

Faculté de médecine et médecine dentaire

**Mechanisms of liver regeneration in *RAPID* –
a novel model of auxiliary liver transplantation**

The role of endothelial progenitor cells

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1. List of abbreviations

ALDAPT	<i>Associating Living-Donor Auxiliary partial orthotopic liver transplantation and right Portal vein ligation for Total staged hepatectomy</i>	Id1	<i>Inhibitor of differentiation 1</i>
ALPPS	<i>Associating Liver Partition and Portal vein ligation for Staged hepatectomy</i>	IL	<i>Interleukin</i>
ANOVA	<i>Analysis of variance</i>	IMDM	<i>Iscove's Modified Dulbecco's Medium</i>
BM	<i>Bone marrow</i>	INR	<i>International normalised ratio</i>
BM-SPC	<i>Bone marrow sinusoidal progenitor cell</i>	IRS	<i>Immunoreactivity score</i>
CA9	<i>Carbonic anhydrase 9</i>	LSEC	<i>Liver sinusoidal endothelial cell</i>
CD	<i>Cluster of Differentiation</i>	LT	<i>Liver transplantation</i>
Clec (-4g, 4m)	<i>C-type lectin domain family member</i>	MAC	<i>Myeloid angiogenic cell</i>
diff.	<i>difference</i>	MCP1	<i>Monocyte chemoattractant protein 1</i>
DL	<i>Deportalised liver</i>	M-CSF	<i>Macrophage-colony stimulating factor</i>
DRL	<i>Deportalised right liver</i>	ML	<i>Marginal liver</i>
ECFC	<i>Endothelial colony-forming cell</i>	OLT	<i>Orthotopic liver transplantation</i>
ECM	<i>Extracellular matrix</i>	OPS	<i>Orthogonal Polarisation Spectral (imaging)</i>
EPC	<i>Endothelial progenitor cell</i>	PBMC	<i>Peripheral blood mononuclear cells</i>
EPC-CFA	<i>Endothelial progenitor cell colony forming assay</i>	PHLF	<i>Post-hepatectomy liver failure</i>
EPO	<i>Erythropoietin</i>	POD	<i>Post-operative day</i>
FACS	<i>Fluorescent-activated cell sorting</i>	RAPID	<i>Resection And Partial liver segment 2–3 transplantation with Delayed total hepatectomy</i>
FGF	<i>Fibroblast growth factor</i>	RCF	<i>Relative centrifugal force</i>
FISH	<i>Fluorescent in situ hybridization</i>	RPM	<i>Revolutions per minute</i>
FLR	<i>Future liver remnant</i>	SDF1a	<i>Stromal cell-derived factor 1</i>
G-CSF	<i>Granulocyte-colony stimulating factor</i>	SFSS	<i>Small-for-size syndrome</i>
GFP	<i>Green fluorescent protein</i>	TGF-β1	<i>Transforming growth factor-beta 1</i>
GRWR	<i>Graft-to-recipient weight ratio</i>	uPA	<i>urokinase-type Plasminogen Activator</i>
HABR	<i>Hepatic artery buffering response</i>	VAP	<i>Vascular adhesion protein</i>
Hb	<i>Hemoglobin</i>	VCAM	<i>Vascular cell adhesion molecule</i>
HCC	<i>Hepatocellular carcinoma</i>	VEGF(a)	<i>Vascular endothelial growth factor (alpha)</i>
HGF	<i>Hepatic growth factor</i>	VEGFR	<i>VEGF receptor</i>
HIF	<i>Hypoxia-inducible factor</i>	WAT	<i>White adipose tissue</i>
HUVEC	<i>Human umbilical vein endothelial cells</i>	Wnt	<i>Wingless-type MMTV integration site family member</i>
ICAM	<i>Intercellular adhesion molecule</i>		

2. Abstract

2.1. In English

Background

The RAPID¹ surgery represents a major breakthrough in liver transplantation, enabling the successful transplantation of small grafts well below conventional size thresholds (GRWR² = 0.8-1%) without inducing small-for-size syndrome (SFSS). This innovative two-stage procedure begins with the resection of the recipient's left liver, which is replaced with a small graft (GRWR < 0.5%) from a living donor. Simultaneously, the right portal branch is ligated, exposing the graft to pro-regenerative portal hyperflow. Approximately 14 days later, the graft regains sufficient volume and functionality to allow completion hepatectomy without triggering SFSS.

SFSS is a form of post-transplant hepatocellular failure arising from two primary vascular phenomena: first, the abrupt redirection of portal flow to a reduced parenchymal mass induces shear stress that damages the hepatic sinusoids. Subsequently, liver regeneration is marked by an imbalance between excessive hepatocyte proliferation and insufficient or delayed neoangiogenesis. Therefore, the clinical success of RAPID in avoiding liver dysfunction, despite significant graft growth and portal hyperflow, prompts a comprehensive exploration of the mechanisms underlying sinusoidal restoration.

In this context, endothelial progenitor cells, known for their potent pro-angiogenic activity, emerge as potential key mediators. This term encompasses both bone marrow-derived myeloid angiogenic cells (MAC), which contribute to angiogenesis in a paracrine manner, and endothelial colony-forming cells (ECFC), which are derived from the vessel wall and physically integrate into new vessels.

Methods

RAPID transplant recipients were included in our prospective single-centre research protocol, called ALDAPT³. They were transplanted for liver metastases from colorectal or neuroendocrine cancer. Biological samples, including liver biopsies and peripheral blood, were collected between the two surgical stages. Living donors undergoing a 40% hepatectomy served as a control group for conventional liver regeneration.

During the critical interstage time frame of the RAPID, systemic levels of pro-angiogenic and chemotactic cytokines were measured by ELISA. The number of circulating endothelial progenitor cells was assessed by flow cytometry, and their ability to form colonies by cell culture. The integration of exogenous sinusoidal cells into the graft was assessed in a sex-mismatched transplantation setting, using a dual FISH X and Y chromosome labelling combined with endothelial immunostaining.

Results

After RAPID transplantation, ALDAPT recipients exhibited a significant increase in G-CSF levels compared to donors (+8.29 pg/mL, $p=.02$), particularly on the first post-operative day (POD) ($p=.04$). However, no systemic elevation of VEGFa or chemotactic cytokines was observed.

Additionally, the number of circulating MACs increased as early as POD2 ($p=.03$), with more robust recruitment occurring in the recipients than in the donors (absolute increase +14.7% of blood mononuclear cells, $p=.04$). Similarly, ex-vivo MAC colony-formation demonstrated a significant increase, albeit with some delay, starting around POD5 ($p=.03$). Colony counts remained comparable between donors and recipients.

In contrast, ECFC levels remained unchanged. They scarcely engrafted into the graft's vascular network, accounting for only 3.06% of sex-identified endothelial nuclei.

In conclusion, our study highlights a systemic pro-angiogenic conditioning in RAPID recipients, characterised by a predominance of paracrine MACs over engrafting ECFCs in sinusoidal restoration mechanisms. In the innovative context of this transplantation, our results encourage future in-depth characterisation of the endothelial physiology, which could orchestrate both graft regeneration and function.

¹RAPID: Resection And Partial liver segment 2–3 transplantation with Delayed total hepatectomy

²GRWR: Graft-to-Recipient Weight Ratio

³ALDAPT: Associating Living-Donor Auxiliary partial orthotopic liver transplantation and right Portal vein ligation for Total staged hepatectomy

2.2. En Français

Introduction

L'intervention chirurgicale RAPID représente une avancée majeure dans le domaine de la transplantation hépatique, car elle permet de réaliser avec succès des greffes de petite taille, bien en-deçà des seuils conventionnels (GRWR = 0,8-1%), sans induire de small-for-size syndrome (SFSS). Cette procédure innovante se déroule en deux étapes. Elle débute par la résection du foie gauche du receveur, qui est remplacé par un petit greffon (GRWR < 0,5%) issu d'un donneur vivant ; simultanément, la branche portale droite est ligaturée, exposant ainsi le greffon à un hyperdébit portal qui stimule sa régénération. Environ 14 jours plus tard, le greffon récupère un volume et fonctionnalité suffisants que pour permettre la totalisation de l'hépatectomie sans déclencher de SFSS.

Le SFSS est une forme de défaillance hépatocellulaire post-greffe résultant de deux phénomènes vasculaires principaux : initialement, la déviation subite du flux portal vers une masse parenchymateuse réduite induit des contraintes de cisaillement endommageant les sinusoides hépatiques. La régénération du foie qui s'ensuit est marquée par le déséquilibre entre une prolifération hépatocytaire excessive par rapport à une néoangiogenèse insuffisante ou retardée. La transplantation RAPID, permettant d'éviter ce dysfonctionnement hépatique en dépit d'une croissance importante du greffon et d'un hyperdébit portal, incite donc à une investigation approfondie des mécanismes qui sous-tendent la restauration sinusoidale.

Dans ce contexte, les cellules progénitrices endothéliales, réputées pour leur puissante activité pro-angiogénique, pourraient jouer un rôle crucial. Ce terme englobe d'une part les cellules angiogéniques myéloïdes (MAC) issues de la moelle osseuse, qui contribuent à l'angiogenèse par voie paracrine ; et d'autre part les cellules endothéliales formant des colonies (ECFC), qui proviennent des parois vasculaires et s'intègrent physiquement dans les nouveaux vaisseaux.

Méthodes

Nous avons inclus les transplantés RAPID dans notre protocole de recherche monocentrique et prospectif nommé ALDAPT. Les indications de transplantation comprenaient des métastases hépatiques de cancer colorectal ou neuroendocrine. Des échantillons biologiques, notamment des biopsies hépatiques et du sang périphérique, ont été prélevés entre les deux étapes chirurgicales. Les donneurs vivants, subissant une hépatectomie de 40 %, ont servi de groupe de contrôle représentant la régénération hépatique conventionnelle.

Pendant la période critique de régénération entre les deux stades chirurgicaux, les taux systémiques de cytokines pro-angiogéniques et chimiotactiques ont été mesurés par ELISA. Le nombre de cellules progénitrices endothéliales circulantes a été évalué par cytométrie de flux, et leur capacité à former des colonies par culture cellulaire. L'intégration de cellules sinusoidales exogènes au sein du greffon a été évaluée dans un contexte de transplantation non appariée par sexe, en utilisant un double marquage FISH des chromosomes X et Y combiné à une immunohistochimie endothéliale.

Résultats

Après la transplantation RAPID, les receveurs ALDAPT ont présenté une augmentation significative des taux de G-CSF par rapport aux donneurs (+8.29 pg/mL, $p=.02$), survenant dès le premier jour post-opératoire (POD) ($p=.04$). Cependant, aucune élévation systémique de VEGFa ou de cytokines chimiotactiques n'a été observée.

En outre, le nombre de MACs circulantes a augmenté dès le POD2 ($p=.03$), avec un recrutement plus important survenant chez les receveurs que chez les donneurs (augmentation absolue de +14,7% des cellules mononucléaires sanguines, $p=.04$). De même, la formation de colonies de MACs ex vivo s'est également significativement majorée au cours de la période post-opératoire ; bien qu'avec un certain délai, à partir de POD5 ($p=.03$). Le nombre de colonies est demeuré comparable entre les donneurs et les receveurs.

En revanche, les taux d'ECFC sont restés inchangés. Leur intégration au sein des parois vasculaires du greffon est demeurée minime, représentant seulement 3.06% des cellules endothéliales sexuellement identifiées.

En conclusion, notre étude met en évidence un conditionnement systémique pro-angiogénique chez les receveurs RAPID, avec prédominance des MACs paracrines par rapport aux ECFC dans la restauration sinusoidale du greffon. Dans le contexte novateur de cette transplantation, nos résultats encouragent une future caractérisation approfondie de la physiologie endothéliale, susceptible d'orchestrer à la fois la régénération et la fonction du greffon.

3. Introduction

3.1. The liver

The liver, weighing approximately 1.5 kilograms in healthy adults, stands as the largest glandular organ within the human body. Its anatomical position and histological microstructure contribute to its critical role in fulfilling numerous vital functions (1).

Anatomy of the liver

Successful hepatic surgery relies on a precise understanding of the functional anatomy of the liver. Back in 1897, Cantlie was the first anatomist to propose a functional left-right division of the liver based on the distribution of the portal branches. This separation, known as Cantlie's line, runs from the gallbladder to the inferior vena cava (1,2). However, this partition isn't uniform and usually reflects a right-left parenchymal weight ratio of 2/3-1/3 or even 3/4-1/4 (3). This particular mass repartition poses specific challenges in living donor transplantation.

In a further development, French surgeon Couinaud presented a classification that divides the liver parenchyma into eight distinct functional vascular units, called segments. These can be individualised according to their specific connections to the vascular and biliary systems (4).

Each of these segments is vascularised by a dual blood source consisting of a single branch of the portal vein and of the hepatic artery. After processing by the liver, the blood is then collected by three hepatic veins that run along the boundaries between the segments, before finally emptying into the inferior vena cava. In addition, a single bile duct is responsible for draining the bile produced in each segment and directing it to the hepatic hilum (4) (*Fig. 1*).

Microstructure and histology of the liver

The liver parenchyma is organised into microscopic functional units called lobules (*Fig. 1bis A&B*). Roughly hexagonal in shape, these lobules consist mainly of rows of hepatocytes also referred to as Remak spans. These hepatocyte cords are interspersed with highly permeable and porous capillaries known as liver sinusoids. At each corner of the lobule lies a portal space containing a bile duct, a branch of the hepatic artery and from the portal tract, a vagal nerve bundle, and lymphatic vessels all surrounded by connective tissue. The core of the lobule is represented by a centrilobular vein, which ultimately drains into a hepatic vein (4,6).

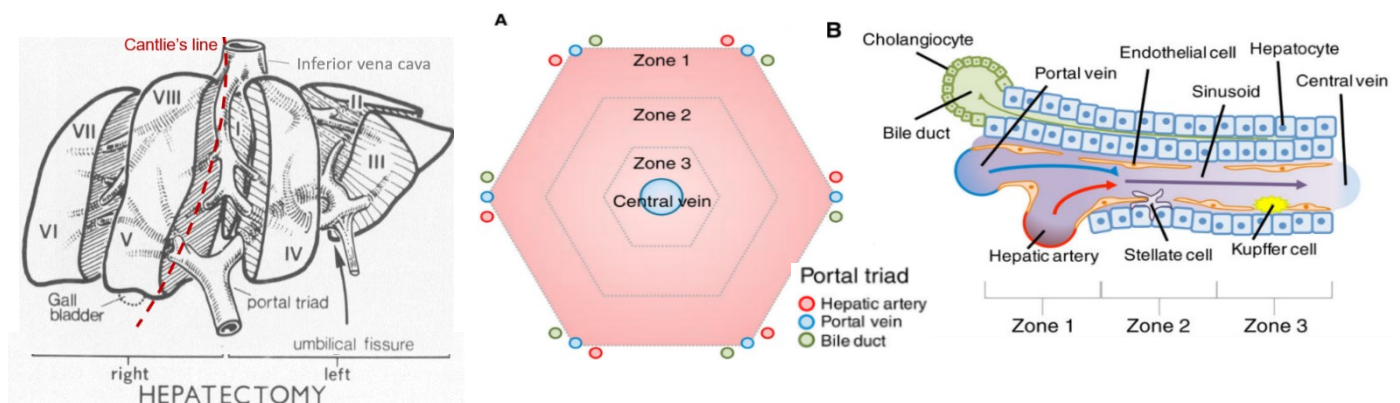


Figure 1. Exploded view showing the segments of the liver (I to VIII). Adapted from Blumgart, 1988 (5)

Figure 1bis. Organization of the liver. From Trefts et al., 2017 (6)
A. Geometric representation of a hepatic lobule
B. Schematic representation of a sinusoid within the liver

Thus, this microstructural organisation provides each lobule with a remarkable dual blood supply, which plays an important role in liver physiology. While the portal branches deliver deoxygenated but nutrient-rich blood from the digestive organs and the spleen, the artery rather delivers oxygenated blood and serves as the primary oxygen supply to the liver (7).

Moreover, a regulatory mechanism known as the Hepatic Artery Buffering Response (HABR) adds complexity to this vascular interaction (8). This compensatory phenomenon aims to prevent excessive shifts in perfusion by balancing arterial blood flow in response to changes in portal pressure. As portal flow increases, the artery undergoes vasoconstriction to balance the overall downstream perfusion; conversely, a decrease in portal venous flow triggers arterial buffering. As arterial blood represents the main source of oxygen to the liver, an excessive triggering of the HABR phenomenon could therefore result in tissular hypoxia (9,10). This mechanism holds particularly relevance in the context of liver surgery where excessive portal flow occurs, as it is thought to contribute to the development of small-for-size syndrome (SFSS), a type of liver failure that can arise after transplantation or major hepatectomy.

Then, at the edge of the portal mesenchyme, the mixed blood enters the liver sinusoid network. The latter is lined by liver sinusoidal endothelial cells (LSECs), which play a significant role in modulating vascular tone. This function complements the HABR as a secondary defence mechanism against fluctuations in blood flow and pressure. This vasoregulation involves Ito stellate cells acting as contractile pericytes, along with endothelial cells secreting vasoactive substances (11,12).

Beyond their role in vessel tone modulation, LSECs play a crucial role in many other hepatic functions. Their fenestrated structure and lack of a basal membrane facilitate direct communication between the blood and the underlying hepatocytes, allowing efficient nutrient uptake, hormone and metabolite trafficking (13). This close proximity also enables LSEC to modulate hepatocyte function, zonation, and proliferation through paracrine signalling (14).

Finally, LSECs partake in various immune functions, which makes them central in the response to liver injury - including surgery. For instance, they actively contribute to the recruitment of inflammatory cells by expressing adhesion molecules like VCAM and ICAM-1, CD31, VAP1 (12). In collaboration with specialized Kupffer macrophages, LSECs also clear diverse wastes from the blood, including pathogens translocated from the digestive tract (15). To efficiently perform this function, they exhibit a high endocytic capacity, facilitated by the membrane expression of scavenging receptors such as CD36, stabilin 2, Clec4g and Clec4m, C206 mannose receptor) (16–18). Notably, these distinctive features prove valuable for identifying LSECs by immunostaining.

Main physiological and metabolic functions of the liver

Hepatocytes, also known as parenchymal cells, constitute approximately 80% of the liver mass and therefore represent its predominant cell type. Within the architecture of the liver lobule, hepatocytes align along the sinusoids and encounter a decreasing gradient of oxygen concentration toward the centrilobular vein. This spatial arrangement, known as zonation, determines three distinct zones that are each characterized by different predominant functions (19).

