UNIVERSITAT POMPEU FABRA

UNIVERSITAT POMPEU-FABRA

PhD THESIS:

"EFFECT OF THE IRREVERSIBLE ELECTROPORATION IN A MURINE MODEL OF COLORECTAL LIVER METASTASES"

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Summary

Irreversible electroporation is a novel technique growing in popularity over the last years among the ablative modalities. Its unique action mechanism produces irreversible nanopores in the membrane of the cell leading to apoptosis; therefore irreversible electroporation can be used to ablate substantial volumes of tissue without the undesirable thermal effects as the ‘‘heat sink effect’’. Moreover the extracellular matrix is left unperturbed, thus sparing the structural architecture of surrounding structures such as bile ducts and blood vessels. In the last years its use has been widespread in both liver and pancreatic ablation. Irreversible electroporation has shown its safety with however some caution, feasibility and favorable outcomes in clinical settings such as unresectable locally advanced disease in which the surgical and therapeutic options are very limited

Resumen

La electroporación irreversible es una entidad que ha ganado en popularidad entre las técnicas de ablación tumoral en los últimos años. Debido a su innovador mecanismo de acción, ya que induce poros en la membrana celular que conducen a la apoptosis celular, ha demostrado su capacidad para destruir el tejido sin presentar los efectos indeseables dela ablación térmica tales como el «heat sink effect». Así mismo mantiene la integridad de la matriz extracelular, por lo que estructuras como los vasos sanguíneos y los ductos biliares no son afectadas por la electroporación irreversible. Su utilización se ha ido extendiendo en los últimos años tanto en la ablación hepática como en la pancreática mostrando su seguridad y resultados prometedores en escenarios clínicos en los que la cirugía en el momento actual no puede ofrecer otras opciones terapéuticas.

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INTRODUCTION

1. HEPATIC TUMORS:

In Europe, between 4-17% new cancer cases diagnosed in 2012 were liver cancers [1]. In Asia, for example, a total of 582,420 incident cases were recorded in the year 2012 and this incidence was absolutely not related with the degree of Development of the country [2]. It is predicted that if management strategies, in these areas will continue to be stable, the number of new cancer cases in Asia will rise by 2020. Worldwide, the burden of cancer is rising, what means stimulating efforts to prevent these diseases and to find alternative therapies in cases of local advanced or metastatic disease.

1.1. Primary liver tumors

Primary liver cancer (PLC) is the fifth cause of death worldwide in men, the eighth in women and it has risen through the last decades [3]. Estimates from the year 2000 indicate that liver cancer remains the fifth most common malignancy in men and the eighth in women worldwide. The number of new cases is estimated to be 564,000 per year, including 398,000 in men and 166,000 in women.

Primary liver cancer includes hepatocellular hepatocarcinoma (HCC), intrahepatic cholangiocarcinoma (ICC) and some other extremely rare ones. The trends in incidence are very variable comparing the different countries and also change along the years. A recent prevalence study collecting data from more than 30 countries showed that the incidence of primary liver tumors was highest in eastern and south-eastern Asia (Japan, China and Singapoure) whereas in south-western and northern European countries was lowest [4,5].

1.1.1. Hepatocellular carcinoma

Among all types of PLC the Hepatocellular carcinoma is the first one in frequency, accounts for the 80-90% of all malignant tumors [6,7]. HCC is a major health problem worldwide, which continues to increase because of the association of HCC with hepatitis B and C viruses. The world trends are unevenly distributed, finding the

highest incidence in eastern Asia, followed by middle Africa, South-East Asia and the Pacific Islands [3]. The ratio of incidence between males/females ranges from 4:1 to 1.3:1 but the reason is still unknown. Concerning age distribution it varies also depending the geographic situation.

HCC in 80-90% of cases is developed as a consequence of a chronic liver disease including chronic hepatitis B virus infection (HBV), chronic hepatitis C virus infection (HCV), alcohol consumption, aflatoxin-contaminated food ingest and other minority etiologies. All these chronic hepatitis conduce after many years of evolution to liver cirrhosis as substrate of the HCC. The most frequent risk factor for hepatocellular carcinoma is chronic HBV infection counting with more than 50% of cases and which is the main risk factor in Eastern-Asia. It has been reported that incidence of hepatocellular carcinoma increases with viral load and duration of infection [8]. Hepatocellular carcinoma HBV-related can also be easily prevented by vaccination, as was shown in a study carried out in Taiwan which demonstrated a decrease in the incidence of new cases of HCC in new-born after implementation an universal program of vaccination [9].

The incidence of hepatocellular carcinoma in individuals with HCV cirrhosis is 3–5% per year. HCC is a complication of HCV-related cirrhosis, particularly in the United States, Europe, Australia and Japan. Progression from infection to cirrhosis takes average 20 years. Alcohol is an important risk factor for development of hepatocellular carcinoma and exerts a synergistic effect in individuals with chronic infection with HBV, HCV, or both. The mechanisms by which alcohol causes HCC are not already known. As stated before, there also other minority causes for developing a HCC as the hemochromatosis disease or exposure to *Aflatoxin* but both with low incidence rates [6].

Histologically, hepatocellular carcinomas present a range of appearances. A common feature is the presence of fibrosis and inflammation as hepatocellular carcinoma often develops in the liver of patients with late stages of chronic hepatitis. In well-differentiated tumors, tumor cells resemble normal hepatocytes

and may be difficult to determine as malignant. The more poorly differentiated tumors display marked pleomorphism, with tumor giant cells showing little resemblance to normal hepatocytes. Intracellular bile production is sometimes a feature of tumor cells that aids in their classification. A sinusoidal growth pattern and absence of intracellular mucin are characteristic features of hepatocellular carcinomas.

There special variants of hepatocellular carcinoma: *fibrolamellar carcinoma (FL-HCC)* and *sclerosing hepatocellular carcinoma*. The fibrolamellar variant occurs normally in young patients without structural cirrhosis [10]. It is thought to be a rare variant of conventional hepatocellular carcinoma (HCC), accounting for 0.85 to 16 % of all hepatocellular carcinomas [10]. The tumors are solid, and frequently contain a central fibrous scar, but it only occurs in less as 5% of the cases (Fig. 1B) Long-term survival of this subtype of HCC is significant better compared to classical HCC. Survival 1, 3, and 5 years after liver transplantation ranged from 63 to 100 %, 43 to 75 %, and 29 to 55 %. However, disease recurrence after complete surgical resection of the FL-HCC is high in this patient population ranging 33–100 %

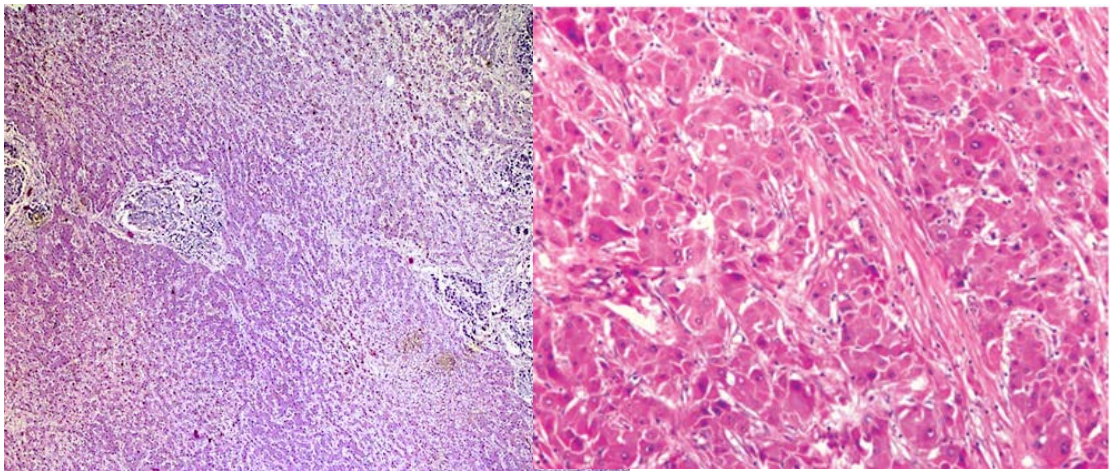

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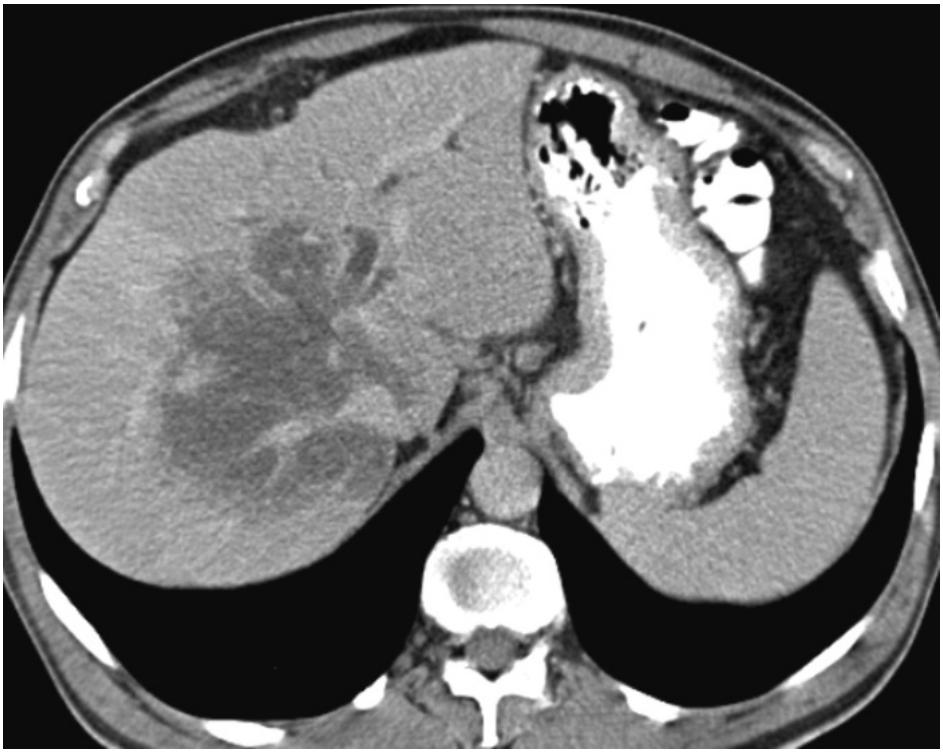
Fig 1. Hepatocytes in the well-differentiated HCC have normally a similar appearance to the normal liver (A). The fibrolamellar variant present a strong connective stroma among the hepatocytes (B).

1.1.2. Intrahepatic Cholangiocarcinoma

Cholangiocarcinoma (CC) is a type of tumor that grows from the biliary epithelium. Its frequency within the PLC is very limited, ranging about 10-15% of the total [6]. The spectrum of Cholangiocarcinoma includes intrahepatic, perihilar and extrahepatic tumors, which have a different epidemiology and therapeutic approaches. As happened with HCC the highest incidence rates of CC is found in northeast Thailand [11]. It seems to be well established that the ethiology of the CC is highly related with a situation of chronic inflammation. There are some entities as primary sclerosing cholangitis (PSC), choledochal cysts, *Opisthorchis viverrini* infection, hepatolithiasis or biliary papillomatosis, which present a strong association with the development of a CC [12].

Microscopically these tumors present themselves firm because of its prominent desmoplastic stroma. The most common microscopic pattern is a well to moderately differentiated adenocarcinoma forming small tubular glands and duct-like structures. This tumor has an aggressive biology and the prognosis is normally poor even in those cases in which a surgical resection is possible [13].

CC is often diagnosed by MRI or CT-scan, however the PET-CT scan present a accurate sensibility in the screening of lymph node metastases and extrahepatic disease, which would play an important role in the staging and therapy [14].



http://liveratlas.org/media/cache/01/85/0185dec278f039a6269d7af5e0938277.jpg

Fig 2. CT-scan in portal vein phase shows a big intrahepatic cholangiocarcinoma involving the right hemiliver and partly the middle hepatic vein.

1.1.3. Hepatoblastome

It is the most common PLC in young children, accounting for over 50% the total. It is composed of tissue resembling fetal liver cells, mature liver cells, or bile duct cells. Alpha-fetoprotein (AFP) is used as a biomarker to help determine the presence of liver cancer in children [15]. Pathologically the tumors are often unique with a surrounding normal structure of the liver. It spreads in the regional nodes, but also can produce metastasis in the lung, adrenal gland and bone.

