DOCTORAL THESIS

To obtain the degree of Doctor

École Doctorale Biologie-Santé
Université de Lille

Evaluation of *in-vitro* and *in-vivo* 5-Aminolevulinic Acid Photodynamic Therapy on human Hepatocellular Carcinoma: Impact on Immune System Regulation

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Presented and defended by
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10th June 2021

Discipline: Molecular and Cellular Biology
Specialty: Oncotherapy and immunology

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THÈSE DE DOCTORAT

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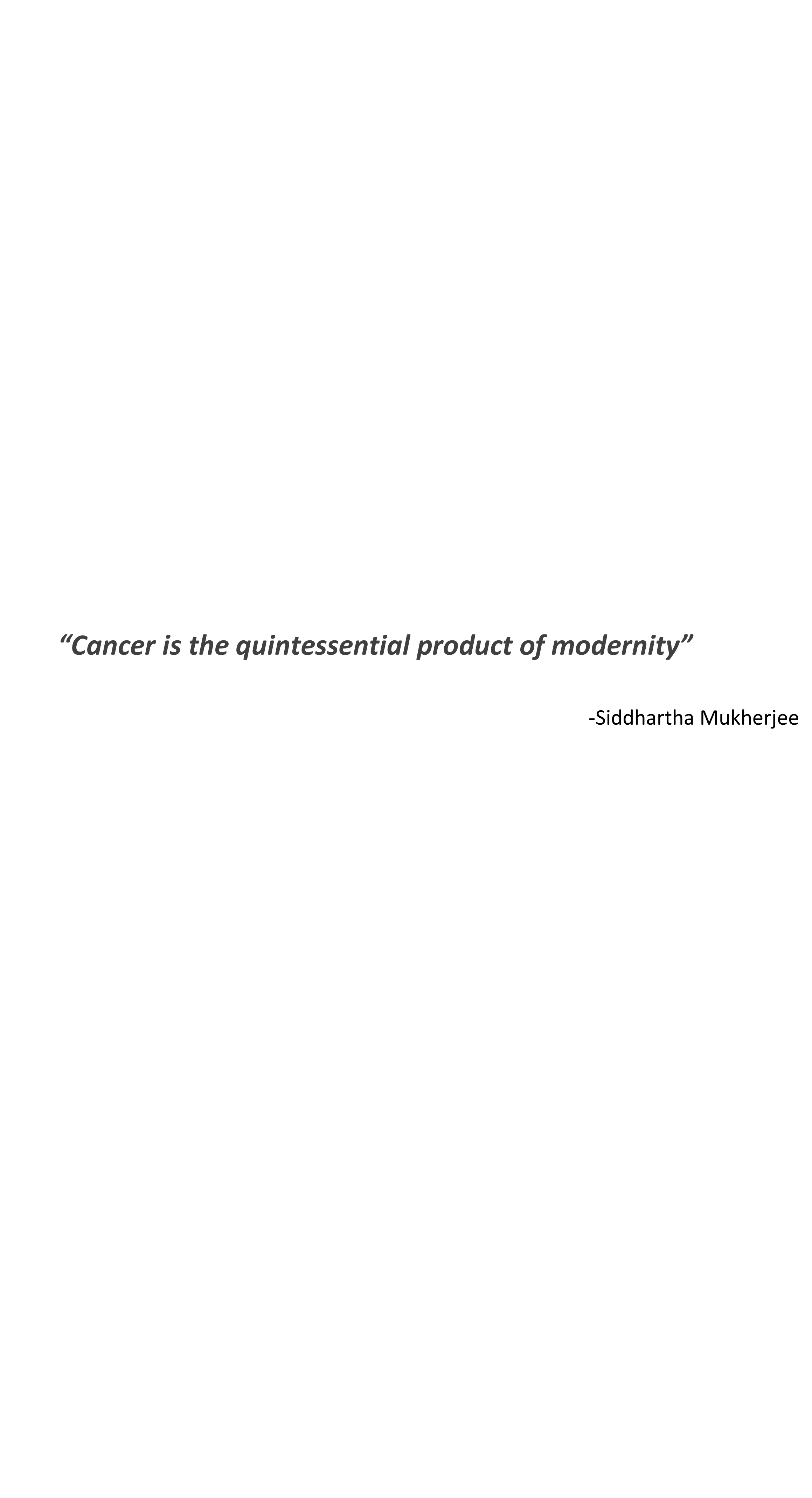

“Cancer is the quintessential product of modernity”

-Siddhartha Mukherjee

For Amma and Baba

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2. **28 November 2019**: 2nd PDT Day at Lille, France "Evaluation of Photodynamic Therapy in Hepatocellular Carcinoma and Consequences on the Regulation of the Immune Response" (National).

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2. **15-17 May 2019**: 12th Annual Scientific Day by Canceropole of North West 2019 at Deauville, France "Impact of Photodynamic Therapy on the Regulation of human Immune System in the context of Hepatocellular Carcinoma" (National).

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1. E. Thecua, L. Ziane, G. Baert, P. Deleporte, B. Leroux, **A. Kumar**, M. Baydoun, O. Morales, N. Delhem, S. Mordon “Devices based on light emitting fabrics dedicated to PDT preclinical studies” Proc. SPIE 11070, 17th International Photodynamic Association World Congress 2019, 110705P.

2. L. Ouaguia, O. Moralès, L. Aoudjehane, C. Wychowski, **A. Kumar**, J. Dubuisson, Y. Calmus, F. Conti, N. Delhem “Hepatitis C Virus Improves Human Tregs Suppressive Function and Promotes Their Recruitment to the Liver” Cells 2019, 8, 1296.

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ABBREVIATIONS

Numbers

1-PA One-Photon Absorption

2-PA Two-Photon Absorption

5-ALA 5-Aminolevulinic Acid

8-OdG 8-Oxo-2'-deoxyguanosine

A

α-SMA α Smooth Muscle Actin

ABCG ATP Binding Cassette group

ADI-PEG20 Pegylated Arginine Deiminase 20

AFP α-Fetoprotein

AIF Apoptosis -Inducing Factor

Akt Protein Kinase B

ALAD 5-ALA Dehydratase

ALAS 5-ALA Synthase

AP-1 Activator Protein-1

Apaf-1 Apoptotic Protease Activating Factor-1

APCs Antigen Presenting Cells

ARID Adenine Thymine-Rich Interaction Domain

ASIR Age-Standardized Incidence Rate

ASMR Age-Standardized Mortality Rate

ATG Autophagy Related

ATP Adenosine Triphosphate

B

BAK	BCL-2 homologous Antagnoist Killer
BAX	BCL-2 like Protein 4
BCL2	B-cell Lymphoma 2
BCLC	Barcelona Clinic Liver Cancer
bFGF	Basic Fibroblast Growth Factor
BID	BH3 Interacting Domain Death Agonist
BIM	BCL-2-like protein 11
BOK	BCL-2 related Ovarian Killer
BOLD	Blood Oxygenation Level Dependent
BRCA1	Breast Cancer Type 1

C

CAFs	Cancer-Associated Fibroblasts
CCL	C-C motif chemokine Ligand
CCR	C-C motif chemokine Receptor
CD	Cluster of Differentiation
CDKN2A	Cyclin-Dependent Kinase Inhibitor 2A
CI	Internal Conversion
CIS	Inter-system Crossing
CK7	Cytokeratin 7
CLIP	Cancer of Liver Italian Program
c-MET	Mesenchymal Epithelial Transition factor
COX-2	Cyclooxygenase-2
CPOX	Coproporphyrinogen
CR	Complement Receptor
CSCs	Cancer Stem Cells

CSP Circumsporozoite Protein

CT Computed Tomography

CTLs Cytotoxic T Lymphocytes

CTLA-4 Cytotoxic T Lymphocyte-Associated Antigen 4

CTNNB1 β-Catenine

CXCL C-X-C motif chemokine Ligand

CXCR C-X-C motif chemokine Receptor

D

D-PDT Daylight PDT

DCP Des-γ-Carboxy Prothrombin

DCS Diffuse Correlation Spectroscopy

DCs Dendritic Cells

DIABLO Direct IAP-Binding protein with Low Isoelectric point

DNA Deoxyribonucleic Acid

DRS Diffuse Reflectance Spectroscopy

E

ECM Extracellular Matrix

ECOG Eastern Cooperative Oncology Group

EGF Epidermal Growth Factor

EMT Epithelial Mesenchymal Transition

EPR Enhanced Permeability and Retention

ER Endoplasmic Reticulum

ERK Extracellular signal-Regulated Kinases

F

FAP	Fibroblast Activation Protein
FcR	Fragment of Crystallization Receptor
FECH	Ferrochelatase
FGF	Fibroblast Growth Factor
FLT-3	Fms like Tyrosine kinase 3
FLVCR1	Feline Leukemia Virus Subgroup C Receptor 1
fMRI	Functional Magnetic Resonance Imaging
FOLFOX4	Fluorouracil, Leucovorin [folinic acid], and Oxaliplatin
FOXP3	Forkhead box P3

G

GITR	Glucocorticoid Induced TNF Receptor

H

HA	Hyaluronic Acid
HAIC	Hepatic Arterial Infusion Chemotherapy
HAL	Hexaminolevulinate
HBeAg	Hepatitis B e-Antigen
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HDL	High Density Lipoprotein
HGFs	Hepatocyte Growth Factors
HIF-1	Hypoxia Inducible Factor-1
HLA	Human Leukocyte Antigen

HMBS Hydroxymethylbilane Synthase

Hp Hematoporphyrin

HpD Hp Derivatives

HPPH 2-[1-hexyloxyethyl]-2-divinyl pyropheophorbide-a

HSCs Hepatic Stellate Cells

HSP Heat Shock Protein

I

IAP Inhibitor of Apoptosis

ICG Indocyanine Green

IDO Indoleamine 2,3-Dioxygenase

IFN Interferon

Ig Immunoglobulin

IL Interleukin

IRF2 Interferon Regulatory Factor 2

ITIM Immunoreceptor Tyrosine-based Inhibitory Motif

J

JIS Japan Integrated Staging

JNK c-Jun N-terminal Kinases

K

KEAP1 Kelch-like ECH-Associated Protein 1

Kras Kirsten rat sarcoma

L

LAG-3 Lymphocyte Activation Gene-3

LDL Low-Density Lipoprotein

LOXL2	Lysyl Oxidase Like 2
LPS	Lipopolysaccharide
LSECs	Lympho-Endothelial Sinusoidal Cells
LSECtin	Liver Sinusoidal Endothelial Cell Lectin

M

MCL-1	Induced Myeloid leukemia cell differentiation protein
M-CSF	Macrophage Colony Stimulating Factor
MDM2	Mouse Double Minute 2
MAL	Methyl Aminolevulinate
MAPK	Mitogen-Activated Protein Kinase
MDCT	Multidetector Computed Tomography
MDSCs	Myeloid Derived Suppressor Cells
MELD	Model for End-Stage Liver Disease
MFRN1	Mitoferrin 1
Mfsd2a	Major facilitator superfamily domain-containing protein 2a
MHC	Major Histocompatibility Complex
miRNA	Micro-Ribonucleic Acid
MLL	Myeloid/Lymphoid or Mixed-Lineage Leukemia
MMPs	Matrix Metalloproteinases
MRI	Magnetic Resonance Imaging
mTOR	Mammalian Target of Rapamycin
MWA	Microwave Ablation

N

NADPH Nicotinamide Adenine Dinucleotide Phosphate

NAFLD Non-Alcoholic Fatty Liver Disease

NASH Non-Alcoholic Steatohepatitis

NF-κB Nuclear Factor-κB

NF-E2 Nuclear Factor Erythroid 2

NGS Next-Generation Sequencing

NIR Near Infrared

NK Natural Killer

NKTs Natural Killer T cells

NOS Nitric Oxide Synthase

NOX-2 NADPH Oxidase-2

NPe6 Talaporfin Sodium (N-aspartyl chlorin e6)

NRF2 Nuclear Factor Erythroid 2-Related Factor 2

O

OS Overall Survival

OX-40R OX-40 Receptor

P

PC4 Silicon phthalocyanine

PD-1 Programmed Cell Death Protein-1

PDD Photodynamic Diagnosis

PDGF Platelet Derived Growth Factor

PDT Photodynamic Therapy

PEI Percutaneous Ethanol Injection

PI3K Phosphoinositide-3-Kinase

PLP	Pyridoxal Phosphatase
PpIX	Protoporphyrin IX
PPOX	Protoporphyrinogen IX Oxidase
PPP	Phosphate Pentose Pathway
PS	Photosensitizer
PTEN	Phosphatase and Tensin Homolog
PTT	Photothermal Therapy
PUMA	p53 Upregulated Modulator of Apoptosis
PUVA	Psoralens and UV A
PVE	Portal Vein Embolization
PVR	Poliovirus Receptor

R

Raf	Rapidly Accelerated Fibrosarcoma
RBCs	Red Blood Cells
RFA	Radiofrequency Ablation
RIP-1	Receptor Interacting Protein-1
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RV	Vibrational Relaxation

S

SHARP	Sorafenib Hepatocellular Carcinoma Assessment Randomized Protocol
SIRT	Selective Internal Radiation Therapy
SLC25A38	Solute Carrier Family 25 Member 38

SOD	Superoxide Dismutase
SPF	Sun Protection Factor
SPR	Surface Plasmon Resonance
STAT6	Signal Transducer and Activator of Transcription 6

T

TAA	Tumor Associated Antigen
TACE	Trans-Arterial Chemoembolization
TAMs	Tumor Associated Macrophages
TARE	Trans-Arterial Radio-Embolization
Tc1	Type 1 CD+ T cells
TERT	Telomerase Reverse Transcriptase
TGF	Tumor Growth Factor
Th17	T Helper 17 cell
TIGIT	T-Cell Immunoreceptor With Ig And ITIM Domains
TILs	Tumor Infiltrating Lymphocytes
TIM-3	T Cell Immunoglobulin and Mucin Domain-3
TIMPs	Tissue Inhibitor of Metalloproteinases
TLR	Toll Like Receptor
TME	Tumor Microenvironment
TNF	Tumor Necrosis Factor
TNM	Tumor, Node Metastasis
TP53	Tumor Protein P53
TRAIL	TNF-Related Apoptosis-Inducing Ligand
Tregs	Regulatory T Cells

TTV Total Tumor Volume

U

UICC Union International Cancer Center

UROD Uroporphyrinogen Decarboxylase

UROS Uroporphyrinogen III

US-FDA US- Food and Drug Administration

UV Ultraviolet

V

VEGF Vascular Endothelial Growth Factor

Z

ZnPc Zinc phthalocyanine

PREAMBLE

Dear reader,

In order to facilitate your reading and understanding, this thesis has been divided into 5 parts:

Hepatocellular Carcinoma: Here we shall first discuss the liver regarding its structure and architecture, the key cellular components along with liver regeneration and fibrosis. We shall then proceed to primary liver cancer and hepatocellular carcinoma, highlighting its epidemiology, etiology, and carcinogenesis. We shall also pinpoint the cellular and non-cellular components of the tumor microenvironment and their role in underlying tumorigenesis. At last, we shall discuss the staging systems for HCC, its diagnosis, and various treatment methods.

Photodynamic Therapy: Here, we shall begin with a general introduction and the history of this modality, followed by a discussion about its most frequent application in the treatment of various dermatological malignancies. Thereafter, we shall describe the principle and mechanism of the therapy, with a detailed discussion on its three key mediators. At last, we shall underscore the cell death mechanism by the therapy.

Photodynamic Therapy and Hepatocellular Carcinoma: Here, we shall refer to our review article, which underlines various studies involving the application of the therapy for the treatment of hepatocellular carcinoma.

Thesis objectives: Here, we present our main objectives by proposing to evaluate **(i)** the *in-vitro* efficacy of 5-ALA PDT over three HCC cell lines; **(ii)** the *ex-vivo* efficacy of 5-ALA PDT on primary HCC patient-derived tumoral hepatocytes; **(iii)** the impact of PDT on healthy donor liver myofibroblasts; **(iv)** the impact of 5-ALA-PDT on human immune system regulation and finally **(v)** the pre-clinical *in-vivo* efficacy of the 5-ALA PDT in a humanized SCID mice model of HCC.

Thesis Results: Here, we shall refer to our main research article based on this thesis work, where we have mentioned all the experimental protocols and described all the results obtained.

Thesis Discussion, Conclusion, and Future Perspectives: Here we have given the global picture of our work, and proposed the treatment strategy to be used by using Photodynamic Therapy. We have also described our future goals, raised some critical questions concerning the study and also some areas for future research.

Collaborative works: Here, we present four collaborative articles, in which I am a co-author. The three first one is directly linked to PDT: **(i)** one dedicated to PDT preclinical studies; **(ii)** one dedicated to targeted-PDT treatment for human pancreatic adenocarcinoma and **(iii)** one dedicated to targeted-PDT impact on peritoneal ovarian cancer cells and its impact on the regulation of the human immune response. Finally, I have contributed to a work on the huge role of hepatitis C virus on regulatory T cells suppressive function and their recruitment to the liver

"I really hope you would enjoy reading this thesis and understand the key notion of our study".

CHAPTER 1

HEPATOCELLULAR CARCINOMA

A. The liver

The liver is the heaviest organ and the largest gland in the human body accounting for 2 to 3 percent of the total body weight, weighing around 1.5 kg. It is the organ only found in vertebrates. The liver is a reddish-brown wedge-shaped organ with two lobes divided by falciform ligament and is located at the upper right quadrant of the abdominal cavity right under the hemidiaphragm and to the right of the stomach. It is protected by the rib cage and held to its position by ligamentous attachments. It is connected with two large blood vessels called the portal vein and the hepatic vein. The portal vein carries the nutrient-rich blood from the gastrointestinal tract to the liver, whereas the hepatic vein carries the oxygen-rich blood from the aorta. These veins then sub-divide into several small capillaries called liver sinusoids which then lead to the lobules, the functioning unit of the liver [1]–[3].

The liver performs essential physiological functions which include bile production, detoxification of various metabolites, synthesis (extramedullary hematopoiesis) and decomposition of red blood cells, production of clotting factors, and thrombopoietin (regulates platelets formation by the bone marrow), and hormone synthesis. The liver plays a central role in the metabolism of carbohydrates, amino acids, and lipids. The liver converts glucose into glycogen, the storage form of glucose, *via* glycogenesis, and then converts it into glucose by glycogenolysis, when needed. It is also responsible for gluconeogenesis, synthesizing glucose from some of the amino acids. Also, the fat is broken down into glucose by the liver and adipose cells. It is also a center for heme-biosynthesis along with bone marrow. This gives the unique pigmentation to the liver. All these suggest the central role of the liver in the human body [1], [2], [4].

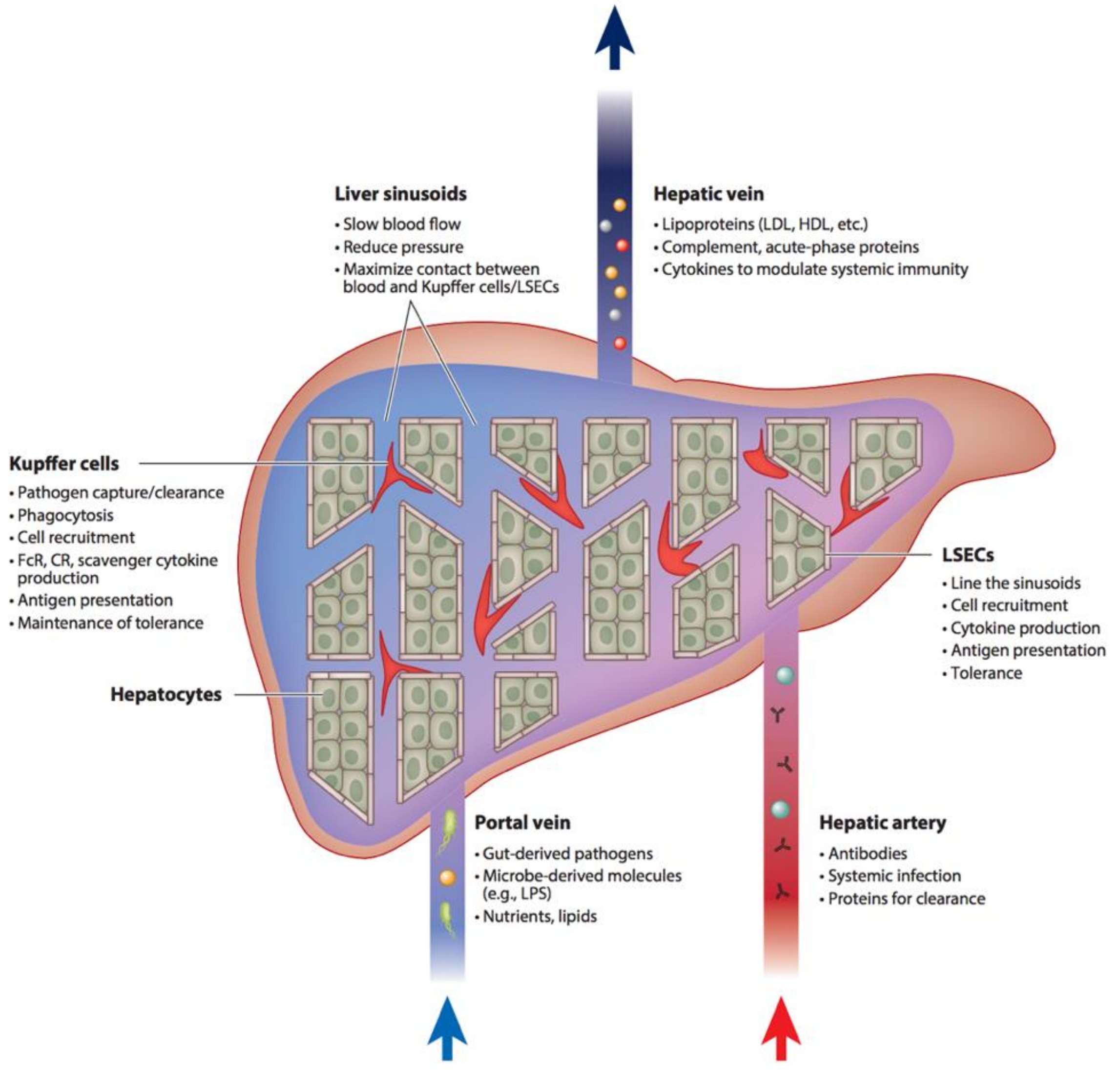

Figure 1: *Anatomical organization of liver, with the function of key components. CR: complement receptor; LSECs: lympho-endothelial sinusoidal cells; FcR: Fc receptor; HDL: high-density lipoprotein; LDL: low-density lipoprotein; LPS: lipopolysaccharide. [4]*

The liver consists of four different types of cells: the hepatocytes, the biliary and lympho-endothelial sinusoidal cells, hepatic stellate cells (HSCs), Kupffer cells. Each of these cells serves a specific function in normal liver physiology to cooperatively regulate hepatic function.

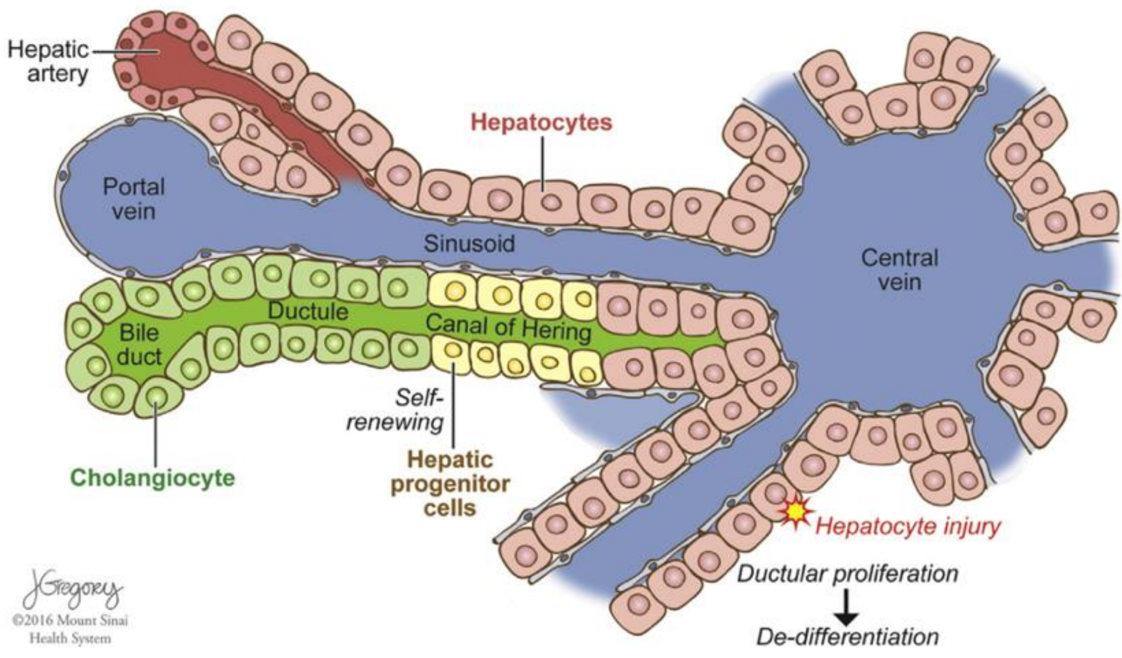

Figure 2: *Structure of a liver lobule, the functional unit of the liver. ©Mount Sinai Health System*

Hepatocytes are the principal parenchymal cell population of the liver. Their unique Golgi system and rough Endoplasmic Reticulum (ER) are the biosynthetic engines of the liver. These hepatocytes are arranged in single-cell-thick plates in a hexagonal sponge-like pattern called lobules, which are the functional units of the liver. Along with HSCs, they are involved in liver regeneration by giving cellular mass and organ function restoration. Hepatocytes also secrete a variety of proteins responsible for activation of innate immune response (like CCL2, CXCL1), which can be elevated during bacterial infection and secreted in the bloodstream for activation of an immune response [3], [5].

The liver sinusoidal endothelial cells are specialized endothelial cells that act as a barrier between the blood and the hepatocytes and comprise the non-parenchymal cells of the liver. They form a fenestrated sieve-like plate at the sinusoidal plates which is critical for the exchange of protein and other molecules within the size range of other liver cells while maintaining the barrier at the same time. They also express molecules to promote antigen uptake and antigen presentation. These cells show similar endocytosis, antigen processing, and presentation to the Dendritic Cells (DCs) [3].

The HSCs represents the majority of the non-parenchymal cells in the liver and is quite dynamic as they can exist in either quiescent or activated state, and reside in the perisinusoidal space of Disse. In case of a liver injury, HSCs are activated and proliferate to

become the main source of extracellular matrix (ECM), responsible for deposition and organization of collagen [1], [3]. They help in liver regeneration by promoting the maturation of hepatic progenitors and secrete different morphogens which may contribute to hepatic regeneration. Studies have revealed its role in intra-hepatic bile duct development. Some of the HSCs also express stem cell markers, suggesting that they might have pluripotent potential. Besides, HSCs are responsible for retinoid storage and transport, secretion of various cytokines, growth factors, and lipids. Recently, HSCs have been recognized as mediators of hepatic immunoregulation as they can amplify the local inflammatory response by secretion of various chemoattractants, produce complement protein C4, and can act themselves as professional Antigen Presenting Cells (APCs) [6], [7].

Kupffer cells, the resident hepatic macrophages, are the largest resident macrophage population in the body. They are found in the sinusoids of the liver and play a crucial role in the innate immune response against the pathogens entering the liver through the portal or hepatic vein. Thus, they serve as the first line of defense against the pathogens or immunoreactive materials entering through the gastro-intestinal tract. They also clear out the dead or dying cells in the systemic circulation, like Red Blood Cells (RBCs), and in hepatic parenchyma. Even though they are associated with a variety of protective functions, any dysregulation in their activity can contribute to chronic inflammation in the liver. Kupffer cell activation can be an important contributor to hepatocyte injury during such inflammation conditions. At the same time, however, they can play a protective function by initiating hepatocyte proliferation in response to hepatotoxic injury [4], [7]–[9]. Besides the Kupffer cells, the liver has some other immune cells as well which can contribute to liver inflammation, like DCs, Neutrophils, T lymphocytes, and recruited bone-marrow-derived macrophages [4], [9].

1. Liver Regeneration

The existence and role of liver stem cells are quite controversial. Unlike other body organs, the cells of an adult liver have a very low turnover and a hepatocyte lifespan ranges from 200 to 300 days. But this quiescent state of hepatocytes can be transformed in case of a liver injury or after liver mass reduction by partial hepatectomy. Once activated, hepatocytes have enormous proliferative capabilities, with evidence suggesting a higher contribution of

hepatocytes in hepatic regeneration than the stem cells. In case of an impaired replication by hepatocytes, epithelial cells with intermediate hepatocyte-cholangiocyte phenotype serve the purpose [10].

Based on the type and extent of the liver injury, the origin of new hepatocytes involved in hepatic regeneration can vary, since not all hepatocytes are the same. As illustrated in Figure 2, each liver lobule consists of a portal vein, hepatic artery, and bile duct, known as a portal triad. The microenvironment at the portal triad and central vein is different since the former is supplied with more oxygen, nutrients, and hormones to the hepatocytes than the latter, which generate different metabolic zones, thus different signaling pathways. This metabolic zonation gives a regeneration response to an injury based on its spatial coordinates. There are different subsets of hepatocytes in the lobule, like *Axin2*$^{+}$*, Mfsd2a*$^{+}$ (Major Facilitator Superfamily Domain-Containing Protein 2a), and *Sox9*$^{+}$ hybrid hepatocytes, each responsible for liver repopulation at different stimuli and localized at different parts of the lobule. A subset of hepatocytes in the lobule which have high expression of Telomerase Reverse Transcriptase (*TERT*^{High}*)* play a significant role in regeneration post-injury. Acting like stem cells, these cells undergo clonal expansion through self-renewal and differentiation mechanisms, thus constituting most part of the uninjured liver. Different toxins result in different zonally restricted cell death, causing different subsets of the population to replenish. During an acute or chronic injury triggered by viral infection or toxins, there is zonal damage to the lobule, which releases signals to promote hepatocyte proliferation and differentiation and thus recover the functional parenchyma [10], [11].

Globally, hepatocyte injury followed by liver inflammation and activation of the innate immune system activates the HSCs to activate and induce fibrosis by ECM secretion and deposition [7]. We shall discuss more about this in the upcoming sections, where we shall consider it during tumorigenesis.

2. Liver fibrosis

Hepatic fibrosis is due to a chronic and repeated liver injury and wound-healing process [12]. As discussed above, during the regeneration process, the necrotic and apoptotic

cells are replaced during which an inflammatory response is activated and controlled deposition of ECM begins. However, if the injury is sustained, the chronic inflammation and ECM remodeling persist thus the hepatocytes are replaced by abundant ECM due to increased synthesis and decreased degradation, which leads to the formation of a permanent fibrotic scar [12]. The activity of ECM-degrading Matrix Metalloproteinases (MMPs) is decreased due to over-expression of their inhibitors, tissue inhibitors of metalloproteinase (TIMPs) [13]. At the advanced stage, the liver contains 6 times more ECM than a healthy liver, accumulating collagens (I and III), fibronectin, undulin, elastin, laminin, hyaluronan, and proteoglycans. As the quantity and composition of ECM alter during the liver disease progression, the liver transitions from fibrosis to cirrhosis, which has a poor prognosis and high mortality [12].

The main source of ECM is the HSCs, which get activated and trans-differentiated into myofibroblasts to accumulate at the site of liver injury and secrete large amounts of ECM, and regulate ECM degradation [14]. ECM deposition in the space of Disse by HSCs disrupts the characteristic architecture of the endothelial lining thereby impairing the bidirectional metabolic exchange between the hepatocytes and the venous flow. This is called as capillarization of sinusoids [15]. Besides HSCs, myofibroblasts derived from small portal vessels (portal myofibroblasts) can also proliferate and initiate fibrosis around hepatic portal tracts [16]. Other cellular sources of fibrosis are bone-marrow-derived circulating fibrocytes, and epithelial cells (through epithelial-mesenchymal transition) [15].

Inflammation plays a crucial role in fibrosis, as it is more a product of dysregulated inflammation than dysfunctional fibroblasts [7]. Kupffer cell-derived Tumor Growth Factor β1 (TGF-β1) is the main fibrogenic agonist and promotes HSC activation by Nuclear Factor-κB (NF-κB) dependent manner. TGF-β1 also stimulates collagen production through Smad pathway to replace collagen IV and VI with collagen I and III. Leptin, an adipocyte-derived hormone, is shown to activate HSCs, which can also stimulate Kupffer cells to secrete TGF-β1 to activate HSCs [15], [17]. Progressive liver injury can cause hypoxic conditions resulting in the expression of Platelet Derived Growth Factor (PDGF) and Vascular Endothelial Growth Factor (VEGF) which can cause fibrosis and angiogenesis [15]. Additionally, Reactive Oxygen Species (ROS) generated by damaged hepatocytes can activate collagen I expression in HSCs and may stimulate signaling downstream of Toll-like receptor 4 (TLR). Kupffer cells target the ligand of TLR4, lipopolysaccharide (LPS), to activate HSCs. This TLR4/LPS signaling is considered

as the link between inflammation and fibrosis. Further, LPS induces HSCs to express chemokines and adhesion molecules to recruit Kupffer cells and also enhance TGF-β1 signaling [7]. Thus, the inflammatory cells activate HSCs, and HSCs in return increase migration and activation of the inflammatory cells thereby stimulating each other [18]. CCR5, CCR1, and CXCL4 expression by HSCs can cause fibrosis. PDGF also activates HSCs through Ras-MAP Kinase pathway [17].

The cellular markers of fibrosis or fibroblast activation are increased cell proliferation, increased expression of α-Smooth Muscle Actin (α-SMA), collagen-1 and TIMP1, PDGFR-β and Fibroblast Activation Protein (FAP) [19], [20]. Since collagen accumulation is a hallmark of fibrosis, hence extra-cellular secretion of collagen-1 is regarded as a very common pro-fibrosis marker. Decreased expression of various MMPs is also observed [21]. Furthermore, Heat Shock Protein (HSP) 47, a collagen-specific chaperone, is also increased during fibrosis [22].

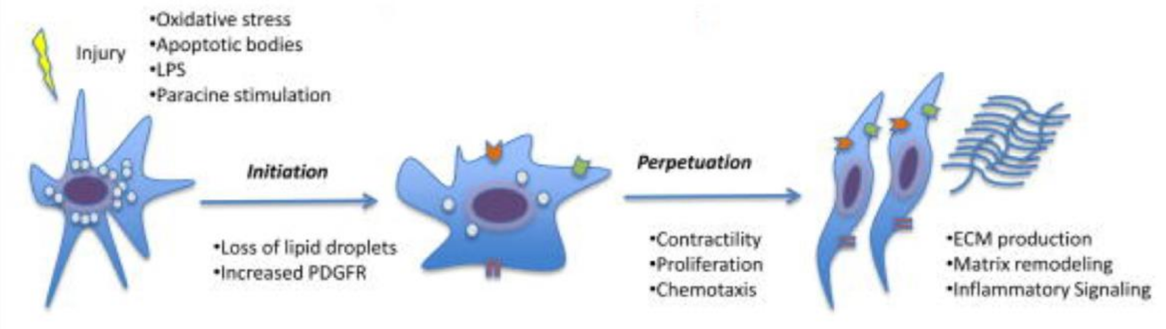

Figure 3: *HSC activation and fibrosis [14]*

B. Primary Liver Cancer

Liver cancer is the sixth most commonly diagnosed cancer worldwide and has high cancer-related death following lung, colorectal, and stomach cancer [23]. As we discussed previously, the liver has highly regulated regenerative properties. But such regulation can go haywire especially in case of a hepatocyte injury in presence of chronic inflammation. Such conditions can arise from different liver-associated diseases like Non-Alcoholic Fatty Liver Diseases (NAFLD), or Non-Alcoholic SteatoHepatitis (NASH), or Hepatitis. In many cases, this could lead to uncontrolled hepatocyte growth, thus giving rise to primary liver cancers.

Primary liver cancers include all the neoplasms which originate from the liver and its associated tissues and organs. It is a major global health issue with the rising number of cases and associated mortality, especially in many developing nations. Liver cancer comprises a heterogeneous group of neoplasms with varying histological properties. Different types of primary liver cancers are Hepatocellular Carcinoma (HCC), cholangiocarcinoma, and hepatoblastoma. We shall discuss HCC in depth further in this section. Cholangiocarcinoma originates from the bile duct of the liver and is closely related to HCC. Cholangiocarcinoma can be of two types: intra-hepatic and extra-hepatic. The former is termed when cancer is formed within the bile duct inside of the liver, while the latter forms outside the liver can hence not be accounted for primary liver cancer. Hepatoblastoma is a rare malignancy of immature hepatocytes and occurs mostly in infants [24].

C. Hepatocellular Carcinoma

1. Epidemiology and etiology

HCC is the most prevalent primary liver cancer, with around 75% of primary liver cancer being represented by HCC [23]. Between 1990 and 2018, the incidence rates have decreased in high incidence rate areas like Asia and Italy, but are increasing in the low rate areas like India, Europe, Oceania, the Americas. The age-standardized HCC incidence rates (ASIR) are depicted in Figure 4. The age-standardized mortality rate (ASMR) of 2018 suggests that Eastern Asia has the highest mortality (16.0 cases per 100,000 person-year), followed by Northern Africa (13.9) and South-Eastern Asia (13.2). Regions like South Central (2.3) and Western Asia, Northern, Central, and Eastern Europe (around 3.8 to 4.0) have low mortality rates. Mongolia and Egypt have high mortality rates [23], [25], [26]. Worldwide, the ASIR (9.3) is close to ASMR (8.5) in 2018, thereby highlighting that HCC is a deadly disease. All these data suggest that even though the incidence rate is decreasing in Asian countries, the mortality remains high. Meanwhile, for developed countries in Europe and America, the number of incidence cases is rising but the mortality remains lower than the Asian counterparts. This highlights a key difference in the level of medical facilities available in developing and developed economies. But at the same time, the decreasing numbers in Asia highlights increased awareness, the spread of education, and good lifestyle practices to avoid HCC spread.

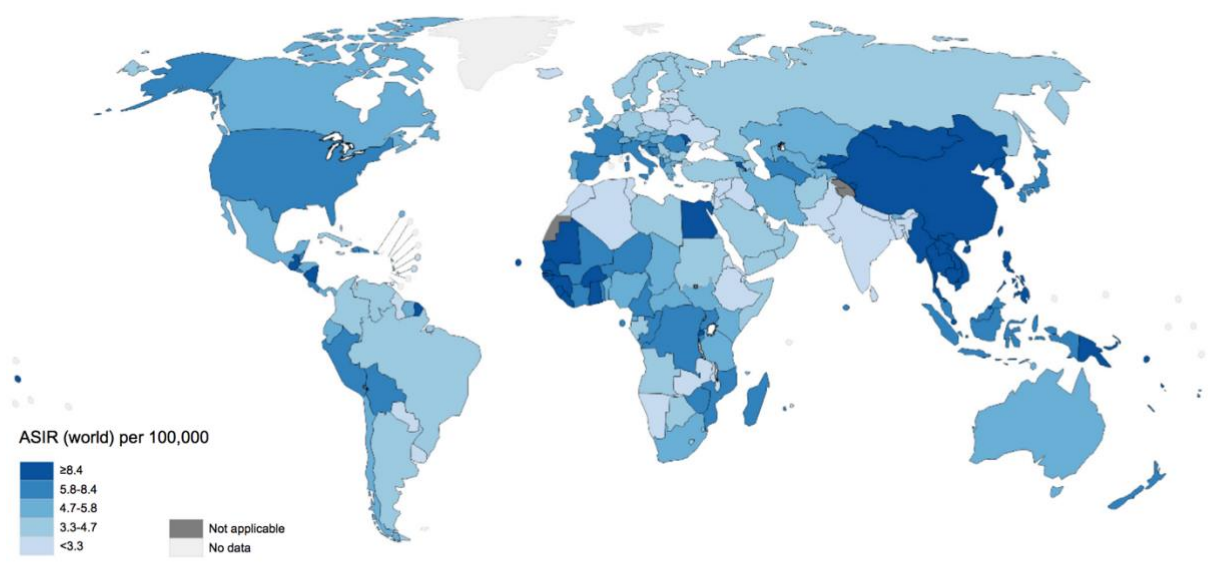

Figure 4: *Worldwide age-standardized HCC incidence rates (2018). Data source: GLOBOCAN 2018. Graph production: IARC (http://gco.iarc.fr/today), World Health Organization. ASIR, age-standardized incidence rate; HCC, hepatocellular carcinoma. [23]*

For most countries, HCC incidence rates are high at the age of around 75 years. The median age at diagnosis is lesser. For example, in the US, the median age at diagnosis among men is between ages 60 and 64 years, while the median age among women is 65-69 years. The gender-wise incidence rates are varying, with men generally more susceptible than women where the rates can be two to four folds higher for men than in women. The wide variation in the incidence rates of HCC by geographical region, age, and sex is due to the prevalence and age at acquisition of the major risk factors [23], [25].

The main risk factors of HCC are chronic Hepatitis B virus (HBV) or Hepatitis C virus (HCV) infection, alcohol abuse, diabetes, NAFLD, NASH. Indeed, underlying chronic liver disease is the common characteristic among all the risk factors leading to HCC [26], [27].

HBV is the leading cause of liver cancer worldwide, where HBeAg (Hepatitis B e-Antigen) seropositive, high viral load, and genotype C are independent predictors of HCC progression [23]. Implementation of HBV vaccination programs in East Asia has resulted in decreased HBV-related HCC cases. For HCV, similar risk factors (like HCV genotype) have been reported, with the presence of cirrhosis and sustained virological response as the strongest indicator. Unfortunately, except for a few anti-viral medications like sofosbuvir, velpatasvir, a lack of HCV vaccine causes regular surveillance a necessity prior to sustained virological response, and even thereafter [23], [25]–[27].

Excessive alcohol consumption is widely regarded as a risk factor for HCC with over three drinks per day shown to increase HCC risk by 16%. Furthermore, HCC risk due to alcohol is four folds higher in women than in men, probably due to the difference in alcohol dehydrogenase activity. No associations with lower levels of alcohol consumption were demonstrated [25]. Thus, an increased risk of developing HCC was reported especially for countries with cultural alcoholic habits [23].

Various health conditions, like insulin resistance, abdominal obesity, hypertension, and atherogenic dyslipidemia, collectively called metabolic syndromes can be associated with an 81% increased risk for HCC. Similarly, diabetes is associated with a two to three-fold increase in HCC risk with men being more susceptible than women. Treatment of diabetes type 2 with metformin is reported to decrease the risk while insulin increases the risk. Obesity is also linked with an increased HCC incidence rate [25].

Studies have demonstrated that NAFLD and NASH, diseases associated with abnormal accumulation of fat in the liver, contribute to HCC development. Around 10 to 30% of NAFLD cases proceed to cirrhosis, and even though these patients are at lower risk of developing HCC than those with NASH cirrhosis, the higher number of NAFLD cases makes it a major risk factor of HCC. Hence patients with NASH or NAFLD cirrhosis are kept under HCC surveillance but non-cirrhotic NAFLD patients are not recommended for the same [23].

Besides these, Aflatoxin B1, a mycotoxin produced by *Aspergillus* fungi, can increase the HCC risk by six-folds, whereas it could reach 54-fold if in combination with HBV. This fungus contaminates a variety of foodstuffs, for example, maize, groundnuts, and tree nuts. Tobacco and cigarette smoking can contribute to 70% increased HCC risk. Interestingly, coffee has been shown to decrease the risk by 35% possibly due to lower liver enzyme levels, slower progression of fibrosis, and lower risk of diabetes along with reducing inflammation, fibrosis, insulin resistance, and oncogenesis. Some host genetic factors are also associated with HCC risk factors [23], [25]–[27].

2. Hepatocarcinogenesis

It is widely accepted that neoplastic development is a multi-step process and includes the incorporation of two or more mutations to facilitate tumor growth, invasion, and metastasis. From the etiology of HCC, we can understand that an impaired hepatic regeneration marked by cirrhosis during a chronic liver disorder replaces the normal liver architecture with fibrotic tissues, hepatitis marked by inflammation, uncontrolled cell proliferation, and cholestasis due to biliary overload, ultimately causing HCC. The emergence of cirrhosis favors the accumulation of somatic gene alterations along with epigenetic modifications to add molecular heterogeneity and impart proliferative, pro-survival, and invasive properties to the tumor. These changes parallelly increase liver function impairment. However, HCC can arise in absence of cirrhosis and marked inflammation [26]. The recent developments in next-generation sequencing (NGS) methods have enabled scientists to rapidly sequence the entire genome and transcriptome, thereby highlighting the key genetic alterations and modifications in signaling pathways giving rise to HCC.

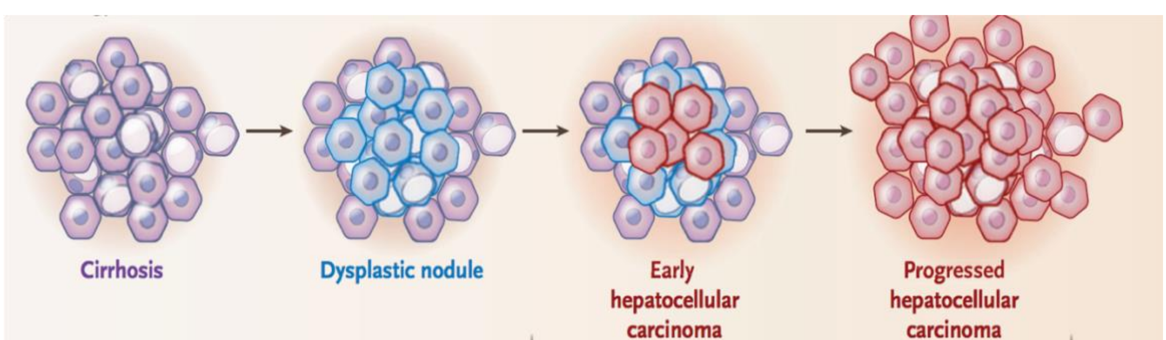

***Figure 5:** Histological progression of HCC. [28]*

With NGS data from HCC patients, we now know that the frequency of replication errors in HCC is low, due to which mutations induced by such errors are unlikely to be the major mechanism of HCC carcinogenesis. But there is a high prevalence of chromosome copy alteration and translocations in human HCC. Chromosomal deletions, re-arrangements, aneuploidy, gene amplifications along with epigenetic modifications activate pathways for cell proliferation while inactivating the inhibitors, including tumor suppressor genes. These changes are further amplified by the tumor heterogeneity, due to which multiple pathways can be involved to produce different subsets of HCC tumors. NGS data have revealed that alterations in the *TERT* promoter are the most frequent genetic alterations found in HCC.

Other mutations of *Tumor protein P53 (TP53), Cyclin-dependent Kinase Inhibitor 2A (CKDN2A), IRF2, β-catenin (CTNNB1), AXIN1, AT-Rich Interaction Domain (ARID) 1A, ARID2* genes affect cell cycle, Wnt signaling pathway, or chromatin remodeling [28], [29].

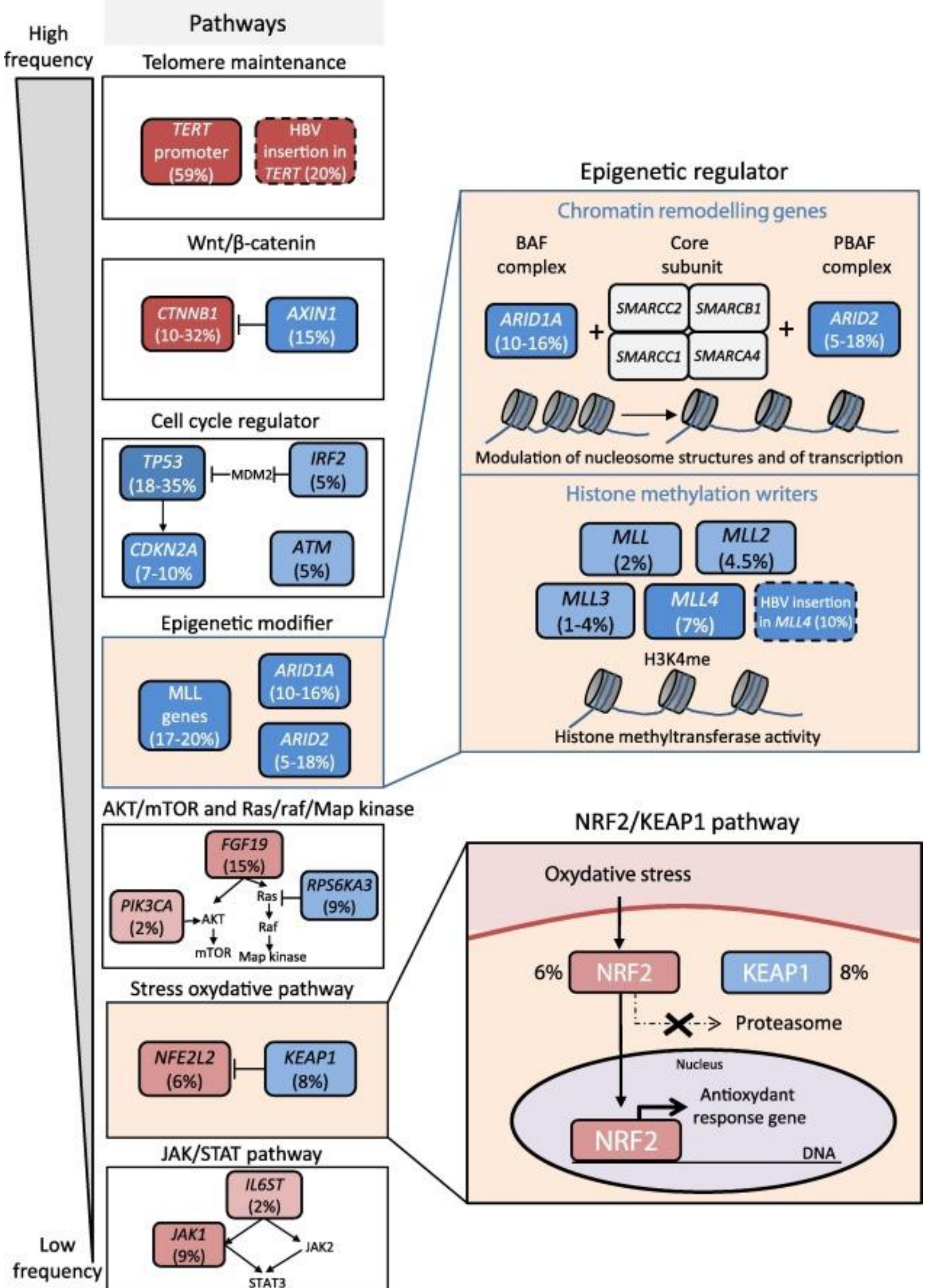

Figure 6: Frequently altered major signaling pathway in HCC. The oncogenes are indicated in red and tumor suppressor genes in blue. The respective lighter shades highlight lower frequency of alterations. [29]

TERT is the main limiting factor of the telomerase complex, which is responsible for telomere synthesis during hepatic regeneration. Telomerase is the key enzyme accountable

for the prevention of telomere shortening after each cell division. Telomere shortening could limit the proliferative capacity of the human cells, which could lead to cell cycle arrest, DNA damage signals, senescence, or cell death [30]. Malignant hepatocyte samples from humans have demonstrated shorter telomere length than the cells in cirrhosis and normal hepatocytes, with similar results for *TERT* expression [28], [31]. From this, we could conclude that telomerase reactivation by mutations in the *TERT* promoter sequence could lead to telomere shortening. The cells, thus, avoid senescence and cell death which is the first step for hepatocarcinogenesis. *TERT* promoter mutations are less frequent in HBV-infection-associated HCC, probably due to *TERT* promoter insertion by the virus itself [28], [32]. Furthermore, these mutations can be correlated with *CTNNB1,* suggesting pro-tumoral cooperation between telomerase and Wnt/β-catenin pathway [32]. Studies have revealed that Wnt/β-catenin signaling pathway upon aberrant activation could lead to initiation and progression of HCC [28], [33]. This pathway is associated with embryonic development and tissue homeostasis, by regulation of cell cycle, cellular differentiation, cell motility, and polarity along with cell death [34]. *CTNNB1,* a regular mutation reported in HCC patients, could lead to over-activation of the Wnt pathway [26], [35]. Similarly, p53 and phosphoinositide-3-kinase (PI3K)/ mammalian Target of Rapamycin (mTOR)/ Protein Kinase B (Akt) (are also critical proteins responsible for various tumor-suppressive actions of the cell, and their mutation could lead to cellular growth and proliferation [33]. With the loss of the *TP53* gene, mature hepatocytes can also yield Nestin-positive progenitor-like cells that can develop into HCC after procuring specific mutations like in the Wnt pathway [10], [26]. More and more studies using lineage-tracing mouse experiments have revealed that HCCs originate from mature hepatocytes rather than progenitor or HSCs [36]. A small subset of HCC possesses Kirsten rat sarcoma (Kras) activating mutations, which are responsible for activation of the Mitogen-Activated Protein Kinase (MAPK) pathway, which results in increased proliferation and resistance to apoptosis. Similarly, Phosphatase and Tensin homolog (PTEN), which is known to negatively regulates PI3K/Akt/mTOR signal transduction, is shown to have inactivating mutations, deletions, or insertions in some HCC patients. These signaling pathways are associated with cell proliferation, apoptosis inhibition, cell metabolism and angiogenesis control [26], [29], [35], [37], [38]. In 6% of HCC, NRF2 is mutated, which causes it to dissociate from its complex with KEAP1, thereby translocating to the nucleus and activate transcription of various anti-oxidant genes. Normally this process is activated in response to

increased ROS, and thus HCC acquires a survival advantage during hypoxic conditions. This is also alarming for therapies that rely on inducing oxidative stress or mediate ROS-dependent cytotoxicity [29], [37]. As for the epigenetic modifications in the HCC, recurrent mutations in Myeloid/Lymphoid or Mixed-Lineage Leukemia (*MLL*), *MLL2, MLL3,* and *MLL4* have been reported which are involved in methylation, acetylation, and nucleosome remodeling. In combination with *ARID2* and *ARID1A* mutations, they link the genomic defects with epigenetic alterations in HCC. *ARID2* and *ARID1A* are Adenine Thymine-rich interactive domain proteins responsible for chromatin remodeling thereby regulating transcription of certain genes. They are considered as a tumor suppressor for HCCs [29], [37].