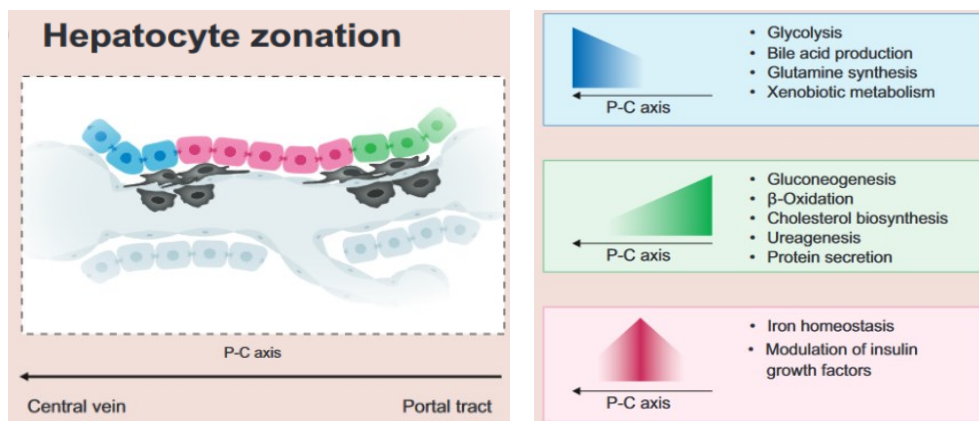


Figure 2. Liver zonation and predominant hepatocytic functions in relation to their spatial distribution. From Manco et al., 2021. (19)

Nevertheless, hepatocytes are responsible for meeting the metabolic needs of the organism by performing the full spectrum of essential liver functions. First and foremost, these cells synthesise numerous plasmatic proteins, including albumin, mediators of the immune response and almost all coagulation factors (6).

In addition, they orchestrate the homeostasis and storage of vital nutrients and vitamins. For instance, they regulate glycemia through glycogen synthesis and neoglucogenesis, participate in lipid metabolism, and control iron metabolism through hepcidin secretion (20). These processes are facilitated by the liver's particular anatomical position, as it receives nutrient-rich portal blood directly downstream from the digestive tract (6).

Moreover, hepatocytes manage the detoxification and elimination of a wide array of metabolites, ranging from xenobiotics (particularly in zone 3) and drugs to endogenous metabolites such as bilirubin or neurotoxic ammonium. Furthermore, the liver assumes an exocrine role, continually releasing bile components from the apical pole of hepatocytes into the bile canaliculi they delineate. The liver also performs several endocrine functions such as production and release of IGF1 and hepcidin amongst others, as well as up to 10% of the overall EPO production (21). The liver is also a site for biotransformation of thyroid hormones (6).

Given the vital nature of these functions, the organism meticulously maintains the functional parenchymal liver mass in precise proportion to total body mass and metabolic demand. Thus, in response to injury, the liver has the unique ability to regenerate rapidly up to 100% of its original volume (22).

In the context of liver surgery, this imperative liver function is reflected in the necessity for a minimal parenchymal mass to remain after resection or transplantation - a construct termed the future liver remnant (FLR). For safe hepatectomy, a minimum FLR of at least 20% of the initial liver volume is deemed essential, although this requirement may increase in cases of underlying liver dysfunction (e.g., chronic liver disease or patient characteristics). In the context of liver transplantation, the FLR is calculated in relation to the recipient's body weight; an appropriate FLR is typically defined by a graft-to-recipient weight ratio (GRWR) exceeding 0.8%. Falling below these thresholds increases susceptibility to a post-operative liver failure, known as small for size syndrome (SFSS) (23).

3.2. Mechanisms of liver regeneration

While knowledge of the mechanisms of liver regeneration has been derived primarily from animal studies, particularly in rodent models, human-based evidence in this area remains scarce. Nevertheless, several hypotheses based on extrapolation are supported by the observation that human hepatocytes in cell culture respond to similar growth stimuli as their rodent counterparts (24).

Multiple overlapping pathways have been identified in liver regeneration. Orchestrated by various cell types, they collectively aim to restore a precisely controlled mass of functional parenchyma, giving rise to the concept of the “*hepatostat*” as coined by Michalopoulos et al. (22). For example, in rats subjected to a 70% hepatectomy or the transplantation of a 30% FLR, the liver fully recovered its initial volume within 10 days (24).

This increase in volume is achieved through different mechanisms depending on the extent of parenchymal resection. In cases of minor hepatectomy, where the FLR represents approximately 70% of the initial volume, regeneration occurs primarily through hypertrophy without significant involvement of cell division. However, in instances of major hepatectomy or small liver transplantation where the FLR is less than 30%, a substantial proportion of hepatocytes enter the cell cycle (up to 95% in the particular ALPPS⁴ surgical setting (25)) and roughly half complete the mitotic process (26).

Participation of liver sinusoidal endothelial cells in acute liver regeneration

As mentioned above, LSECs represent the most abundant non-parenchymal cell type in the liver, playing a critical role in supporting and regulating liver function (27). Studies have unsurprisingly shown that endothelial apoptosis can impede liver regeneration, while the administration of angiogenic inhibitors or stimulators can significantly modulate it (28). Therefore, our primary focus will shift to the involvement of LSECs in liver regeneration after hepatectomy or partial liver transplantation.

⁴ ALPPS: Associating Liver Partition and Portal vein ligation for Staged hepatectomy

Origin of LSECs

Typically quiescent under homeostatic conditions, LSECs respond to acute liver injury or resection by initiating proliferation to restore an appropriate sinusoidal network. Cell damage and shear stress caused by change in flow and pressure are thought to be the stimuli for such a response.

The origin of proliferative LSECs has been attributed to three potential sources (9,29). The primary pool includes mature LSECs residing within the capillary walls, which undergo division and migration to restore sinusoidal integrity (30–33). Intrahepatic endothelial progenitors represent another contributor to the LSEC population. However, their role appears to revolve around homeostatic cell turnover rather than acute regeneration (14). Thirdly, endothelial progenitor cells (EPC) primarily from the bone marrow are recruited to the liver and have been proposed to play an essential role in acute liver regeneration after injury (34–36).

However, disentangling the individual contributions of these cellular contingents remains challenging due to their indistinguishable morphology once they differentiate within the liver microenvironment (37).

Contribution of LSECs to parenchymal restoration

Liver regeneration is a finely tuned process in which LSECs play a crucial role in regulating hepatocyte proliferation. The growth of liver parenchyma involves an interplay of partially redundant molecular pathways, including complete mitogens such as hepatocyte growth factor (HGF) and additional accessory mitogens (22).

Of particular note, HGF is widely recognised as the most potent complete hepatocyte mitogen. Under normal physiological conditions, HGF is sequestered in large amounts in an inactive monomeric form within the extracellular matrix (ECM) (38,39). However, when homeostasis is disrupted by an acute parenchymal loss, such as in hepatectomy or partial liver transplantation (LT), several consequential events occur.

The immediate events involve hemodynamic changes: as the entire portal flow is redirected towards a diminished vascular network, sinusoidal pressure increases. This subjects LSECs to shear stress, triggering mechanotransduction and initiating various priming mechanisms for hepatocytic proliferation (40).

During the initiation phase, LSECs release nitric oxide (NO) in response to shear stress, sensitising hepatocytes to the mitogenic effects of HGF (41). Moreover, LSECs indirectly release active HGF by upregulating their production of urokinase-type plasminogen activator (uPA). This metalloproteinase activator degrades the ECM scaffold, liberating trapped HGF and activating it into its functional heterodimeric form (38,39).

Proliferating hepatocytes then initiate the secretion of vascular endothelial growth factor (VEGF), further supporting regeneration through several pathways. Primarily, VEGF binds to the VEGFR2 expressed by LSECs ; this triggers an intracellular transduction cascade resulting in the upregulation of the inhibitor of differentiation 1 (Id1) transcription factor (42), which is responsible for the secretion of hepatotropic mitogens. The latter encompass HGF and Wingless-type MMTV integration site family member 2 (Wnt2), both of which further enhance hepatocyte proliferation (40,42). It is important to note that some researchers attribute the stimulation of LSEC-derived HGF secretion to the VEGFR1 pathway (43).

Moreover, rising VEGF acts as a potent endocrine stimulus to attract bone marrow-derived progenitor cells that further enhance the regenerative process. Interestingly, Wang et al. suggested that recruited bone marrow-derived progenitors may serve as the primary source of endothelial HGF and Wnt2, whereas mature LSECs produce negligible amounts, similar to most other endothelial cells (36,44). Finally, VEGF is known for triggering intrahepatic LSEC proliferation via the VEGFR2 receptor (32,33), initiating the subsequent angiogenic phase.

The second phase of regeneration is dominated by angiogenic restoration. During this period, typically peaking between postoperative days 3 and 6 in rats (45), the vascular network adapts and expands to accommodate the increased parenchymal mass. Interestingly, regeneration is therefore marked by a temporal mismatch between early hepatocyte- and delayed LSEC proliferation.

Parenchymal cells play an active role in this process; in particular, clusters of newly proliferating hepatocytes encounter a relatively hypoxic microenvironment, triggering the activation of the HIF1a signalling pathway. As a result, several pro-angiogenic and vasculotropic factors are secreted, including VEGF, angiopoietins, FGF1 and 2. Correspondingly, the receptors for these factors are upregulated on LSECs. This orchestrated response, driven by hypoxia-associated growth signals, not only stimulates the proliferation of pre-existing mature LSECs but is also thought to attract bone marrow-derived progenitor cells that further enhance the regenerative process (28,31,46).

Ultimately, the termination of liver regeneration occurs when the parenchymal mass has been restored. Though the 'hepatostat' sensing mechanisms regulating this step remain incompletely understood, LSECs are thought to play a role in halting the proliferative process, notably through their upregulation of TGF- β 1. In addition to its direct mitostatic effect on hepatocytes (47), TGF- β 1 also stimulates the secretion of ECM proteins that bind HGF, effectively attenuating its local activity (22) and contributing to the restoration of a homeostatic state.

In conclusion, this intricate interplay involving LSECs at all stages of liver regeneration underscores their indispensable role in orchestrating the regenerative process. A marked disruption of this delicate balance may result in reduced parenchymal growth, highlighting the importance of studying and optimising LSEC function during hepatic regenerative events.

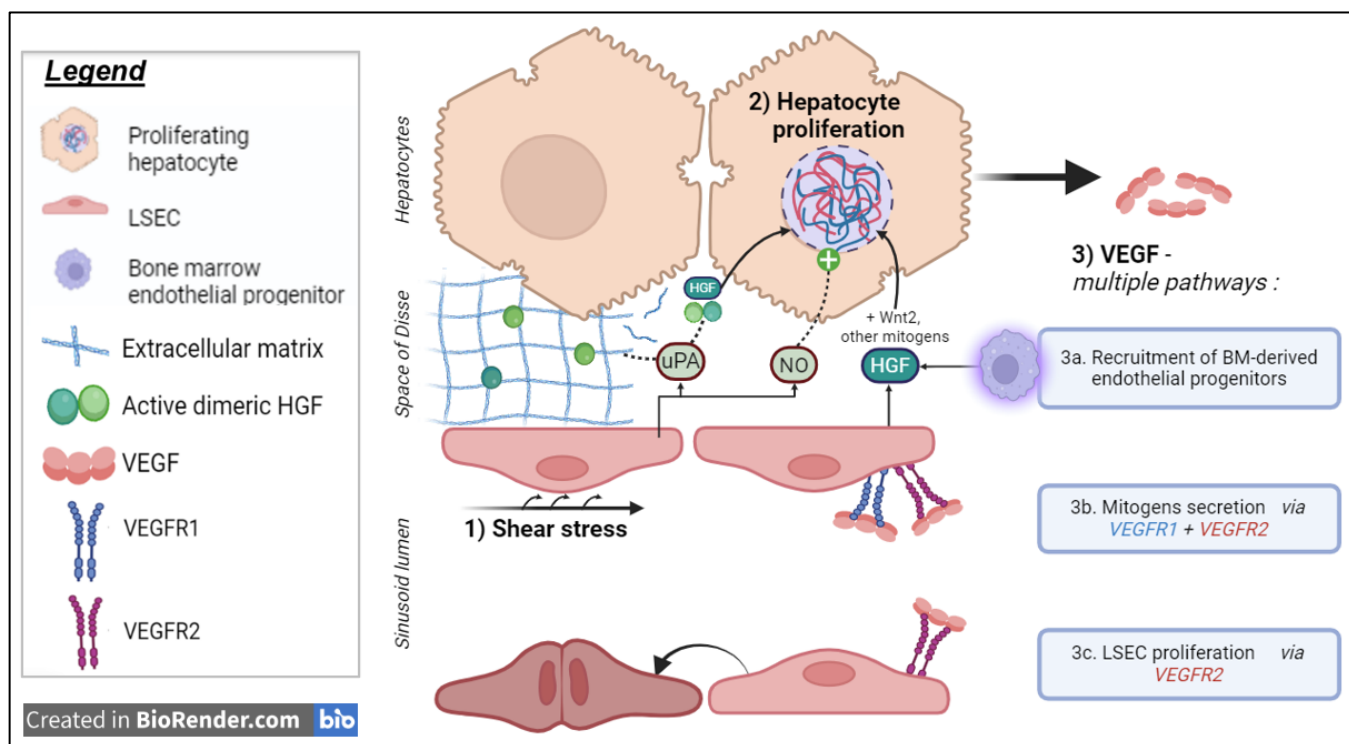


Figure 3. Illustration of the role of VEGF in liver regeneration (as detailed above).

(1) Following the increase in portal flow, LSECs experience immediate **shear stress**, which triggers mechanotransduction. This results in the secretion of both uPA, which degrades the ECM in the space of Disse, allowing for the release of active HGF; and NO, which sensitises hepatocytes to mitogenic signals. (2) Under mitogenic stimulation, **hepatocytes proliferate** and subsequently release VEGF. (3) **VEGF** further enhances hepatocyte proliferation and initiates the angiogenic repair phase through three main mechanisms: (3a) recruitment of bone marrow-derived progenitor cells with potent paracrine function, (3b) induction of mitogens secretion in LSECs, (3c) induction of LSEC proliferation.

3.3. Small-for-size syndrome

While liver regeneration can effectively compensate for a certain degree of parenchymal loss, it may falter in extreme surgical scenarios, or in the presence of underlying chronic diseases that impede regenerative processes. In such situations, a life-threatening liver failure known as small-for-size syndrome (SFSS) may manifest. This common post-transplantation complication has been documented in up to 20% of patients receiving reduced size grafts (GRWR < 0.8%), including cases of partial living donor LT and split LT (48). Consequently, SFSS represents a significant barrier to the wider adoption of living donor liver transplantation (LDLT) (49).

Definition

This clinical entity was characterised by Dahm et al. as the presence of at least two of the following findings, on three consecutive days: hyperbilirubinemia exceeding 100 µM, coagulation abnormalities with an INR above 2, and hyperammonemia causing grade 3 or 4 encephalopathy. The diagnosis requires the exclusion of technical complications, infection, and immunological etiologies. Associated morbidity may include an increased risk of haemorrhage, cholestasis, ascites, and portal hypertension, which may progress to sepsis and multi-organ dysfunction (49).

A similar syndrome, referred to as post-hepatectomy liver failure (PHLF), may occur following approximately 8 to 12% of major liver resections (50). PHLF has been formally defined by the International Study Group of Liver Surgery as the concurrent presence of hyperbilirubinemia and an elevated INR on or after postoperative day 5 (51). This condition is linked to a 5% mortality rate within 90 days of surgery, establishing it as the leading cause of postoperative morbidity and mortality (52).

To mitigate the occurrence of SFSS, it is imperative to ensure that the FLR is large enough to maintain adequate liver function. As previously discussed, the benchmarks are set at 20% of the initial healthy liver volume for hepatectomy, and at a GRWR of 0.8% for transplantation (9,53). Innovative techniques for auxiliary transplantations, such as the RAPID procedure, allow for the use of a smaller graft and have attracted considerable interest worldwide.

Physiopathology of SFSS

The onset of SFSS is classically thought to result from major hemodynamic changes associated with liver surgery. A primary mechanism proposed in its pathogenesis is excessive portal hyperperfusion. Following resection of a significant portion of the liver parenchyma, all portal flow is redirected to a relatively small implanted graft (54). While increased portal flow is essential for liver regeneration, excessive flow also generates significant shear stress that can damage the sinusoidal structures (55,56). Accordingly, histopathological findings in SFSS cases include the denudation of the periportal endothelial lining, focal hemorrhage, sinusoidal congestion and collapse of the space of Disse (57,58).

Additionally, portal hyperperfusion triggers the compensatory hepatic arterial buffering response. The subsequent vasoconstrictive and inflammatory responses, including the upregulation of endothelin-1 (59), create an ischemic environment that is thought to further impede liver regeneration and participate in the development of SFSS (8,60). Histologically, this phenomenon translates into arterial vasospasm, cholangitis sequelae or parenchymal infarcts (57).

The second prevailing hypothesis describes SFSS as a manifestation of functional hepatocellular failure resulting from insufficient hepatocyte proliferation following liver surgery. Studies conducted in a mouse model of SFSS have indicated that, despite many hepatocytes initiating the cell cycle, they fail to complete mitosis due to the upregulation of cell cycle inhibitors such as p21, and to insufficient levels of cell cycle drivers such as c-fos (61). Similarly, in rats, a diminished hepatic regenerative activity was observed following extended hepatectomy (62).

However, the latter simplistic view was challenged by several findings more suggestive of hepatocyte overproliferation. In the context of LDLT, Gruttadauria et al. described an association between the overexpression of the cell cycle entry marker Ki67 and adverse postoperative outcomes such as an increased likelihood of SFSS (63). Furthermore, when comparing a standard hepatectomy with a rat model of SFSS, Ninomiya et al. reported an increase in the number of hepatocyte

duplications. Interestingly, they were also able to improve survival by administering a proliferation inhibitor, whereas the injection of pro-mitogenic HGF showed no beneficial effect (64).

As suggested by the findings of these two authors, SFSS appears to stem from an intensification of the physiological proliferative mismatch, characterised by the excessively rapid hyperproliferation of hepatocytes compared to a relatively insufficient vasculature (63,64). This disproportion results in the formation of avascular and dysfunctional clusters of hepatocytes, disrupting the lobular structure. Ultimately, it is these microarchitectural disturbances that are held responsible for SFSS-associated liver dysfunction, rather than a deficient hepatocyte proliferation. Intriguingly, this phenomenon can manifest even in the absence of significant histopathological injury (65).

3.4. Liver resection and transplantation: a growing medical need

Taking advantage of the liver's inherent regenerative abilities, liver surgery remains the primary therapeutic approach for several severe liver diseases, depending on a favourable assessment of its risk-benefit ratio. For example, it offers a potential cure for patients presenting with primary liver tumours such as hepatocellular carcinoma (HCC) or a slow-growing secondary neoplasms (most commonly colorectal or neuroendocrine cancer metastases) (21–23). The two surgical procedures discussed further are partial hepatectomy and transplantation, with a focus on their application in oncology.