1.1.4. Others: sarcoma, primary hepatic lymphoma

These are extremely rare tumors. Primary hepatic lymphoma is a very uncommon lymphoproliferative malignancy. It accounts for only 0.4 % of all extranodal non-Hodgkin lymphoma and 0.016 % of all cases of non-Hodgkin disease [16]. Primary

hepatic sarcomas are also extremely unusual, as only are reported 20-30 cases per year in the literature [6]. Microscopically the most frequent pattern is the angiosarcoma, which grows by extending along preexisiting vascular channels in the liver.

1.2. Secondary liver tumors

Liver is the most common site of metastatic disease at most becoming from the gastrointestinal tract, lung or neuroendocrine tumors. In the following subsections it would be detailed the different types of secondary tumors.

1.2.1. Liver metastasis of neuroendocrine tumors

Neuroendocrine tumors (NT) produce liver metastasis in a 75% of the cases and can result in an extensive liver disease without finding the primary tumor [6]. From an embryological point of view NT are derived from the embryonic neural crest and their typical feature is to have the capacity of secrete hormones. The World Health Organization (WHO) classifies these tumors as well-differentiated vs. poorly-differentiated NT.

The concept of differentiation is linked to the grade of the tumors, but there are some differences between the two concepts. Differentiation refers to the extent to which the neoplastic cells resemble their non-neoplastic counterparts. On the other hand, grade refers to inherent biologic aggressiveness of the tumor. The grade of a tumor depends on the rate of proliferation, which is measured by the percentage of tumor cells that are positive for Ki-67 antigen per 10 high-powered microscope field (hpf). Gastroenteropancreatic NET are considered low grade (G1) when < 2 mitoses / 10 hpf and <3% Ki67 index; intermediate grade (G2) 2-20 mitoses / 10 hpf or 3%-20% Ki67 index; high grade (G3) >20 mitoses / 10 hpf or >20% Ki67 index. [17]

The clinical course of patients with metastatic gastroenteropancreatic NET is highly variable. They are usually asymptomatic but may present with symptoms as a result of hormone hypersecretion. Well-differentiated tumours are the most common and

they are thus 50 % of all NETs in the gastrointestinal tract [18]. In the magnetic resonance imaging hepatic metastases of neuroendocrine tumors had a typical hypervascular pattern in 73% of patients [19] and contrast enhancement in the computer tomography (CT). The most sensitive imaging is the 18F-DOPA-PET/CT, which has a high sensitivity and specificity in detecting NET lesions, and has demonstrated to be superior to other conventional staging modalities.

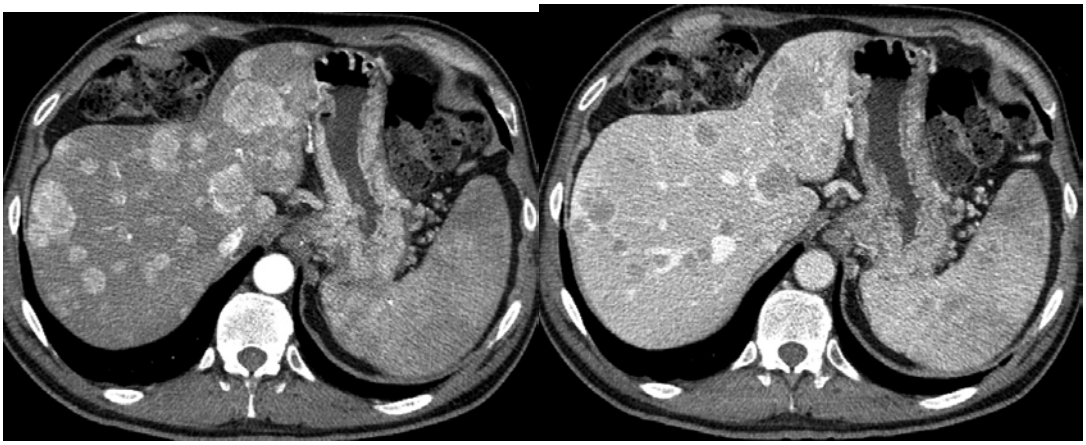


Fig. 3: Multiphase CT scan shows multiple liver metastasis of a neuroendocrine tumor, which present enhancement in the arterial phase and wash-out in the venous phase

They present often with a pattern of multiple liver small liver metastases. These have normally a more indolent course, as their growth rate is very low in comparison with the CRLM.

1.2.2. Colorectal liver metastases

Metastatic colorectal cancer (MCRC) represents the most common indication for liver resection as colorectal cancer represents the 4th most common cancer in western countries. Five- year survival after treatment ranges from 25%-37% [20,21] and more as 50% of the patients would present liver metastasis during the evolution of the disease [22] or other type of metastatic disease [23]. Until recently, patients with stage IV liver disease were relegated to palliative treatments or surgeries. However, since the last decade it has been established that the first and foremost goal of

oncological surgery is to provide local control of the disease and even to treat the local or peritoneal recurrence is nowadays no contraindication for re-do surgeries [24].

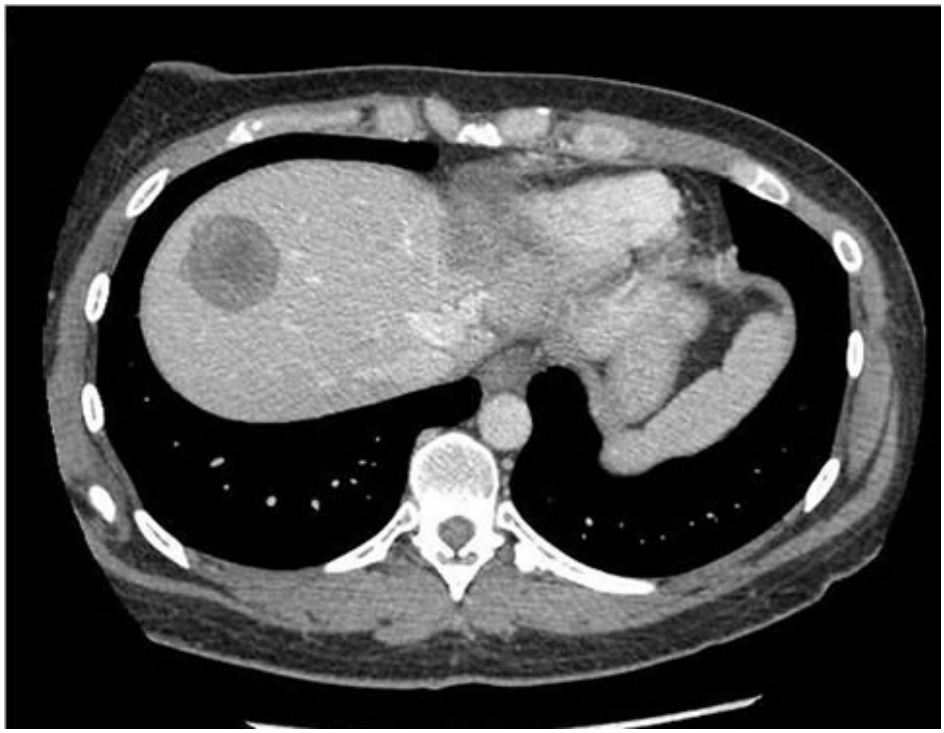


Fig. 4: CT scan shows a solitary liver metastasis of colorectal carcinoma in segment VIII. MCRC could appear very differently in the imaging

From a histological point of view, sometimes is very difficult to differentiate a metastasis from a PLC. One of the most difficult ones is to distinguish an intrahepatic cholangiocarcinoma from a metastatic adenocarcinoma. Some inmunohistochemical stains can help but the sensitivity is very low. Liver metastases from CCR are normally no unique and rarely metastasize to bile ducts nor grow as intrabiliary tumor.

Data concerning tumor growth is very complex and different cell cascades and pathways are involved in this process [6]. The natural history of the colorectal cancer starts with the transformation of a normal intestinal epithelial cell into a polypoid lesion. The process continues with the sequenced transformation from an adenoma

to a carcinoma as a conclusion of accumulated mutations. There are at least two well-described genetic pathways that may lead to colorectal metastases.

The most common, which mediates up to 60% of carcinomas, is the chromosomal instability pathway. This is the result of mutations of the p53, APC and protooncogene K-Ras, allelic loss of 18q and aneuploidy [25]. The role of the gene APC in the pathogenic of colorectal cancer is well known, as 100% of the patients with familial adenomatous polyposis (FAP) would develop a colorectal cancer in their lifetime without a proper treatment. The second pathway is the microsatellite instability, which suppose a mutation in DNA mismatch repair genes and affects about a 5-6% of the patients who suffer from non-hereditary colorectal cancer.

The further distant dissemination from the tumoral cells depends on a lot of different molecular pathways and the interrelationship from these cells with the stroma (endothelial cells, fibroblasts, immune cells) as well as the angiogenesis process. Specifically, the colorectal metastasis dissemination in the liver consists on four phases: phase of liver-infiltrating malignant cells; interlobular micrometastasis phase; angiogenic micrometastasis phase and established hepatic metastasis phase.

Hepatic sinusoidal immune system involves the hepatic-specific natural killer cells (NK) (pit cells), Kupffer cells (KC) and hepatic endothelial cells (HEC), and is proven to play an important role in protecting the liver from invading colon carcinoma cells. Kupffer's cells (KC) are the hepatic macrophages that have a relevant capacity of arresting tumoral cells from the bloodstream and avoid its penetrance into the parenchyma. However, KCs may contribute to liver colonisation if their tumouricidal ability is saturated due to excessive numbers of invading cells or when metastases are already established [26]. Similarly, the sinusoidal endothelial cells also could aid the tumoral cells to penetrate the liver tissue under cytokine activation by inducing sinusoidal endothelial cells to express intercellular adhesion molecules and increase adhesion of the tumor cells and retention in the liver [27,28].

After endothelial adhesion of the tumoral cells within the sinusoids, strong intercellular bonds allow cells to resist the attractive forces of plasmatic flow and circulating blood cells [29] and they are allowed to penetrate across the Disse space and achieve the hepatocyte cytoplasm [30]. The tumoral cells would cause micrometastases in the hepatic parenchyma and they could remain in a dormant state ("sleep metastases") for an unknown period of time. To conclude the establishment of the liver metastasis it is probable that these micrometastases will be reactivated after an unspecified time period and will create macrometastases.

When metastatic colorectal cancer is treated with a combination of chemotherapy (5-FU, oxaliplatin, irinotecan) and antiangiogenic drugs median overall survivals now extend beyond 24 months [31]. At the diagnosis one-third of patients have advanced stage disease without the option of curative resection. After a surgical resection by relapse the liver is normally the main affected organ [32], that's why new therapies which target this potential recurrences have to be envisioned.

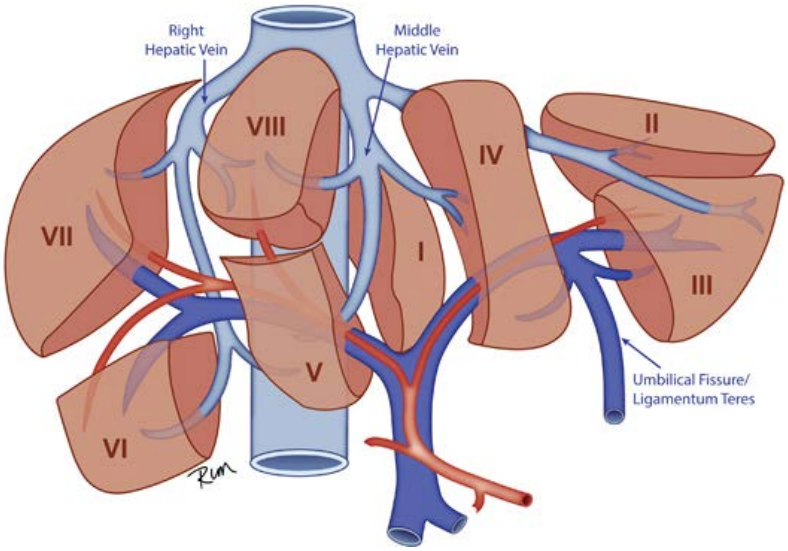
2. TREATMENT OPTIONS OF THE HEPATIC TUMORS

2.1. Resection: surgical anatomy and technique

Liver resection is often the only potentially curative technique in the treatment of primary and secondary hepatic tumors. With the development of the preoperative diagnostic techniques and the improvement of intra- and postoperative patient management liver resections can currently be performed with low mortality and relatively low rate of complications [33]. There are some general technical considerations concerning to liver resections, which can be applied universally to safely perform a liver procedure no matter what the underlying cause is.