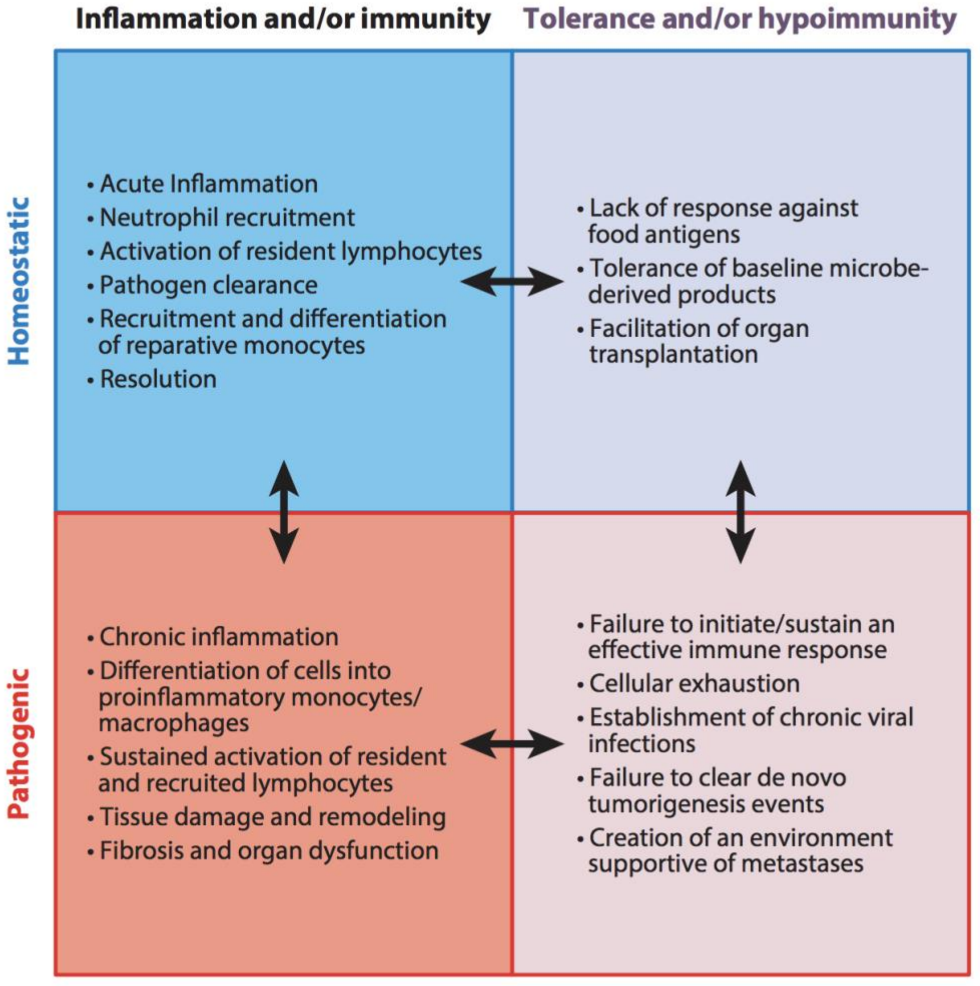

Figure 7: *Immune response in the healthy liver is normally tolerogenic (top-right), to avoid response against food antigens, or low-level microbe-derived products. During a pathogen invasion, a rapid acute inflammation response is activated for pathogen clearance and liver restoration. During a chronic inflammation, there can be substantial liver injury with tissue remodeling, fibrosis, and creation of a pro-tumoral microenvironment (bottom-right). [4]*

3. HCC tumor microenvironment

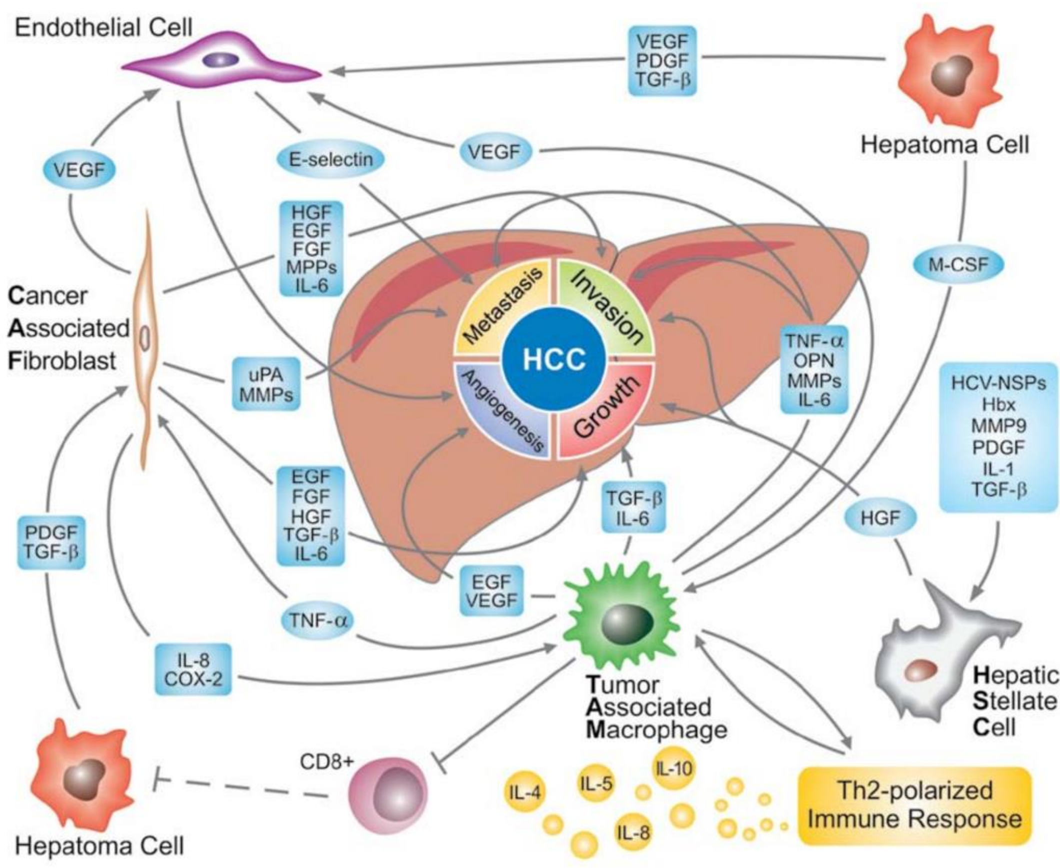

Figure 8: HCC Tumor Microenvironment at a glance. [39]

Irrespective of the cell types from which the HCC originates, tumor progression requires a specific and favorable microenvironment. The tumor microenvironment (TME) is a dynamic system that dictates the behavior of cancer not by the tumor's intrinsic modifications, but by the surrounding milieu which supports the tumor by giving pro-survival, growth, and proliferation signals [40]. Changes in the liver ECM, cross-talk between the parenchymal and non-parenchymal cells of the liver, and chronic inflammation are some of the key factors that contribute to HCC growth [10].

HCC is a very classic example of inflammation-related cancer, as it grows and develops at the backdrop of a chronic inflammation characterized by the expression of chemokines and cytokines along with recruited immune cells. These immune cells in turn assist in neoplastic transformation by releasing free radicals like ROS, nitric oxide, to induce DNA damage which can be helpful for cancer progression [41]. Additionally, this chronic inflamed state can lead

to activation of liver fibroblasts to induce fibrosis, which leads to HCC and poor prognosis [40]. Studies have demonstrated that HCC inducing mutations can only generate hepatocellular adenoma, a benign liver tumor, in absence of chronic liver inflammation [42], [43]. It is therefore clear that there is a cross-talk between the cancer cells and the "innocent bystanders" of the TME.

The TME comprises of two types of components: (a) the cellular components which include cancer-associated fibroblasts (CAFs), HSCs, endothelial cells, and various immune cells; (b) the non-cellular components which include the ECM, MMPs, exosomes, cytokines, various inhibitors, and growth factors [44]. In the upcoming sections, we shall highlight the role of different components of TME in HCC progression. We shall certainly not discuss in-depth but will highlight the well-established interactions and their overall impact on patient prognosis.

a. Cellular components of TME

i. Hepatic Stellate Cells

HSCs at quiescent state accumulate vitamin A lipid droplets in a healthy liver. PDGF, TGF-β1, MMP-9, c-Jun N-terminal Kinases (JNK), insulin-like growth factor binding protein 5, HBV X protein and HCV can activate the HSC whereas adiponectin suppresses its activation. Once activated, these cells exhibit reduced intracellular lipid levels, increased cellular proliferation, increased αSMA expression, along with ECM production, and morphological changes. During liver injury, HSCs trans-differentiate into activated myofibroblast-like cells to secrete cytokines and ECM proteins in order to protect the liver. Therefore, these cells are held responsible for collagen synthesis and fibrosis in the liver [12], [45]. These activated HSCs can also infiltrate the HCC stroma to localize around tumor sinusoids, fibrous septa, and capsules. There they help in HCC proliferation, activate NF-κB transcription factor and Extracellular signal-Regulated Kinases (ERK) pathway. This helps in HCC tumor growth, invasiveness and inhibits necrosis. HSCs secrete growth factors and cytokines like Hepatocyte Growth Factors (HGFs) and Interleukin 6 (IL6) which change cancer cell phenotype. Interaction between HSCs and HCC has been demonstrated to form pro-angiogenic microenvironment by VEGF-α and MMP9 secretion [44], [46], [47].

ii. Cancer-Associated Fibroblasts

Fibroblasts are a common cell lineage in connective tissues and are responsible for ECM secretion and maintaining structural integrity along with wound healing. CAFs are a specialized group of fibroblasts in cancer, which are the most prominent cell types in a lot of tumor stroma, and are extremely critical in the HCC microenvironment due to the underlying fibrosis and cirrhotic nature of the tumor. CAFs and HCC are interdependent [48]. HCC tumoral growth, intravasation, and metastatic growth are CAF-dependent through up-regulation of CCL2, CCL26, and Lysyl Oxidase Like 2 (LOXL2) genes [46]. While HCC stimulates CAF proliferation probably by Tumor Necrosis Factor α (TNF-α) and PDGF secretion [44]. Activated CAFs can render Natural Killer (NK) cells dysfunctional *via* PGE2 and indoleamine 2,3-dioxygenase (IDO), thereby helping HCC in immune escape [49].

iii. Endothelial Cells and Tumor Vasculature

Tumor-associated endothelial cells play a crucial role in blood vessel formation and its migration for tumor neovascularization. The tumoral endothelial cells have irregular shapes and sizes. The tumor blood vessels thus formed have an abnormal morphological structure with leaks and gaps assisting the tumor cells to enter the mainstream and spread further. HCC is known to be hypervascularized. VEGF is known to induce angiogenesis and has been demonstrated to be up-regulated in HCC [44], [46]. Yamaguchi *et al.* demonstrated that the secretion of VEGF by HCC cell lines was increased by inflammatory cytokines IL-1β, Interferon α (IFN-α), IFN-γ, and TNF-α; and by growth factors, epidermal growth factor (EGF), PDGF-BB (two B-subunits), basic fibroblast growth factor (bFGF), and TGF-α, with TNF-α showing highest secretion levels [50]. This VEGF is a mitogen for tumoral endothelial cells resulting in their survival, proliferation, migration, and induction of angiogenesis [51]. Furthermore, the endothelial cells have high TGF-β1 and CD105 (Cluster of Differentiation) expression. This TGF-β1 acts as a chemo-attractant for CD105+ cells and thereby inducing angiogenesis in HCC. The HCC associated endothelial cells are further shown to manifest increased angiogenesis and drug resistance [52]. A tyrosine kinase inhibitor, sorafenib, is the most widely used therapy for advanced HCC. It acts by targeting VEGF, which we shall discuss later.

iv. Immune cells

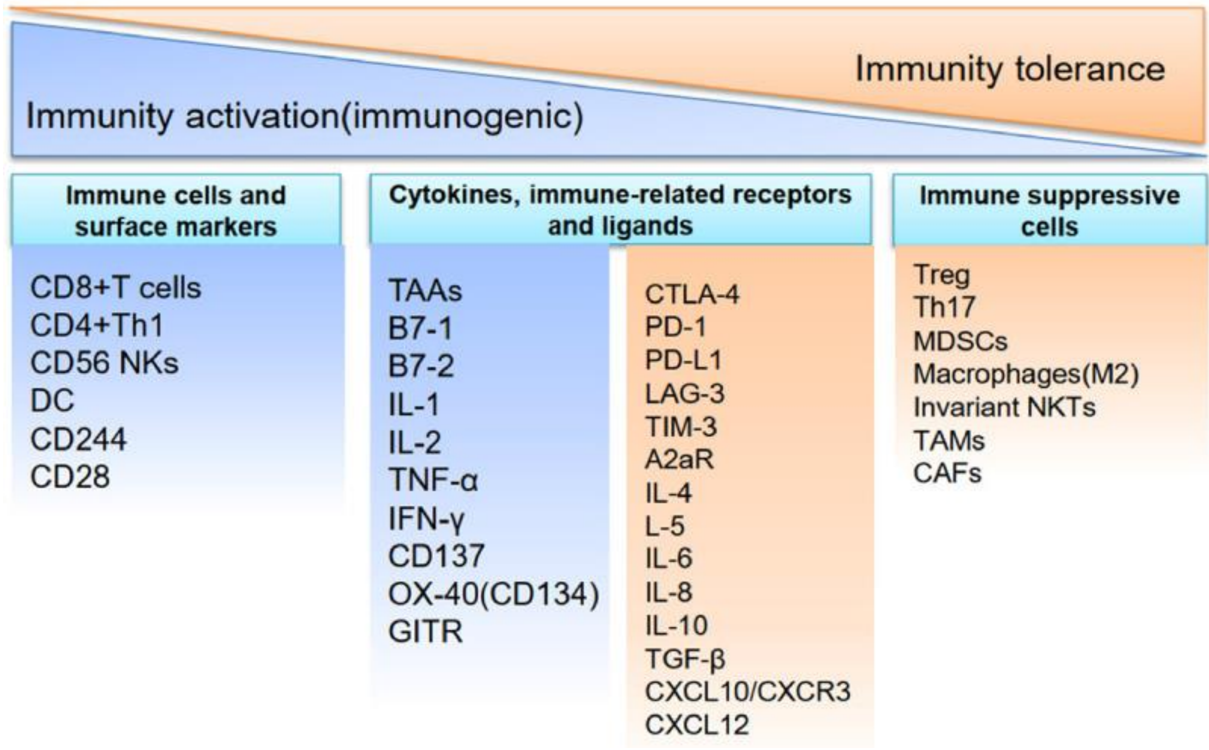

Figure 9: *The balance of immune status in HCC, regulated by various immune cells, cytokines, and receptors-ligand interactions. TAAs: tumor-associated antigens; TILs: tumor-infiltrating lymphocytes; CTLs: cytotoxic lymphocytes; DC: dendritic cell; TNF- α: tumor necrosis factor α; IFN-γ: interferon γ; OX-40R: OX-40 receptor; Treg: T regulatory cell; Th17: T helper 17 cells; MDSCs: myeloid-derived suppressor cells; NKTs: natural killer T cells; TAMs: tumor-associated macrophages TGF-β: transforming growth factor-β; GITR: glucocorticoid-induced TNF receptor; CAFs: cancer-associated fibroblasts. [53]*

Generally, the function of the immune system is to detect neoplasms and eliminate them from the body. But during tumorigenesis, the immune landscape evolves from highly inflamed to a pro-tumoral microenvironment which is immunosuppressive in nature. We have already discussed some key immune cell populations native to the liver and their role in the development of chronic inflammation during liver injury leading to HCC.

The most prevalent immune population in the HCC TME is Tumor Associated Macrophages (TAMs), recruited by HCC signals which include VEGF, PDGF, TGF-β, CCL2, and Macrophage Colony Stimulating Factor (M-CSF), either from residing Kupffer cells or by differentiating circulatory monocytes into macrophages [40], [54]. HCC also has a high surface expression of glypican-3 which is associated with TAM recruitment [55]. TAMs are localized in the peritumoral region with high HLA-DR (Human Leukocyte Antigen), IL-1β, IL-6, and IL-23. In the tumor core, they possess a more exhausted phenotype with HLA-DR^{low} and IL-10^{low} thereby failing to trigger an effective anti-tumoral response [43]. This indicates that tumoral

cells can effectively re-educate the macrophages by changing their phenotype according to their necessity, and depending upon their localization. However, studies have also revealed that TAM infiltration and increased pro-inflammatory cytokine levels after tumor resection have resulted in the survival of patients with reduced recurrence [56], [57].

T lymphocytes, both $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells, have been known to play a crucial role in tumor recognition and impeding its growth, thus correlated with a favorable outcome. A significant decrease in $CD4^+$ T cell population in patients with cirrhosis and HCC has been demonstrated [58]. For $CD8^+$ T cells, their infiltration in the HCC TME is decreased compared to the adjacent tissue along with a more exhausted T cell phenotype [59]. Any increase in the infiltration of $CD8^+$ T cells in the HCC, might or might not lead to increased patient survival [60], [61]. The T cell exhaustion can be due to tumor antigen load or immunoinhibitory receptors expressed on exhausted T cell surface like programmed cell death protein 1 (PD-1), lymphocyte activation gene-3 (LAG-3), CD-244, T cell immunoglobulin, and mucin domain 3 (TIM-3), cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) [53]. This impaired functionality of these cells can also be due to a decrease of granzyme B, IFN-γ, and TNF-α.

Regulatory T cells (Tregs) are a subpopulation of $CD4^+$ T cells which are responsible for tolerance to self-antigen by suppressing the recruitment and proliferation of $CD8^+$ cytotoxic T cells. These naturally occurring or thymic derived Treg cells are identified as $CD4^+CD25^+FOXP3^+$ (Forkhead box P3) population. They induce cell death to NK cells and cytotoxic T cells by granzyme B or perforin-dependent manner and by IL-10 and TGF-β [53]. Besides, various checkpoint inhibitors like CTLA-4 are also involved in the suppression mechanism. Treg infiltration gradually increases during various stages of hepatocarcinogenesis [60]. This infiltration might be affected by TAM density in the HCC by secretion of chemokines CCL17, CCL18, and CCL22 [53]. These Tregs are more prevalent in the HCC TME than $CD8^+$ T cells. Various studies have demonstrated an increased Treg frequency in peripheral blood and HCC, which can lead to poor prognosis, and reduction in their levels could lead to increased patient survival [59], [62], [63]. Their accumulation after liver transplantation could lead to HCC recurrence by CXCL10/CXCR3 signaling pathway [64]. HCC derived Tregs induce immunosuppression primarily by impairing $CD8^+$ T cells and can inhibit DC function by CTLA-4 [53], [65]. Overall, Tregs mediate their suppressive effect in HCC by excessive regulatory

activity, insufficient B7 co-stimulation, inhibition by ligands (like PD-1, CTLA-4), or TGF-β mediated T cell impairment [44].

Th17, another subset of $CD4^+$ T cells, are known for their pro-inflammatory role by secretion of cytokines such as IL-17, IL-17F, and IL-22, IL-21 [53]. Due to the underlying inflammatory conditions, the increased prevalence of Th17 in HCC is eminent. In HCC, Th17 has shown increased infiltration leading to low patient survival and post-operative HCC recurrences, thereby suggesting their role in HCC progression [66]. These increased levels of Th17 can thereby serve as a prognostic marker for HCC. Th17 can work in cooperation with Tregs as studies have shown higher levels of Th17 in less differentiated HCC with lower Treg infiltration and high $CD8^+$ T cell, thereby enabling an immunosuppressive microenvironment and assisting in tumor progression [67]. However, it is unknown if Th17 could mediate the pro-tumoral effect by itself or through an indirect pathway. *In-vitro* studies have demonstrated the IL-17 secreted by Th17 cells could activate HSC thereby prompting cirrhosis and also induce the production of other pro-inflammatory cytokines such as IL-6 and IL-8 [68]. IL-17 has also been shown to induce hepatocarcinogenesis *via* Kupffer cell activation in a NASH mouse model [69].

Reduction in NK cell population in the cirrhotic liver leads to HCC development and progression [46]. NK cells with $CD56^{low}CD16^+$ have been shown to have low frequency in HCC liver and peripheral blood, with impaired IFN-γ production and associated cytotoxicity. CD16 is known to have a key role in the cytotoxic function of NK cells *via* its antibody-dependent cell-mediated cytotoxicity. This highlights the local environment-mediated disruption of the anti-tumoral function of hepatic NK cells in HCC patients which can further correlate to increased Tregs in TME [70], [71]. Increased expression of PD-1, TIM-3, and CD96 checkpoints could inhibit the activity of tumor-infiltrating NK cells in HCC, which can correlate to poor outcomes [71], [72]. Immune checkpoint inhibitor-based immunotherapy can yield a positive outcome in such situations, which shall be discussed in upcoming sections. Other immunosuppressive populations in TME, like myeloid-derived suppressor cells (MDSCs), Tregs, or CAFs can render NK cells dysfunctional by soluble immunomodulators like TGF-β and IDO [71].

Besides these, there are other cell populations like benign hepatoma cells, cancer stem cells, adipocytes, and other subpopulations of differentiated and un-differentiated immune cells. Together they can have varying impacts on the tumor progression, but largely their role is yet to be evaluated.

b. Non-cellular components of TME

The ECM is a network of extracellular macromolecules which provides structural support to surrounding cells. Changes in the ECM assist in the tumoral transformation of healthy hepatocytes. High levels of hyaluronic acid (HA) have been detected in the serum of patients with fibrosis and advanced cirrhosis. It is synthesized by synovial lining cells, HSCs, and Mesenchymal stem cells during liver wound healing [73]. During chronic liver injury, there is a steady increase in HA levels in the TME, which leads to HA-CD44 interaction, a potential cancer stem cell marker, and facilitates tumor progression by their self-renewal, differentiation, and chemoresistance [74], [75]. HA binds to IL-6 to retain and increase the local concentration of the cytokine which is known for HSC trans-differentiation to myofibroblasts and local inflammation, to help in HCC progression [76].

Proteoglycans are associated with fibrosis and liver cirrhosis. They are mainly secreted by activated HSCs, during chronic liver diseases [77]. Decorin, a proteoglycan, is expressed in low levels by healthy liver fibroblasts and myofibroblasts but fibrogenesis and chronic hepatic injury could lead to increased levels of decorin which results in increased TGF-β secretion, a known inducer of fibrosis [78]. The role of glypican-3, a proteoglycan, in TAM recruitment has been discussed in previous section. Its overexpression is restricted to a malignant liver; thus, it is used as a marker for HCC along with others [78].

Laminin-5, a subtype of laminin which is responsible for cell adhesion, is expressed in metastatic HCC and promotes epithelial-mesenchymal transition (EMT) for HCC in presence of TGF-β1 [79], [80]. Together with Integrin $\alpha3\beta1$ and $\alpha6\beta4$, they mediate adhesion, proliferation, migration, and invasion of the cancer cells. Integrins are intracellular proteins that are responsible for cell-matrix and cell-cell adhesion and they are responsible for various pro-tumoral as well as anti-tumoral responses. Another ECM protein, collagens are associated with structural support, tumor migration, proliferation, and growth for HCC [44].

Exosomes are membrane-bound extracellular vesicles of diameter 30 to 120nm loaded with various messenger RNA (Ribonucleic Acid), microRNA (miRNA), non-coding RNA, lipids, or proteins as part of cell-cell communications. Tetraspanins like CD9, CD63, CD81, and CD82 are the biomarkers of exosomes. A growing number of studies have demonstrated that exosomes secreted by tumor cells, also known as tumoral exosomes, can carry vital information for cancer progression and can regulate the nearby microenvironment through these nano-vesicles especially in immune escape. HCC cells under ER stress secrete exosomes loaded with miR-23a-3p and miR-146a-5p miRNA which targets M2 type TAMs and increase PD-1 surface expression *via PTEN* inhibition [81], [82]. miR-92b loaded exosomes secreted by HCC cell line Hep3B can induce an immunosuppressive effect on NK cells by CD69 dysregulation [83]. miR-92b is considered as cancer-associated miRNA, or oncomir, for HCC where it aids in HCC growth *via* SMAD7 targeting and at the same time inhibiting tumor immune surveillance [83], [84]. Exosomes derived from various HCC cell lines and patient samples rich in miR-210 are shown to induce angiogenesis by targeting the negative regulators, SMAD4 and Signal Transducer and Activator of Transcription 6 (STAT6) [85]. Exosomes secreted under hypoxic conditions contain miR-155 which is associated with angiogenesis and poor prognosis in HCC [86]. miR-10b and miR-21 loaded exosomes can increase the HCC cell proliferation by increasing vimentin and Snail expression and decreasing PTEN and E-cadherin [87]. These studies highlight the role of exosomal miRNA in mediating a cross-talk between HCC and the surrounding cells to create a pro-tumoral microenvironment primarily by immune-escape, drug resistance, and tumor growth [88].

Besides, there are various cytokines, growth factors, and MMPs in the HCC TME. IL-6, IL-1, and TNF-α are the well-established cytokines involved in HCC tumor progression by mechanisms previously explained. Further, MMPs can be of different sub-types, such as collagenase, gelatinase, membrane-type, stromelysins, and matrilysins. These MMPs are involved in liver fibrosis, tissue remodeling, inflammation, tumor growth, migration, and invasion of the tumor. Due to their increased proteolytic activity, MMPs can degrade the tumor surrounding the stroma and thus facilitate tumor invasion. MMPs can activate TGF-β1 which can initiate EMT in HCC [89]. Growth factor TGF-β can play both an anti-tumoral as well as a pro-tumoral role in HCC. It is primarily involved in fibrosis and tumorigenesis, but at premalignant state, it inhibits cell proliferation by cyclin-dependent kinase inhibitors c-Myc

oncogene suppression and down-regulation of anti-apoptotic proteins. TGF-β1 promotes growth, survival, migration, and invasion of HCC cells. HCC patients with higher TGF-β levels resulted in decreased survival period. Other growth factors such as Fibroblast Growth Factor (FGF), HGF, VEGF, PDGF, EGF are involved in angiogenesis, tissue regeneration, wound healing, fibrosis, activation of a specific liver cell population, inflammation, and drug resistance which ultimately help in HCC progression [39].

D. Diagnosis, staging, and therapeutic options for HCC

1. Diagnosis of HCC

Oftentimes, HCC arises silently during prevailing liver disease, due to which the neoplasm goes undetected at an early stage and advances to the terminal stage. An early diagnosis of HCC is crucial for best treatment results which can also result in reduced disease-related mortality. Hence, asymptomatic high-risk patients are often recommended for routine surveillance, to identify tumors prior to hepatic decompensation or other complications [90]. For diagnosis of HCC, there are primarily three types of screening modalities that are widely used in the clinic. This includes various imaging techniques, analysis of serum tumor markers, and biopsy-based histological analysis [90], [91]. A diagnostic algorithm for HCC is provided in Figure 10.

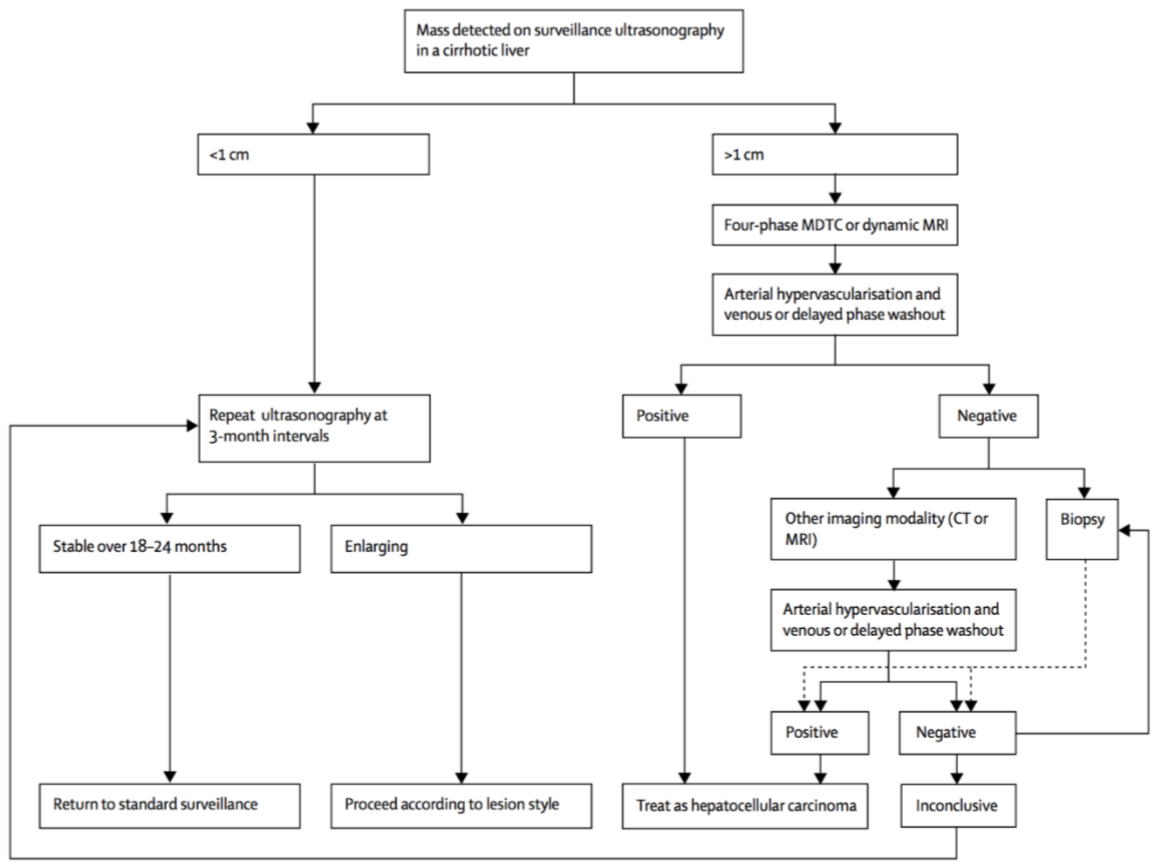

Figure 10: *Diagrammatic representation of HCC diagnostic algorithm. MDCT: Multidetector Computed Tomography; MRI: Magnetic Resonance Imaging; CT: Computed Tomography [92]*

a. Diagnostic imaging of HCC

Imaging techniques are very vital not only for the diagnosis of HCC but also for the identification, characterization, and localization of lesions. They also facilitate surgical procedures and other therapies with curative intent, along with further tumor follow-up post-treatment. However, detection of small tumors and differentiation of HCC from benign lesions remain a challenge with these procedures [90]. The key principle in these methods is the dynamic radiological behavior (*i.e.,* a vascular shift during hepatocarcinogenesis where benign lesions are supplied by portal vein branches while malignant nodules by hepatic artery causing an early washout in the portal phase and thus creating a contrast used to distinguish neoplasms from non-cancerous liver parenchyma). The main imaging techniques which are widely accepted or recommended for HCC diagnosis are ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), and angiography [93].

Ultrasound is primarily used for HCC surveillance of high-risk patients for monitoring of cirrhosis to distinguish a regenerative nodule from a small HCC. HCC is detected as it has

different echogenicity from its surrounding tissue [91]. Here, if a lesion is detected by ultrasound, it is further evaluated by other advanced imaging tests. Ultrasound has low-cost, non-invasive, well-tolerated, and widely available, but has low sensitivity, operator dependent, unsatisfactory diagnostic accuracy, and positive predictive value with coexisting cirrhosis [93], [94]. Sonographic contrast agents like carbon dioxide and helium have promising accuracy [90]. Contrast-enhanced ultrasound can be used to characterize liver nodules but it is not regarded as non-invasive and cannot differentiate intra-hepatic cholangiocarcinoma from HCC [92].

CT scan-based diagnosis of HCC is based on the principle explained above where imaging is conducted at various time intervals for phase-contrast enhancement [91]. These four phases include one without and three with contrast enhancement, where one is in the arterial phase, one in the portal phase, and one in the late phase. The arterial phase provides chest imaging. The portal phase investigates portal hypertension and provides staging assessment by imaging the abdominal and pelvic cavities [90], [93], [94]. The arterial phase enhancement can increase the HCC detection sensitivity by 10% [95]. The sensitivity of this approach lies from 60-94.4% for tumors larger than 1 cm and reduces to roughly 40% for tumor size below 1 cm. This method can also identify complications of HCC, like vascular thrombosis, portal or hepatic invasion, bleeding, and hemoperitoneum [93]. The fibrous structures within or encapsulating the tumor will retain the contrast and hence are visible during the delayed phase. However, the invasive nature and high cost limit the applicability of this method [90].

MRI scan uses a similar concept as in CT scan and is considered as the best imaging tool for HCC. MRI is superior to CT scan for characterizing tumor masses as it has better contrast resolution (*i.e.,* the ability to distinguish differences between similar but non-identical tissues) and can obtain more time points due to absence of ionizing radiations. During MRI, the scanner generates a strong magnetic field at an appropriate resonance frequency in order to excite the hydrogen nuclei from water molecules in the tissues. The excited atoms emit a radio-frequency signal which is measured by the scanner's receiving coils to create a signal. This signal is then processed to generate 3-D image of the body in terms of the density of the hydrogen nuclei. The contrast between the tissues is generated by the rate at which the excited atom returns to its equilibrium state. Similar to CT scan, exogenous

contrasting agents can be used for enhancing image quality [96]. Furthermore, these excited nuclei return to their equilibrium state by the independent relaxation processes of T1 and T2. Typically, HCC is hypointense on T-1 weighted images, and hyperintense on T-2 weighted images, thus distinguish cirrhotic nodule from HCC [90], [94]. Its sensitivity is lowest when the tumor size is less than 2 cm [93]. Normally, large ascites volume is removed prior to imaging, in order to avoid movement artifacts [94].

Angiography is more commonly used to determine the liver anatomy before hepatic resection or catheterization (trans-arterial chemoembolization). It is used as a diagnostic tool primarily due to its vascular nature, but it is unable to detect tumors below 2 cm in size [90].

b. Serum tumor markers

Serum tumor markers are an alternative for surveillance and early detection of HCC. These diagnosis methods are non-invasive and reproducible. α-Fetoprotein (AFP) is a common serological marker for HCC diagnosis [97]. It is found to be elevated above 20 ng/mL in 70% of patients, but it can be elevated up to 1000 ng/mL during high neuroinflammatory activity, which does not correlate to HCC [98]. Usually, AFP serum levels of 400-500 ng/mL are considered as positive predictive value for high-risk patients. The sensitivity of the AFP test ranges from 41-65% and specificity from 80-94% [99]. It is used for monitoring treatment response and recurrence detection.

Due to limitations of AFP measurement, other serological markers are also used alone or in combination. AFP-L3 is an isoform of AFP and has been reported as a marker, and can be helpful in patients with low serum AFP levels, and early HCC detection. Des-γ-carboxy prothrombin (DCP) is an abnormal form of prothrombin, and its levels are elevated in HCC since they do not metabolize it properly and thus secrete it. DCP can be elevated in other conditions such as jaundice, intra-hepatic cholestasis, and ingestion of drugs such as warfarin or wide-spectrum antibiotics. A number of studies have reported high specificity with serum glypican-3 in HCC patients. TGF-β1 is also considered as a serum marker for HCC [90], [92], [93]. All these markers can have low specificity and sensitivity for HCC but can be used for disease surveillance in combination.

c. *Liver biopsy*

Liver biopsy-based histological analysis of hepatic lesions has been considered as one of the most reliable and wide opted diagnostic approaches for HCC detection. Here, cytologic and histologic samples are obtained by percutaneous fine-needle aspiration and needle core biopsy, respectively. Together, they provide greater accuracy than alone with more than 96% sensitivity. But it should be kept in mind that the sensitivity of biopsy largely relies upon location, size, and expertise. The samples are recuperated user the guidance of ultrasound or CT scan. In case of unclear diagnosis, the sample should be stained with CD34, Cytokeratin 7 (CK7), glypican 3, HSP-70, glutamine synthetase, markers of progenitor cells, or neovascularization to improve diagnostic accuracy [90], [91], [100], [101]. Most of the time, a biopsy is used for patients whose suspicious lesion does not necessarily meet the characteristic radiographic or serum features of HCC. However, the potential of the spread of tumors from the needle track is of great concern.

2. HCC Staging and Classifications

After HCC diagnosis, the classification of the patients is the next step for their management. The patients are stratified based on underlying liver disease, liver function impairment, tumor size, spread to hepatic vessels, tumor invasiveness, and presence of metastasis. Such classification or staging system assists clinicians in organizing patients into groups based on their prognostic assessment and thereby facilitates them in choice of therapy, along with patient selection and randomization for clinical studies. An HCC staging system should have a prognostic prediction paired with the treatment outcome, and thus balance the benefits of the treatment with the potential harms of the intervention [102].

The most relevant and widely accepted staging systems for HCC are Tumor, Node Metastasis (TNM); Okuda system; Cancer of Liver Italian Program (CLIP); Japan Integrated Staging (JIS) score; Union International Cancer Center (UICC), and Barcelona Clinic Liver Cancer (BCLC) [102], [103]. Here we shall discuss the BCLC staging system only, which is at the forefront of many clinical trials used to demonstrate the drug efficacy against HCC and thus became the de facto system to be employed. However, we would like to highlight that the

BCLC staging system is more to consider as a pre-therapeutic algorithm, rather than a real determinant of prognosis in terms of tumor recurrence and patient survival. There is no consensus as to which staging system is better in predicting HCC patient survival and no system is superior to the rest.

BCLC staging system was first proposed in 1999 in Seminars in Liver Disease, and since then it has evolved with time to incorporate emerging changes [104], [105]**.** The system considers four crucial elements, *i.e.*, tumor extension, liver functional reserve, physical status, and cancer-associated symptoms. The tumor extension includes the size and number of tumors along with portal vein invasion and extrahepatic spread. It incorporates traditional Child-Pugh grade for assessment of liver dysfunction, which scores liver function based on total bilirubin, serum albumin, prothrombin time, ascites, and hepatic encephalopathy measurement. Physical status is analyzed by Eastern cooperative oncology group (ECOG) performance. Based on this, patients are assigned categories 0, A, B, C, and D [104], [106].

Stage 0 represents a very early-stage tumor, with a single HCC of ≤ 2 cm with well-compensated cirrhotic liver, well-preserved liver function (Child-Pugh A), and absence of portal hypertension. Such patients represent *in situ* carcinoma and can be treated by transplantation, resection, or ablation. Stage A represents an early-stage tumor with a single nodule of any size or a maximum of three nodules < 3 cm diagnosed with Child-Pugh A or B grade liver function. However, patients with Child-Pugh B status patients are contraindicated for transplantation due to poor liver function as a prognostic marker for lower survival. These patients are asymptomatic and are suitable for radical therapies. Patients with intermediate-stage HCCs are classified as stage B. These patients are asymptomatic, but have large multifocal tumors without vascular invasion or extrahepatic spread. The liver function is preserved with Child-Pugh score A or B. BCLC stage C represents advanced-stage patients. Such patients may have symptoms, with probable vascular invasion and extrahepatic spread. The presence of ascites, diuretic requirement, and increased bilirubin level can indicate a worse prognosis. Finally, patients with severe liver dysfunction (Child-Pugh grade C), ECOG performance status of > 2, and extremely grim prognosis are terminal/end-stage patients or BCLC stage D patients. Best supportive care is recommended for patients at stage D [28], [92], [102]–[104], [106]. Figure 11 highlights the treatment option available at each BCLC stage along with respective indications and special aspects.

However, the system has some shortcomings. Primarily, as stated above, the BCLC system only presents a treatment algorithm rather than a prognostic evaluation. Additionally, stage B includes a heterogeneous HCC patient group with varying tumor size and liver function. At last, the system lacks external validation. Hence, different suggestions have been made for the BCLC system, namely sub-classification of stage B (B1-B4) incorporating other staging systems and more treatment options; and sub-classification of stage A according to AFP levels, which is associated with poor prognosis. The prognostic role of AFP in staging systems has recently been reappraised. AFP has even been integrated into liver transplantation allocation rules in some countries, including France [107]. Other tumor markers like VEGF, angiopoietin 2 and KIT can be used as for prognostic assessment [91], [92], [102], [103], [106].

3. Treatment of HCC

Given the complexity, various treatment options, and intricate staging system, HCC treatment and patient management require a multi-disciplinary team including hepatologists, radiologists, pathologists, medical oncologists, transplant and hepatobiliary surgeons, and hepatobiliary surgeons, to ensure the best therapeutic outcome and long-term patient survival. However, the treatment options with curative intent for advanced-stage HCC patients are very limited and do not ensure long-term survival or improvement in patient quality of life. The investigations and evidence of the success of novel therapeutic options for HCC are very limited and are largely based upon small cohort studies with insufficient randomized controlled trials. The key therapies available for HCC which have proven patient survival benefits are surgical resection, liver transplantation, ablation, chemoembolization, and tyrosine kinase inhibitors (sorafenib, lenvatinib, regorafenib). Therapies like arterial embolization (without chemotherapy), external radiotherapy, and radio-embolization have shown decreased tumor burden but no significant impact on patient survival. However, available treatment options should be evaluated as per individual patient, and if the patient is not suitable for a candidate first-line therapy, then the next most suitable option for the same stage or advanced stage must be considered for migration [92].

Here we shall discuss some of the first-line treatment modalities for HCC with their treatment mechanism and standard treatment protocol or dose. We shall also highlight the efficacy of the therapy in terms of various factors like anti-tumoral toxicity, impact on patient survival and quality of life along with some major side-effects associated. We will also discuss the current status of check-point inhibitor-based immunotherapy for HCC treatment. Some key points to be noted are most of these therapies are applicable only to a subset of patients of a particular HCC stage, as highlighted in Figure 11, and hence we will discuss the therapy primarily for those cases.

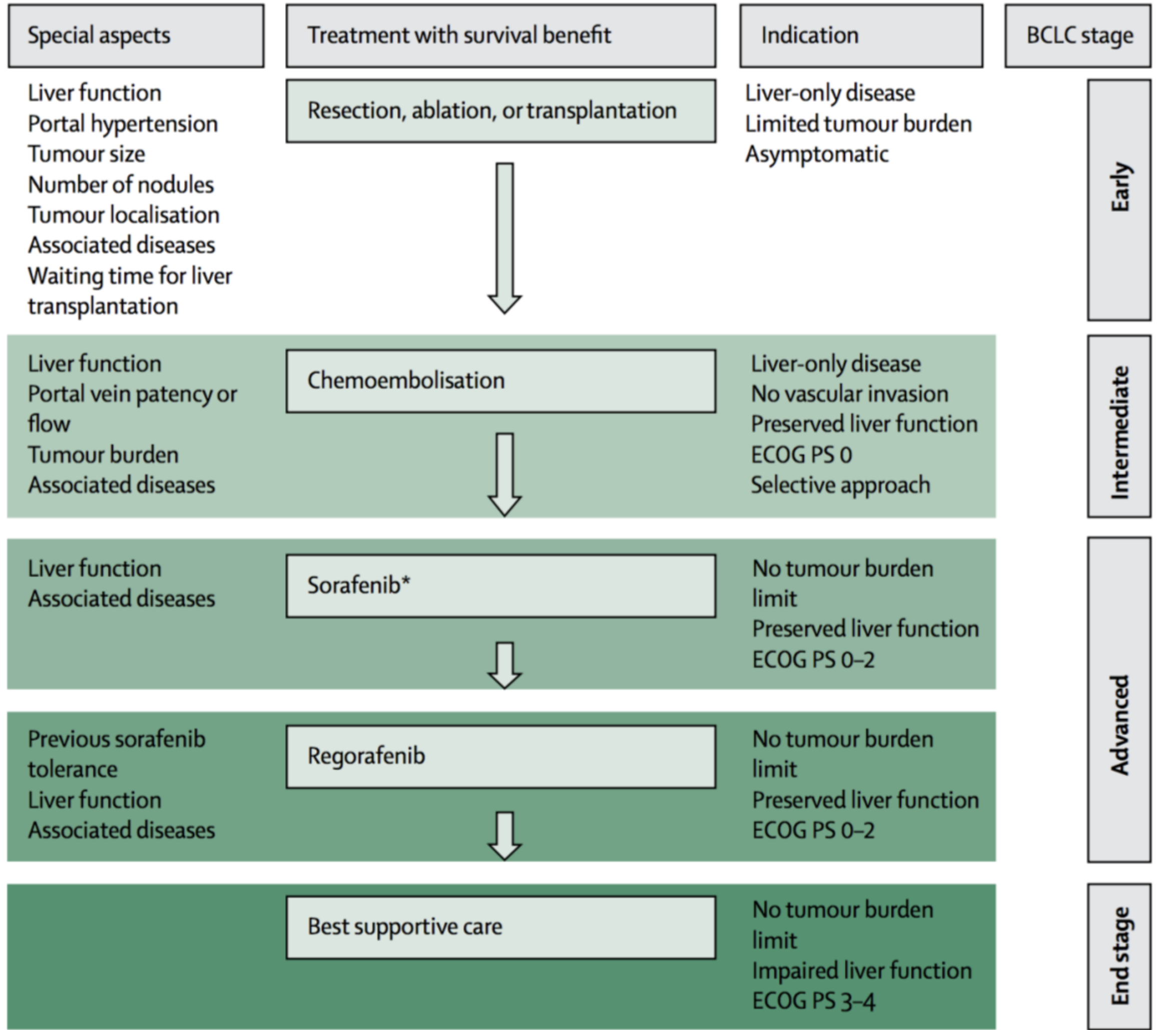

Figure 11: *Sequence of treatment options available for HCC, based on BCLC staging system. ECOG: Eastern Cooperative Oncology Group; PS: Performance Status. *Lenvatinib has been shown to be non-inferior to sorafenib, but no second-line option after lenvatinib has been explored. [92]*

a. Liver Transplantation

From an oncologic standpoint, orthotopic liver transplantation is the best HCC treatment since it simultaneously removes the neoplasm and restores hepatic function as well as the underlying cirrhosis and the other risk factors for tumor recurrence, including the unrecognized sites of intra-hepatic spread [91], [92], [108], [109]. Even though a direct comparison is challenging, orthotopic liver transplant with living and deceased donors has continued to demonstrate better long-term survival than partial hepatectomy or locoregional therapies [92]. The outcome after total hepatectomy for 10-year recurrence-free survival of patients is 50-70%, with only 11-18% recurrence rate [110], [111]. The BCLC system provides guidelines on whether a patient should be considered for transplant versus resection or any other option. The Milan criteria proposed by Mazzaferro *et al.* in 1996, are a set of considerations of HCC patients with cirrhosis waiting for liver transplantation and have been proven to be a strong predictor of survival outcomes after post-transplant. Under this system, the threshold for liver transplantation is a single nodule of ≤ 5 cm in diameter or up to three nodules of ≤ 3 cm, along with extrahepatic spread or macrovascular tumor invasion [112]. Adjuvant therapies can be utilized to down-stage the tumors exceeding the limits of Milan criteria. These therapies can also help in reducing the number of dropouts due to tumor progression where the waiting period is more than 6 months [28], [113]. A list of different criteria based on morphometric (based on tumor size and number) and biological (using AFP level and total tumor volume) parameters along with patient survival rates are highlighted in Figure 12. A gap between the increasing number of HCC patients on the waiting list and available donors is the major setback for liver transplantation.

Criteria	Criteria Content	Survival
Extended morphometric criteria for hepatocellular carcinoma		
Milan Criteria Mazzaferro *et al.* 1996	Solitary nodule ≤5 cm or up to 3 nodules, each ≤3 cm; Absence of macroscopic vascular invasion or distant disease	4-yr OS: 85%
UCSF Criteria Yao *et al.* 2001	Single nodule ≤6.5 cm, or ≤3 nodules ≤4.5 cm each, with a total tumour diameter ≤8 cm	5-yr OS: 72.4%
Navarra Criteria Herrero JI 2001	Single nodule ≤6 cm, or 2-3 nodules ≤5 cm each	5-yr OS: 79%
Valencia Criteria Silva M *et al.* 2008	1-3 lesions ≤5 cm and total tumour diameter ≤10 cm	5-yr OS: 63%
Up-to-7 Criteria Mazzaferro *et al.* 2009	Sum of the size of the largest tumour in cm and the total number of tumours ≤7; Absence of tumour microvascular invasion	5-yr OS: 71.2%
Tokyo Criteria Sugawara *et al.* 2007	Nodule no larger than 5 cm and no more than 5 nodules (5-5 rule)	5-yr OS: 75%
Shanghai Criteria Fan J *et al.* 2009	Solitary nodule ≤9 cm or ≤3 lesions with the largest ≤5 cm with a total tumour diameter of ≤9 cm; Absence of macrovascular and lymph node invasion; Absence of extrahepatic metastasis	5-yr OS: 78.1%
Asan Criteria Lee SG *et al.* 2008	Nodule ≤5 cm, ≤6 nodules and free of gross vascular invasion	5-yr OS: 81.6%
Extended biologic criteria for hepatocellular carcinoma		
Hangzhou Criteria Zheng SS *et al.* 2008	Total tumour diameter ≤8 cm without macrovascular invasion or if >8 cm, histology from tumour biopsy without poor differentiation (grade III) and AFP level ≤400 ng/ml	5-yr OS: 78.3%
Kyoto Criteria Ito T *et al.* 2007	≤10 nodules, all nodules ≤5 cm and a serum DCP ≤400 mAU/ml	5-yr OS: 86.7%
Extended Toronto Criteria Sapisochin G *et al.* 2008	Biopsy of largest nodule is not poorly differentiated and no cancer-related symptoms	5-yr OS: 69%
LT French Study Group AFP Model Duvous C *et al.* 2012	Points assigned base on tumour diameter, number of nodules (1-3 *vs.* ≥4), AFP	5-yr OS low risk: 67.8% high risk: 47.5%
TTV/AFP Toso C *et al.* 2015	Total tumour volume ≤115 cm^3 and AFP ≤400 ng/ml	4-yr OS: 74.6%

Figure 12: *Extended criteria for liver transplantation in hepatocellular carcinoma [108]. AFP, alpha-fetoprotein; DCP, des-gamma-carboxyprothrombin; HCC, hepatocellular carcinoma; OS, overall survival; TTV, total tumor volume.*

b. Surgical resection

Unlike liver transplantation, hepatic resection treats HCC by partially removing the HCC affected liver by surgery. Patients with an early stage solitary non-cirrhotic tumor (BCLC stage 0 or A), with good liver performance (Indocyanine green (ICG) retention, elevated bilirubin, and low portal hypertension) and no radiological evidence of vascular invasion are considered for hepatic resection. Additionally, resectability is also dependent upon tumor size and location and whether the remaining liver volume could affect morbidity and mortality. Although the tumor size does not necessarily affect the outcome, provided that the residual liver volume and technical aspects of the surgery are achieved, the risk of vascular invasion and dissemination increases with tumor diameter [92], [114]. 5-year patient survival after partial hepatectomy for the solitary tumor is above 60% with less than 3% post-operative mortality, and thus a reliable alternative to liver transplantation.