Irrespective of the surgical modality used, oncological liver surgery must always meet two fundamental requisites (24). On the one hand, the resection should be sufficiently extensive to ensure complete tumour removal with a so-called R0-margin (microscopically negative for residual tumoral cells). On the other hand, the residual parenchymal volume should be sufficient to ensure postoperative liver function and subsequent long-term viability. Failure to meet this latter criterion places the patient at risk of potentially fatal postoperative complications, such as SFSS in transplantation (as discussed previously), or its counterpart phenomenon PHLF following extensive resections. These considerations hold major clinical significance, as they may lead to several restrictions in surgical indications and consequently affect the patients' prognosis (21).

Although partial hepatectomy has proven to be effective in the treatment of localised malignancies, it is hampered by several limitations. Firstly, the spatial distribution of neoplastic lesions can pose surgical challenges, particularly in the vicinity of the hilum and hepatic veins. In cases of multiple or bilobular tumours, it becomes difficult to achieve oncological resection without compromising the minimal necessary parenchymal remnant (25). Furthermore, HCC frequently occurs in the setting of an underlying chronic liver disease, which in itself increases the patients' susceptibility to PHLF (20).

To overcome these functional limitations, liver transplantation (LT) emerges as an alternative. Initially a standard of care for end-stage liver failure (26), the applications of LT have expanded greatly over the years due to a better understanding of liver regeneration and continuous improvements in surgical management. With decreasing procedural risks, LT has become accessible to a broader range of patients who were formerly deemed high-risk candidates (e.g., comorbid, older, frailer patients) (21).

Furthermore, LT has emerged as a valuable treatment option for an expanding range of malignant indications. These encompass HCC falling within the Milan criteria, specific Klatskin tumours post- neoadjuvant chemotherapy, as well as surgically challenging colorectal or neuroendocrine metastases (21).

However, despite these significant improvements in LT, the availability of suitable donor grafts remains a critical limiting factor. Certain regions (e.g. Hong Kong) contend with restrictions due to a limited supply of liver grafts, leading to a careful selection of their surgical indications (21). Although the situation is comparatively less severe in western countries, the shortage of grafts remains a significant issue. For instance, the Eurotransplant network has seen a substantial increase in the number of LTs performed over the last 25 years (doubling from 1993 to 2018), with a decreasing waiting list since 2010. Despite these improvements, while 1603 patients underwent transplantation in 2022, 1293 remained on the waiting list; tragically, approximately one third (n=420) passed away while awaiting a graft (66).

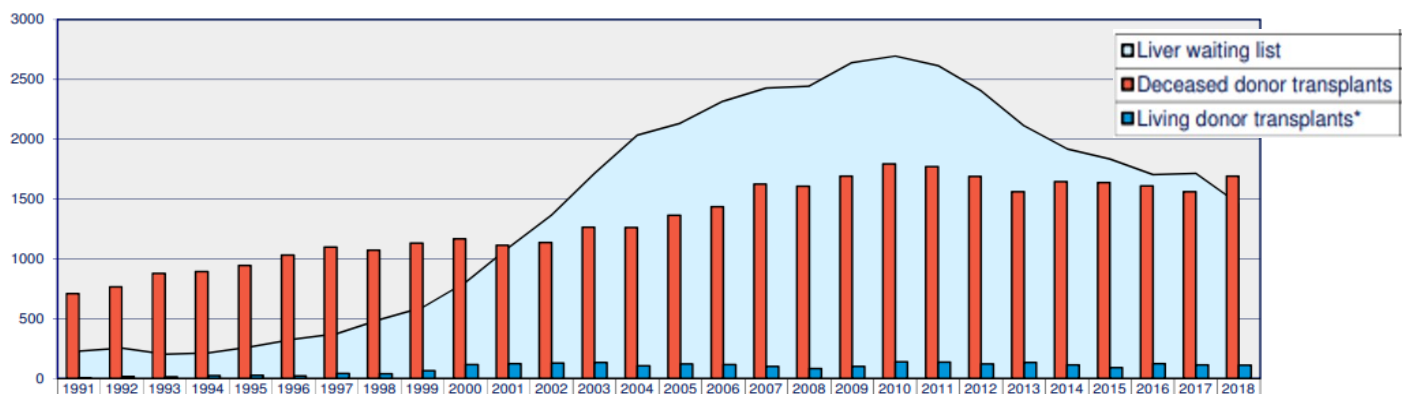


Figure 4. Dynamics of the Eurotransplant liver waiting list and liver transplants between 1991 and 2018. Issued from Eurotransplant statistics, 2023 (66).

3.5. Strategies to improve the access to liver transplantation

Several options have been proposed to address the limited supply of liver grafts. The first approach aims to expand the deceased donor pool by considering so-called “marginal liver” (ML) grafts, which meet less stringent donor quality criteria. However, the literature on this topic presents conflicting evidence. Some authors have cautioned against the use of ML grafts, attributing a two-fold increase in recipient mortality (67). Conversely, Halazun and colleagues have advocated for the use of ML grafts, claiming that comparable survival rates to standard LT can be maintained while achieving a reduction in waiting list mortality (68).

The second option is to optimise the use of the conventional deceased donor pool by segmenting a single liver for the benefit of two recipients. However, this parenchyma-sparing approach is limited by the prerequisite that both segments should be sufficiently substantial to ensure adequate post-operative liver function and to minimise the risk of SFSS for the recipients (69,70).

A third strategy focuses on living donor liver transplantation (LDLT), which offers the advantage of reducing waiting list mortality and dropout rates (similar to ML transplantation), without compromising on the quality of the donor grafts (71). In LDLT, a partial liver transplant is performed using the left hepatectomy from a living donor. Unfortunately, due to the unequal distribution of parenchyma between the right and left hemi-livers, LDLT may expose the recipient to SFSS (3). However, attempting to mitigate this size discrepancy by transplanting the donor’s right liver would jeopardise the donor’s own residual function and carry unethical risks (72).

Finally, recent advances in liver surgery have focused on regenerative techniques that allow for more extensive hepatectomies by stimulating the growth of the FLR. Several techniques take advantage of the liver’s innate ability to regenerate after vascular deprivation or tissue loss (53), including strategies such as portal vein embolization or ligation, or staged hepatectomies with an intervening regenerative interval.

For instance, in the context of major hepatectomy, the ALPPS⁵ procedure consists of two distinct surgical phases. First, the right portal vein is ligated to divert portal flow to the left liver, and a parenchymal split is performed to eliminate intrahepatic vascular collaterals. These manipulations stimulate the growth of the left liver (or FLR) in the interstage period. In the second surgical step, the residual diseased liver segment is resected through an extended right hepatectomy (53).

These concepts derived from ALPPS have subsequently found application in transplantation, uniting the advantages of regenerative surgery with the optimisation of graft availability from split or living donors. The RAPID procedure, initially developed by Line et al. in Oslo (73), integrates a living donor transplantation with a staged surgery strategy. In the initial stage of RAPID, the left liver is resected and substituted by a small living donor auxiliary graft (GRWR usually < 0.5%), and the right

⁵ ALPPS: Associating Liver Partition and Portal vein ligation for Staged hepatectomy

portal vein is ligated. After approximately 14 days, the graft has regained sufficient volume (GRWR >0.8%) and functionality to complete the hepatectomy while avoiding the onset of SFSS (74).

In our institution, the implementation of the RAPID technique paved the way for our research protocol called ALDAPT (3,75). Preliminary results from L.Coubeau show promising clinical outcomes; in particular, 8 patients underwent RAPID transplantation with small-for-size grafts, their GRWR falling below 0.5% and reaching as low as 0.26%. Remarkably, none of these cases experienced SFSS.

As these innovative approaches require a smaller FLR, they hold promise for improving transplant availability and oncological surgical outcomes. In order to increase the efficacy and safety of regenerative surgery, push its boundaries to mitigate the impact of organ shortages, it is essential to further our understanding of liver regeneration in this specific surgical context.

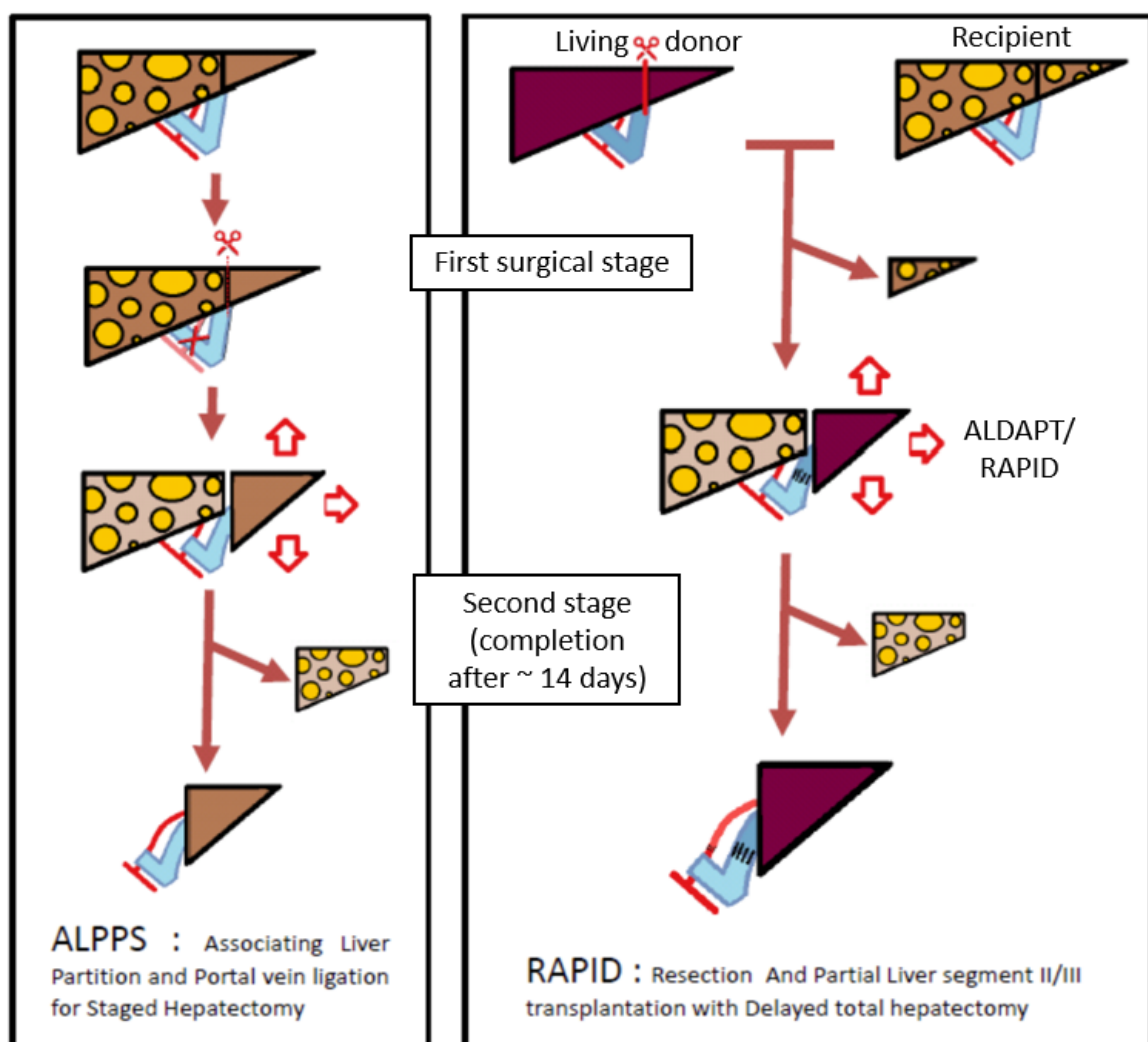


Figure 5. Schematic comparison of ALPPS and RAPID surgeries. Issued from Van Den Eynde M, Coubeau L, 2023 (76)

3.6. Endothelial progenitor cells

In the pursuit of developing both safer alternatives to liver transplantation and effective therapeutic options for its complications, particularly SFSS, a profound knowledge of angiogenic restoration appears crucial. Beyond the scope of surgical refinements, the realm of cell therapy holds promise as an alternative or complementary approach to liver transplantation (77). Within this field, endothelial progenitor cells (EPC) have emerged as potential candidates, particularly in light of the challenges previously encountered with parenchymal cell transplantation, including issues with hepatocyte viability in culture and complications related to cryopreservation or re-engraftment (78,79). In this context, a better understanding of EPC biology is needed for developing their potential as a therapeutic modality.

Since their discovery by Asahara two decades ago, EPCs have received increasing attention in various fields of research (80). In recent years, several clinical trials have been conducted to explore their potential for therapeutic revascularisation by mobilising treatments or cell therapy. Relatively promising results were reported in ischemic diseases such as leg ulcers, limb ischemia, pulmonary arterial hypertension and coronary heart disease, as reviewed by Chong et al (81). In addition, EPCs have attracted interest in the context of acute liver regeneration, following significant findings by several authors.

This section will first address the complex identity of EPCs, highlighting the challenges in defining their precise characteristics. Subsequently, their specific role in liver regeneration following hepatectomy or transplantation will be reviewed.

Endothelial progenitor cells: a complex definition

Firstly, it is important to establish a precise definition of EPCs. This issue has generated considerable debate within the scientific community, due to factors such as terminological discrepancies and conflicting results between different studies in this field (82). Broadly speaking, EPCs can be described as cells with the capacity to proliferate, differentiate into mature endothelial cells and actively participate in angiogenesis by integrating into the endothelial lining (83).

Accumulating evidence suggests that so-called EPCs cannot be considered as a uniform cell population, but rather comprise two distinct subsets known as myeloid angiogenic cells (MAC) and endothelial colony-forming cells (ECFC). Nevertheless, both types of EPCs efficiently contribute to vascular regeneration in an ischemic context, albeit with differences in isolation methods and angiogenic mechanisms (82,84).

Endothelial progenitor cell isolation methods

The identification of EPCs relies on two primary techniques. The gold standard approach involves the analysis of surface antigens using flow cytometry. In numerous studies, the classical antigenic profile typically used to include at least one marker of immaturity, like CD133 in humans, in combination with a marker linked to endothelial commitment, such as VEGFR-2 or CD31 (83).

However, there is evidence indicating that cells exhibiting the CD34+CD133+VEGFR2+ phenotype may not differentiate into endothelial cells as anticipated, but rather remain committed to the hematopoietic lineage (85).

Alternatively, in vitro colony formation assays (86) allow the estimation of circulating EPC numbers. In addition, they provide valuable insight into the clonal expansion, overall proliferative capacity, and hierarchical differentiation process of the cultured cells. However, it is important to recognise that culture conditions can influence cell fate and may not fully reflect their behaviour in vivo. Therefore, combining both identification techniques may strengthen the validity of results (83).

Myeloid angiogenic cells

The first cellular subset, now more accurately termed myeloid angiogenic cells (MACs), was originally classified of endothelial progenitor nature. Yet, a recent consensus on nomenclature advises against this label (82), as studies have shown that MACs originate from the hematopoietic lineage and are unable to differentiate into mature endothelial cells (87,88).

Instead of integrating into the endothelium, they stimulate vessel formation through the paracrine secretion of potent angiogenic growth factors (88–90). This mechanism of action has proven effective in rescuing ischemic conditions and has subsequently been investigated in several promising cell therapy clinical trials (81).

When peripheral blood mononuclear cells (PBMCs) are cultured in a specific endothelial-conditioned medium, MACs constitute the earliest visible colonies, forming clusters of small round cells that appear visible after one week in culture (86,91). By flow cytometry, MACs typically co-express classic hematopoietic and monocytic markers such as CD45 and CD14, together with endothelial markers such as CD31 and VEGFR2 (82). They may also lack expression of CD34 and CD133 (82).

Initially, the co-expression of endothelial features led to their identification as EPCs, a title which has been revoked since Ohtani et al. demonstrated that the genes typically associated with endothelial function are epigenetically silenced in MACs (92). It has therefore been debated whether the endothelial antigens detected may be due to microparticle contamination during cell culture, potentially leading to false-positive events in fluorescence-activated cell sorting (FACS) analysis (93).

However, some studies suggest that this overlapping phenotype may result from intercellular communication, whereby hematopoietic macrophages are truly reprogrammed to an endothelial fate through the transfer of epigenetic material (94).

Endothelial colony forming cells

In contrast to MACs, ECFC are considered to be the “bona fide” endothelial progenitor due to their consistent commitment to an endothelial identity and their robust proliferative capacity (87). They have the remarkable ability to differentiate into mature endothelial cells both in vitro and in vivo (91), and physically contribute to the formation of new capillaries under ischemic conditions (82,95).

However, isolating ECFC poses a significantly greater challenge compared to MACs. Indeed, their inherent scarcity in peripheral blood is further compounded by patient-specific factors such as age, cardiovascular disease, or diabetes. These can lead to a drastic reduction in the number of circulating ECFC, dropping to 2-3 or even 0 per millilitre of blood (96–99). This rarity limits their availability for therapeutic and clinical applications.

Similar to MACs, ECFC can be identified by colony forming assays. These large cells become visible after 18 days in culture and exhibit a characteristic cobblestone appearance (86). Flow cytometric analysis of ECFC reveals the expression of the full set of canonical endothelial antigens, but importantly lacks hematopoietic lineage markers (CD34, CD45 or CD14). Although they may also express typical progenitor markers such as CD133, the latter is a non-specific antigen. It can also be expressed by mature endothelial cells, which confounds the precise identification of ECFCs (83).

Interestingly, this observation spurred the hypothesis that ECFC may actually originate from a subset of highly proliferative mature endothelial cells rather than from true progenitors. Evidence suggests that they could shed from the endothelium itself (100) or from specific niches within the vessel wall (101,102). Some have hinted at alternative sources of ECFCs such as the intestine, liver, and white adipose tissue (WAT) (103,104). Importantly, it has now been established that ECFCs do not originate from the bone marrow (105,106).

A continuum: the hematoendothelial lineage?

The absence of a hematopoietic signature is often highlighted as an important criterion for distinguishing true progenitor cells (ECFCs) from their myeloid counterparts (MACs), which provide supportive paracrine functions. However, an ongoing debate revolves around the possible existence of a hybrid hemato-endothelial phenotype.

This hypothesis originates from the discovery of a hemogenic endothelium, known as the hemangioblast. Initially present during embryonic development, this hemangioblast has the capacity to generate both hematopoietic and endothelial lineages (107). However, it remains uncertain whether adult angiogenesis can reinstate this dual endothelial-hematopoietic phenotype, especially under ischemic conditions (83,108,109).

An intriguing perspective emerges from the work of Masuda et al., which hints at the possible existence of such a continuous phenotype within adult endothelial progenitors. Using an in vitro single-cell clonogenic assay, they demonstrated a hierarchical relationship between an immature CD133+ MAC equivalent and ECFC-like cells. Extended culture of “MACs” resulted in the emergence of outgrowing cells that not only shared morphological similarities with ECFCs, but also displayed a more mature endothelial antigenic profile, reduced expression of hematopoietic markers, and enhanced angiogenic properties compared to their MAC precursors. Remarkably, in a hemato-endothelial commitment assay, cultured cells could be induced towards the endothelial lineage by conditioning the culture medium with VEGF (86).