Classically the liver division was considered in two lobes anatomically separated for the *falciform ligament* [34]. In the last century the hepatic division has evolved towards a functional division based on the vascular distribution of the portal and hepatic veins branches. In 1950s two authors Couinaud [35] and Healey [36] proposed a similar system, which divided the liver into two hemilivers along the middle hepatic vein (corresponding to *Cantlie* line) and each of one consequently subdivided into 4 liver segments. Portal and hepatic vein segmentation (french segmentation) was preferred by Couinaud over the arteriobiliary segmentation described by Healey [37]. All studies agree about the division of the liver in two hemilivers. When taking into account what are customarily called the main ramifications of the triad, most investigators further subdivide the 2 hemilivers into 2 further parts each, leading to a quadripartition of the liver or sectors.

In year 2000 at Brisbane Conference of the I.H.P.B.A., was reconsidered the hepatic segmentation by the introduction of the term of “sectional anatomy”, a unitary surgical nomenclature and an updated terminology of liver resections were unanimously recommended and currently the most used.



http://www.nature.com/modpathol/journal/v27/n3/fig_tab/modpathol201380f5.html

Fig. 5: Liver segments according to the actual terminology for liver division based on the functional distribution of the segments.

In the last decades, indications in liver surgery have been expanded due to technological and technical improvements. Within the hepatic resections we distinguish between anatomic resections including hemihepatectomy, segmentectomy/bisegmentectomy that are supposed to follow this pre-established liver segments or atypical resections, which do not follow it. Anatomic resections are performed often in case of large or deep tumors while atypical resections in those peripheral situated. Each patient would require a single strategy and a laparoscopic approach could be also assessed in selected patients [38]. In the last twenty years the laparoscopic approach has gained in popularity and currently even the major hepatic resections can be safely performed laparoscopically [39,40] Concerning the surgical technique there are plenty of different mechanisms for splitting the liver parenchyma and there is different data on what of the mechanisms had demonstrated to be superior [41,42]. Classically it had been stated that the resection margin must be >1cm, but in latest publications has been demonstrated no relationship between overall survival and the width of the resection margin [43,44].

With regard to the extension of the indications of the liver resections, new techniques have been described lately to wide them. Adam et al. described first in 2000 a two-stage hepatectomy addressed to patients whose initially irresectable metastases are down-staged by neoadjuvant chemotherapy [45]. In the year 2006, the Associating Liver Partition and Portal vein ligation (ALPPS) procedure was also introduced by Schnitzbauer et al. [46] as an alternative to patients with bilobar metastases and since then several groups have published their results presenting favourable surgical and oncological outcomes [47,48].

Hereafter we would detail the specific considerations of liver resection for each type of tumor:

2.1.1. Resection of primary liver tumors

HCC and intrahepatic cholangiocarcinoma are the most common causes of liver resection in PLC. Despite the cholangiocarcinoma has a more aggressive biology compared to HCC, with an appropriate surgical approach can achieve a longer survival in its initial stadiums. Liver resection indications in patients with HCC depend on the staging. There are several staging systems for the HCC and the Barcelona Clinic Liver Cancer (BCLC) is the only system that links prognosis with treatment recommendations, and thereby BCLC staging system has been proposed as a standard for the assessment of prognosis in Europe and the United States [49,50]. Liver resection is normally recommended in patients in stage 0-A with normal liver function (Child-pugh A, occasionally B) and no portal hypertension [8,51,52]. The rest of the patients may be candidates to liver transplantation, ablative techniques or systemic therapies. Different studies have compared surgery for HCC with ablative therapies such as RFA and the results suggest that patients from surgery group may achieve a longer overall survival and lower recurrence [53,54]

Surgical resection by intrahepatic cholangiocarcinoma is only curative in few cases, as the majority of the patients have a spread disease at the moment of diagnostic and the tumor has an aggressive biology. Only 30% of them are potentially resectable

and the surgery involves a major/ extended liver resection. Despite a curative resection the 5-year survival rate ranges between 10-40% [6]. In the last decade the liver transplantation has been introduced as a reasonable curative approach in patients with intrahepatic cholangiocarcinoma after receiving a determined radio-chemotherapy and having responsed to the therapy, but that is still controversial [55–58].

2.1.2. Resection of colorectal liver metastasis

MCRC is the most frequently indication of liver resection as 10-20% of the patients presents synchronous liver metastases at the moment of diagnostic and 20-30% develop metachronous liver metastases [44]. Surgical resection appears to be the option of choice with a well-demonstrated benefit regarding long-term survival in MCRC, and perhaps in the neuroendocrine tumors but this statement is not so clear in other types of tumors [59].

Indications for liver resection by MCRC have been expanded in the last decades. Traditionally, extrahepatic disease and bilobar metastases were contraindications to perform an extended liver resection but currently it is accepted that a R0 situation and potentially cure could be achieved by pulmonary colorectal metastases resection or even by peritoneal carcinomatosis [23,32,60].

Currently the possibility of a redo surgery or repeat hepatectomy by recurrent disease is a reasonable alternative and several studies have published favourable long-term outcomes [61]. Lee H. et al describe a 5-year disease free survival (DFS) of more than 40% after secondary hepatectomy for recurrent CRLM, however the recurrence rate was significantly higher compared with the first surgery [62]. In addition each redo surgery adds a risk of complication when the surgery is not addressed to selected patients. In a nordish cohort of patients the possibility of liver transplant for patients with CRLM has also demonstrated favourable long-term outcomes when the patients fulfil the following premises: diameter of the largest CLM <55 mm, interval >2 years between colorectal and transplant operations, pre-LT carcinoembryonic antigen level <80 ng/ml, and responsive or stable disease under chemotherapy [63].

Therefore, in patients who would not be candidates to surgical treatments other therapeutic options have to be envisioned, such as the ablation

2.2. Ablation of liver tumors

In the last 10-20 years a number of ablative treatments for liver tumors have been described as an alternative to liver resection. As above stated, most patients whose liver function may be compromised by a liver resection would benefit of a minimally invasive approach and would avoid thus a liver impairment. These patients are generally not surgical candidates due to their inability to tolerate general anaesthesia or due to widespread metastatic disease.

Radiofrequency (RFA) and microwave ablation (MWA) are all ablative therapies based on thermal tumour damage and have emerged in the last years as promising techniques for tumor ablation [64,65]

2.2.1. Radiofrequency ablation (RFA)

In RFA, which involves the placement of one or more electrodes directly into the centre of the tumour, heat is produced through the application of a high-frequency alternating current. Biological cells are sensitive to temperature [66]. This leads to thermal coagulation and protein denaturation of the plasmatic membrane, nucleid acids, and thus tumour destruction. Temperature arises to 60-100ºC to produce the expected necrosis effect. Interestingly, temperatures >100 °C are less effective, as the desiccation that results at these temperatures, which manifests as water vapour and burnt tissue, increases the tissue impedance and therefore limits further electrical conduction through the remaining tissue [67]. There have been described 3 zones of action depending on the circumferential distance from the tip: a central one, which undergoes ablation-induced coagulative necrosis (temperature around 60ºC), a peripheral zone of sublethal hyperthermia and the surrounding healthy tissue (Fig. 6).

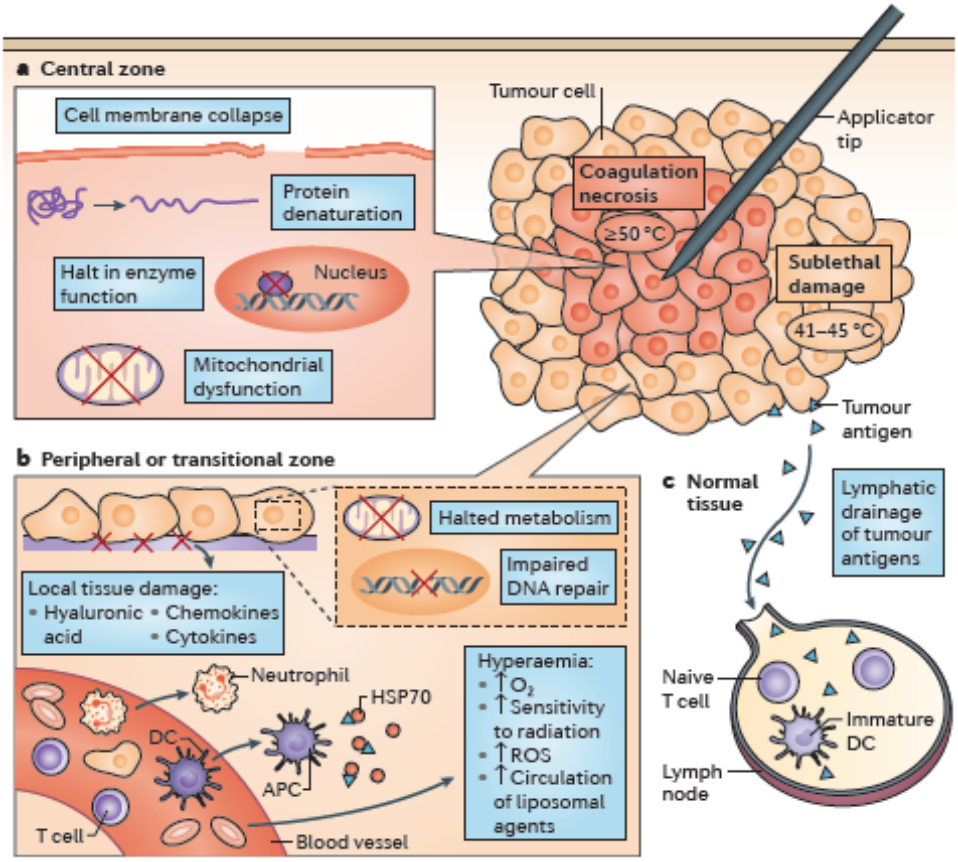


Fig. 6: It shows the different treatment zones depending on the achieved hyperthermia level (http://www.ncbi.nlm.nih.gov/pubmed/24561446). The longer the distance from the tip is, the lower temperature achieved would be.

At this point it is important to remark the importance of the size of the ablation target to achieve an optimal treatment, which is actually one of the greatest limitations of the RFA. As shown in the figure 6, RFA is normally delivered by applying needle electrodes. Because of the rapid radial decrease in the amount of heat delivered to tissue (by a factor of approximately d^2) the risk of falling outside of the lethal energy threshold increases with distance from the source [68]. In fact, in a recent meta-analyses evaluating the contributing factors of recurrence after RF ablation the multivariate analyses showed that the tumor size > 3cm was an independent factor of greater recurrence [69]. Therefore, to correctly ablate a tumor > 3 cm several electrodes would be needed in this approach, as shown in figure 6.b

Other limitation of the RFA is the so-called *"heat sink effect"*, which means that when RFA is delivered close to a large vessels it produces a refrigerating effect and dissipates part of the heat, decreasing the ablative effect in the target tissue. It has been described in RFA suboptimal outcomes in those tumors situated in the proximity of the main portal, hepatic veins or main bile ducts [69]. However, those are in fact the undesirable locations to a surgical resection and to perform RFA.

2.2.2. Microwave thermal ablation (MWA)

MWA is also a thermal ablation technique, which uses electromagnetic radiation to achieve the tumor death with frequencies ranging between 915-2450 MHz. Microwave ablation utilizes dielectric hysteresis to produce heat. Tissue destruction occurs when tissues are heated to lethal temperatures from an applied electromagnetic field, typically at 900–2500 MHz [70]. Polar molecules in tissue (primarily H_2O) are forced to continuously realign with the oscillating electric field, increasing their kinetic energy and, hence, the temperature of the tissue Clinical studies are scarce as MWA is a relatively new technique and the greatest experience has been reported in Japan and China. Shibaba et al. reported in a comparative cohort between RFA and MWA no differences in recurrence or in complication rates [71]. MWA has been proposed to decrease the "heat sink effect", but to date there is not enough available data to support this theory. However, there are some groups who defend a longer overall survival in the patients treated with MWA vs RFA [72]. On the contrary, other authors have drawn our attention to a potential higher rate of complications of the RFA compared to MWA when applied in peribiliary location [73]

2.2.3. Irreversible electroporation (IRE).

Next chapter (C.3) is entirely dedicated to IRE.

3. FUNDAMENTS OF THE IRREVERSIBLE ELECTROPORATION (IRE)

3.1. Molecular bases of the Electroporation/ electropermeabilization

Electroporation (EP) is a biophysical phenomenon becoming increasingly used as an ablation technique. Although the mechanism of EP is not yet fully understood, it is widely accepted that high electric field pulses delivered across tissue induce instabilities in the cell bilayer membrane thereby increasing its permeability [74]. This technique and phenomenon is called reversible electroporation or electropermeabilization (EP).