However, significant portal hypertension and impaired liver function can increase patient mortality [114], [115]. For patients with underlying cirrhosis, who are the vast majority, strict guidelines-based selection is required to avoid resection-related complications and post-

operative liver failures leading to death [116]. For such patients, operative mortality is increased in comparison with non-cirrhotic patients and may range from 5% to 14%, in some studies. Different tools and algorithms have been reported to select candidates for liver resection in cirrhotic livers. The most commonly used are Child-Pugh score, Model-for-End-stage-Liver-Disease (MELD) score, indocyanine green clearance, liver transient elastography, and hepatic venous pressure gradient [117]–[121]. Recently, a new score has been proposed to evaluate the risk of post-hepatectomy liver failure in cirrhotic patients undergoing liver resection [122]. This score only involves three pre-operative variables: the platelet count, the estimated volume of the remnant liver, and the feasibility of a laparoscopic approach to perform liver resection. Peri-operative portal vein embolization (PVE), hypertrophy induction of the anticipated residual liver remnants, can allow more extensive liver resection and thereby increase liver volume, with fewer major complications and 90-day mortality [114], [123]. Furthermore, laparoscopic liver resection is more efficient and safer than traditional open surgery due to its lower invasiveness [124], [125].

Despite a better understanding of post-operative liver failure and better patients' selection before surgery, partial hepatectomy remains a difficult challenge in patients with HCC occurring in a cirrhotic liver. Moreover, those patients experience a tumor recurrence rate of about 70% at 5-years along with a higher risk at microvascular invasion and satellite nodules, which can be observed in the peri-tumoral surrounding parenchyma [27], [114]. Thus, the role of an adjuvant treatment that could deal with this peri-tumoral involvement becomes obvious, since the extension of the surgical margins is limited by the presence of cirrhosis and the risk of hepatic decompensation. Unfortunately, no adjuvant treatment has been found to have a significant impact on tumor recurrence and patient survival after liver resection for HCC. In particular, the sorafenib-based adjuvant therapy (STORM Trial) has failed to demonstrate any clinical improvement [126]. Hence, there is a necessity to develop new therapeutic modalities which, associated with surgical resection, may not only eliminate microscopic peri-tumoral seeding but also decrease tumor recurrence by the development of an anti-tumor immune response.

c. Tumor Ablation

Percutaneous local ablation is a standard treatment for BCLC stage 0 or A HCC patients, who are not suitable for resection. Ablation can be achieved by two means, chemical (absolute alcohol or trichloroacetic acid) or physical means (Radiofrequency Ablation (RFA) and Microwave Ablation (MWA)) [28].

Ethanol perfusion or alcohol ablation or Percutaneous Ethanol Injection (PEI) has been associated with fairly impressive success rates. It is achieved by percutaneous injection of 8-10 mL of absolute alcohol guided through CT or ultrasound. The needle injects alcohol at the distal end of the tumor which slowly progresses proximally to the whole lesion, to induce necrosis to all tumors smaller than 2cm [91]. Multiple sessions might be required for bigger tumors. It can be the procedure of choice for patients with a single lesion of < 5 cm in diameter or three lesions of < 3 cm [27]. Without any serious complications, the common problems are pain, feeling of intoxication, and fever [91]. Studies have shown similar patient survival rates to resection with 83% and 34% at 1-year and 4-years, respectively [127].

During RFA, high intratumoral temperature is attained by inserting a needle into the tumor through which an alternating current generates a heating effect to induce tumoral necrosis. This method can destroy tumors of diameter up to 5 cm, but the necrotic damage decreases with increasing tumor size and drops significantly for tumors larger than 4 cm in diameter [91]. However, RFA cannot be used for tumors adjacent to blood vessels or tough to reach liver segments (*i.e.* Segment 1) or tumors with multinodular cirrhosis [114]. RFA can be a preferred treatment option than resection for solitary tumors of less than 2 cm and in a favorable location in the liver parenchyma. Complications include bleeding from the puncture site, fever, abdominal pain, and transient elevation of serum transaminase [91]. The 5-year survival rate for patients treated by RFA is from 33% to 55%, depending on initial tumor size [128]. RFA has been used to treat recurrent tumors after partial hepatectomy [129]. RFA has shown higher complete tumor necrosis than ethanol infusion with 90% and 80%, respectively, with a lesser number of sessions. Although RFA is a safe and effective procedure, it is associated with a high risk of tumor persistence in the target site and hence it should not be considered as an independent HCC modality for large nodules [27]. Similar to RFA, MWA uses

microwave with frequency > 900 kHz to induce rapid temperature elevation and tumor ablation [114].

Ethanol infusion is used for tumor proximity to the gallbladder, stomach, colon, or other viscera, which cannot be treated by RFA [92]. In any procedure, local ablation has shown initial tumor clearance similar to resection with comparable 5-year patient survival rates in randomized controlled trials in patients with a single HCC of up to 3 cm [130]. Such local ablations can serve to limit the tumoral growth or downsize the tumor size for patients waiting for liver transplantation and can be considered as first-line therapy for very early-stage HCC patients.

d. Trans-arterial chemoembolization (TACE)

TACE is the most commonly used treatment for downstaging of tumors which exceeds the transplantation or resection criteria and locoregional HCC [131]. Patients with the intermediate stage of HCC (BCLC stage B) are considered for TACE. The key principle for the therapy is the hypervascularization and neoangiogenic properties of HCC along with the hepatic arterial supply of the lesion. HCC obtains its blood from the portal vein, and as it progresses the supply arterializes due to which even the well-differentiated HCCs rely on the hepatic artery for blood supply. Embolization, or blood vessel blockage, of the hepatic artery branch by image-guided catheter-based insertion of particles, can lead to tumor-selective hypoxia and ultimately to necrosis [132]. The process needs catheterization of the targeted hepatic artery along with an arteriogram [91]. This can be coupled with the infusion of a chemotherapeutic agent like doxorubicin, cisplatin, mitomycin, and epirubicin to enhance the efficacy, and thereby called TACE [92]. Various drug-eluding beads, like doxorubicin-eluting beads, are also developed to enhance the treatment response rate and lesser side effects [133], [134]. The adjacent healthy liver tissue is protected from TACE since its blood supply comes from both the portal vein and the hepatic artery [28]. Thus, TACE provides improved drug delivery with increased drug-tumor contact time and high rates of the first-pass extraction, due to which the drug is concentrated in the target site and induces minimal side-effect [91].

Contraindications for TACE include decompensating cirrhosis, tumor with extensive replacements on both liver lobes, reduced portal flow, portal vein tumor thrombosis, and

creatinine clearance of < 30 mL/min [135]. Survival rate for TACE treated patients with unresectable HCC is 82% and 63% at 1 and 2 years, respectively, with morbidity rate below 5% [114], [136], [137]. Depending upon patient selection, the median survival with TACE ranges from 26 to 40 months [134]. However, studies have also demonstrated no enhanced survival by TACE vs other supportive therapies for unresectable HCC, over 4 years [138]. The decision to stop TACE is complex. It cannot be repeated when an insufficient response is obtained, or for untreatable progressions [92]. Common complications by TACE include liver failure, formation of liver abscess, pain, nausea, ileus, and fever [91]. The combination of TACE with sorafenib has not yielded any positive outcome in terms of progression-free survival [139].

The chemotherapeutic agent can be substituted with radioactive isotope Y90-labelled microspheres to induce an anti-tumoral activity, which can be infused through the hepatic artery to be used for Transarterial Radio-embolization (TARE). Clinical studies have shown similar patient survival with TARE with respect to TACE and with around 40 to 90% tumor response rate. TARE can be used with portal vein thrombosis, which is a contraindication for TACE. Further studies are needed to show its effect and compare it with other contemporary modalities [140].

e. Systemic Therapies

A majority of HCC patients are diagnosed at an advanced stage. For advanced-stage HCC patients (BCLC stage C) or intermediate-stage patients (BCLC stage B) with progression after TACE, systemic therapies are recommended. Systemic therapies are quite new as HCC modality. Prior to 2008, none of the systemic therapies have shown any increase in patient survival rate. But through the Sorafenib Hepatocellular Carcinoma Assessment Randomized Protocol (SHARP) trial, a new ray of hope was given. Sorafenib is an oral multi-kinase inhibitor for Raf (Rapidly Accelerated Fibrosarcoma) serine/threonine kinase isoforms, as well as the receptor tyrosine kinases (Like VEGFR 2 and 3, PDGFR-β, c-KIT, FLT-3 (Fms like Tyrosine kinase 3), and RET), thereby impede tumor angiogenesis and tumor cell proliferation [141]. Through the multicenter, phase 3, double-blind, placebo-controlled SHARP trial, Llovet *et al.* investigated the overall survival, symptomatic and radiologic progression, and safety of sorafenib for advanced HCC patients who had not received any previous systemic treatment [142]. They highlighted a median survival from 7.9 months with placebo to 10.7 months with

sorafenib. And thus, sorafenib became the first systemic drug approved by the US-Food and Drug Administration (US-FDA) for the treatment of patients with advanced HCC. Similar trials in the Asia-Pacific region demonstrated the efficacy, safety, and tolerability of sorafenib, due to which it can be recommended as a first-line treatment for advanced HCC [141]. Sorafenib is recommended for patients with advanced HCC, preserved liver function who are not eligible for liver transplantation or hepatic resection, and other locoregional therapies have failed to respond [143]. The side-effects are skin-associated toxicity, thromboembolism, and bowel perforation, and others including anorexia, nausea, vomiting, weight loss, hoarseness of voice, esthesia, and hypertension due to which the therapy is not well tolerated and dose reduction or treatment interruption is often demanded [144].

However other systemic therapy agents tested in a phase III trial have failed to demonstrate any advantage in patient survival either as first-line or second-line treatment. Some of these agents are erlotinib, brivanib, sunitinib, linifanib, everolimus, pegylated arginine deiminase (ADI-PEG20), doxorubicin, FOLFOX4 (fluorouracil, leucovorin [folinic acid], and oxaliplatin), and tivantinib (for patients with overexpression of MET) [28]. A graphical summary of these results has been represented in Figure 13.

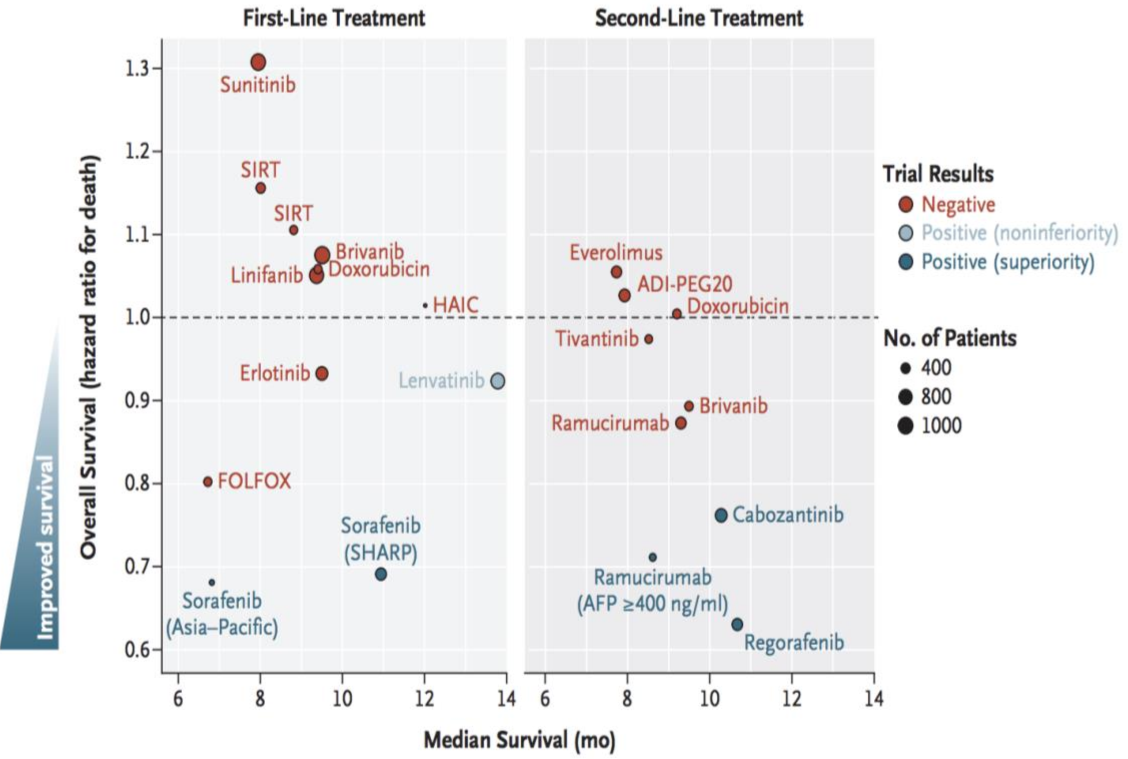

Figure 13: *Systemic Therapies Tested in Phase III Trials for advanced HCC. ADI-PEG20: pegylated arginine deiminase 20, HAIC: hepatic arterial infusion chemotherapy, SHARP: Sorafenib Hepatocellular Carcinoma Assessment Randomized Protocol, and SIRT: selective internal radiation therapy. [28]*

Through phase III clinical trial, another multi-tyrosine kinase inhibitor, Regorafenib, has been demonstrated as an effective second-line treatment for advanced HCC patients who have progressed during sorafenib treatment [145]. Cabozantinib, an inhibitor of receptor tyrosine kinases, including VEGFR, MET, and AXL, has increased patient survival in a placebo-controlled trial as second-line therapy [146]. An anti-VEGFR 2 antibody, Ramucirumab, has also been tested as a second-line treatment for advanced HCC patients in a randomized, placebo-controlled phase III trial after a failed sorafenib treatment. The results highlighted improved survival with 1.6 months for placebo vs 2.8 months for the Ramucirumab group along with an objective response rate of 1.1% and 4.6%, respectively [147].

Besides, there are a few chemotherapeutic agents which are largely used for a palliative purpose, rather than with curative intent. Most of them are anti-angiogenic with a few being immunomodulatory. Usually, systemic chemotherapy is associated with low response rates [91]. Some of the drugs which are being evaluated for HCC treatments are listed in Table 1. As a summary, the above-mentioned HCC treatments are summed up in with patient survival and recurrence rates, in Table 2.

Table 1: List of drugs in evaluation for HCC treatment. [27]

Type of drugs	
Chemotherapeutic agents	5-Fluorouracil, Capecitabine, Gemcitabine, Doxorubicin, Epirubicin, Etoposide, Cisplatin, Mitoxantrone
Hormones and anti-hormones	Megestrol acetate, Tamoxifen, Octreotide
Others	Pravastatin, Cox-2-inhibitors, Troglitazone

Table 2: List of HCC modalities with their survival and recurrence rates. [140]

HCC Treatment Modalities	Survival	Recurrence
Liver Transplantation	> 70% (5 yrs)	< 15% (5yrs)
Hepatectomy	41%-74% (5 yrs)	70% (5 yrs)
RFA/PEI	70% (5 yrs) lesions <2 cm	2%-50% (3 yrs)
TACE/TARE	20%-60% (2 yrs)	TACE is a noncurative treatment; response rates, 6% to 60%

Sorafenib	Median survival is 3 months longer than with placebo	Time to progression is 3 months longer than with placebo

f. Immunotherapy

The role of immune escape for tumor proliferation has become widely recognized in recent years, and hence it has paved the path for the development of new therapeutic strategies based on boosting or reviving an immune response, strong enough to control neoplastic growth. The human immune system works on a very intricate balance of on-and-off stimulus-based reactions. The reaction generated by cancer cells can create an activating signal for immune response to counter the growing neoplasm which is negatively regulated by various cells, receptors, and ligands. This tightly regulated immune suppression is crucial to avoid over-stimulation and provide self-tolerance. These negative regulators of the immune response are called immune checkpoints [148]. We have previously highlighted how HCC TME expresses various immune check-points in order to escape the immune reaction. Some of the most studied check-points are CTLA-4, PD-1, PD-L1, TIM-3, LAG-3, IDO, and T-Cell immunoreceptor with Ig And Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM) domains (TIGIT). A summary of all these negative regulators is provided in Table 3. We shall now discuss some of the key immunotherapy strategies, and their application for HCC treatment.

Table 3: A summary of T-cell surface receptors associated with immune inhibition. [149]

Receptors	Expressing cells	Ligands	Ligand-expressing cells
PD-1	CD4 (activated/exhausted, follicular), CD8 (activated/exhausted), B cells, DCs, monocytes, mast cells, Langerhans cells	PD-L1, PD-L2	Antigen-presenting cells, CD4+ T cells, non-lymphoid tissues, some tumors
CTLA-4	CD4 (activated/exhausted, Tregs), CD8 (activated/exhausted), some tumors	CD80, CD86	Antigen-presenting cells
LAG-3	CD4 (including Treg and exhausted), CD8 (including exhausted), NKs	Major Histocompatibility Complex (MHC) class II, LSECtin (Liver Sinusoidal Endothelial Cell Lectin)	Antigen-presenting cells, liver, some tumors
TIM-3	CD4 (Th1, Th17, Treg), CD8 (including exhausted and Type 1 CD8 cells (Tc1)), DC, NK, monocyte, macrophages	Galectin-9, phosphatidylserine, high mobility group protein B1, Ceacam-1	Endothelial cells, apoptotic cells, some tumors
TIGIT	CD4 (including Treg, follicular helper T cells), CD8, NK	CD155 or Polivirus Receptor (PVR), CD122 (PVRL2, nectin-2)	APCs, T cells, some tumors

CTLA-4, or CD152, is localized in the intracellular compartments of the naïve T cells and arises at the later stages of the CD4+ T cell activation. Since it shares homology with T-cell co-stimulatory protein, CD28, they both bind to CD80 (B7-1) and CD86 (B7-2) on antigen-presenting cells. However, CTLA-4 can out-compete CD28 and transmit the inhibitory signal to T-cells alongside indirectly diminishing the CD28 co-stimulatory signal. Thus, CTLA-4 can decrease T-cell activation and increase their activation threshold thereby reducing response against tumor-associated antigens. CTLA-4 is primarily expressed by Tregs but can be expressed by exhausted T cells and intracellular vesicles. Tregs are CD4+, CD25+, FOXP3+ T cells which are the inhibitors of immune response *via* various mechanisms [149]–[152]. PD-1, also homologous to CD28, is another immune checkpoint expressed by activated CD8+ and CD4+ T cells, B cells, NK cells, Tregs, monocytes, and MDSCs, with PD-L1 and PD-L2 as its ligands. Upon binding with PD-L1, PD-1 can inhibit CD8+ T cell activation by TCR signaling blockade, and inhibit CD4+ T cells by increased IL10 secretion. The T-cells targeting tumor-associated antigen, secrete IFN-γ which induces PD-1 expression on these reactive T cells and PD-L1 expression by APCs and tumoral cells [149], [151]–[153]. TIM-3 is the surface receptor on various immune cells which can interact with galectin-9, a soluble β-galactoside lectin protein. Cancers use these checkpoints to escape immune recognition. Studies have demonstrated that galectin-9 can cause T cell suppression thus indicating the check-point role of TIM-3. TIM-3 may act synergistically with PD-1 to promote CD8+ T cell inactivation [152], [154]. Similarly, LAG-3 is also expressed on activated T cells and can cooperate with PD-1 in T cell inhibition [152], [155]. In 2018, Pr. James Patrick Allison and Pr. Tasuko Honjo were awarded Nobel Prize for Physiology for their work on immune checkpoint inhibitor-based cancer immunotherapy.

Immunotherapy in HCC has largely seen the implication of anti-CTLA-4 and anti-PD-1/PD-L1 based immune checkpoint inhibitors, as we now highlight some of the key clinical studies associated. Tremelimumab, a fully human IgG2 monoclonal antibody against CTLA-4, was the first of such check-point inhibitors to be tested for HCC treatment. In a phase II, non-controlled, multi-center trial for evaluation of tremelimumab efficacy on HCV infected HCC patients demonstrated improved diseases control rate (76.4%), partial response rate (17.6%), and time to progression (6.48 months) with respect to the placebo group. Also the immunotherapy was well tolerated by patients, except a few symptoms of skin rashes [156].

Later, in a Phase I/II trial, Duffy *et al.* combined tremelimumab with locoregional treatments for HCC to investigate immunogenic cell death amplified by anti-CTLA-4 based therapy. Even though this pilot study enrolled only 32 patients, the combinatorial approach showed a diseases control of 89% and median time to progression of about 7.4 months [157]. Both these studies had smaller patient groups but highlighted the anti-tumor effect with no adverse effects observed. In a randomized, double-blind, multi-center phase III trial (KEYNOTE 240), 413 advanced staged HCC patients previously treated by sorafenib were treated with pembrolizumab (anti-PD-1) or placebo along with best supportive care. The results showed median overall survival and progression-free survival of 13.9 months and 3 months, respectively with respect to the placebo group (overall survival of 10.6 and progression-free survival of 2.8 months). The study did not highlight any significant difference by pembrolizumab alone [158]. Checkmate 459, a randomized, multi-center, phase III trial, also failed to show significant improvement in overall survival by nivolumab (anti-PD-1) vs sorafenib [159]. However, nivolumab in combination with levatinib showed improved anti-tumor activity and better tolerance in patients with unresectable HCC [160]. Similarly, atezolizumab (anti-PD-L1) plus bevacizumab also showed better efficacy and progression-free survival of advanced HCC patients compared with bevacizumab alone [161]. IMBrave 150 study further compared the efficacy of this combinatorial approach with sorafenib alone, to demonstrate longer overall survival and progression-free survival [162]. Thus, bevacizumab combined with atezolizumab was approved as a first-line treatment for advanced HCC. More such immune checkpoint inhibitors are being evaluated alone or in combination with other therapies.

That said, it is pretty too early to comment on anything about the efficacy of immune checkpoint inhibitor-based immunotherapy for HCC since the field is still young and more and more evidences are being gathered to understand the mechanism and resistance of tumor against immunotherapy. Furthermore, even though we have listed some crucial clinical trials here, we still have not considered immunotherapy for early-stage HCC, primarily for patients on the waiting list for transplantation in order to hold the tumor growth. We also have to investigate the impact of immunotherapy on tumor-infiltrating lymphocytes, and hence an immune-surveillance study could be crucial for prognostic assessment. But most importantly, we need to complement immunotherapy with a more immunogenic anti-tumoral therapy for

HCC, where we could obtain the full potential of immunotherapy to diminish the tumor progression and not just stall it.

Besides these, there are other immunological targets in HCC that are being evaluated like anti-TGF–β and anti-AFP along with oncolytic virus, peptide vaccines, and adoptive cell transfer therapy [163]. The discussion of these strategies lies beyond the scope of the present work.

CHAPTER 2

PHOTODYNAMIC THERAPY

A. Introduction

The success of new emerging therapies for various cancers has given a ray of hope for the patients. The notion of cancer as an untreatable disease is somewhat weakening with the introduction of various targeted therapies, immunotherapies, hormone therapy, and improvements in existing surgical and chemotherapeutic modalities [164], [165]. Scientific research on new frontiers of therapies, namely Stem cell therapy, oncolytic viruses, anti-cancer vaccines, and precision medicine is also keeping up with the pace [165]. This race to develop a more effective and tumor-selective therapy is continuous, as more anti-cancer drugs and modalities are being approved each year by the regulatory authorities of different countries. In recent decades, scientists have investigated the use of nanoparticle-based anti-cancer therapies, in order to increase the efficacy, drug delivery, and targeting of existing chemo and radiotherapy along with various other promising combinations with immunotherapy and surgery [166]. Additionally, many other therapies are being proposed as an adjuvant to existing treatments thereby increasing overall patient survival by decreasing tumor relapse. One key mention goes to immunotherapy, which has gained the spotlight in recent decades, primarily due to our increased understanding of the immune system and its role in tumorigenesis. Anti-CTLA-4 and anti-PD-L1 are becoming a standard treatment for a few cancers, but their potential as a solo treatment is still questionable.

Despite such a tremendous development in cancer diagnostics and therapy, we have only a few modalities that are effective and useful in the clinic or have shown potential. There are still a lot of cancers that cannot be treated completely or have very limited options available for intermediate and advanced-stage patients. Hence the search for a novel therapeutic approach for the treatment and management of cancer patients is still ongoing.

Photodynamic Therapy (PDT) is a light-based modality based on systemic or local administration and accumulation of a photosensitizing agent or photosensitizer (PS) at the target site. This agent is in turn activated by light of an appropriate wavelength which will give rise to a triplet state which further reacts with the intracellular oxygen to form ROS, like superoxide anions ($O_2^{\bullet -}$), singlet oxygen($^1O_2$), peroxides (O_2^{2-}), hydroxyl radicals ($OH^\bullet$) [167]. These ROS are known to create oxidative stress, DNA damage, oxidation of amino acids and polyunsaturated fatty acids, and activate the cell death mechanism [168]. The combined

effect of optimal drug concentration and optimal illumination dosage at an appropriate wavelength makes this therapy highly selective and thus imparts lesser side effects. The therapy has been tested and proven to be an effective and safe treatment option, hence it is approved for various skin disorders and microbial infections, and now it is being tested to treat various superficial and internal malignancies [167].

B. History of Photodynamic Therapy

Photochemotherapy in different forms has been practiced in ancient civilizations. The ancient medicine system of India, *Ayurveda*, described the use of psoralens (a DNA intercalant that induces apoptosis on UV exposure) and sun-light for re-pigmentation of vitiliginous skin. Ancient Egyptians used psoralens for the treatment of leukoderma. Similar evidences have been found in Chinese and Greek scriptures as well [169].

In the modern era, such therapy was first described by German scientists Raab and von Tappeiner in 1900. This entire finding was serendipity when Raab while investigating the impact of acridine orange at low dilutions on *Paramecium caudatum* observed that a prevailing thunderstorm had caused the toxicity to the protozoa, thus interpreting the role of light in the underlying toxicity. With subsequent experiments, they concluded that acridine orange or a light source alone had no effect, but it is their combined effect that induced cytotoxicity to the target cells [170]. Hence, the first evidence of PDT was reported where a PS, acridine orange in this case, in a combination of light could successfully induce cytotoxicity to target cells, *Paramecium caudatum*. In the following years, von Tappeiner coined the term 'Photodynamic action' and showed that oxygen is an important modulator for this action [171].

Together with Jesionek, von Tappeiner also performed PDT for the treatment of non-melanoma skin cancer, lupus vulgaris, and chondylomata lata using topical 5% eosin as a PS and white light as an illumination source. They, hence, concluded that once the PS is incorporated into the cells, it could induce a cytotoxic effect when exposed to an appropriate

light source, and thus became the first to perform photochemotherapy on patients [172]. These were the earliest work done in PDT, hence making Von Tappeiner a pioneer in this field, and his studies established protocols for future scientific research. Contemporary work from other scientists using subcutaneous injections of erythrosine solution for treatment of lupus vulgaris resulted in severe pain during illumination [173].

These results were promising but yet unsatisfactory, due to which the therapy failed to establish itself as a mainstream modality. Hence, the search for new PSs began, which can potentially improve the efficacy of the novel therapy. Hausmann used chlorophyll extracts to study PDT on Red Blood Cells. Hausmann later used hematoporphyrin (Hp), a ferric-ion free derivative of heme, as a PS revealed phototoxicity when exposed to light [174]. Friedrich Meyer-Betz and Hans Fischer studied the impact of the porphyrin structure on the efficacy of Photodynamic activity and found the mesoporphyrin is not as phototoxic as Hp, while uroporphyrin is almost the same [169]. Later, Meyer-Betz conducted self-experiments by injecting himself with Hp and reported swelling and severe pain at light-exposed skin for until 2 months. This became the first study to analyze the impact of Hp-mediated PDT on humans [175]. Accumulation and retention of Hp were analyzed by using fluorescence in rat sarcoma, which was later confirmed in patient tumors, thus showing a high tumor-localizing efficacy of PS [169], [176]–[178]. But Hp requires high doses for consistent uptake and fluorescence, which thereby caused phototoxicity. Later studies also revealed that Hp is a mixture of various porphyrins with pure Hp showing the poorest tumor localization capabilities. Hence, Schwartz developed Hp derivatives (HpD), derived from the acetylation and reduction of Hp, which showed enhanced tumor localization despite being an impure mixture [169], [179]. Later, R. L. Lipson from Mayo Clinic used HpD mediated PDT on animal models followed by clinical trials to detect and treat patient cancer [180]–[182]. Following the success of HpD mediated PDT for *in-vivo* models and small patient studies, Thomas J. Dougherty started the first human trial of 25 cancer patients. He intravenously injected HpD at 2.5 mg/kg and 5 mg/kg and illuminated the treatment region using red light 1-7 days post-injection. He demonstrated partial or complete reduction of 111 tumors out of 113 of varying nature including squamous cell, basal cell angiosarcoma, melanomas, breast, prostate, colon carcinomas. They also found that highly pigmented and larger subcutaneous tumors require more doses of HpD (5 mg/kg) than non-pigmented and superficial tumors (2.5 mg/kg). Additionally, skin damage was reduced by

decreasing the light exposure and increasing the time interval between the injection and illumination [183]. This was a groundbreaking study as it revealed the efficacy and potential of PDT utilizing HpD as an anti-tumor modality, highlighting its clinical success in the large patient group. This was followed by a surge in studies to evaluate the potential of PDT for the treatment of different cancers, by using the more purified form of HpD, Photofrin II.

Even with these optimistic outcomes, PDT was still facing criticism, mainly in dermatology, due to the underlying phototoxicity of the HpD. In 1990, J.C. Kennedy introduced 5-Aminolevulinic Acid (5-ALA) as a topical porphyrin derivative. 5-ALA is a prodrug that will incorporate into the cells and then metabolized by the Heme-biosynthetic pathway into protoporphyrin IX (PpIX) [184]. 5-ALA was the first PDT pro-drug to be approved by regulatory authorities for the treatment of actinic keratoses by utilizing blue light. Kennedy demonstrated that topical 5-ALA administered PDT can completely eliminate superficial basal cell carcinoma in 90% of treated patients, with partial response for 8%. More about this study will be discussed later. A brief timeline depicting the major breakthroughs in the field of PDT are highlighted in Figure 14.

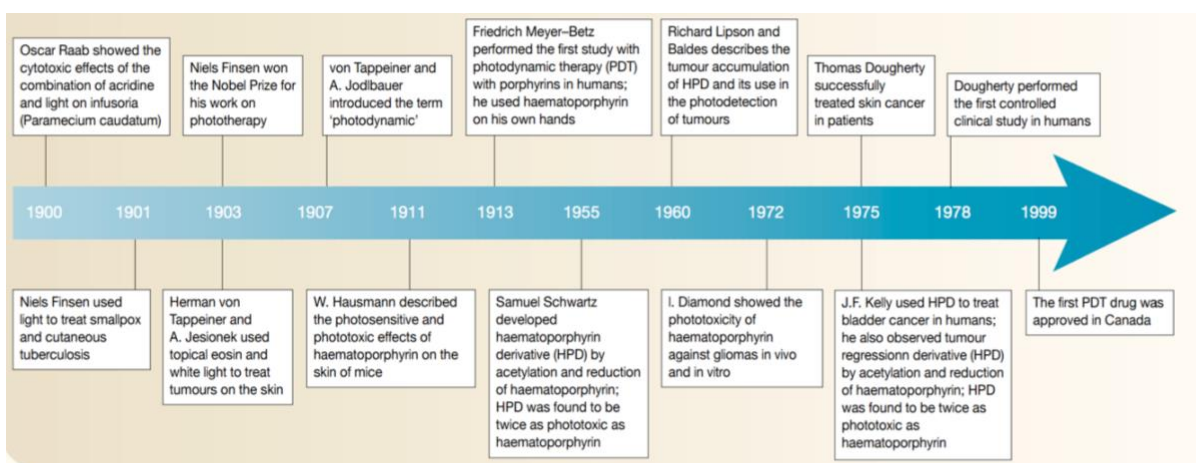

Figure 144: *A timeline of PDT, highlighting major developments in the field. [185]*

C. PDT in Dermatology

Initially, PDT was used for treating various dermatological disorders, including similar malignancies. PDT is a widely approved therapy in dermatology. Due to its higher efficacy, easy to use, relatively cheaper, and better aesthetic outcome, PDT has been applied or tested for almost all skin disorders. Hence, we hereby highlight the application of the therapy in this field, in order to discuss the implementations and execution of PDT in general.

1. Acne

Acne is a common dermatological disorder, affecting a significant amount of the young population, giving rise to psychological stress due to aesthetic concerns and facial scars [186], [187]. Acne is an inflammation of the pilosebaceous unit along with excess sebum production, follicular hyperkeratosis, and *Propionibacterium acnes* overgrowth.

In 2000, Hongcharu *et. al.* demonstrated the first use of topical 5-ALA mediated PDT for acne treatment in 22 patients. Previous studies had already established that *Propionibacterium acne* could metabolize porphyrins, hence Hongcharu and his colleagues proposed that topical administration of 20% 5-ALA and red-light excitation 3 h after with a dose of 150 J/cm^2 could significantly decrease acne for up to 10 weeks with a single PDT session and up to 20 weeks with multiple sessions [188]. This inhibition was primarily by inhibition of sebum, decreased inflammation, and toxicity to *P. acne*. Other studies further confirmed that a single session 5-ALA PDT might not be sufficient to induce a long-lasting impact, and hence proposed for multiple sessions [189]. PDT using ester form of 5-ALA, Methyl Aminolevulinate (MAL), has shown a similar impact upon PDT at high-dose [187]. Generally, low dose PDT for acne, characterized by low drug concentration and light fluence, short interval between drug administration and illumination along with blue light illumination, results in a mild response in terms of an immune response or microbial inhibition. However, a high dose gives a more long-lasting response. Due to porphyrin accumulation in the epidermis, side effects like pain, skin reactions, hyperpigmentation, dyschromia, acneiform eruption, erythema, edema, milia, and blistering are observed [186], [187]. MAL is shown to induce lesser pain during illumination and developed lesser erythema, pustular eruptions, and

epithelial exfoliation after treatment than compared to 5-ALA for acne treatment, due to better accumulation in the target tissue [190]. Similarly, topical ICG solution-mediated PDT for acne treatment of Asian patients has been shown to induce acne lesions, with minor short-lived side-effects and no significant difference between single or multi-session PDT [191]. The use of Pulsed-light sources like lasers is lesser painful than LED-based sources. A shorter incubation period, topical anesthetics, cooling devices, have been shown to reduce the other side-effects [186], [187].

2. Actinic Keratosis

Actinic Keratosis is cutaneous premalignant lesions due to long-term exposure to Ultraviolet (UV) radiations. These lesions cause scaly reddish papules due to the light exposure, surrounded by photodamaged area, called cancerization field, and possess molecular alterations [192], [193]. Fair skin, frequent sun exposure, immunosuppression, and age are some of the common risk factors. Actinic Keratosis is the earliest clinically detectable form of Squamous Cell Carcinoma and with similar genetic alterations, Actinic Keratosis might develop into carcinoma [193].

Topical 5-ALA PDT is an effective and non-invasive treatment for Actinic Keratosis and is being tested on different skin types and colors. Kennedy *et. al.* first assessed the application of 20% topical 5-ALA mediated PDT for 10 Actinic Keratosis patients, where they found complete elimination for nine patients after a single session [194]. PDT with 5-ALA and MAL have been reported to have a better outcome for Actinic Keratosis when compared with other treatment modalities in terms of efficacy, lesion clearance, cosmetic outcome, and patient satisfaction [192]. However, studies also reported remission, and hence Calzavara-Pinton *et al.* studied 5-ALA mediated PDT on 50 Actinic Keratosis lesions, with treatment being repeated every day until the area appeared treated and the recurrent lesions were later subjected to multiple cycles of PDT. They showed 100% elimination of lesions followed up to 36 months post-treatment [195]. Mordon *et al.* demonstrated the application of a optic fiber based light emitting fabric for treatment of patients with Actinic Keratosis by two different illumination devices. They highlighted a better or a similar lesion complete response rate when compared to conventional PDT at 3 months post treatment [196]. The efficacy of 5-ALA PDT for treating

Actinic Keratosis at extremities was improved by employing heat during incubation (38°C for 1h) [197]. Daylight PDT also has been proposed for its treatment with lesser pain, long-term efficacy, and a possibility to be used for immunosuppressed patients and in combination with other modalities [198]. Clementoni *et al.* used plastic rollers embedded with stainless steel needles (108 µm in width and 300 µm in length), which were gently rolled a couple of times over the patient treatment area to provide uniform erythema without bleeding. This needling provided fine holes to assist penetration of topical 5-ALA cream through the dermal-epidermal barrier, and hence significantly improving the overall clinical efficacy [199]. A similar microneedle patch can be employed for such pre-treatment [200]–[203]. All in all, PDT is now considered as a preferred treatment modality for Actinic Keratosis, and further improvements are being done to enhance this non-invasive treatment method.

3. Bowen's disease

Bowen's disease is an *in situ* squamous cell carcinoma. It is a slowly growing erythematous patch with a scaling and crusted surface. It is commonly located in extremities like lower limbs and on the head and neck with a very low chance of developing into invasive carcinoma. Some common factors for the disease are irradiation, immunosuppression, carcinogen exposure, and dermatoses. Classical treatment methods include Cryotherapy, excision, curettage, 5-Fluorouracil, and radiotherapy [204].

Bowen's disease is highly responsive to PDT, and clinical guidelines recommend PDT as first-line therapy, while surgery remains a secondary choice [205]. In 1988, Robinson *et al.* used HpD mediated PDT to treat over 500 lesions of two patients and concluded that PDT could be better therapy for Bowen's disease than other options [206]. Later, Kennedy *et al.* used 5-ALA induced PDT can successfully treat six lesions with a complete response [194]**.** Salim *et al.* demonstrated that 5-ALA mediated PDT is more effective than 5-Fluorouracil for patients with Bowen's disease, in terms of lesion complete response, pain, and other adverse effects [207]. Morton *et al.* further conducted a placebo-controlled randomized multicenter trial for MAL-PDT to compare its efficacy against cryotherapy and 5-Fluorouracil, with a follow-up of 3 to 12 months post-treatment. Their results highlight superior lesion complete response and tolerability with better cosmetic outcomes for MAL-PDT than with the other two

modalities [208]. For immunocompromised patients, the response of 20% topical 5-ALA mediated PDT is not satisfactory, as it failed to provide a long-term cure rate [209]. For invasive squamous cell carcinomas, PDT is not recommended as it has been shown to have a higher average recurrence rate than with other treatment modalities, but it can reduce or delay the occurrence of the carcinoma [210], [211].

4. Basal cell carcinoma

Basal cell carcinoma is the most common malignancy with a very low mortality rate. However, underlying local destruction can cause significant morbidity. Carcinogenesis is dependent on environmental factors, namely UV radiation exposure, but patient factors, like skin types, hair and eye color, family history of cancer. Other risk factors include immunosuppression, exposure to carcinogens, ionizing radiations [212]. There are 3 major variants of basal cell carcinoma: superficial, nodular, and infiltrative [213]. Common treatment methods include surgery, radiation therapy, Imiquimod and 5-Fluorouracil based topical therapies, and Hedgehog pathway inhibitor-based systemic targeted therapies [212].

According to the guidelines of the European Dermatology Forum, 5-ALA or MAL mediated PDT is should be considered for low risk, non-aggressive, “easy to treat” basal cell carcinomas. While PDT can be a preferred choice for recurrent small or large superficial carcinomas, the pigmented, micronodular and deeply penetrated ones should not be treated with PDT. For a nodular form of basal cell carcinoma or those with intermediate-risk, PDT is more of a secondary or tertiary treatment [214]. A clinical study for comparative treatment efficacy and cosmetic outcome between MAL-PDT and excision surgery for superficial basal cell carcinoma highlighted that the two modalities have similar clinical lesion responses but the former gives better cosmetic response over 1 year [215]. Similar results were demonstrated in another clinical setting for treating superficial carcinoma by MAL-PDT or cryotherapy and highlighted both therapies have equal recurrence rates but PDT offers better cosmetic outcomes for 5-years [216]. Additionally, MAL-PDT showed similar residual tumors and aesthetic outcomes in patients treated by imiquimod or 5-Fluorouracil, and overall imiquimod was superior to MAL-PDT [217]. PDT using other PSs, namely Foscan®, Photofrin®,

and Hypericin, for superficial basal cell carcinomas have demonstrated similar efficacy as of other classical therapies with better cosmetic outcome or remission or adverse effects [218].

5. Psoriasis

Psoriasis is an immune-mediated genetic disorder that affects the skin and is associated with physical as well as psychological factors. An individual with psoriasis is vulnerable to other chronic diseases like psoriatic arthritis, cardiovascular disorders, metabolic syndrome, Crohn's disease, anxiety and depression, non-alcoholic fatty liver disease, and lymphoma. These psoriatic skin lesions occur due to impaired interaction of the immune system with the resident subcutaneous cell types. Plaque psoriasis, or psoriasis vulgaris, comprises the highest number of cases. Classical treatments include glucocorticosteroids, vitamin D derivatives, or combinations of both, systemic drugs like methotrexate, ciclosporin, acitretin. Inhibitors of TNFα and Interleukin 12 and 23 have shown promising results and are currently regarded as second-line treatment [219].

Clinical data reveals that PDT could have anti-psoriatic efficacy. By topical administration of HpD followed by the visible or ultraviolet radiation of the psoriatic plaques resulted in the subsequent reduction of the degree of lesions [220]–[222]. Boehncke and his colleagues demonstrated that peripheral mononuclear cells isolated from six psoriasis patients, when subjected to psoralens mediated phototherapy (Psoralens and UV A (PUVA)) or photosan-3 mediated PDT, caused a decrease in the secretion of IL-6, TGFβ, and IL-1β. Further, they also demonstrated localization of the PS in the target region only, when applied topically. They did not discuss why the PS accumulation is strictly limited to the target site, but it could probably due to direct application of PS based ointment on the target site, along with Enhanced Permeability and Retention (EPR) effect. This study also highlights the ability of PDT to modulate the immune system. These findings were later confirmed for TNF-α and IL-8 as well [223]. Interestingly, topical 5-ALA PDT has shown more clinical variation and side-effects than PDT using topical hypericin and methylene blue or systemic 5-ALA and verteporfin which is more efficacious and better tolerated [224], [225]. The present state of PDT for psoriasis treatment is very inconclusive as it shows huge therapeutic potential but lacks sufficient clinical studies and a better approach for application.

6. Photorejuvenation

PDT is also being proposed as a treatment for photodamaged skin. Several studies have suggested that topical 5-ALA or MAL mediated PDT is safe and effective and thus could be used for repairing the photodamaged tissue by using different light sources like red light, blue light, intense pulsed lasers [192], [226]–[229]. PDT causes a decrease in fine lines, skin tightness, and roughness with no pigmentation. However, it caused side effects like erythema, edema, and scaling with mild to moderate discomfort during treatment [226]. MAL-PDT has shown effectiveness for the treatment of photodamaged, mottled pigmentation, sallowness, and roughness on patients, which was further improved by using microneedle-assisted MAL-PDT [230]. The damage inflicted by Intense Pulse Laser mediated 5-ALA PDT causes thermal damage along with free-radical based destruction resulting in augmented collagen I formation and thus leading to dermal remodeling [231]. However, the stratum corneum remains a challenge here for adequate penetration of the topical 5-ALA or MAL. Hence use of microneedles or penetration enhancers could prove to be vital in increasing the overall efficacy of the therapy [228], [230]. Daylight PDT using MAL was equally proven to be effective and safe with lesser pain to treat skin facial photodamage [232].

D. Principle of PDT

As described previously, PDT is a therapy that relies on the combined effect of three key components: a PS, light, and intracellular oxygen [167], [233]. Individually, these three components have minimal to no impact on target or healthy cells. But when combined, they exhibit a photochemical reaction to initiate cytotoxicity [167]. Now, we shall discuss all these individual components in the following section, and later we shall discuss how these three components act together to elicit a photodynamic effect.

1. The Photosensitizer

The interaction of light and matter is governed by two laws, known as laws of photochemistry. According to the first law of photochemistry, also known as Grotthuss–Draper law, the light must be absorbed by the chemical substance, in order to initiate a

photochemical reaction [234]–[236]. As illustrated in Figure 14, when photons, or small energy packets of electromagnetic radiations, are absorbed by the atoms of these chemical substances, which in turn excite the electron of the atoms from its stable ground state, S_0, to an unstable excited state, S_1 or S_2, by a transition of the electron from its lower vibrational level to a higher level [233], [235]. In some cases, this excited electron does not affect any other molecules in the system but relaxes the electron from the highest to the lowest vibrational energy level thereby releasing photons, of energy resembling the difference of the energy levels, and causes fluorescence. However, in other cases, the electron alters the surrounding molecules, and thus causes photosensitization. The molecules that absorb the photons are called PS [235].

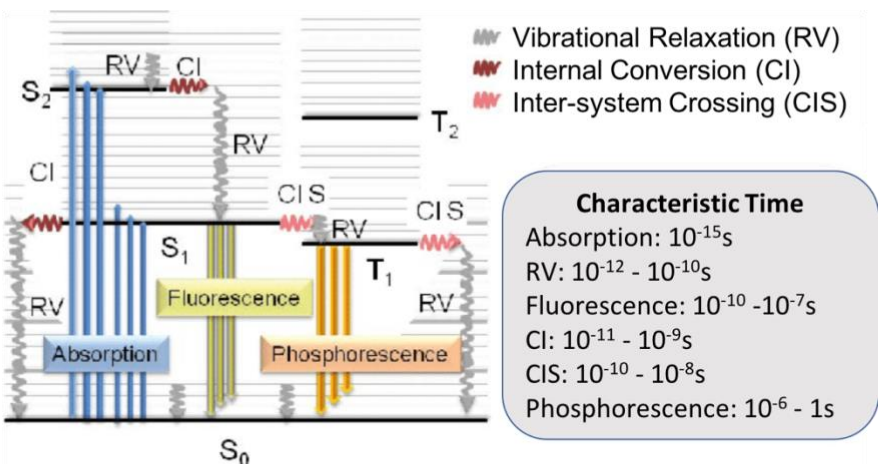

Figure 15: *Perrin-Jablonski energy level diagram illustrating the mechanism of PDT. RV: Vibrational Relaxation; CI: Internal Conversion; CIS: Inter-system Crossing. [237]*

In a photochemical reaction, the photons are absorbed by the PS, and thus the energy-rich PS alters the surrounding molecules in the system [233], [235]. According to Pauli's Exclusion principle, these excited PS molecules are always in the singlet state (^{0}PS), in which all the electrons are paired or have the electron of opposite quantum spin [235]. Since the excited state (^{1}PS) has the same spin as the ground level, they have a very short lifetime (~1-100 ns) and can easily decay back to the ground state thereby emitting light in form of fluorescence [235], [238]. But the electrons in S_1 state can also undergo a change in the direction of their spin, thus giving an excited triplet state, T_1 (^{3}PS*). Now since the electrons are spinning in the same direction, it is forbidden for them to transit back to the stable singlet ground state, S_0 (^{0}PS). This process is called Intersystem Crossing (CIS) [235]. This phenomenon might occur due to an encounter with a heavy atom or a triplet ground state oxygen molecule

(most stable and common allotrope of oxygen) [233], [235]. The $^3PS^*$ excited triplet state can return back to ground state by emitting phosphorescence. But, this excited triplet molecule can also give two types of photochemical reactions, which shall be discussed in the later sections. Besides the radiative decay which occurs under the emission of light, there are also non-radiative decay where the energy is released in the form of heat. Such decay occurs when the energy difference between the levels is very small and occurs faster than the radiative decay.

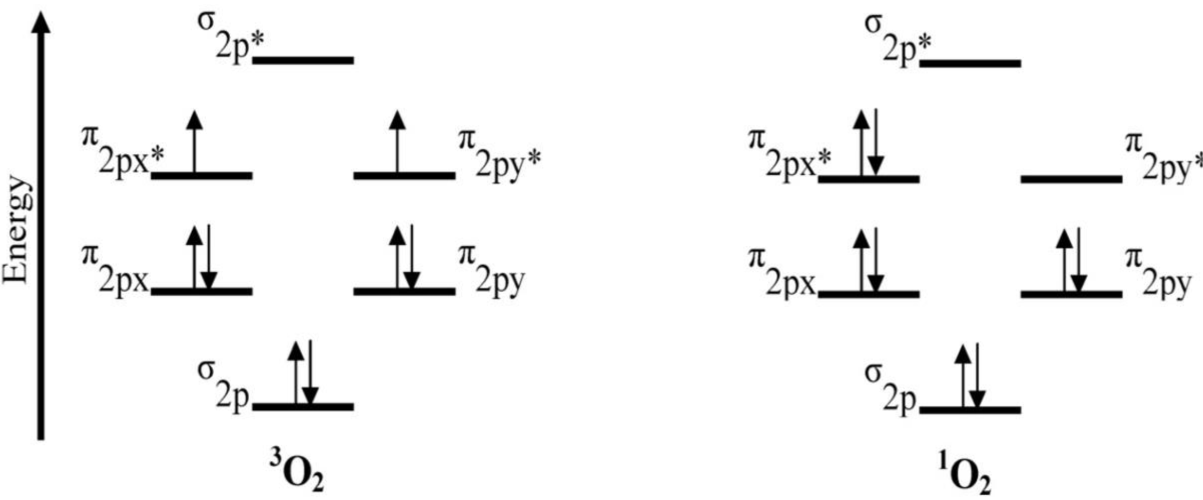

Figure 16: *Electron configuration of Triplet (3O2) and Singlet (1O2) Oxygen. [239]*

a. Types of photosensitization reactions

In general, there are two types of photosensitization, depending on whether an exchange of electron or energy is involved and the type of PS utilized [235].

i. Type I Photo-chemical Reaction (Radical based)

In these types of reactions, the excited triplet state PS ($^3PS^*$) can receive or donate electrons to surrounding molecules (substrate, Sub), thereby generating free radical products which are highly reactive. These free radicals can thereby react with oxygen to give rise to chemically reactive species containing oxygen. In some reactions, the ground state of the PS can be regenerated by these reactions, hence acting as a catalyst and readily available for another reaction [235], [238]–[240]. When the PS reacts with triple oxygen, it forms superoxide with low efficiency.

Table 4: List of Reactions by Type I reactions; SOD: Superoxide Dismutase. [241]

Reactants	Products	Note
$^3PS^* + {}^3PS^* \longrightarrow$	$PS^{\cdot+} + PS^{\cdot-}$	
$^3PS^* + Sub \longrightarrow$	$PS^{\cdot-} + Sub^{\cdot+}$	
$PS^{\cdot-} + {}^3O_2 \longrightarrow$	$PS + O_2^{\cdot-}$	($O_2^{\cdot-}$ = Superoxide anion)
$^2O_2^{-\cdot} + 2H^+ \xrightarrow{SOD}$	$O_2 + H_2O_2$	(SOD = SuperOxide Dismutase)
$O_2^{-\cdot} + H_2O_2 \longrightarrow$	$^3O_2 + OH^- + OH^\cdot$	($OH^\cdot$ = Hydroxyl radical)

These reactions occur more effectively in a high concentration of substrate and low oxygen concentrations. The substrate here can be highly oxidizable or reduceable, hence facilitating the easy and quick exchange of electrons [235], [238]–[240].

ii. Type II Photo-chemical Reaction (Energy-based)

In type II reactions, $^3PS^*$ will transfer its energy to a triplet ground state oxygen molecule ($^3O_2$), thereby giving a chemically reactive singlet state of oxygen ($^1O_2$) and ground state of the PS (^0PS) [235], [238]–[240].

$$^3PS^* + {}^3O_2 \rightarrow PS + {}^1O_2$$

Since the spin of $^3PS^*$ and $^3O_2$ is the same, it is an allowed transition (triplet-triplet annihilation). This changes the spin of the outermost electron of the oxygen molecule, which ultimately gives a lower-energy form of excited singlet oxygen ($^1O_2$) which is responsible for further cellular damage [235], [238]–[240].