Similarly, the liver endothelium is also subjected to this phenotypic ambiguity. Indeed, LSECs share phenotypic resemblances with leucocytes, exhibiting characteristics related to immune and scavenging functions. Notably, in a rat model, DeLeve's team identified a distinct subset of LSECs expressing the common leucocyte antigen CD45, distinguishing them from other CD45⁻ mature endothelial counterparts (12,14,16,110,111). Additionally, they characterised a unique population of liver-specific EPCs termed bone marrow sinusoidal progenitor cells (BM-SPCs). These expressed CD31 and CD45 akin to LSECs, alongside the progenitor marker CD133 (14,36).

While CD45 expression in human LSECs has not been convincingly documented (18), a subset of mature LSECs expresses the CD14 marker, also commonly associated with monocytes and thus used to differentiate true ECFC from MACs (18). Overall, these complexities caution against overly stringent exclusion of hematopoietic markers in the identification of LSEC progenitors.

In summary, these findings highlight the important role of hematopoietic and endothelial interactions, and even suggest the speculative existence of an overlapping phenotype that bridges these two lineages in adult hematopoiesis. However, in our quest to standardise findings within the field, it becomes imperative to use common nomenclature. Specifically, the term "EPC" should be avoided or reserved exclusively for ECFC, which represent the circulating progenitors devoid of hematopoietic markers. On the other hand, pro-angiogenic cells sharing both endothelial and hematopoietic features should be appropriately categorised as MACs. The following schematic representation synthesises commonly used features to distinguish between MACs and ECFC.

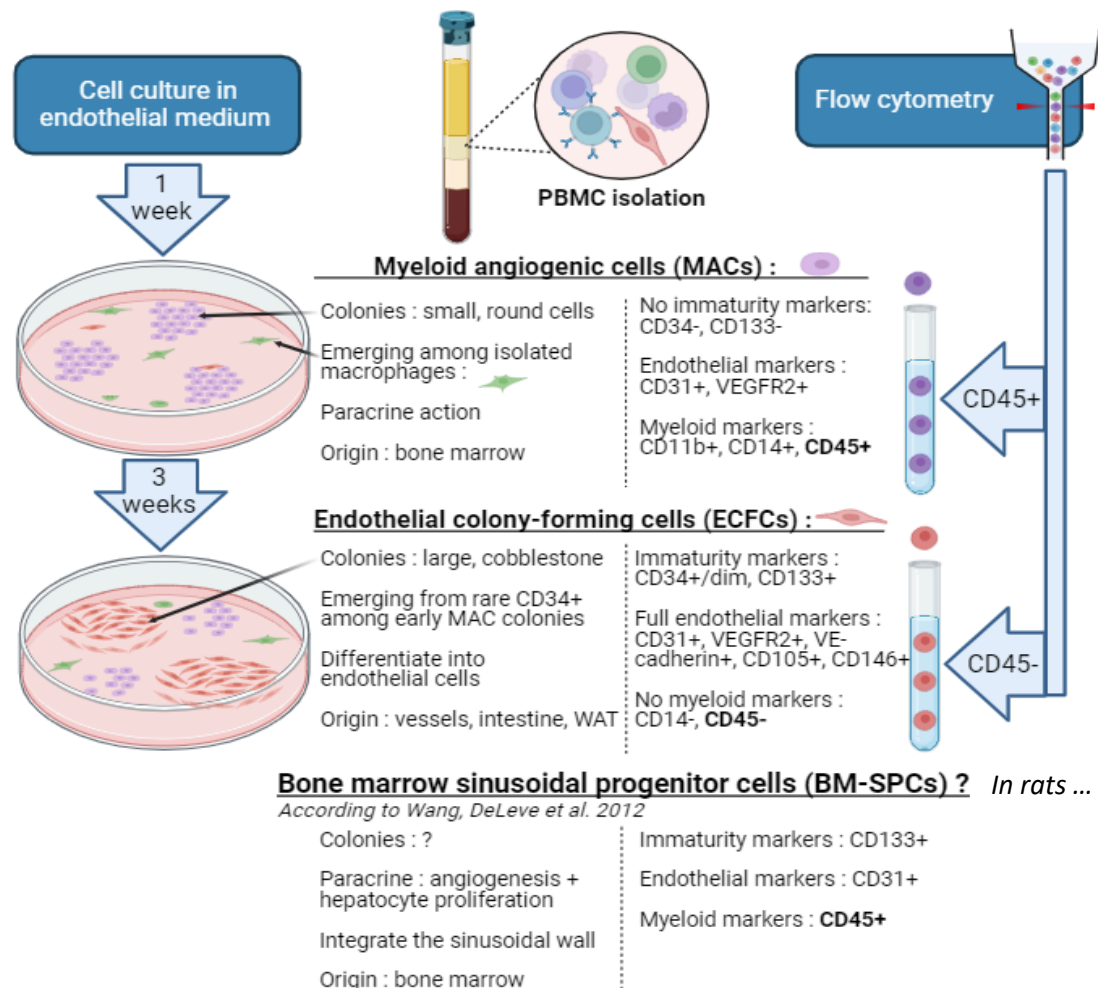


Figure 6. Synthetic comparison of EPC subtypes. Created in BioRender.com

Implication of endothelial progenitors in liver regeneration

The importance of EPCs in post-hepatectomy regeneration has drawn attention due to its innovative insights and clinical potential. However, the field faces challenges stemming from limited data availability (9), compounded by inconsistencies in nomenclature and identification techniques across preclinical studies. Their generic use of the term 'EPC' has led to ambiguity, contributing to confusion regarding the nature and properties of specific pro-angiogenic subtypes.

In the quest to unravel the role of EPCs in liver regeneration, pioneering work by Fuji et al. introduced the concept two decades ago that bone marrow (BM) -derived progenitors could be mobilised to the regenerating liver through marrow transplantation experiments (35). Subsequently, Harb et al. demonstrated that BM-derived CD133+CD45+ progenitors replaced injured LSECs in a rat model of toxic-induced sinusoidal obstruction syndrome, suggesting their critical role in vascular repair. Conversely, bone marrow irradiation impaired vascular recovery (112).

Other researchers studied EPCs in a 70% hepatectomy mouse model, which reflects a major hepatectomy scenario without inducing SFSS. For instance, Beaudry et al. showed that BM cells labelled with green fluorescent protein contributed to 12% of the LSEC pool after hepatectomy, and this percentage remarkably increased to almost half of the LSEC upon administration of mobilising VEGF treatment (34).

Similarly, albeit in a rat model, Wang et al. reported that approximately 25% of the total LSEC pool after hepatectomy was derived from endothelial progenitors, defined in their experiments as CD133+CD45+CD31+ triple positive cells. These cells not only differentiated into mature, fenestrated LSECs, but also exhibited higher HGF expression and proliferation rates compared to resident LSEC progenitors (36). To validate this concept, isolated triple-positive progenitors were reinfused into myeloablated mice; this cell therapy facilitated successful liver regeneration when performed on day 1 after hepatectomy but failed when performed on day 3. This suggests that while these EPCs contributed to later angiogenesis, they may also play a critical role in early paracrine-mediated stimulation of hepatocyte proliferation (29).

Finally, Lee et al. took advantage of a unique sex-mismatched, human dual LDLT context. They observed that male recipient progenitor cells, defined as XY+ / CD34+ cells, were mobilised and integrated into female donor XX+ liver grafts. Not only did these progenitors contribute significantly to the formation of neovascular networks, but their greater engraftment also correlated with accelerated liver regeneration; these observations were exacerbated in cases with a FLR falling below the conventional threshold of 0.8% GRWR (113).

Together, these findings underscore the likely importance of BM-derived cells in liver regeneration, particularly in response to potent regenerative stimuli. However, given the recent advances in EPC classification and nomenclature, there is an urgent need to further characterise the precise nature and role of the contributing pro-angiogenic cells.

3.7. Current hypotheses and work questions

Regenerative surgical techniques hold the promise of revolutionising liver transplantation, potentially offering innovative solutions to organ shortage and surgical treatment availability. Among these techniques, the RAPID procedure provides a unique opportunity to investigate the mechanisms underlying efficient and extensive liver regeneration without inducing SFSS despite a too small for survival liver remnant. While the mechanistic details of human liver regeneration remain largely unexplored, the following key observations in the field of regeneration and small liver transplantation have prompted our research project based on the RAPID setting:

1° In physiological liver regeneration, the sinusoidal network plays a pivotal role both in the initiation phase by priming hepatocyte proliferation, and in the angiogenic phase by restoring adequate tissular oxygenation.

2° “Endothelial progenitor cells” are critical drivers of this endothelial-mediated liver regeneration, especially when the transplanted FLR is small. They contribute significantly to paracrine HGF secretion and may integrate into the vessel wall. However, their exact nature and origin has not been thoroughly investigated in the existing literature. Most of the existing studies may evaluate the presence of MACs, which cannot be considered as bona fide progenitors.

3° Small-for-size syndrome, a potentially fatal postoperative liver failure, is associated with portal hyperperfusion causing excessive sinusoidal damage and tissular ischemia. It occurs predominantly when the FLR falls below the conventional cut-off of 0.8% GRWR. Despite mimicking the pathogenic conditions of SFSS, such as the infra-threshold FLR and particular hemodynamic settings, **the RAPID procedure** maintains favourable clinical outcomes.

Therefore, this project aims to elucidate the mechanisms driving liver regeneration and function maintenance after RAPID, with a primary focus on the role of endothelial progenitor cells. The following hypotheses will be investigated:

1° The deportalised right liver contributes to graft regeneration through pro-angiogenic humoral signalling, thereby preventing the occurrence of SFSS. Our investigations include:

- Evaluating the presence of endothelial progenitor recruitment signals, by quantifying plasmatic bone-marrow mobilising, pro-angiogenic and chemotactic cytokines.
- Locating a potential hypoxia-associated endocrine signalling source for systemic EPC mobilisation, through the assessment of hypoxia markers within the deportalised right liver (DRL) and graft.

2° The RAPID procedure is associated with enhanced recruitment of pro-angiogenic cells.

Within the two main angiogenesis-promoting cell types, specifically MACs and ECFCs, one may predominate in active sinusoid restoration mechanisms. Determining whether this occurs through paracrine signalling or by integration into the vessel wall will require:

- Comparative analysis of circulating MAC and ECFC levels between ALDAPT recipients after surgery, and their donor control group undergoing partial hepatectomy.
- Verification of endothelial progenitor engraftment within the sinusoidal network of the transplanted FLR, using a sex mismatch model. Recipient-derived cells displaying the XY chromosome phenotype will be identified among the endogenous cells from the female donor liver (XX).

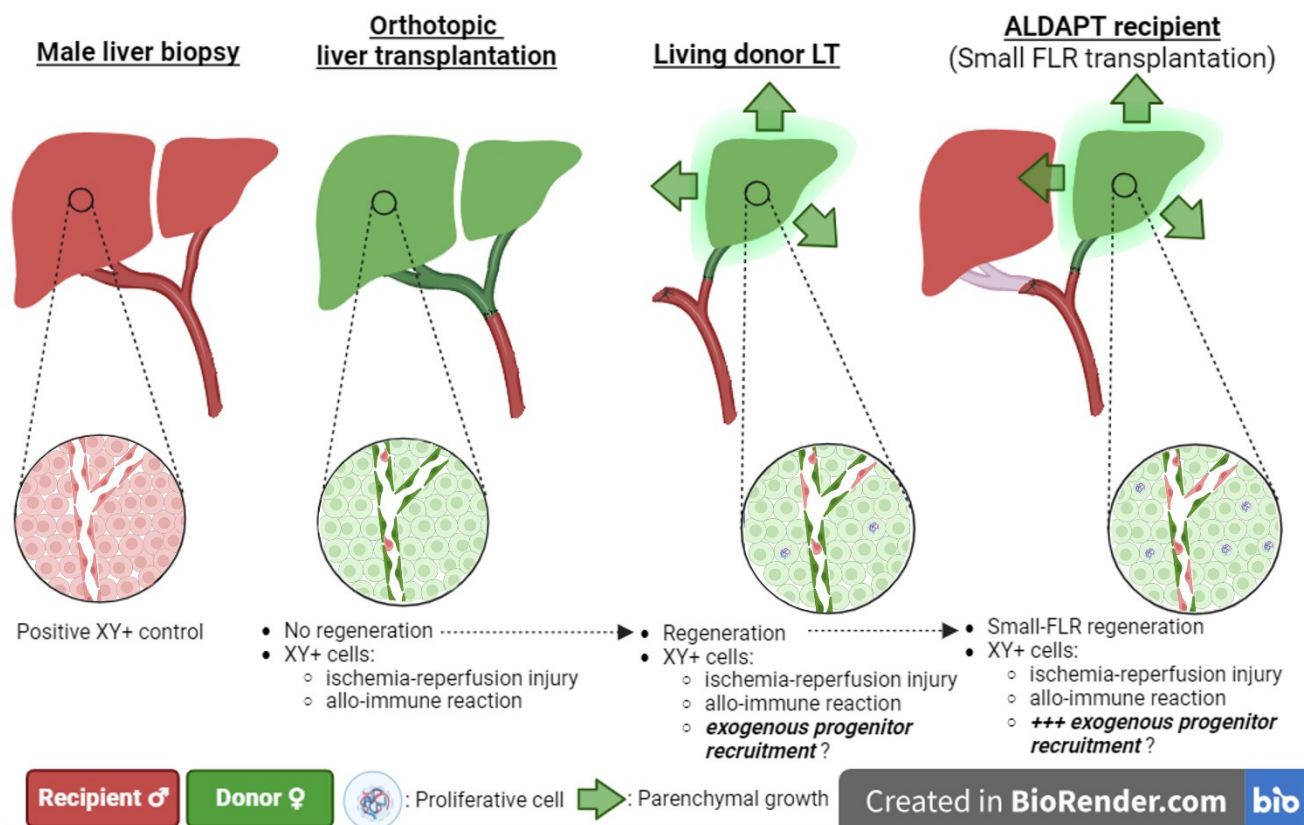


Figure 7. Compared groups for the analysis of RAPID-associated liver regeneration. Created in Biorender.com

3° Recruitment of endothelial progenitors may contribute to sinusoidal restoration in the graft, thereby supporting regeneration. This hypothesis entails the following investigations:

- Assessing the integrity of the vascular network in the ALDAPT recipients' grafts, measured by sinusoidal density and diameter.

Notably, our histological results will be the first available for this type of auxiliary transplantation.

4. Materials and methods

4.1. Patients and samples

Samples were collected during the previously described RAPID transplantation protocol. Briefly, the first surgical step consisted in the ligation of the right portal branch concomitant to an auxiliary living donor transplantation, while the second allowed for completion of the hepatectomy. Our prospective phase 2 clinical trial, named ALDAPT, included 8 patients. They were transplanted for an indication of either colorectal or neuroendocrine cancer liver metastases.

For the analysis of circulating EPC and plasmatic cytokine levels, peripheral blood was drawn from the recipients and their matched donors at the first step of surgery, then daily until postoperative day (POD) 7. Additional samples of portal blood were collected during the transplantation stage.

For histological analysis, ALDAPT recipients were subjected to surgical liver biopsies. Fragments of the implanted left graft were collected both at reperfusion stage on POD0, and on POD14. Biopsies were also taken from the deportalised right liver (DRL) on POD0 and 14. Control groups included macrobiopsies from healthy livers, postoperative percutaneous needle biopsies obtained at POD7 after orthotopic whole liver transplantation (OLT), and after right LDLT.

Regarding ethical requirements, this study was approved by the Ethical Committee of the Université Catholique de Louvain (CEHF 2020/19FEV105). Written informed consent for publication was obtained from the patient.

4.2. Enzyme-linked immunosorbent assay

Plasma from matched donors and recipients undergoing RAPID surgery was subjected to ELISA. The samples were obtained from per operative systemic blood (POD0-Sys) and portal blood (POD0-Port) samples, at POD 1 to 7, and POD14. They were not diluted prior to analysis.

Levels of EPO, G-CSF, IL8, IL6, M-CSF, MCP-1, SDF- α , VEGF- α were quantified by utilizing the multiplex U-Plex assay® (*Meso Scale Discovery™ platform, Meso Scale Diagnostics, Rockville, Maryland, USA*), according to the manufacturer's specifications.

4.3. Peripheral blood mononuclear cells isolation

16mL of blood was drawn from ALDAPT recipients and donors at first time of surgery and during the 7 following days, using BD Vacutainer® CPT™ Mononuclear Cell Preparation Tubes anticoagulated with sodium citrate (*ref.362761, BD Biosciences, Erembodegem, Belgium*). CPT Tubes were immediately centrifuged at room temperature for 30 min in a horizontal rotor, at relative centrifugal force (RCF) of 1600, with an acceleration and deceleration set to 1-1.

PBMC were then resuspended in the plasma by inversion. This suspension was transferred into a sterile conical Falcon, then centrifuged at room temperature for 15 min, at a speed of 1300 revolutions per minute (RPM), with an acceleration and deceleration of 9-9. The supernatant plasma was transferred to another 15mL falcon, and the remaining cells were washed twice with 10mL of ice-cold PBS (with the same centrifuge settings).

The PBMC pellet was resuspended in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with Glutamax (*ref.12539089, Gibco, ThermoFisher, Waltham, Massachusetts, USA*), along with 10% Human Serum (*ref.H5667, Merck, Darmstadt, Germany*) and 1% Penicillin-Streptomycin (*ref.15140122, Gibco, ThermoFisher*). The cells were either cultured immediately in colony-forming culture assays or frozen at -80°C with the addition of 10% DMSO.

4.4. Endothelial progenitor cells colony forming assay (EPC-CFA)

This assay was performed according to the methodology previously developed by Masuda et al (86). We conducted the EPC-CFA on two occasions of RAPID transplantation, using samples from the both the recipient and their matched donor, in concomitant cultures. Freshly isolated PBMC were cultured in duplicate dishes for each subject and timepoint. EPC-CFA was performed for each of the following samples: per-operative systemic blood (POD0 Sys), per-operative portal blood (POD0 Port), and systemic blood from POD1 to 7.