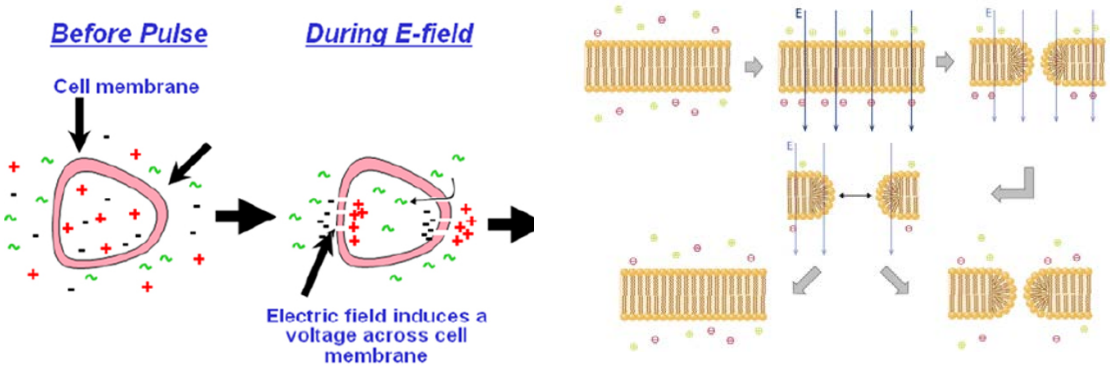


Fig. 7: Electropermeabilization creates nanopores across the membrane cell allowing free diffusion of molecules between intracellular and extracellular medium.

This technique and its effect on lipid bilayer was first described in the 1970s [75], however was not till the last two decades that its use has been spread out. Electroporation was commonly used in medicine and biotechnology to introduce in the cells small molecules such as drugs, antibodies or fragments of DNA [76]. EP application to increase the permeability of the cell membrane in tissue was introduced by Okino and Mohri in 1987 and by Mir et al. in 1991 [77], who independently discovered that combining an anticancer drug with reversibly

permeabilizing electric pulses greatly enhanced the effectiveness of the treatment rather than either one alone [78]. When EP is applied in combination with a chemotherapy agent the technique is known as electrochemotherapy (ECT) [79].

Plasmatic membrane of the cell is responsible of its shape and it is the gatekeeper, acting as a regulatory barrier for transport into and out of the cell. Due to its lipid bilayer surface it is permeable to hydrophobic molecules such as lipids, but polar components (DNA, aminoacids, ions…) are not allow to freely diffuse. These molecules are transported through the membrane by endocytosis (macromolecules) or by the aid of proteins embedded in the membrane. Ion's channels control permeability to specific ions, for example, Na+□, Ca2+, K+□and Cl−□and permit to create transmembrane gradients. Cell homeostasis is maintained by the plasmatic membrane, which assures a chemical gradient between Na+ and K+ across itself. Great part of the energy required by the cell is used to guarantee this gradient through the Na-K-ATPase.

Transmembrane potential in the cell remains normally on the order of 70 mV [80] but if this potential drop across the membrane exceeds 1V, the lipid bilayer suffers a rearrangement, pores are created and consequently ions diffuse freely, i.e., irreversible electroporation [81].

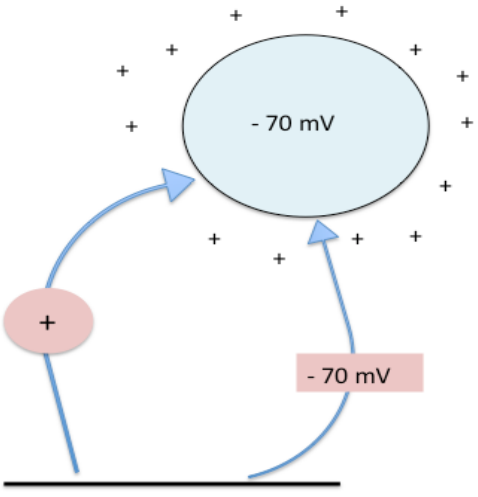


Fig. 8: This energy-consuming mechanism polarize the cell so that the interior of excitable cells has a potential of -70mV with respect to the extracellular electrolytes

The cell is able to repair sometimes this damage. The role of the Ca^{2+} concentration together with the loss of ATP are the determine factors involved in this process. Ca^{2+} is a fundamental signalling molecule implied in the cellular pathways; thus, if control of Ca^{2+} is lost, pathological signals can be activated, leading to necrotic or apoptotic cell death. The concentration of this ion is directly dependent on the availability of ATP since the low concentration of Ca^{2+} in the cytoplasm is maintained by pumping Ca^{2+} into Ca^{2+} storage in the sarcoplasmic or endoplasmic reticulum (SR or ER) or out across the membrane by ATP-ases. If the repair mechanisms overcome the damage before metabolic depletion occurs, the cell may regain control and survive the insult; however, if the degradative mechanisms take over, the cell dies.

If the electric field is maintained in a sufficient voltage the pore sizes gradually increase and stabilize being unable to reseal after the procedure, therefore destroying the cell's ability to maintain homeostasis. It has been shown that as a function of the electrical potential strength, number and duration of the pulses the effects of the EP are reversible or not. Energy is delivered using square wave direct current (DC) pulses whose amplitude, length, number and frequency can be independently modified. Studies developed in mouse muscle showed how the cytoplasmic concentration of the ions amply differed depending on the pulsing regimens [82]. Eight short high voltage pulses (8HV) achieved a higher permeabilization than one short high voltage pulse followed by one long low voltage (HV+LV) or eight low voltage pulses (8LV). Nevertheless, the thresholds for transient and stable pore formation are very likely to be dependent on membrane composition.

It is not clear the mechanisms underlying this cellular death, as two major types of cell death were recognized: necrosis and apoptosis [75]. Apoptosis is a controlled reaction triggered by normal and healthy processes, which requires ATP and turns the cell into encapsulated apoptotic bodies that are easily removed by macrophages without leaving a trace. Necrosis involves an inflammatory reaction and it is usually triggered by external factors. Apoptosis, which can also occur as a defence

mechanism during healing processes, is almost always normal and beneficial to an organism, while necrosis is always abnormal and harmful.

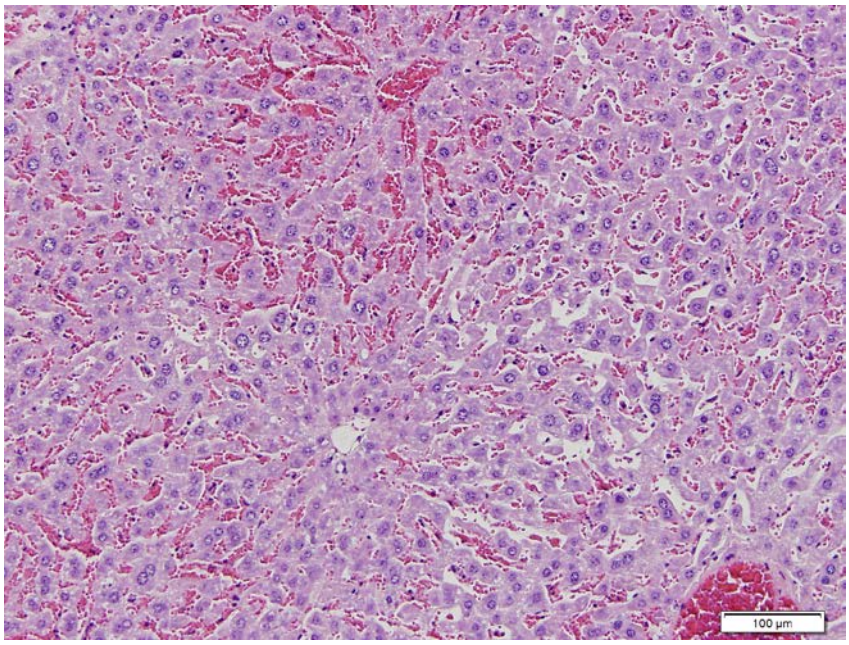


Fig. 9 Representative image of the liver parenchyma after IRE, which shows areas of sinusoidal congestion more evident in the areas around the vessels as a result of rupture of the intercellular joins and the extravasation of molecules from the intracellular to the extracellular medium.

In the histopathologycal analyses of the liver after de IRE, widespread pale eosinophilia and congestion in the sinusoids are usually evident in the frozen sections (fig. 9). However, cell death during the IRE seems to be follow an apoptosis pathway [83]. Cell apoptosis is associated with tissue regeneration and less inflammatory response and therefore the most preferable effect. Long et al. performed a histological examination in a model of pig liver harvested 6 hours after performing the IRE procedure. They intentionally created a lesion, which contained thermal damage as well as cell death due to IRE. Circular lesions, each with tissue effects in a concentric zoned pattern were examined. There were clearly 2 demarcated patterns. The zone 1 and 2 immediately surrounding the electrode presented tissue destruction by necrosis and lost of the cellular architecture, corresponding to thermal damage. However, in the zones 3 and 4 (approximately five to six lobules thick apart from the

electrode) NBT stain for NADH was positive as well as TUNEL stain for fragmented DNA, which means that in these zones the cells suffered an apoptosis process. The study concludes that apart from a limited region around the electrodes, death occurs by a non-thermal cell membrane poration mechanism.

Interestingly, the liver seems to recover from IRE more quickly than following thermal ablation [84]. Golberg et al. have assessed these changes in the histological structure of the liver 6 months after the ablation [85]. They demonstrated that the ablation of a normal liver by IRE preserves the extracellular matrix, liver architecture and triggers the regenerative processes. In general terms it is known that the hepatocytes grow after performing a hepatectomy following different mechanisms and due to its tremendous restorative capacity is able to regenerate even after removal of 90% of organ mass [86]. In the particular case of the IRE, it seems that the extracellular matrix and the liver architecture is preserved and the IRE ablation would trigger the liver regeneration. In this study they showed how 3 days after IRE the tissue was intensively positive stained with Ki67 as marker of hepatocytes regeneration and the Masson's Trichrome stain showed secretion of new extracellular matrix. This regeneration changes were shown till even 1 month after the ablation.

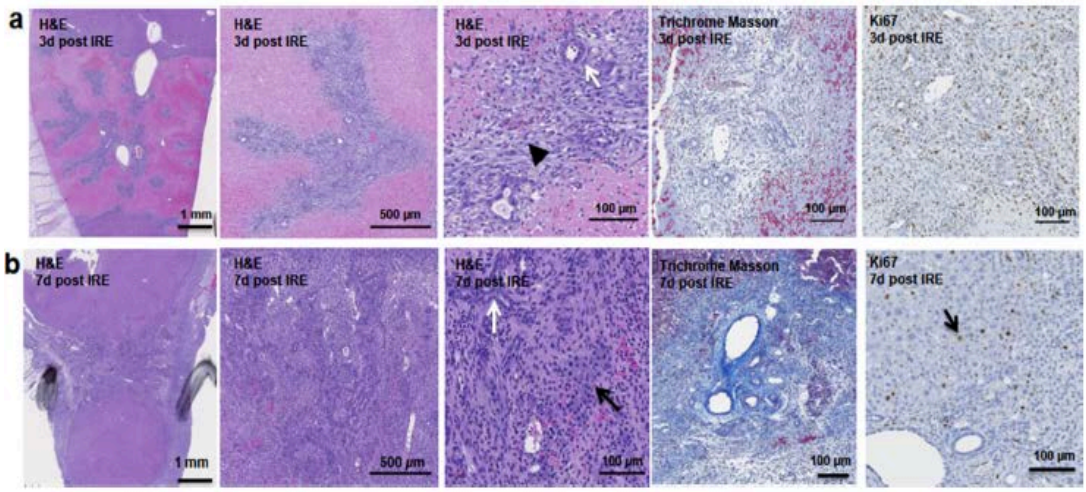


Fig. 10: Representative image from Goldberg et al. [85] which shows how the hepatocytes and the extracellular matrix would be regenerating 3 and 7 days after IRE.

3.2. Physical basic concepts and definitions

In order to understand the biophysical principles of the electroporation it is necessary to define some basic concepts, which are important for both comprehension and interpretation of otherwise complicated data [75].

Coulomb's Law is a law of physics describing the electrostatic interaction between electrically charged particles.

3.2.1. Coulombs law: It states that between two state charges there is a force proportional (F) to the product of the charges and inversely proportional to the square of the distance between them (r).

$$F = k\frac{q_1 \cdot q_2}{r^2} \qquad (1)$$

where ***k*** is a proportionality factor which is usually written as $1/4\Pi E_0$ and has a value of 9.0×10^9 and the unit V·m/C. q_1 and q_2 are two point charges, *r* is the distance between the charges, and "r-hat" is a unit vector parallel to the line connecting the charges.

At this point it is important to highlight the importance of the *principle of superposition*, as in the biological systems there are normally more than two charges present. It states that the force on any charge is the sum of the Coulomb forces from each of the other charges.

