80.7 ± 13.7% *vs* 18.4 ± 9.8%, p< 0.001 and 20.0 ± 5.6 days *vs* 41.6 ± 15.5 days, *p*< 0.001, respectively) (Table 6.3).

	PVE (n= 24) No chemo 4; chemo 20	**RALPPS** (n= 26) No chemo 4; chemo 22	*P value*
Time from first stage operation to second CT (median, range in days)	35 (21-75)	20 (14-36)	<0.001
Pre-intervention FLRV (Mean±SD)			
no chemo	23.7 ± 2.9	23.1 ± 2.5	0.74
chemo	33.1 ± 1.5	33.8 ± 1.8	0.2
Post intervention FLRV (Mean±SD)			
no chemo	28.5 ± 9.4	44.6 ± 5.6	0.04
chemo	40.4 ± 6.6	59.4 ± 4.3	<0.001
Increase FLRV post intervention (%)	18.4 ± 9.8	80.7 ± 13.7	<0.001

Table 6.3. Future liver remnant volume (FLRV) before and after PVE or RALPPS. The future liver remnant volume (FLRV) was calculated depending on the type of hepatic resection

needed to achieve tumor clearance by the proportion of future liver volume to total liver volume (TLV) minus total liver tumor volume (FLRV= FLRV/TLV-TLTV). Keys: chemo, preoperative chemotherapy; no chemo, no preoperative chemotherapy.

6.3.4 Second intervention or stage

After volumetric analysis and successful increase of the FLRV, 21 patients in the PVE group and 24 in the RALPPS group underwent the stage-2 operation for completion of hepatectomy (Table 6.4).

6.3.4.1 PVE arm

Majority of the completion hepatectomies were open (15 patients, 93.75%) with 3 cases needing extra RFA or wedge resection. Intraoperatively, 5 patients did not proceed to completion hepatectomy (31.25%) as 2 of them were found to have disease progression and 3 patients had insufficient increase in FLRV and therefore only RFA was performed (Figure 6.1 and Table 6.4). There was a median blood loss of 500ml, and 6 patients needed perioperative transfusion (25%). From those who underwent hepatectomy, 13 patients (61.9%) had minor postoperative complications ($\leq$ Dindo IIIa) and only one patient (4.8%) developed a serious complication on postoperative day eight: a chest infection associated with pleural effusion which required intravenous antibiotics and ultrasound-guided drainage (Dindo IIIb). There was no 90-day mortality. The median length of hospital stay was 7 days (Table 6.4).

6.3.4.2 RALPPS arm

In the RALPPS group, all 24 patients who succeeded the first intervention, underwent liver resection (Figure 6.1 and Table 6.4). Five patients underwent minimally invasive resections from which one was performed robotically. There was a median blood loss of 300 ml and 10 patients (38.5%) required blood transfusion perioperatively. Three patients suffered from serious complications in the postoperative recovery (11.4%): one patient developed an intra-abdominal collection which was drained radiologically and renal failure needing haemofiltration; another patient had a supraventricular tachycardia and chest infection with pleural effusions requiring radiological drainage, and third patient had a prolonged post-operative ileus successfully managed with total parenteral nutrition. There was one patient who died from a sudden cardiorespiratory arrest on postoperative day 13 after having a relaparotomy with bowel resection for peritonitis secondary to bowel ischaemia on day 10 after open right hepatectomy (3.8%). The median length of stay was 8 days (Table 6.4).

6.3.4.3 PVE *vs* RALPPS

No significant differences were found in terms of type of hepatic resection, operative time, blood loss, blood transfusion, length of hospital stay, morbidity, resection margin positivity and liver function blood tests on day 1, 3 and 5 post hepatectomy (Table 6.4, Supplementary Tables A.2 and A.3).

With an intention to treat, a significant failure rate of PVE was found (PVE, 33.3% *vs* RALPPS, 7.7%, p= 0.007). Furthermore, fewer patients in the PVE arm had the final completion hepatectomy in comparison with RALPPS (PVE, 66.7% *vs* RALPPS, 92.3%, p= 0.007) (Table 6.5).

	PVE (n= 24)	RALPPS (n= 26)	*P value*
Type of second operation			
Right hepatectomy (open/lap/robotic)	8 (7/1/0)	18 (14/3/1)	
Extended right hepatectomy (open/lap/robotic)	5 (5/0/0)	5 (4/1/0)	
Right hepatectomy with wedge resection/RFA (open/lap/robotic)	3 (3/0/0)	1(0/1/0)	
RFA	3	0	
Abandoned intraoperatively	2	0	
Length of operation (median, range in mins)	180 (100-420)	180 (110-390)	0.87
Blood loss (median, range in mls)	500 (50-2850)	300 (50-3200)	0.3
Perioperative blood transfusion[&] (%)	6 (25.0)	10 (38.5)	0.18
Postoperative complications (%)	14 (66.7)	14 (53.8)	0.75
Dindo I	4 (19.0)	0	
Dindo II	9 (42.9)	9 (34.6)	
Dindo IIIa	0	1 (3.8)	
Dindo IIIb	1 (4.8)	0	
Dindo IVa	0	2 (7.7)	
Dindo iVb	0	1 (3.8)	
Dindo V	0	1 (3.8)	
90-day mortality (%)	0 (0)	1 (3.8%)	0.99
Length of stay (median, range in days)	7 (5-27)	8 (4-32)	0.25
Resection margin (%)			
R0	11(68.7)	18 (75.0)	0.87
R1	5 (31.2)	6 (25.0)	0.71
R2	0	0	

Table 6.4. Details of stage 2 operation. †Laparoscopic. [&] Number of patients transfused.

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	PVE (n= 24)	**RALPPS** (n= 26)	*P value*
Failure of procedure (%)	8 (33.3)	2 (7.7)	0.007
Failure to progress to stage 2 resection (%)			0.661
Disease progression	2 (8.3)	2 (7.7)	
Insufficient FLRV	1 (4.1)	0	
Cross over (%)			0.103
PVE to RALPPS	0	0	
RALPPS to PVE	0	3 (12.3)	
Final resection achieved (%)	16 (66.7)	24 (92.3)	0.007

Table 6.5. Failure of procedures and cross over among PVE and RALPPS groups. Keys: NA, not available.

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6.4 Discussion

Nowadays, hepatobiliary biliary surgeons are seeing an increased number of referrals for liver resection of patients with primary and secondary liver cancer. A sizeable proportion of these referrals would traditionally have been deemed inoperable due to insufficient remnant liver volume following hepatectomy. While the technique of portal vein embolization has enabled

liver hypertrophy and subsequent hepatectomy for many previously inoperable patients, there is still significant scope from improvement. Specifically, both the final volume of liver hypertrophy and the time taken to develop such hypertrophy are important factors. An alternative method to this gold standard was proposed by Schnitzbauer *et al.* in 2012 (25). With a simultaneous right portal vein ligation combined with in situ liver splitting in patients with small FLRV, the ALPPS procedure has demonstrated a faster and greater volume hypertrophy. The mean hypertrophy rate of the FLRV reported was 74%. Unfortunately, not only morbidity was observed in 68% of the patients, including a high rate of postoperative bile leak (24%), but also an unacceptable mortality rate of 10%. To avoid bile leaks, a modification of the technique wrapping the right ischaemic liver lobe in a hermetic bag was described (370, 371), but its morbidity rate remained high at 58% (327). Surprised by the enhanced and much faster liver regeneration but high morbidity reported with this novel technique, an international online registry was set up (http://www.alpps.net). Data from this registry showed a liver failure rate as defined by the ISGLS criteria of 30% and a mortality rate of 8.8% (373). Not discouraged by these results, various groups developed alternative methods to minimize the complications. The so called “variant ALPPS” includes: Tourniquet-ALPPS, mini-ALPPS, partial ALPPS, hybrid ALPPS and the one evaluated in the present chapter, RALPPS (329, 331, 397-402). It was concluded that although there was no clear evidence that ALPPS or its variants could replace PVE or that variant ALPPS were superior to conventional ALPPS (690), there seemed to be a significant reduction in morbidity and mortality rates when comparing variant ALPPS with pure ALPPS (329). Although RALPPS

has been demonstrated as superior to PVE in a small pilot study, the full comparative efficacy profile in the medium and long term between RALPPs and PVE remains unknown (331). The RALPPS procedure is based on the same principle as ALPPS. A ligation of the contralateral portal vein is performed but avoids the *in situ* liver splitting by generating a necrotic line of demarcation with radiofrequency energy thus reducing the risk of potential visceral damage, bile leaks and bleeding. The currently presented randomised controlled trial was designed to assess these aspects in a more controlled and unbiased manner.

Up to date, this is the first randomized controlled trial comparing a modification of ALPPS technique with the gold standard PVE and the second only randomized controlled trial on ALPPS since the procedure was described. By capitalizing the main benefits of ALPPS and without increased morbidity and mortality, RALPPS achieved a significantly greater increase in liver regeneration of the FLR in a much shorter interval compared to PVE.

To increase resectability rates and rescue patients with unresectable primary and secondary liver tumours, PVE and more recently, ALPPS with its variants, are being used to induce hypertrophy of the insufficient FLRV. By occluding the portal vein of the liver lobe to be resected during both methods, the portal venous flow is diverted into the FLR and two phenomena occur: an atrophy of the occluded lobe and an increase in volume of the contralateral lobe. The predecessor of PVE was first demonstrated in the 1920s by Rous and Larimore. They showed a compensatory hypertrophy in the contralateral hepatic lobe following portal vein occlusion in a rabbit model (341). It was in

1986 when the first preoperative PVE was undertaken by Kinoshita *et al.* in patients with hepatocellular carcinoma (351).

A meta-analysis from Abulkir *et al.* included 1088 patients who underwent PVE prior to hepatic resection (355). This research work showed a mean hypertrophy rate of the FLRV after PVE of 11.9% after an average of 29 days, while in other systematic review, a volume increase as high as 39% could be achieved (326). In this meta-analysis, major morbidity from PVE was seen in 2.2%, with no mortality. After hepatic resection, the morbidity rate was 16% with a 1.7% mortality rate.