PBMC were isolated as described above (*see 4.23*), then cultured for 18 days in an adjusted semi-solid culture medium prepared as follows. First, the semisolid stock medium was prepared following recommendations from the StemCell Technologies® Technical Manual (114). Specifically, MethoCult™ SF H4236 medium (*ref.4236, StemCell Technologies, Vancouver, Canada*) was supplemented with 30% fetal bovine serum (*ref.A3160801, Gibco, Thermofisher*), 1% penicillin-streptomycin, 2 U/mL heparin (*ref.D67797, LEO, Liege, Belgium*), and the following growth factors : 100 ng/mL Recombinant Human SCF (*ref.300-07*), 50 ng/mL Recombinant Human VEGF165 (*ref.100-20*), 50 ng/mL Recombinant Human FGF-basic 154 a.a. (*ref.100-18B*), 20 ng/mL Recombinant Human IL-3 (*ref.200-03*), 50 ng/mL Animal-Free Recombinant Human EGF (*ref.AF-100-15*), 50 ng/mL Recombinant Human IGF-I (*ref.100-11*) (*all from Peprotech, London, UK*). This stock medium was then diluted at a ratio of 2 to 3 parts IMDM-Glutamax / FBS30% to obtain the final working medium, which was aliquoted and stored at -20°C until use.

For every sample and timepoint, duplicate cultures were performed. In each culture dish, 500 000 live PBMC concentrated in 100µL of IMDM/10% Human Serum were added to 1mL of thawed working medium, resulting in cell mixture that was dispensed into a 35-mm culture dish (*first assay: ref.27100, StemCell Technologies; second assay: ref.734-2317, VWR Europe*) using a 16-gauge blunt-end needle. This process yielded a seeding concentration of 500,000 live cells per dish. The cultures were subsequently incubated at 37°C with 5% CO₂ and 95% humidity for a duration of 18 days without changing the culture medium.

At day 18 of cell culture, all culture dishes were scanned using a Zeiss LSM800 microscope at a 5x magnification in bright field. Subsequently, the colonies were visually quantified by two independent operators who were blinded to the samples. They were defined as cohesive cell clusters measuring at least 300µm in diameter. Cell morphology was used to classify them between Small EPC (SEPC), corresponding to MAC colonies, and Large EPC (LEPC) considered as ECFC.

4.5. Flow cytometry

Flow cytometry analysis was performed on PBMC isolated from matched ALDAPT donors and recipients. A cryovial containing isolated PBMC was initially thawed by immersion in a 37°C water bath and then resuspended in 10 mL of IMDM-10% FBS. Following two washes in PBS, 10^5 cells were resuspended in 100 μ L of PBS for subsequent analysis. Cultured human umbilical vein endothelial cells (HUVEC) were used as a positive control. The samples were subjected to a 10-minute staining procedure utilizing anti-CD31 (*ref. 130-110-807*), CD34 (*ref. 130-120-522*), CD11b (*ref. 130-110-556*), CD14 (*ref. 130-110-583*), CD45 (*ref. 130-110-775*), CD133 (*ref. 130-110-614*), and VEGFR2 (*ref. 130-117-911*) antibodies (*Miltenyi Biotec, Leiden, The Netherlands*). Data regarding VEGFR2 expression was excluded from our analysis due to an absence of detection in the positive control.

Data acquisition and analysis were performed in a BD FACS Canto II flow cytometer. The laser configuration and voltage were calibrated prior to running the samples. Compensation was optimised using single stained controls. Live and dead cells were discriminated using the forward and side scatter plot morphology. A minimum of 10^4 events was recorded for each sample.

The data were further analysed using FlowJo software (*v10.8.1, BD Biosciences*).

4.6. Immunohistochemistry and immunofluorescence

To perform the immunostaining, 4 μ m-thick tissue sections were deparaffinized in xylene and rehydrated in isopropanol. Endogenous peroxidase activity was blocked by immersing the sections in a solution of methanol and 0.3% hydrogen peroxide for 15 minutes. Antigen retrieval was achieved by heat-induced unmasking for 30 minutes at 95°C, using either a citrate buffer (pH6) or Tris-EDTA (pH8). To permeabilize the cytosolic and nuclear membranes, the sections were treated with a 0.03% Triton-PBS solution for 15 minutes, before incubating for 30 minutes in a BSA-based blocking solution.

Subsequently, the samples were incubated overnight at 4°C with the primary antibody (*anti-ERG rabbit mAb [A7L1G], ref. 97249, Cell Signalling Technology, Danvers, USA; anti-Ki67 recombinant rabbit antibody [SP6], ref. ab16667, and rabbit Anti-LSEctin antibody [EPR13724], ref. ab181196, Abcam, Cambridge, UK*). After washing with TBS-tween, the adequate secondary antibody (*Dako EnVision + system-HRP labelled polymer, either anti-Rabbit, ref. K4003; or anti-Mouse, ref. K4001, Agilent Technologies Inc., Santa Clara, USA*) was added for 30 minutes.

For immunohistochemistry, the labelled antigens were finally visualized using Dako liquid DAB + substrate chromogen system (*ref. K3468, Agilent Technologies Inc., Santa Clara, USA*). The sections were counterstained with hematoxylin and eosin and observed using bright-field microscopy.

For immunofluorescence, the antigens were revealed using TSA AlexaFluor tyramide reagent λ 595 (*ref. B40957*) or λ 647 (*ref. B40958*) (*Life Technologies Corporations, Eugene, OR, USA*) in borate buffer with 0.003% H_2O_2 . Nuclei staining was performed with DAPI. The sections were examined using fluorescent microscopy.

4.7. Quantification of tissular hypoxia

To evaluate the degree and extent of tissular hypoxia in the DRL compared to the implanted left graft, immunostaining for carbonic anhydrase 9 (*anti-CA9 mouse mAb [2D3], ref. ab107257*) a HIF pathway downstream effector, was conducted. A colon biopsy served as a positive control. The immunoreactivity score (IRS) was calculated as previously described in the literature (115), through the multiplication of an intensity score (ranging from 1 to 2) by a proportion of marked cells score (from 1 to 4).

4.8. Assessment of sinusoidal morphology

Sinusoidal morphology was assessed through the examination of LSEctin-stained specimens, which were scanned at a magnification of 20x using a Zeiss Axioscan slide scanner. Analysis was performed using the Zen Blue 3.7 software. To determine sinusoidal density, 500µm lines were randomly drawn at five locations within each specimen, and the total number of sinusoid crossings along these lines was recorded. Concurrently, the sinusoids' diameter was determined by identifying their minimum cross-sectional diameter within five squares of a 200µm length, traced at random locations on each specimen (minimum 200 sinusoids measured per condition).

4.9. Combined immunofluorescence and fluorescent in-situ hybridization (FISH)

Anti-ERG immunofluorescence staining was performed as previously described, using TSA 647 fluorochrome for revealing. The sections were then imaged on a Zeiss Axioscan Slide Scanner. Subsequently, the samples were immersed in water for 10 minutes such as to solubilize the mounting medium, then progressively dehydrated in ascending ethanol series for 15 minutes.

FISH was then performed on the samples according to the manufacturer's instructions, using a Vysis CEP X SpectrumOrange / Y SpectrumGreen direct labelled fluorescent DNA probe kit (*ref. 07J22-050, Abbott, Illinois, USA*).

After staining, the tissue was scanned at 40x magnification using a Zeiss Axioscan, using a 5-level Z-stack to produce a maximal intensity projection of the FISH signal. Finally, the image for analysis was created by numerically superposing both the scans displaying the IF and the FISH signals, using the HALO Image Analysis software (*Indica Labs, Albuquerque, USA*).

4.10. Statistical analysis

Graph creation and statistical analyses were performed using GraphPad Prism version 8.0.1 for Windows (*GraphPad Software, San Diego, California USA*). Continuous variables are graphed using mean and standard deviation, while categorical variables are presented as frequencies and corresponding percentages. Dots represent individual data points.

Statistical analysis was performed using either repeated-measures two-way analysis of variance (ANOVA) or one-way ANOVA, followed by multiple comparisons with Bonferroni's correction. A linear mixed-effects model (restricted maximum likelihood - REML) was fitted to accommodate uneven intra-group variance whenever presented with missing values. Paired or unpaired Student's T test was used to determine the correlation between two variables.

Significance levels were set at $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), and $P < 0.0001$ (****).

5. Results

1° ALDAPT recipients exhibited significantly increased levels of granulocyte-colony stimulating factor (G-CSF), especially on the first post-operative day. In contrast, no systemic increase in VEGFa or chemotactic factors was noted.

Plasma samples obtained from both donors and recipients after the initial RAPID procedure were analysed through ELISA. The aim was to quantify several cytokines and growth factors that could be implicated in either bone marrow stimulation, progenitor chemotaxis or angiogenesis. Data regarding perioperative portal blood samples is provided in the supplemental materials section.

We performed linear mixed effects (REML) analysis to assess the effect of postoperative timing ("timing" factor) and donor or recipient status ("group" factor) on G-CSF levels (*Fig. 8a*). Simple main effects analysis showed a significant effect of group ($p=.02$, $F=9.42$), with recipients having higher overall G-CSF levels compared to donors (mean difference 8.29pg/mL). In addition, timing showed a significant effect on G-CSF levels ($p=.01$, $F=10.04$), with post-hoc pairwise comparisons revealing an increase in G-CSF on POD1 (mean diff. 18.51pg/mL, $p=.04$). There was no significant interaction between these variables ($p=.23$, $F=1.40$).

In addition, recipients showed a trend towards higher overall erythropoietin (EPO) levels compared to donors (*Fig. 8b*), although this group effect fell just short of statistical significance ($p=.06$, $F=5.57$). Post-operative kinetics also showed a sub-significant effect on EPO levels ($p=.20$, $F=1.93$). No interaction was found between these independent variables ($p=.83$, $F=0.53$).

Other cytokines tested included VEGF-a, IL6, IL8, MCP1, M-CSF, and SD1-a; none of which showed a statistically significant increase in either donor or recipient during the post-operative period.

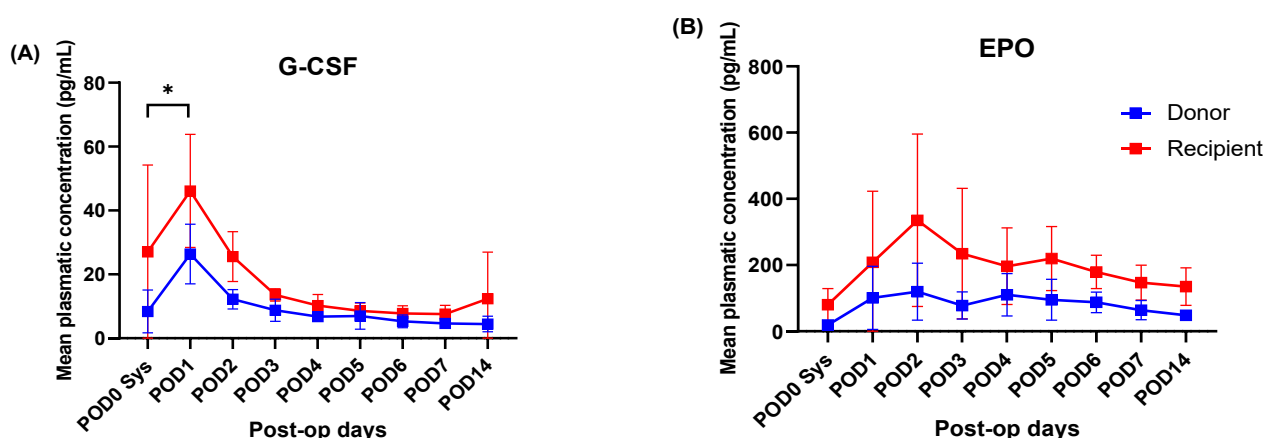


Figure 8. G-CSF and EPO levels in ALDAPT donors and recipients.

Levels of G-CSF and EPO were measured by ELISA in the plasma of ALDAPT donors and recipients ($n=3$ to 4).

(A) G-CSF levels exhibited a significant peak at POD1, as indicated by post-hoc multiple comparisons (mean diff. 18.51 pg/mL, $p=.04$). (B) A trend towards EPO elevation was observed in recipients, although it remained sub-significant.

2° Both the deportalised right liver and graft exhibited an increased expression of hypoxia marker CA9 at POD14.

The presence of tissular hypoxia in the recipients' livers was assessed by immunostaining for CA9 in both the DRL and the left graft, at reperfusion (POD0) and at the second stage of RAPID (POD14) (*fig.9b*). The intensity and extent of CA9 staining was quantified using an immunoreactivity score (IRS).

A one-way ANOVA was performed to examine the effect of the above conditions on CA9 expression (*fig.9a*). There was a statistically significant difference in mean IRS between groups ($F=5.43$, $p=.006$), with post-hoc multiple comparisons showing a significant increase in both ALDAPT DRL and grafts at POD14 compared to healthy controls ($p=.02$ and $p=.04$ respectively).

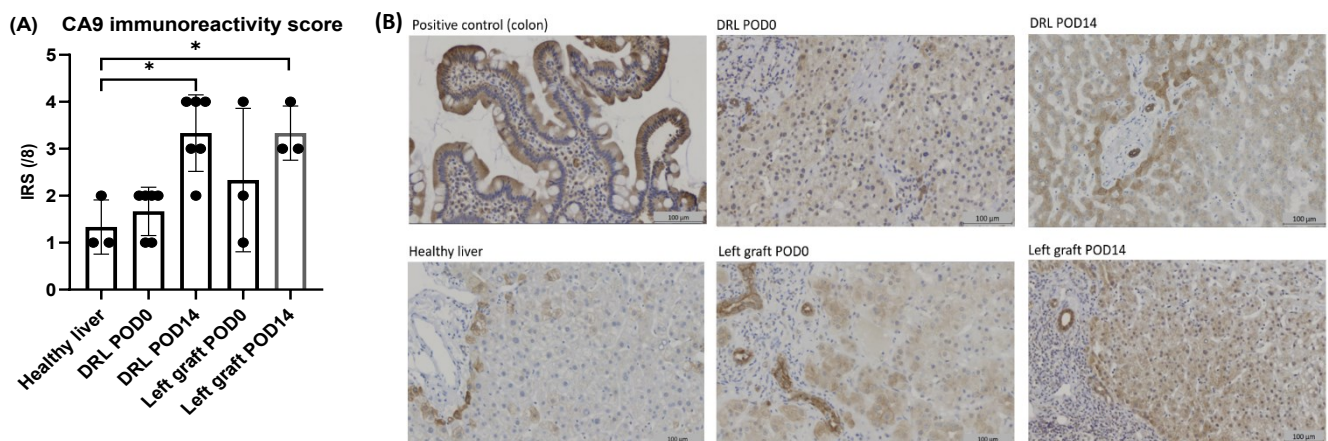


Figure 9. Expression of CA9 in the liver of recipients at POD0 and 14.
 (A) CA9 expression quantified by IRS ($n=3$ for healthy liver and left grafts; $n=6$ for DRL).
 (B) Illustration of representative CA9 staining in each group (scale bar representing 100µm).

3° Circulating MAC numbers increased after RAPID transplantation, with the recipients experiencing a recruitment of higher magnitude than the donors. ECFC levels however, displayed no significant variation.

Flow cytometric analysis was performed on cryopreserved PBMC from four ALDAPT donor-recipient pairs. Myeloid angiogenic cells were identified by assessing the co-expression of the myeloid markers CD45 and CD11b together with the endothelial marker CD31. Conversely, ECFC levels were estimated by quantifying CD31+CD34+ cells, with critical exclusion of CD45.

Statistical analysis through repeated measures two-way ANOVA revealed a significant post-operative increase in absolute MAC levels ($F=4.35$, $p=.046$), maximal on POD2 (mean diff. 27.83 pg/mL, $p=.03$). There was no significant effect of group, but most strikingly a marked intersubject variability ($F=4.54$, $p=.006$) (*fig. 10a*).

After normalising absolute cell counts to baseline levels (as measured pre-surgery on POD0), both time and group effects proved significant, with notable recruitment occurring from POD2 (mean diff. +27.83% of PBMC, $p=.03$), and of significantly greater magnitude in recipients (mean diff. +14.77% of PBMC, $p=.04$).

On the other hand, ECFC levels were comparable between donors and recipients and did not undergo significant recruitment after liver surgery (*fig. 10b*).

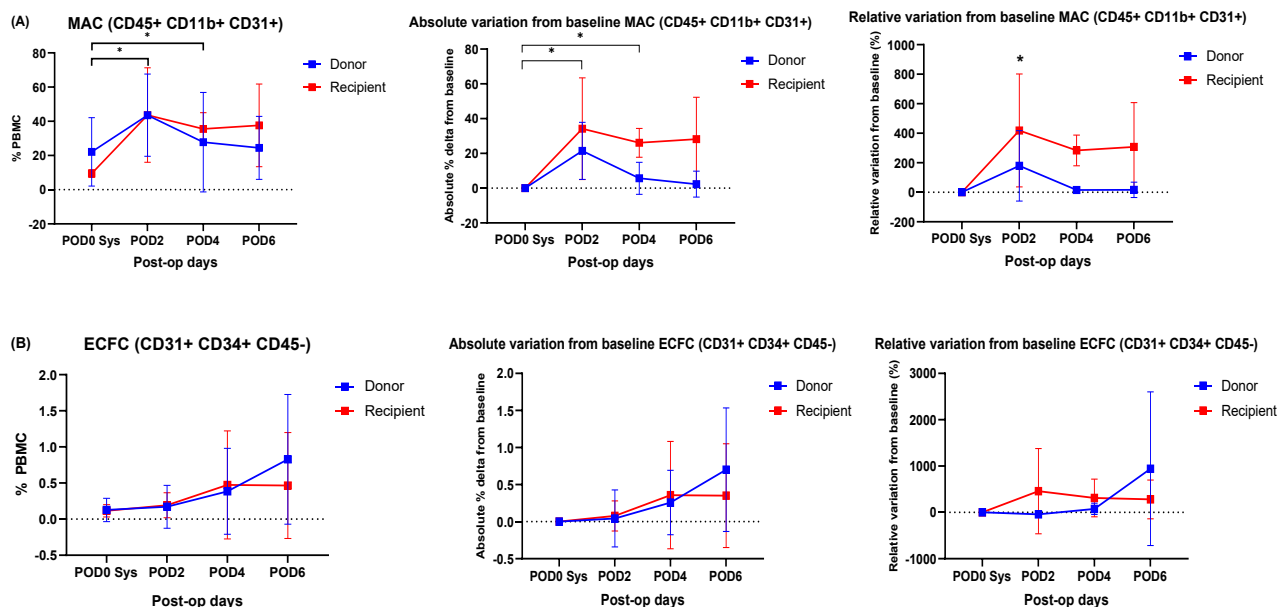


Figure 10. Flow cytometric analysis of post-operative MAC and ECFC levels, data represented as mean and SD (n=4). (A) MAC levels after the 1st stage of RAPID in recipients and donors, expressed as (left to right): proportion of total PMBC, absolute increase from baseline percentage, relative increase normalized by baseline percentage (time factor $p=.07$, group factor $p=.04$ with mean diff. +199.5% in recipients compared to donors). (B) ECFC levels after the 1st stage of RAPID in recipients and donors, expressed as (left to right): proportion of total PMBC, absolute increase from baseline percentage, relative increase normalized by baseline percentage

3° Both the donors' and recipients' cultured PBMCs exhibited a similar enhancement of endothelial-related colony formation abilities following liver surgery.