Both type I and type II reactions can occur simultaneously in different proportions, which further depends on the type of PS used, along with external factors like concentration of oxygen and substrate and illumination [235], [238], [240]. Because of the high reactivity and short lifetime of these ROS, they react only with the molecules in close proximity and often in the same organelle [238], [240], [242]. The $^1O_2$ diffuse at an order of 0.01-0.02μm with a lifetime of 0.01-0.04μs [238]. The impact of these ROS on other biomolecules and cellular mechanisms will be discussed later.

b. Properties of a Photosensitizer

Some key characteristics of a good PS, which could be relevant clinically are described below:

Selectivity: One key feature of a PS is its ability to selectively accumulate at the site of action, and only in the target cells or tissue with minimal or low accumulation in healthy tissue. It is also to be noted that it should be retained by the target tissue or else, a low or no PDT effect would be observed. Primarily this is attributed to EPR effect, due to which molecules of certain sizes accumulate more in the tumoral tissues than in healthy. Due to abnormal fluid transport dynamics (since cancer has irregularly formed blood vessels) and lack of effective lymphatic drainage system, the macromolecules accumulate in the TME. Furthermore, this selectivity can be enhanced by conjugating the PSs with targeting or localizing ligands, specific to the targeted cancers. Some examples of such coupling can be by binding of short peptide sequences, antibodies, ligands, or encapsulating the PSs with tumor targeting nanoparticles [240], [243]–[245].

Dark Toxicity: The PS should remain inert or inactive in absence of an appropriate light source, hence it should exhibit cytotoxicity only in the presence of light. Additionally, further metabolism of the PS should not produce any harmful or toxic byproducts [240], [243]–[245].

ROS Generation: Since ROS are the key mediator of the therapy, the PS must have a high ($^1O_2$) quantum yield ($Ø_\Delta$) [240], [243]–[245].

Elimination: Bio-clearance of any external drug molecule is always important clinically. Hence a good PS must be eliminated or excreted out from the body to avoid any toxicity due to light exposure [243]–[245], [245], [246].

Wavelength: The PS must absorb photons efficiently. This means it should have a longer wavelength for its activation with a high molar extinction coefficient (ability to absorb light at a given wavelength, per molar concentration) as it allows deeper tissue penetration of the light with energy high enough to produce $^1O_2$. Preferred range is between 600 to 800 nm [240], [243]–[245]. Figure 16 shows the relative penetration of light through human skin at different wavelengths.

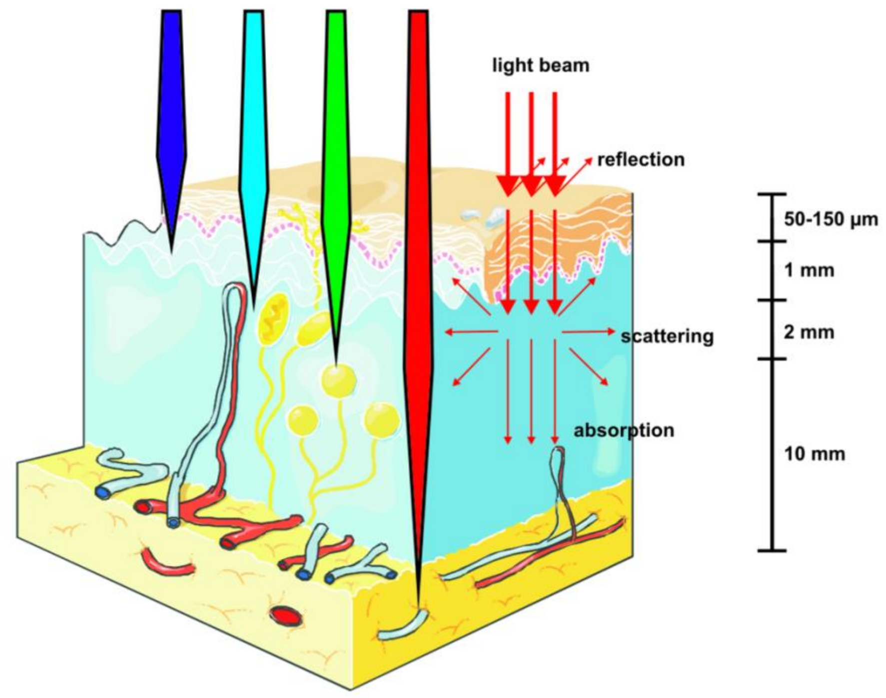

Figure 17: *The relative penetration of light at different wavelengths; blue light is the least able to penetrate tissues while infrared light is the best for tissue penetration at depth. Here Dark blue represents light of wavelength 245-390nm, Cyan blue represents light of 425 to 475nm, Green light represents light of 495 to 550nm and Red light represents light of 650 to 990nm. [247]*

Solubility: The PS should be water-soluble as it helps in the easy transport of the PS in the body. The PS might be modified to enhance its hydrophilicity [240], [243]–[245].

Availability: The PS should be a single pure compound, to facilitate its manufacturing and ensure regular availability [240], [243], [244].

A list of various PSs approved for clinical use or under PDT-related clinic trials is provided in Table 5.

Table 5:List of various PSs approved for clinical use or under PDT-related clinic trials. [248]

PSs	Drug	λ max (nm)	ε_{max} (M^{-1} cm^{-1})	ϕ_{Δ}	Application	Marketed by
Porfimer sodium	Photofrin	632	3000	0.89	Canada-bladder cancer (1993); USA-esophogeal cancer (1995); USAlung cancer (1998); USA -Barrett's esophagus (2003); Japan-cervical cancer; Europe, Canada, Japan, USA, UK-endobroncheal cancer	Axcan Pharma, Inc. (QC, Canada)
5-aminolevulinic acid (5-ALA)	Levulan	632	5000	0.56	USA-actinic keratosis (1999)	DUSA Pharmaceuticals, Inc. (MA, USA)
Methyl aminolevulinate (MAL)	Metvixia	635	-	-	USA-actinic keratosis (2004)	Galderma Laboratories, L.P. (TA, USA)

N-aspartyl chlorin e6 (NPe6)	Laserphyrin	664	40,000	0.77	Japan-lung cancer (2003)	Meiji Seika Pharma Co., Ltd. (Japan)
Meta-tetra(hydroxyphenyl)chlorin(m-THPC)	Foscan	652	35,000	0.87	Europe-neck and head cancer	Biolitec Pharma Ltd. (Dublin, Ireland)
2-(1-Hexyloxyethyl)-2-devinylpyropheophorbide (HPPH)	Photochlor	665	47,000	-	Clinical trials-esophogeal cancer, basal cell carcinoma, lung cancer, Barrett's esophagus	Roswell Park Cancer Insitute (NY, USA)
Palladium bacteriopheophorbide Palladium bacteriopheophorbide	Tookad Tookad-Soluble, Stakel	763 753	108,000 > 100000	0.50 -	Clinical trials-prostate cancer Europe-prostate cancer	Steba Biotech (Luxembourg) Steba Biotech (Luxembourg)
5,10,15,20-Tetrakis(2,6-difluoro-3-Nmethylsulfamoylphenyl)-bacteriochlorin	Redaporfin	750	-	-	Clinical trials-head and neck cancer	Luzitin SA (Portugal)

Verteporfin	Visudyn	689	34,000	0.84	Clinical trials-melanoma (skin), breast cancer, pancreatic cancer and prostate cancer	QLT Inc. (Canada)
Disulfonated tetraphenyl chlorin	Fimaporfin	652	-	-	Clinical trials-cutaneous or subcutaneous malignancies, cholangiocarcinoma	PCI Biotech (Norway)
silicon phthalocyanine PC4	PC4	675	40,000	-	Clinical trials-primaryor metastatic cutaneous cancers	Not licensed and produced by NCI (USA)
Aluminum phthalocyaninetetrasulfonate	Photosens	676	110,000	0.38	Russia- stomach, skin, lips, oral cavity, tongue, breast cancer	General Physics Institute (Russia)
ruthenium-based dipyridyl coordination complex	TLD1433	530	-	-	Clinical trials- bladder cancer	Theralase (Canada)

Besides these key features, other properties like its ability to be integrated with other therapies, cost, safety, less pain, easy administration, stability, and shelf-life, should not aggregate in biological systems, short drug-to-illumination duration, and ability to generate an immune response are also considered beneficial add-ons [240], [243], [244]. Methods for PS dosimetry by fluorescence and photobleaching, to monitor PS accumulation and disease follow-up pre-and post-PDT, are also desirable [240].

c. Structure of Photosensitizers

The structural characteristics of individual PS play an important role in their functionality as a sensitizer. PSs can be categorized based on their chemical structure: Tetrapyrrole-based PS, and other naturally occurring PS.

i. Tetrapyrrole based PS

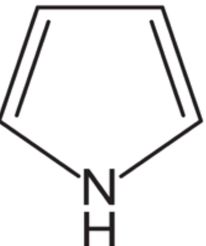

Figure 18: *Molecular structure of a pyrrole ring. [249]*

A large number of PSs currently in use are derived from an aromatic core based on tetrapyrroles. Tetrapyrrole structures are prosthetic groups that have four pyrroles or pyrrole-like rings, linked by a methine (=CH-) or methylene (-CH_2-) functional groups in linear or cyclic orientation [240], [250], [251]. These structures have wide functions like light absorption, electron transfer, and oxygen binding [250]. They are quite common in various pigment molecules and other biomolecules found in nature, including heme, chlorophyll, cytochromes, chlorins, Vitamin B12, and bacteriochlorins [251]. Tetrapyrroles generally produce Type II reactions, with an exception of bacteriochlorins [240].

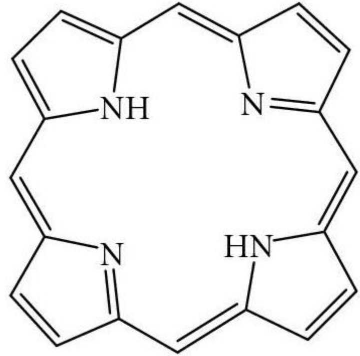

Figure 19: *Molecular structure of a tetrapyrole ring. [239]*

Porphyrins: Porphyrins are aromatic macrocyclic compounds, in which the *meso* or β position hydrogens of tetrapyrrole structure are substituted by other atoms or functional groups. Due to their high $^1O_2$ generation capacity and excellent fluorescence, they are highly advantageous in PDT. They have typically a narrow Soret band with several Q-bands (explained later, Figure 31). The previously discussed HpD and Photofrin®, first-generation PS, belong to this class. However, lower chemical purity, poor tissue penetration and accumulation, prolonged skin hypersensitivity, and weak absorbance with red light ultimately led to their limited success. However, they are still approved and used in many countries for treating various disorders. This was followed by the second generation of porphyrin-based PSs which include texaphyrins, 5,10,15,20-Tetrakis(1-methylpyridinium-4-yl) porphyrin tosylate, and PpIX. Out of these, PpIX is the most widely studied and clinically used. PpIX is the final intermediate of the Heme biosynthesis pathway, at the end of which the tetrapyrrole macrocycles of PpIX chelate with iron to form heme using ferrochelatase enzyme. Generally, it is used in the form of its pro-drug, 5-ALA, or its derivatives. Due to their clinical relevance and 5-ALA being the focus of our study, we would now discuss about it, its metabolism along with some key studies.

5-Aminolevulinic Acid and its derivatives: 5-Aminolevulinic Acid or 5-ALA is a naturally occurring non-proteinogenic amino acid, which is a precursor for the Heme-biosynthetic pathway (Figure 19) [194], [233], [244], [252]. When 5-ALA is endogenously incorporated, it acts as a pro-drug where it becomes the precursor for one of the metabolites of the pathway, PpIX [233], [244]. This eight-step process, occurring partly in the mitochondria and partly in the cytoplasm, is a universally conserved pathway [233], [252]. Hence all human cells are capable to metabolize 5-ALA at varying extent. However, some malignant and ailing cells

show higher internalization of 5-ALA, possible due to EPR effect. This thereby causes increased accumulation of intracellular PpIX, thereby exhibiting higher toxicity than the healthy surrounding cells. As explained earlier, PpIX has a porphyrin-based structure and hence has the potential to be an effective PS.

Figure 20: *Molecular structures of (a) PpIX and (b) 5-ALA. [239]*

The biosynthesis begins with the combination of glycine and succinyl-CoA into 5-ALA in the mitochondria which is a rate-limiting step (Figure 20) [252]. This thereby controls the overall levels of Heme and PpIX in the cells. However, with exogenous administration of 5-ALA, this barrier is overcome and hence the cells continue to metabolize 5-ALA into PpIX [194]. Thereafter, 5-ALA is transported to the cytoplasm where two 5-ALA molecules are condensed by ALA Dehydratase to form porphobilinogen. They are further metabolized into hydroxymethylbilane by hydroxymethylbilane synthase, and then into cyclic uroporphyrinogen III by uroporphyrinogen synthase. After the removal of a carboxylic group, coporphyrinogen III is formed which is transported back into mitochondria. After another decarboxylation followed by oxidation, PpIX is formed. The final conversion of PpIX to Heme requires the enzyme ferrochelatase and ferric ion, which can be down-regulated in certain conditions. This ultimately leads to the accumulation of PpIX. However, some studies reveal that PpIX can be excreted out of the cells by a member of the Adenosine Triphosphate (ATP) Binding Cassette Group (ABCG), ABCG2 [252], [253]. This issue can be resolved by inhibiting ABCG2 activity, thereby causing increased retention of PpIX [254].

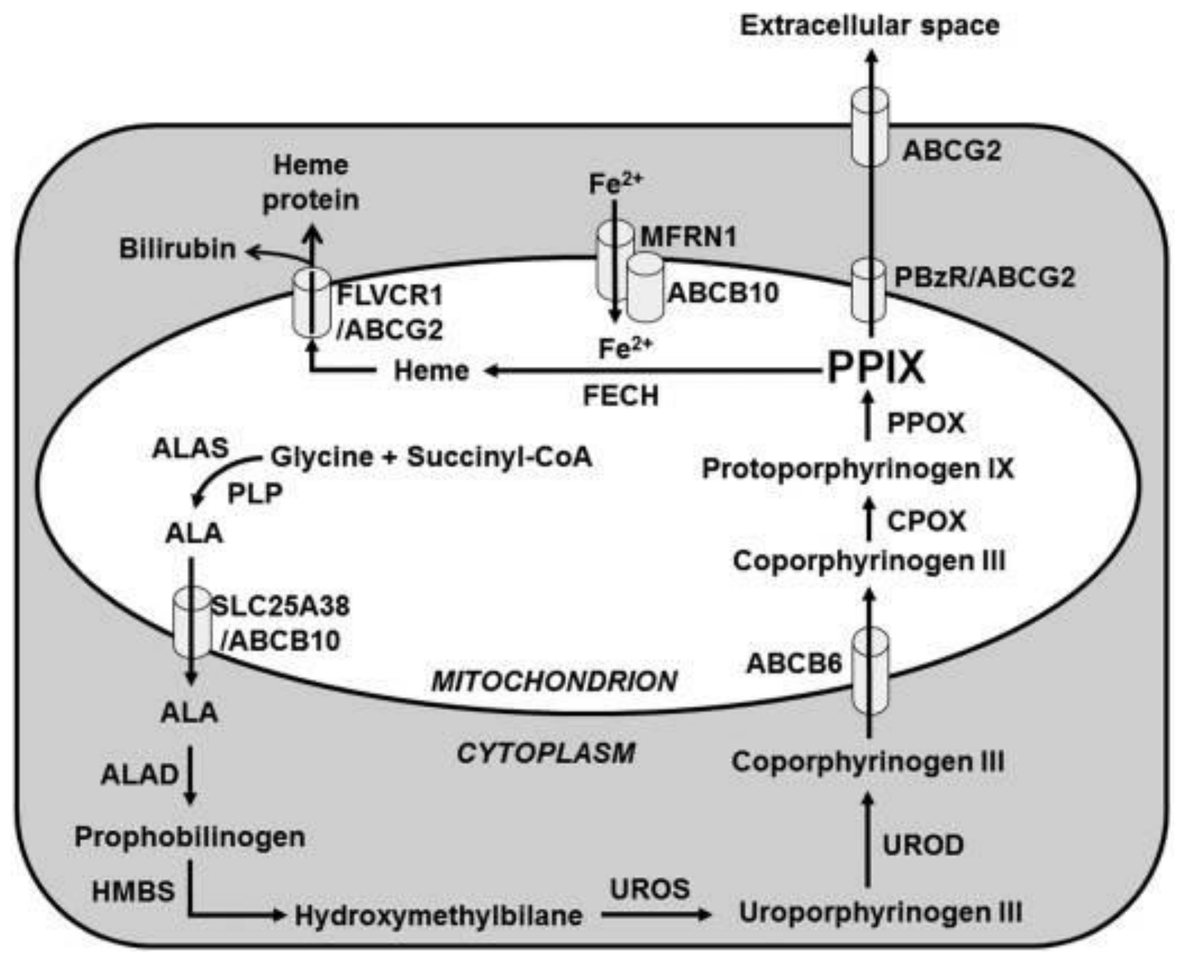

Figure 21: *Heme biosynthesis pathway in mammalian cells, with all the intermediates, enzymes, cofactors, and transporters involved. ALA, 5-Aminolevulinic Acid; ALAS, ALA Synthase; PLP, pyridoxal phosphate; ALAD, ALA Dehydratase; HMBS, hydroxymethylbilane synthase; UROS, uroporphyrinogen III synthase; UROD, uroporphyrinogen decarboxylase; ABCB6, ATP-Binding Cassette subfamily B6; CPOX, coproporphyrinogen oxidase; PPOX, Protoporphyrinogen IX Oxidase; FECH, Ferrochelatase; MFRN1, Mitoferrin 1; FLVCR1, feline leukemia virus subgroup c receptor 1; SLC25A38, solute carrier family 25 members 38. [252]*

5-ALA has a higher accumulation rate than other PSs. Additionally, its rapid bioclearance makes it safer than others. Normally, 5-ALA takes from 3 to 8 hr to accumulate and metabolize, depending on the target tissue and the mode of administration. Once accumulated, PpIX can be activated by illumination at 635 nm wavelength. We would discuss more the underlying photophysics in the later sections. 5-ALA has been approved by FDA for various superficial and cutaneous diseases, like Bowen's Disease, superficial Basal cell carcinoma, Actinic Keratosis, and others. Investigations for various solid tumors are also underway. *In-vivo* experiments demonstrated low or minimal 5-ALA mediated toxicity and low neurotoxicity at higher doses, highlighting its safety [194], [233]. Additionally, PpIX emits red fluorescence when excited by light of wavelength 400-410nm, which thereby correlates to PDT mediated toxicity [194], [252]. This makes 5-ALA a strong candidate for a pro-drug-

mediated PDT. It is commercially sold for therapeutic purposes by the name Levulan by DUSA Pharmaceuticals [238]. However, it is sold in different forms and formulations by names such as Ameluz, Gleolan, and Gliolan [239].

Figure 22: Molecular structures of (a) Methyl Aminolevulinate (MAL) and (b) Hexaminolevulinate (HAL). [239]

However, the esterified derivatives of 5-ALA are also very commonly used due to their better bioavailability and pharmacological properties. The *n*-hexyl ester of 5-ALA, Hexaminolevulinate (HAL), was approved for PDT by US-FDA in 2010 [239], [243]. HAL-mediated PDT has been shown to have higher efficacy than exogenous PpIX mediated PDT, and cause cell death by apoptosis compared to necrosis by the latter [255]. It is commonly marketed as Hexvix® or Cysview®. The methyl ester form of 5-ALA, MAL, has higher cell penetration than 5-ALA and is thus more effective than 5-ALA. It is being marketed by the name of Metvix® by Photocure ASA [239], [243]. A benzyl derivative of 5-ALA is also developed by Photocure and is being currently investigated.

Chlorins: Chlorins are partially hydrogenated porphyrins, which shifts their longest absorption band towards the 650-690 nm region with an increase in the magnitude well. This makes them better PSs than porphyrins. Chlorins are the derivates of naturally occurring pigment, chlorophyll, hence the name. This group includes some of the most promising PSs like, m-tetrahydroxyphenylchlorin (Temoporfin or Foscan), benzoporphyrin and its derivatives, radachlorin, pheophorbide and its derivatives (including (2-[1-hexyloxyethyl]-2-divinyl pyropheophorbide-a) (or HPPH)), tin(II) etiopurpurin, Talaporfin Sodium (or NPe6, or (mono-L-aspartyl chlorin e6)).

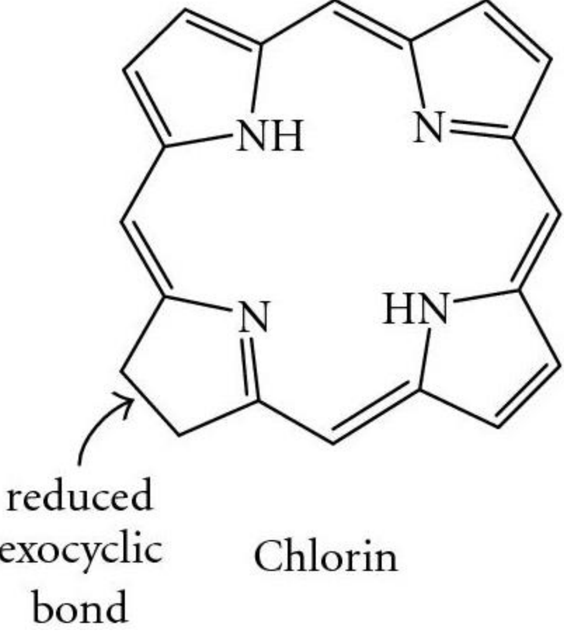

Figure 23: Molecular structure of chlorin. [239]

Bacteriocholorins: In bacteriochlorins, another double bond of the tetrapyrrole ring is hydrogenated, thus shifting the absorption band even further into the red region and increasing the height of the peak. However, there are only a few candidates from this more efficient group and are probably lesser stable. They are shown to be effective against pigmented tumors like melanoma and used at a wavelength of around 700 to 800 nm. TOOKAD® is one prime example of this group.

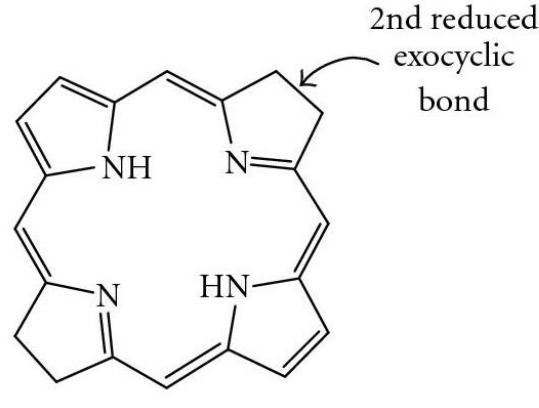

Figure 24: Molecular structure of bacteriochlorin. [239]

Phthalocyanines: PSs of this group have four isoindole rings, linked by nitrogen. The structure is very similar to tetrapyrroles and is related to them, hence they can be placed in the same

category. But they are commonly regarded as synthetic dyes. Due to lesser solubility in water, their applicability is restricted. Phthalocyanines are activated by a far-red light of approximately 670 nm. Some key examples include Zinc phthalocyanine (ZnPc), Silicon phthalocyanine (PC4), RLP068.

Phthalocyanine

Figure 25: *Molecular structure of Phthalocyanine. [239]*

ii. Other Naturally Occurring Photosensitizers

Besides the above-mentioned tetrapyrrole-derived molecules, many other naturally occurring molecules can be used as PSs with varying activation wavelength and photodynamic effect potential. Some of the key PSs of this class are being discussed here.

Anthraquinones: Used as dyes and antibiotics, some members of this group have shown photosensitive potential with a high yield of singlet oxygen on excitation. Most of the members of this group are perylene quinoid pigments and exhibit photodynamic action due to the 3,10-dihydroxy-4,9-perylene quinone moiety [256]. However, an absorption peak at a lower wavelength limits their application potential. One key example of this group is Hypericin, which is extracted from a weed named St. John’s wort. It acts through both type I and types II reactions. With a peak at around 595 nm, Hypericin has been shown to accumulate in ER and thus initiating a PDT reaction involving an immune response [257]. However, most of the members of this group are still under study to optimize dosage, targeting, and delivery. Hypocrellin A and B also belong to this group, which are derived from the parasitic fungus *Sinarundinaria* sp. After internalization and with excitation at 632 nm,

Hypocrellins can induce ROS generation in cell lines as well as mouse models and have shown effective and safe for PDT usage [256].

Indocyanine Dyes: ICG is a tricarbocyanine with amphiphilic properties. ICG was developed during the Second World War for photography. Later on, it was used for cardiac output monitoring, liver clearance test, and retinal angiography. The absorption and fluorescence spectrum of the dye is in the Near-Infrared (NIR) spectrum, in the tissue optical window, and is the only NIR dye approved by US-FDA for clinical applications. Nowadays, it is also being used in surgery, for imaging and monitoring blood circulation [258], [259]. However, ICG has shown potential for PDT [260]. Due to its higher patient safety in terms of toxicity, shorter life in blood circulation, NIR absorption spectrum, and amphiphilicity, ICG is being used for NIR PDT for several superficial and solid tumors. Besides, it also provides better fluorescence-based imaging with lower background noise. They are also capable to give a deeper PDT effect, due to higher penetration of the light [258], [260]. However, ICG has a lower quantum yield for ROS, lack of selectivity, and aggregates in biological systems [260]. Thus, ICG is being encapsulated with various nanoparticles for efficient drug delivery and being coupled with antibodies for increased targeting [258], [261], [262]. Various other derivatives of ICG are also synthesized comprising a family of indocyanine dyes [245]. ICG and its derivatives mediated PDT is being investigated for various skin disorders along with cancer types like cervical, breast, leukemia, and pancreas [260].

Figure 26: *Molecular structure of Indocyanine Green (ICG). [239]*

Phenothiazine and derivatives: Phenothiazine is organic compound, used for a wide variety of medical applications, namely insecticidal, anti-helminthic and anti-bacterial (Figure 26). Its

wide range of derivatives is employed for anti-histamine and anti-psychotic effects, anti-cancer, and anti-malarial drugs, and neurodegenerative diseases [263]. Members of this family have alkyl side chains attached to nitrogen. Some key members of this group are Methylene Blue, Promethazine, and Chloropromazine, out of which methylene blue has shown potential for PDT [264]. It is excited at 666 nm and has shown potential for treating melanomas, basal cell carcinoma, Kaposi's sarcoma, bladder cancer, and adenocarcinoma [239], [263], [265]. It is hydrophilic, absorption in the red spectrum, and has a high quantum yield of reactive oxygen [265]. Another member of this group, Toluidine Blue, excites at 630 nm which is also being used for PDT [239]. Chloropromazine, promethazine, and other derivatives are also being investigated [264].

Figure 27: *Molecular Structure of Phenothiazine [263]*

Xanthene dyes: Xanthene dyes contain xanthylium or dibenzo-γ-pryan nucleus, which acts as a chromophore. Thus, they exhibit brilliant hues and are often fluorescent [266]. Rhodamine, eosin Y, and Rose Bengal are used as PSs [239], [266]. Buck *et al.* demonstrated that Rose Bengal is more efficient as a PS amongst the other xanthene dyes by inducing higher cytotoxicity and photo-oxidation of uric acid [267]. Rose Bengal acetate, which has increased permeability due to the added acetate group, has been tested for PDT on various cancers. Rose Bengal acetate-mediated PDT has been used to induce cell death in HeLa cell lines by apoptosis and autophagy [268], [269]. One study has demonstrated that the PDT successfully induced phagocytosis of the dead cancer cells by macrophages, which further released immunogenic factors thereby highlighting its immunogenic potential [270]. The addition of a halogen further enhances their quantum yield potential for ROS generation. In a clinical study, fluorescein angiography-guided PDT was demonstrated safe and effective for the treatment of central serous chorioretinopathy [271]. Blue light-activated eosin Y can be used for antibacterial PDT [272].

Besides the above-mentioned natural PSs, Siewert and Stuppner have summed up a whole list of such PS which includes furanocoumarins, polyacetylenic molecules and thiophenes, curcumins, alkaloids (Chinolin-alkaloids, Pterin, Benzylisoquinolines, Oxoglaucine) and phenalenones [273].

There are synthetic PSs, which are being developed based on our understanding and knowledge of the mechanism of the photodynamic action. These primarily include the third generation of PS which are either the enhanced version of existing PS or newly synthesized PSs developed by nanotechnology. For example, PS could be coupled with various HCC targeting peptides, antibodies or ligands, which could enhance the PS targeting and localization, thereby increasing PDT efficacy. Their efficacy and safety are yet to be identified. Some novel targets for HCC could be Mesenchymal epithelial transition factor (c-Met), synthetic peptide HCBP1, and Circumsporozoite protein (CSP) I plus [274]–[276].

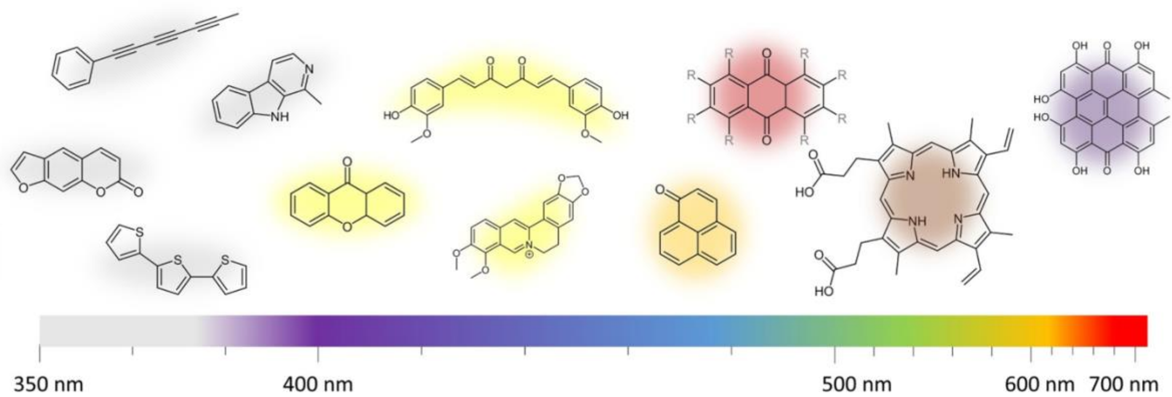

Figure 28: Overview of natural PS scaffolds with their approximate absorption max given below. [273]

2. The Light

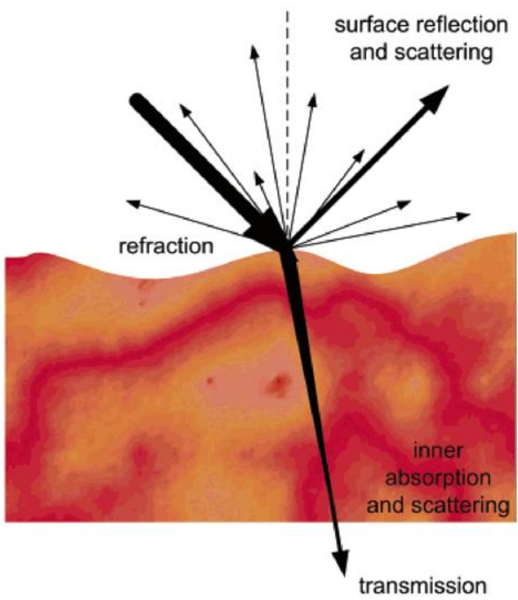

Figure 29: *Interaction of incident light with a tissue. [277]*

The interaction between the light and the PS is key for any photodynamic reaction, as it is the primary requirement for PS activation. Thus, homogenous light delivery and distribution are essential. Moreover, the stronger the overlap of the light source's spectrum with the absorption spectrum of the PS, the stronger will be the photoactivation of the PS and thus the photodynamic efficacy. This interaction imparts dual selectivity, but at the same time adds more variables for the success or failure of the therapy. The light to be utilized decides the therapeutic efficacy of PDT, as it has to penetrate the superficial layer of the tissue to reach the target site as well as to activate the PS. The light upon interaction with any tissue surface can be refracted, reflected, scattered, or absorbed (Figure 27). This depends on the properties of the incident light and the optical properties and composition of the tissue. The heterogeneity of the tissue and the biomolecules limit the penetration of light. The most abundant chromophores in our tissues are hemoglobin ($Hb(H_2O)$), oxyhemoglobin ($Hb(O_2)$), melanin, and cytochromes [278]. As shown in Figure 28, the absorption spectra of different chromophores define the optimal absorption range which PDT could be, called as 'Optical therapeutic window', which is from 600 nm to 900 nm. However, the light beyond 850nm does not have sufficient energy to activate the PS to its triplet state and generate singlet oxygen [277], [279]. Thus, for the majority of PDT applications, the wavelength range lies

between 620 nm to 850 nm, thereby allowing optimal light penetration and PS activation [277]. Besides, different tissues have different homogeneity and structural components which can limit the penetration of light. Generally, light fluence through a tissue decreases exponentially as the distance increases. Overall, the effective penetration depth is inversely proportional to the effective attenuation coefficient (how easily a light beam travels through a volume of material) [280].

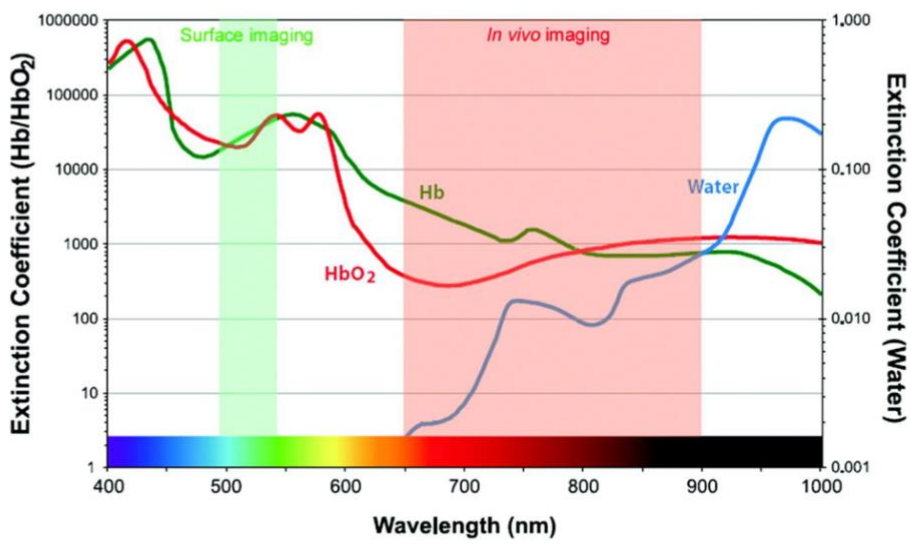

Figure 30: *Illustration highlighting the absorption spectrum of key endogenous tissue chromophores, and thus demarcating the in-vivo optical window for PDT. [281]*

As for the light, the attenuation coefficient, photon energy, power density, and exposition time are the factors that determine its properties. Photon energy is the energy carried by a single photon and is inversely proportional to the wavelength of the light. It is denoted in joules (J). The number of photons falling per unit area denotes the power, or fluence, and is denoted by J/cm^2. And the fluence per unit time is called fluence rate, or power density, and is denoted by $J/s/cm^2$ or W/cm^2. Exposition time is the duration for which the target site was subjected to illumination. All these factors together decide the illumination protocol, where varying fluence, fluence rate, and exposition time can define a high or low dose of illumination [282]. Also, the PS itself can limit the light penetration where the PS at the periphery would shield the core of the target site and inhibit the photodynamic effect. This process is called PS self-shielding. Furthermore, photobleaching also affects the overall efficacy of the therapy, where the PS will lose its photosensitizing ability upon interaction with light. However, this would also inhibit PS self-shielding, and therefore the light can

penetrate deeper into the tissue [280], [282]. Additionally, light fractionation, or splitting the light dose with single or multiple dark pauses, can also play a critical role [283]. The precise role and function of fractionation are still unknown but it is speculated to be the combined effect of re-oxygenation and reduction of PS self-shielding [284]. Some studies have highlighted that light fractionation can improve the penetration and overall efficacy of the therapy, while some have rejected the notion [283], [285], [286]. All in all, light while being a critical component of PDT, also makes the therapy more complex and an intricate process.

When the light falls on a tissue and excites the PS, there can be either radiative decay or non-radiative decay. For the former, there is no intersystem crossing, and the excited PS directly goes back to its stable state by radiating photons to give fluorescence, as discussed previously (Figure 14). This phenomenon is used for photo-diagnosis. For the latter, the electron will not radiate photons but transfer energy either to nearby atoms by intersystem crossing or to nearby surroundings in form of heat by internal conversion. This thereby gives three types of interactions with the tissue possible: photochemical, thermal, and electromechanical. Since this phenomenon has been previously discussed, we will now proceed directly to the role of light in this and different phototherapies including PDT.

a. Different types of light-mediated therapies and diagnostic procedures

i. Photothermolysis

In absence of any exogenously administered PSs, irradiation in the range of 380 nm to 1000 nm can alter the function and structure of some cellular components and biomolecules [277]. It can activate or inactivate enzymes, for example, UV radiations causing DNA (Deoxyribonucleic Acid) damage might induce, activate and enhance DNA repair enzymes. Similarly, such illuminations can induce bio-stimulation where the light is absorbed by various chromophores of the respiratory chain (like cytochromes, Nicotinamide Adenine Dinucleotide Phosphate (NADPH), and other enzymes) to thereby cause changes in the redox status of the mitochondria. This leads to increased ATP generation, the Ca^{2+} ion influx, and ultimately increased DNA and RNA synthesis to modulate cellular proliferation [287], [288]. This can further be implicated in the induction of electron transfer, changes in protein folding, ROS generation, or a heating effect [277], [288]. Low-energy and low-intensity optical irradiation of non-specialized endogenous photoreceptors can induce a highly localized hyperthermal

effect to damage the tissue, which is known as photothermolysis. Unlike PDT, this type of cellular destruction relies on endogenous levels of chromophores like heme, melanin, hemoglobin, water, cytochromes. The radiation-less decay of these excited chromophores leads to heat evolution and ultimately the damage to the surrounding target area[277].

The thermal destruction of the target site requires sufficient deposition of heat energy into the absorbing area followed by heat dissipation into the nearby surroundings. The heating effect decreases with the distance, which is in term dependent upon the absorption coefficient of the chromophore and heat transmission coefficient between the absorbing area and the intervening tissue. However, at high temperatures, there is an energy loss due to bleaching, melting, boiling, and bubble formation. Also, to induce more permanent tissue destruction, the temperature has to be above a certain limit. This gives us a range within which the temperature of the tissue should be, in order to induce a more lasting effect with minimal energy loss and negative effects [289]. Using this information, along with the visible-range absorption band of the targeted chromophore, clinicians can selectively target the small tissue section thereby giving a precision-based approach. Huang *et al.* demonstrated that a tightly focused NIR laser beam can precisely target single blood vessel closure by spatially selective photothermolysis. They pulsed a super-high-power density laser for femtoseconds, which caused vessel closure by the heat generated. With the process, they can get higher light penetration, with lesser side-effects to the surroundings due to the NIR used where the power in the surrounding tissues would be insufficient to induce damage [290].

ii. Photodynamic Diagnosis (PDD)

As described previously, exogenously administered PS can emit photons upon excitation by light of sufficient wavelength and energy, by a process called internal crossing. Here, the light emitted by the release of photons is called fluorescence. In clinical practices, there are five aims for fluorescence-based diagnosis: (1) detection of pre-cancerous lesions; (2) detection of early-stage neoplasms; (3) detection of recurrences; (4) monitoring the therapy; (5) exclusion of neoplastic diseases. PDD is similar to fluorescence-guided surgery, where PDD provide increased intensity of fluorescence and duration along with cytotoxic effect due to PDT. PDD can be performed either by using the endogenous molecules (where it is described as autofluorescence diagnosis) or exogenous fluorophores. The method of

autofluorescence diagnosis is not very sensitive and effective in diagnosing small changes. Red fluorescence may be used for tumor tissues, in contrast to green for normal tissue. Various endoscopy techniques use red-to-green fluorescence ratio for the detection of cancerous lesions, where blue light is used to detect both green and red autofluorescence [291].

On the other hand, administration of exogenous fluorophores or PSs can yield better results in terms of fluorescent signal, visualization, lesser background autofluorescence, along easy detection and analysis techniques [291]. Additionally, these exogenous PSs are very well studied, and hence their excitation and absorption spectra are well known, which further increases their efficiency. However, their tissue selective accumulation, mode of administration, excretion, impact of patients, and cost makes them underutilized [291], [292]. But since most of the PSs used for PDD can be utilized for PDT as well, due to which they either get photo-degraded, photo-bleached, or metabolized, their safety is thereby increased.

By measuring fluorescence intensity, numerical color value red-to-green color intensity, and three-dimensional imaging we can observe the accumulation of PS, for example PpIX, in the target site which further helps in the identification and localization of the malignant and benign neoplasms. PDD, thus, aids in imaging and detection of pre-malignant or micro-neoplastic nodules which can't be visualized by white light [292]. Besides, PDD can be useful for medical institutes which are not equipped with intra-operative MRI facilities, and thus providing cost-effective substitutes for surgeons [293]. Various clinical studies have validated the use of 5-ALA and other porphyrins-mediated PDD for various cancer in comparison with traditional white light cytoscopy [217], [293]–[303]. New PSs are also being developed which are excited at a NIR wavelength for deeper tissue penetration.

Besides, there is another exogenous PSs mediated thermal therapy, called Photothermal therapy (PTT). However, it remains out of context for our current study, hence we shall not discuss it here.

b. Light Sources

An ideal light source will be the one that can photo-activate the PS, provide sufficient tissue penetration, is easy and flexible in clinical use especially for internal application, have

no risk of pigmentation, and with minimal erythema [304]. The selection of a light source for the PDT depends on two key factors: (1) the absorption spectrum of the PS; and (2) the wavelength and fluence of the light. A typical absorption spectrum of porphyrins, the most common PSs, has an intense and narrow peak, which is called the Soret band. This peak is typically at around 400 nm and arises due to the transition of the electrons from the ground state to a higher energy level. But this is followed by a series of smaller longer wavelengths with weaker absorptions, called Q-bands. These bands originate due to weaker excitation energy and thus the electrons are excited to lower energy levels [238]. Based on this, the optimal photo-excitation wavelength of the PS is determined. At the Soret band, the PS has high absorption but it has lesser tissue penetrating capability. However, for Q-bands, we obtain lesser absorption at the red light and higher tissue penetration, thereby giving a higher quantum yield [304].

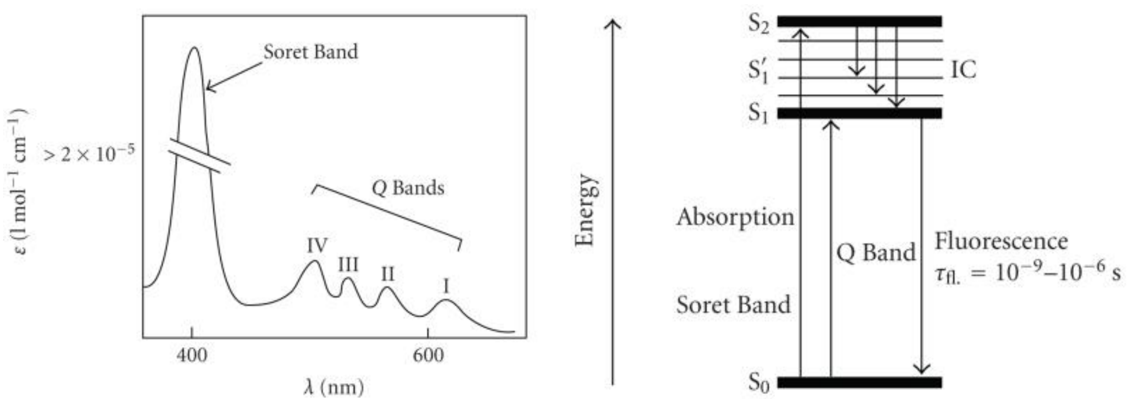

Figure 31: Absorption spectrum of a porphyrin (Left); Jablonski diagram highlighting the Soret and Q bands (Right). [238]

In the early days of PDT, a broad-spectrum light source was used in combination with systemic administration of the PS. Light sources such as quartz lamp, xenon arc lamp, slide projectors, halogen lamps, and other non-coherent sources were used, which emitted light at a broad range of wavelength, instead of a specific wavelength corresponding to the PS [304]. Later on, light sources fitted with red filters were employed to emit light in the red range of the spectrum [194], [304]. But since PDT was most often used to treat superficial skin malignancy, especially Actinic Keratosis, hence the therapy was approved by US-FDA by using blue light and 5-ALA providing a more superficial effect than deeper light penetration [305]. Interestingly, MLA mediated PDT has shown improved efficacy with a red light at various clinical sets ups and hence suggesting that both red and blue lights can be effectively

used for the PDT treatment [304]. The selection of blue or red-light largely depends on the type of malignancy and its nature.

However, with the advent of laser for clinical applications, the illumination system for PDT developed immensely. Lasers were employed for PDT in the 1990s, predominantly of red wavelength. Later, with the development of blue light-emitting diodes-based illumination systems, which was awarded the Nobel Prize for Physics in 2014, was also employed for PDT [306], [307]. Due to lasers, PDT could now have increased tissue penetration depth, PS specific wavelength which could limit the side-reactions, higher flexibility in terms of fluence and fluence rates, along with the ability of the laser to be connected with fiber optic systems for treatment of internal tumors [307]. We will now briefly discuss the different laser systems available for PDT.

i. Argon/dye lasers

The argon laser uses a noble gas, Argon, as an active medium, emitting light in the blue-green spectrum. A dye laser is then pumped by the argon laser to deliver a continuous wave of 1 to 7 W at 630 nm. This amount of energy by the laser could help to connect the laser with a beam splitter for fiber optics, thus helpful for illumination of a wider area with uniform energy [307]–[310].

ii. KTP:YAG/dye lasers

A KTP/YAG laser operates at 1064 nm and 532 nm during the frequency-doubled phase. With the help of a dye laser head, the wavelength could then be set to 630 nm with a power of 3.5 W and 7 W. This system produces pulsed light instead of continuous light of argon/dye lasers. However, this could lead to different photobiological impacts due to different laser-tissue interactions. The later studies confirmed that the impact was minimal or not much different from the continuous laser systems [309], [310].

iii. Gold vapor lasers

A variant of the argon/dye laser system, this laser system is of the category of metal vapor lasers. The gold vapor laser is particularly of interest since it can generate a 627.8 nm wavelength laser, without using any dye laser coupling. Additionally, this laser is portable and do not need specialized electrical supply or water cooling. However, the gold charges required

to maintain the laser output are essential, and thus make the therapy expensive [309]. Additionally, this laser produces pulsed illumination, which as previously described, can have varying impacts.

It is well known that the delivery of PSs activating light to the target tissue is critical as it dictates the overall efficacy and healthy tissue toxicity. An efficient light delivery system imparts flexibility in usage along with selective, adequate, and uniform illumination of the target site, with minimal or low transmission loss of energy, and determines the actual fluence by the laser system [309]. Dupont *et al.* developed a novel light delivery system for intra-operative PDT protocol. Here a single-use balloon is inserted into a trocar, which is usually used during endoscopic operations to separate the tissues in the extraperitoneal space. This assembly is then connected to a sterilizable optical fiber guide which ensures positioning of the optical fiber at the center of the balloon and helping in permeability. When inserted inside the patient cavity, the balloon is filled with a low absorption emulsion so that the balloon reaches the brain-patient boundary. At last, an optical fiber is inserted in the optical guide for illumination. They tested this system for glioblastoma treatment in order to achieve a therapeutic fluence rate by 5-ALA mediated PDT in the patient cavity and its surrounding area [311]. A clinical study is also under process (clinical trial: NCT03048240). Similar approaches have been reported by other studies for PDT and light dosimetry by using different PSs and protocols for internal tumors [312]–[314].

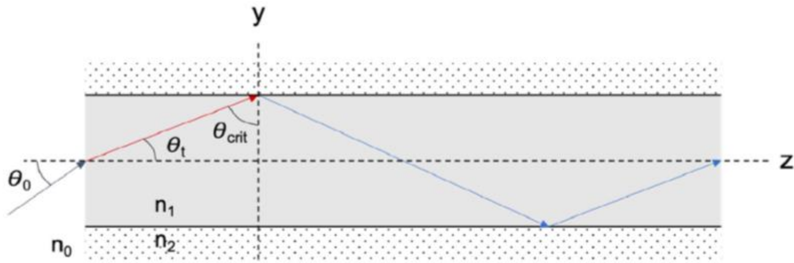

Figure 32: *Light Propagation in an optical fiber. [196]*

iv. Optic fiber-based light delivery systems

Optic fiber-based light delivery system has been widely used for PDT. Optical fibers are flexible transparent fibers that allow incident light from an optical source to be transmitted by a series of total internal reflections with minimum loss. When these optical fibers can be knitted or woven into a flat structure like conventional yarn, thus we can obtain

homogenous illumination over a flat surface [196]. Thecua *et al*. developed knitted light-emitting fabric technology, where the optical fibers were knitted into a fabric. They reduced macro bending and yarn tensions to give homogenous illumination over the surface. The two sides of the fabric were then connected with two beam expanders, from which a laser source injected light thus reducing side emission [196], [315]. This fabric system was thereby used to developed different *in-vitro* and *in-vivo* illumination sets which demonstrated the effectiveness of the illumination system [196], [316], [317]. Vignion-Dewalle *et al.* used this light-emitting fabric system in a randomized, controlled, multicenter study for the treatment of AK by MAL PDT. During the study, they applied MAL cream under a transparent occlusive dressing and used the light-emitting fabric lining inside a cap for illumination, with a 30-minute drug-to-illumination period followed by 2.5 hr of photo-activation at 37 J/cm^2 fluence with an irradiance of 12.3 mW/cm^2. They demonstrated that this new protocol is as effective as classical PDT protocol at 3 months post-treatment and imparts superior tolerability than the latter [308], [309]. Further, Thecua *et al.* used light-emitting fabric technology to develop a flexible, flat tissue to provide diffused illumination for the treatment of Paget's disease by MAL PDT, following the above-mentioned treatment protocol [196], [320], [321].

c. Light Dosimetry

In-situ measurement and control of light fluence is an important aspect during the PDT protocol, as it ensures homogenous illumination and proper targeting of the tissue. While the illumination of a broad flat surface is easy, and hence easy to detect and measure, such conditions are barely available in clinical practice. The fluence rate depends on the distance to the surface and thus can be varying. The net fluence (*i.e.,* the sum of the scattered and non-scattered incident lights) at the target site might differ due to the light scattering by the tissue and the body fluids. This scattering may differ from organ to organ and possibly resulting from variations caused by rapid muscular contractions, squeezing blood out of the tissue, and changes in the blood volume due to treatment response [322]. Because of this scattered light, the probability of activating a PS can increase. Studies have reported an increase in the fluence at least by a factor of 2, which can cause unwanted side-effects due to over-illumination and unnecessary discomfort to the patient during the treatment [323].

Optic fiber light detectors are employed for the reliable data of light fluence and uniformity. Primarily, two methods for light dosimetry are utilized for PDT. The first method is by linear diffusor, where a fluorescent probe is attached with the optic fiber and then inserted by a needle to measure fluence, as a function of penetration. This is an invasive method. An alternative non-invasive method is based on quantitative reflectance spectrophotometry where the reflected light intensity of the illuminated spot is measured as the function of the radial distance along the surface [324]. D'Hallewin *et al.* demonstrated that with proper light dosimetry, they could measure the fluence *in-situ* during Photofrin® PDT of patients with bladder cancer, due to which all patients received the same light dose, thus getting the maximum anti-tumor response and with minimal side-effects [325]. For hollow cavities, elastic scattering adds to the total dose received, and thus dose should be determined by actual fluence rate measurement inside the cavity. For interstitial PDT, hollow needles are employed with multiple fibers in the tissue to help in illumination and in real-time measurement of the fluence, which could be adjusted according to the variations in the tissue [322].

d. Two-Photon PDT

Two-photon absorption (2-PA) is an optical process in which two photons are simultaneously absorbed and thus their combined energy is used for photo-excitation of the molecule. Here, the combined energy of the two photons will correspond to the energy of a single photon used to excite the molecule at normal light. But due to the lower energy of the photons, wavelengths of NIR can be used to excite PSs which has excitation in the visible spectrum of the light. This thereby provides higher tissue penetration potential for the light to be used. Furthermore, 2-PA occurs at a high intensity of light thus the treatment zone can be highly focused and confined in a smaller volume than the traditional illumination methods. Due to recent developments in 2-PA associated PDT, different PSs which were previously excluded from traditional one-photon absorption (1-PA) PDT, were being re-evaluated [326], [327].