More recently, a new interventional radiology technique named the "liver venous deprivation" has achieved a volume increase comparable to ALPPS by simultaneously embolizing both right portal and hepatic veins (323).

In the REBIRTH trial, a high hypertrophy rate of 80.7 ± 13.7% in the RALPPS arm compared to 18.4 ± 9.8% in the PVE arm is shown. These results are similar to that reported in the literature after PVE (326, 355) and the 68.0 ± 38.0% increase of FLRV in the ALPPS group of the LIGRO trial (380) (Table 6.6). Postoperative complications were also comparable with PVE. There were no bile leaks after the first stage in RALPPS patients. Regarding the patients who accomplished the final hepatectomy in the second stage, all RALPPS patients were successful but this was not possible in five patients in the PVE arm. In keeping with the MD Anderson report where a significant improvement of liver hypertrophy was observed in segment 2 and 3 after additional embolization of segment 4 branch, 18 patients within the PVE group had this segment further embolized (366).

	RALPPS (n= 26)	**ALPPS**[380] (n= 48)
Stage 1		
Type of operation		
Open	0	48
Laparoscopic/Robotic	24/2	0/0
Length of operation (median, range in mins)	115 (60–225)	NA
Length of stay (median, range in days)	3 (2–17)	NA
Morbidity	23.0	NA
Mortality	0	NA
FLRV Increase (Mean±SD, %)	80.7 ± 13.7	68.0 ± 38.0
Time from Stage 1 to Stage 2 (Mean±SD, days)	20.0 ± 5.6	11.0 ± 11.0
Stage 2		
Complications grade ≥ 3b (%)	15.3	11.0
30 (90) day mortality (%)	3.8 (0)	9.1(0)
Total length of stay (Mean±SD, days)	15.3 ± 9.7	23.0 ± 17.0
Resection Rates (%)	92.3	92.0

Table 6.6. Comparison of two randomised control trials: RALPPS *versus* ALPPS (380). NA: not available.

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An important question that remains unanswered is the timing of liver resection after PVE, ALPPS or its variants. This could be explained due to the

lack of a single and wide available technique able to predict the multiple functions that the liver accomplishes. From one hand, a reduced time period between stage one and two operations could be ideal by decreasing the formation of adhesions and facilitating the second stage. From the other hand, this rapid increase of the liver volume in a short interval have shown a hypertrophy of the hepatocytes rather than a true functional gain by hyperplasia in animal models (333). Given this fact as well as the high liver failure rate of 30% reported by the ALPPS registry (373), many hepatobiliary surgeons are inclined to perform staged hepatectomy with previous PVE (691).

The RALPPS procedure showed a median of 20 days before second CT scan and completion hepatectomy, likely allowing time for a true functional gain rather than a simple increase in volume.

The insights of the greater effect in liver regeneration observed in ALPPS and its variants remains unknown. Some authors have hypothesized that this may be related to parenchyma transection with reduction in shunting or collateralization as it is the main difference with the pure portal vein occlusion either by ligation or by embolization (386, 405). In the RALPPS procedure, there is not such transection, but a virtual splitting of the hepatic parenchyma divided by an area of necrosis achieved with the radiofrequency ablation probe. Furthermore, liver regeneration has been shown to be greatly enhanced by RFA itself when compared with PVE alone in animal models (692).

It seems that either by splitting liver or dividing it with a line of necrosis, both methods may stimulate further the liver regeneration process than a portal vein occlusion alone. This is supported by the rapid regeneration

response achieved by the REBIRTH trial RALPPS group and the LIGRO trial ALPPS group, compared with the REBIRTH trial PVE patients (20, 9 and 35 days, respectively). As mentioned before by shortening the time between the two stages, there could be a benefit of a less demanding completion hepatectomy with fewer adhesions and what it can be more interesting from the oncological point of view, less time to develop *de novo* metastasis. In fact, two patients in the PVE arm had disease progression and resection was abandoned. Whether the growth of the tumours was secondary to induction of liver regeneration it is not clear.

Some limitations must be taken into consideration in this RCT.

To start with, this is a two-arm prospective single centre RCT which emerged from a small pilot study published in Annals of Surgery comparing RALPP and PVE patients with inadequate FLRV prior to hepatectomy (331). Being a novel alternative to the gold standard PVE in patients with small FLR, the RALPPS procedure was not widely spread among HPB surgeons at the time of the RTC planning and recruitment. Hence, a multi-centre or international study was not possible to be carried out.

Furthermore, the sample size was calculated from the aforementioned pilot data. This was based on the mean rates of increase in FLRV on the assumption that a RCT should be able to detect a clinically meaningful increase in FLRV. The null hypothesis was that there was no difference between the intervention and standard treatment in the primary endpoint. The sample size calculation assumed two-sided testing. The sample size of each arm was calculated using the equation designed for two proportions; α was set at 0.05 to control for

type I error (false-positive result) and β at 0.10 to control for type II error (false-negative result). Based on these data a power calculation estimated a total sample size of 32 patients, however due to the relatively small sample size of the pilot data it was decided to aim to recruit a minimum of 25 patients per arm to the trial. A final recruitment of 57 patients randomised into PVE (n= 28) and RALPPS (n= 29) techniques was achieved.

Aiming to compare two liver regeneration techniques, RALPPS and the current gold standard PVE, in patients with unresectable liver tumours and small FLR, an inclusion of a control group in this two-arm RCT was not possible.

Regarding the type of patients, different types of liver tumours were included as the primary endpoint was increase of FLRV. Furthermore, some aspects which affect the liver regeneration process in the clinical practice such as nutritional status were not investigated in this study. This may imply a potential heterogeneity in the liver restoration mechanism amongst the recruited patients independently of their allocated arm.

Additionally, patients should have been randomized to RALPPS or PVE as closer as possible as the intervention and not shortly after the HPB MDT meeting discussion.

Other methods such as the standardized FLR using the Vauthey formula, the ratio of FLRV to body weight or the kinetic growth ratio could have been used to calculate the volumetric measurement instead of the FLRV (450, 693, 694). A weakness of the trial is that there was no functional evaluation with indocyanine green test or mebrofenin scintigraphy before both stages as

volume measured by CT scan does not equal function nor conventional liver function test in blood. With regards to the PVE technique, other groups have demonstrated a greater induction of regeneration by a better occlusion and less re-vascularization using other materials such as glue (326, 695, 696). Although there was cross-over from the RALPPS group to the PVE group, there were no cross-overs from PVE to RALPPS. The three RALPPS patients who had a technically demanding laparoscopic first stage could have been converted into an open operation instead of abandoning and performing a PVE postoperatively.

Finally, outcome measures of survival and oncological benefit were not investigated in this trial, but the percentage of increase of FLRV and postoperative complications. To unveil the true effect on the oncological treatment with these procedures, future work will include a long-term survival and oncological outcome analysis between the RALPP and PVE patients recruited in the REBIRTH trial.

CHAPTER 7: GENERAL DISCUSSION

The liver is a multifunctional solid organ present in all mammals with the remarkable property of recovering from acute disasters. It is the only visceral organ able to regenerate after partial resection or chemical injury with a rapid restoration of its volume and function.

Liver regeneration constitutes a very enigmatic and complex process that has been investigated extensively in both animals and humans. In the first chapter of the present thesis, an overview of the complexity of this process including its phases, biological mechanisms and an extensive variety of factors that may alter its course is described. A summary of these factors is listed in Table 7.1 where the present work constitutes the first full genetic characterization of an ALPPS variant, the RALPPS technique and its comparison with PVE liver regeneration induction.

The intrinsic biological mechanisms involved in liver regeneration are still great a focus of interest. Rather than following the blastema-mediated epimorphic regeneration that occurs on other mammals, two different mechanisms for repair have been described depending on the type of injury. In the case of chemical or viral injury, proliferation of stem/progenitor cells (oval cells) will restore the liver and its functions. On the other hand, after trauma or partial resection, a compensatory increase in cell volume (hypertrophy) followed by replication of cells (hyperplasia) is observed (13).

Factors influencing liver regeneration process	
Patient-related	Age (lysosomal disfunction, lipid accumulation, fibrosis), nutritional status, diabetes mellitus (hepatotrophic role of insulin)
Liver-related	Biliary obstruction, intrinsic liver disease: steatosis, steatohepatitis, fibrosis and cirrhosis
Drugs and chemotherapy	Hepatic steatosis. Sinusoidal obstructing syndrome. Chemotherapy associated steatohepatitis
Type of injury/LR method	Chemical/viral (proliferation of stem/progenitor cells)
	PVL: in-flow haemodynamic changes (blood flow redirection)
	PVE: in-flow haemodynamic changes, type of embolic material, extension of embolised parenchyma (addition of segment 4 portal branches)
	Hepatectomy: trauma from manipulation/mobilization. Haemodynamic changes (clamping manouver, ischemia reperfusion injury and blood flow redirection). Interruption of liver-gut axis. Hypertrophy/hyperplasia depending on FLRV. Extent of hepatectomy. ALPPS and its variants (blood flow redirection, extension of transection line, necrotic line with RFA of microwave ablation, deportalized liver)
	Liver venous deprivation: in-flow and out-flow haemodynamic changes, cell damage, abrogation of porto-porto collaterals
	Selective internal radiation therapy: in-flow haemodynamic changes
	Liver transplant: graft's size, concomitant hepatitis B or C, portal hypertension, poor venous drainage, immunosuppression
Bile acid metabolism	Bile interaction with Farnesoid X receptor
Inflammation, cell damage and growth factors	Complete mitogens (HGF and EGFR ligands); auxiliary mitogens (IL6, FGF, TNFα, VEGF, COX-2, complement signalling) and mito-inhibitors (TGFβ)
	Bacterial infections (controversial in animal models)
	Redox status (ischemia reperfusion, cholestasis, concomitant liver

	disease)
Extrahepatic	Lung, kidney, duodenum, bone marrow, circulating platelets
Genetic	Different genetic pattern expression profiles during LR phases (animal models)
	Novel miRNA and lncRNA with different roles
	In ALPPS technique: no full genetic characterization of LR in ALPPS or its variants. Possible upregulation mTOR/AMPK pathway in ALPPS
	Current study: first full genetic characterization of LR induced by RALPPS and its comparison with PVE

Table 7.1. Summary of described factors influencing the liver regeneration process.