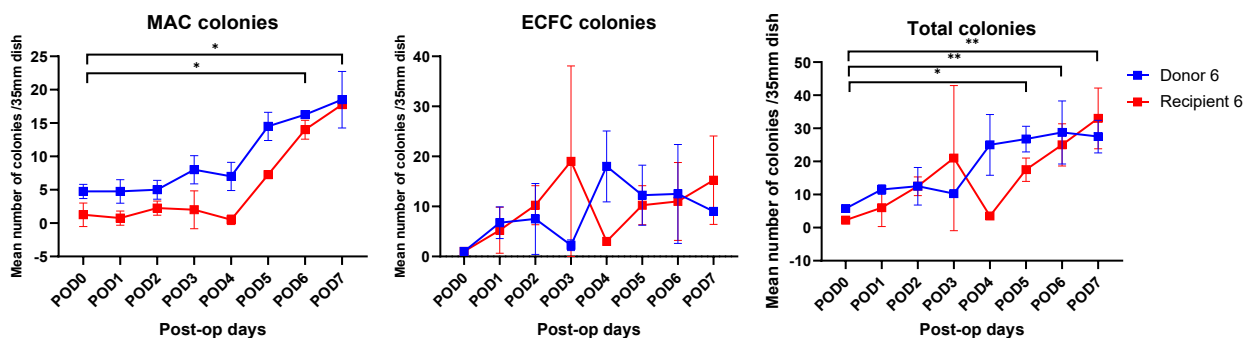
EPC-CFA was performed on PBMCs from recipients and donors of the 6th and 7th ALDAPT cases. After an 18-day culture, distinct colonies emerged, comprising either small (MAC) or large (ECFC) cells. Due to methodological and interindividual specificities leading to discrepancies in absolute colony counts between experiments, we opted to analyse group- and time-related variation patterns in both conducted experiments separately.

Statistical analysis using two-way ANOVA revealed no significant simple main effect of the group on colony numbers. However, post-operative timing significantly influenced total colony formation in both conducted EPC-CFA (respectively: $F=5.25$, $p=.004$; and $F=7.21$, $p=.0009$).

In the ALDAPT6 experiment (*fig. 11b*), a notable increase in MAC colony numbers occurred by POD6 ($p=.01$), and in total colonies by POD5 ($p=.03$). The impact of post-operative timing on total colonies was further affirmed in the ALDAPT7 assay (*fig. 11b*); however, surprisingly, its kinetics were marked by a transient decrease in total colony counts initially, followed by a significant increase eventually restoring similar-to-baseline levels by POD4.

In contrast, no significant variation in ECFC colony counts was observed after surgery.

(A) EPC-CFA in ALDAPT 6



(B) EPC-CFA in ALDAPT 7

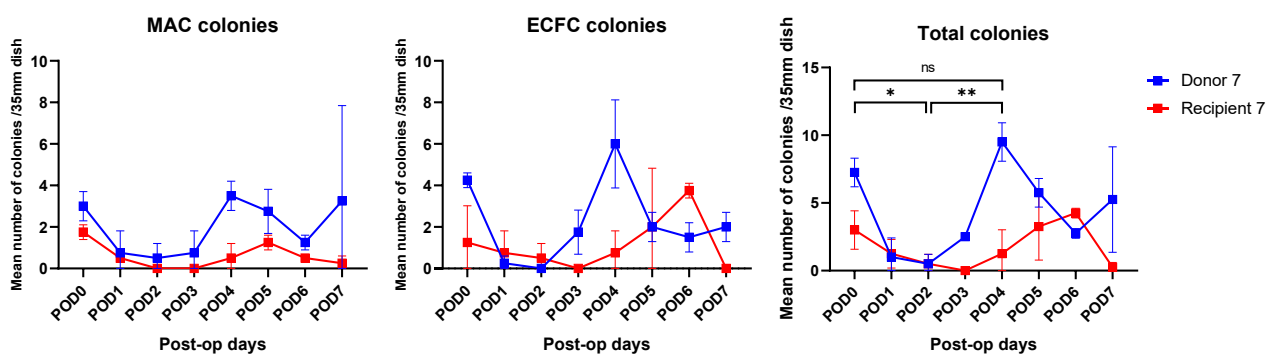


Figure 11. Colony counts in twice-conducted EPC-CFA, with data expressed as the mean of duplicate cultures for each timepoint (n=2).

(A) EPC-CFA conducted on the 6th ALDAPT pair, marked by a significant increase in both MAC colonies on POD6 (mean diff. 12.13, $p=.01$) and POD7 (mean diff. 15.3, $p=.02$); and total colonies on POD5 (mean diff. 18.13, $p=.03$), POD6 (mean diff. 22.88, $p=.007$), and POD7 (mean diff. 26.25, $p=.002$).

(B) EPC-CFA on the 7th ALDAPT pair, showcasing an initial drop in total colony count reaching minimal levels at POD2 (mean diff. -4.62, $p=.01$). Subsequent increase occurred from POD2 to POD4 (mean diff. 4.86, $p=.007$), becoming comparable to baseline.

4° The post-operative surge in angiogenic-related colony formation was confirmed to specifically affect myeloid angiogenic cells.

Differentiating colonies formed by either MAC or ECFC based on simple morphological analysis proved challenging. To address this issue, we phenotyped cultured cells by dispersing them on a glass slide using a cytopspin device. After an 18-day culture, cells were collectively detached and stained (see Fig.12). We then attempted to selectively harvest cells derived from MAC or ECFC colonies by mechanical aspiration with a micropipette under visual microscopic control. However, this technique did not yield enough cells for staining, nor was it sufficiently selective.

Our qualitative characterisation of cultures containing both small and large cell colonies by immunofluorescence staining revealed that a significant proportion of cells expressed the endothelial markers CD31 (estimated 22.5%) and VE-cadherin (estimated 64%), with almost all expressing CD45 (see Fig.12 - right). The latter homogeneous expression of MAC-associated CD45 suggests that all our colonies were predominantly MACs.

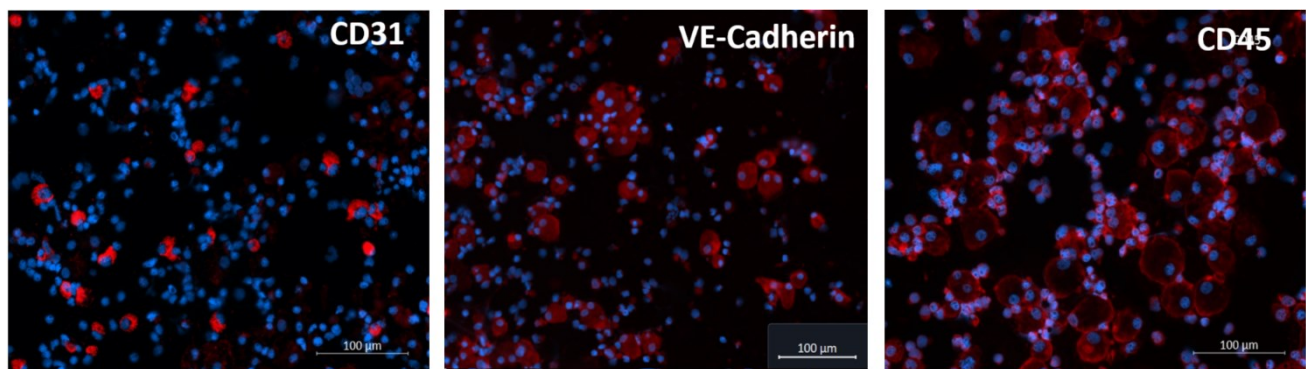


Figure 12. Expression of Endothelial and Hematopoietic Cell Surface Markers. Cultured cells derived from both MAC and putative ECFC colonies exhibit the expression of both endothelial and hematopoietic cell surface markers. Scale bar representing 100μm. DAPI staining is represented in blue. From left to right, red represents CD31, VE-cadherin, and CD45.

5° Engraftment of exogenous LSECs in the graft's sinusoidal network remained anecdotal.

To identify recipient-derived LSECs engrafted within the graft at POD14, we performed dual X/Y chromosome FISH and immunofluorescence staining for the nuclear endothelial marker ERG (*fig.13*). In our preliminary analysis of one sex-mismatched ALDAPT transplant at POD14, we identified 475 ERG-positive nuclei, of which 54.95% were sex-identified (displaying two sexual chromosomes). Out of these, 3.06% showed XY chromosome co-staining marking their exogenous origin, while 96.94% were of XX phenotype. The remaining 45.05% ERG+ nuclei either displayed a single X or Y, or no FISH signal.

In contrast, a sex-mismatched orthotopic whole liver transplantation biopsy at POD10 showed no XY+ERG+ nuclei.

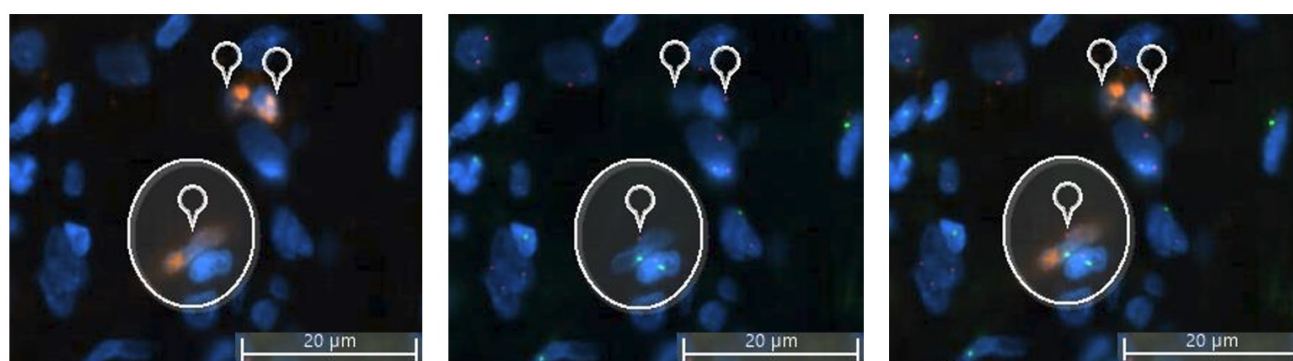


Figure 13. Illustration of a XY+ endothelial cell in a graft of female origin, transplanted into the male recipient.

Endothelial nuclei designed by pointers. Nucleus of interest circled. Scale bars represent 20μm.

From left to right, DAPI in blue and ERG in orange; DAPI in blue, X chromosome in red and Y in green; all colours merged.

6° Sinusoidal diameter was restored in ALDAPT grafts at POD14, unlike sinusoidal density.

LSEctin staining was performed to facilitate the identification and analysis of sinusoids in samples from various liver transplantation scenarios. This C-type lectin, also known as Clec4g, is expressed by LSECs and some immune cells. It plays a role in their immune function, binding to carbohydrate ligands expressed by various pathogens.

We first evaluated vascular density. One-way ANOVA showed a significant impact of different liver transplantation contexts - namely whole liver OLT, right LDLT, RAPID - on sinusoidal density ($F=9.79$, $p=.0003$) (*fig.14a*). Further comparisons revealed that, within 7 to 14 days post-transplantation, all biopsies exhibited a similar reduction in vascular density compared to a healthy sinusoidal network (mean diff. ranging from -4.75 to -6.15, $p=.001$ to $.0007$). Interestingly, while sinusoidal density in right (r-)LDLT decreased from reperfusion to POD7 (mean diff. -3.8, $p=.04$), this phenomenon did not progress in RAPID ($p>.99$) to the end of the interoperative period and was not different from that of OLT ($p>.99$).

Microcirculatory integrity was further assessed by measuring the mean sinusoidal diameters in graft biopsies (*fig.14b*). One-way ANOVA showed a significant impact of transplantation on these

diameters ($F=9.78$, $p=.0003$). Specifically, post-hoc multiple comparisons revealed that RAPID transplantation was associated to sinusoidal dilation at reperfusion (mean diff. $+1.7\mu\text{m}$, $p=.03$), which resolved by POD14 similar to healthy livers ($p=.73$). In contrast, right LDLT was associated to a decrease in mean sinusoidal diameter from reperfusion to the follow-up biopsy at POD7 (mean diff. $-1.9\mu\text{m}$, $p=.045$). rLDLT diameters at POD7, though sub-significantly inferior to those of a normal liver, were significantly reduced in comparison to ALDAPT POD14 specimens (mean diff. $-1.99\mu\text{m}$, $p=.02$). Examination of the vascular diameter distribution confirmed these trends, showcasing a superimposable profile between ALDAPT grafts at POD14 and healthy livers, while rLDLT was associated to a shift towards lower values at POD7 (*fig.14c*).

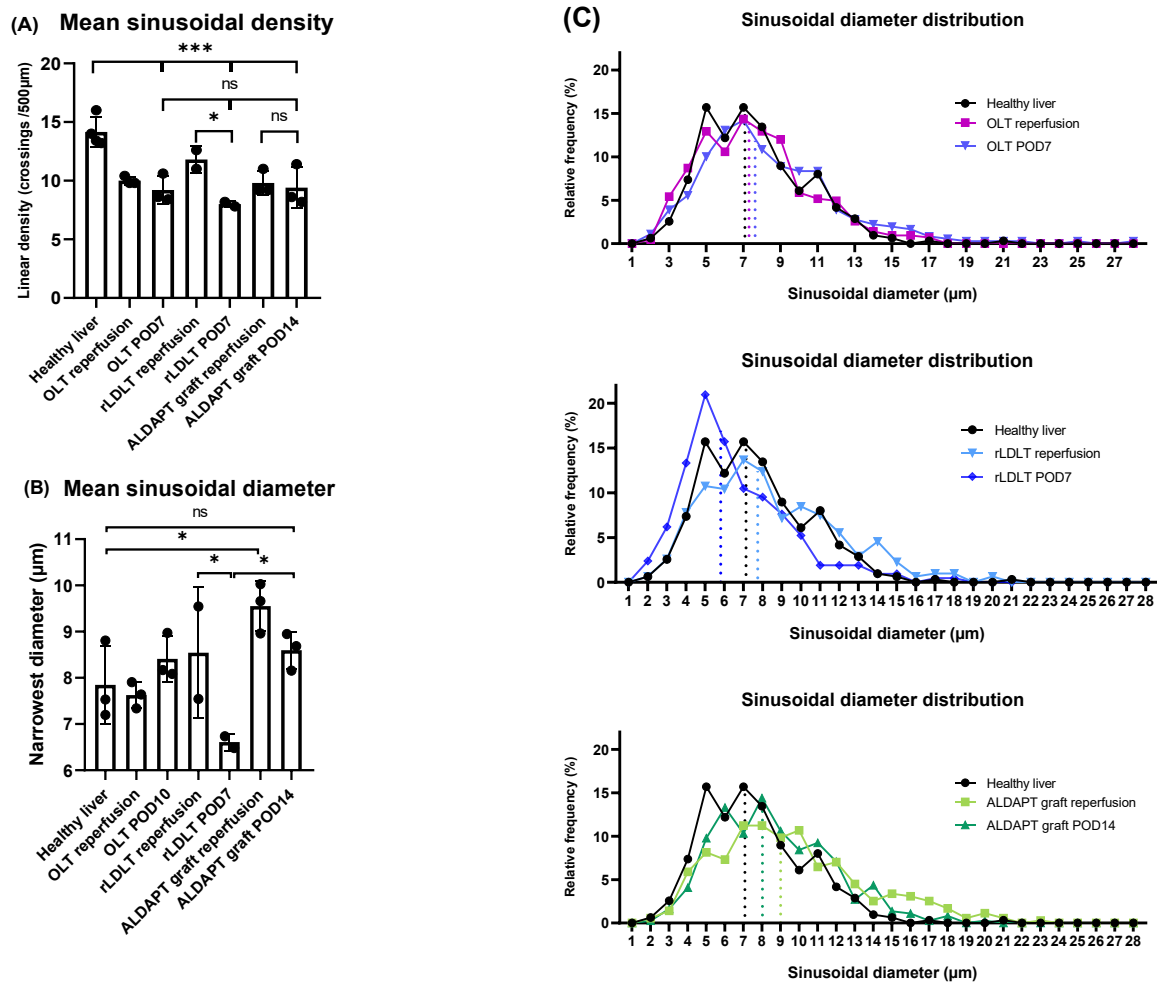


Figure 14. Microcirculatory and sinusoidal morphology assessment through density and diameter.
 (A) Sinusoidal density, dots representing the mean of individual values measured in 2 to 3 biopsies per condition ($n=2-3$).
 (B) Narrowest sinusoidal diameter, with dots representing the mean of individual values measured in 2 to 3 biopsies.
 (C) The distribution of post-operative vessel diameters at reperfusion and post-operative control biopsy is expressed as the relative percentage of sinusoids per given narrowest diameter, with data measured over 2 to 3 biopsies. Dotted lines represent the median. In healthy livers, the median diameter is $7.11\mu\text{m}$ with a range of $1.85-20.78$. In OLT, the sinusoidal diameter distribution pattern remains similar to healthy liver from reperfusion (median $7.34\mu\text{m}$, range $1.69-17.47$) to POD7 (median $7.65\mu\text{m}$, range $2.37-27.55$). In rLDLT, while no apparent diameter variation was found at reperfusion (median $7.75\mu\text{m}$, range $2.30-19.87$), the POD7 biopsy showed a skewed distribution towards smaller diameters (median $5.90\mu\text{m}$, range $1.82-17.85$). In RAPID, the pattern observed at reperfusion was characterised by a slight shift towards sinusoidal dilation (median $9.02\mu\text{m}$, range $2.06-22.91$), which resolved to a curve similar to that of a healthy liver at POD14 (median $8.11\mu\text{m}$, range $2.33-20.19$).

6. Discussion

With the limited availability of liver grafts, living-donor liver transplantation is gaining increasing attention. Historically underutilised due to the challenges associated with transplanting a reduced parenchymal volume, this approach increases the risk of post-operative complications, with small-for-size syndrome (SFSS) being a major concern. The RAPID procedure is emerging as a promising solution, offering clinical efficacy by avoiding SFSS while improving safety for both donor and recipient. Consequently, this research project aims to elucidate the mechanisms underlying the remarkably efficient regeneration and sustained function of the liver after RAPID, with a particular focus on the pivotal role of pro-angiogenic vascular restoration mechanisms.

6.1. Systemic endothelial progenitor recruitment stimuli

To account for regenerative efficacy observed in RAPID transplantation and its counterpart hepatectomy, ALPPS, it is proposed that the DRL may perform a dual role by supporting hepatic metabolic function during the graft regeneration phase, and by secreting endocrine factors promoting the graft's growth (116). Thus, we aimed to identify the latter pro-regenerative cytokines in peripheral blood samples issued from ALDAPT recipients.