The first study using 2-PA mediated PDT was published by Andreoni *et al.*, where they demonstrated the efficiency of 2-PA PDT over 1-PA PDT by using HpD and a pulsed laser [328]. Later studies showed that 2-PA successfully induced fluorescence of HpD at 1064 nm. Further,

different PSs had been tested for 2-PA based PDT, namely Photofrin®, pheophorbide, phthalocyanine, PpIX, Hypocrellin A, and B. However, these classical PSs have low efficiency for 2-PA PDT [326]. Hence newer conjugates or compounds are being developed which can have higher $^1O_2$ generation capabilities with 2-PA resonance wavelength in the therapeutic window. Since 2-PA can be highly focused, Collins *et al.* suggested 2-PA mediated PDT for targeted closure of blood vessels of cancers and other organs. They used a verteporfin-based new PS for 2-PA PDT to target closure of blood vessels of diameter 40±5 µm of an *in-vivo* model with 920 nm light, thus highlighting new possible applications of PDT [329].

e. Daylight PDT

In dermatology, classical PDT is an indoor protocol where the patients are kept in occlusion with a light-proof dressing for 3 to 4 hr after a topical application of 5-ALA or MAL, followed by a short illumination period for PpIX photo-activation [192]. Despite a good therapeutic outcome, this protocol results in pain, stinging, and burning sensation during the illumination [330]. Alongside, longer clinic visits for patients and the occlusion procedures are some major setbacks for the therapy.

Daylight PDT (D-PDT) is an effective alternative to the classical PDT where the treatment zone is exposed to natural daylight instead of an artificial light source [198], [331], [332]. Daylight or sunlight contains the entire spectrum of electromagnetic radiations with very high intensity. Of the visible spectrum of light, a range of 400 to 700 nm is efficient for photo-activation of PpIX, as discussed previously. Due to blue light, 87% of the PpIX is activated, while with red light a deeper light penetration is obtained. Therefore, by using daylight as an illumination source, we could obtain similar results as with classical PDT [333]. Further, D-PDT does not require a special light source, provides greater practicality and easy application, with lesser treatment cost. D-PDT offers a shorter drug-to-light period of 30 minutes followed by a longer illumination period of approximately 2 to 2.5 hr, which allows a more continuous PpIX production. This continuous slow PpIX activation with red and blue lights is probably the reason for higher tolerability by patients during D-PDT [334], [335]. Several studies have concluded that D-PDT is as effective as classical PDT protocol for the treatment of Actinic Keratosis, for short- and long-term follow-up [331], [334]. Further, during a clear sunny day, 2 hr of daylight exposure is sufficient to induce an effective response

against thin Actinic Kertosis to provide a minimum light dose of 3 J/cm^2 [336]. And the weather and latitudes play no role in determining the efficacy of D-PDT, however, clinicians recommended not to perform D-PDT during rainy weather [331], [334]–[336].

Some standard treatment protocols and guidelines for D-PDT are as follows [198], [334], [337]:

1. The treatment zone should be cleaned, prior to application of topical PS, and a sunscreen of Sun Protection Factor (SPF) 30+ should be applied to all sun-exposed areas. The sunscreen products with physical filters like zinc oxide, iron oxide, and titanium dioxide, should be avoided in order to allow effective light penetration. Sunscreen is recommended 15 minutes before pre-treatment.
2. A pre-treatment with micro-needling, curettage or chemical peels can be done prior to application of PS, in order to remove scales and crust and roughen the skin surface for increased cream penetration.
3. A thin layer of the MAL cream or 5-ALA nano-emulsion should be applied to the treatment area for 30 minutes. The usage of 5-ALA or MAL is varying in different countries.
4. The patients should keep the treatment zone exposed to daylight for 2 hr and avoid shade during the illumination period. The PS cream should be washed off at the end of the period.
5. Regular monitoring for at least up to 3 months with regular moisturizing of the treatment zone is highly recommended. Repeat sessions can be necessary, as per the follow-up.

However, the treatment has its shortcomings. Even though there is sufficient light dose for most of the locations, the ambient temperature for the recommended procedure can be too low, *i.e.,* 12-18°C. This can be a limiting factor, especially at the start of the year for the northern hemisphere. Further, changing weather conditions can be tough to accommodate with patient appointments. Hence, Mordon *et al.* recommend artificial or simulated light sources with the standard D-PDT protocol as an alternative [338].

3. The Oxygen

As discussed previously, PDT-mediated tumor destruction is arbitrated by energy transfer from photo-activated PS to $^3O_2$, thereby producing ROS to activate cell death pathways. Even though the PS and light play an important role in efficacy, the overall therapeutic output of PDT against the malady is determined most likely by its potential to yield ROS from the intracellular oxygen. For most PSs used in PDT, with the exception of some cyanine dyes, PDT is strongly oxygen dependent. Studies have demonstrated that the PDT mediated cytotoxic effect is reduced during hypoxic conditions, while anoxic conditions can totally eliminate it, and thereby the cells attain resistance against the therapy [339]–[344]. Intratumoral hypoxia can be of two types: diffusion-limited and perfusion-limited hypoxia. In diffusion-limited hypoxia, the available oxygen is consumed by rapidly proliferating tumoral cells at the periphery, while the latter is caused by temporary obstruction, lower blood flow rate, and high interstitial blood pressure in the tumor vessels [345]. In any case, the efficacy of the PDT is widely affected by the Spatio-temporal distribution of oxygen in the tumor periphery and the core. Furthermore, during PDT, both the consumption of oxygen as well as vasculature shutdown can lead to depletion of molecular oxygen, thus causing insufficient tumor destruction. The damaged tumor vasculature, however, in combination with hypoxic or anoxic conditions may lead to favorable long-term response [344]. Thus, intracellular oxygen is an absolute requirement for photo-inactivation of the cells, and an important parameter determining the resistance against the treatment.

During PDT, the tissue oxygenation of the target site changes. It is done in three phases [346]:

1. During the first phase of PDT, there is a dynamic and reversible depletion in the levels of oxygen which is proportional to the fluence rate. This process is initiated due to the photo-oxidation reactions by PDT. At this stage, since there is no change in blood flow, the variations in the levels of oxygen are indicative of oxygen consumption by PDT and ROS formation.

2. In the subsequent phase, there is an occlusion of the blood vessels and an increase in hypoxic conditions due to oxygen-induced physiological alterations. At a low fluence rate, this phase is reversible.

3. Ultimately, there is an irreversible vascular collapse and depletion of oxygen levels along with the initiation of cell death processes. However, some studies using *in-vivo* model have stated that there is a transient reoxygenation suggesting that some tumor vasculature was preserved during the treatment [347].

We will now discuss the key intermediate formed after the illumination, *i.e.,* ROS.

a. Reactive Oxygen Species

ROS are highly reactive partially reduced or activated form of molecular oxygen, formed due to oxygen's higher electron acceptability. Some examples of ROS are superoxide ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), peroxide (O_2^{-2}), hydroxyl radical ($OH^{\bullet}$) [167]. $^1O_2$ and $OH^{\bullet}$ can react with organic substrates to form intermediate substrates capable to generate more ROS (explained later). ROS are considered an unavoidable byproduct of aerobic metabolism. As discussed previously, ROS accumulation in cells can lead to oxidative damage to lipid membranes, proteins, RNA, DNA, along with enzyme deactivation and oxidative stress [168]. They cause damage to proteins by targeting amino acids like histidine, tryptophan, methionine, cysteine, and tyrosine. Guanidine is the target of nucleic acids. They can also oxidize the unsaturated fatty acids and cholesterol [348].

All ROS reactions have a primary reaction to produce peroxide, which then causes a chain of auto-oxidation reactions to produce more oxidized species [348]. A simple scheme of reactions is described below:

$$X\cdot + RH \rightarrow R\cdot + XH \quad \text{(initiation)}$$

$$\left.\begin{array}{l} R\cdot + O_2 \rightarrow RO_2\cdot \\ RO_2\cdot + RH \rightarrow ROOH + R\cdot \end{array}\right\} \quad \text{(propagation/ auto-oxidation)}$$

Figure 33: *List of reactions to produce ROS; X: Substrate; R: Oxygen Radicals. [348]*

By these reactions, oxygen radicals like peroxy, alkoxy, and hydroxyl, along with $^1O_2$ and $O_2^{\bullet-}$ are formed. The lifetime of these species is quite varying, depending on their reactivity. $OH^{\bullet}$ are quite reactive, due to which their life is short and their diffusion is limited. $RO^{\bullet}$ are less reactive, hence diffuse further, followed by $RO_2^{\bullet}$ radicals. $^1O_2$ can traverse farther

and is capable to diffuse into the cell membrane. $O_2^{\bullet-}$ are less reactive and can diffuse long distances [348].

The main intracellular sources of ROS are mitochondria, NADPH oxidase, cytochrome P450, ER, lysosomes, peroxisomes, and chloroplasts [168], [349], [350]. External stimuli like UV and ionizing radiations, TNF-α, IL-1β, hypoxia, and various xenobiotics are the exogenous sources of ROS production [349]. Living organisms, however, have developed pathways to mitigate the toxic effects of ROS, primarily by eliminating them from the cells. A different class of enzymes, like SOD, catalase, peroxidases, and peroxiredoxins, can catalyze ROS to yield more stable and lesser toxic products. Plants have molecules like carotenoids, tacopherols, and plastoquinones, which can quench $^1O_2$, formed during photosynthesis [351]. Other molecules, known as anti-oxidants, are ascorbic acid, uric acid, melatonin, and glutathione [168], [350].

Redox equilibrium is crucial for cells physiology. While the excessive accumulation of ROS can lead to oxidative stress and cell death, lower levels of ROS are beneficial for cells as they act as redox signaling messengers required for various pathways. ROS activate NRF2/KEAP1 (nuclear factor erythroid 2 (NF-E2)-related factor 2/Kelch-like ECH-associated protein 1) pathway, by modification of cysteine residues of KEAP1, which disrupts KEAP1-dependent NRF2 degradation and its cytoprotective function [349]. ROS also influence the activation of the NF-κB signaling pathway. NF-κB is a crucial transcription factor responsible for a wide range of actions like cell adhesion, cell death, immune response, and inflammation [352]. ROS affect both the canonical and non-canonical pathways of NF-κB activation, by causing the phosphorylation or ubiquitination of the intermediate molecules, ultimately causing NF-κB inactivation or decreased function. In terms, the NF-κB pathway activates the expression of antioxidant enzymes and molecules to reduce the cellular levels of ROS. ROS activate PI3K to amplify the downstream signaling and also inhibit the negative regulators of the pathway thus leading to cell cycle progression, proliferation, protein synthesis, and response to growth factors. ROS also influence MAPK signaling cascades, which altogether are responsible for cell growth, proliferation, survival, and death. ROS activate the various receptor, protein phosphorylation, oxidize enzymes, and triggers protein detachment for MAPK pathway. For the PI3K-Akt pathway, ROS amplify downstream signaling and inactivate the inhibitors of the pathway. Similarly, ROS are associated with regulation with a lot of other

signal transduction mechanisms, hence highlighting their pivotal role in cellular physiology and pathology [350], [353].

For cancer, ROS are considered oncogenic where they can arbitrate tumorigenesis, tumor growth, invasiveness, and metastasis. The levels of ROS in cancer cells are normally elevated due to internal and external alterations [350]. Carcinogens like UV radiation and smoking disturb the intricate balance of ROS levels in the cells [354], [355]. The increased metabolism instigated by cancer results in further increased levels of ROS. Other oncogenes like *Kras, c-myc,* and BRCA1 (Breast Cancer Type 1) also increase ROS concentrations [356]. An accelerated glycolysis (Warburg effect) and Pentose Phosphate Pathway (PPP) in cancer cells could decrease dependence on mitochondrial oxidation and increase levels of anti-oxidants (like NADPH and glutathione) thereby reducing levels of ROS. This effect is augmented during oxidative stress [357]. A persistent oxidative stress would result in a metabolic shift from glycolysis to PPP, which can favor cancer cell de-differentiation into Cancer Stem Cells (CSCs) [358]. The prime target of ROS for tumor initiation is DNA damage where it can assist in modification of 8-Oxo-2'-deoxyguanosine (8-OdG) DNA base thus causing mutation [359]. Myeloid cell-derived ROS can induce epithelial mutation to initiate tumor development, thus highlighting the role of increased oxidative stress in chronic inflammation [360]. ROS also activate or enhance the accumulation of CAFs. These CAFs play an important role for EMT by ROS-dependent expression of genes like cyclooxygenase-2 (COX-2), Hypoxia-Inducible Factor-1 (HIF-1), and NF-κB, thus assisting in metastasis. Similarly, TAMs induce ROS generation, by activation of NOX-2 (NADPH Oxidase 2) and Nitric Oxide Synthase (NOS), promote tumorigenesis by recruiting CAFs and MMP activation. These TAMs also secrete pro-inflammatory factors to create a pro-tumoral immune-suppressed microenvironment [361]. Finally, ROS have been shown to induce anoikis resistance to cancer cells. Anoikis is a programmed cell death that occurs in anchorage-dependent cells when they lose matrix attachment. Hence, ROS promote uncoupling of the cancer cells and thereby enabling metastasis [356].

However, ROS can act as anti-tumoral as well, where the increased genetic instability of cancer cells can trigger cell death or senescence [362]. This effect is highly dependent on the ROS concentration. Most of the pro-tumoral adaptive strategies by cancer are indeed anti-oxidant responses by normal cells, thus an anti-oxidant therapy may result in tumor

aggressiveness. Studies have demonstrated that anti-oxidant therapy, which decreases ROS levels, can result in poor prognosis and decreased survival since the therapy disrupts the cancer-suppressive levels of ROS due to which the cancer cells do not undergo cell death mechanism [363]. This can lay an assumption that an increased short-term oxidative stress can in term lead to tumor destruction.

b. Oxygen Dosimetry

There are oxygen concentration gradients within the single cells, particularly near the mitochondria which are the center of cellular respiration. Similarly, for a tissue, there can be variations in oxygen concentration over small distances based on the biological activity and metabolism, overall oxygen consumption, tissue vascularization, and state of the tissue. This can be further aggravated for advanced solid tumors, where hypoxic conditions and heterogeneity of oxygenation are quite prevalent. Such heterogeneous distribution of oxygen can, thus, render PDT ineffective. Furthermore, the PDT process itself will consume the oxygen, which will thereby inhibit its further effectiveness. Hence strategies for tumor oxygenation and real-time oxygen level measurements are important. The spatial and temporal profiling of oxygen concentration in the tumor during and post-illumination can help us to understand the post-treatment tumor response to the therapy.

Most of the tissue profiling for oxygen levels is done by monitoring the hemoglobin saturation and blood flow. To correlate the levels of oxyhemoglobin with cellular oxygen levels, a mathematical model of oxygen diffusion and consumption is required. Incorporating different factors that affect tissue oxygenation, these mathematical models were developed to help visualize the radial distribution of oxygen as a function of distance from the capillary. They can predict distance-dependent oxygen depletion from an isolated capillary, and thus showed the theoretical aspect of the impact of high fluence rates on oxygen depletion during PDT [364]. Such models, along with a real-time feedback system could help us to develop a more flexible illumination protocol tailored according to the prevailing conditions of the patient. Hence, a number of methods has been developed to monitor tissue oxygenation during PDT. Here we discuss some widely used and tested methods for oxygen dosimetry used during clinical PDT protocols.

i. Polarography

Oxygen polarography is a direct method for the detection of tissue oxygenation and is based on Clark's cell principle. A clark cell is an amperometric cell (*i.e.,* detection of ions in the solution by changes in electric current) that is polarized at around -650 mV. Here, the detection sensor is a platinum electrode consisting of a gas-permeable membrane through which oxygen diffuses into the measurement chamber containing potassium chloride solution. The detection sensor is tightly attached to the skin surface, while the other electrode is made of silver or silver chloride, and electric current flows between the two electrodes to measure the level of diffused oxygen [365].

Some key limitations of this method are oxygen consumption by the electrodes, low sensitivity, constant movement of the probe for measurement, variation in oxygen consumption at different electrode application sides, and insufficient detection of ischemia caused by chronic peripheral arterial obstruction [346].

ii. Magnetic Resonance Oximetry

Due to two unpaired electrons in the anti-bonding orbitals of $^3O_2$, its ground state is a triplet state, which imparts inertness to molecular oxygen and its paramagnetic properties. Thus, oxygen yields strong magnetic resonance at the gas phase. From a physiological point of view, this bestows paramagnetic nature to oxyhemoglobin, while deoxyhemoglobin is diamagnetic. This change in magnetic resonance can be detected by Blood Oxygenation Level Dependent (BOLD) functional Magnetic Resonance Imaging (fMRI) [366]. This gives a non-invasive and non-destructive method for the detection and localization of oxygen in the tissue. However, MRI scanners employed for the purpose are expensive and tough to mobilize. This approach has been demonstrated to monitor oxygen consumption during PDT by using *in-vivo* models, where the oxygen levels dropped to 75% during illumination and remained stable at approximately 60% of the pre-treatment value when checked 20 minutes post-illumination [365]. This highlights the applicability of such methods for monitoring oxygen levels during PDT, especially for intra-operative protocols.

Besides this, paramagnetic probes can also be used for oximetry through their electron paramagnetic resonance spectrum. Here, the paramagnetic probe quenches the other paramagnetic species for effective electron paramagnetic resonance oximetry. Electron

paramagnetic resonance is the resonant absorption of microwaves by paramagnetic particles in presence of a static magnetic field. Such probes are inert in nature and can be implanted in the tissue, where they could be removed later. They provide higher sensitivity and thus could be more useful for monitoring variations in oxygen levels. Some of the electron paramagnetic resonance probes are lithium phthalocyanine, nitroxide spin probes, and perfluorocarbon (19F) [346], [365]. Lithium phthalocyanine has been used for online tissue oxygen monitoring for verteporfin-mediated PDT of *in-vivo* fibrosarcoma [367].

iii. Fluorescence and Phosphorescence based Oximetry

Oxygen in biological systems can be used to quench different injected fluorochromes or phosphors for tissue oximetry. Usually, they are suitable for surface detection of oxygen levels, with a penetration depth of roughly 1 mm. Luminescence quenching-based probes have been used for such probes are injected intravenously in order to measure the microvascular oxygen pressure. Short pulses of blue light excitation by a fiber optic probe attached with immobilized ruthenium or pyrene-based fluorochrome at the probe tip, can be also be used for oximetry. Such methods, known as dynamic fluorescence quenching, determine oxygen concentration which is inversely proportional to the fluorescence lifetime. There are more sensitive than polarographic methods, do not consume oxygen while measurement, and do not require an injection of the probe. However, the detection probes used here have to be inserted inside the tissue, and mechanical damage or physical changes could influence the measurements [346], [365].

Phosphorescence quenching-based spatial oxygen measurement was performed on normal rat liver during 5-ALA PDT. Dynamic fluorescence quenching-based method was evaluated while hypericin and bacteriochlorophyll-serine mediated PDT for different tumor models. The studies validated the applicability of this approach [365].

iv. Spectroscopy based Oximetry

NIR spectroscopy allows the measurement of total hemoglobin concentration and blood oxygen saturation. Diffuse NIR can penetrate the tissues where its spectroscopic properties can be used for the detection of chromophores like hemoglobin while fluctuations in its temporal intensity can be used for blood flow detection. This is called Diffuse Correlation Spectroscopy (DCS). DCS is a non-invasive, deep tissue penetrating, method for temporal

oximetry which can be used at the patient bedside. DCS can be combined with Diffuse Reflectance Spectroscopy (DRS), another NIR spectroscopic oximetry method, for measurement of tissue blood oxygenation where DCS will measure tissue blood flow [365], [368]. Different studies have evaluated the possibility for the application of a real-time *in situ* monitoring of the oxygen levels by using diffuse light spectroscopy [369], [370].

c. *Re-oxygenation Strategies during PDT*

Since oxygen plays such a critical role in the success of a PDT protocol, it becomes necessary that such protocols must incorporate steps to ensure proper tissue oxygenation monitoring and re-oxygenation strategies. Hence, the above-mentioned real-time oxygen dosimetry methods coupled with steps that couple either decrease oxygen depletion or increase oxygenation rate while illumination could be helpful for clinicians. Here, we are discussing some widely studied re-oxygenation strategies which have resulted in improved efficacy of PDT. Before we proceed, it is important to note that as soon as the tumor microvasculature is destroyed directly or indirectly by PDT, the cycle of oxygen depletion and re-oxygenation would be disrupted, thus such strategies would render ineffective.

i. Low Fluence Rate

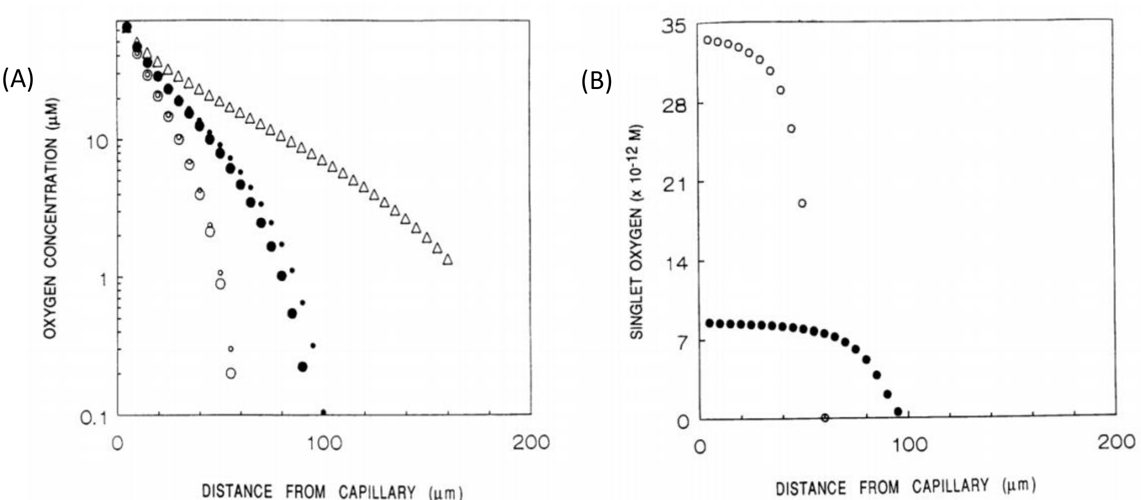

Figure 34: *The effect of tissue oxygenation in the vicinity of an isolated capillary. (A) Radial oxygen concentration (in μM) by two different fluence rates, i.e., 200 mW/cm² (●) and 50 mW/cm² (○) along with metabolic consumption (△). The smaller circles represent the mathematically calculated oxygen concentration using formula derived by the authors; (B) Radial singlet oxygen concentration with two different fluence rates 200 mW/cm² (●) and 50 mW/cm² (○). [361]*

Mathematical modeling has revealed that the rate of oxygen consumption during PDT is critical for its success. By using various *in-vivo* models, studies have demonstrated that the

intensity of photoirradiation influences the degree of tumor response against the PDT [371]. High fluence rates result in oxygen consumption rates that exceed the rate at which the oxygen is resupplied by the vasculature [372]. Thus, increased oxygen consumption during PDT would lead to a decreased radial distance of tissue oxygenation. This added oxygen stress will certainly outclass the regular metabolic consumption but by varying the intensity of irradiation, or fluence rate, the radial diffusion distance of oxygen from the capillary will be affected, as demonstrated in Figure 32. By increasing the fluence rates, the oxygen concentration decreases as a function of distance from the capillary. This further increases the overall $^1O_2$ turnover rate and the distance from the capillary. Decreased fluence rates would result in decreased $^1O_2$ generation, but would increase the radial distance from the vicinity of a capillary [371].

Such studies prove that a high fluence rate PDT could convert a significant volume of tumors into a temporary hypoxic state, which could protect them from PDT action and impart resistance against the therapy. Lower fluence rate also increase the time required to deliver the fixed amount of effective therapeutic dose to the tumor, which can be hypothesized for enhanced effect. Additionally, this would cause delayed toxicity to the microvasculature, which would keep the oxygenation process online, as prompted earlier [371]. This microvasculature would ultimately be destroyed slowly by lower fluence rates than at higher and thus causing thrombosis and hemorrhage due to disruption of microvasculature perfusion [372].

This approach has been employed in metronomic PDT, where the PS and the light are given continuously at very low doses over a prolonged period stretching from hours to days or weeks. The work over this approach is fairly new and hence we would not discuss this any further.

ii. Light Fractionation

Another method to enhance the re-oxygenation process in the tumor core is the administration of light dose with an intermittent short dark period. This is called fractionated light exposure. The dark periods range from 30 to 300 seconds, based on tumor type, the PS utilized, and the fluence rate [283], [373]. Similar approaches have shown increased survival of patients treated by hyper-fractionated radiation therapy for non-small cell lung cancer

[374]. During the dark periods, effective re-oxygenation takes place depending on the hypoxia-induced and microvasculature damaged during the illumination [372], [375]. Additionally, it has been reported that PS might be re-localized or replenished during the dark pause, thereby increasing the effective therapeutic outcome [376]. Using oxygen microelectrodes, Curnow *et al.* demonstrated that light fractionation during 5-ALA PDT on the colon on normal Wistar rats can include a partial recovery in pO_2 levels of the illumination zone when compared with continuous illumination at 1 mm and 3 mm away from the irradiance fiber, as shown in Figure 33 [375].

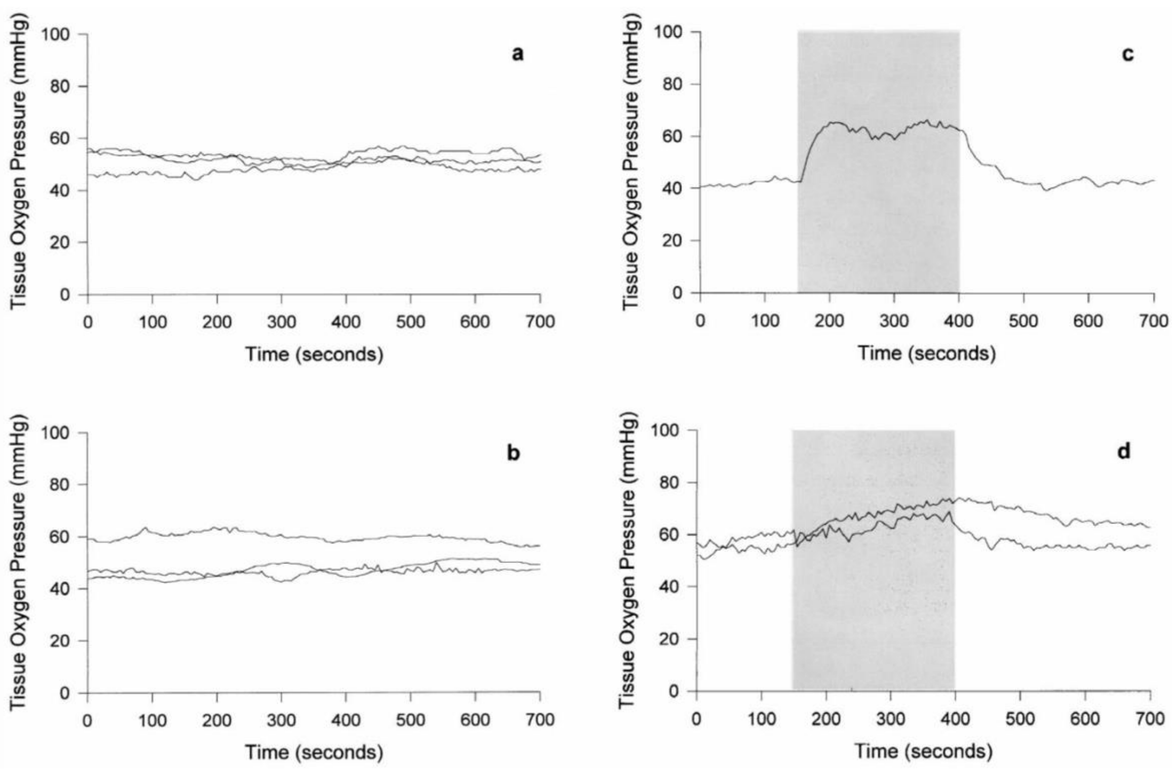

Figure 35: *Tissue oxygen pressure (mmHg) of the normal rat colon plotted as a function of time (s) for groups of three animals receiving (a) neither 5-ALA nor light (surgery only blank controls); (b) 200 mg/kg 5-ALA i.v. 2 hr prior to surgery with no light administration (drug only controls) and (c and d) 25 J of continuous light administration (635 nm, 100 mW) without any 5-ALA (light only controls) and the oxygen measured 1 mm from the irradiation fiber (c) or 3 mm (d). The period of illumination is denoted by the shaded area. [375]*

By mathematical modeling, Foster *et al.* proposed that determining the metabolic oxygen consumption and the average intercapillary spacing in the tumor an optimal fractionation protocol can be devised [371]. In a pre-clinical study involving glioblastoma model treated by 5-ALA PDT with different fractions, Leroy *et al.* demonstrated that the group treated with 5-fractions (167 seconds of illumination and 150 seconds of pause) showed higher apoptosis ratio, peri-tumoral edema, and macrophage infiltration than 2-fraction (167

seconds of the first illumination with 667 seconds of the second illumination interrupted by 150 seconds of pause) at same PDT dose [377]. Some studies for PDT on dermatological diseases have suggested dark periods of 2-24 hr. However, such PDT protocols are not very feasible, as the dermatologists have to accommodate the incoming patients for a longer duration or have patients have to undergo multiple sessions. These limitations will not be very beneficial for the success of PDT in the clinic.

iii. Other Methods

There are some other interesting approaches for re-oxygenation that have not been thoroughly tested and thus will not be discussed here in length.

Weiss *et al.* demonstrated that pre-treatment with VEGF receptor targeting tyrosine kinase inhibitor, axitinib, can successfully induce a transient increase in tumor oxygenation which can slightly improve the anti-tumoral effect of Visudyne®-PDT [378]. Inhalation of hyperbaric oxygen has also been proposed for increasing the level of tumor oxygen, which cannot eliminate the possibility of reduced oxygenation due to vascular damage and potential toxic effects of excessive oxygen [379], [380]. Oxygen-carrying materials like hemoglobin and perfluorocarbons have been used to deliver oxygen but due to limited oxygen binding sites on hemoglobin and lower solubility of perfluorocarbons, the approach has not got much attention. Besides, the incorporation of oxygen-releasing substances is also being studied. These methods use enzymes to convert H_2O_2 of the tumor cells into oxygen. However, it is not sufficient to cause reoxygenation post-PDT [372]. Larue *et al.* have discussed in-depth about various re-oxygenation strategies that could improve PDT efficacy [381].

E. PDT mediated Cell Death

For designing a potent PDT protocol, a thorough understanding of the mechanism of cell death elicited by the therapy is important. The direct cytotoxic impact of PDT induces oxidative stress in key organelles namely mitochondria, lysosomes, and ER. Since the ROS have low diffusibility, the photodamage inflicted is primarily impacted by the localization of PSs as these are the first cellular compartments to observe oxidative stress. The PS localization depends upon the characteristics of the PSs, thus the cellular response and

subcellular targets associated with PDT are different for different PSs. Even the illumination protocol and the target cells can show variation in terms of cellular cytotoxicity.

In any condition, PDT induces direct cytotoxicity by classical cell death mechanisms: apoptosis, necrosis, or autophagy. The ability of PDT to activate multiple cell death pathways overcomes the problem of apoptosis-resistant tumors. The direct cytotoxicity will decide the possibility of an indirect cytotoxic effect. This can be by vasculature shutdown, targeting of other microenvironment components, or induction of an anti-tumoral immune response [279], [382]–[385]. In the upcoming sections, we will discuss both the direct and indirect cytotoxicity induced after the PDT-mediated photodamage. However, we shall not discuss the anti-tumoral immune response by PDT, as it has already been considered in the review we have presented.

1. Direct Cytotoxicity

With increasing attention in PDT, numerous *in-vitro* and *in-vivo* studies have demonstrated the cytotoxicity of PDT and elucidated the mechanism of cell death both at the cellular and molecular levels. Damage to the cellular membrane has been shown. Membrane effects post-PDT have shown increased cellular attachment to the substratum and decreased attachment of the suspended cells, highlighting reduced metastatic capabilities of the PDT treated cells [386]. PDT can damage only a fraction of DNA, which is close to the nuclear membrane since most of the anionic PSs cannot enter the nucleus causing little to no nuclear damage [387]. Hydrophilic PSs can damage the microtubule assembly and thus arrest cells in mitosis to ultimately induce cell death [388]. Besides, the PS accumulating in mitochondria induce apoptotic cell death, while those localized in lysosomes and ER can induce *via* necrosis or apoptosis. Such organelle-specific internalization has been used to develop targeted and selective PSs, like lysosomal photo-chemical internalization.

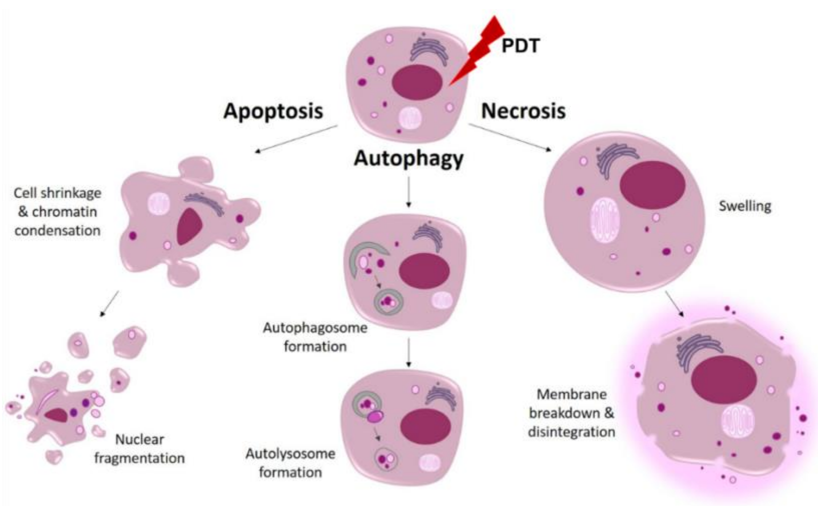

Figure 36: Three key mechanisms of direct cytotoxicity by PDT (apoptosis, necrosis, and autophagy). [389]

At the molecular level, the photo-activated PS generates ROS which activates a complex cascade of signaling pathways causing irreversible damage to vital subcellular compartments. The oxidation of cellular molecules like nucleic acids, proteins, lipids would cause cell death, if unrepaired. If the cells do not die with the direct PDT mediated effect, the photo-oxidative stress will lead to activation of genes associated with stress response and cytokine genes, which would lead to cell death by an alternative mechanism. Like normal cells, the transformed cells can turn on or off various redox-sensitive signaling pathways as a response to hypoxia and oxidative stress. This leads to a transient increase in early response genes *c-jun* and *c-fos* which combine to form Activator Protein-1 (AP-1), an early response transcription factor. However, Photofrin® mediated PDT does not induce AP-1 and NF-κB. But studies have confirmed up-regulation in the downstream targets of NF-κB, post-PDT. NF-κB promotes tumorigenesis by preventing apoptosis and promoting angiogenesis, and some of its downstream products like IL-6 and TNF-α are anti-tumoral. So, its implication in PDT is quite debatable and difficult to elucidate [390]. HIF-1, another transcription factor that is activated during hypoxic conditions, can lead to resistance to tumoral cells after PDT. All in all, these transcription factors of survival pathways have a very complicated role in PDT, as studies have revealed PDT sensitivity even with up-regulation in levels of pro-tumoral agents.

To put it all together, when there is an imbalance between the rise in cellular ROS level and its detoxification pathway, different cell death pathways are triggered. Different pathways can be activated simultaneously and thus initiate an overlapping but complex mechanism and make it difficult to identify a single pathway at the tissue level. However, *in-*

vivo and *in-situ* studies have revealed that tumor periphery and regions close to the vessels, show necrosis due to prolonged and stronger PDT action. While the deeper regions show apoptosis and autophagy due to lower levels of PDT dose and ROS [389].

a. Apoptosis

Apoptosis, or programmed cell death, is the main cell death mechanism in PDT. As stated previously, regions of lower doses of PDT and ROS undergo apoptotic cell death. Apoptosis can be mediated by either activation of death receptors (*i.e.,* extrinsic pathway) or by the mitochondrial release of cytochrome c (*i.e.,* intrinsic pathway). The extrinsic pathway is triggered when a death ligand (like FasL, TNF-α, TNF-related apoptosis-inducing ligand (TRAIL)) binds to its extracellular receptor. Here, extracellular perturbations are detected by the plasma membrane through the death receptors, thus inducing mitochondrial membrane permeabilization by caspase 8 [383]. In the case of PDT, this pathway is mainly triggered by cytokines or death ligands released by PDT-sensitized cells or dying cells [391]. Intrinsic apoptotic pathway depends either directly on mitochondrial damage or secondary pathways inducing mitochondrial damage [383]. Since most of the commonly used PSs, including PpIX, localizes in mitochondria, the intrinsic pathway is the most common pathway observed during PDT [391]. It is a highly regulated pathway that is activated by B-cell Lymphoma 2 (BCL-2) family. This family has both pro-apoptotic (BCL-2 like Protein 4 (BAX), BCL-2 homologous Antagnoist Killer (BAK-1), BCL-2 related Ovarian Killer (BOK), p53 Upregulated Modulator of Apoptosis (PUMA), BH3 Interacting Domain Death Agonist (BID), BCL-2-like protein 11 (BIM), NOXA) and anti-apoptotic protein (BCL-XL, Induced Myeloid leukemia cell differentiation protein (MCL-1), BCL-W, BFL-1) members [383]. In the case of PDT, the intrinsic pathway is shown independent of tumor suppressor protein p53 status, but some studies have shown p53-dependent photo-killing. For PpIX mediated PDT, interestingly, p53 is partially responsible for the apoptotic activity. PpIX binds to p53 and disrupts its interaction with the negative regulator, Mouse Double Minute 2 (MDM2), as shown for PpIX PDT against human colon cancer [392].

For the intrinsic pathway, mitochondria play a central role. The anti-apoptotic BCL-2 family members are associated with the mitochondrial, nuclear, and ER membrane and prevent cytochrome c release stored in inter-mitochondrial space. When these proteins

encounter death stimuli, they induce permeabilization of mitochondrial membrane and cytosolic release of cytochrome c. This cytochrome c then binds to Apoptotic protease activating factor 1 (Apaf-1) and this complex then binds to pro-caspase 9 in presence of ATP. This activated caspase 9 then cleaves and activates caspase 3 and 7 to mediate cell death. Mitochondria also release other proteins like Smac/DIABLO (Direct Inhibitor of Apoptosis (IAP)-Binding protein with Low Isoelectric point), HtrA2, Apoptosis-Inducing Factor (AIF), and endonuclease G. AIF and endonuclease G translocate to the nucleus to induce apoptosis independent of caspase cascade and DNA fragmentation [383]. PDT has been shown to damage anti-apoptotic proteins like BCL-2, thus increasing BAX/BCL-2 ratio and facilitating the pro-apoptotic proteins. When PS is localized in organelles other than mitochondria, PDT increased cytosolic Ca^{2+} levels to cause activated proteins up-stream of BH3-only to ultimately induce apoptosis [383], [385], [389].

b. Necrosis

While apoptosis is a caspase-dependent cell death mechanism, there is an alternative pathway during caspase inhibition to elicit PDT-mediated cell death, called necrosis, and is initiated during extensive and fast cellular damage. Necrosis, often termed as accidental cell death, is a passive, rapid and unregulated mechanism resulting from a strong acute physical or chemical insult. It is characterized by cytoplasmic swelling, leaky plasma membrane, destruction of organelles, and decomposition by proteolytic enzymes. The release of intracellular content makes necrosis a potent inducer of local inflammation and immunological cell death which can elicit indirect cytotoxicity, discussed later. Mitochondria, lysosomes, and ER play important role in the initiation and progression of necrosis [383], [391].

For PDT, necrosis is observed when the site of PS localization is the plasma membrane (like Photofrin®, PC4). High dose PDT induced quick photo-damage to the plasma membrane causes leakage of intracellular material. This also causes cell death mediated by TNF and Fas receptors which activate receptor-interacting protein 1 (RIP-1) when the caspases are inhibited. Besides, Ca^{2+} level increase, lysosomal damage, and ROS production together contribute to necrotic cell death [385], [391]. Recently, PDT is being associated with a more regulated and programmed form of necrosis, called necroptosis. Necroptosis is activated

when PDT triggers death receptors like TNFR1, Fas, but under the condition of caspase inhibition [389]. More studies are being conducted to understand this mode of cell death and its association with PDT.

c. Autophagy

Autophagy is a catabolic mechanism through which cells recycle long-lived, degraded, or dysfunctional cellular products. Normally, it is a cytoprotective mechanism through which the cells promote survival by eliminating damaged cell organelles and toxic metabolites by self-digestion during stress, starvation, and high ROS levels. But autophagy can act as a cell death mechanism as well, in the absence of apoptosis and when the cellular damage inflicted is strong and permanent. Thus, this mechanism can act as both pro-survival as well as lethal. During autophagy, a double layer membrane vesicle, called autophagosomes, is formed which engulfs the damaged material, thus separating it from the cytoplasm. Thereafter, it fuses with lysosomes, forming autolysosomes, where the cargo is hydrolyzed by the lysosomal enzymes [389], [391], [393].

For PDT, it is still unclear if autophagy may facilitate or impede the cell death induced by photo-damage. Autophagy can be a defense mechanism against PDT where it can lyse the photo-damaged organelles, oxidized biomolecules, and sequester the ROS generated, thus trying to preserve cellular viability [389]. PDT can inactivate the negative regulators of autophagy (like BCL-2, and mTOR) instead of activating pro-autophagy proteins [383]. Studies have revealed that the knockout of autophagy genes (like Autophagy Related (atg) *7* and *atg5*) can increase the sensitivity of the cells against PDT [385]. Autophagy is activated at low PDT doses, just like apoptosis, and thus both can be triggered simultaneously. However, the fate of autophagy is decided based on the photo-damaged organelles and the cargo of the autophagosomes. The mitochondrial and ER targeted PSs often generate pro-survival autophagy, while lysosome targeted PS will inhibit autophagy obviously due to loss of activity of the key autophagy organelle [279], [385]. However, this also suggests that autophagy is independent of PS localization. Further, autophagy can play a pro-death role in absence of apoptotic capabilities and thus inducing autophagic cell death [383], [385].

2. Indirect Cytotoxicity

PDT is not very selective in terms of its cytotoxic effect. However, as discussed previously, the two-step cell death method based on partially selective PS accumulation and effective illumination dose together can increase the specificity of the therapy. This does not mean that PDT could not target the tumor surrounding tissue. However, this can turn beneficial for the overall efficacy of the therapy, since a lot of tumors grow at the backdrop of a tumor-supportive stroma. Furthermore, PDT induces high tumoral toxicity within several hours after illumination, which might reduce the overall effectiveness at a longer perspective. Hence direct toxicity alone cannot be sufficient to eradicate the tumor, and secondary responses like inflammation are deemed necessary. PDT has been shown to induce an inflammatory response or a full-fledged anti-tumoral immune response which could further limit the growth of untreated tumoral cells. In this section, we will discuss two key secondary effects of PDT, which limit the growth of the tumor and ultimately cause tumoral cytotoxicity: the vasculature shutdown and the induction of anti-tumoral immune response.

a. Impact of PDT on tumor stroma and vasculature

The TME is comprised of some key proliferation supporting cells which include fibroblasts, various inflammatory and immune cells including myeloid-derived suppressor cells, mesenchymal stem cells along with vascular endothelial cells, adipocytes, pericytes where the tumoral cells constitute the parenchyma of the tumor. The ECM comprises various signaling molecules, exosomes, and proteins like vimentin, collagens, glycoproteins, proteoglycans fibronectin, and other secretory proteins [394], [395]. Since the tumor parenchyma is in direct contact with the stroma, any change in the latter will affect the former, thus the cross-talk between the tumor and its microenvironment plays a critical role in its growth, invasiveness, and motility. By a dynamic feedback regulatory mechanism, the stroma assists the tumor to adapt according to the changes induced. All these factors are known to limit the efficacy of any classical treatment against cancer.

The effect of PDT on the ECM and the cell adhesion is not completely understood. As stated previously, PDT treatment decreases the adhesiveness and invasiveness of the cells, thus limiting their metastatic ability [395]. Further, the stroma can limit the distribution of PS

throughout the core due to impaired vascularity and some pigmented molecules can decrease the effective light penetration, as in the case of the liver [279]. However, the tissue insult inflicted during PDT initiates a host response like wound healing. This can initiate a cascade of complex host reactions which can induce changes either helping the tumor to re-grow by the supply of growth factors *via* the tumor vasculature or cause permanent damage [385]. Therefore, local vascular damage is crucial for the tumor response against PDT. PS localization in the collagen induces vascular photo-damage, thus the tumors with high collagen content show better results making it an important microenvironmental factor for overall therapeutic outcome [396]. But as stated previously, early vascular destruction can lead to disruption in tumor oxygen supply thus limiting the therapeutic outcome. Hence a proper design of the PDT targeting and protocol is deemed necessary.

The vascular damage inflicted by PDT could cause angiogenesis by the stroma, thereby causes tumor recurrence. Hence, a combination of PDT with anti-angiogenesis therapy could prove to be an effective strategy [397], [398]. An anti-angiogenic pre-treatment could limit the expression of HIF-1 and VEGF by PDT-treated tumor cells, which are the activators of angiogenesis, and thereby such a combination could decrease potential activation of angiogenesis [397]. Star *et al.* demonstrated through *in-vivo* studies that direct tumor destruction is not sufficient unless vascular destruction is initiated by HpD based PDT. In the model used in their study, all the tumors recurred, unless there is micro-circulation damage thus postulating that tumor destruction is secondary to vasculature destruction by PDT [399]. *In-vivo* PDT by verteporfin over prostate cancer and chondrosarcoma induced damage to the vasculature is increased by decreasing the drug-to-light interval. This is primarily due to the rapid accumulation of PS in blood vessels (in 15 minutes post-injection), while a more uniform distribution throughout the tumor requires more time (3 hr as reported), and thus more permanent tumor destruction was observed [400], [401]. Similar results were demonstrated using benzoporphyrin derivative-based PDT where it was stipulated that PDT could target the endothelial cells of the neovasculature thereby causing tumor destruction [402]. This also prompts that targeting tumor vasculature by short-interval PDT could give long-lasting results. Ben-Hur *et al.* demonstrated that PDT treatment of endothelial cells, which line the blood vessels and regulates exchange between the bloodstream and surrounding tissue, causes photo-damage and release of clotting factors which together can decrease the tumor

blood flow and cause thrombosis and tumor destruction [403]. Using Photofrin® mediated PDT, Fingar *et al.* demonstrated that platelet activation and eicosanoid release are necessary for vessel constriction and blood flow disruption during and after PDT [404]. Studying and implementing strategies that could affect the hyper-vascularized stroma of tumors like HCC could prove to be beneficial for the overall and long-term success of the therapy.

b. Induction of anti-tumoral immune response by PDT

"*I have explained and discuss the importance of PDT induced inflammation and immune response in our review. We kindly refer to that for this section".*

CHAPTER 3

PHOTODYNAMIC THERAPY AND HEPATOCELLULAR CARCINOMA

Based on the submitted Review (*in Cancers, May, 2021*)

Title: "**Could Photodynamic Therapy be a Promising Therapeutic Modality in Hepatocellular Carcinoma Patients:** A critical Review of Experimental and Clinical Studies**"**

Could Photodynamic Therapy be a Promising Therapeutic Modality in Hepatocellular Carcinoma Patients? A Critical Review of Experimental and Clinical Studies.

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List of Abbreviations

HCC: Hepatocellular Carcinoma

BCLC: Barcelona Clinic Liver Cancer

PDT: Photodynamic Therapy

PS: Photosensitizer

ROS: Reactive Oxygen Species

5-ALA: 5-Aminolevulinic Acid

MAL: Methyl aminolevulinate

HMME: Hematoporphyrin Mono-Methyl Ether

PBMC: Peripheral Blood Mononuclear Cells

VEGF: Vascular Endothelial Growth Factor

PDAC: Pancreatic Adenocarcinoma

CTLA4: Cytotoxic T Lymphocyte Antigen 4

PD-L1: Programmed Death Ligand 1

PpIX: Protoporphyrin IX

mTHPC: meta-tetra(hydroxyphenyl)chlorin

mTHPBC: 5,10,15,20-tetrakis(m-hydroxyphenyl)bacteriochlorin)

NIT: Near Infrared

ICG: Indocyanine Green

PTT: PhotoThermal Therapy

ICD: Immunogenic Cell Death

ATP: Adenosine Triphosphate

HMBG1: High Mobility Group Box 1

HSP90: Heat Shock Protein 90

HSP70: Heat Shock Protein 70

DAMPs: Damage Associated Molecular Patterns

HPPH: 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a

MIP-2: Macrophage Inflammatory Protein 2

HIF: Hypoxia Inducing Factors

DC: Dendritic Cells

SCID: Severe Combined Immunodeficiency Syndrome

Tregs: Regulatory T Lymphocytes

MDSCs: Myeloid derived suppressor cells

Abstract

Photodynamic Therapy (PDT) is a minimally invasive phototherapy which relies on local or systemic administration of a light sensitive dye, called photosensitizers, to accumulate into the target site followed by its excitation with light of appropriate wavelength and power. This photo-activated molecule will thereby react with the intracellular oxygen to induce selective cytotoxicity of targeted cells by generation of Reactive Oxygen Species, and simultaneously generating fluorescence. Initially used for various dermatological diseases, PDT is now being investigated for neoplasms of varying types and locations. Hepatocellular carcinoma, one of the leading causes of cancer associated mortality worldwide, has insufficient treatment options available for patients of various stages. The inability of these therapies to induce a sufficient anti-tumoral immune response, causes tumor recurrence for up to 70% of patients treated with a curative intent. In this review, we discussed the mechanism and merits of PDT along with its recent developments as an anti-cancerous therapy. We have also highlighted the application of this novel therapy for diagnosis, visualization and treatment of HCC. We examined where does PDT stand amongst the other HCC modalities, along with the underlying challenges, some pre-clinical and clinical studies associated and possibilities of future studies. At last, we discussed the mechanism of an active immune response by PDT and thereafter explored the role of PDT in generation of anti-tumor immune response in context of HCC, with an emphasis on check-point inhibitor-based immunotherapy. The objective of this review is to propose PDT as a plausible adjuvant to existing therapies for HCC, highlighting feasible combinatorial approach for HCC treatment.

Introduction

For hepatocellular carcinoma (HCC) treatment, the Barcelona Clinic Liver Cancer (BCLC) staging system is the most commonly used staging classification, which classifies the patients into Early, Intermediate, Advanced and End-Stage (1–4). Based on this staging system, curative options are only considered in early-stage HCC. Patients with solitary HCC and preserved liver function are referred to either surgical resection or percutaneous ablation while liver transplantation, when feasible, is reserved for up to 3 nodules of no more than 3 cm in diameter. In every other situation, which concern the majority of patients with HCC, only palliative options are available, ranging from locoregional therapies such as chemoembolization to targeted therapies, if not best care support. The median survival gain obtained with such treatments, although statistically significant, does not exceed one year for patients with advanced tumors (5–7). Furthermore, even in early stages and after treatment with curative intent, HCC recurrence rates are quite high and re-treatment is not always feasible. However, in the therapeutic armamentarium used in managing HCC, other emerging therapies have shown encouraging results. Those based on tumor radiation, *i.e.* stereotaxic body radiation therapy (8) and trans-arterial radio-embolization with ^{90}Ytrium (9) are still under clinical evaluation, and it is unknown whether radiation therapies would outperform surgery or thermal ablation in the future. In addition, those treatments have specific contraindications. Therefore, there is still a need to explore or develop novel therapeutic modalities for HCC, either alone or combined with locoregional treatments.

Photodynamic Therapy (PDT) is a clinically approved anti-cancer treatment. It relies on the systemic or topical administration of a non-toxic dye called photosensitizer (PS) followed by accumulation of the PS for a predetermined time (called drug-to-light interval) and

illumination of the tumor by light of a wavelength and energy corresponding to the PS in order to elicit cytotoxicity (10). This photochemical process stimulates reaction with the intracellular oxygen to form Reactive Oxygen Species (ROS) like peroxide, singlet oxygen and hydroxyl species, to induce cytotoxic effect finally. These PS must exhibit high and selective accumulation in the tumor along with low or minimal dark toxicity (*i.e.* the toxicity induced by the PS in the absence of illumination), high bio-stability and high bio-clearance (10,11). PDT can either directly induce cell death by necrosis or apoptosis or both, or indirectly by targeting the tumor microenvironment and vasculature to induce an inflammatory and immune response against the tumor (11,12).