It has been proposed that post hepatectomy liver regeneration is a product of both hepatocyte hyperplasia and hypertrophy depending on the future liver remnant volume. Regeneration terminates precisely when the normal liver-to body-weight ratio is reestablished, gaining the organ its original size. After minor partial hepatectomy (PH) of 30% in mice, it seems that hypertrophy alone is responsible for the restoration of lost liver mass. In a 70% PH, hypertrophy precedes proliferation and at the end of the regeneration process, half of the regeneration is due to hypertrophy and the other half due to cell proliferation (697). In an experimental study of regenerative dynamics of hepatocyte hyperplasia and hypertrophy in rats subjected to different partial hepatectomies sizes (30%, 70% and 90%), general hypertrophy of hepatocytes was followed by hepatocyte proliferation only in the remnant of

70% and 90% partial hepatectomy. After 30% PH, only hepatocyte hypertrophy was induced (30).

From the genetic point of view, the process of liver regeneration can be considered crucial to maintain the liver functions and internal homeostasis of the body after diverse hepatic injury. Interestingly, genetic knockdowns in animal models have often revealed delays in the course of regeneration and no single signal has been identified to be both sufficient and necessary for liver regeneration.

Over the years, different clinical applications in both medicine and surgery have been developed taking advantage of the regenerative ability of the liver. In the surgical setting, hepatic resections for removal of liver tumours, manipulation of venous portal vein flow to increase the size of the future liver remnant for unresectable tumours or liver transplantation depend on this intrinsic phenomenon of liver restoration.

Currently, individualized surgical planning is crucial to identify optimal resection strategy and diminish the risk of post hepatectomy liver failure (PHLF) and tumour recurrence in extended liver resections for primary or secondary hepatic tumours. Preoperative investigations including liver function tests and volumetric studies may give a rough estimation of the future liver remnant (FLR) volume and function (449). Although several studies have documented peripheral blood increase of similar signals as seen in rodents (HGF, IL6, TNF, norepinephrine, serotonin), in the daily practice, induction of liver regeneration in humans is more commonly assessed by CT 3D volumetric studies and serial liver function tests in blood. Volumetric

analysis of the macroscopic atrophy and hypertrophy has been described as a tool to predict PHLF and death in patients with liver tumours, as well as post-therapy assessment of the graft following liver transplant. Patients with a FLR <20% following major surgical resection has been found to be associated with postoperative liver insufficiency and higher morbidity. The exact FLR cut-off has been proposed in various publications. Around 25% of FLR could be enough in patients with healthy liver whereas in patients in high-grade steatosis or cirrhosis, FLR needs to be >40% and 50%, respectively (16, 427).

Different techniques to preoperatively optimize an insufficient small FLR in patients with initially unresectable liver tumours have been developed. These procedures aim to avoid liver failure, improve morbidity and mortality rates and, in turn, increase resectability rates. At the present time, the most used procedure in this scenario of preoptimization of the small FLR, is the radiological procedure, portal vein embolization (PVE). Unfortunately, tumoral progression has been observed after inducing liver regeneration precluding the potential curative hepatectomy.

In 2012, the work from Schnitzbauer *et al.* of associating a liver partition to a right portal vein ligation in patients with insufficient future liver remnant revealed an unprecedented acceleration of liver regeneration. This novel surgical technique named ALPPS (associated liver partition with portal vein ligation for staged hepatectomy), revealed a greater hypertrophy of the liver remnant in comparison with the gold standard, PVE (25). However, several controversies have been related to the ALPPS technique requiring further investigations. Firstly, this method was initially associated with a high

morbidity and mortality which prompted the need of technical modifications developed by diverse groups (ALPPS variants) and the instauration of an online platform to support the data management, the ALPPS registry. Secondly, a critical analysis of the indications of ALPPS for colorectal liver metastasis, including more than four hundred cases from the international ALPPS registry, revealed that its indications seemed to be unjustified in 15% of the cases based on based on liver-to-body-weight index (LBWI >0.5) prior to stage 1 and absence of chemotherapy (<12 cycles) (396). Therefore, some authors have suggested that despite of the great potential of inducing liver growth achieved with the ALPPS technique, due to its high perioperative risks, its indications should be carefully reviewed. Thirdly, the mechanistic insights of the accelerated liver regeneration observed in the novel technique, ALPPS and its variants, are unknown. In an attempt to unveil its underlying mechanisms of accelerated regeneration, multiple animal models and different variations have been used making difficult to interpret the results. In 2019, a systematic review including 107 research articles focused on portal vein occlusion and/or associating liver partition and portal vein ligation for staged hepatectomy techniques in animals stated the need to improve the methodical quality to allow correct interpretation of preclinical findings (395). Furthermore, as described by Schadde *et al.*, some authors may have been tempted to simplify the mechanistic conclusions about ALPPS just as a difference of liver flow between the two liver lobes. It seems clear that creating a real or virtual line abrogates the communication between the right and left lobe of the liver, enhancing the regeneration observed after portal vein occlusion on its own (440). Furthermore, along with the description of the first mice model for

ALPPS in 2014, Clavien *et al.* hypothesized that there seems to be a systemic release of circulating factors during the first stage of ALPPS procedure enhancing the normal process of liver regeneration.

Nonetheless, other authors claim the advantages of the rapid regenerative process over the gold standard, PVE, despite of the main pitfalls described with the original ALPPS technique. Firstly, major tumour progression seems to be unlikely due to the short interval between the induction of liver regeneration and the final resection and, secondly, there is less time for development of troublesome adhesions facilitating the surgical dissection during the second step. Also, this short treatment may allow faster recovery and possibly a quicker start of adjuvant chemotherapy (592).

Despite of its enhanced liver regeneration, the ALPPS technique has not been widely accepted by some hepatic surgeons given its high morbidity and mortality rate, in particular from bile leaks and postoperative liver failure. Different ALPPS variants have been developed to minimize these potential complications (329, 331, 397-399, 401, 402). Among these variations, the two-stage RALPPS technique, an association of portal vein ligation with a virtual partition of the liver parenchyma by a line of necrosis generated with radiofrequency ablation is further investigated in the present research. This novel technique was firstly described by Professor LR Jiao's group in 2015 as a safe alternative for rapid liver regeneration (331). In order to compare this technique in the clinical practice with the current gold standard, PVE, a randomized clinical trial was undertaken: the REBIRTH trial. This RCT is the first clinical trial comparing the gold standard PVE with an ALPPS variant and

the second in the general setting of ALLPS and its variants. Its results are explained in more detail in Chapter 6. REBIRTH trial revealed a comparable morbidity and mortality rates with a greater increase in FLRV over a much shorter period of time with the RALPPS technique than PVE (80.7 ± 13.7% *vs* 18.4 ± 9.8%, $p< 0.001$ and 20.0 ± 5.6 days *vs* 41.6 ± 15.5 days, $p< 0.001$, respectively). Furthermore, several benefits can be applied to the novel RALPPS technique. Firstly, both first and second stages can be performed either robotic or laparoscopically. Secondly, the first stage of RALPPS allows not only enhancing the liver regeneration on the contralateral side with the portal vein ligation and the virtual splitting of the liver with radiofrequency ablation, but also the clearance of the FLR in patients with bilobar liver metastasis and small FLR. However, liver size and function are not necessarily equivalent (413). Unfortunately, certain limitations described in depth the Discussion section of Chapter 6 need to be taken in consideration from this RCT as these may affect the validity of conclusions based on the observed data and not being able to generalise to general populations. Besides, neither oncological outcomes nor long-term survival were investigated representing an essential future research work arising from this PhD.

It is interesting to mention that some authors have suggested that the degree of hypertrophy after ALPPS is not unprecedented. A kinetic growth rate observed in the FLR from living donors has been shown to be similar or even greater than the one observed after ALPPS. These authors suggested that the FLR growth rate correlates directly with its size preoperatively, the smaller the FLR, the faster the process of regeneration (698). Furthermore, a recent

comparison of volume changes and histopathologic and immunohistochemical findings in hepatocytes and bile ductules between ALPPS and living-donor liver transplantation (LDLT) with a risk for small-for-size syndrome (SFSS) suggested a similar hepatic regenerative process. There were no significant differences in the hypertrophy ratios FLR between the first stage ALPPS and in small-for-size grafts (1.702 +/- 0.407 in ALPPS *vs* 1.948 +/- 0.252 in LDLT) (*p*= 0.205). Interestingly, histologic grades for sinusoidal dilation (*p*= 0.896), congestion (*p*= 0.922), vacuolar change *(p*= 0.964), hepatocanalicular cholestasis (*p*= 0.969), and ductular reaction (*p*= 0.728) within the FLR at the second-stage operation during ALPPS or implanted graft were all similar between the groups (699).

Even if there are strong proponents to the conventional PVE such as additional embolization of segment IV portal venous branches or of the corresponding hepatic vein (367), ALPPS and its variants will still have a role to play as a potential curative tool in selected patients with advanced hepatobiliary tumours.

The knowledge of serial changes of growth factors and cytokines in peripheral blood after partial hepatectomy in humans is scarce and mainly extrapolated from animal models (46). During the priming phase, quiescent hepatocytes are prepared to move into mitosis by IL6 and TNFα secreted by macrophages. Hepatocyte cell cycle progression will be triggered by the release of the complete mitogens, HGF, EGF and TGFα, and enhanced by other auxiliary mitogens. Proliferating hepatocytes will then produce many other growth factors targeting other non-parenchymal cells such as TGFα, FGFs,

TNFα and VEGF. To ensure an adequate internal homeostasis with well-adjusted liver volume and functions, antiproliferative factors like TGFβ will stop cell proliferation during the less researched termination phase (16, 208).