The presence of electric charge in a region of space modifies the characteristics of the surrounding space resulting in an *electric field* (E). So, we can consider an electric field as a region of space whose properties have been modified by the presence of an electric charge, so that by introducing into this electric field a new electric charge, this will experience a force.

The best definition of the electrical field comes of the Coulomb's Law:

3.2.2. Electric field: If we divide the electric force by charge, we get F/q

$$E = \frac{F}{q} \tag{2}$$

where its SI units are Newtons per coulomb ($N \cdot C^{-1}$) or, equivalently, volts per metre ($V \cdot m^{-1}$).

A field is any physical quantity that takes on different values at different points in space. So in addition to a number, the electric field also has a direction at every point in space. This is known as a vector field E. The electric field strength (size of the vector) and orientation is, in most cases in clinical situations, very significant [75].

The understanding of the biophysical phenomenon in the IRE is crucial for the success of the treatment as there are so many factors that can alter the electrical field in the biological systems and consequently affect the level of induced permeability of the cell membrane. The effectiveness of IRE is highly dependent upon the distribution of the electric field in the tissue [87]. In the clinical application of the electropermeabilization is important to take in consideration how these variables would potentially influence in the cell permeabilization.

First of all, the applied *voltage* by the generators is the most well controlled way of influencing the electric field strength. An increase in the applied voltage would result on a linear increase of the electric field strength.

Even more significant in the electric field is the geometry of the applied electrodes and the distance between them. Currently, IRE can be performed with different electrodes and protocols all affecting significantly size and shape of the treatment. From a theoretical point of view, plate electrodes may be more effective for surface tumors due to their ability to produce more uniform electric fields (Fig. 11). The analytical solution for the electric field between two infinite parallel plate electrodes is:

$$E = \frac{U}{d} \tag{3}$$

where *U* is the applied voltage and d is the distance between the electrodes.

Fig. 11: As shown in the theoretical model, plate electrodes are able to produce a more homogeneous Electrical field (E) (Kee et al., 2011)

Concerning pin electrodes it is known that the electric field strength is inversely proportional to the distance (r) from the centre of the electrode [75]. IRE application for soft tissue tumors is currently delivered using needle electrodes, since they are the only commercially available. According to this paradigm, the volume of electroporated tissue is limited, with a size and shape difficult to predict with the available software technologies and probes [88–91].

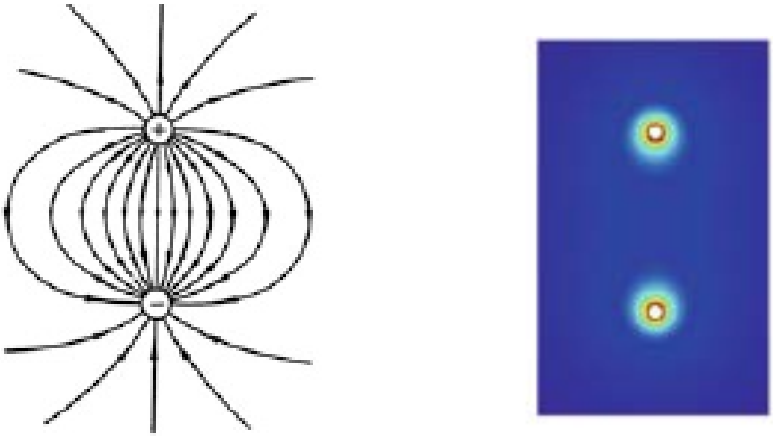


Fig. 12 (A) Electric fields are highest near the electrodes and rapidly drop off with distance, which means that the greatest amount of energy is delivered near the electrodes. (B) Figure bellow (in the next page) shows how with the increase of the spacing of the electrodes the ablating zone becomes less homogeneous (Image from Appelbaum et al., 2014)

Appelbaum et al. state in their study how the interelectrode spacing (cm), the number and duration of the pulses resulted in variations of the ablated zone in a liver model and they recommend the settings for a four-electrode Array [92]. It must be taken in consideration that in some cases would not be possible to place these electrodes within the tumor, and meanwhile preserving critical structures. Ben-David et al. demonstrated that slight discrepancies in the needle electrodes orientation or the target tissue could result in different sizes of ablation despite using exactly the same protocols [93].

Tissue heterogeneity and morphology can have tremendous impact on the electric field. As previously mentioned the effectiveness of the IRE is highly depending of the homogeneity of the electrical field. Therefore, the size of the bulk target volume and electrical resistance of the tissue types involved are important parameters. Liver for example is classically considered an isotropic organ, with a homogeneous parenchyma distribution [94]. However, Golberg et al. demonstrated the existence of electric field sinks, corroborated in both the numerical simulations as well as in vivo, as a result of the heterogeneity of the liver parenchyma [95]. Heterogeneity of the

electrical field has tried to be predicted with different imaging methods but with poor outcomes [96,97].

Reported healthy liver conductivities go from 0.03 S/m to 0.12 S/m [98]. Interestingly, it is commonly reported a significantly higher conductivity of tumor than healthy tissue. According to the reported conductivities, the sufficient voltage to produce irreversible electroporation of the whole tumor tissue (about 2750 V), also would suppose the overtreatment of the whole healthy tissue.

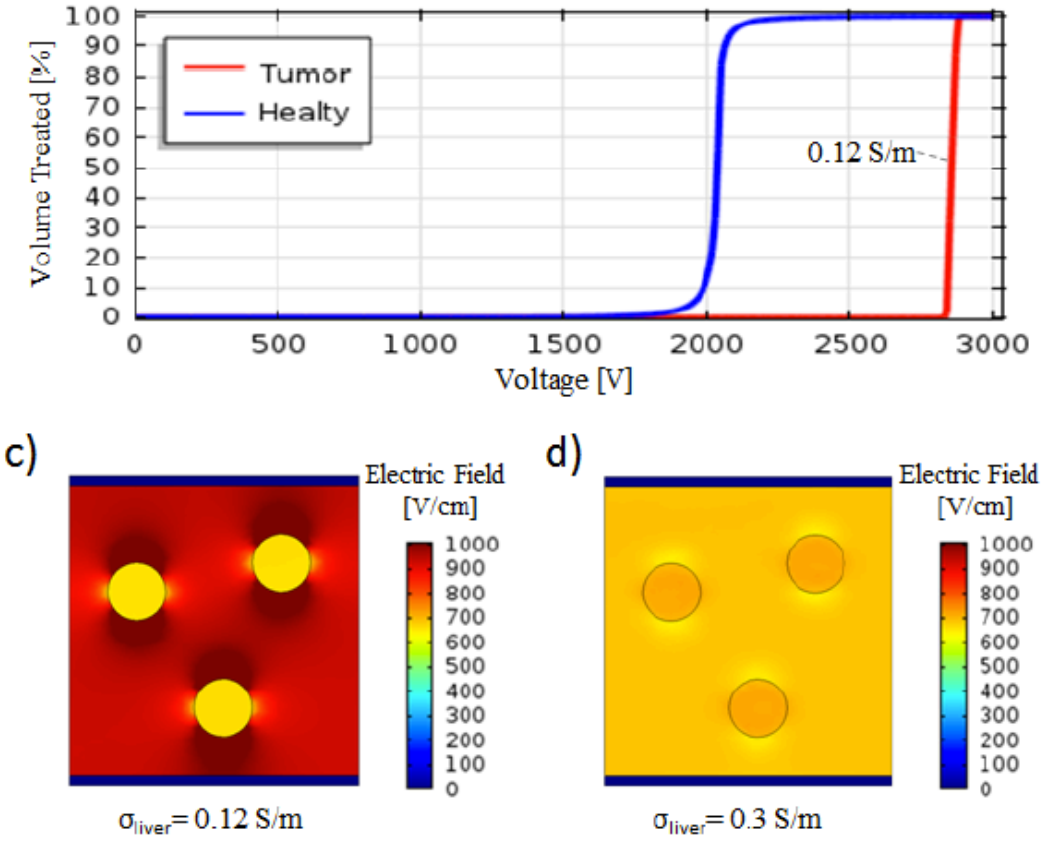


Fig. 13: Relative volume of tissue irreversibly treated by electroporation in relation to the voltage applied between electrodes. (σ liver = 0.12 S/m). Effects for 0.3, 0.6 and 1.2 S/m liver conductivity are represented at the same time. c) Electric field distribution within liver applying 2750 V between electrodes (σ liver = 0.12 S/m). d) Electric field distribution within liver with a high conductivity applying 2050 V between electrodes (σ liver = 0.3 S/m).

To conclude this chapter of basic concepts, several aspects must be considered to ensure a correct delivery of the electric field to the target tissue: the prescription of

the correct electric field strength according to the target tissue, the calculation of the electric field for specialized electrode devices, required geometrical margins, and the involvement of critical normal tissue.

3.3. Effects of the IRE on tumor blood flow

Underlying mechanisms that conduct to the cell death in the IRE remain still unclear as well as its vascular effects. These vascular effects would act separately from the cell death produced by the IRE. Several authors have demonstrated that the application of EP pulses lead to a local blood flow decrease ("vascular lock") and a vascular permeabilization of the tumors [99–103].

This blood flow modification consists in two phases depending on two different physiological mechanisms: the first rapid (lasting 1-3 min) attributed to sympathetically mediated reflex vasoconstriction of afferent arterioles; and the second mechanism (lasting up to 30 min.) based on cell permeabilization is a consequence of transient changes in cellular cytoskeleton [104]. The cell cytoskeleton provides a basic infrastructure for maintaining cell shape and function and it relies on microtubules, actin filaments, and intermediate filaments.
Kanthou et al. demonstrated that short, high-voltage square-wave electric pulses across adherent human endothelial cells results in an immediate but transient disruption of interphase microtubules and actin filaments, loss of contractility, and loss of VE-cadherin from cell-to-cell junctions [105]. These lead subsequently to changes in endothelial barrier function and could, therefore, account for the increase in endothelial monolayer permeability, which was observed in response to electroporation.

This increase in the endothelium permeability has important implications for blood flow *in vivo* since macromolecules, proteins and other oncotic substances are allowed to freely diffuse between extra- and intravascular medium leading to interstitial edema, increased IFP and as a result a compromise of the blood flow (Fig 13). Both statements contribute to this decrease of the blood flow after the EP.

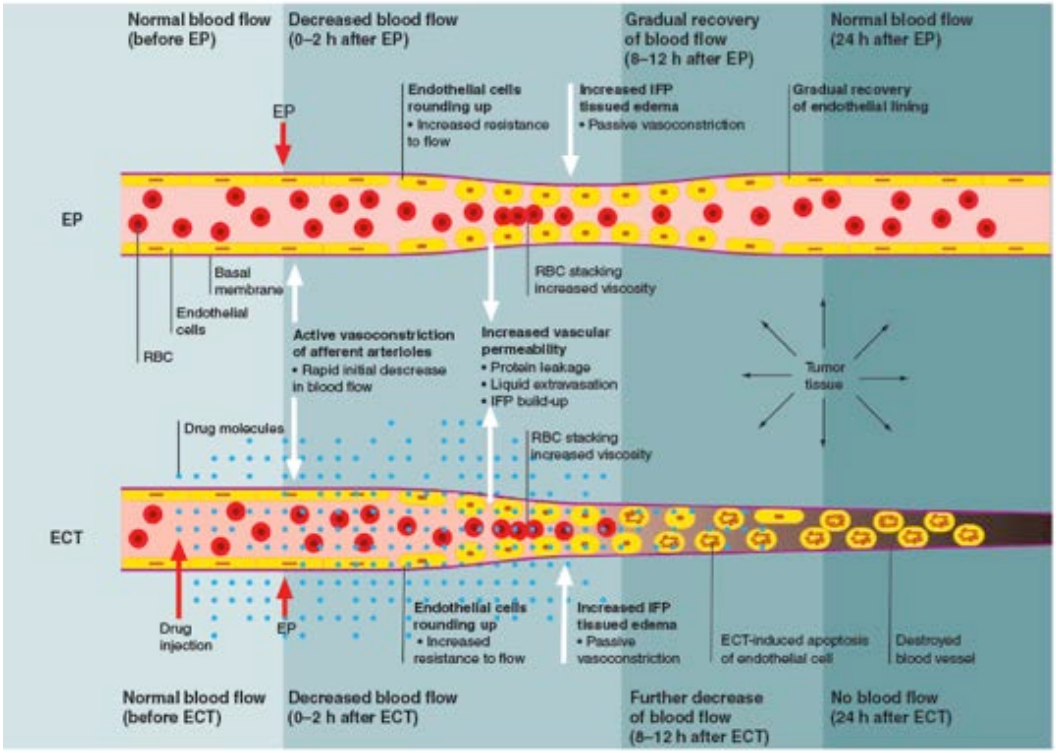


Fig. 15: It represents how the blood flow decreases through the pulse application by different synergic mechanisms (Image from Jarm et al. 2010)

The clinical implications of this "vascular lock" have been amply investigated in relation with the ECT therapies. There is evidence of reduced tumour oxygenation, endothelial cells swelling, apoptosis, and extensive tumour cell necrosis after electroporation and electrochemotherapy with Bleomicin [99]. Sersa et al. demonstrated this effect in a murine model of sarcoma in which the blood flow in the vessels supplying the tumor was measured immediately after the pulses by Doppler ultrasono-graphic imaging. This rapid increase in tumor vascular permeability and sustained reduction in tumor blood flow conducted to the induction of hypoxia of the tumors.