In this review, we will unveil the role of PDT as an anti-cancer therapeutic modality and describe how the main obstacles against its development have been or could be countered. We will then review the use of PS and PDT for HCC diagnosis and treatment, and discuss possible future research endeavors in this field, including the impact of PDT for inducing an anti-tumor immune response.

Development of photodynamic therapy as an anti-cancer treatment

PDT was first coined by Hermann von Tappeiner in 1903 (13), and was initially used to treat cutaneous disorders. PDT soon gained widespread usage in the field of dermatology to treat various skin disorders namely, papillomavirus infections, cutaneous leishmaniasis, actinic keratoses, acne, viral warts, photo-rejuvenation, psoriasis, hypertrophic, keloid scars and port wine stains, with guidelines and recommendations being developed by agencies of different countries (14). Some of the classical PS used here are pro-drug 5-Aminolevulinic Acid (5-ALA) and its hydrolyzed methyl ester Methyl Aminolevulinate (MAL), along with Photofrin®, chlorin, pthalocyanine and Hematoporphyrin Mono-Methyl Ether (HMME) (14).

A major breakthrough to introduce PDT as a potential anti-cancer therapy came in 1978, when T.J. Dougherty *et al.* used Hematoporphyrin Derivative based PDT for treatment of cutaneous or sub-cutaneous tumors including breast, colon and prostate metastases (15). This hence led to the clinical development of PDT for melanomas and squamous cell carcinomas. Since the pioneering work of Dougherty *et al.*, PDT is now proposed as an alternative tool in cancer treatment with the introduction of laser and optic fiber based light delivery systems and the discovery of new PS. The past decade has observed a huge number of PS being developed and tested which include nanoparticles and chemically conjugated PS. Some of them have been approved by the regulatory authorities of various countries for clinical studies, leading to a surge in the number of publications for PDT unravelling its various aspects ranging from its mechanisms of action to the possible activation of an anti-tumor immune response. Interestingly, almost all the PS are degraded by illumination upon forming singlet oxygen which adds to an important aspect of drug bio-clearance (16,17). Besides the usual use as an anti-cancerous therapeutic agent, the fluorescence of the PS can be utilized either as a diagnostic agent or as an aid during surgery as it could delimit tumor burden (12,17).

The effectiveness of the therapy depends on the accumulation of the PS into the neoplasm, adequate uniform dosage, power of the light, its penetration into the tissues and availability of the intracellular oxygen (10,12,18). With improved understanding in the biology and mechanism of the therapy along with development of targeted PS and efficient light delivery systems, the overall efficacy of the therapy has increased by overcoming some obstacles. PS penetration for various skin disorders has been enhanced by various physical and chemical pre-treatments which include enhanced drug formulation, stratum corneum removal,

iontophoresis, and temperature modulations (19). Further, the conjugation of classical PS with monoclonal antibodies, ligands, biomolecules, liposomes and nano-carriers to increase hydrophobicity and selective accumulation of the PS has given improved results (17). Azaïs *et al.* used a new folic-acid coupled PS based intraperitoneal PDT to specifically target the epithelial ovarian cancer, which has a higher folate receptor expression. Their study highlighted higher PS accumulation than other PS, along with increased human peripheral blood mononuclear cell (PBMC) proliferation (20). Such highly targeting PS could prove to be a real asset for ovarian cancer treatment and management since most of the patients also show microscopic peritoneal metastases, which are tough to visualize and remove by surgery. For delivery of homogenous illumination, optic fiber woven-based light emitting flexible fabrics has been shown to give higher output in terms of fluorescence rate, illumination homogeneity and flexibility (21). Additionally, fractionation of the illumination dosage further increases the effectiveness of the PDT, since it results in continuous supply of an important modulator of PDT mediated cell death, oxygen; along with increased influx of the PS in certain cases (22). This accompanied with an optimal illumination dose, decreases the heat generated and the underlying pain, which is a common issue during PDT treatment of skin disorders.

The cytotoxic ROS generated by PDT not only kills the tumor cells, but also damages the microvasculature of the tumor. The damages inflicted upon vascular basement membrane causes permeability of the vessels along with vasoconstriction which ultimately leads to tumor destruction (23). Since PDT consumes the oxygen of the tumor micro-environment, this might lead to release of angiogenic growth factors, to favor angiogenesis and facilitate the growth of remaining tumor cells, thus reducing the efficacy of the therapy (24). However, this impact can be reduced by combining the therapy with an anti-Vascular Endothelial

Growth Factor (VEGF) targeted therapy. Such combinatorial approaches with classical therapies to improve the overall cytotoxicity of PDT has given promising results (25,26).

In clinical practice, however, PDT has remained a rescue therapy in patients presenting with advanced disease, not eligible or irresponsive to conventional treatments, or being too sick to undergo surgery (12,18). A prime reason for this is the difficulty to establish standard treatment conditions. Given the possible variation of several parameters, including the type of PS used, light dosage, power of illumination, light fractionation, drug-to-light interval; it becomes a challenging process to standardize treatment conditions for a clinical set-up in different types of cancer. Additionally, only a few PS have been approved for clinical trials, which include Porfimer sodium, Temoporfin, 5-ALA and MAL, which thereby limit the usage of new third generation of PS being developed.

However, some studies have demonstrated the benefit of PDT in a clinical setting, not only in palliative situations, but also in early stages and even as an adjuvant therapy associated with surgery. Cuenca et.al. used Photofrin® PDT in a small group of patients with chest wall progression of breast cancer. They observed a significant decrease in tumor size in all patients (27). Moole *et al.* pooled the outcomes of 10 different clinical studies of PDT in patients with non-resectable cholangiocarcinoma, and conclude that PDT, in combination with biliary stenting, improves biliary drainage and thereby increases patient survival (28). Gonzalez-Carmona *et al.* used a combination of PDT with different porphyrin derivatives with a systemic chemotherapy in patients diagnosed with extrahepatic cholangiocarcinoma, and demonstrated a significantly higher median survival (29) compared with the chemotherapy-alone group, which also had a lower survival when compared with the PDT-alone group. This study gives crucial evidence for the role of PDT as a local therapy to control the progression

of extrahepatic cholangiocarcinoma and increase overall survival. A Photofrin® II mediated PDT in patients with breast cancer showed that 50% of the patients were responsive for the therapy with higher efficacy in case of minimal or moderate tumor extent when compared with advanced stages (30). In another clinical study, 5-ALA injection 5 hours prior to a laparoscopic surgery of ovarian cancer patients, aided tumor visualization by fluorescence through protoporphyrin IX (PpIX) providing higher sensitivity for the detection of intra-peritoneal metastases (31). A recent clinical trial coordinated by Pr. N. Reyns in our department is currently ongoing (NCT03048240) aimed to analyze the impact of intraoperative 5-ALA PDT delivered in the tumor bed during resection of glioblastoma.

Otherwise, a very recent investigation from our team has suggested that PDT using folate coupled PS (demonstrated by Azaïs *et.al.*), is an effective therapy in the treatment of pancreatic adenocarcinoma (PDAC), also activates the immune system and could be considered as a real adjuvant for anti-cancer vaccination. Folate binds to FOLR1, in a specific way, expressed in 100% of ADKP or over-expressed in 30% of cases. In a very interesting way, they observed a significant increase in the proliferation of activated-human PBMC and T cells when cultured with conditioned media of PDAC cancer cells subjected to PS-FOL/PDT (32). This is quite an important study, as it highlights the applicability of a targeted PDT for a solid tumor in the abdominal cavity, similar to liver cancers.

Overall, PDT has a mixed success-failure story in clinics and waits for more clinical studies with higher number of patients to be more conclusive.

Is photodynamic therapy applicable in patients with HCC?

Recent advances in HCC treatment rely on physical therapies, like trans-arterial radio-embolization with ^{90}Ytrium, improved molecular targeted therapies like multi-kinase inhibitors, and immune-modulation by anti-Programed Death Ligand 1 (anti-PD-L1) or anti-Cytotoxic T-Lymphocyte Antigen 4 (anti-CTLA4). In this context, PDT, as being a complex product of physics, chemistry and biology, may provide a combined local and systemic approach to HCC treatment. However, the use of PDT for treatment of liver tumors has been limited so far. Generally, the major issues with the first generation of PS were short wavelength of absorbance, poor *in-vitro* aqueous stability with short circulation half-life, lesser tumor selectivity and skin phototoxicity (10,11). This further aggravates when using PDT on the liver, where the high vasculature make certain PS accumulate not only in the tumor but also in the healthy parenchyma. For instance, during 5-ALA PDT for HCC, we can observe higher accumulation in the healthy liver, since liver is the center for heme synthesis, which is used to metabolize the pro-drug 5-ALA to the actual PS, protoporphyrin IX (PpIX) (33). This heme biosynthesis pathway is further responsible for liver pigmentation thus altering the light penetration which decreases as the function of distance (34).

The latter issue may be addressed by using PS being more homogeneously distributed throughout the tumor at an optimal concentration and requiring higher wavelength for their activation (33). For instance, a study on rat liver reveals that meta-tetra(hydroxyphenyl)chlorin (mTHPC) requires less PS dosage than Photofrin® and other hematoporphyrin derived PS, since it activates at higher wavelength (33–35). Moreover, this cytotoxic effect was further increased with near-infrared PS 5,10,15,20-tetrakis(m-

hydroxyphenyl)bacteriochlorin (mTHPBC), a PS belonging to the same class of hydroporphyrins as mTHPC but with higher wavelength of activation light (33–35).

Tumor selectivity and targeting could be facilitated by coupling PS with nano-carriers. With the recent advances in nano-carrier technology, a lot of PS are being modified and tested for enhanced efficacy. Wang *et al*. demonstrated that PDT mediated by IR780 and near infrared (NIR) illumination could induce higher cell growth inhibition of HCC cell lines when delivered by a nanoparticle complex (Pullulan, Pluronic F68 and phospholipid) also encapsulating paclitaxel, with respect to IR780 or paclitaxel alone (36). *In-vivo* studies further demonstrated reduced tumor growth and angiogenesis (36). Zhang *et al*. further combined multiple approaches of hypoxia, PDT, and chemotherapy with an efficiently designed drug delivery system based on DNA aptamers and gold nanoparticle, to develop a targeted and effective HCC therapy (37).

Indocyanine green (ICG), a water soluble tricarbocyanine dye, is a widely used agent in clinical practice for intraoperative HCC visualization (38) and liver function assessment (39). Additionally, it has also been used for NIR PDT of several cancer models including HCC. Interestingly, when photoactivated, ICG also generates heat, which thereby contributes for a tumor-suppressive effect, known as PhotoThermal Therapy (PTT). Under PTT the PS is photo-excited, to generate vibrational energy in form of heat, thereby inducing a cytotoxic effect (40). An ICG-Lactosome nanoparticle complex has been developed showing higher accumulation in HCC, improved tumor visualization and causing higher cell death when compared with ICG alone (41). Therefore, ICG has potentiality to become the best candidate for PDT in HCC treatment. Nevertheless, the mixed impact of PDT and PTT has its equal drawbacks. As summed up by Giraudeau *et al*., ICG exhibits a phototoxicity *via* PDT at low

power dose, and *via* PTT at high dose, which are both relying on different molecular and cellular mechanisms (40). It is still controversial to comment which effect is superior to other, but efficiency of ICG-induced phototoxicity, especially by PDT, is not particularly effective, due to various reasons. Coupling it with nano-carriers might improve the targeting by binding to specific receptors, but it requires more modifications so as to improve its ROS yield and stability at physiological conditions and also avoid agglomeration (40,41).

In-vitro studies present major setback for PDT research, the most significant being oxygen availability, since most of the cancers, including HCC, develop in a hypoxic background. Use of hypoxic chambers and organoids based 3-D cultures might prove beneficial for HCC modelling. Such systems, however, will not be cost-effective and require high skill sets. That is why a pre-clinical set-up, using various humanized mouse models can help us understand the applications of PDT by giving a more detailed effect on the 3-D microenvironment. The most widely used models for this purpose are the subcutaneous tumors developed by either injecting human or murine HCC cell lines beneath the skin; or transplanting small tumor pieces from one mouse to another. Many teams have developed orthotopic mouse models where the tumors are injected to the organs of origin, which gives a better model of the cancer. Another approach can be the use of specific carcinogens to induce organ specific cancers. However, these models can have major drawbacks as the light might not penetrate to its full efficiency thereby limiting the effectiveness of the therapy (42). Our unpublished data have revealed that the fluorescence from PpIX could not be observed from the exterior, but was successfully detected after the sacrifice and recuperation of the tumor from the mice. Studies by two independent groups using 5-ALA PDT for different pre-clinical HCC model demonstrated a fluorescence based selective accumulation of PpIX in the tumor, along with

an anti-tumor effect (42,43). The most interesting feature of these studies was PpIX accumulation and necrosis in the tumor core (up to 8mm for mouse model). This reflects the penetrating capabilities of 5-ALA PDT, rather than a mere superficial effect.

Hepatic resection has become a standard HCC treatment for early stage patients, even in the presence of liver cirrhosis. However, long-term survival is often limited by intra-hepatic recurrences, which are not always prevented by anatomical resection or adequate surgical margins. Furthermore, small satellite nodules are hardly detected through visual inspection and intraoperative ultrasonography, especially in liver cirrhosis (42). Hence, PDT can introduce itself as an adjuvant to hepatic surgery, where it may be used both as a simple and rapid real-time fluorescence-based visual aid, and as a complementary treatment targeting the tumor surrounding parenchyma. Fluorescence-guided hepatectomy using ICG has already become a standard, and one can take advantage of its potential as a PS to perform PDT during ICG-guided surgical resection. Most importantly, PDT will induce an anti-tumor immune response, which might further eliminate the possibility of tumor recurrence thereby giving a long-lasting protection through the development of an immune memory, which will be discussed in the following section.

Photodynamic therapy may induce an anti-tumor immunity

For most of the anti-neoplastic modalities, there is a change in the organization of the infiltrating immune cells in the tumor microenvironment, which can be crucial for the overall follow-up of the therapy. This change can be either pro or anti-tumoral, which thereby gives the tumor either a resistance or hypersensitivity, respectively. An ideal anti-cancer modality will not only destroy the tumor but also trigger the immune system to work against the neoplasm, either primary or malignant, by inducing Immunogenic Cell Death (ICD). As

postulated by Kroemer *et al*., ICD is induced by cytotoxic therapies which induce calreticulin exposure, ATP secretion, and release of HMGB1, HSP70 and HSP90 among others, which are preceded by either Endoplasmic Reticulum based stress, ROS production or autophagy (44). These Damage Associated Molecular Patterns (DAMPs) induce activation and maturation of innate immunity and thereby induction of an immune response. Since ROS production is the *modus operandi* for PDT, the induction of such immune stimulatory effects by PDT is quite obvious and relevant for involution and control of the neoplastic lesions. Various *in-vivo* studies have concluded that the efficacy of PDT is reduced in absence of an active immune system, thereby highlighting that PDT has an immune-stimulatory impact which may have some clinical influence.

Like any host response to an external stimulus, PDT induced immune response will rely on intricate network of inflammatory cytokines, chemokines, transcription factors and release of DAMPs by the PDT treated tumor. After treating colon cancer with pyropheophorbide-a methyl ester-based PDT, two waves of transcription factor NF-κB activation were observed. NF-κB regulates the expression of a wide range of genes responsible for activation of inflammation and immune response (45). Along with transcription factor AP-1, NF-κB induces expression of cytokines such as IL-1α, IL-6, IL-8, TNFα (45). Thus, PDT activates pro-inflammatory mediators thereby generating an acute inflammatory response.

Studies involving various PS and cancer models, have demonstrated the direct impact of PDT on immune components can be activating, suppressive or lethal (46). Initially, Korbelik and Cecic demonstrated with Photofrin® that PDT coupled with mycobacterium cell-wall extracts, can decrease the re-occurrence or caused relapse of mammary sarcoma in BALB/cJ mice. This study highlights that the inflammatory response triggered by PDT can be augmented by an

adjuvant, thereby giving a surge of anti-tumoral cytokines like TNF-α, IL6 (47). Later it was demonstrated that PDT mediated by another PS, 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a (HPPH), can induce a similar inflammatory response by local secretion of Macrophage Inflammatory Protein 2 (MIP-2) and E-selectin which causes an influx of neutrophils in the micro-environment capable of tumoral cytotoxicity, and thereby recruitment of other immune cells by secretion of cytokines and chemokines (48). These cytokines can have other impacts, for example, decreased IL-10 secretion which further inhibits skin contact hypersensitivity (49). This thereby highlights the role of cytokines and chemokines along with other secretory factors, in inducing an inflammation and overall action-reaction scenario for PDT.

Generally, PDT induced tissue damage causes infiltration of innate immune cells due to underlining oxidative stress, which leads to increased expression of Hypoxia Inducing Factor (HIF) (50). Additionally, since this lowered level of oxygen typically resembles a site of wound or infection, HIF also causes secretion of other inflammatory cytokines and co-stimulatory factors to enhance the function of these infiltrating innate cells (50). Being the first mediators of an immunologic response, they primarily include neutrophils, macrophages, natural killers, dendritic cells (DCs) and mast cells. Zhang *et al*. demonstrated that DCs matured and activated by deuteporfin mediated PDT on mice Hepatomas could significantly decrease the tumor growth along with higher survival rates, when compared with PDT alone (51). Here, DCs, the professional antigen presenting cells, engulfed the tumor-associated antigen released by the PDT, which thereby activated and presented effector T cells to induce an anti-tumor immune response finally. This was further proven by Garg *et al*. who showed *in-vitro* that when cancer cells are treated with reticulotropic PS, Hypericin based PDT, there is an exposure of

calreticulin and HSP70, which then facilitate the tumor cell phagocytosis by DCs, thereby highlighting the underlying mechanism of ICD by PDT (50,52,53).

However, activation of adaptive immunity is the most important aspect, in order to impart a long-lasting tumor growth control. In light of that Korbeliek *et al.*, demonstrated an adoptive transfer of splenocytes from mice treated with Photofrin® PDT against mammary sarcoma, resulted in increased tumor regression post-PDT in the recipient SCID mice than to the mice receiving just the PDT dose. This highlights that the presence of tumor sensitized T cell in spleen can have a significant impact on augmenting the impact of PDT (54). Furthermore, Kabingu and Korbeliek demonstrated that after treatment of a sub-cutaneous mammary tumor with Photofrin® PDT in BALB/cJ mice resulted in tumor decrease of primary as well as secondary tumor site in the lungs by increased infiltration by CD8+ T cells (55). These results highlight that besides the direct cytotoxicity, PDT induces anti-tumor vaccine, by generation of memory CD8+ T cells.

PDT and immune response in HCC

Due to viral infection and cirrhosis, a majority of patients suffer from a chronic inflammation in HCC. In tumor micro-environment, there is a high prevalence of immuno-suppressive regulatory T cells (Tregs) and Myeloid derived suppressor cells (MDSCs) (monocytes, macrophages, and dendritic cells), along with an increased expression of immune check point regulators (56). Due to these immune suppressive populations, the tumor infiltrating CD8+ T cells population get exhausted and their capacity to present tumor associated antigen is impaired, which further leads to tumor progression and poor prognosis (56–58). All this develops a network of cytokines, chemokines and other factors which results in an intricate microenvironment. With the recent development in immune check-point inhibitor-based

immunotherapy, the influence of the suppressive population in tumor has decreased. The two key targets are, Programmed Death Ligand 1 (PD-L1) and Cytotoxic T Lymphocyte Antigen 4 (CTLA4). When these inhibitory signals bind to their receptors on T cells (CD8+ and/or CD4+), it reduces their proliferation. At the same time, they also reduce Treg apoptosis and contribute to their inhibitory function (56). These signals are often overexpressed in tumor microenvironment, thus contributing to immune escape mechanism. Blockage of these signals, by using anti-PD-L1 and anti-CTLA4 antibodies, has given improved results in clinic for a wide range of solid tumors, in sole or in combination with existing chemo or radiotherapy. However, it remains to be tested with PDT. Therefore, immune checkpoint blockade-based immunotherapy along with increased infiltration of tumor specific effector T cells can lead to lower tumor recurrence and increased patient survival rate. Studies have shown that after PDT, there is a less secretion of TGFβ, an immunosuppressive cytokine secreted by Tregs which have an autocrine role (45).

The basic rationale for HCC treatment is the targeting of the primary tumor site along with suppression of pro-tumor factors. The current treatment regimens only target one of the aspects of the rationale, while the persistence of immune-suppressive microenvironment remains a hurdle. Since PDT, causes a tumor insult, which results in a tissue injury and release of tumor antigen. This initiates a host-tumor reaction, which results in infiltration of Tumor Infiltrating Lymphocytes to induce an anti-tumoral immune response and can be combined with immunotherapy to augment its impact. Hence, PDT will not only target the HCC, but it will also transform its microenvironment from a pro-tumoral to anti-tumoral. This was proven in HCC based *in-vitro* study where it was reported that Pheophorbide based PDT induces immunogenic cell death by triggering phagocytosis by macrophages (59).

Conclusion

Recently, PDT has received growing attention in international community, which is evident from the rising number of publications, but even though the therapy has seen a lot of advances in almost all of the fields, a lot of work still needs to be done especially with the combinatorial approach of PDT. PDT-induced anti-tumor effects include direct tumor cytotoxicity, tumor infiltrating immune cells, innate immune cell recruitment and vasculature shut down. Since HCC occurs in the background of a chronic inflammation with a complex micro-environment, the role of PDT becomes of interest as it has shown potential to transform an immuno-suppressive environment to an anti-tumoral one.

For HCC treatment, we propose PDT as an adjuvant of hepatectomy as an intraoperative procedure. The PS, along with proper drug formulation which assist in higher tumoral accumulation, could be injected right into the tumor bed with the help of catheter, a practice commonly used during Trans-arterial chemo-embolization. After ambient drug-to-light interval, the patient shall undergo hepatectomy, where the florescence generated by PS excitation shall provide a visual-aid to the hepatologist. Finally, at the end of the surgical procedure, the cavity shall be illuminated by light of appropriate power and wavelength, using optimal optic-fiber and laser-based illumination devices. This shall not only kill the undetected residual tumor, but also activate a possible PDT-induced immune response which shall further limit the possibility of tumor recurrence and thereby increasing patient life expectancy.

Through this review, we would like to emphasize the need for standardization in PDT protocols for HCC treatment and to suggest that PDT should be part of a combinatorial approach with immune check point blockade-based immunotherapy in HCC patients. We would also like to accentuate the necessity of developing smart illumination devices for such

intraoperative procedure, PS with enhanced tumoral selectivity, along with more novel therapeutic strategies for treatment of HCC. The application of improved protocols for adoptive therapy in combination with PDT, which could be facilitated by the chemotactic factors secreted by the treated tissue, may yield higher efficacy for HCC treatment.

However, current clinical data regarding the use of PDT in HCC patients are scarce, consisting only of small patient groups with short-term follow-up. This is certainly a field where the clinicians and researchers should come up together to design relevant clinical trials in the future.

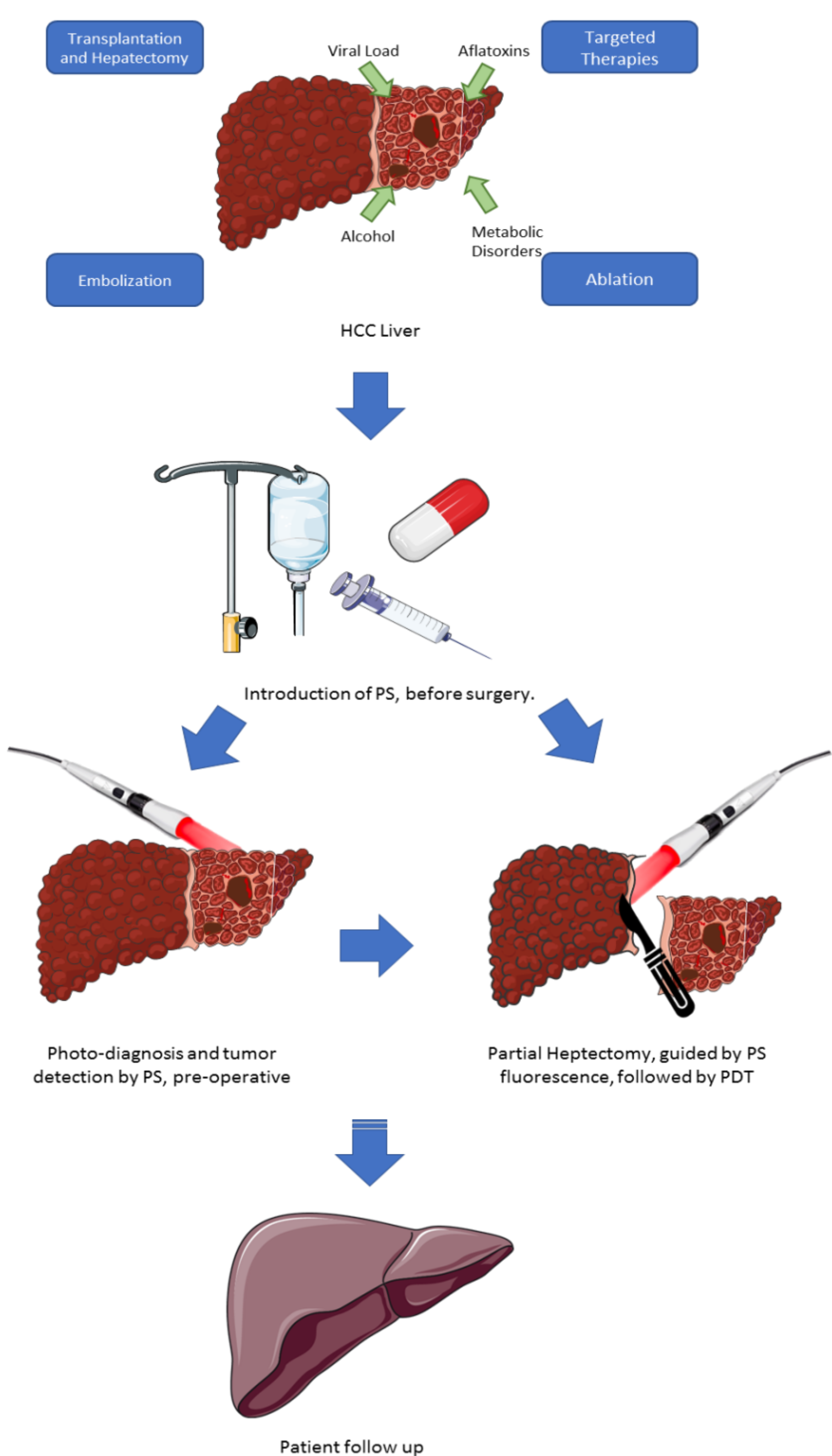

Figure 1: Proposed strategy for treatment of HCC using PDT as an adjuvant during partial hepatectomy

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Authors names in bold designate shared co-first authorship

CHAPTER 4

THESIS OBJECTIVES

Until now, we have discussed the evolution of HCC and its various characteristics along with different treatment options available. We have highlighted the shortcomings of the treatment options, along with the main therapeutic strategy employed. Further, we discussed the mechanism and various advantages of PDT, as an anti-tumoral modality. We underlined how different shortcomings of the therapy can be overcome by smart designing, development of new technology and improved therapeutic strategy. At last, we also talk about the role of an effective anti-tumoral immune response and how PDT could activate an immune response that could improve the overall efficacy of the therapy.

This led us to pose the following questions:

- Can 5-ALA mediated PDT be used for the treatment of HCC with a curative intent?

- What could be the effective treatment strategy to be employed for the treatment?

- How PpIX mediated fluorescence be utilized for intra-operative procedure during hepatectomy?

- What is the impact of 5-ALA mediated PDT on human immune system?

- What could be the potential side-effects or other cells that could be affected by 5-ALA PDT?

In light of that, we hypothesize 5-ALA mediated PDT as an adjuvant for partial hepatectomy through an intra-operative procedure. Thus, through our study, we aim to evaluate the following goals:

I. *In-vitro* efficacy of 5-ALA PDT over three HCC cell lines with different p53 state: HuH7, Hep3B and HepG2.

II. Impact of 5-ALA PDT on primary healthy donor liver myofibroblasts, in terms of their viability and induction of fibrosis. Various fibrosis markers or fibroblast activation were investigated, namely, cellular proliferation, collagen I secretion and change in the gene expression of collagen-1, HSP47, αSMA, TIMP1 and MMP2 by RT-qPCR.

III. *Ex-vivo* efficacy of 5-ALA PDT on primary HCC patient derived tumoral hepatocytes over a period of 12 days post illumination.

IV. Impact of 5-ALA PDT on human immune system.

V. *In-vivo* efficacy of the 5-ALA PDT over a SCID mice model of HCC

During our study, we utilized the laser and illumination set-up developed by the Physico-PDT team in our laboratory, and thereby validated its efficacy on our model. Furthermore, we used clinically established 5-ALA dose to study the *in-vivo* efficacy of the therapy.

CHAPTER 5

THESIS RESULTS

An Efficient 5-Aminolevulinic Acid Photodynamic Therapy Treatment for Human Hepatocellular Carcinoma

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Simple Summary

The goal of our research is to assess the potential of 5-Aminolevulinic acid (5-ALA) mediated Photodynamic therapy (PDT) as an adjuvant for existing HCC modalities. Here, we confirm that 5-ALA PDT can effectively reduce cancer cell viability in a dose dependent manner, along with secretion of factors which can potentially induce anti-tumoral immune response and reduction in tumor proliferation. We validated our findings by testing it over primary HCC patient samples and on a robust *in vivo* SCID mice model. We also highlighted the safety of the therapy by testing it over healthy donor liver myofibroblasts. Overall, we propose PDT as an intraoperative modality where it should be used immediately after hepatectomy to illuminate the cavity and eliminate any residual undetected tumors, thereby reducing chances of tumor recurrences.

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ABSTRACT

***Background**:* Photodynamic Therapy (PDT) is a two-stage treatment relying on cytotoxicity induced by photo-excitation of a non-toxic dye, called Photosensitizer (PS). Using 5-Aminolevulinic acid (5-ALA), the pro-drug of PS Protoporphyrin IX, we investigated the impact of PDT over Hepatocellular Carcinoma (HCC).

Methods: Optimal 5-ALA PDT dose was determined on three HCC cell lines by analyzing cell death after treatment with varying doses. HCC patient derived tumoral hepatocytes and healthy donor liver myofibroblasts were treated with optimal 5-ALA PDT dose. Proliferation of cancer cells and healthy donor immune cells cultured with 5-ALA PDT treated conditioned media was analyzed. Finally, therapy efficacy on humanized SCID mice model of HCC was investigated.

***Results**:* 5-ALA PDT induced a dose-dependent decrease of viability, with up to 4-folds reduced viability of patient tumoral hepatocytes. The 5-ALA PDT treated conditioned media increased immune cell clonal expansion. 5-ALA PDT has no impact on myofibroblasts in terms of viability, and their activation, meanwhile decreased cancer cell proliferation, and reduced tumor growth rate of *in-vivo* model.

***Conclusion**:* For the first time, 5-ALA PDT has been validated on primary patient tumoral hepatocytes and healthy donor liver myofibroblasts. 5-ALA PDT can be an effective anti-HCC therapy, which might induce pro-tumoral immune response.

Keywords: humanized SCID mice model, primary tumoral hepatocytes, primary healthy donor liver myofibroblasts, anti-tumoral immunity, anti-tumor therapy

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary liver cancer and has the second highest cancer associated mortality worldwide (1). Generally arising from chronic liver cirrhosis, a recurrent cycle of cell death and regeneration in liver, HCC exhibits a highly complex Tumor Microenvironment with hypervascularization (2,3). Despite advances in diagnostic, locoregional and surgical therapies, complete HCC treatment has still remained a challenge (4–6). The inability of available therapies to induce an effective anti-cancer immune response causes the tumor recurrence in several cases. Therefore, this field seeks development of novel therapies, which can not only induce tumor cell death, but also activate anti-tumor immunity, and supportive for tumor visualization before and during surgery and later in prognosis.

Photodynamic Therapy (PDT) is a minimally invasive treatment that uses a light-sensitive drug called photosensitizer (PS). Following the accumulation of the PS in the tumor, it is excited by light of appropriate wavelength which results in the production of Reactive Oxygen Species capable of inducing cytotoxicity (7). The two-step treatment process makes the therapy highly specific thereby giving a localized destruction of tumor with minimal side-effects (8). Additionally, the florescence property of the PS could aid in tumor visualization and diagnosis (9,10). The identification of small tumors is of high importance for resection and improvement of patient outcomes. Its ability to induce an anti-tumor immunity by induction of immunogenic cell death has been demonstrated in some key studies (11,12). One of the most widely studied PS is Protoporphyrin IX (PpIX), which is metabolized from pro-drug 5-Aminolevulinic Acid (5-ALA) using the heme-biosynthetic pathway. 5-ALA mediated PDT has become a standard in dermatology for skin disorders like Actinic keratoses, along with other

malignant and premalignant diseases (13,14). Clinical trials for several superficial and cutaneous cancer types is underway (15).

Here, we investigated the use of 5-ALA mediated PDT as a potential anti-HCC therapy. The aim of our study was to use 5-ALA PDT to induce *in-vitro* tumor cell death for HCC cell lines, and study its impact on immune system. We validated our findings by evaluating the impact of 5-ALA PDT on primary tumor hepatocytes from patient explants. We also studied the impact of the therapy on primary healthy donor liver myofibroblast, in terms of their cellular viability and activation state, to observe a possible fibrosis by the therapy. Subsequently, we supported our study by evaluating the impact of 5-ALA PDT on a subcutaneous SCID mice model of HCC. Our results will help the future development of PDT application for HCC and evolution of a better treatment strategy.

MATERIALS AND METHODS

In-vitro cell models

HCC cell lines

Three human HCC cell lines were used: HuH7, HepG2 and Hep3B. HuH7 and HepG2 were provided as courtesy by Pr. Filomena Conti (UPMC, Paris). Hep3B was provided as a courtesy by Pr. Jean Dubuisson (CIIL, Lille). The cell lines were cultured in RPMI 1640 (Gibco, Waltham, USA) supplemented with 10% (v/v) decomplemented and filtered Fetal Calf Serum (FCS) (Eurobio, Les Ulis, France) along with 100 units/mL of Penicillin, 100μg/mL of streptomycin (Gibco, Waltham, USA). Cell were maintained at 37°C, 5% CO_2 and 95% humidity. Luciferase

expressing HuH7 cell line (HuH7-Luc), developed and provided as a courtesy by Pr. Antoine Galmiche (Lymphocyte Normal - Pathologique et Cancers Lab, Amiens, France), were cultured in the above-mentioned media along with frequent passage with Neomycin (Sigma Aldrich, Saint Louis, USA) at 10µg/mL for 5 to 7 days.

Tumoral hepatocytes from HCC patients

Primary tumoral hepatocytes were isolated from HCC patient explants, given as a courtesy by Pr. Emmanuel Boleslawski (CHU of Lille, France), with due consent from the donors. The explant was washed twice with PBS (Gibco, Waltham, USA) and Betadine 10% (MEDA Pharma GmbH, Wangen-Brüttisellen, Switzerland), before undergoing mechanical shredding, and enzymatic digestion, using collagenase 1A (5mg/mL, Sigma Aldrich, Saint Louis, USA), and dispase (10mg/ml, Life Technologies, Carlsbad, USA). This suspension was thus incubated for 1h at 37°C, with regular shaking. The solution was then centrifuged (800g, 5 min, 20°C), pellet washed with PBS, and centrifuged again (800g, 5 min, 20°C). Thereafter, the cells were washed with Red Blood Cell Lysis Buffer (Miltenyi Biotec, Bergisch Gladbach, Germany) for 10 minutes. At last, the cells were centrifuged again (800g, 5 min, 20°C) to recuperate the pellet in complete DMEM culture medium (Gibco, Waltham, USA) (10% FCS + Gentamicin), counted, and seeded at 50,000 cells/100µL of media in a white wall 96-well plate (Corning, Corning, USA). The cells were used after at least 48 hours of culture.

Liver myofibroblasts from Human healthy donor (HLMFs)

HLMFs from three healthy donors were provided as a courtesy by Pr. Filomena Conti (UPMC, Paris), with due consent from the donors. The cells were maintained in DMEM (Gibco, Waltham, USA) supplemented with 10% (v/v) of decomplemented and filtered FCS (Eurobio, Les Ulis, France), 1% Sodium Pyruvate (Gibco, Waltham, USA), 100 units/mL of Penicillin, 100μg/mL of streptomycin (Gibco, Waltham, USA) and 1% Zell Shield (Minerva BioLabs, GmBH, Berlin, Germany). The cells were cultured at 37°C, 5% CO_2 and 95% humidity and changed media every 48 hours.

Peripheral Blood Mononuclear Cells from Human Healthy donor (PBMCs)

Human peripheral blood samples were collected, from healthy adult donors with informed consent in accordance with the approval of the EFS board (Etablissement Français du Sang). PBMCs were isolated by density gradient centrifugation of the blood using a lymphocytes separation medium (Eurobio, Les Ullis, France) and 50mL Leucosep tubes (Greiner Bio One, Courtaboeuf, France). In a 96-well plate (Corning, Corning, USA), 100,000 PBMCs were activated or not by 1µg/mL of Phytohaemagglutinin (PHA) (Sigma Aldrich, Saint Louis, USA) cultured for 5 days with 50:50 of conditioned media (see PDT section) and ML10 media (RPMI 1640 medium, sodium pyruvate (1 mM), nonessential amino acids MEM 1×, HEPES (25 mM), 2-mercaptoethanol(50µM), gentamicin (10µg/mL) (Thermo Fisher Scientific, Waltham, USA), and 10% FCS (Gibco, Waltham, USA)), to measure their viability and cellular proliferation at regular time points.

***In-vivo* SCID mice model of HCC**

All procedures were approved by the local Ethical Committee of the Institut Pasteur de Lille performed with required permission of the National governing ethical board (approval number 2019041015585930), and the mice received humane care. All male mice aged 6 to 8 weeks were used, kept in a pressurized and individually ventilated cages with regular mice diet of 10% animal fat. Anesthetized SCID mice were subcutaneously injected with 10 million HuH7-Luc cells in 100µL Matrigel (Corning, Corning, USA) and observed for tumor growth by using intraperitoneal injection of 100µL of D- luciferin (30 mg/mL, Perkin Elmer, Waltham, USA) over an IVIS LUMINA XR reader (Caliper LifeSciences, Hopkinton, USA), and analyzed under Living Image 4.1 software (CaliperLife Sciences, Hopkinton, USA). Results were obtained after spectral unmixing according to the manufacturer's instructions, and expressed in normalized bioluminescence.

Photodynamic therapy

For *in-vitro* assays, 5000 cells per 100µL of media for each cell lines were seeded in a 96-well plate (Corning, Corning, USA) in triplicate and incubated for 4h with or without 5-ALA (Sigma Aldrich, Saint Louis, USA) at various concentrations (0.01mM to 0.6mM) following irradiance by laser set-up at 635nm with varying dosage (0 J/cm^2 to 3.6J/cm^2) (16,17). The power output of laser was set to 1mW/cm^2 with a fractionated illumination consisting of 2 minutes of illumination and 2 minutes of pause, as a standard illumination protocol. All the experiments were performed at dark conditions. For recuperation of conditioned media, cells were cultured in T75 culture flasks (Sarstedt, Nümbrecht, Germany) till 70% confluence with 10mL complete media, followed by 5-ALA PDT at optimal dose and recuperation of media 24h post illumination.

For *in-vivo* experimentations, mice were divided into four groups: Non-Treated, 5-ALA Only, Light Only, 5-ALA PDT Treated. For 5-ALA only and 5-ALA PDT condition 100µL of 5-ALA (Sigma Aldrich, Saint Louis, USA) solution at 200mg/kg of mice dissolved in distilled water was injected intraperitoneally, 18h prior to illumination. For Light only and 5-ALA PDT group, mice were subjected to illumination by laser-set at 635nm with 32.4J/cm^2 (16,17). The power output was set to 12mW/cm^2 with 2 minutes of illumination followed by 2 minutes of pause, as a standard protocol. Images were then analyzed under the Living Image 4.1 software (CaliperLife Sciences, Hopkinton, MA, USA) and results were obtained after spectral unmixing according to the manufacturer's instructions. Results were expressed in normalized bioluminescence.

Viability assay

The cells were cultured, in a white wall 96-well Costar plates (Corning, Corning, USA) as per required cell density in at least triplicate for each condition (Non-treated, Light only, 5-ALA Only, PDT Treated). Celltiter-Glo mix (Promega, Madison, USA), prepared according to manufacturer's instructions, was added 100µL to each well, incubated at room temperature for 10 minutes in dark. Luminescence reading was taken with the Luminometer centro LB960 (Berthold Technologies, Oak Ridge, USA) driven by MikroWin software Version 4.41 (Mikrotek Laborsysteme GmbH, Overath, Germany). Results were expressed in Relative Luminescence Unit (RLU) or Normalized RLU. Normalized RLU= RLU of the sample/ Average RLU of Non-Treated Control.

Fluorimetry

5000 cells per 100μL of media for each cell lines were seeded in a black wall 96-well plate (Corning, Corning, USA) in triplicate. 24 hours after seeding, 5-ALA was added to the wells with a final concentration of 0.6mM for varying incubation time points (0, 1, 2, 4, 8, 18, 24 hours). Throughout the experiment, the cells remained in total darkness. At the end of incubation period, the media was recuperated to measure the extracellular levels of PpIX fluorescence while fresh media was added to the cells to measure the intracellular levels. Non-Treated controls, containing no 5-ALA, were carried out. PpIX fluorescence was measured by an excitation wavelength of 400±10nm and an emission wavelength of 650 nm, by using FLOUstar OPTIMA (BMG Labtech, Champigny sur Marne, France) driven by OPTIMA software version 2.20R2 (BMG Labtech, Champigny sur Marne, France). The results were expressed in Relative Fluorescence Unit (RFU).

Confocal Microscopy

20 000 cells were cultured on sterile cover slips 18mm in diameter (Paul Marienfeld GmbH, Lauda-Königshofen, Germany) in 12-well plates (Dutscher, France). 24 hours after culture, 5-ALA was added to the cells at a final concentration of 0.6mM. Negative controls, not containing 5-ALA, were carried out. After 4 hours and 18 hours of incubation with 5-ALA, the cells were washed three times with PBS and then fixed with 4% paraformaldehyde (PFA) (Santa Cruz Biotechnology, Dallas, TX, USA). The cells incubated 10 minutes with 50ng/mL 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, Saint-Louis, MO, USA) and then washed with PBS. The slides containing the cells were then mounted on slides using Mowiol (Sigma-Aldrich, Saint-Louis, MO, USA) and stored at 4°C until analysis. Throughout the experiments, the cells remained in total darkness. The analysis was carried using a confocal microscope LSM 880

(Carl Zeiss, Oberkochen, Germany) with a magnification of 64X. The images were acquired with an excitation wavelength of 405 nm and an emission wavelength between 640 and 695nm powered by Zen Lite 2.3 (Zeiss, Oberkochen, Germany). Further image analysis and fluorescence quantification was performed using Image J software version 1.52q (ImageJ, Wayne Rasband, U. S. National Institutes of Health, Bethesda, MD, USA)

Proliferation assay

Proliferation assays were set up in a round bottom 96-well plate (Corning, Corning, USA) in triplicate and measured by adding radioactive [^{3}H] thymidine (1µCi/well) (PerkinElmer, Courtaboeuf, France) to each well, 18 hours before harvesting. At the end of culture, the cells were harvested on a glass fibre filter (PerkinElmer, Courtaboeuf, France) using a Tomtec harvester (Wallac, Turku, Finland), sealed in a sample bag (PerkinElmer, Courtaboeuf, France) with scintillation liquid (Beckman Coulter, Brea, USA). Radioactive thymidine, was measured by scintillation counting using a β-counter (1450 Trilux, Wallac, Finland). Proliferation was estimated in Count Per Minute (CPM), and expressed in CPM or Normalized CPM. Normalized CPM= CPM of the sample/ Average CPM of Non-Treated Control (18).

Culture of cancer cell lines with conditioned media

5000 cells from HCC cell lines were seeded in 96-well plate (Corning, Corning, USA) with 50:50 of conditioned media of the respective cell line and fresh media to monitor their cellular proliferation and viability for a course of 5 days.

RNA extraction and RT-qPCR

Total RNA extraction from different samples were done using method previously described (18) and quantified using UV spectrophotometer. The RT-PCR reactions were performed, for selected genes (Table 1), according to the manufacturer's instructions using 2×MESAGREEN qPCR MasterMix Plus for SYBR 258 Assay (Eurogentech, Liège, Belgium) in a 96-well qPCR plate (Sarstedt, Nümbrecht, Germany), and the Mx3005P™ sequence detection system (Agilent technologies, Santa Clara, USA). Quantitative analysis was made based on the cycle threshold (Ct) value for each well and calculated using the MxPro software (Agilent technologies, Santa Clara, USA). The results were normalized by three housekeeping genes (HKG) (Table 1) and data are represented as dCt or as fold differences by the 2^{-ddCt} method, where dCt = Ct target gene – Ct HKG.

Cytotoxicity assay

5000 cells of each of the cell lines were seeded in a 96-well plate (Corning, Corning, USA) and treated with the above-mentioned *in-vitro* 5-ALA PDT protocol, followed by recuperation of the media at 24 hours post illumination. This media was immediately used to analyze cytotoxicity by using Cytotoxicity Detection (LDH) kit (Roche, Sigma Aldrich, Saint Louis, USA), according to manufacturer's instructions. The measurements were done using UV spectrophotometer (Multiskan RC, Thermo Fischer Scientific, Waltham, USA) at 492nm powered by Ascent™Software v2.06 (Thermo Fisher Scientific, Waltham, USA). The cytotoxicity is represented as Relative %Cytotoxicity = [(Sample value – Non-Treated Control) / (Positive Control – Non-Treated Control)] * 100.

ELISA of Collagen I

HLMFs were seeded in a 6-well plate (Corning, Corning, USA), treated with 5-ALA PDT and conditioned media was recuperated 24h post illumination. Collagen I levels were analyzed for the three controls and the test condition using Quidel MicroVue CICP EIA kit for ELISA (Quidel, San Diego, USA), following manufacturer's instructions. The measurements were done using UV spectrophotometer (Multiskan RC, Thermo Fisher Scientific, Waltham, USA) at 405nm powered by Ascent™Software v2.06 (Thermo Fisher Scientific, Waltham, USA). Concentration was determined in ng/mL.

Statistical analysis

All data were analyzed using the statistical package GraphPad Prism for windows 3.0.1 (GraphPad, San Diego, USA). All quoted *p*-values are two-sided, with $p<0{\cdot}05$ and $p<0{\cdot}01$ considered statistically significant and highly significant, respectively.

RESULTS

RT-qPCR based gene expression study of PPOX and ALAD

RT-qPCR based expression analysis of Delta-aminolevulinic acid dehydratase (ALAD) and Protoporphyrinogen oxidase (PPOX), for the three HCC cell lines was performed. Fig.- 1 demonstrates that HuH7 has higher mRNA expression of ALAD and PPOX enzymes, than the other two cell lines. However, this difference is not significant, hence the mRNA levels of the enzymes of three cell lines is similar.

In-vitro phototoxicity on HCC cell lines

In Fig.- 2(a), cellular viability at 24h post illumination for the three HCC cell lines is demonstrated when they are treated with different 5-ALA PDT doses in terms of 5-ALA concentration and illumination power. Despite slight variations among different doses, we did not observe any significant change in the normalized viability of the cell lines for Non-treated, 5-ALA or Light only controls. The cellular viability started to decrease with 0.1mM of 5-ALA and 0.6J/cm^2 of power for HuH7; and 0.2mM of 5-ALA and 1.8J/cm^2 for Hep3B and HepG2. From this dose dependent effect, we, hence, obtained an IC50 dose (half maximal inhibitory concentration) of 0.6mM of 5-ALA with 0.6J/cm^2 for HuH7 and 0.6mM of 5-ALA with 1.8J/cm^2 for Hep3B and HepG2.

Fig.- 2(b) shows a significant decrease in cell proliferation of all the cell lines treated with their respective IC50 dose of 5-ALA PDT. While all the cell lines showed significant decrease when treated with 5-ALA only, along with decrease for Light Only control of HuH7, it is the combined impact of 5-ALA and illumination which exhibit higher and significant decrease for all the cell lines. When analyzed for cytotoxicity (Fig.- 2(c)) via LDH release, we observed a significant increase of approximately 80-90% for PDT treated cells, at 24h post illumination. There was also an increase of 60-80% for cells treated with light only control, suggesting a photo-toxicity. 5-ALA has no impact on the cytotoxicity of the cells.

PpIX accumulation in HCC cell lines

In Fig.- 3, the fluorimetry based intracellular accumulation of PpIX in the three HCC cell lines is demonstrated when cultured with 0.6mM 5-ALA for varying time points. We observed a

continuous increase in the levels of PpIX fluorescence at an earliest of 1 hour of incubation for HuH7 and 2 hours for Hep3B and HepG2. The intracellular fluorescence levels kept on increasing until 18 hours after which the levels saturated for all the cell lines. Interestingly, starting from 4 to 8 hours, we started to observe an increase in the levels of extracellular PpIX fluorescence which kept on increasing till 24 hours. There was no change in intracellular or extracellular PpIX fluorescence levels of Non-Treated control.

Fig.- 4(a) highlights the confocal microscopy based PpIX fluorescence in the three cell lines cultured with 0.6mM of 5-ALA for 4 hours and 18 hours. DAPI was used as a nuclear stain. Fig.- 4(b) depicts semi-quantitative estimation of PpIX fluorescence in the three cell lines at 4 hours and 18 hours of incubation with 0.6mM of 5-ALA.

Impact of 5-ALA on HCC patient explant

Tumor hepatocytes harvested from HCC patient tumors were subjected to two different doses of 5-ALA PDT of 0.6J/cm^2 and 1.8J/cm^2 with 0.6mM of 5-ALA, to analyze their viability at different time points post illumination. Our results (Fig.- 5) demonstrate that at 3 days post illumination, there is a significant decrease in the viability of the tumoral hepatocytes treated with both the doses of 5-ALA PDT, along with those treated with 5-ALA only control. The viability continued to decrease till 10 days post illumination. No viability decrease was observed for Light Only control group, with respect to Non-Treated condition.

Impact of 5-ALA PDT on healthy human myofibroblasts

Primary healthy donor human liver myofibroblasts were subjected to two doses of 5-ALA PDT to analyze their viability and activation state 24 hours post illumination. As shown in Fig.- 6(a), despite slight insignificant variations in normalized viability, we observed no statistically significant decrease of normalized viability for the three donors with respect to Non-Treated control. Further, we analyzed the activation state of these myofibroblasts by studying their cell proliferation, collagen I secretion and gene expression for Collagen I, HSP47, αSMA, TIMP1, and MMP3. As demonstrated in Fig.- 6(b), despite decrease in fold change of mRNA transcripts of the genes, we did not observe any significant difference. When analyzed for collagen I secretion by ELISA, we observed a slight significant decrease of approximately 100ng/mL for both the PDT doses. Similarly, 5-ALA PDT showed a significant decrease in cell proliferation of myofibroblasts, along with slight significant decrease in 5-ALA control group.

Impact of 5-ALA PDT treated conditioned media on human activated PBMCs and HCC cell lines

In order to assess the impact of HCC cell lines secretome on the proliferation of human PBMCs, we cultured activated PBMCs with the conditioned media of the cell lines from different conditions. Fig.- 7(a) shows that 5-ALA PDT treated conditioned media harvested 24h post illumination could significantly increase the proliferation of human activated PBMCs starting from day 2 of culture for the three cell lines. This increased proliferation maxes at Day 3 by 2 to 3 folds of Non-Treated condition, after which it starts to decrease. We observed minor but significant changes in the normalized viability levels of different groups for the cell lines (Fig.- 7(b)).

Furthermore, we used this conditioned media to culture respective cancer cell lines. Our results in fig.- 7(c) shows a significant decrease in cell proliferation of these cancer cells which were cultured with 5-ALA PDT treated and control 5-ALA only conditioned media, with no significant difference between them. Fig.- 7(d) shows slight significant change in normalized viability.