A few groups have investigated these biomarkers' variations post hepatectomy either after resections of liver tumours or after living liver donations (569, 574-579). Furthermore, there is even more limited literature focused on the serial changes of circulating growth factors and cytokines during induction of liver regeneration by either portal vein occlusion or with the more novel technique ALPPS and its variants (580). Clavien *et al.* generated a rat ALPPS model and hypothesized the presence of circulatory growth factors responsible for a much faster liver regeneration than PVL on its own (32). Only a recent comparison has been made for the assessment in peripheral plasma of growth factors associated with liver regeneration in patients who underwent ALPPS and PVE procedures by Sparrelid *et al.* (580).

In Chapter 4, a prospective observational experiment aimed to find differences in the peripheral plasma levels of relevant circulating factors for liver regeneration including HGF, EGF, FGF, IL6, TNFα, TGFα, VEGF and TGFβ before and after liver regeneration with either PVE or RALPPS in a total of 23 patients.

In keeping with the observations made by Sparrelid *et al.* where plasma levels of IL6 increased on postoperative day one after ALPPS in comparison to baseline (p= 0.004) (580), similar findings were observed in the RALPPS group. Concentrations of IL6 were significantly higher post stage one in the RALPPS procedure than preoperatively in the RALPPS group (p= 0.047). A raise of IL6

levels were also detected post stage one in the PVE group, although this change did not reach statistical significance (p= 0.417) (Table 4.12). This modification of IL6 was also found post right lobe hepatectomy in healthy liver donors by Sasturkar *et al.* (575).

On the contrary, no significant differences were found in the comparisons of peripheral concentrations levels before stage one and before stage two in both groups PVE and RALPPS (Table 4.13). The lack of statistic difference could be explained for some reasons. Firstly, a small sample size of patients could have been not enough to reveal subtle differences of concentrations in the peripheral plasma. Secondly, peripheral blood levels may not represent a more accurate intrahepatic or portal blood levels. Thirdly, and likely the most plausible explanation, could be that the process of liver regeneration with its associated modifications in plasma levels occurred between the two time points, returning to similar baseline levels prior second stage.

Unexpectedly, a higher baseline level of HGF in the PVE group than the RALPPS group was detected (p= 0.006) (Table 4.14). The nature of this finding is difficult to explain as none of the patient had underlying hepatic disease. Looking into the raw data, all RALPPS patients had HGF concentrations below 100 pg/ml in comparison with the majority of the PVE patients where the minimum and maximum quantities detected were 60.92 pg/ml and 245.97 pg/ml, respectively (Tables 4.10 and 4.11). Whether plasma levels before stage one are different between RALPPS and PVE groups within this mitogenic factor, it is arduous to establish whether the differences between pre stage

one and other time points (less than 48 hours and pre second stage) can be a bias.

Comparisons within 48 hours after inducing liver regeneration with RALPPS or PVE, revealed no statistically significant differences among all the cytokines or growth factors plasma concentrations, except for VEGF. Interestingly, levels of VEGF, a factor produced by proliferating hepatocytes and involved mainly in neoangiogenesis during later stages of regeneration (16, 47), were higher post stage one in PVE in comparison with the undetectable levels of VEGF at the same time point in RALPPS (p= 0.037). Regarding this biomarker, Sparrelid *et al.* found significantly diminished VEGF levels after both stages of ALPPS to then increase after four weeks post stage two (580).

In the present experiment, regarding the most potent and complete mitogens, HGF and EGF, both had higher peripheral plasma concentrations after stage one of RALPPS procedure in comparison with the PVE group, although this did not achieve statistical significance. Observing HGF levels, the group of Sparrelid *et al.* detected an increase on day one post ALPPS with a peak before the second operation without statistical significance (580). In contrast, in the RALPPS group, we observed an increase of HGF after stage one with a decline before the second operation. In the PVE group, levels of HGF decreased after occluding the portal vein and increased before the second operation. None of these changes reached statistical significance. The other potent mitogen, EGF, decreased on day one post op and was also lower before the second operation in the Sparrelid's ALPPS cohort. In this research, the PVE group showed a similar trend regarding the plasma EGF levels, but not the

RALPPS group as EGF increased after first stage and decreased before second stage.

The inhibitor of hepatocyte proliferation, TGFβ, expected to have higher levels at the end of the liver regeneration process, was found to have higher levels in the PVE group at all times in comparison with the RALPPS group although these differences did not reach statistical significance (Tables 4.5 ,4.6 and 4.7).

Prior the second stage, none of the biomarkers showed any significant changes within the two different techniques. High levels of IL6 and TNFα were observed in the PVE group in comparison with the RALPPS group (Table 4.8).

To unveil further the liver regeneration process, genetic expression profiles of liver regeneration after partial hepatectomy have been explored mainly in animal models. Very little here is known about the liver transcriptome during liver regeneration in humans including the underlying genetic mechanisms behind the accelerated liver regeneration observed after ALPPS or its modifications. To date no full genetic characterisation of liver regeneration in ALPPS or its variants has been performed in humans. Only a few groups have investigated in more detail, mainly animal models, the genetic changes observed in the future liver remnant after ALPPS to improve the understanding of this accelerated liver regeneration and to help in refinement of the procedure for clinical benefit (32, 581).

In Chapter 5, changes of the liver transcriptome achieved after RALPPS and PVE are presented. In addition, the effect of liver regeneration by RALPPS in

the metastatic tissue from the deportalized liver lobe was investigated at a genetic level.

In different group comparisons, some of the significantly expressed genes lack of specific known functions and very little information was found in the current literature. This is the case in the RALPPS group for the significantly downregulated *RPL23AP2* and *SNORD3D* in the regenerated tissue; and the upregulated *RPL41P1* and downregulated *AC159540.1* in right-sided colorectal liver metastasis after liver induction. In addition, the downregulated *C3P1* and *RP11-108K14.4*, along with upregulated RPL41P1 genes in the comparison of regenerated tissue after RALPPS *vs* PVE, had no function stablished. Therefore, as none of these genes have been associated to the course of liver regeneration previously, these findings suggest possible novel roles within the restoration process depending on the different scenarios.

The effect of RALPPS technique was investigated in both liver lobes, regenerated and non-regenerated or deportalized. As mentioned earlier, two genes with no stablished roles in liver regeneration were found significantly downregulated in the regenerated tissue in comparison with the normal liver parenchyma, *RPL23AP2* and *SNORD3D*. None of these genes were significantly deregulated in the regenerated tissue post PVE, conferring them a more specific role in the accelerated liver regeneration stimulated by RALPPS.

The influence of the deportalized liver lobe in PVE and ALPPS in the regeneration process remains unknown. Necrosis, hypoxia and subsequent inflammatory response have been some of the mechanisms aiding the regeneration process described by different groups after portal vein occlusion,

ALPPS and its variants. With a complete lack of direct communication between the two liver lobes in ALPPS procedure or the presence of a necrotic line between the liver lobes in RALPPS, the influence of the deportalized liver in the speed observed after these techniques remains unclear. Unfortunately, in the present research, no significant differences at a genetic level were identified between the normal liver parenchyma and the occluded liver lobe tissue after RALPPS. The downregulated *MT-CO2,* also named *COX-2*, which in normal situation promotes cell growth and inhibits apoptosis, could explain a logical inhibition of cell proliferation and angiogenesis in favour of cell apoptosis in the occluded liver lobe after RALPPS, although this finding was not statistically significative (*adjusted p-value=0.094).*

Tumour progression after induction of liver regeneration has been a worrisome situation precluding potential curative hepatic resections. When investigating the effect of RALPPS in the colorectal liver metastasis in the ligated liver lobe at a genetic level, three genes were found to be differentially expressed genes: *RPL41P1*, *CLDN2*, and *AC159540.1*. The first two were found upregulated and the latter, down regulated. As already mentioned, *RPL41P1* and *AC159540.1* have not been mentioned in the literature and its function has not been described. Interestingly, the protein coding *CLDN2* gene has been extensively investigated. Its encoded protein, Claudin 2, is a paracellular channel protein localised at tight junctions and overexpressed in liver tissue with roles in proliferation and migration. This is an interesting finding in line of how liver regeneration can induce or trigger tumour progression. Therefore, upregulation of *CLDN2* may reflect a possible induction of metastatic

progression produced by RALPPS. It is important to stablished that in the present experiment, the effect in the tumoral progression of metastatic tissue in PVE tissue was not investigated so we cannot conclude that the liver regeneration provoked by RALPPS influences in the tumour progression of the already present metastatic disease more than PVE.

When analysing the regenerated liver tissue by PVE at a genetic level, the pathway analysis of the significantly expressed 32 genes revealed enrichment in lipid metabolism pathways as well as PPAR signaling pathways. Accumulation of lipids from adipose tissue lipolysis is a well-known phenomenon to provide energy substrate for the cell proliferation during hepatic regeneration. Furthermore, both lipid metabolism as *PPAR* signaling have been linked to termination of the liver regeneration process suggesting that the liver tissue regenerated by PVE at the moment of the specimen collection was in a termination phase of the liver regeneration process. Whether liver regeneration induce by PVE induces tumour proliferation or progression with the present results, a pathway involved in the tumourigenic process, the transcriptional misregulation in cancer pathway was found to be enriched using KEGG database in PVE group. This finding is consistent with the already known evidence about the tumour progression observed and investigated after induction of liver regeneration, with portal vein occlusion, either with embolization or ligation at staged hepatectomies (338, 362, 465-469).