G-CSF

G-CSF is a pleiotropic cytokine secreted by several cell types, of which fibroblasts and endothelial cells (125). It is renowned for its immune-related functions, including granulopoiesis and mobilization of hematopoietic stem cells (117–119). Interestingly, G-CSF has demonstrated additional implication in liver regeneration and angiogenesis (120–123). For example, administration of exogenous G-CSF improved survival and increase sinusoidal perfusion in rodent models of SFSS (120,124).

Our investigation into RAPID liver transplantation revealed a significant increase in G-CSF levels among recipients compared to donors, peaking at POD1. Importantly, this surge appeared independent of any systemic inflammation related to group differences, as evidenced by similar levels of IL6 and CRP (*respectively, fig.15c and fig.15d – available in supplemental material*). These findings suggest that G-CSF elevation may stem from a recipient-specific localised immune response to liver injury, rather than a generalised inflammatory response. Supporting this, previous studies have specifically linked increased G-CSF to liver regeneration in rats (122).

Moreover, our study found a correlation between heightened post-operative G-CSF levels in recipients and increased MAC levels, suggesting a potential contribution of induced bone marrow hematopoiesis to liver regeneration. This is consistent with previous findings in the field of coronary and peripheral artery disease, where G-CSF priming was shown to improve MAC levels, function and proliferative capacity (126–128), while it failed to effectively mobilise ECFC (117).

While we acknowledge the potential for liver-derived G-CSF secretion, our analysis warrants caution regarding two considerations. Firstly, our plasmatic measurements lack data on key systemic inflammation-related G-CSF inducers such as IL-1 and TNF. Additionally, the recipients' post-operative immunosuppressants, such as tacrolimus, may influence early inflammatory responses.

Furthermore, since intrahepatic data was not collected, further investigation is needed to ascertain the source of G-CSF production. For instance, exploring whether hepatic fibroblasts and endothelial cells may contribute to G-CSF secretion could help clarify the recipient's systemic neutrophilic and bone marrow response (125). In situ hybridisation offers a means to localise intrahepatic G-CSF mRNA and identify the cells responsible for its production (123).

Lastly, it should be noted that the clinical relevance of G-CSF in human liver regeneration remains controversial. Despite its potential, G-CSF has not yet demonstrated a clear benefit in treating severe liver injuries such as acute-on-chronic liver failure and SFSS (129,130).

VEGFa

While G-CSF plays a crucial role in MAC recruitment, VEGFa is also recognised for priming pro-angiogenic properties (86,117). Given its significant involvement in typical liver regeneration (*previously detailed in section 3.2*) and its induction under hypoxic conditions (131), we expected an increase in VEGFa levels in recipients following portal ligation of the right liver. Surprisingly, no such observation was made in either recipients or donors after surgery (*fig.16 - available in supplemental material*), leading to conflicting results that could be attributed to several factors.

First, our small cohort of patients (n=4 per group) showed marked inter-individual variability, making statistical analysis challenging. The potential impact of anti-VEGF therapies on the recipients' data adds another layer of complexity. Bevacizumab, which binds 97% of VEGFa, has a prolonged mean half-life of 20 days, ranging from 11 to 50 (132). Thus, caution has been advised in interpreting VEGF levels due to significant persistence up to 3 to 6 months after administration (133).

Consistent with this pharmacodynamics, the two recipients who maintained a flat post-operative VEGFa curve had both received standard treatment plans for colorectal metastases, including bevacizumab, between 4 and 7 months prior to surgery. In contrast, the other two did show a post-operative increase, one treated for neuroendocrine tumour metastases with somatostatin and the other for colorectal cancer metastases with a last administration of bevacizumab 9 months prior.

In addition, the absence of a systemic increase in VEGFa doesn't negate a differential intrahepatic production dynamic. For instance, in the ALPPS setting, Robles-Campos et al. previously showed an inhomogeneity in intrahepatic hypoxia-related factors expression. The FLR showed rapid gene expression changes, with both HIF1a and VEGFa increasing within one hour of contralateral portal vein ligation, which had normalised by the second stage of hepatectomy. In contrast, no upregulation of VEGFa was found in the deportalised liver (134).

In conclusion, despite challenges in detecting systemic angiogenic signalling in our limited cohort, various confounding factors hindered rigorous statistical analysis. Further investigations on a larger scale would benefit from a subgroup analysis considering the administration of anti-angiogenic therapies. In addition, alternative pro-angiogenic pathways, both systemic and intrahepatic, such as angiopoietins or FGF1 and 2, warrant investigation.

EPO

During the postoperative period, we observed a slightly sub-significant trend towards globally increased EPO levels in ALDAPT recipients, in comparison to their donors. This finding holds relevance given EPO's known pro-angiogenic properties that complement its role in erythrocyte differentiation. Indeed, it exerts direct effects on endothelial cells (135–137), and notably enhances MAC levels and function (138,139). Numerous studies also suggest that EPO contributes to liver regeneration and improves survival after hepatectomy or partial liver transplantation (140–144).

The factors driving increased EPO production in the context of RAPID surgery remain elusive. Perioperative blood loss is likely to be involved, acting as a significant renal stimulus for EPO secretion. Accordingly, our data showed a trend towards higher perioperative blood loss and lower post-operative total hemoglobin (Hb) levels in recipients, albeit not reaching significance (*fig.17a, 17b - supplemental material*). However, surprisingly, the post-operative drop from baseline Hb was comparable between recipients and donors (*fig.17c - supplemental material*), leaving the complete explanation for the recipients' rise in EPO levels partly unresolved.

An alternative hypothesis points to the intrahepatic secretion of EPO. Given its induction by hypoxia through the HIF pathway (145), it is conceivable that a hypoxic environment in the DRL or graft could contribute to EPO elevation. Although the fraction secreted by the liver usually constitutes only 10% of total EPO after fetal development, this proportion can increase under certain conditions, such as observed in chronic kidney disease (146).

Further investigations are necessary to understand the mechanisms responsible for the increased EPO levels observed in RAPID recipients. To determine whether the DRL or graft contribute to the upregulation of this humoral pathway, it would be necessary to investigate intrahepatic production, which was not explored in this study.

Chemotactic factors - SDF1a, MCP1, IL8

Several chemotactic cytokines, including SDF1a and IL8 (147–149), are known for attracting pro-angiogenic monocytes from the bone marrow to ischemic regions. Moreover, MCP1 exerts direct chemotaxis on endothelial cells (150,151), making it a crucial angiogenic mediator.

Due to their unfeasibility of evaluating local MAC recruitment through engraftment, given their paracrine-mediated contribution to angiogenesis, we aimed to measure chemokine levels as surrogate markers for MAC intrahepatic homing signalling. In this regard, we were confronted with the absence of any substantial post-operative increase in peripheral blood. Our results remain rather

unsurprising, as chemokines are expected to follow a concentration gradient that reaches its peak at the local ischemic (hence, intrahepatic) recruitment site even when they are overexpressed (152,153).

In conclusion, these preliminary findings imply the existence of bone marrow-stimulating agents in recipients that could aid in MAC recruitment and/or function. Among the limitations of this study figure the limited cohort size, preventing us from carrying out robust statistical analyses. In addition, our control group was represented by healthy living donors undergoing 40% partial hepatectomy; this introduces possible confounding variables in our analysis, including preoperative oncological treatments, differing initial graft or FLR volume, presence of deportalised parenchyma, ...

Consequently, to distinguish the pro-angiogenic factors resulting from a small FLR volume from that of the ligated right liver, samples issued from subjects undergoing portal vein ligation and others a left LDLT, at comparable time points, may provide the most informative control. A comprehensive analysis of local cytokine gradients in subhepatic blood or hepatic parenchyma would also benefit the understanding of both chemotaxis and DRL contribution in ALDAPT. Finally, we suggest studying levels of other important pro-angiogenic factors besides VEGFa, such as angiopoietins, FGF1 and 2, which may be less subjected to antagonism by antitumoral therapies.

6.2. Source of systemic bone-marrow mobilising signals

Corroborating the notion that the DRL may act as an endocrine pro-angiogenic signalling source, our observations suggest its involvement in hypoxia-induced pathways. We noted an elevation in the hypoxia marker CA9 by POD14 in both the DRL and the graft, compared to a healthy liver. This aligns with the previously reported upregulation of hypoxia-induced HIF1a in the DRL and FLR following ALPPS (134,154).

Moreover, yet unpublished hemodynamic data from our ALDAPT recipients (*issued from Dr L. Coubeau*) indicate decreased O₂ supply to the DRL with an increase in O₂ extraction, implying tissular adaptation to hypoxia. In contrast, the graft exhibited signs of hypoxia despite increased O₂ delivery compared to the native liver, without concomitant adaptation of extraction. This phenomenon could not be attributed to HABR, ruled out through hemodynamic evaluation of the arterial flow; neither did it result from increased oxygen consumption due to hepatocyte hyperproliferation, as KI67 expression was not significantly upregulated by POD14 (*fig.18 - supplemental material*). Thus, hypoxia in the graft may result either from sinusoidal insufficiency, or from a sudden escalation in metabolic demand associated with assuming increasing vital hepatic functions. Accordingly, preliminary clinical data (*courtesy of Dr L. Coubeau*) showed a subsignificant yet noteworthy trend towards increasing graft function from POD7 to 14, as assessed through hepatobiliary scintigraphy (HBS) with mebrofenin-labelled Technetium-99m. In 8 RAPID scenarios, the mean relative gain in graft function from POD7 to 14 was 42.31% (paired T-test: $p=.07$; $\pm 56.44\%$).

Certainly, a direct examination of intrahepatic hypoxia-induced factors is crucial to validate our preliminary and surrogate findings.

6.3. Circulating endothelial progenitor cells

In our analysis of myeloid angiogenic cells and endothelial colony-forming cells levels after liver surgery, we used both cell culture and flow cytometry to assess peripheral blood samples from donors and recipients. Though both techniques indicated a post-operative increase in MAC levels, they provided slightly discrepant kinetics. Absolute MAC counts were found to rise from POD2, with recipients experiencing a significantly greater recruitment than the donors; while the colony-forming potential peaked in both groups at POD5 to 6. This observation is further supported by previous findings from Stroncek et al, who described a delay of 4-5 days from G-CSF treatment to peak MAC levels and function (129). Thus, our data suggests that pro-angiogenic cells are rapidly recruited and progressively acquire a more proliferative phenotype over time.

This discovery holds clinical relevance, as previous studies have emphasised the pivotal role of MACs in liver regeneration (35,36). Encouraging preclinical investigations have further supported this, associating the presence of hematopoietic progenitor cells (although not fully characterised) with a substantial enhancement in FLR growth following selective portal vein embolization (155–157). Additionally, our preliminary data suggest a correlation between the upward trend in graft function as evaluated by hepatobiliary scintigraphy from POD7 to 14, and the delayed increase in in-vitro MAC proliferation. However, caution is warranted in interpreting these findings, due to limitations including the absence of available baseline for HBS function data (at POD0) and the limited cohort.

In contrast to myeloid contributors, a concurrent increase in ECFC levels was not observed in either recipients or donors. This may be attributed to their inherent scarcity in the bloodstream, and analysed PBMC numbers may not have been sufficient to detect subtle changes in ECFC levels. Given uncertainties in preliminary data regarding the clinical relevance of exogenous LSEC engraftment within the graft, we restrained from refining our analysis.

Additionally, ECFC counts are influenced by individual factors such as general cardiovascular health and prior exposure to anti-tumour therapies, complicating the comparison between donors and recipients. Major confounding factors, including treatments such as bevacizumab and Folfex known to affect ECFC and MAC levels and function (158), should be considered in future subgroup analyses.

To improve the reliability of MAC and ECFC identification in future analyses, several technical refinements should be considered. Increasing the volume of analysed PBMCs would improve the sensitivity of detecting rare ECFC. In addition, specificity could benefit from expanding the flow cytometric antigen panel, to include a wider range of endothelial-related markers such as endoglin, CD146 and VCAM1. Furthermore, we acknowledge the limitation of excluding VEGFR2 expression from our analysis due to its absence in the positive control (HUVECs). Testing of alternative VEGFR2-specific detection antibodies may address this issue.

In conclusion, our findings suggest that the RAPID is associated with increased MAC recruitment. They highlight the need for future investigations to focus on intrahepatic chemotactic

gradients influencing local MAC recruitment, and to consider comparisons with other control groups (LDLT, ALPPS and Portal vein ligation). In addition, standardisation of cell identification across studies is essential for a comprehensive understanding of EPC-related mechanisms.

6.4. Endothelial progenitor recruitment to the implanted liver graft

Due to the rarity of adequately sex-mismatched transplantations in the clinical setting, our exploratory analysis focused on a single available sample issued from a sex mismatched RAPID transplantation, using an OLT biopsy as control. We were able to successfully confirm the recruitment of exogenous LSECs into the RAPID graft's sinusoidal network, constituting 3.06% of the total sex-identified LSEC pool at POD14, while none was found in OLT at POD10. However, this relatively minor proportion of engraftment may not singularly account for a globally improved RAPID graft vascularisation. We propose that our observation stems rather from an epiphenomenon of rare ECFC recruitment, while MAC in more substantial amounts contribute more significantly to angiogenesis.

Our investigation into endothelial engraftment aligns with the findings of a study by Lee et al. in the context of dual lobe human LDLT. They demonstrated the integration of exogenous CD34+ cells in the sinusoidal endothelium, contributing to neovascular network formation in inverse correlation with the FLR volume (113).

In both Lee's study and our own, pinpointing the exact origin of these engrafted cells beyond their exogenous nature poses a considerable challenge, primarily due to the inherent limitations of human-based observational research. The prevailing understanding suggests that bone marrow-derived MACs generally do not integrate into vascular walls. However, the straightforward conclusion that engrafted endothelial cells solely originate from ECFC might be an oversimplification. Notably, certain rodent-based studies, including from the GAEN laboratory (UCLouvain) (159), employed GFP-tracing experiments to successfully identify a substantial engraftment of LSECs derived from the bone marrow (34,36).

Importantly, we addressed an important concern raised in Lee et al.'s study about potential fusion events between hematopoietic stem cells and endogenous LSECs without true differentiation, a mechanism previously described in hepatocytes (160). Our analysis permitted to rule out such a phenomenon by controlling for the presence of a single concomitant X chromosome, excluding fusion events. In counterpart, our FISH analysis may have led to an underestimation of Y+ exogenous LSECs numbers due to the precise intranuclear localization of the Y probe, whose signal was only partially detected using gross Z-stack imaging. This approximation led to a lack of complete chromosomal data regarding near half of our endothelial nuclei. Our technique could therefore be refined through several strategies, including technical imaging optimisations (finer Z-stack), or introducing a correction factor for the nuclear cutting effect (determined using a positive male control).

Ultimately, the main limitation of our analysis stems from the scarcity of sex-mismatched transplanted patients, which precluded statistical analysis.

6.5. Impact on the sinusoidal network

The microcirculation in the liver is often disturbed after transplantation, mainly due to ischaemia-reperfusion events (161,162). We assessed whether this temporary damage was exacerbated in RAPID, a context additionally characterized by portal hyperperfusion. Furthermore, we aimed to investigate whether sinusoidal morphology was restored during the surgical interphase, in correlation with the observed increase in MAC levels.

We specifically focused on evaluating sinusoidal density and diameter, which are crucial parameters used in orthogonal polarisation spectral (OPS) imaging to assess microvascular integrity in vivo (162–164). Indeed, these two factors could be estimated in fixed biopsies, while assessing other perfusion parameters such as erythrocyte velocity and volumetric blood flow proved challenging in ex vivo conditions.

Sinusoidal diameters in ALDAPT grafts initially showed a slight dilation, likely attributable to increased portal flow. Remarkably, by POD14, the diameters had normalised to align with those found in healthy livers. This stands in contrast with rLDLT cases, where diameters had significantly decreased from reperfusion to POD7, falling below those observed in ALDAPT POD14 specimens. Similar to these observations in rLDLT, previous studies in the ALPPS setting had reported sinusoidal narrowing in the FLR in 50% of cases, correlating with parenchymal proliferation and hepatocyte immaturity (165). However, as both rLDLT and ALPPS data in literature was collected at an earlier timepoint (POD7) than the RAPID, we acknowledge that interpreting our findings requires nuance. Indeed, the observed differences in vascular morphology could arise from the biopsy timing, with POD7 potentially falling within the parenchymal proliferation phase or preceding the completion of the angiogenic repair phase. Supporting this timing-related explanation, our data showed that RAPID grafts had likely already concluded the hepatocyte proliferation phase by POD14, as indicated by the absence of increased Ki67 expression (*fig.18 - supplemental material*). Therefore, a comparative analysis of RAPID biopsies at earlier timepoints, such as POD7, could provide additional insights into the quality of vascular regeneration.

In contrast to these encouraging data regarding diameters, the second major perfusion factor - sinusoidal density - prompted reserve regarding the overall improvement in liver microarchitecture in ALDAPT grafts. At POD14, sinusoidal density decreased compared to a healthy liver. Importantly, this reduction was not exclusive to RAPID but was also observed in the other transplantation scenarios, including non-regenerative orthotopic whole liver transplantation (162). Consequently, our data doesn't specifically link reduced sinusoidal density to impaired vascularisation in small liver transplantation. Further evaluation of in vivo parenchymal flow velocity, for instance using orthogonal polarisation spectral imaging (OPS) or Doppler parameters, is warranted to further enhance our understanding of the overall sinusoidal function.

7. Conclusion and perspectives

The RAPID procedure represents a revolutionary approach to living-donor transplantation with profound clinical, ethical, and mechanistic implications. From a clinical perspective, the RAPID's remarkable efficacy in transplanting small grafts opens new doors for curative treatment, particularly for oncological and metastatic patients who are often side-lined on traditional transplant waiting lists. The procedure also offers an ethical advantage by subjecting donors to a standardised and safer left hepatectomy, mitigating the risk of PHLF.

From a mechanistic point of view, our ALDAPT protocol within the RAPID context represents a unique opportunity for human-based translational research, providing insights into liver regeneration and its pathological counterpart, SFSS. By closely mimicking its physiopathogenic factors such as portal hyperflow and extremely low FLR, RAPID provides a window into SFSS without increasing the risk of liver failure in recipients. The inclusion of a sex-mismatched transplantation context also allows for the tracking of exogenous cells within the liver, enriching our understanding of the contributions of different cell populations to regeneration - areas that have traditionally been difficult to explore in observational human studies, and often rely on limited data extrapolated from rodent models.

Given the proposed link between SFSS and hepatic microcirculatory dysfunction, our primary focus in this study was to unravel the pro-angiogenic mechanisms in the context of RAPID, compared to normal liver regeneration as represented by healthy donors undergoing partial hepatectomy. Our investigation in this process has provided valuable insights.