In Vivo Evaluation of the 5-ALA PDT efficiency in the Humanized SCID Mice Model of HCC

We performed 5-ALA PDT on a SCID mice model of HCC, along with other control groups. We observed a 6 to 8-fold decrease in normalized bioluminescence on this group, when compared with Non-Treated group, which reflects a decrease in tumor growth (Fig.- 8(a)). Overall, there is a decrease in the rate of tumor growth. 5-ALA or Light does not seem to affect tumor growth. Fig.- 8(b) demonstrates a significant decrease in the average normalized bioluminescence of the mice group treated with 5-ALA PDT. Additionally, de did not observe any change in the mice weight of all the groups (Fig.- 8(c)).

DISCUSSION

In the present study, we highlighted the impact of 5-ALA PDT on HCC, where we tested the therapy on HCC cell lines, as well as primary HCC patient and healthy donor samples, and at last on a humanized SCID mice model for the same. We also studied the impact of the secretome on human immune cells and cancer cells.

As a preliminary study, we demonstrated that the three HCC cell lines have similar expression patterns of the enzymes for the Heme-biosynthesis pathway, and thus any difference in the impact of PDT on these cell lines, will not be due to the difference in the levels of those enzymes. Further, when tested for varying doses of 5-ALA PDT, we demonstrated a dose dependent effect on these cell lines. Interestingly, our results demonstrate that the three HCC cell lines exhibit varying sensitivity for the therapy, with HuH7 evincing higher cell death than Hep3B and HepG2. We further investigated the impact of 5-ALA PDT on cell proliferation and cytotoxicity. Our results showed that 5-ALA PDT decreased proliferation, and increased cytotoxicity, 24 hours post illumination. This confirms that 5-ALA PDT induces cell death to the cell lines. Previously, Abo-Zeid *et al.* showed a cytotoxic and genotoxic impact by 5-ALA PDT on HepG2 cell line (19). Since we used in-house developed laser illumination system along with light fractionation, we did not use any previously published PDT dose for this study. A series of illumination and pause, called light fractionation, has been demonstrated to enhance the overall efficacy of the therapy by improving the influx of intracellular oxygen (20).

In order to observe the intracellular PpIX accumulation in the three cell lines at the optimal 5-ALA concentration, we cultured the cells with 0.6mM 5-ALA for varying incubation durations to measure PpIX fluorescence. Our results highlight an increase in the levels of intracellular PpIX fluorescence starting from 1 hour and kept on increasing until 18 hours. Interestingly, we also observed an extracellular increase in the PpIX fluorescence levels from roughly 4 hours which kept on increasing until 24 hours. From this, we can conclude that the cells maintain a constant metabolism of the exogenous 5-ALA until 18 hours, after which they get saturated and thus the cells started to secrete the PpIX out of the cells. Instantaneously, an equilibrium of PpIX synthesis and secretion might have been established. When confirmed with confocal

microscopy, we found that the intracellular PpIX fluorescence levels for the three cell lines are similar.

Taking in consideration the similar levels of ALAD and PPOX enzymes, along with similar intracellular PpIX accumulation in the three cell lines, it seems to be that the varying sensitivity of the cell lines for 5-ALA PDT is not associated to PpIX levels. There can be other intrinsic factors responsible like variation in p53 expression. The activation of p53 is a central factor for regulation of cellular proliferation, cell death and resistance to chemotherapy (21). p53 signaling is altered in roughly 70% of HCC patients, along with WNT signaling and telomerase promotor, and can affect the sensitivity of a patient for a therapy (22). Previous studies have shown that HuH7 has an over-expression of transcription factor p53, while Hep3B and HepG2 are p53 null and wild type, respectively (21). Yow *et al.* demonstrated that 5-ALA PDT on HepG2 causes cell death and upregulation of p53 (23). Similarly, p53 is upregulated in HuH7 after Fosgen-PDT (24). Tong *et al.* had hypothesized that p53 affects the sensitization of human fibroblasts for PDT mediated by Photofrin, also known as Sodium Porfimer (25). All these studies, thus, highlight the crucial role of this transcription factor in mechanism of PDT in general. Understanding its role in the efficacy of a therapy, could give crucial information about the mechanism of the therapy and thereby facilitate the clinicians in deciding a better therapy for the patients.

When the above-mentioned two doses of 5-ALA PDT were tested on primary tumoral hepatocytes, we observed a significant decrease in the viability of cells treated with 5-ALA PDT and 5-ALA alone over the time, with no significant difference between each other. This means that 5-ALA itself is causing toxicity to these tumoral hepatocytes and not the combined effect. Furthermore, when analyzed the impact of the therapy on healthy donor liver myofibroblasts

from three donors, we observed no significant difference in the viability, 24 hours post illumination. We also did not observe any significant increase in gene expression of activation markers, i.e. Collagen I, HSP47, αSMA, TIMP1, and MMP3. Similarly, 5-ALA PDT induced no significant increase in cell proliferation and increase in collagen I levels of HLMFs were detected with respect to non-treated cells. All these are the key markers of fibroblast activation. Hence, we can conclude that 5-ALA PDT does not exhibit any impact on HLMFs, in terms of viability and their activation. Previous studies had demonstrated, that fibroblast activation causes fibrosis in the HCC tumor microenvironment, which ultimately leads to poor prognosis (26).

To our knowledge, no one has presented impact of PDT on primary patient tumoral hepatocytes. We understand that our tumoral hepatocytes are not exclusive of other cellular components of tumor microenvironment. But to eliminate this possibility, we specifically harvested cells from the core of the tumor, which were later confirmed to be tumoral by biopsy. Additionally, these hepatocytes were cultured in high density to avoid proliferation of fibroblasts. Alongside, we are the first to demonstrate the impact of 5-ALA PDT on human healthy donor myofibroblasts, where we performed *in vitro* studies to examine the cellular viability, as well as activation of fibrosis markers.

Tumor secretome is one of the key components of its microenvironment which can limit the overall efficacy of a therapy by immune suppression, resistance to the therapy or future tumor recurrence. Hence, we studied the impact of 5-ALA PDT treated secretome, represented by conditioned media, on human activated PBMCs and cancer cells. Our results demonstrated that the 5-ALA PDT Treated secretome, can significantly increase the proliferation of PBMCs by two folds with no change in viability. This highlights clonal expansion where a certain

activated immune population rapidly proliferates when encountered by an antigen, which means immune activation. However, the immune population activated and its activation state remains to be studied. This is crucial as this will define the type of immune response generated by the therapy and further induction of immunogenic cell death. When tested for cytokine release, we did not detect any change for IL-2, IL-6, IL-10, TGF-β, TNFα and IFNγ (results not shown). It has been shown that an *in vitro* Pheophorbide-a mediated PDT on HepG2 cells can improve the efficacy of the therapy by activating an anti-tumoral immune response (27). Recently, Baydoun *et. al.* demonstrated that extracellular vesicles secreted by ovarian cancer cell lines treated with a novel folate coupled PS mediated PDT, could induce a CD4+ or CD8+ T cell activation (28).

Further, the conditioned media from 5-ALA PDT and the control 5-ALA Only induced a significant two to four-fold decrease in the proliferation of cancer cells with no impact on their viability. This reflects that 5-ALA or its intermediates is responsible for secretion of factors by the cancer cells, which are responsible for cytostatic effect on other cancer cells, by an autocrine or paracrine signaling process. These results are very important in context of a solid tumor like HCC since effective penetration of light decreases as a function of distance. Hence it is important to understand how PDT over the treated region will impact the untreated region of the cancer.

The efficacy of PDT is limited by PS bioavailability, light penetration, along with availability of intracellular oxygen. The liver pigmentation further decreases the light penetration. Additionally, liver is the center of heme-biosynthesis, due to which the healthy liver might actively incorporate exogenous 5-ALA to metabolize to PpIX. However, cancers are known to have disrupted metabolic pathways, which can affect the bio-transformation of 5-ALA to PpIX

in positive or negative direction. All these factors can contribute immensely to the success or failure of 5-ALA PDT. Hence, we treated a subcutaneous humanized SCID mice model of HCC with 5-ALA PDT where we demonstrated a decrease in the rate of tumor growth. On the whole, when compared for total average bioluminescence of each group during the experiment, we found a significant decrease for 5-ALA PDT treated group, with respect to Non-Treated group. We observed no change in the weight of the mice throughout the experiment, suggesting the biosafety of the treatment. For these *in-vivo* experimentations, we used 200mg/kg of 5-ALA which is used commonly in clinic with 18 hours of incubation, along with 45mW/cm^2 of illumination dose with 2 minutes of illumination and 2 minutes of dark.

The illumination device used here has been published recently while the protocol used has been standardized by our unpublished study (17). The 18 hours drug-to-light interval is based on our *in vitro* results which suggest higher PpIX accumulation in HuH7 cells until 18 hours. This rationale is also supported by other publications. Egger *et al.* had previously established the *in-vivo* efficacy of 5-ALA PDT on subcutaneously implanted rat hepatoma model, where they demonstrated that tumoral tissues had higher accumulation of PpIX and for longer duration, and thus, showed higher degree of necrosis, than the control groups (29). These findings were later verified by Otake *et al.*, where they demonstrated higher tumor selective PpIX based Photodynamic Diagnosis (PDD) and PDT for a chemically induced HCC (30). Nishimura *et al.* demonstrated 5-ALA mediated Photodynamic Diagnosis (PDD) to detect real-time fluorescence of PpIX in the tumor of all the 12 patients (10). Inoue *et al.* validated these results with a larger patient group (9). In context of other solid tumors of abdominal cavity, Wagner *et al.* highlighted the efficacy of Temoporfin mediated PDT for advanced biliary tract carcinoma, a type of liver cancer, for 29 patients in a phase II study (31). Recently, Quilbe *et.*

al. investigated the impact of a novel folate coupled PS mediated PDT on humanized SCID mice model of Pancreatic Adenocarcinoma, and demonstrated a decrease in growth of tumor over time (18). This, along with our study, highlights the possible application of PDT on other solid tumors. In summary, the present study demonstrates that PDT using 5-ALA pro-drug, can be an effective treatment for HCC, as proven by our *in vitro* and *in-vivo* experimentations. The therapy does not induce any effect on liver myofibroblasts. We also demonstrated an immune-stimulatory impact by the secretome of 5-ALA PDT treated cells which can also inhibit the proliferation of cancer cells. Nevertheless, 5-ALA mediated PDT needs to be tested on *in-vivo* mouse models in the presence of an immune system, in order to analyze its clinical functionality, and development of future pre-clinical and clinical studies. One of the most important application of our study is the development of 5-ALA PDT in combination with partial hepatectomy, where it could not only work as anti-tumor therapy, but also a visual aid during surgery. Additionally, the immune-stimulatory effect of 5-ALA PDT can help enhance the effect of various immunotherapies currently in trial for HCC (6). Our study is a crucial key for development of PDT for HCC treatment, thereby providing more therapeutic arsenal for fight against HCC.

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Author Contributions

A.K. carried out all the *in-vitro* experiments, collected and analyzed the data, and assured the writing of the manuscript. M.B. and A.K. planned and carried out the *in-vivo* experiments. A.Q. participated in various biological experiments. O.M. supervised the experiments being undertaken, and helped in data analysis. L.Z., B.L. and E.T. developed and calibrated the illumination devices. L.A. and F.C. provided the primary healthy donor liver myofibroblasts, along with technical expertise. S.M., E.B. and N.D. scientifically led the project, participated in data analysis, paper writing, and project funding. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

Ethics Statement

All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Institut Pasteur de Lille. Accordingly, all the animal experiments of the study were approved by the Ethics Committee of Institut Pasteur de Lille (approval number 2019041015585930).

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Authors names in bold designate shared co-first authorship

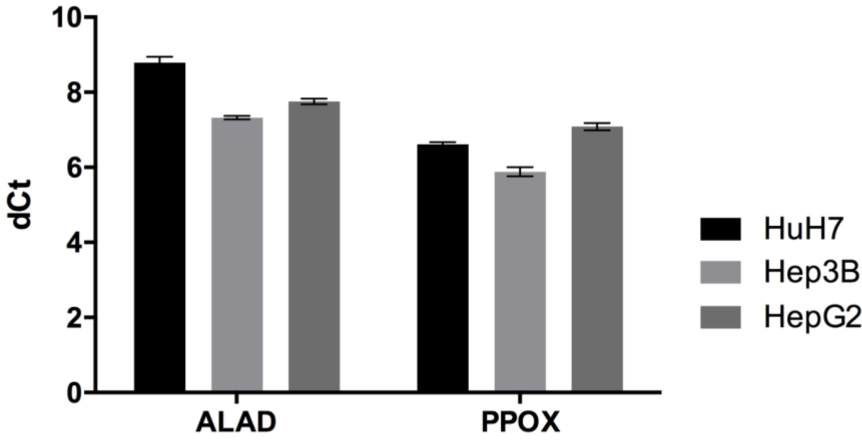


Figure 1: RT-qPCR based mRNA expression analysis of 5-Aminolevulinic acid Dehydratase (ALAD), and Protoporphyrinogen Oxidase (PPOX) genes in the three HCC cell lines. Results are presented in dCt. (n=3)

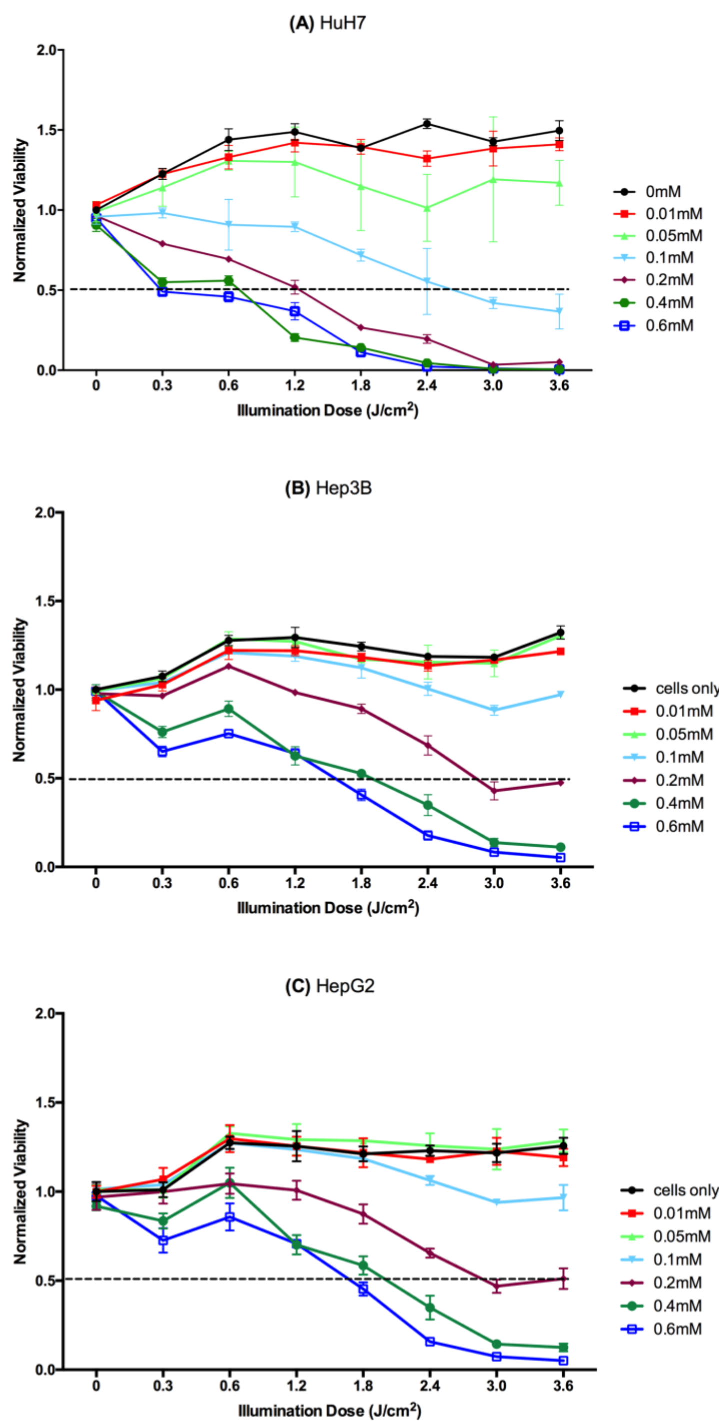


Figure 2(a): Viability analysis of HCC cell lines treated with different concentrations of 5-ALA and illumination doses, at 24hrs post illumination. The cells were Non-Treated, or subjected to different 5-ALA Concentrations (at 0J/cm2), or to different illumination doses, at 24hrs post illumination. The cells were Non-Treated, or subjected to different 5-ALA Concentrations (at 0J/cm²), or to different Illumination doses (at 0mM 5-ALA), or PDT Treated. The viability readings were then normalized by Non-Treated Control. (n=3).

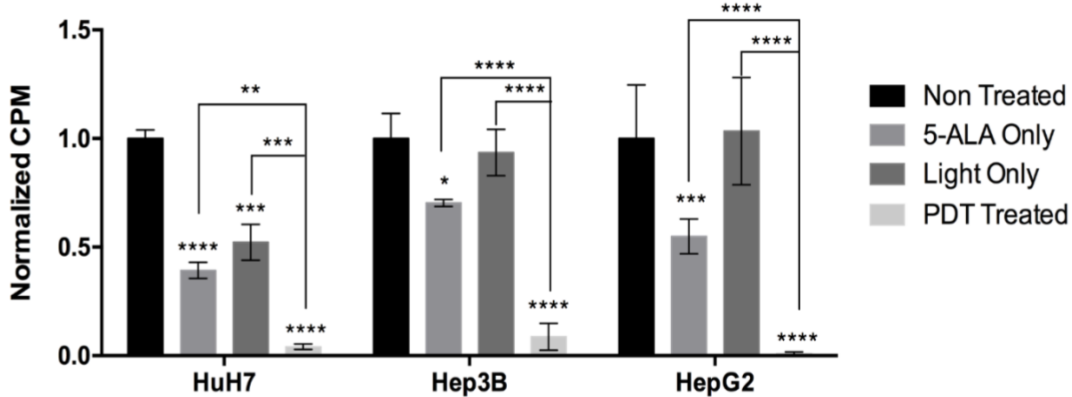


Figure 2bi): Proliferation assay of HCC cell lines treated by their respective IC50 dose, 24hrs post illumination. The cells were subjected to no treatment (Non-Treated), 0.6mM of 5-ALA Only (5-ALA Only), 0.6J/cm2 or 1.8J/cm2 of illumination (Light only) or PDT Treated. Two-way ANOVA Test was performed with $p \leq 0.05$ (*), $p \leq 0.001$ (**), $p \leq 0.0001$ (***) and $p \leq 0.00001$ (****) being considered statistically significant for the first and highly significant for the others. (n=3)

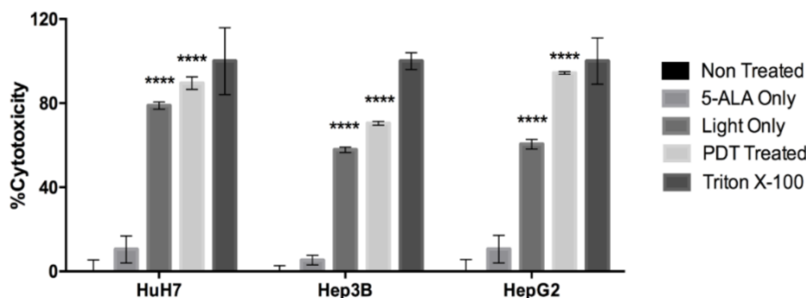


Figure 2(c): Lactate Dehydrogenase (LDH) release-based cytotoxicity was analyzed for HCC cell lines treated by 5-ALA PDT at 0hr and 24hr post illumination. The values are expressed as % Cytotoxicity where the values were compared with Non-Treated and apositive control (treated by Triton-X). Two-way Anova test was performed, with $p \leq 0.05$ (*), $p \leq 0.001$ (**), $p \leq 0.0001$ (***) and $p \leq 0.00001$ (****) being considered statistically significant for the first and highly significant for the others. (n= 3).

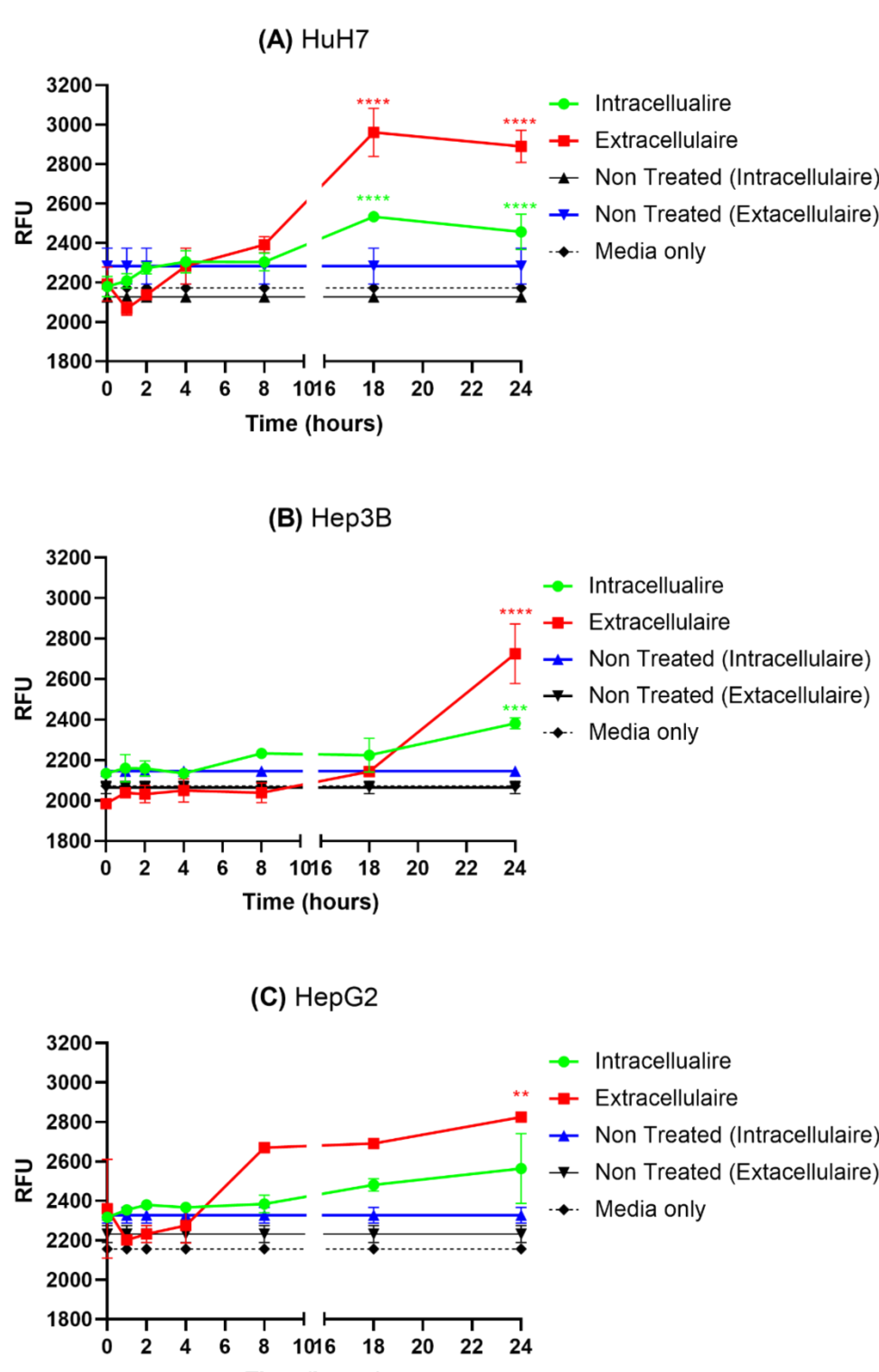


Figure 3: Fluorimetry based Protoporphyrin IX (PpIX) quantification of three HCC cell lines treated by 0.6mM of 5-ALA for different incubation periods (0, 1, 2, 4, 8, 18 and 24 hours). Extracellular PpIX levels were determinded by fluorimetric analysis of the conditioned media, while intracellular levels were measured by cells in fresh media. The values are represented in Relative Fluorescence Unit (RFU). One-way Anova test was performed, with p ≤ 0.05 (*), p ≤ 0.001 (**), p ≤ 0.0001 (***) and p ≤ 0.00001 (****) being considered statistically significant for the first and highly significant for the other.

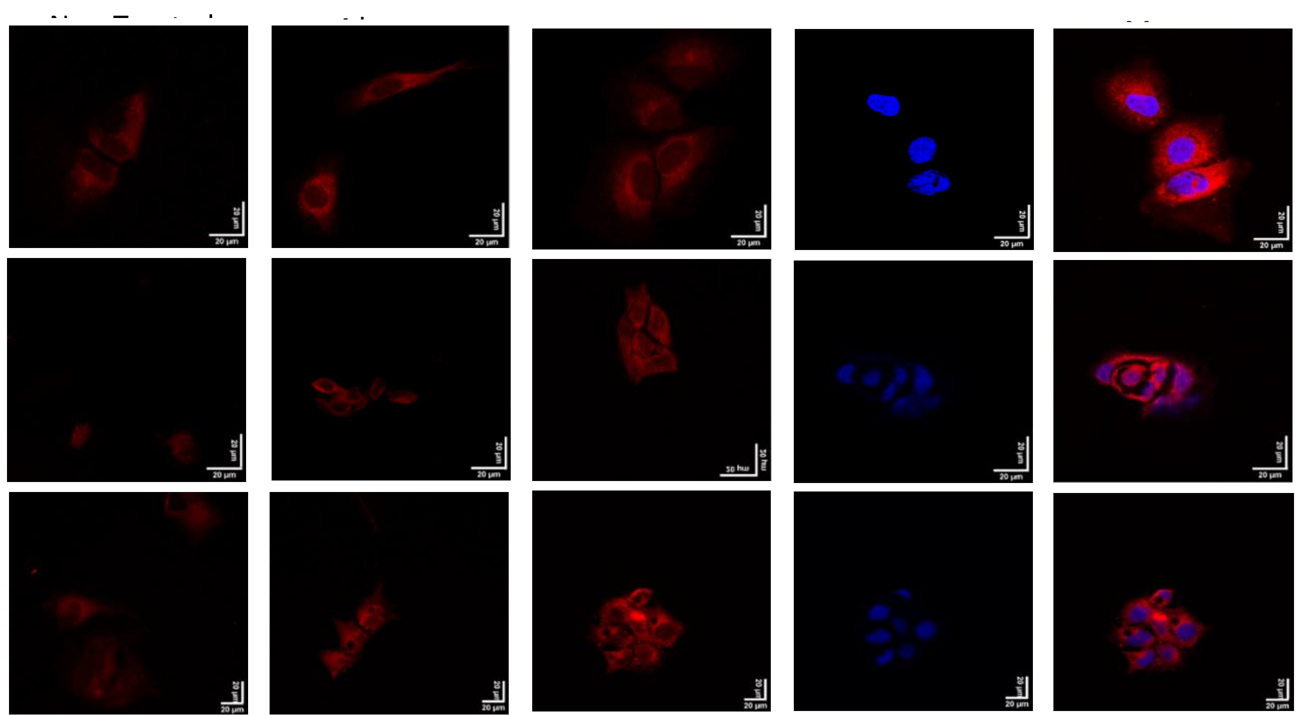


Figure 4(a): Confocal microscopy based PpIX accumulation in three HCC cell lines cultured with 0.6mM of 5-ALA for 4 hours or 18 hours.

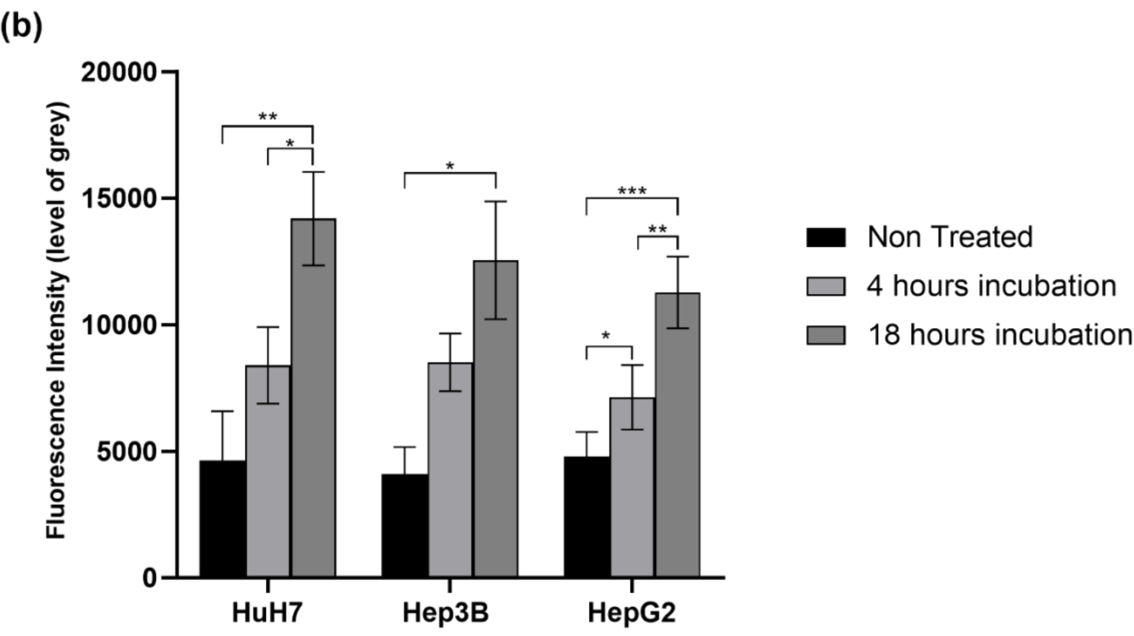


Figure 4(b): Confocal microscopy based semi-quantitative estimation of PpIX fluoresence in three HCC cell lines cultured with 0.6mM 5-ALA for 4 hours or 18 hours. The values are represented in Level of Grey. One-way Anova test was performed, with p ≤ 0.05 (*), p ≤ 0.001 (**), p ≤ 0.0001 (***) and p ≤ 0.00001 (****) being considered statistically significant for the first and highly significant for the other.

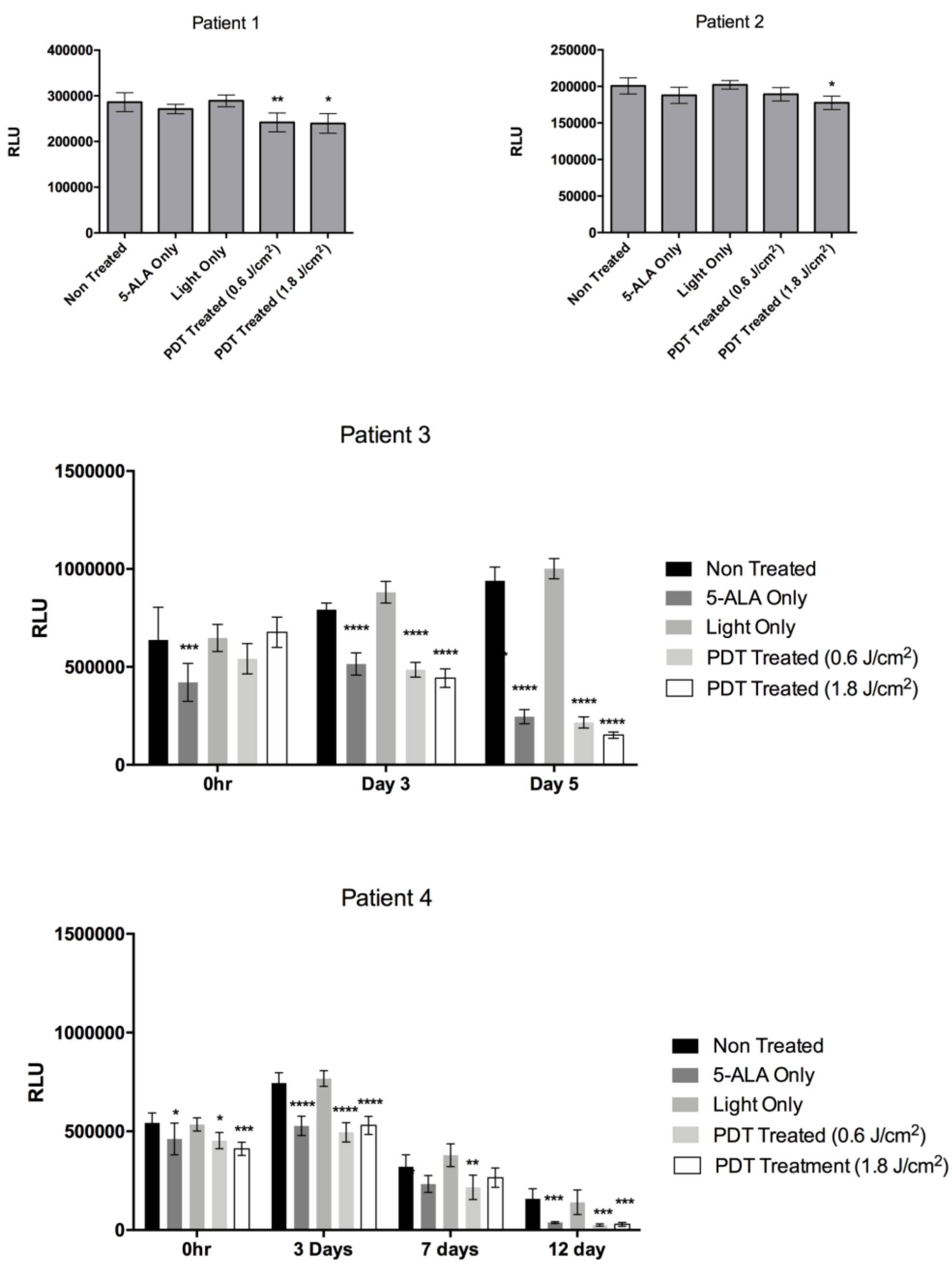


Figure 5: Viability analysis of tumoral hepatocytes treated by two different doses of 5-ALA PDT (0.6mM of 5-ALA with 0.6J/cm2 and 1.8J/cm2 of illumination dose) on four different HCC patient samples, at different time points post illumination. The values are expressed as Relative Luminescence Unit (RLU). Two-way Anova test was performed, with p ≤ 0.05 (*), p ≤ 0.001 (**), p ≤ 0.0001 (***) and p ≤ 0.00001 (****) being considered statistically significant for the first and highly significant for the others.

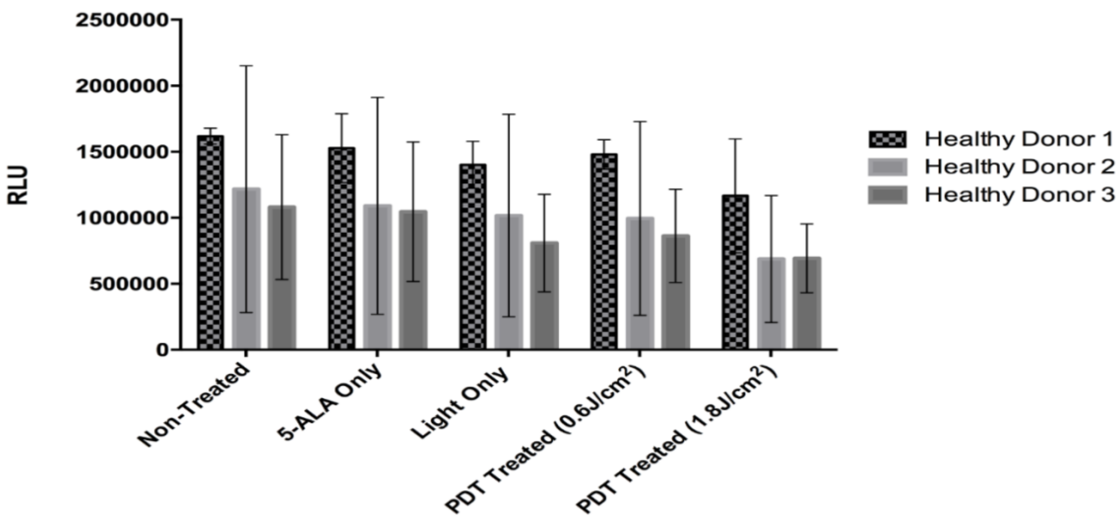
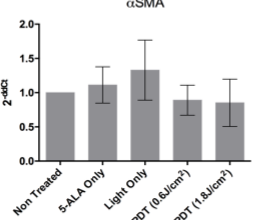
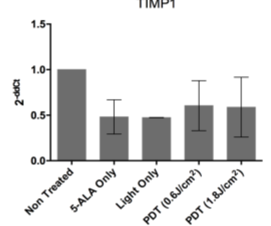


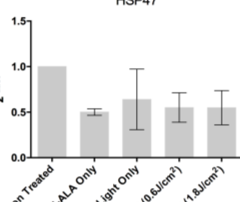
Figure 6(a): Viability analysis of healthy donor liver myofibroblasts (HLMFs) treated by two doses of 5-ALA PDT (0.6mM of 5-ALA with 0.6J/cm2 and 1.8J/cm2 of illumination dose) at 24hr post illumination. The values are expressed as Relative Luminescence Unit (RLU). Two-way Anova test was performed, with $p \leq 0.05$ (*), $p \leq 0.001$ (**), $p \leq 0.0001$ (***) and $p \leq 0.00001$ (****) being considered statistically significant for the first and highly significant for the others. (n=3)

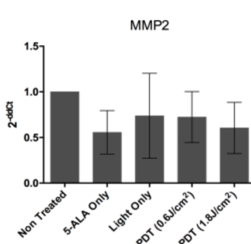
(A) RT-qPCR based gene expression analysis

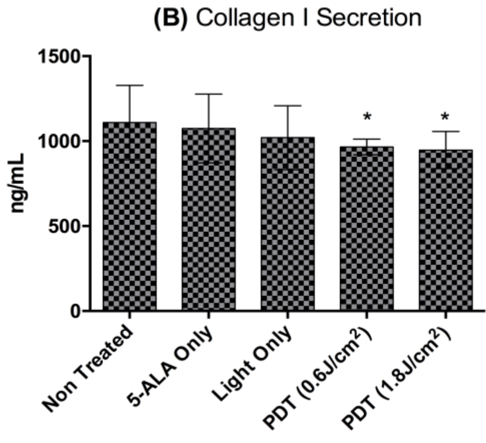
Collagen 1











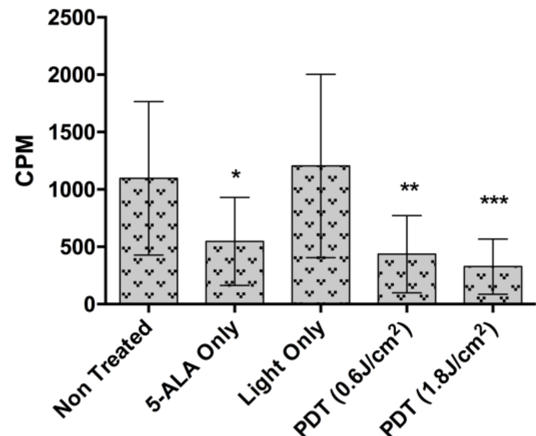


Figure 6(b): Analysis of fibrosis induced by 5-ALA PDT at two doses, 24hr post illumination, by (A) ***RT-qPCR based gene expression analysis of Collagen 1, αSmooth Muscle Actin (SMA), Tissue Inhibitor of Metalloproteinase 1 (TIMP1), HSP47, Matrix Metallopeptidase 2 (MMP2) for different conditions, 24hrs post illumination*** *(B)* ***ELISA based analysis of Collagen I secretion****; (C)* ***Cellular proliferation analysis****. Two-way Anova test was performed, with $p \le 0.05$ (*), $p \le 0.001$ (**), $p \le 0.0001$ (***) and $p \le 0.00001$ (****) being considered statistically significant or highly significant respectively. (n=3)*

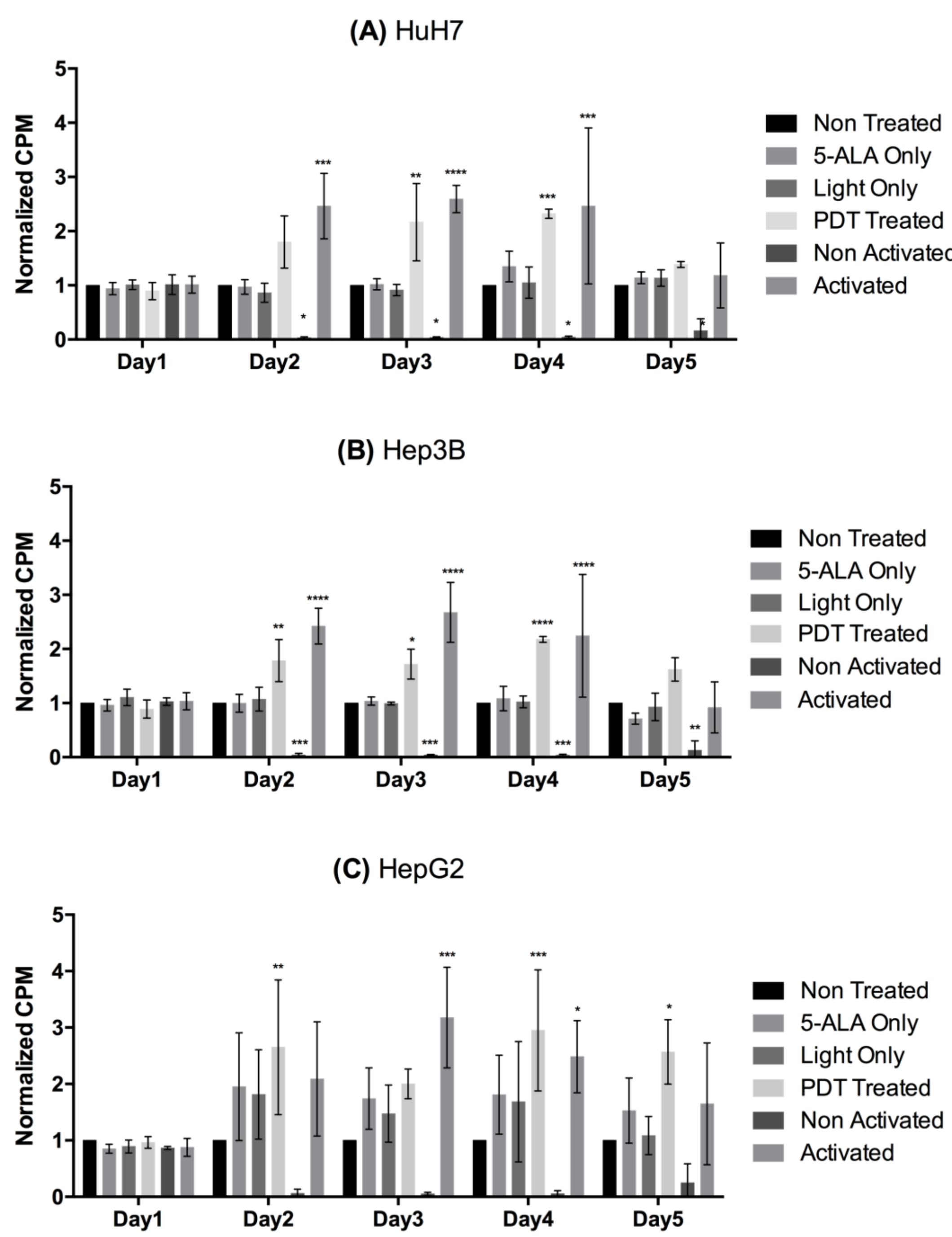


Figure 7(a): Impact of conditioned media on proliferation of activated human PBMCs. Two-way Anova test was performed, with p ≤ 0.01 (*), p ≤ 0.001 (**), and p ≤ 0.0001 (***) being considered statistically significant for the first and highly significant for the others. (n=3)

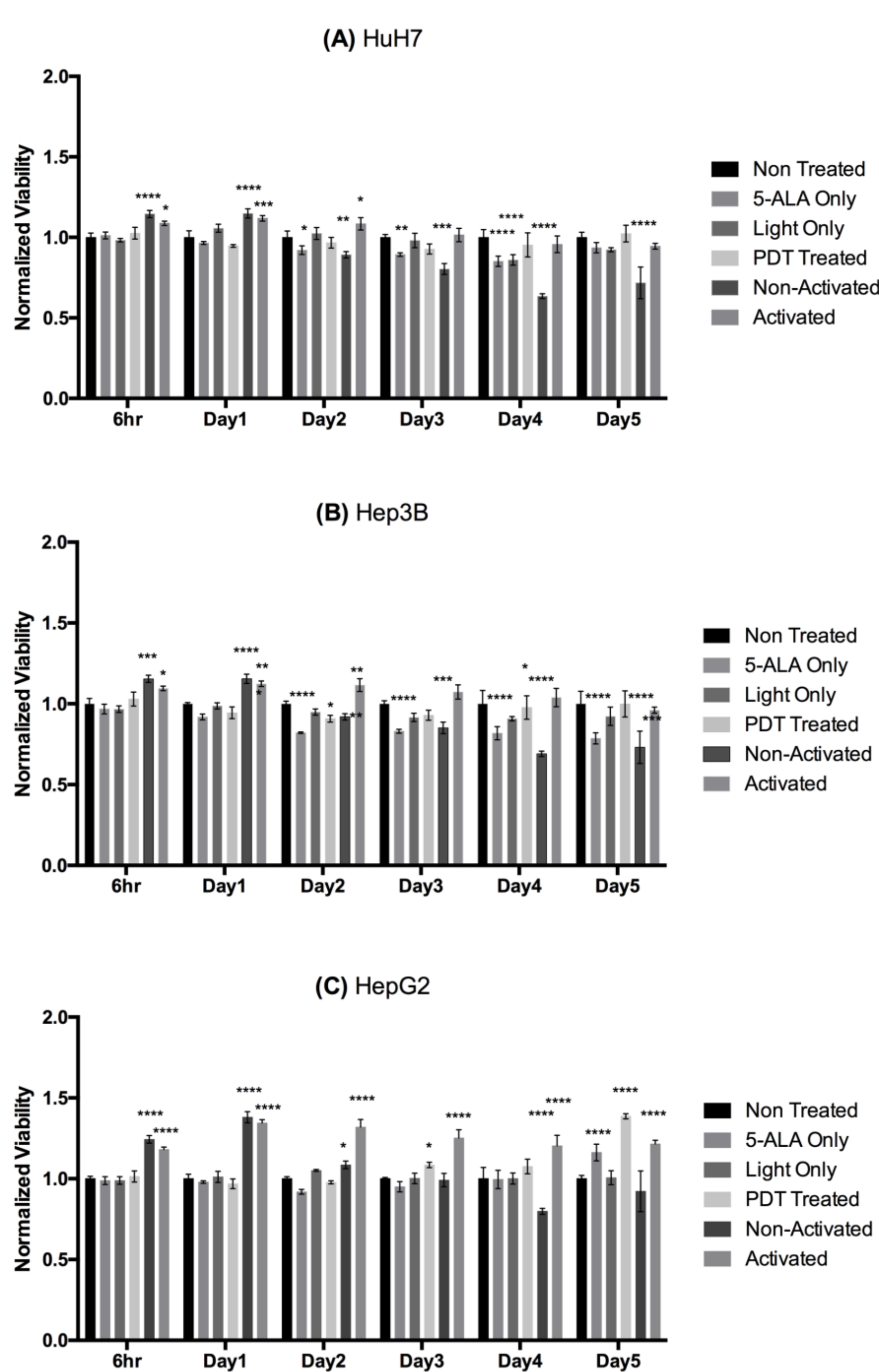


Figure 7(b): Impact of conditioned media on viability of activated human PBMCs. Two-way Anova test was performed, with $p \leq 0.01$ (*), being considered statistically significant.