As shown in the presented RCT, RALPPS procedure achieved a faster liver regeneration with a greater increase of the FLRV when compared to PVE

(Table 6.3). In order to unveil its underlying differences, genetic profiles of regenerated tissue after RALPPS and PVE were compared. Fourteen genes were significantly deregulated in these two procedures. Amongst the upregulated genes found in RALPPS tissue, *CCL3* and *HLA-H* are involved in inflammatory response, suggesting a possible higher inflammatory response secondary to RALPPS than in PVE. The upregulated *RPL12* gene encoding a ribosomal protein which plays an important role in "rRNA processing in the nucleus and cytosol" and, in turn, in protein synthesis, may reflect a higher need of protein synthesis in the RALPPS group than in PVE. Furthermore, upregulated *SSPO* may avoid protein degradation as protein synthesis may be reduced in PVE. A very interesting finding is the upregulation of several genes involved in tumourigenesis in the PVE group in comparison with RALPPS: *LINC00319; TRIM29* and *TACC2.* This suggest that PVE could trigger tumourigenesis, tumour progression and from the clinical point of view, a poorer prognosis. In line with this finding the REBIRTH trial revealed final completion hepatectomy in the PVE arm in comparison with RALPPS (PVE, 66.7% *vs* RALPPS, 92.3%, *p*= 0.007) with a significant failure rate of PVE was found (PVE, 33.3% *vs* RALPPS, 7.7%, *p*= 0.007) (Table 6.5). From the 5 patients who did not proceed to completion hepatectomy, two patients had tumoral progression and three did not achieve an adequate increase of FLR. Two genes upregulated in the liver tissue of patients with non-alcoholic fatty liver disease, *PLIN5* and *SNORD3D,* were also upregulated in the PVE group in comparison with RALPPS. This is in keeping with the physiological steatosis observed after partial hepatectomy and the enhanced lipid metabolism observed in latter phases of liver regeneration.

Considering the results presented in this thesis, the enhanced liver regeneration observed with RALPPS indicates that this is a safe two-staged procedure with some benefits over the current gold standard for liver induction, PVE. Although PVE may induce a more mature liver regeneration and a lesser inflammatory response, significant expression of genes favouring tumourigenesis were identified along with a worse final completion hepatectomy rate than RALPPS.

CHAPTER 8: CONCLUSIONS AND FUTURE DIRECTIONS

The liver is the only visceral organ that can fully regenerate after injuries caused by viruses, toxins, ischemia, surgical resections or transplantation and furthermore, it is capable in maintaining its multiple functions and internal homeostasis according to the body needs.

Liver regeneration is a very complex but well-orchestrated process requiring the involvement of many intra and extrahepatic factors.

The focus on unveiling the intrinsic mechanisms of liver regeneration and its applications in medicine and surgery has greatly increased in the past years.

Several methods are used to induce liver regeneration and preoptimize patients with small liver remnants requiring extended hepatectomy for clearance of primary or secondary liver tumours in the clinical practice.

Among these methods, the novel two-stage technique described by Schnitzbauer *et al.*, ALPPS procedure, has shown an unprecedented liver hypertrophy with restoration of the liver mass enabling resection of otherwise unresectable liver tumours in a shorter period of time in comparison with the current gold standard, portal vein embolization (PVE).

Postoperative complications such as liver failure along with inability to complete the second resectional stage associated initially with ALPPS resulted in the need for development of modifications on the technique, the ALPPS variants.

The RALPPS procedure emerged as a modification of the ALPPS technique where the transection of the parenchyma is substituted by a necrotic line generated with a radiofrequency ablation probe during the first stage. Both stages of RALPPS can be performed with minimally invasive surgery, laparoscopic or robotically.

Currently, there is little insight to explain the molecular and genetic mechanisms associated to the accelerated regeneration observed in ALPPS and its variants in comparison with the portal vein occlusion on its own.

In the present thesis, mechanisms of liver regeneration for preoperative induction of liver hypertrophy prior to major liver resection in PVE and RALLPS patients were investigated including serial changes of plasma levels of relevant biomarkers for liver regeneration at different time points, genetic characterization of liver tissue before and after the procedures and a randomised clinical trial comparing the two techniques PVE and RALPPS.

Comparisons of serial changes of plasma levels of relevant biomarkers for liver regeneration in RALPPS and PVE patients showed statistically significant difference ($p \leq 0.05$) in three situations. Firstly, IL6 levels were found to be significantly higher <48h after stage one in comparison with baseline levels in the RALPPS group ($p = 0.047$) suggesting a major inflammatory response at this stage in the RALPPS technique. Secondly, unexpected higher HGF levels were found in the PVE group in comparison to the RALPPS group before inducing liver regeneration ($p = 0.006$) of unknown significance. Thirdly, VEGF levels were higher after 48 hours in the PVE group than the RALPPS group ($p = 0.037$).

The rest of the biomarkers' comparisons amongst the groups did not reach statistically significant difference ($p \leq 0.05$).

Alike ALPPS, RALPPS is a good model to unfold the molecular and genetic mechanisms that govern the accelerated liver mass restoration as it enables taking liver biopsies at both stages, before and after induction of liver regeneration. This can also be performed in patients who undergo a two stage hepatectomy with preoperative PVE. Genetic characterization of liver tissue before and after PVE and RALPPS was undertaken in this research work. Deregulations of expression of several protein coding and non-coding RNA genes, including pseudogenes, were identified after NGS.

Two novel genes in the field of liver regeneration were significantly downregulated in the regenerated liver after RALPPS procedure *RPL23AP2* (*adjusted p-value*= 0.038) and *SNORD3D* (*adjusted p-value=* 0.038) suggesting a possible role in inhibition of cell proliferation.

The effect of RALPPS in the occluded liver lobe did not reveal any statistically significant differences at a genetic level. Although downregulated *MT-CO2* (*adjusted p-value*= 0.094) could imply enhancement of cell death and suppression of hepatocyte proliferation.

The influence of ALPPS and its variants in the tumoral tissue from the deportalized liver lobe remains unclear. In this PhD, CRLM in the deportalized liver lobe after RALPPS revealed a statistically significant upregulation of *CLDN2* (*adjusted p-value*= 8.60E-05), a gene involved in proliferation and migration. A similar role could be conferred to the novel downregulated

pseudogene *RPL41P1* (*adjusted p-value*= 1.62E-05) and roles in tumour suppression or cell death to the long-noncoding gene *AC159540.1* (*adjusted p-value*= 0.006). Hence, as other types of liver regeneration techniques, RALPPS *per se* could induce tumour progression in the metastatic tissue.

Significantly deregulated genes in favour of tumourigenesis and lipid metabolism were demonstrated in the PVE group. Enriched lipid metabolism pathways in PVE suggest the timing of terminal phases of liver regeneration.

When comparing RALPPS and PVE at a genetic level, the upregulated genes in the restored liver tissue by RALPPS, *CCL3* and *HLA-H*, along with the downregulated *NECAB2,* could imply a higher inflammatory response in RALPPS than the generated after PVE. A lesser protein synthesis with an enhanced peptidase inhibitor activity to avoid protein degradation could be suggested in PVE given the downregulated *RPL12* and upregulated *SSPO*. Upregulation of genes involved in tumorigenesis (*LINC00319; TRIM29*; *TACC2)* in PVE indicate a pro-oncogenic tendency of PVE in comparison with the liver regeneration induced by the RALPPS procedure. Upregulation of *PLIN5* confirms the importance of lipid metabolism during liver regeneration and potential higher lipid accumulation in the PVE than in RALPPS. Furthermore, enriched lipid metabolism pathways present in terminal phases of liver regeneration in the PVE group suggests a more mature phase of liver regeneration than RALPPS. In addition, two pseudogenes with no assigned function, *C3P1* and *RP11-108K14.4*, were found upregulated in PVE suggesting a role in the process of liver regeneration. Regenerated RALPPS tissue seems to be in a less mature phase than PVE It can be possible that the LR induced by

RALPPS is in an earlier stage than PVE where tumourigenesis genes are not yet expressed. It is possible that RALPPS achieves a more immature regenerative status than PVE where tumourigenesis genes are not yet expressed.

Results of the first randomized clinical trial (RCT) on PVE and an ALPPS variant since the ALPPS procedure was first described in 2012 are presented in this PhD, the REBIRTH trial. In line with the unprecedented speed in liver regeneration observed after ALPPS, this RCT demonstrated a greater and faster increase of the FLRV with similar morbidity and mortality in the RALPPS arm than in the PVE arm (FLRV 80.7 ± 13.7% *vs* 18.4 ± 9.8%, p< 0.001; and time from liver induction to CT volumetry prior second stage (20.0 ± 5.6 days *vs* 41.6 ± 15.5 days, *p*< 0.001; in RALPPS and PVE, respectively) (413). In order to apply its results to the general population, certain limitations must be taken in consideration.

In the same manner as during the ALPPS procedure, both first and second stages in RALPPS can be successfully performed with minimally invasive surgery and furthermore, their first stage not only allows a faster liver regeneration in a small FLR than PVE, but also a clearance of tumoural tissue in the FLR in patients with bilobar liver disease (413).

In line with the genetic findings where PVE seems to enhance tumourigenesis, a significant failure rate of PVE was found (33.3%) where two PVE patients had tumour progression, compared to RALPPS (7.7%) (*p*= 0.007). As a consequence, fewer patients in the PVE arm had the final completion hepatectomy in comparison with RALPPS (PVE, 66.7% *vs* RALPPS, 92.3%, *p*= 0.007) (413).

In light of the results presented in this thesis, RALPPS is a safe technique which induces a faster liver regeneration with likely higher inflammatory response and protein synthesis than PVE. In addition, RALPPS seems to achieve a less advanced phase of LR where tumourigenesis genes are not yet expressed in comparison with PVE.

Further studies are needed to address the molecular and genetic mechanisms of the observed acceleration of liver regeneration with ALPPS and its variants, including the RALPPS technique, in comparison with the gold standard for preoperative liver induction, PVE. With this regard, future work could include similar research methods than the presented experimental work, but with a greater sample size. Addition of extra time points for patients' blood collection (e.g. 6h and 12 hours after inducing liver regeneration) could be beneficial in the identification of potential changes in plasma concentrations of biomarkers between the two mentioned techniques. A histologic analysis of hyperplasia and hypertrophy within the liver cells to enlighten the controversy whether LR is secondary to hypertrophy or hyperplasia in RALPPS could also be included. Given that the degree of hypertrophy observed after ALPPS is not unprecedented and a similar or even higher kinetic growth has been demonstrated in living donors than the one observed in ALPPS, it would be interesting to see if there are any differences between three scenarios of liver restoration: living donors, ALPPS and RALPPS with both volumetric and genetic profiling studies. Furthermore, this study should take into consideration other known factors that may affect the liver regeneration process *per se* in the clinical practice such as nutritional status,

age, diabetes or even drugs. Finally, outcome measures of survival and oncological benefit were not selected as endpoints of interest in the RTC protocol, but the percentage of increase of FLRV and postoperative complications in RALPPS and PVE patients. Hence, given its clinical relevance and to capture the true effect on the treatment for these oncological patients, future work arising from this PhD will include a long-term survival and oncological outcome analysis of the RALPP and PVE groups.