First, we found increased bone marrow stimulatory signals in RAPID recipients, particularly G-CSF. Together with the enhanced recruitment of myeloid angiogenic cells, our data therefore suggest a systemic pro-angiogenic conditioning in RAPID. However, further investigation of the intra-hepatic paracrine contribution of these cells to liver regeneration is warranted. As MACs lack distinctive features to identify them within the transplanted liver, we would recommend further documenting intrahepatic homing factors that may trap circulating MACs into the graft, such as to strengthen the association between MACs and liver regeneration.

In contrast, our analysis revealed no significant increase in bona fide endothelial progenitor cells (ECFC), as assessed by both circulating levels and intrahepatic engraftment. Consequently, we argue that their role may be less substantial compared to bone marrow-derived cells. However, our preliminary results in this regard require validation in a larger cohort of sex-mismatched pairs, the study of ECFC being notably and inherently marked by inter-individual variations.

The third aspect of our study examined the potential link between EPC recruitment and the restoration of sinusoidal architecture, which is critical for graft growth and function. Importantly, our analysis of vessel structure revealed a normalised sinusoidal diameter in RAPID recipients at completion hepatectomy stage. While sinusoidal density remained reduced, this singular factor may not solely account for sinusoidal dysfunction, particularly considering clinical and scintigraphic

indications of enhanced graft function. Instead, it could stem from preceding parenchymal compartment growth. Thus, further analyses integrating detailed histological examination and in vivo microcirculation imaging (like OPS) would offer deeper insights into our findings.

The presence of a deportalised liver remnant represents the second key feature of RAPID, and may exert a significant influence on graft regeneration, either through endocrine stimulation or as a supplementary functional entity during the interim period. While this study did not comprehensively delve into the underlying mechanisms, it hinted at a plausible endocrine role of the ligated right liver through hypoxia-related pathways.

Our observation of increased expression of the hypoxia marker CA9 in the DRL suggests a potential source of hypoxia-associated signalling from the ligated right liver. However, the absence of systemic VEGFa signalling within our cohort, hindered by the limited number of patients and their heterogeneous clinical history, necessitates further investigation. Intrahepatic hypoxia mapping and refined characterisation could be achieved through the use of specific markers such as HIFs and ERO1a. Additionally, we advocate for the exploration of additional systemic angiogenic pathways, including angiopoietins and FGF1 and 2 levels. Finally, integrating supplemental sampling to evaluate hepatic venous blood, downstream of both the DRL and the graft, would enhance our understanding.

The alternative hypothesis proposes a functional role for the DRL during liver regeneration. Further investigation is necessary to determine whether it actually enhances graft regeneration or acts as a tutor, facilitating vital liver functions and allowing time for the critical angiogenic phase to unfold. Clinical data regarding differential liver function are currently being collected using mebrofenin-labelled Tc99 scintigraphy. Future results will reveal whether a function shift occurs between the DRL and the graft during the post-operative period, guiding the design of further mechanistic investigations into the regulation and shift of the liver's functional load.

For instance, considering the crucial role of LSECs in regulating hepatocytic function, it would be valuable to investigate whether intercellular communication drives LSECs to influence hepatocyte proliferative and functional activity. Exploring the mechanisms potentially involved in promoting functional programming, such as epithelial-to-mesenchymal transition pathways, would enhance the understanding of liver regeneration dynamics and help develop further clinical opportunities.

In conclusion, our study contributes significantly to the unravelling of pro-angiogenic mechanisms in RAPID and highlights the need for further investigation, particularly focusing on intra-hepatic signalling. The precise contribution of the DRL to graft regeneration and overall maintenance of liver function remains an intriguing area for future investigation. Elucidation of these mechanisms and their relative contribution to the success of RAPID has the potential for wider clinical application, thereby improving outcomes in liver transplantation. The particular focus on addressing small-for-size syndrome and improving graft regeneration highlights the translational impact of this research.

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9. Supplemental material

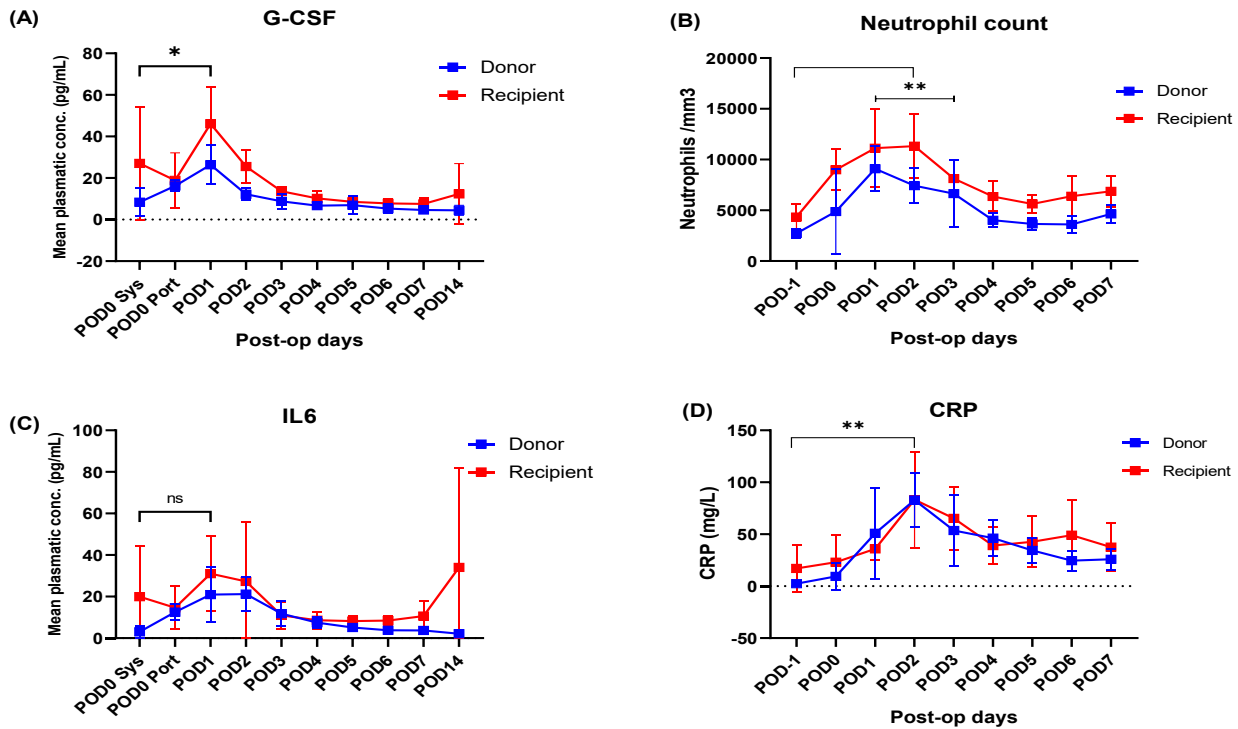


Figure 15. Relationship between G-CSF levels and inflammation-related parameters.

(A) Significant post-operative G-CSF elevation, particularly in recipients (refer to fig.8a).

(B) Elevation in neutrophil counts following the 1st stage of RAPID (n=6), correlating with G-CSF stimulation. Mixed-effects model analysis revealed a significant effect of post-operative timing ($p < .0001$), specifically on POD1 to 3 ($p = .003$ to $.009$). Group effect was also significant ($p = .005$), with recipients exhibiting a greater recruitment than donors (mean diff. + 2525/ μ L, SE 706.4).

(C) IL6 levels following the 1st stage of RAPID (n=4). Mixed-effects model analysis showed no significant effect of group ($p = .16$) or post-operative timing ($p = .17$) on IL6 levels.

(D) Elevation in CRP levels following the 1st stage of RAPID (n=6). Mixed-effects model analysis indicated a significant effect of post-operative timing ($p < .0003$), specifically on POD2 ($p = .005$, mean diff. +71.83mg/L, SE 13.9) and POD3 ($p = .009$). However, no inter-group variations were found ($p = .44$).

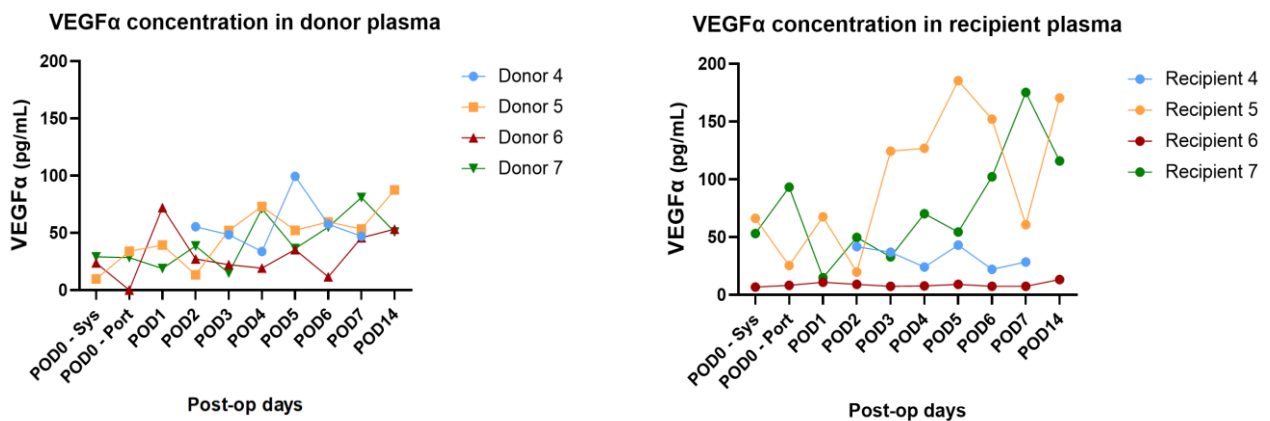


Figure 16. Post-operative plasmatic VEGFa in ALDAPT recipients and donors (n=3 to 4).

Statistical analysis, performed using a mixed-effect model, revealed no significant effects of post-operative timing ($p = .20$) or group ($p = .68$) on plasmatic VEGFa.

Qualitative assessment of individual values revealed diverse post-operative kinetics among recipients.

Notably, recipients 5 & 7 exhibited a marked increase in VEGFa, while others displayed a flat curve. Interestingly, no donor achieved an increase of such magnitude.

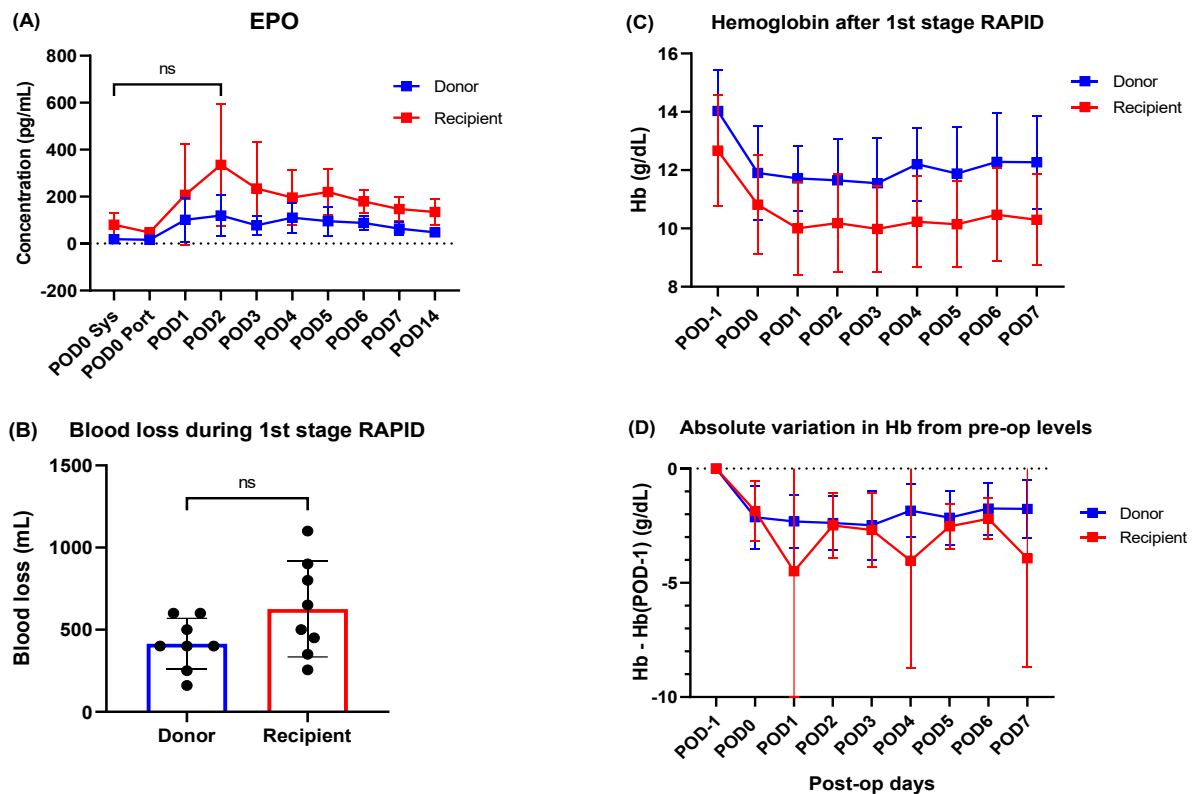


Figure 17. Relationship between EPO levels and perioperative blood loss-related parameters.

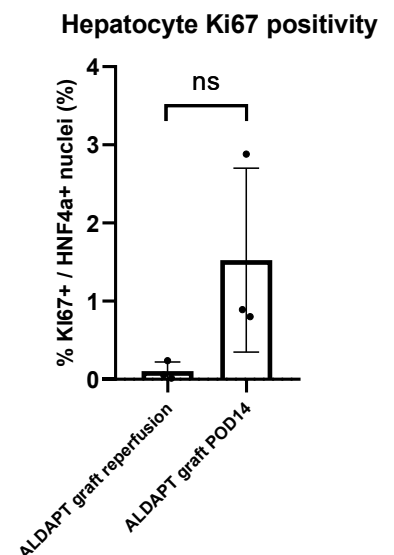
(A) Trend towards post-operative EPO elevation, particularly in recipients, although not reaching significance (see fig. 8b).
 (B) Blood loss during the 1st stage of RAPID in recipients and the donating procedure in donors (n=8). Unpaired T-test indicated a non-significant variation between groups but suggested a trend towards greater blood loss in recipients ($p=.09$, mean diff. 212mL, SE 116mL).
 (C) Blood Hb levels in RAPID recipients and donors after the 1st stage of surgery (n=6 to 7). Mixed-effects model analysis showed a significant effect of post-operative timing ($p=.0001$), and a trend towards lower total Hb in recipients ($p=.07$).
 (D) Absolute drop from pre-operative Hb levels in ALDAPT recipients and donors after the 1st stage of surgery (n=6 to 7). Mixed-effects model analysis revealed a significant effect of timing ($p=.049$), but no significant effect of group ($p=.30$). Post-operative Hb kinetics are therefore similar between recipients and donors.

Figure 18. Hepatocyte proliferation index in the graft after RAPID (n=3)

Proliferation was assessed using dual immunofluorescent staining, with Ki67 expression indicating proliferative activity and nuclear HNF4a expression used for hepatocytic lineage identification.

The quantification of double-positive nuclei was performed using the HALO software.

At the reperfusion stage, the proliferation index is near zero (mean 0.10%). Following the 14-day period of graft growth, hepatocytic proliferation is not significantly greater than at reperfusion (Paired T-test: $p=.15$).



10. Author's contributions and acknowledgments

Contributions

The field of medical research has been a source of continuous curiosity and fascination for me since the early stages of my medical studies. Initially, I discovered the discipline as a hobby during my free time, participating to the study of atopic dermatitis under the supervision of Pr. Poumay at the LabCeTi, UNamur. Consequently, when I joined the UCLouvain master's program three years ago, the opportunity to formally undertake a research memoir appeared like a natural progression.

This experience began with an immersive introduction to daily laboratory practices at the GAEN laboratory (IREC, UCLouvain), which provided a stimulating and intellectually engaging environment for medical research. Interactions with fellow lab members, participation in journal and data clubs, and hands-on experiences helped me develop critical thinking, planning, rigorous methodology, and problem-solving skills. During this journey, I have noticed a significant improvement in these areas, which has enhanced my ability to contribute actively to meaningful research. Overall, this experience has been intellectually fulfilling, offering me a growing sense of accomplishment and fulfilment that I sometimes felt lacking during my purely theoretical courses.

To initiate my work, I conducted a thorough literature review to identify any gaps and establish clear objectives. This process not only acquainted me with a new topic, but also honed my skills in navigating medical search engines and exploring a diverse array of existing literature.

Moreover, it enabled me to undertake the design of experiments as a personal endeavour, which promoted my autonomy from conception to implementation and contributed to improving the study's foundational framework. In this process, collaborating with experienced scientists from various IREC labs has enriched my learning experience, fostering a comprehensive approach to the multifaceted research questions at hand.

My hands-on involvement began with the critical step of collecting and preserving samples, including tasks such as isolating PBMCs, embedding tissue samples in paraffin or OCT, and cutting blocks for subsequent analysis. Dr Coubeau is acknowledged for his crucial role in collecting surgical samples, providing access to historical cohorts, and offering valuable assistance.

As the main collector of experimental data, I performed various techniques such as immunohistochemistry, immunofluorescence, cell culture, FACS, ELISA, and various microscopy applications. The FISH staining makes exception, as it was carried out in collaboration with the Pathology department (Pr Galant - Cliniques Universitaires Saint-Luc, Woluwe).

Subsequently, I delved into the process of data analysis, navigating several software applications such as ZEN or HALO for image analysis. I was also responsible for performing statistical analysis to extract meaningful conclusions from the collected data. I also gathered pertinent information from patients' clinical records using the Epic system, integrating clinical considerations

important for a comprehensive understanding of our human-based research. Collaboration with Dr Coubeau included his analysis of clinical hemodynamic and functional aspects within the expansive ALDAPT project, which further enriched the fundamental study's clinical context.

To conclude this research project, I undertook the redaction of the present manuscript. Additionally, I presented my results at various laboratory meetings, which enhanced my public speaking skills, as well as my confidence, communication, and synthesis skills.

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I would like to express my gratitude to all individuals and entities who have been essential to the successful completion of this research. Among those figures, I thank Maxime de Rudder for his careful proofreading and comprehensive mentoring, providing invaluable guidance on both practical and technical aspects; Dr Laurent Coubeau for his inventive problem-solving skills; Pr Alexandra Dili for her expertise and for laying the foundation of this project through her earlier thesis work. The support and investment from the GAEN team, especially Pr Isabelle Leclercq, have been instrumental.

I also thank the personnel at 2IP and CytoFlux (C. Bouzin, D. Brusa, A. Daumerie) for their valuable assistance, support, and patient teaching. Similarly, the collaboration with the CUSL Pathology Department and Biobanque proved a success.

Finally, I express my sincere appreciation to all encouraging friends and family. A special nod to family members who went above and beyond, extending their unconditional yet somewhat preposterous solidarity to joining the memoir-writing bandwagon, a few years away from retirement.