Interestingly, this study provides a mathematical modelling of the field distribution provided by the IRE around the endothelium of the blood vessels. Electric field distribution was determined by calculating the gradient of the electric potential between blood and the surrounding tissue. Blood conductivity is higher in

comparison to the endothelial cells, due to which the electric current is locally concentrated in the vicinity and inside the blood vessel. Consequently, the model predicts that endothelial cells lining the blood vessels are exposed to up to 40% higher electric field than the surrounding tumour cells (fig.16)

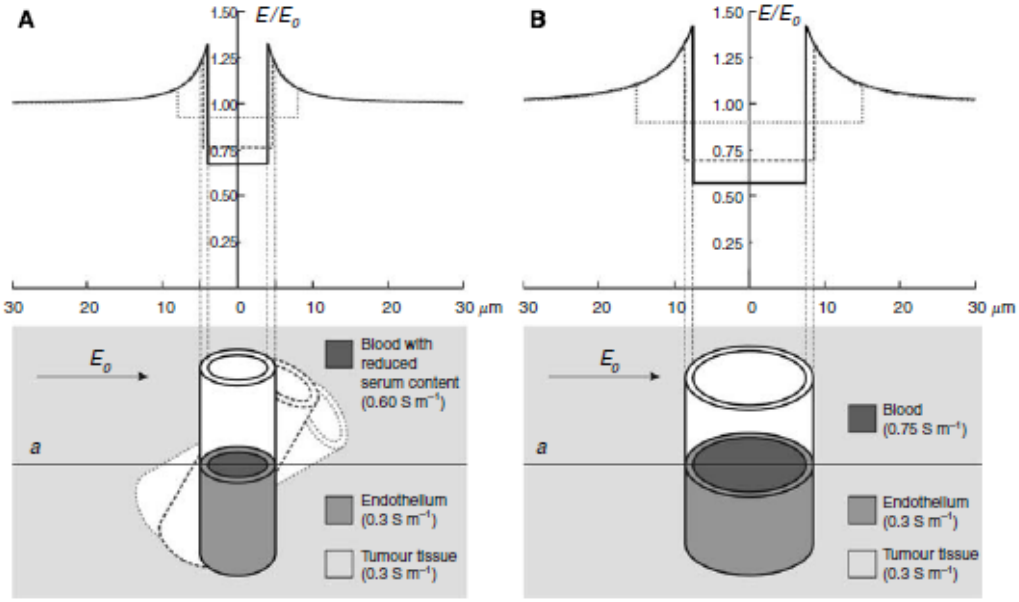


Fig.16: As consequence of the different conductivity among the blood and the surrounding tissue, the edge of the blood vessels lumen would receive an increased electrical field.

As stated before, biological tissues are not homogeneous so that it is expectable that the electric field distribution would be uneven (see chapter 3.2). The study of Sersa et al. gives a new insight into the potential effects of the IRE in the blood vessels and it is consistent with the results from Goldberg et al. [95] who explained the presence of "electric field sinks" in the biological tissues.

Besides its direct lethal effect in cell membrane, IRE would target its effect on small vessels, specifically on vascular endothelium, and together with the described "vascular lock" would contribute to the cytotoxic effect leading the tumor to ischemia. However, this effect seems to be produced preferably on small vessels as multiple studies have demonstrated that IRE does not affect the large vessels [106–108].

All these phenomena are part of the vascular-damaging component of the electroporation-therapies with contributes together with the direct cellular death to the effects that IRE produces in the biological tissue.

3.4. Advantages of the IRE comparing to other ablative techniques. Thermal damage.

In contrast to the other physical ablation modalities, IRE has a distinctive advantage: it is bellow the thermal damage threshold of 50ºC so that it does not involve thermal damage [66] and therefore it is not influenced by the so called "heat sink effect" that appears in the vicinity of large vessels, which is the greatest limitation of the RFA[109]. As a general rule, the allowable pulse lengths for which there is no part of the tissue exposed to 50 °C are much longer for plate electrodes than for needle electrodes.
Thermal ablation causes the cell death by denaturalization of the proteins, which leads to destruction of the tissue and coagulative necrosis (see figure 6). Due to this propriety of destroy the tissue, the RFA could be dangerous in some clinical scenarios when tumors are located in the proximity of vital structures. Complications leading the RFA procedures include thermal injury of colon, small bowel or bile ducts, which could be even more difficult to assess in the percutaneous approach.

Cell death in the irreversible electroporation is a consequence of the electroporation of the cell membrane, not due to a thermal damage despite some level of thermal damage could be achieved under some circumstances. Faroja et al. [110] have evaluated this statement by applying different protocols with high-doses and many pulse repetitions. There was a linear correlation between the increase in temperature with overall IRE “energy dose,” defined as the product of the voltage (maximum by 2900V) and pulse number (maximum by 360 pulses). They showed that ablations in which the temperature overcame 60ºC, the tissue presented the typical signs of coagulative necrosis (“white zone”), however by temperatures under 42ºC only IRE-associated phenomenon were noticed [110] as apoptosis, denoted by caspasas-3 positivity in the staining, congestion and haemorrhagic infiltrate.

In a similar study Van den Boss et al. [111] demonstrated that the asymmetrically distribution of the needle electrodes focuses the thermal effect in the area with the shortest interelectrode distance (fig.15). Yet, it may be unavoidable that IRE causes collateral, coagulative necrosis to some extent.

Therefore, in the clinical practice an individual assessment of each patient with individualized protocols and electrodes placement is highly recommended in order to avoid this potential thermal damage.

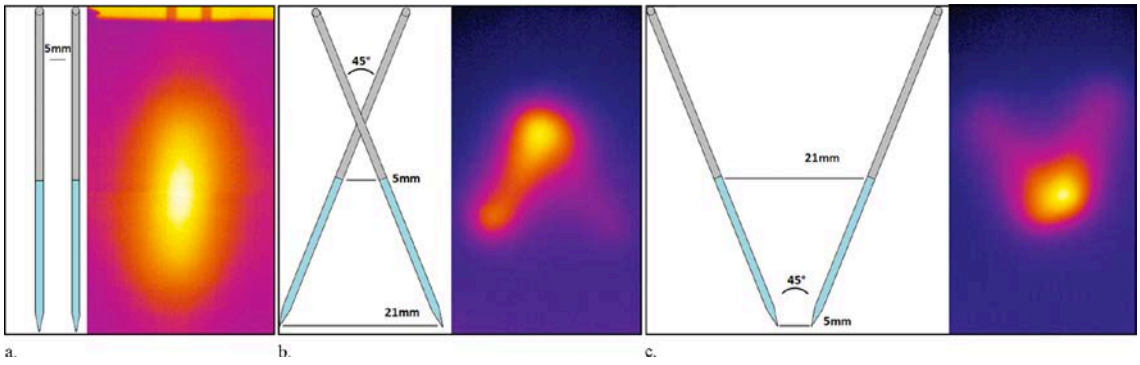


Fig. 17: representative image from Van den Boss et al. shows how the temperature distribution is homogeneous when the electrodes are parallel (a), however in the cases of convergent (c) or divergent (b) electrodes the temperature is maximum in areas of minimum distance.

There are not currently studies that compare the outcomes of the IRE to RFA. Bulvit et al. [112] demonstrated recently that the IRE ablation produces an enhanced systemic inflammatory response compared to RFA. As a reason is presented that vascular patency of the IRE, which makes that the cytokines and inflammatory factors are delivered into the bloodstream. On the other hand the study from Narajan et al. compared the pain experienced by the patients depending on the therapy the had become. They saw that there was no significant differences in the cumulative hydromorphone dose (1.54 mg (IRE) vs. 1.24 mg (RFA); $p = 0.52$) between both groups [113].

Nevertheless randomized clinical trials are still required to compare the different ablation techniques regarding their potential benefits and oncological outcomes.

4. CLINICAL IMPLICATIONS OF THE IRE IN THE SURGERY.

Irreversible electroporation is quite a young technique and its origins as tumor ablation technique the clinical setting date back to 1990s. First animal studies were addressed to evaluate the efficacy of the electrochemotherapy to ablate a large variety of murine subcutaneous implanted tumours [114–116]. Ramirez et al. demonstrated in 1998 the ability of the ECT with bleomycine to treat a VX2 hepatic carcinoma in a rabbit model with favourable outcomes [117]. ECT slowly grew up in the clinical practice as an innovative technique to ablate subcutaneous and cutaneous melanomas, head and neck tumors and thereafter followed by several others [79,118]. ECT was even proposed to treat deeply located tumours as liver metastases [119], pancreas [120–123] and brain tumors [124].

In 2005, Davalos et al. first proposed that irreversible electroporation could be used as a minimally invasive surgical procedure to ablate undesirable tissue without the use of adjuvant drugs and achieve similar results in terms of cell ablation [78]. They demonstrated in a mathematical theoretical modelling that irreversible electroporation alone could produce substantial tissue ablation without inducing a thermal damage in the cells. After this theoretical approach, the ability of the IRE to destroy cells was first demonstrated *in vitro* in cellular cultures of primary human hepatocarcinoma by Miller et al. [125] and consequently repeated in several animal experiments [126–128]. Guo et al. for example reported successfully ablation with IRE in a hepatocellular carcinoma tumor model [129]. They reported significant reduction of the tumor size 15 days after treatment and a clear progression from poorly differentiated viable hepatoma tissue pretherapy to extensive tumor necrosis and complete tumor regression in 9 of 10 treated rats.

Results in pre-clinical studies in small and large animal models [90,126–130] were encouraging, and had demonstrated its safety and feasibility [131]. Based on these premises, in the last decade several clinical studies have been expanded to medical field, addressing the advantages of IRE to tumor ablation next to portal or hepatic veins and major bile ducts [132] avoiding the technical limitations of the thermal modalities [107,133–139].

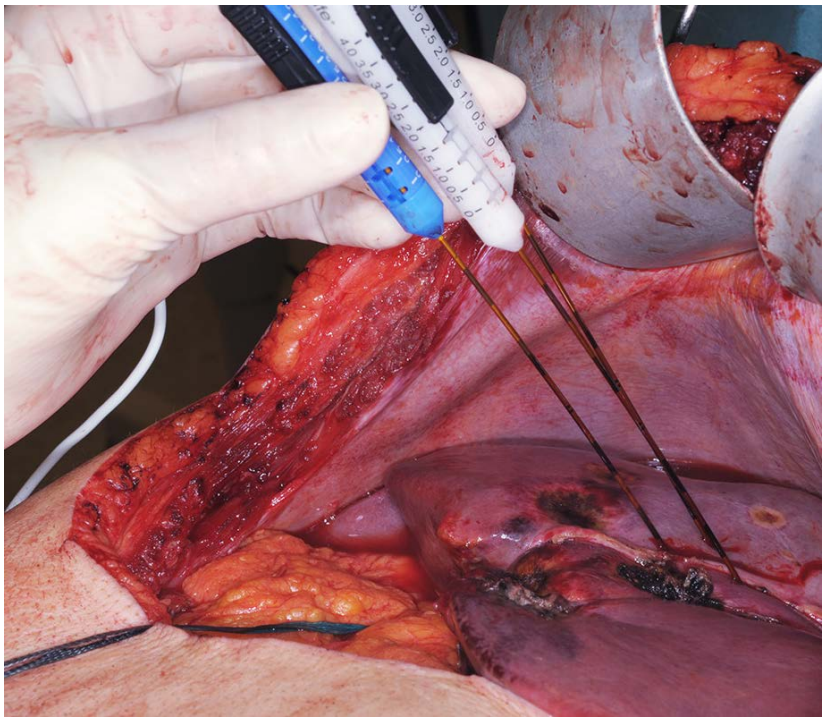


Fig.18: Representative picture of the clinical application of the IRE in the liver with a needle electrode model (picture of Sanchez-Velazquez et al. cir esp)

The study of Phillips et al. showed that even if the small bowel should be affected for the proximity while treating a solid abdominal tumor; it will be able to recover and heal without structural damage [140,141] IRE has also been tested as ablative technique in pancreas with palliative intent [121,122,142–152], kidney [153,154], prostate [155] and breast [156]. Its use in locally advanced pancreas adenocarcinoma has emerged in the latest 5-10 years [151,157,158]. Martin RC. et al reported a serie, which includes 200 patients treated with IRE after a neoadjuvant chemotherapy and the patients presented a prolonged overall survival compared to a historic cohort.