In summary, a better understanding of the intrinsic mechanisms involved in the liver regeneration inherent to the techniques used prior to major liver resections in patients with liver tumours and small FLR, such as PVE, ALPPS or its variants like RALPPS, is crucial to refine these procedures in order to enhance the regeneration process, prevent postoperative liver dysfunction, minimise or eliminate the risk of tumour progression, improve resectability rates and, overall, achieve better patient safety and survival.

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Appendix

	Patient	ARN	ALN	ART	ALT	BRN	BLN	BRT	BLT
RALPPS	**1**	36	35		76	28	30	29	
RALPPS	**2**	37	60				57	58	
RALPPS	**3**	65	39			66	46	47	
RALPPS	**4**	22	23	21	24	54	55		
RALPPS	**5**	27,68	26,69, 74		31	75			1
RALPPS	**6**	25	53	49	50,73	51	45	59	
RALPPS	**7**	33	32			41	43	42	
RALPPS	**8**		34		70				
RALPPS	9	56							
RALPPS*	10	40	44			52	48		
TOTAL		**9**	**9**	**2**	**5**	**7**	**7**	**5**	**1**
PVE	1					15	16		
PVE*	2					6	7		
PVE	3	19	20	17	18				
PVE*	4					67	8		
PVE	5						13		
PVE	6					61	1		
PVE	**7**	63	11	12		38	2	3	10
PVE	**8**	5	4	9	62				
TOTAL		**3**	**3**	**3**	**2**	**5**	**6**	**1**	**1**

Supplementary table A.1. Detailed distribution of numbered tissue samples sent for mRNA sequencing after quality check (marked in yellow). Each sample was assigned a number during RNA extraction. Any empty cell means there is no sample collected. Samples written in red were not amenable for NGS due to inadequate quality/quantity.

Keys:
ALN: first stage, left normal liver tissue; ALT: first stage, left tumoral liver tissue; ARN: first stage, right normal liver tissue; ART: first stage, right tumoral liver tissue; BLN: second stage, left normal liver tissue; BLT: second stage, left tumoural liver tissue; BRN: second stage, right normal liver tissue; BRT: second stage, right tumoural liver tissue.

* Patients with non-colorectal liver metastasis (one ovarian; two cholangiocarcinoma).

	PVE (n= 24)	**RALPPS** (n= 26)	*P value*
Bilirubin			
Pre-op	13.7± 14.2	9.9 ± 5.1	0.211
Post-op			
D1	34.9 ± 20.8	35.1 ± 25.8	0.982
D3	29.6 ± 10.6	36.9 ± 30.1	0.279
D5	34.0 ± 22.6	34.45 ± 29.3	0.961
ALP			
Pre-op	151.2 ± 97.1	132.9 ± 89.7	0.502
Post-op			
D1	98.5 ± 46.7	101.6 ± 52.8	0.849
D3	117.5 ± 56.7	115.7 ± 53.7	0.918
D5	229.8 ± 123.3	228.4 ± 151.1	0.976
ALT			
Pre-op	50.7 ± 60.9	34.6 ± 22.7	0.243
Post-op			
D1	628.6 ± 292.1	532.3 ± 351.4	0.381
D3	458.4 ± 249.6	383.2 ± 268.4	0.388
D5	207.6 ± 108.6	162.6 ± 94.9	0.198
Albumin			
Pre-op	35.9 ± 2.8	33.7 ± 5.4	0.132
Post-op			
D1	25.7 ± 6.7	27.6 ± 6.4	0.363
D3	29.6 ± 5.7	31.1 ± 7.1	0.477
D5	30.5 ± 5.9	31.7 ± 7.7	0.612

Supplementary table A.2. Liver function following major hepatic resection.

Keys:
Pre-op: preoperatively; post-op: postoperatively; D1, 3, 5- Days 1, 3 and 5 post completion major hepatectomy; Bilirubin shown in umol/L; ALT – alanine transaminase, shown in IU/L; ALP - alkaline phosphatase, shown in IU/L; Albumin shown in g/dL; P-values for the Mann-Whitney U test are shown where *p<0.05* is considered statistically significant.
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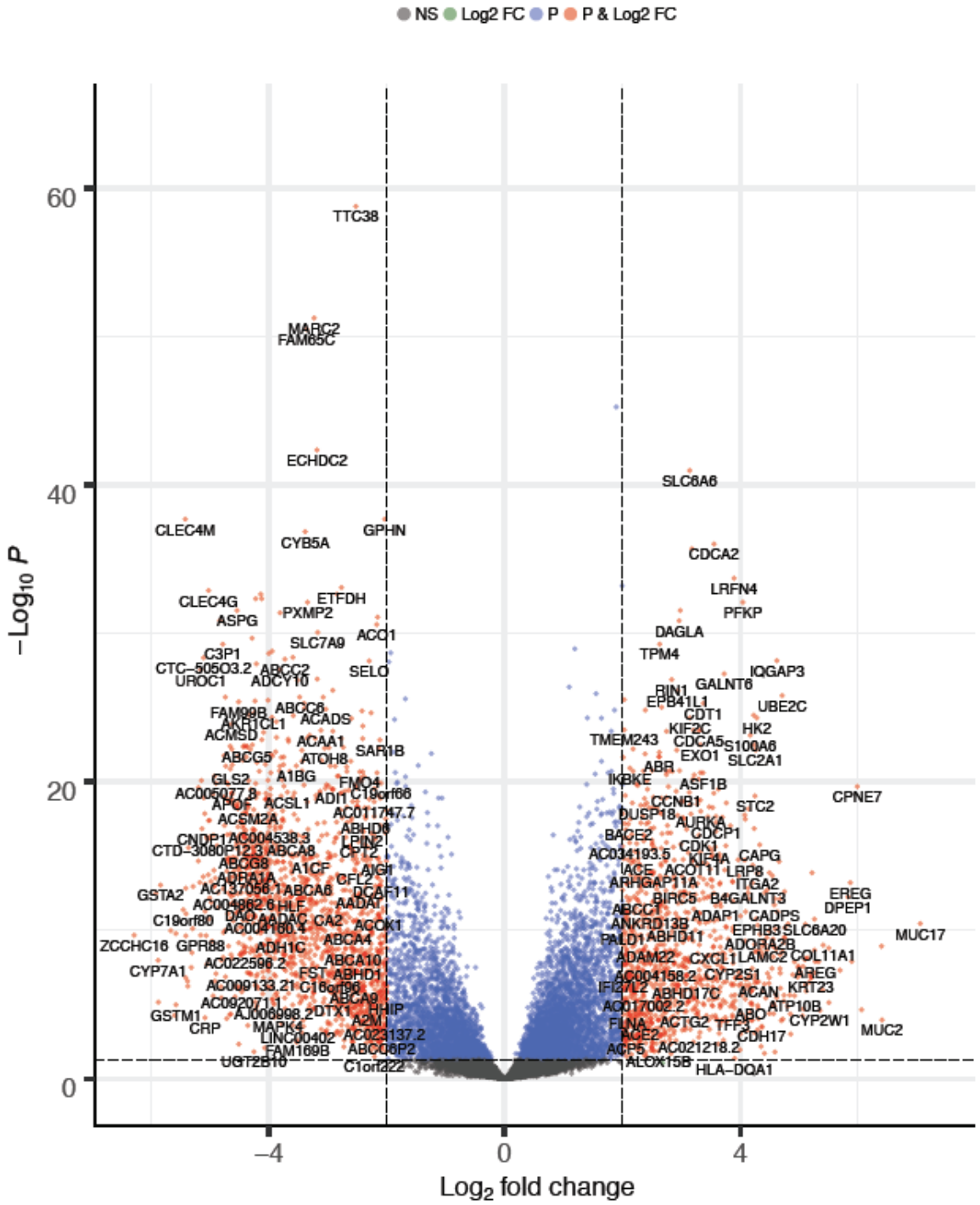
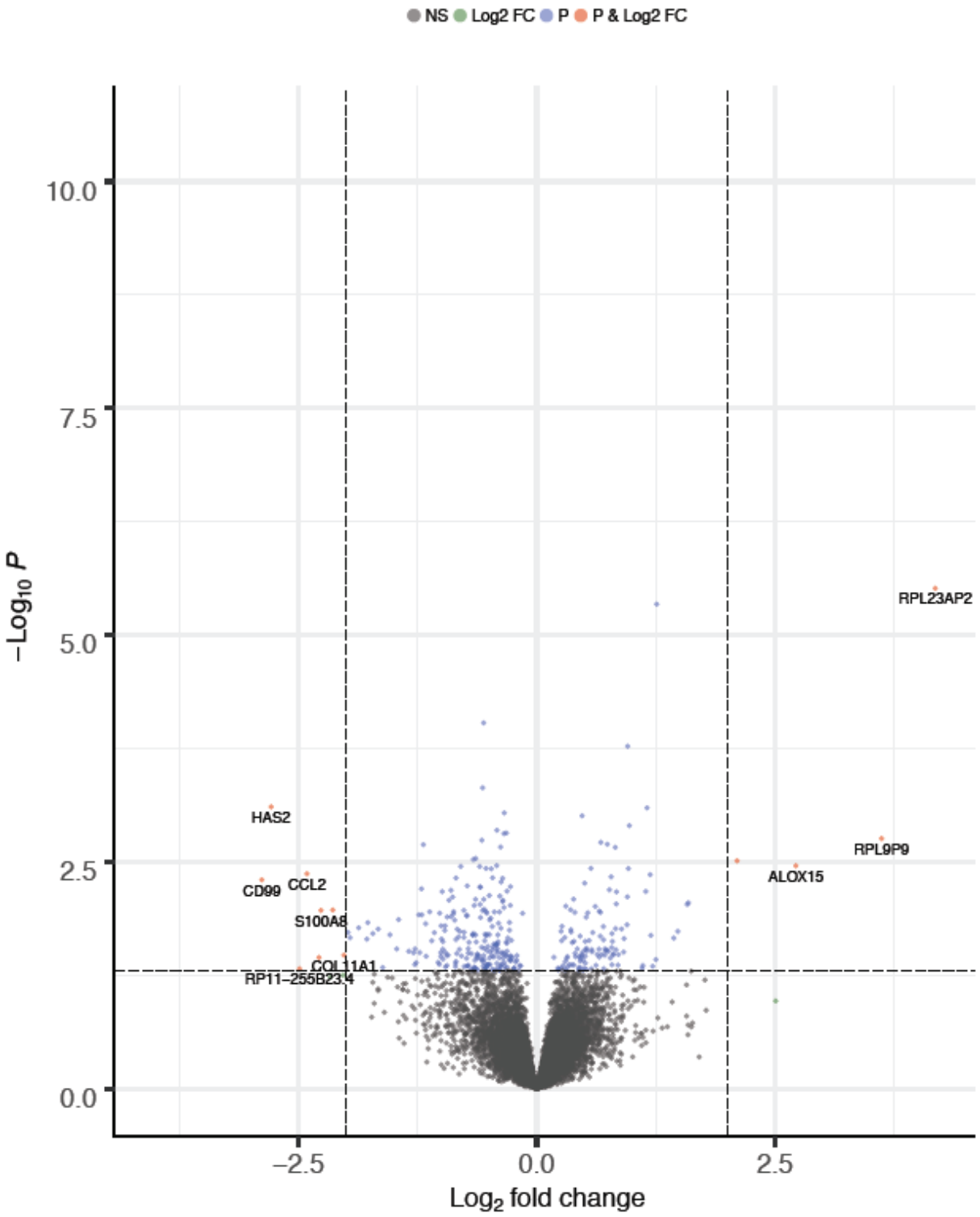
	PVE (n= 24)	**RALPPS** (n= 26)	*P value*
Hb			
Pre-op	122.5 ± 28.3	127.3 ± 14.6	0.456
Post-op			
D1	105.9 ± 20.7	99.1 ± 22.9	0.362
D3	100.5 ± 20.8	95.9 ± 13.1	0.414
D5	102.5 ± 16.3	90.7 ± 21.2	0.077
CRP			
Pre-op	37.2 ± 55.1	18.7 ± 53.7	0.283
Post-op			
D1	66.4 ± 35.8	55.6 ± 29.3	0.309
D3	139.8± 59.8	159.1 ± 69.9	0.38
D5	127.22 ± 61.2	116.6 ± 57.4	0.593
PT			
Pre-op	10.7 ± 0.6	15.9 ±10.7	0.385
Post-op			
D1	14.0 ± 2.2	13.5 ± 1.7	0.479
D3	13.8 ± 2.7	13.9 ± 2.0	0.847
D5	12.7 ± 2.5	12.7 ± 1.4	0.816
APTT			
Pre-op	25.1 ± 2.7	28.1 ± 6.8	0.445
Post-op			
D1	26.4 ± 2.6	26.9 ± 2.9	0.597
D3	29.9 ± 4.4	29.3 ± 4.1	0.659
D5	27.6 ± 3.0	28.8 ± 3.7	0.386