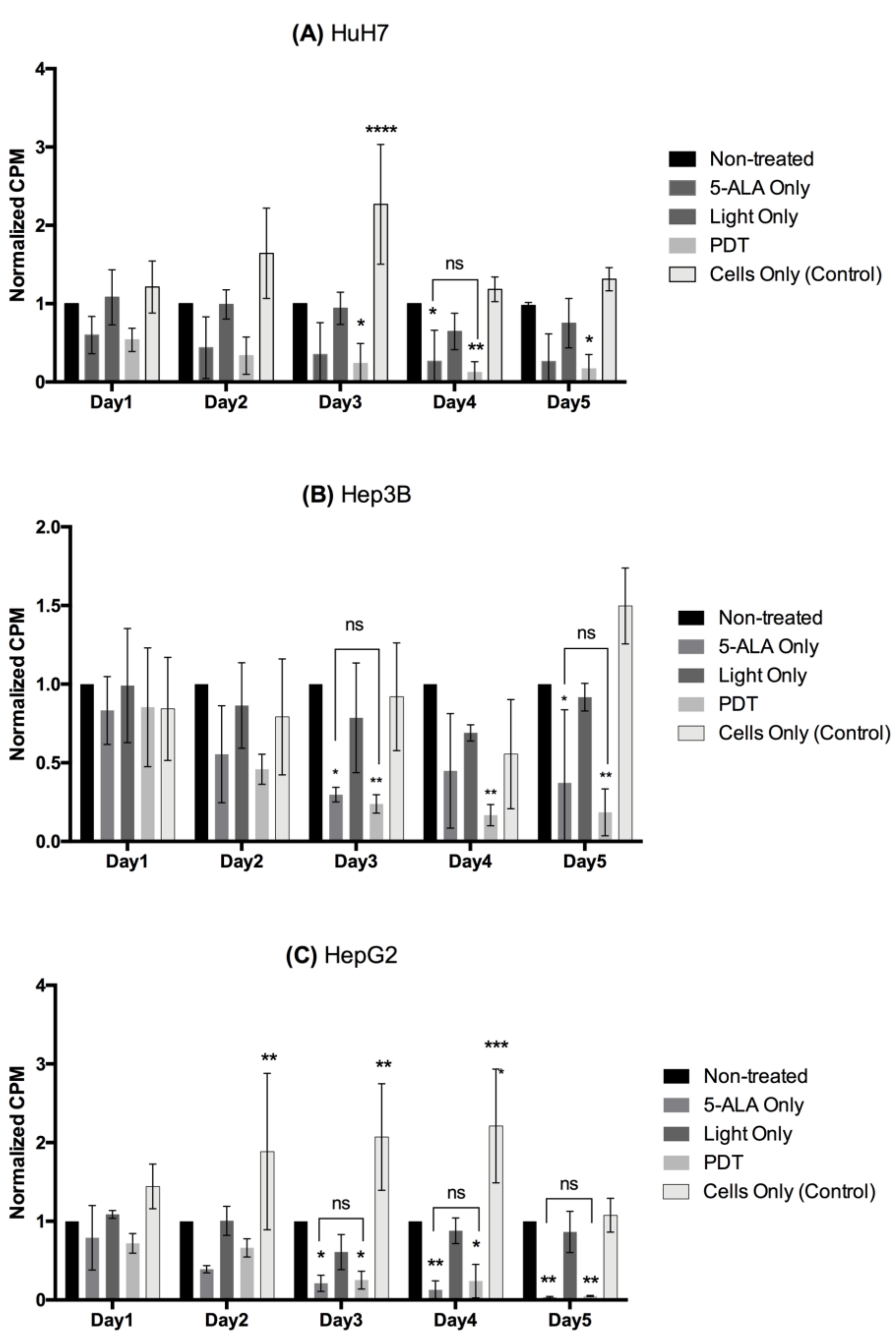


Figure 7(c): Impact of conditioned media on proliferation of cancer cells. Two-way Anova test was performed, with p ≤ 0.05 (*), p ≤ 0.001 (**), p ≤ 0.0001 (***) and p ≤ 0.00001 (****) being considered statistically significant for the first and highly significant for the others. (n=3)

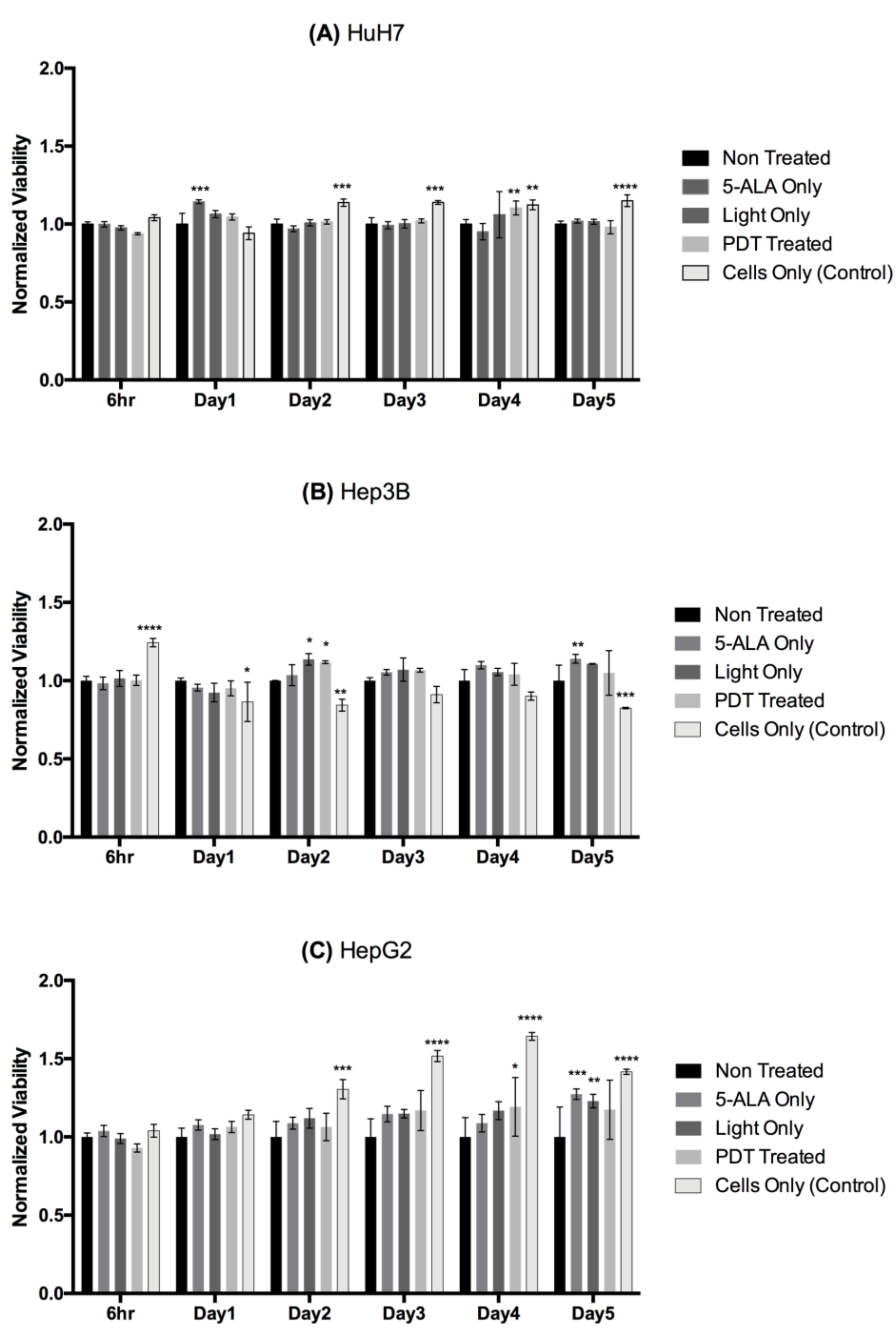


Figure 7(d): Impact of conditioned media on viability of cancer cells. Two-way Anova test was performed. (n=3)

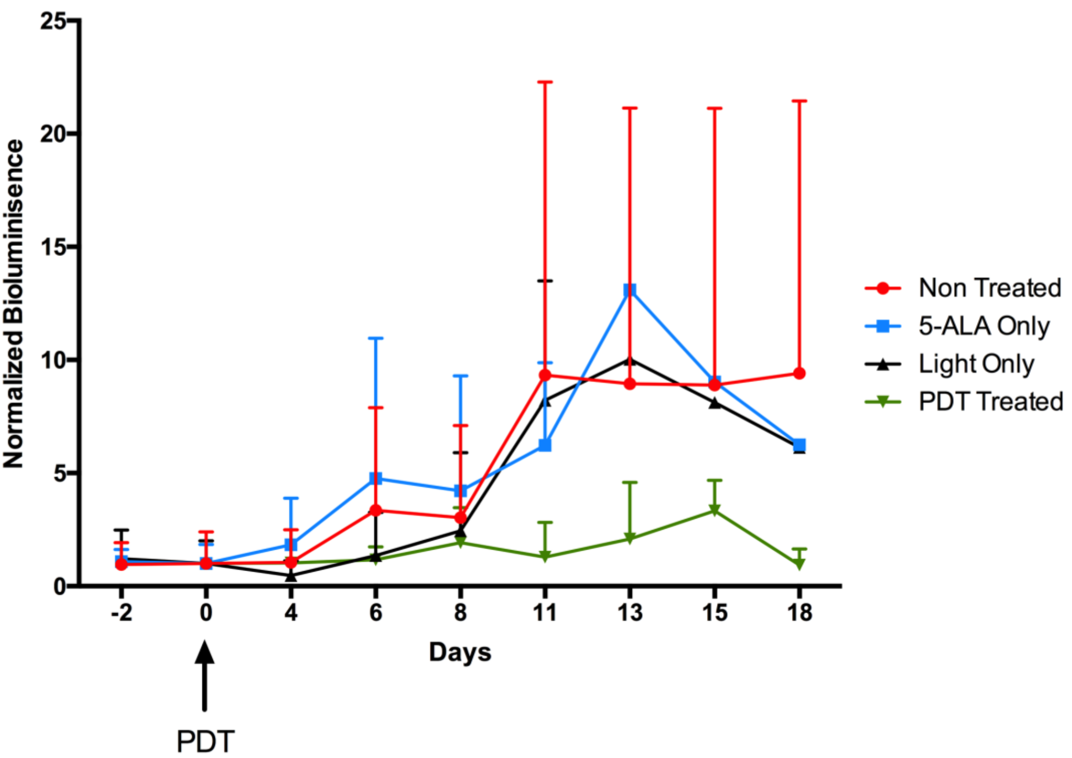


Figure 8(a): Normalized bioluminescence of SCID mice with humanized HCC tumor treated with or without 5-ALA PDT

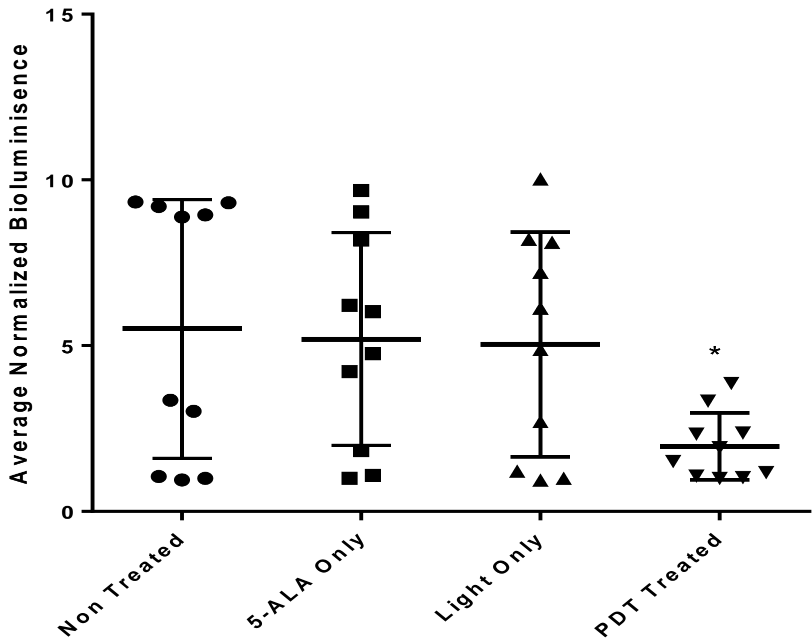


Figure 8(b): Average normalized bioluminescence of different mice groups starting from Day 0 to day 20 post illumination. Two-way Anova test was performed, with p ≤ 0.01 (*) being considered statistically significant.

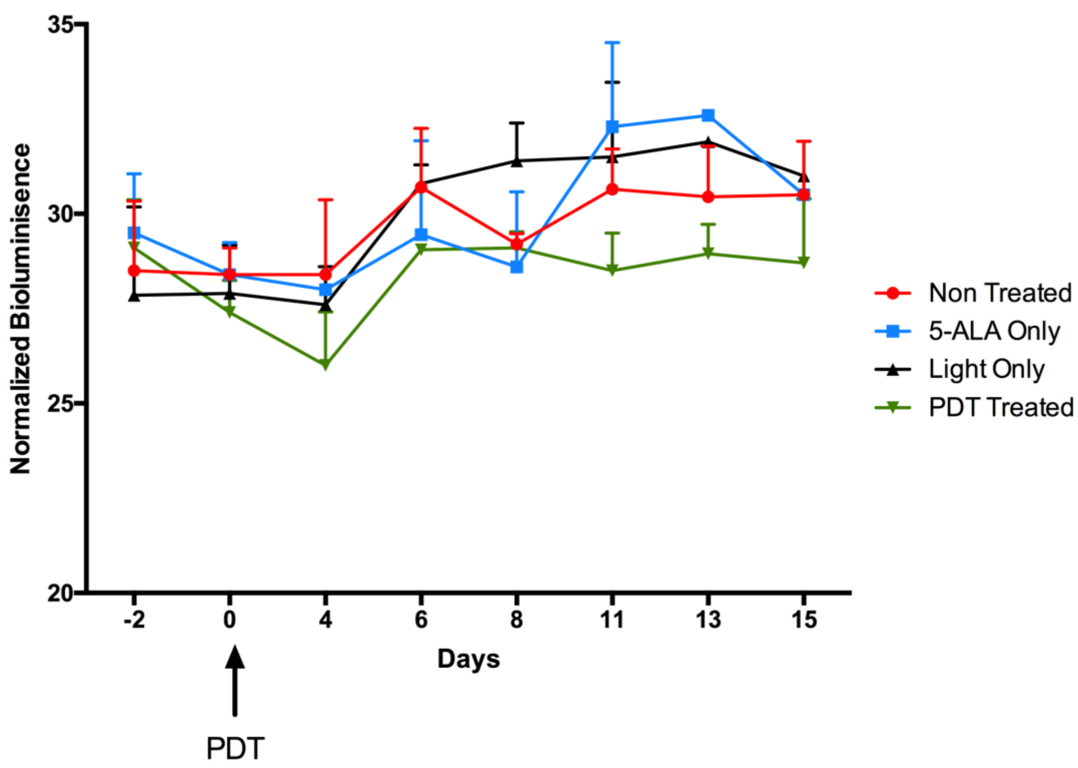


Figure 8(c): Average of mice weight of different groups over a time period from 2 days prior to illumination to 20 days post illumination.

CHAPTER 6

THESIS DISCUSSION AND FUTURE PERSPECTIVES

A. Why PDT for HCC treatment?

We have previously highlighted all the available treatment options for HCC patients at various stages of cancer, along with their contraindications, and possible future combinatorial application. We also discussed the grey area in which the various systemic targeted therapies are at present, and a ray of hope immunotherapy can be. Even with these modalities, however, HCC has a very limited number of possible treatment options available in the clinic. The most widely opted treatment option is the surgical approach by either liver transplantation or partial hepatectomy. A combinatorial approach can endeavor in certain cases, where some potential candidates for surgical resection are treated with a radio or chemotherapy. However, this has a major shortcoming of a limited number of liver donors available, the complexity of hepatectomy, and tumor recurrence.

We also scrutinized various benefits PDT imparts, mainly its two-layer action mechanism which offers higher tumor-selective outcomes, with lesser side effects. Besides, PDT has demonstrated anti-tumoral immune response in various studies and has been approved for clinical use in various dermatological disorders.

We propose PDT as adjuvant therapy for HCC, where 5-ALA mediated PDT shall be used as an intra-operative approach. The patient shall be administered with 5-ALA prior to the hepatectomy, and towards the end of the surgery, the surgeons shall illuminate the liver cavity by appropriate illumination systems. 5-ALA PDT shall act more like a residual tumor clean-up therapy, which is inoperable, too small, close to blood vessels, or undetected during the surgery. These are very common situations during partial hepatectomy, where the location, size, and volume of the tumor decide the success of the resection. Furthermore, 5-ALA PDT can tally up additional benefits where the PpIX based real-time fluorescence can be utilized for tumor detection during the surgical process, providing a visual aid to the surgeon. Surgeons nowadays are equipped with better 3-D surgical visualization systems, which can conceive better images with fluorescence-based tumor detection. Such methods have been successful in breast cancer, glioblastoma, cervical cancer, and other skin disorders where a fluorescent probe-guided surgery has assisted the clinicians in successful identification and eradicate the neoplasm [453].

However, the most crucial impact of 5-ALA PDT is its after-effect. We previously highlighted how the oxidative stress generated during PDT can resemble the wound-healing process thereby inducing the secretion of cytokines and chemokines responsible for the infiltration of immune cells. PDT is already considered as a physical modality, which can induce immunogenic cell death where cancer-associated antigens released by the therapy are taken up by infiltrating immune cells to activate a possible anti-tumoral immune response. The tissue damage and insult induced by PDT can generate infiltration of immune cells, which can replace the exhausted T cells of the TME. Such exhausted T cells or immune-suppressive population is quite prevalent in HCC TME. It can be interesting to combine PDT with an immune check-point inhibitor-based immunotherapy, where these exhausted immune cells can be replenished or re-invigorated. Through our study, we focused on the possible anti-tumoral immune response induced by 5-ALA PDT in the context of HCC.

Our study also highlighted a possible decrease in tumoral proliferation by factors released by 5-ALA PDT sensitized cancer cells. This can be very nifty for HCC patients, which exhibited less response to chemotherapy prior to surgical resection, and thus control the growth of more aggressive and chemo-resistant HCC.

B. What is the proposed strategy for HCC treatment by 5-ALA mediated PDT?

PDT requires proper penetration of light by an optimized light source, in order to activate the PS and induce its cytotoxic effect. Our experience with dermatological diseases has indicated that the red light used for PDT, has a penetration of just a few millimeters. Hence, it is evident that for solid internal tumors, PDT cannot be used as an independent modality.

Therefore, we propose PDT during intra-operative procedures during which the tumoral cavity shall be completely or partially open through which the illumination source can enter and thereby generate the photodynamic effect.

However, there are major problems in the successful implementation of intra-operative 5-ALA PDT for HCC treatment, due to which there is a need to develop a proper strategy for effective execution. Before we discuss any possible treatment strategy for HCC, we should summarize all the key characteristics of neoplasm.

Hypervascularization: HCC is highly vascularized, which exhibit neoangiogenic properties. VEGF expression is up-regulated in HCC, which is responsible for cell migration and angiogenesis. Vascular features are visible for various HCC tumoral explants and histological analyses. Vascularization is crucial for HCC growth as it supports oxygen and nutrition supply along with metastatic advantage to the growing neoplasm. This feature of HCC has been very beneficial for anti-VEGF-based targeted therapies along with some other chemotherapies, which show increased drug accumulation due to HCC's hypovascularized tumor core.

Hepatic arterial blood supply: As we had described previously, the HCC tumor core derives blood through the hepatic artery while the healthy liver tissue obtains blood from the hepatic artery as well as the portal vein. This key feature is utilized in TACE and TARE.

Disrupted bilirubin secretion: This is a crucial marker of liver function, as HCC exhibits bilirubin retention and elevated levels, and is used as a diagnostic and prognostic marker for HCC. ICG, when administered intravenously prior to surgery is used to estimate liver function and safety limit of hepatic volume to be resected. Here, ICG binds to plasma proteins, which emits light with a peak wavelength of around 830 nm when illuminated with NIR light.

Chronic inflammation: HCC progresses in the backdrop of an inflammation induced by chronic liver disease or injury. The malignant hepatocytes themselves secrete cytokines and chemokines, which are responsible for HCC immune escape and increased activation and proliferation of immune-suppressive cells. Here, the most prevalent immune cells are TAMs, Tregs, and Th17 cells. These immune components are the target for various immunotherapies that are being developed for HCC treatment.

Fibrosis: Hepatic fibrosis imparts the characteristic tumoral architecture to the HCC, with high levels of HA, glypican-3, decorin, and laminin-5. Such factors assist in tumoral self-renewal, differentiation, and chemo-resistance, thus fibrosis often leads to poor prognosis.

Therapies capable of reversing the fibrosis process are being developed, but what remains a challenge is the impact of current treatment modalities on fibrosis.

Liver as a center for heme-biosynthesis: Heme is primarily synthesized in the bone marrow and the liver. This means that the exogenously incorporated 5-ALA shall be readily metabolized to PpIX by the healthy hepatocytes as well, which shall thereby induce background fluorescence and possible sensitivity for 5-ALA PDT. However, healthy non-tumoral cells have an active feedback system by which they can regulate the intracellular levels of heme and PpIX, and can also secrete them out of the cells. Additionally, they will convert PpIX into heme, which cannot be photosensitized, and thus limit the overall hepatocyte sensitivity [454].

Liver pigmentation: The liver is a heavily pigmented organ, with its peculiar reddish-brown color due to blood saturation. This pigmentation can certainly limit the overall penetration of the light and thereby limit the efficacy of the therapy.

Based on these issues, we propose intra-arterial infusion of 5-ALA by vessel embolization, similar to TACE/TARE, approximately 18 hr prior to surgery. This shall be followed by partial hepatectomy guided by PpIX fluorescence and photo-excitation by optimal illumination apparatus. With the image-guided catheter-based insertion of 5-ALA, we could ensure highly selective and specific accumulation of PpIX right into the tumor bed, and with vessel embolization, pro-drug would persist in the tumor rather than getting washed-off by the first-pass effect. Here, we propose a higher drug-to-light period, *i.e.* 18 hr, since our study and previous results demonstrated higher PpIX accumulation in-*vivo* HCC model than at clinically used 4 hr period. By this increased incubation, we can assume that the 5-ALA absorbed by healthy hepatocytes can be metabolized and thereby secreted out, to reduce background PpIX fluorescence and healthy hepatocyte sensitivity. We also propose that 5-ALA can be administered with an iron-chelating agent which can eliminate the iron in the microenvironment, thereby inhibiting the metabolism of PpIX into heme [455], [456]. In an *in-vivo* study, Chang *et al.* highlighted the use of iron chelator, 1,2-diethyl-3-hydroxypyridin-4-one (also known as CP94), caused double PpIX-based fluorescence than 5-ALA alone group and exhibit reduced skin photosensitization [457]. Such methods have not been tested for HCC, and can be studied in future. Curnow *et al.* demonstrated that simultaneous light

fractionation and iron chelation by CP94 can considerably enhance the efficacy of the therapy by up to five-folds, roughly twice produced by the independent application [458]. Vitamin D has also been proven to enhance PpIX levels in the cells [459]. Such drug formulations along with a very strategic drug delivery system can significantly improve the efficacy of the therapy.

However, after PS accumulation, the next major step is the development of an innovative light delivery system equipped with oxygen monitoring techniques. Laparoscopic surgery is quite a norm for HCC surgical resection, and hence a light system, which could be coupled with this, could prove to be beneficial for surgeons. At OncoThAI, we have developed innovative illumination methods for various skin disorders and solid tumors like glioblastoma. Recently we have published a clinical application of a balloon-based illuminating device for intra-operative PDT protocol, successfully employed for 5-ALA PDT-based treatment of glioblastoma [460]. We, hence, propose to use the same illumination system for HCC. With an efficient, optic fiber-based laser illumination setup, we could enhance the penetration of light through the pigmented liver and thereby induce a cytotoxic effect. An illustrative description of the proposed strategy is shown in Figure 35.

Even though this is not the scope of the present study, but an immune checkpoint inhibitor-based immunotherapy can be coupled with PDT for patients with increased Treg infiltration of an immune-suppressive TME. Hence, immunotherapy will boost the anti-tumoral immune response that might be generated by the therapy.

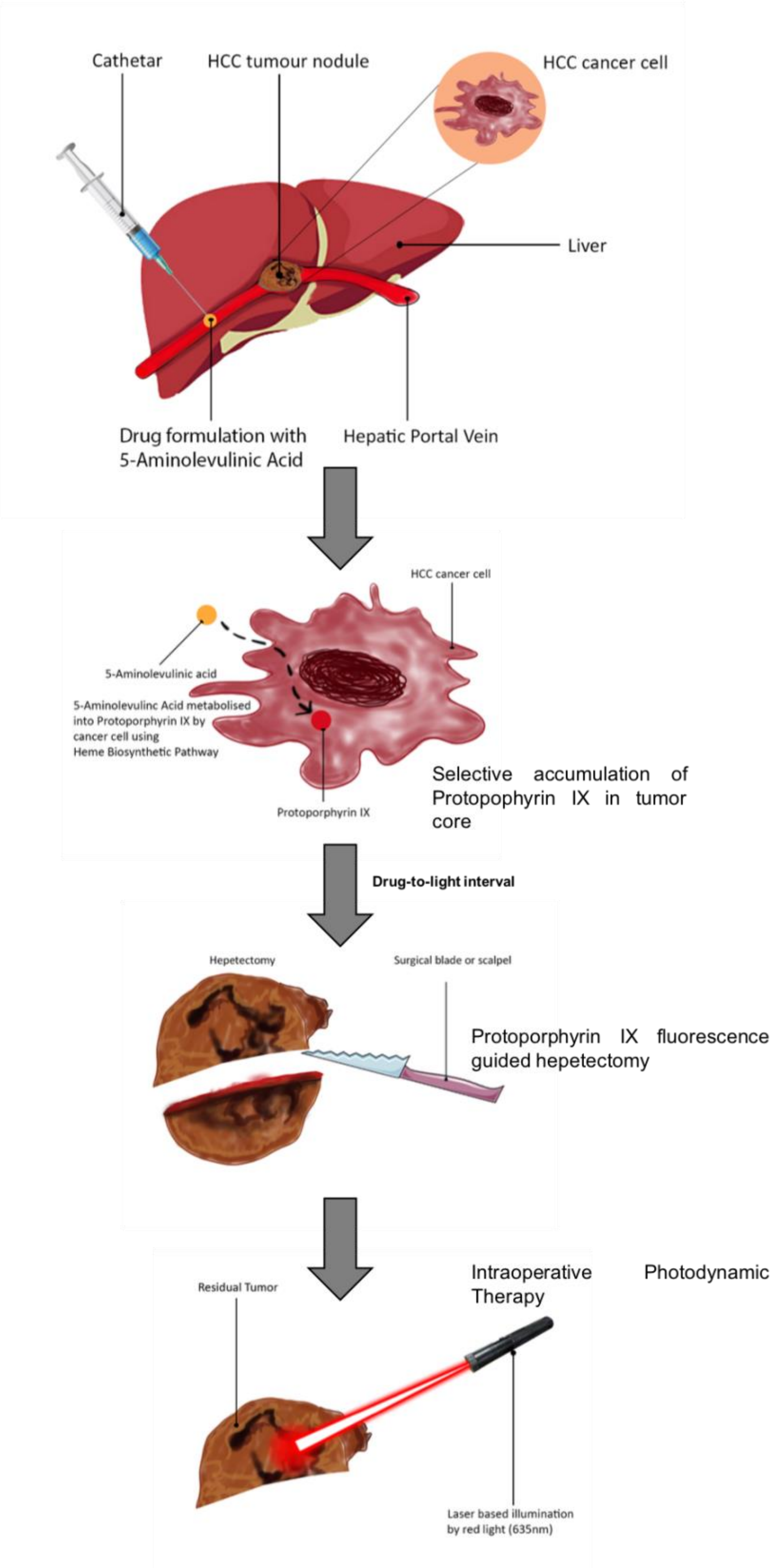


Figure 37: *A proposed strategy for the treatment of HCC. Before hepatectomy, patients will be given a suitable drug formulation of 5-Aminolevulinic Acid through a catheter and thereby embolized, in order to enhance Protoporphyrin IX accumulation in the tumor core. After an ambient drug-to-light interval, a Protoporphyrin IX fluorescence guided hepatectomy shall be performed. In the end, the cavity shall be illuminated by suitable illumination devices along with proper dose and protocol.*

C. What are the challenges and future work to be accomplished?

The current study is the beginning of a possible future treatment option for HCC and hence needs many further experiments before we could discuss a study at clinical setup. For sake of an easy discussion, we have divided our future perspective for Hepato-PDT into two fronts:

(1) Further validation of proof-of-concept by *in-vitro* and *in-vivo* studies; and

(2) Development of illumination devices during intra-operative procedures for Hepato-PDT.

As a part of our study, we proved the efficacy of 5-ALA PDT over HCC cell lines, various healthy donor and patient primary samples, and *in-vivo* model along with its safety and innocuous impact. However, our study opened many other questions, which are yet to be answered:

5-ALA PDT treated conditioned media decreases cancer cell proliferation. Conditioned media is recuperated 24 hr after illumination, and thus it contains all the factors and molecules secreted by the cells due to PDT. How does 5-ALA PDT treated by conditioned media could inhibit the proliferation of the cancer cells? What are the factors responsible for this action?

5-ALA PDT treated conditioned media induces clonal expansion of human activated PBMCs. What are the factors responsible for this action? Which immune population is being activated here?

Our *in-vivo* study in a xenograph based SCID mice model demonstrated a decrease in tumor growth rate by 5-ALA PDT. We now wish to investigate the impact of PDT in presence of a human immune system and compare it with our present results. Furthermore, it would be interesting to validate the results in other preclinical models.

Besides, our study opened many other interesting frontiers, which we shall explore in upcoming years, which are enlisted below:

Role of exosomes in the underlying processes

We have observed that PDT treated conditioned media has an impact on human immune cells and cancer cells. Extracellular vesicles and exosomes are often considered vehicles of intercellular communication, where they can carry different molecules, like microRNA, which can bind to their target cells and transfer the mediated signal. Our research team has knowledge and expertise in exosome harvest and analyzing their impact on various cells in terms of their activation and metabolism. Hence, we would focus on the role of these exosomes and their intracellular impact. This could highlight the indirect impact of PDT, something that is yet to be understood in the field. Furthermore, it could also help to develop markers of poor or good prognosis, post-PDT treatment through regular patient surveillance.

Immunogenicity of 5-ALA PDT

A long-lasting indirect impact of an anti-tumoral modality is the demand of present-day clinicians. Therapies, which can induce immunogenic cell death, are gaining more attention than those which can simply induce a short-term anti-tumoral impact, with no or minimal immune response. In recent few years, PDT has demonstrated the potential to induce heavy tumoral tissue damage that causes a release of tumor-associated antigens and generates hallmarks of immunogenic cell death. However, these limited pilot studies have only investigated one or two aspects of ICD by PDT and did not evaluate if these tumoral antigens could activate the human immune cells. Many of these studies are based on cancer cell lines of immune cells, which are not necessarily from humans. In our laboratory, we have years of experience in the culture of human PBMCs from healthy donors, and to activate and mature them into DCs, the antigen-presenting cells known to induce an anti-tumoral immune response by intake of the tumor-associated antigens. We propose to trigger these DCs with tumor antigens released during PDT, and thereby follow their maturation and induction of cytotoxic T cells for an anti-tumoral response.

Development of HCC based organoids

It is widely recognized that cell lines based *in-vitro* studies are not the best models for analyzing the efficacy of 5-ALA PDT. Conditions like PS targeting, ambient light penetration, and intratumoral oxygen, cannot be regulated and optimized to actual clinical conditions. However, *in-vitro* studies are necessary for understanding cell-to-cell interaction and monitoring, besides being cheap and ethically viable. Keeping this in mind, our team has started focusing on developing organoids and optimizing them for PDT-based studies. Our pilot study to investigate the impact of PDT on organoids of different cancer cell lines has generated positive results (yet to be published). Hence, we would continue expanding in this new domain and develop organoids for HCC, to investigate 5-ALA penetration, PpIX generation, the limit of light penetration, oxygen availability, and understand overall PDT efficacy, something that is tough to replicate in an *in-vivo* model which, moreover, are destined to disappear in time.

Development of robust illumination apparatus for Hepato-PDT

At our unit OncoThAI, we are also working on in-house-developed illumination devices to be used for various PDT procedures. During our study, we used *in-vitro* and *in-vivo* illumination set-ups developed by our team, and we demonstrated robustness, customizability, flexibility, and reproducibility by the set-up. The team has also developed clinical illumination apparatus, which have been successfully utilized during a clinical study for intra-operative 5-ALA mediated glioblastoma treatment. In the future, we have to develop a similar illumination strategy for Hepato-PDT. Knowing that HCC surgical resection is often performed using laparoscopy and is also being evaluated by robotic surgery, we would work on combining our illumination apparatus with them to yield easy clinical applicability. Such laparoscopic devices are coupled with fluorescence-based visualization which could be used for PDD during hepatectomy. We have also worked on various programs and simulations to analyze the oxygen levels during PDT protocols. Applying this knowledge to Hepato-PDT could be beneficial.

D. What is the future work in Hepato-PDT for the PDT community?

The work presented here and before have laid a ground basis for a new innovative therapeutic strategy for the treatment and management of HCC patients. Nevertheless, there is a lot of work, which needs to be done, and hence we seek a helping hand from the research community, including clinicians and physicians, to develop and share their insight for Hepato-PDT.

For clinicians

To our knowledge, there have been some small-scale clinical studies, which tried to evaluate the efficacy of 5-ALA mediated PDT, and we have shared their study outcome previously. However, there has been no study to compare PDT with other non-surgical modalities for HCC, namely TACE/TARE, and other systemic therapies. This is a crucial step in order to understand if PDT could actually add something to the existing arsenal against HCC, in terms of tumor burden reduction, anti-tumor immune response, reduced recurrence, overall patient survival, and improvement in patient life quality. We have already seen many similar studies undertaken for the evaluation of sorafenib and other tyrosine kinase inhibitors. We do understand that a similar study for PDT can be a major challenge as we propose PDT as an intra-operative modality for HCC, and hence there cannot be any concrete method to understand the efficacy of the therapy. However, we believe that we could still compare the intra-operative procedure with regular hepatectomy. Nevertheless, we still seek better ideas to comparing PDT with other non-surgical therapies.

Furthermore, we need strong guidelines for the implementation of PDT for HCC treatment. We understand that there is a long way for PDT to be recommended or approved for HCC, but our clinician friends can initiate discussions on guidelines at various national and international liver associations like the European Association for the Study of the Liver and the American Association for the Study of Liver Diseases. This shall include a standard PDT dose and protocol, namely the 5-ALA dose, the drug-to-light interval, the illumination dose, and protocol. Alongside, we need to consider the stage and patients' pathological condition at which PDT should be recommended, along with a possible combinatorial approach with immunotherapy.

For scientists

PDT is at a stage where it is coming out from its infancy to gain the limelight of academia and industry interest. However, there are a lot of unanswered questions and unventured endeavors for the rest of the community. We still need to know which intracellular factors regulate the cell death elicited by PpIX mediated PDT effect so that we could distinguish between patients who shall be resistant or over-sensitive to the therapy. At the same time, we need to find factors or molecules, which can increase the intracellular PpIX accumulation and methods to increase intracellular oxygen in order to augment the net impact of the therapy. Certainly, we need to study more about the immunogenicity of PDT and understand how it evolves with time, which shall be helpful in post-treatment immune monitoring of the patients. We have already seen a lot of new improved PSs and 5-ALA modifications, which have been shown to ameliorate the efficacy of the therapy. Their patient safety is yet to be determined. However, we also seek new innovative illumination devices, which shall be less invasive, can be customized, easy to employ and sterilize, with appropriate illumination and oxygen level monitoring, and shall be able to give uniform light dose throughout the procedure. For intra-operative procedures, coupling it with laparoscopic machines shall be even more high-yielding.

E. Parting words

The study presented here does not present any ground-breaking or revolutionary ideas, but in fact, try to establish new innovative therapeutic strategies for the treatment of cancer which is in an increase in the surrounding regions. Being a research team associated with immune-oncology and the development of new immunotherapeutic strategies, we believe that PDT has the potential to bridge the gap between targeted anti-cancer therapy and the induction of an effective immune response. Our study, along with those published previously; highlights the proof of concept while answering some key questions about the mechanism of the therapy and at the same time raising new questions and challenges for future researchers. By our study, we proposed a 5-ALA PDT-based therapeutic strategy using low-cost ingenious illumination systems developed in-home by the team. While more studies

and results are requisite for establishing PDT as the main therapy for HCC, we are hopeful and yet skeptical that our current and future work would show the promising outcome, thus implementing this innovative therapy for saving the lives of patients.

CHAPTER 7

COLLABORATIVE WORKS

PRESENTATION BASED ON 4 PUBLISHED ARTICLES

ARTICLE 1 PRESENTATION:

Title: “Devices based on Light Emitting Fabrics dedicated to PDT preclinical studies”

Light plays a determinant role in the efficacy of PDT. Undefined spatial and/or spectral distribution of light, thermal emission during illumination and unable to adapt to varying shape and volume to illuminate could cause inconsistent illumination dose, thus affecting the reproducibility. This study presents our home-made laser and optic fiber-based illumination device for 635nm wavelength utilized during preclinical studies, including both *in-vitro* and *in-vivo* models. Based on knitted light emitting fabric technology, this study demonstrated that our deivce offers homogenous illumination, without thermal emission and is capable to be equipped with various illumination sources, thus allowing investigations at various wavelengths, energy, and irradiance and illumination mode. For *in-vitro* studies, light plates were developed which are capable to illuminate upto four 96-well plates. Mice boxes were developed for *in-vivo* studies, which can illuminate up to three mice at a time with homogenous extracorporeal illumination.

The thesis presented here is based on this illumination set-up, and thus all the results generated during the thesis, thereby validate the effectiveness, reliability, reproducibility and robustness of the illumination device developed by OncoThAI.

Devices based on Light Emitting Fabrics dedicated to PDTpreclinical studies

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ABSTRACT

Whether preclinical studies either involve a cell or animalmodel, the distribution of light plays a determinant role in the reproducibility of results of photodynamic therapy (PDT) studies. Unfortunately, only few illumination devices dedicated to preclinical studies are available and are for the most, very expensive. Mostresearch teams use home-made solutions that may not always be reproducible because of undefined light distribution, additive thermal emission, or unsuitable for shapes and volumes to illuminate.

To address these issues, we developed illumination devices dedicated to our preclinical studies, which embed knitted light emitting fabrics (LEF) technology. LEF technology offers a homogeneous light distribution, without thermal emission and can be coupled with various light sources allowinginvestigation of several PDT modalities (irradiance, wavelength, illumination duration/mode).

For in-vitro studies, we designed light plates,each allowing illumination of up to four 96-cells plates. For in-vivo studies, we designed mice boxes allowing three animals placement in prone position, equally surrounded by LEF and ensuring homogeneous extracorporeal illumination.

Optical validation was performed and reproducibility of both preclinical systems were assessed.

Both systems can deliver homogeneous light with an irradiance that can reachseveral mW/cm^2, with varying durations and wavelengths. First results of preclinical studies demonstrate a high reproducibility, with an easy setup, and a great adaptability of illumination modalities with these devices based on light emitting fabrics.

Keywords: Light emitting fabrics, textile, optical, fibers, bending, PDT, preclinical studies, illumination, MDB TEXINOV

1. INTRODUCTION

Over the last decades, photodynamic therapy (PDT) has proven to be efficient for certain types of cancer [1, 2]. Widely used in dermatological practice, PDT is one of the first-line treatments for the management of actinic keratosis and superficial basal cell carcinomas [3]. Furthermore, several preclinical and clinical trials suggest that PDT in intraoperative conditions represents a promising complementary therapeutic modality for the management of invasive cancers in neurosurgery, pneumology, otolaryngology and gynecology [4-7]. Indeed, in spite of shallow light penetration into biological tissues, photodynamic therapy is particularly appropriate for the treatment of subclinical lesions scattered in the cavity after maximal surgical removal of the tumor [8].

Therapeutic effect of PDT depends on a combination of parameters that include photosensitizer (PS)concentration, drug-light interval, oxygen in cells, fractionation mode, wavelength and total dose of light distributed in biological tissues [9]. To date, PDT parameters are under many investigations, as many for increasing direct tumor cell death rates as for

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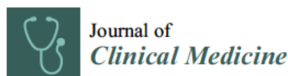
ARTICLE 2 PRESENTATION

Title: "An Efficient Photodynamic Therapy Treatment for Human Pancreatic Adenocarcinoma"

Various studies have demonstrated the Folate Receptor 1 can be a promising target for therapeutic and diagnostic strategies for various cancers. In this study, we evaluated the efficacy for PDT of a newly patented PS (called as PS2), coupled with folate to target Folate Receptor 1 over different pancreatic cancer cell lines followed by illumination with 672nm. We demonstrated cellular accumulation of the PS2 through immunofluorescence and decreased viability up to 95%, 10 minutes after illumination. In the meanwhile, no viability decrease was observed for untreated, PS2-only or illumination-only controls. Human PBMCs (activated or not) when cultured with PDT treated conditioned media could increase their proliferation. Alongside, no change in the cytokine levels of IL-2, IL-10 and TGF-β with decreased IL-6, suggesting reduced inflammation. Following the *in-vitro* studies, we validated our results in an *in-vivo* SCID mice model of pancreatic adenocarcinoma. This study suggests that the PS2 mediated PDT could be successfully utilized for treatment of pancreatic adenocarcinoma.

During the study, I contributed for conceptualization along with establishment of *in-vitro* experimentation protocols. I assisted the lead authors for various *in-vitro* experiments, primarily RT-qPCR, viability assays, ELISA and PBMC culture with conditioned media.



Article

An Efficient Photodynamic Therapy Treatment for Human Pancreatic Adenocarcinoma

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Abstract: To date, pancreatic adenocarcinoma (ADKP) is a devastating disease for which the incidence rate is close to the mortality rate. The survival rate has evolved only 2–5% in 45 years, highlighting the failure of current therapies. Otherwise, the use of photodynamic therapy (PDT), based on the use of an adapted photosensitizer (PS) has already proved its worth and has prompted a growing interest in the field of oncology. We have developed a new photosensitizer (PS-FOL/PS2), protected by a recently published patent (WO2019 016397-A1, 24 January 2019). This photosensitizer is associated with an addressing molecule (folic acid) targeting the folate receptor 1 (FOLR1) with a high affinity. Folate binds to FOLR1, in a specific way, expressed in 100% of ADKP or over-expressed in 30% of cases. The first objective of this study is to evaluate the effectiveness of this PS2-PDT in four ADKP cell lines: Capan-1, Capan-2, MiapaCa-2, and Panc-1. For this purpose, we first evaluated the gene and protein expression of FOLR1 on four ADKP cell lines. Subsequently, we evaluated PS2's efficacy in our cell lines and we assessed the impact of PDT on the secretome of cancer cells and its impact on the immune system. Finally, we evaluate the PDT efficacy on a humanized SCID mouse model of pancreatic cancer. In a very interesting way, we observed a significant increase in the proliferation of activated-human PBMC when cultured with conditioned media of ADKP cancer cells subjected to PDT. Furthermore, to evaluate in vivo the impact of this new PS, we analyzed the tumor growth in a humanized SCID mice model of pancreatic cancer. Four conditions were tested: Untreated, mice (nontreated), mice with PS (PS2), mice subjected to illumination (Light only), and mice subjected to illumination in the presence of PS (PDT). We noticed that the mice subjected to PDT presented a strong decrease in the growth of the tumor over time after illumination. Our investigations have not only suggested that PS2-PDT is an effective therapy in the treatment of PDAC but also that it activates the immune system and could be considered as a real adjuvant for anti-cancer vaccination.

ARTICLE 3 PRESENTATION

Title: "Photodynamic Therapy using a New Folate Receptor-Targetd Photosensitizer on Peritoneal Ovarian Cancer Cells Induces the Release of Extracellular Vesicles with Immunoactivating Properties"

In the article of Baydoun *et al.*, we aimed to study the impact of above-mentioned folate coupled PS2 mediated PDT over ovarian cancer and further evaluate potential consequences of the treatment on human immune system. We firstly studied the folate receptor expression by the ovarian cancer cell lines followed by PDT treatment of the cell lines with a pre-established dose to observe the viability at different time intervals. The study demonstrates that PDT not only successfully reduce cell viability but can also induce secretion of extracellular vesicles, which can increase PBMC proliferation in a dose-dependent manner. The immune population activated by these vesicles are CD4+ and CD8+ T cells. PDT also induce secretion of pro-inflammatory cytokines, as demonstrated by ELISA.

During this study, I assisted in various *in-vitro* experimentations involving proliferation assays, viability assay, PBMC isolation, and flow cytometry.

Article

Photodynamic Therapy Using a New Folate Receptor-Targeted Photosensitizer on Peritoneal Ovarian Cancer Cells Induces the Release of Extracellular Vesicles with Immunoactivating Properties

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Abstract: Often discovered at an advanced stage, ovarian cancer progresses to peritoneal carcinoma, which corresponds to the invasion of the serosa by multiple tumor implants. The current treatment is based on the combination of chemotherapy and tumor cytoreduction surgery. Despite the progress and standardization of surgical techniques combined with effective chemotherapy, post-treatment recurrences affect more than 60% of women in remission. Photodynamic therapy (PDT) has been particularly indicated for the treatment of superficial lesions on large surfaces and appears to be a relevant candidate for the treatment of microscopic intraperitoneal lesions and non-visible lesions. However, the impact of this therapy on immune cells remains unclear. Hence, the objective of this study is to validate the efficacy of a new photosensitizer [pyropheophorbide a-polyethylene glycol-folic acid (PS)] on human ovarian cancer cells and to assess the impact of the secretome of PDT-treated cells on human peripheral blood mononuclear cells (PBMC). We show that PS, upon illumination, can induce cell death of different ovarian tumor cells. Furthermore, PDT using this new PS seems to favor activation of the immune response by inducing the secretion of effective cytokines and inhibiting the pro-inflammatory and immunosuppressive ones, as well as releasing extracellular vesicles (EVs) prone to activating immune cells. Finally, we show that PDT can activate CD4+ and CD8+ T cells, resulting in a potential immunostimulating process. The results of this pilot study therefore indicate that PS-PDT treatment may not only be effective in rapidly and directly destroying target tumor cells but also promote the activation of an effective immune response; notably, by EVs. These data thus open up good prospects for the treatment of micrometastases of intraperitoneal ovarian carcinosis which are currently inoperable.

ARTICLE 4 PRESENTATION

Title: "Hepatitis C virus Improves Human treg Suppressive Function and Promotes their Recruitment to the Liver"

HCV specific CD4+ and CD8+ T cells are capable to limit or control the HCV infection. However, studies have demonstrated a failure of innate and adaptive immune response probably due to elevated levels of intra-hepatic and circulating Tregs found in patients. Differentiation and recruitment of Tregs to the liver, thus, can probably be the lead to disease progression. Through this study, Ouaguia *et al.* demonstrated that HCV virion particles generated by human hepatocytes after HCV induced HCC could cause Treg expansion and recruitment by CCL20. The HCV inoculation further increases mRNA expression of CD5 in Tregs, which facilitates HCV entry in T lymphocytes, thereby making them more sensitive for virus interaction. The HCV infectious particles can also increase Treg suppressive phenotype and IL-2 dependent increase in their survival and proliferation ability. The study establishes an interplay between HCV and Tregs contributing to HCC progression.

I validated some of the results generated during the study, at the beginning of my thesis.

Article

Hepatitis C Virus Improves Human Tregs Suppressive Function and Promotes Their Recruitment to the Liver

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Abstract: Background: The role of regulatory T cells (Tregs) is now well established in the progression of hepatocellular carcinoma (HCC) linked to Hepatitis C virus (HCV) infection. However, nothing is known about the potential interplay between Tregs and HCV. In this pilot study, we have investigated the ability of Tregs to hang HCV on and the subsequent effect on their suppressive function and phenotype. Moreover, we have evaluated how HCV could promote the recruitment of Tregs by infected primary human hepatocytes. Methods: Tregs of healthy donors were incubated with JFH-1/HCVcc. Viral inoculation was assessed using adapted assays (RT-qPCR, Flow Citometry (FACS) and Western Blot (WB). Expression of Tregs phenotypic (CD4, CD25, CD127 and Foxp3) and functional (IL-10, GZMB, TGF-β1 and IL-2) markers was monitored by RT-qPCR, FACS and ELISA. Suppressive activity was validated by suppressive assays. Tregs recruitment by infected primary hepatic cells was evaluated using Boyden Chamber. Results: Tregs express the classical HCV receptors (CD81, CLDN1 and LDLR) and some co-receptors (CD5). HCV inoculation significantly increases the suppressive phenotype and activity of Tregs, and raises their anergy by inducing an unexpected IL-2 production. Moreover, HCV infection induces the expression of chemokines (CCL17, CXCL16, and CCL20) by primary hepatic human hepatocytes and chemokine receptors (CCR4, CXCR6 and CCR6) by Tregs. Finally, infected hepatocytes have a significantly higher potential to recruit Tregs in a seemingly CCL20-dependent manner. Conclusions: Direct interaction between HCV and Tregs represents a newly defined mechanism that could potentiate HCV immune evasion and favor intratumoral recruitment contributing to HCC progression.

Keywords: HCV; HCV/JFH-1; regulatory T cells; chemokines; immune escape

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ENGLISH VERSION

In recent years, there has been a steady increase in patients with Hepatocellular Carcinoma (HCC) mainly in the north of France. Despite a large number of therapeutic options, HCC has a high cancer-related mortality. Hence there is a need to develop novel therapeutic strategies for HCC treatment which could increase the efficacy of existing options and at the same time provide longer tumor free survival for the patients.

Photodynamic Therapy (PDT) is a local anti-tumoral modality relying on systemic administration of a non-toxic dye, called Photosensitizer (PS), and subsequent illumination by light of appropriate wavelength and energy to elicit tumor cell death. Initially used for treatment of dermatological ailments, PDT has shown motivating results as a possible anti-tumoral therapy in last few decades.

Using 5-Aminolevulinic acid (5-ALA), the US-Food and Drug Administration (US-FDA) approved oral pro-drug of Protoporphyrin IX, the actual PS, we investigated the *in-vitro* impact of PDT over HCC cell lines, along with patient tumoral hepatocytes, healthy donor liver myofibroblasts and human immune system. At last, we validated our results on an *in-vivo* model of HCC. The overall aim is to establish 5-ALA PDT as an adjuvant to partial hepatectomy through intra-operative procedures.

When the HCC cell lines were treated with varying 5-ALA concentration and light doses, we observed a dose-dependent decrease of cellular viability. We also observed varying sensitivity for 5-ALA PDT between the three HCC cell lines used (HuH7, Hep3B, HepG2), highlighting the role of p53 for underlying cell death mechanism. Thereafter, an IC50 dose for each of the cell line was obtained which induced decreased cellular proliferation and increased cell death with respect to non-treated control. These results confirm that 5-ALA PDT induce cell death to the HCC cell lines.

Furthermore, in order to validate the *ex vivo* efficacy of 5- ALA-PDT, we studied the impact of this therapy on tumor hepatocytes from four patients with HCC, showing that cell viability decreased continuously up to 4 times, from day 3 after illumination, compared to the untreated control. In addition, we have studied the safety of 5-ALA-PDT in liver myofibroblasts from healthy donors. Our results show that 5-ALA-PDT does not induce any change in terms

of viability and proliferation of healthy myofibroblasts and that the levels of expression of the mRNA of the markers of fibrosis, namely collagen-1, HSP47, αSMA, TIMP1 and MMP2 are not modified. In addition, no modulation of the levels of collagen secretion was observed.

Most interestingly, when we cultured human activated Peripheral Blood Mononuclear Cells (PBMCs), with conditioned media obtained from 5-ALA PDT treated HCC cell lines, we observed increased cellular proliferation with respect to non-treated control. This highlights clonogenic expansion and possible anti-tumor immune response activation by 5-ALA PDT. This conditioned media has further inhibit the proliferation of respective cancer cell lines, reflecting secretion of anti-proliferation factors by cells due to PDT.

At last, our *in-vivo* results demonstrated that PpIX has higher accumulation in the tumor with respect to non-tumoral tissues and a decrease in the rate of tumor growth for humanized SCID mice treated by 5-ALA PDT, with respect to Non-Treated control group.

To date, no other study has evaluated the impact of 5-ALA PDT on primary patient and healthy donor samples. We are also the first to assess the impact of 5-ALA PDT treated conditioned media on PBMCs and cancer cell line proliferation. Our study strengthens the notion of the application of intra-operative 5-ALA PDT for treatment of HCC patients. Further studies will aim to understand the role of p53 in the cell death mechanism, identify the immune population activated by PDT, evaluate the efficacy of the therapy in a humanized SCID mice model and establish a standard treatment protocol to be utilized in a clinical set-up.

FRENCH VERSION

Le carcinome hépatocellulaire (CHC) est le principal cancer primitif du foie avec un taux d'incidence qui a augmenté ces dernières années. Malgré les nombreuses options thérapeutiques disponibles, le CHC est associé à une mortalité très élevée. Dans ce contexte, il apparaît nécessaire de développer de nouvelles stratégies thérapeutiques qui pourraient augmenter l'efficacité des traitements conventionnels, ainsi que la survie sans progression.

La thérapie photodynamique (PDT) est une modalité antitumorale locorégionale reposant sur l'administration systémique d'une molécule non toxique, appelée photosensibilisateur (PS), et l'illumination secondaire par une lumière de longueur d'onde et d'énergie appropriées afin de provoquer la mort des cellules tumorales.

Nous avons évalué l'impact *in vitro* de la PDT **(i)** sur des lignées humaines de CHC, **(ii)** sur des hépatocytes tumoraux de patients, **(iii)** sur des myofibroblastes hépatiques de donneurs sains et **(iv)** sur des cellules immunitaires humaines, en utilisant l'acide 5-aminolévulinique (5-ALA) [la protoporphyrine IX, étant le réel PS]. Dans un second temps, nous avons validé nos résultats sur un modèle *in vivo* de CHC. L'objectif global était d'établir la validité de la 5-ALA-PDT comme adjuvant à l'hépatectomie partielle par des procédures peropératoires.

Lorsque les lignées cellulaires de CHC ont été traitées avec des concentrations variables de 5-ALA et des doses de lumière adaptées, nous avons observé une diminution dose-dépendante de la viabilité cellulaire. Ces résultats confirment que le 5-ALA- PDT induit la mort des lignées cellulaires de CHC humaines.

En outre, afin de valider l'efficacité *ex vivo* de la 5- ALA-PDT nous avons étudié l'impact de cette thérapie sur des hépatocytes tumoraux de quatre patients atteints de CHC, montrant que la viabilité cellulaire diminuait en continu jusqu'à 4 fois, à partir du jour 3 après l'illumination, comparativement au témoin non traité. Par ailleurs, nous avons étudié l'innocuité de la 5-ALA-PDT sur des myofibroblastes hépatiques de donneurs sains. Nos résultats montrent que la 5-ALA-PDT n'induit aucun changement en terme de viabilité, de prolifération et que les niveaux d'expression de l'ARNm des marqueurs de fibrose, à savoir la collagène-1, HSP47, αSMA, TIMP1 et MMP2 ne sont pas modifiés. De plus, aucune modulation des niveaux de sécrétion de collagène n'a été observée.

Plus intéressant encore, lorsque nous avons cultivé des cellules mononuclées du sang périphérique (PBMC) activées, avec des milieux conditionnés obtenus à partir de surnageants de lignées cellulaires de CHC traitées par la 5-ALA PDT, nous avons observé une prolifération cellulaire accrue par rapport au contrôle non traité. Cela met en évidence l'expansion clonogénique et l'activation possible de la réponse immunitaire anti-tumorale par la 5-ALA PDT. Ce milieu conditionné a en outre induit une inhibition de la prolifération des lignées de CHC suggérant une sécrétion de facteurs anti-prolifération par les cellules cancéreuses traitées par la 5-ALA PDT.

Enfin, dans un modèle de CHC chez la Souris SCID humanisée, traitée par 5-ALA PDT, la PpIX avait une accumulation plus élevée dans la tumeur que dans les tissus non tumoraux et on observait une diminution du taux de croissance tumorale, par rapport au groupe contrôle non-traité.

À ce jour, aucune autre étude n'a évalué l'impact de la 5-ALA PDT sur des prélèvements de patients et de donneurs sains. Nous sommes également les premiers à avoir évalué l'impact des milieux conditionnés traités par 5-ALA PDT sur les PBMC et la prolifération des lignées cellulaires cancéreuses. Notre étude renforce le projet d'application la 5-ALA PDT au traitement des patients atteints de CHC. D'autres études ultérieures viseront à identifier la population immunitaire activée par la PDT, à évaluer l'efficacité de la thérapie dans un modèle de souris SCID humanisé et à établir un protocole de traitement standard à utiliser dans un ensemble clinique.