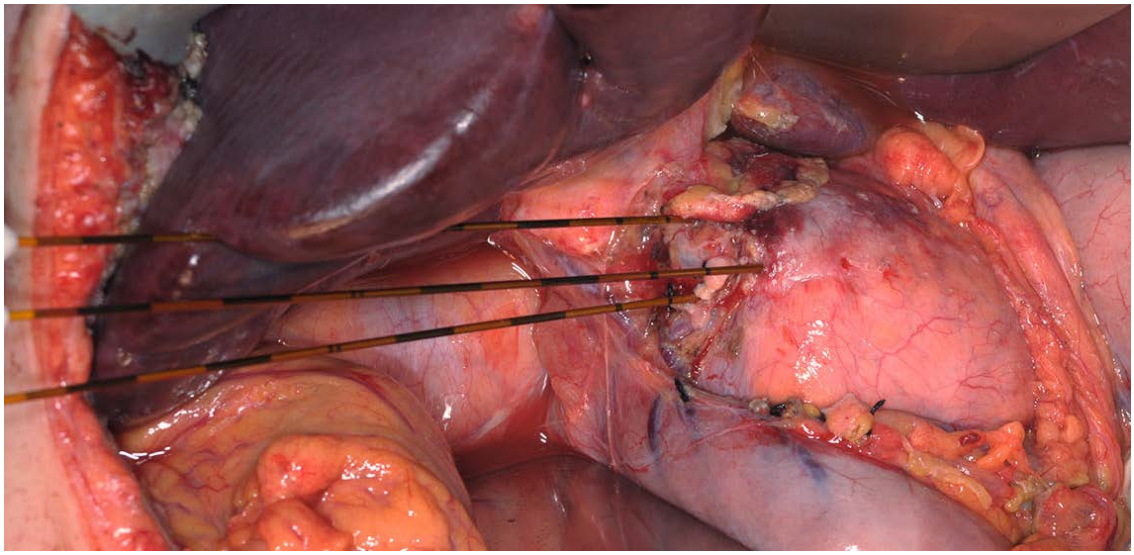


Fig.19: Representative picture of the application of the IRE in pancreas with the representative needle paradigm (Nanoknife). Picture extracted from Sánchez-Velázquez et al. Cir.Esp.

On the contrary other authors as Kluger et al. reported not so good outcomes by a high incidence of major complications including bleeding, duodenal perforations, bile leak and death [159].

4.1. Irreversible electroporation ablation modality and device

There is currently only one FDA-approved device [510 (k) Number: K080376] for clinical use of IRE (Nanoknife® [AngioDynamics, Latham, NY]). Electrical pulses are applied by bipolar or monopolar electrodes in varying number depending on the lesion to be treated and the device can generate a maximum intensity of 70mA or 3000V [160–162]. The application protocols are variable, but most studies deliver between 70-90 pulses with a duration of 70-100 μs. The device supports a maximum of six 19G electrodes whose maximum distance must not exceed 2 cm and whose alignment must be parallel to maintain the uniformity of the electric field. There is no standardized protocol on the number or arrangement of the electrodes, but the uniformity of the electric field plays a key role in the success of ablation.

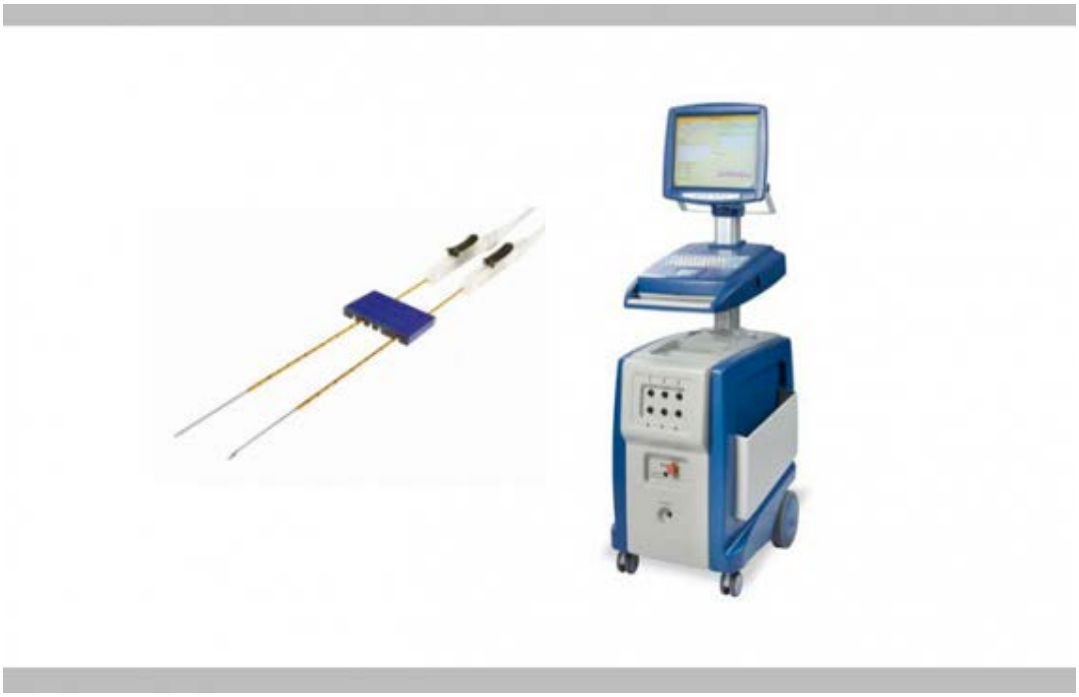


Fig. 20: Nanoknife® [AngioDynamics, Latham, NY] is currently the only commercialized device approved by the FDA to apply the IRE in a clinical setting.

The synchronization of the pulses with the electrocardiogram (ECG) is indispensable for the application of the pulses in order to avoid arrhythmias. The electrical pulse should be applied exactly 50 µs after the R wave to coincide with the absolute refractory period of the myocardium in the cardiac cycle [163]. It is also necessary that patients are under general anesthesia and under the effect of muscle plate relaxants such as rocuronium to avoid muscle contractions that trigger the pulses. Most studies monitor patients with TOF-watch® devices to ensure maintenance of proper muscle relaxation and the placement of defibrillator blades as a preventive measure. Navegation systems applied to the percutaneous or intraoperative application of the pulses [164]

4.2. Safety and feasibility of Irreversible electroporation

Since the introduction of the technique in humans, clinical safety has been the major concern of irreversible electroporation. The most common adverse effect is a cardiovascular event, since the application of the electric field in pulses implies that, as previously mentioned, the occurrence of arrhythmias, including ventricular fibrillation, was frequent [165]. Since the introduction of the ECG synchronizer in a standard way, the occurrence of such arrhythmias was substantially reduced. Rhythm disturbances vary depending on the treated area.

A recent study has shown that cardiovascular events are significantly related to the location of the electrodes. Thus, ablations in the celiac trunk region in the multivariate analysis were a risk factor for developing a cardiovascular event [166]. These arrhythmias do not usually involve a hemodynamic compromise for the patient and only require medical treatment [167]. Also the IRE can produce transient increase of the arterial tension of the patient. Up to 77% of the patients in this serie had an increase in mean blood pressure of 15 mmHg, a fact that correlates with the results of other authors. In some cases, extreme elevation of systolic blood pressure (SAD) has been reported up to 200 mmHg, but these were tumors of the upper renal pole or ablations in relation to the adrenal glands [165]. Hydroelectrolytic alterations have also been reported, such as hyperkalemia or metabolic acidosis. Taking into account the mechanism of action, the hypothesis is that the formation of nanopores in the plasma membrane of cells leads to the massive release of intracellular potassium into the circulatory torrent, similarly to what occurs in tumor lysis syndrome.

IRE application in human trials has reported to date scarce warnings in terms of safety. Ball *et al.* demonstrated slight metabolic acidosis and hyperkalemia in 4 subjects from a group of 28 patients (14.3%) in which IRE was applied for ablation [165]. It was suggested that diselectrolitemia occurred in individuals who underwent ablation of very large tumors. In a recent prospective multicenter study on 150 patients undergoing IRE high morbidity was found but no acid-base balance disorders were described or other life-threatening complications [168]. Niessen et al. in

a recent study reported a complication rate of 27%, which appears to be relatively high, but 16% were minor complications that did not require any further therapy [162].

In a study of Dollinger et al. they reported a rate of major complications of 7.1% of IRE procedures, while minor complications occurred in 18.8%, being the most frequent major complication the postablative abscess. [169]. This work is the special relevance as they exhaustively measured the proximity from the target ablation lesion to a large vessel in the liver, namely portal vein or suprahepatic vein. From all the patients who underwent an ablation, only 9.9% of them presented with a venous thrombosis (see Fig. 19). In some exceptional cases severe complications as arterial thrombosis have been reported, but the majority of the groups with experience in IRE do not report arterial complications [170,171].

Vessel	Total number of vessels/number of vessels with vascular alterations	Number of vessels in location regarding ablation zone/number of vessels with vascular alterations		
		Encased/ altered	Abutting/ altered	Within a perimeter of 0.1–1.0 cm/altered
Main PV	7/0	1/0	0/0	6/0
Right PV or segmental PV branch	39/5	13/5	16/0	10/0
Left PV or segmental PV branch	31/11	10/7	9/3	12/1
Middle HV	36/1	13/1	16/0	7/0
Right HV	38/1	13/0	14/0	11/1
Left HV	22/0	5/0	11/0	6/0
IVC	17/1	0/0	11/0	6/1
TIPS stent	1/0	0/0	1/0	0/0
Total	**191/19**	**55/13**	**78/3**	**58/3**

PV, portal vein; HV, hepatic vein; IVC, inferior vena cava; TIPS, transjugular intrahepatic portosystemic shunt

doi:10.1371/journal.pone.0135773.t003

Fig. 21: Table from the manuscript of Dollinger et al. 2015, which details the IRE application in the vicinity of main venous branches.

As stated before (chapter 2.1), as only 10-20% candidates for liver surgery are potentially resectable [20], ablative techniques are perceived as promising alternatives and assessed in terms of their capability to ablate large volumes [172]. Until now, in clinical scenarios the irreversible electroporation is typically performed in small tumors (diameter < 3cm). However, there are some pioneering studies indicating a growing interest in treating larger volumes. For instance, use of IRE in large tumor

models was experimentally evaluated by Lee et al. who achieved a maximum ablated volume of 22.1 cm^3 in a liver rabbit model (between 25-32% of total volume) [173] with favourable outcomes [174]. Appelbaum et al. demonstrated that IRE application was clinically acceptable addressed to tumors > 6 cm [92]. Furthermore, it has been described that the success rate of IRE rapidly decreases with the tumor size [172]. Furthermore, the results from Mali et al. showed that ECT was less effective on tumors larger than 3 cm in comparison to tumors smaller than 3 cm [175].

In a recent systematic review, Scheffer *et al*. showed that, while from 67 to 100% primary efficacy was obtained in small lesions, efficacy dropped dramatically to 45% in large tumors [176]. It has been suggested that the cause is incomplete ablation of large tumors because of limited coverage by existing electrodes and generators [168,172]. Despite safety has been extensively reported when moderate volumes are ablated [153,165,167,177], little has been published concerning the safety when performing larger ablations. By the way, the efforts should be focused to leverage the IRE's advantages to the maximum extent possible.

4.3. Efficacy of the Irreversible electroporation in the liver. Clinical outcomes.

Initial results in on-going clinical trials in the literature reveal variable rates of response. Early results of these studies demonstrate an enormous variability in terms of tumor recurrence rates, ranging from 5,6% to 100% depending on series [117,136,160,168]. Ablation techniques are now a fundamental part of the treatment of liver tumors. Whether in primary tumors such as hepatocarcinoma (HCC), which is currently 6th worldwide prevalence [178], as in the metastases of tumors of gastrointestinal origin, ablation is a reasonable alternative for non-tributary surgical resection patients.

The first experimental studies were directed towards hepatic ablation in the HCC, since RF ablation is established worldwide as the treatment of choice in its incipient stages. Guo et al. [129] describe for the first time in a murine model of hepatocarcinoma

that IRE ablation demonstrates a significant decrease in tumor size and a higher percentage of treatment-induced necrosis in ablated tumors. In their subsequent translation to the clinic, the studies of Cheung et al. [160] and Bhutiani et al. [161] specifically aimed to the treatment of HCC in limited series of 11 and 55 patients, describe the feasibility of the technique and few adverse effects. Even in patients with Child B cirrhosis, good oncologic results and few complications have been demonstrated [131].