Supplementary table A.3. Haemoglobin, CRP levels and clotting screen following major hepatic resection.

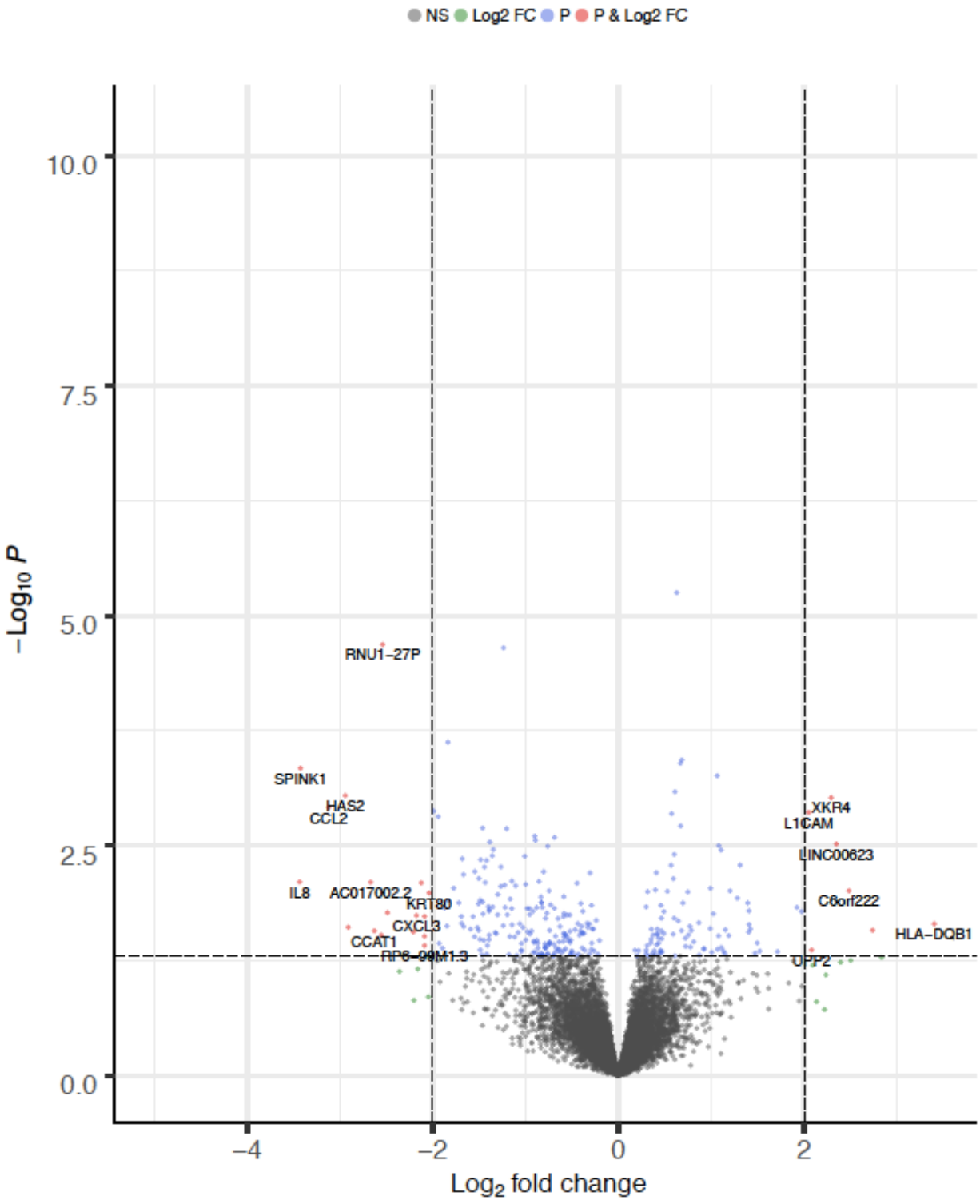
Keys:
Pre-op: preoperatively; post-op: postoperatively; D1, 3, 5- Days 1, 3 and 5 post completion major hepatectomy; Hb-haemoglobin, shown in g/dl; CRP- c-reactive protein, shown in mg/L; PT-prothrombin time, shown in seconds, APTT-activated partial thromboplastin time, shown in seconds. P-values for the Mann-Whitney U test are shown where $p<0.05$ is considered statistically significant.
Adapted from *Jiao LR et al., Cancers (Basel) 2019 Mar; 11(3): 302.* Open access article. Attribution 4.0 International (CC BY 4.0). http://creativecommons.org/licenses/by/4.0/



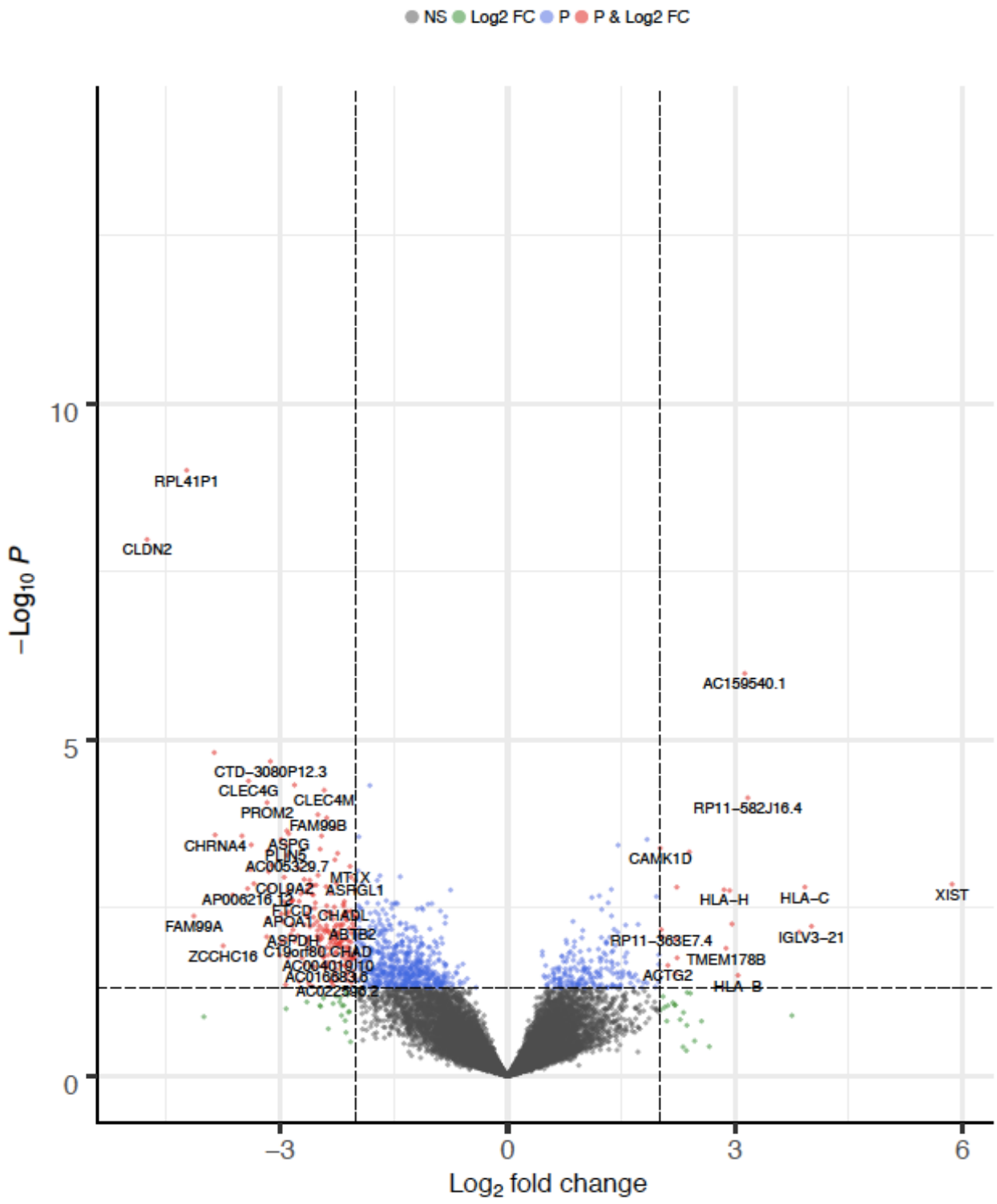
Supplementary figure A.1 Volcano plot of mRNA in normal vs colorectal liver metastatic tissue samples (ALN *vs* ALT/ART comparison).



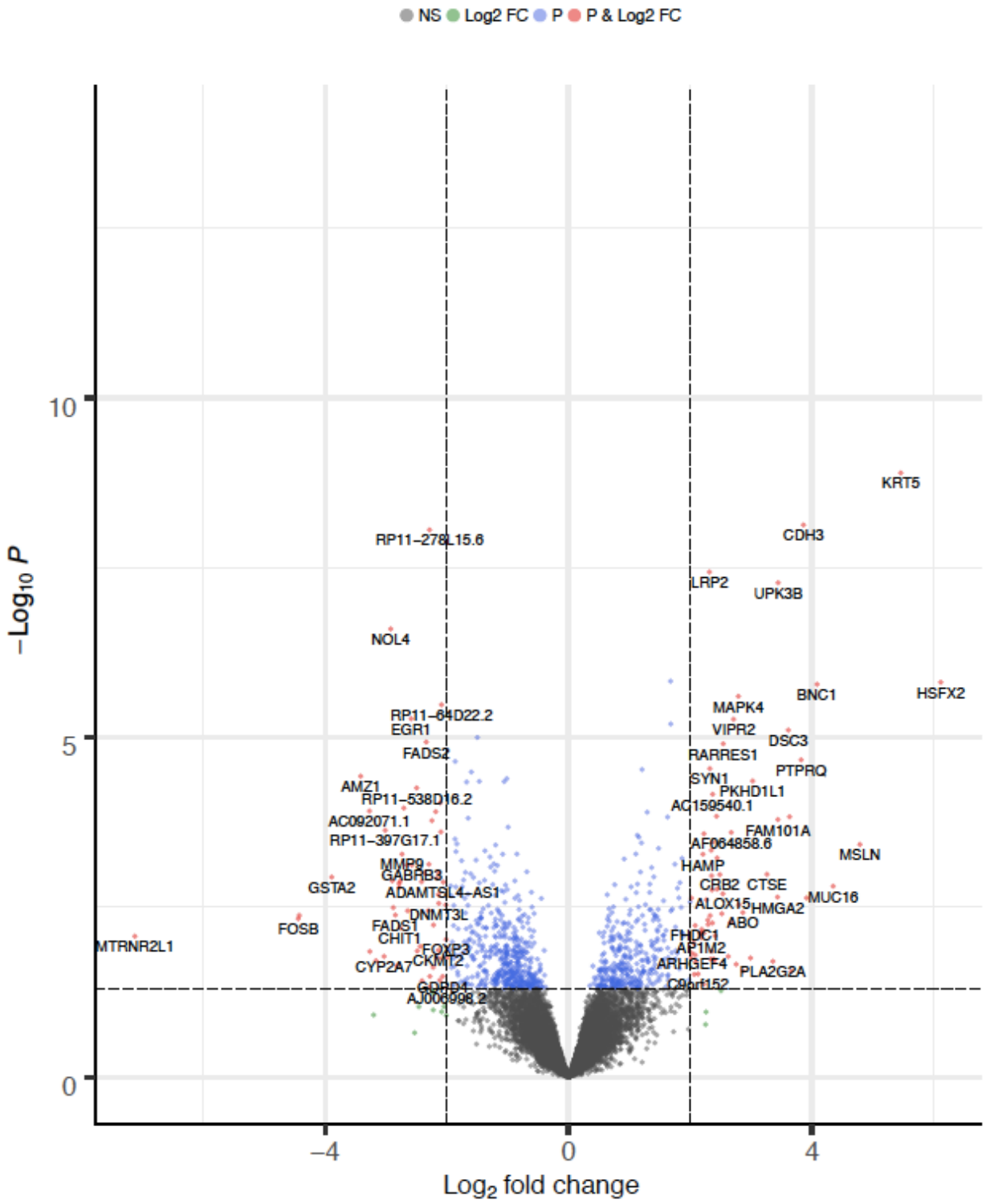
Supplementary figure A.2. Volcano plot of RALPPS ALN (n= 6) *vs* BLN (n= 5) comparison.



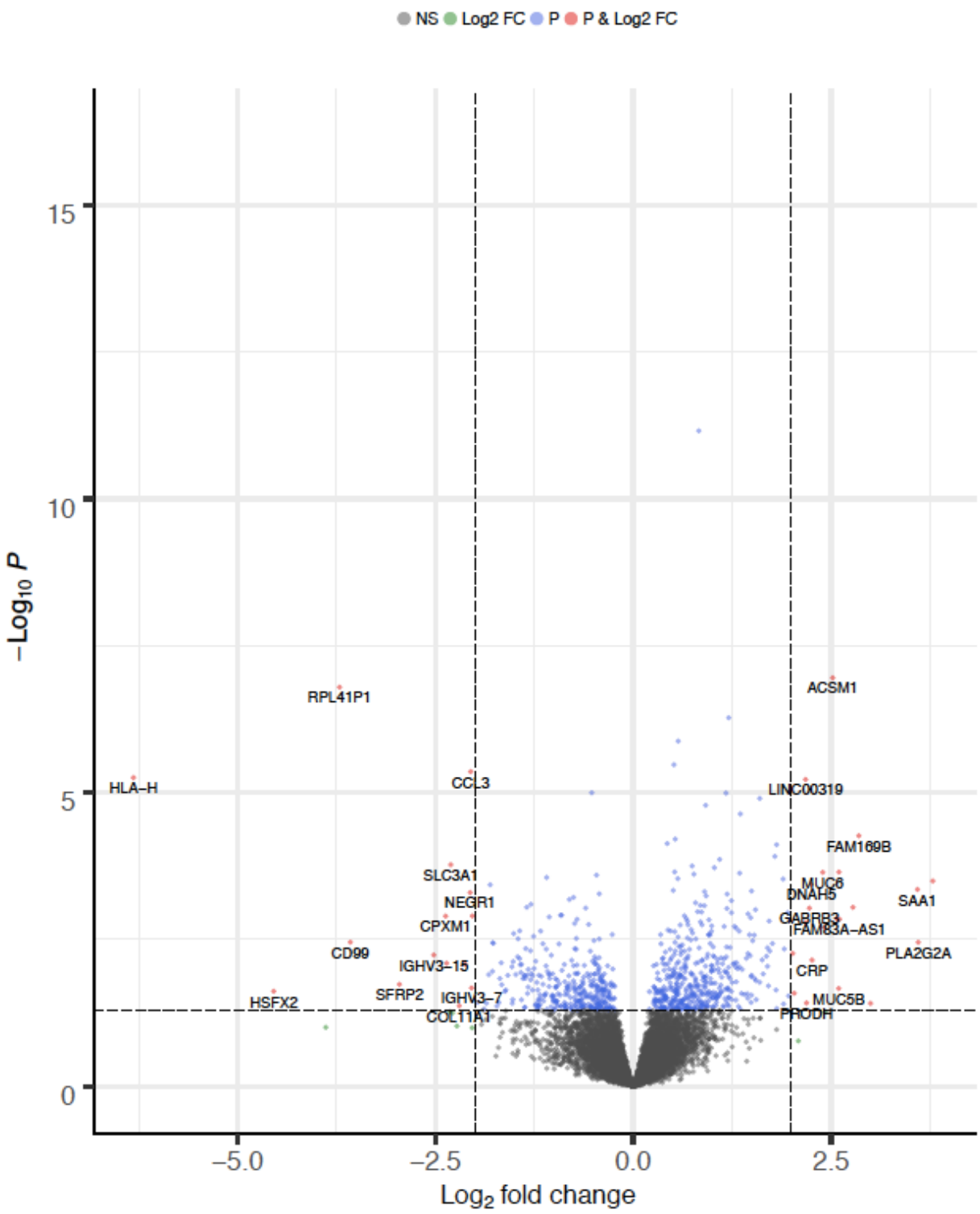
Supplementary figure A.3 Volcano plot of RALPPS ALN (n= 6) *vs* BRN (n= 5) comparison.



Supplementary figure A.4. Volcano plots representing the comparison ALT/ART (n= 6) *vs* BRT (n= 3) in RALPPS patients.



Supplementary figure A.5. Volcano plots representing the comparison ALN (n= 3) *vs* BLN (n= 4) in PVE patients.



Supplementary figure A.6. Volcano plots comparison BLN PVE (n= 4) *vs* BLN RALPPS (n= 5) patients.

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