Similarly, the IRE has a role in the ablation of colorectal-origin metastases (CRLM) that are not feasible for surgery because of its usually central or perivascular location. To date, IRE has demonstrated better outcomes regarding the tumor type, although it has been suggested that the histological subtype may play a role in the success of ablation [172]. In most series, since the number of patients is low the sample is very heterogeneous and this implies a limitation in the interpretation of the oncological results. Only Scheffer et al. [137] within the framework of the COLDFIRE study prospectively included 10 patients with an exclusive diagnosis of CRLM in whom the lesion was ablated and then resected for histological analysis. The results showed extensive areas of non-viable tumor in 80% of patients confirmed by the positivity of the marker of apoptosis caspase-3. This study was extremely relevant, as they reported for the first time a histological analysis after IRE-ablation. However, unfortunately it lacks on long-term follow-up and do not provide data on recurrence rate.

Niessen et al. [172] recent published in a cohort of 25 patients undergoing 48 ablations a local recurrence rate up to 75% depending on the underlying tumor disease. In the subsequent regression analyses they concluded that the tumor volume > $5cm^3$ and underlying disease type were factors independently associated with early local recurrence. Kingham et al. [136] in a retrospective cohort of 28 patients with a median follow-up of 6 months reported 5.7% of local recurrences. In the other hand, the study of Philips et al. describe a total of 31% local and distant recurrences evaluated by RECIST criteria [168]. It has to be realized that this study includes an extremely heterogeneous sample of patients, especially regarding tumor characteristics as

51.3% of patients presented large lesions with great vascular involvement. Moreover, different tumor origins are included which implies different patterns of recurrence and consequently an impact in the survival outcomes.

Edhemovic et al. published their results of 16 patients treated with electrochemoterapy with bleomicin showing that 71% of the metastases were still in complete response at median of 147 days after treatment [134]. The series of Scheffer et al. and of Dollinger et al. included 10 and 43 patients, respectively, but did not specify whether the ablation was complete, nor did they provide clear results about survival. More recently in the study by Niessen et al. [162] they reported that the local recurrence-free survival (LRFS) after treatment at 3 months, 6 months, and 12 months was 87.4%, 79.8%, and 74.8% respectively. Currently, there are at least one running clinical trial to investigate the role of the IRE as neoadjuvant treatment for central colorectal metastases not feasible to surgery or other ablation modalities [179].

It seems that IRE has a better performance by ablating primary tumors as HCC or CCC compared with metastasis and also the results are better to treat unique lesions under 3cm. In the case of the HCC the results are better the earlier the stadium (BCLC classification) and the Child is. [180]

4.4. Evaluation of the efficiency in the follow-up

Grades of oncological response are usually evaluated in the clinical setting by imaging. However, imaging studies regarding intermediate-term and long-term follow-up are scarce and stem from small animal models which makes very difficult its clinical translation and the interpretation of the results. Whereas after successful RFA or MWA, a clear-cut scar is visible on CT or MRI, and the detection of local recurrence is quite straightforward, the situation is more complex, and apparently more difficult after hepatic IRE.

Barbarasch et al. [181] describe the different patterns in the MRI evaluation of the post-ablated lesion, but it is one of the little studies addressed to this interpretation. On the day of the IRE, the ablation zone was visible as an intermediately and homogeneously hyperintense area compared with normal liver parenchyma on T2w TSE images. The following day the lesion turns into hypointese but maintaining a hiperintensity rim on the arterial phase and in the course of four weeks it will be slowly decrease the enhancement until it completely disappears. Within 3 months after IRE in 95% of the cases this enhancement would disappeared. That's why for the long-term follow-up, it is suggested to start at 3 months after the procedure because by that time, signs of inflammation (hyperintense rim and strong contrast enhancement) have subsided. However, IRE is a new technique in the clinical setting, which makes that the radiological response evaluation, is not standardized.

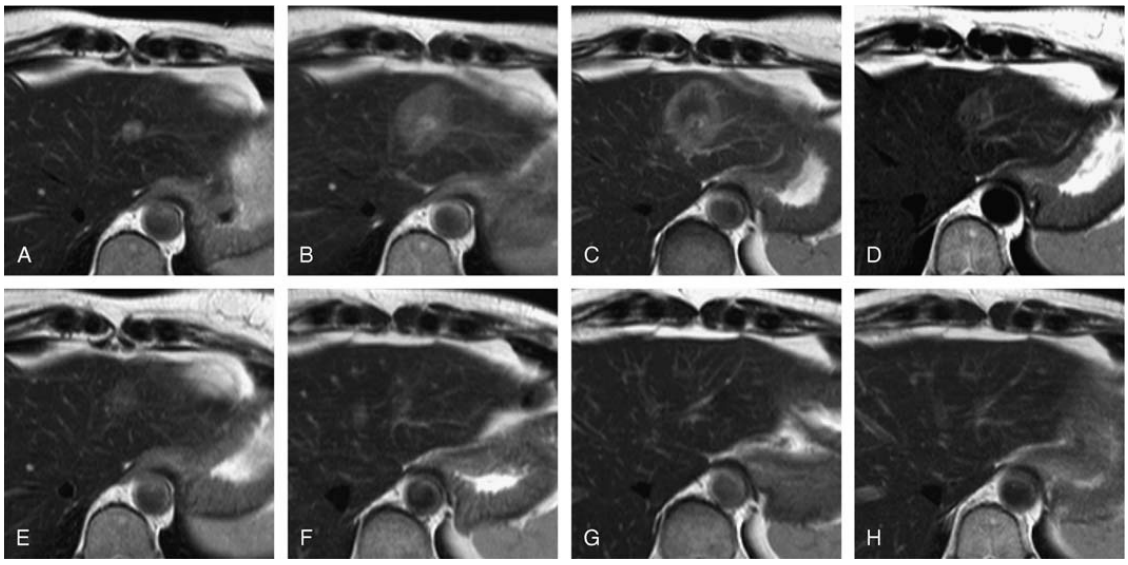


Fig. 22: MRI evolution of the lesion after IRE (imaging from Barbarasch et al.). (A) Lesion pre-IRE. (B-D) Appearance of the hyperintense rim in arterial phase still one week after the treatment. (E-H) Enhancement of the lesion slowly disappears till 1 year after the ablation (H) is not visible any more.

On the other hand, in the clinical practice it is not possible the evaluation of the histological response as most of the ablations are percutaneous and although they were open there is not routinely gain of histology. A recent review, analyses the

effects of IRE on liver tissue among 24 studies performing IRE in vivo which included more than 260 animals [182]. The histopathological analyse of the ablated tissue ranged within the studies between 1-14 days and that is the main limitation of these studies as specimen evaluation was performed at varying times after IRE-ablation. This may be problematic since cell death is a dynamic process by definition, and the question to which extent the time of histological evaluation determines the outcomes remains unanswered.

To the best of our knowledge, there is only one clinical study that evaluates the histological response after IRE in human ablation (see chapter 4.3) and the results are promising, as 80% of the tumor appeared to be necrotic immediately after the ablation. However, this is a marginal result lacking of a follow-up. This leads us to believe that there is an urgent need for a conclusive histological evaluation of the changes experimented in the tumors after a long-term follow-up.

HYPOTHESIS

1. Application of high-voltage electrical pulses of irreversible electroporation between two plane electrodes, which include an implanted tumor together with approximately 40% of the murine liver, is a feasible and safe procedure exempt from preoperative and postoperative complications.

2. Application of high-voltage pulses as before described conduces to twice percentage of tumoral necrosis of the treated animals and therefore an increase in the survival in comparison with the untreated controls. Significance level is defined as $p<0.05$.

3. Intensity of the high-voltage electrical pulses (i.e. 1000 V/cm or 2000V/cm) is directly related with the efficiency of the ablation, the grade of necrosis achieved and the survival.

OBJECTIVES

1. The first objective was to assess the effect of simple *"macroelectroporacion"* (i.e. 40% of liver mice volume between parallel plate electrodes) compared to the control group in terms of safety and feasibility (Phase 1). To develop this objective it was necessary to prolong the Phase 1 into a phase 1.2 in which it was not implanted any tumor since the objective was to evaluate the toxicity of the applied technique.

2. The second objective was to evaluate the effectiveness of the treatment in terms of oncological outcomes. The primary end-point was the overall survival (OS) in both groups: treated and controls; secondary end-points were the histopatological changes given by percentage of tumor necrosis, tumor growth rate, presence or not the distant metastases.

MATERIALS AND METHODS

1. Animal model

The animal research protocol was approved by the Ethics Committee for Animal Experimentation of the PRBB (Barcelona Biomedical Research Park) and by the Government of Catalonia's Animal Care Committee (FBP-13-77-74, DAAM: 7016) according to their guidelines. These guidelines follow Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

Two strains of six-week-old male mice, immunodeficient *athymic-nude* (Harlan laboratories, Indianapolis, IN, USA) and immunocompetent C57-Bl6 (Charles River Laboratories, New York, NY, USA) were acquired and maintained under standard conditions with a laboratory diet and water ad libitum.

Fig 23: Harlan athymic-nude in the left side of the picture. C57-Bl6 in the right side

2. Tumor implantation

The KM12C human colon cancer cell line was implanted in immunodeficent mice. In order to create a locally advanced animal tumor model, the poorly metastatic KM12C human colon cancer cell line was used. This cell line is a poorly differentiated adenocarcinoma, which has a low capacity to develop extrahepatic metastasis [183].

Tumors subcutaneously implanted and passaged in nude mice were extracted to prepare tumor fragments of 2 × 2 × 2 mm^3 and then implanted. Animals were anesthetized with a mixture of isofluorane and inhaled oxygen and analgesia was provided with buprenorphine (0.05-0.1 mg/kg SC) and meloxicam (1-3 mg/kg SC). After anaesthesia, a subxiphoid laparotomy was performed and the left lobe of the liver

exposed. The tumor was stitched to the hepatic capsule using a monofilament suture of 6/0 prolene (fig. 24). The abdominal muscle layer was closed with a running suture of vicryl 5/0, and the skin with a single silk suture.

Following initial implantation, 15 days were allowed for tumor growth. All the animals from the initial 55 implanted animals developed a tumor suitable for an IRE procedure and the treatment was applied after the growth period.

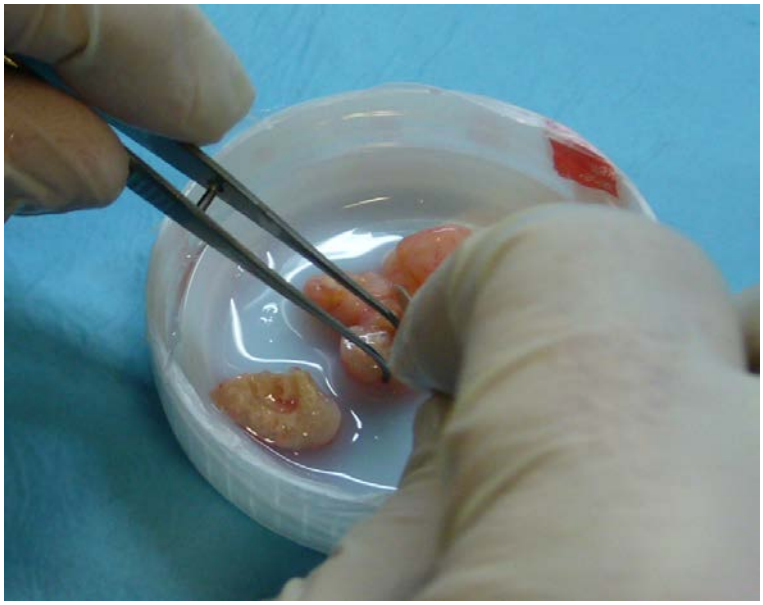


Fig. 24: Fragments of tumor ready to be implanted in the hosting mice. Figure bellow shows the process of implantation in the mouse liver.

3. Irrevers

After exposi[illegible]. Not only the whole tumor [illegible] also both two segments of [illegible]al size [184]. In contrast to ne[illegible]ic field across the treated vo[illegible]

After measuring the tumor, plate electrodes were placed as shown in Fig. 23. In addition to the whole tumor volume between the two electrodes, both lobes of the left liver, which represents around 40% of total size [185,186], were also included in the

ablation zone. As previously published, a premedication was administered to prevent hyperkalemia and enable safe ablation of such a large volume of liver[187].

A thin layer of partially conductive gel (Aquasonic 100 Sterile, Parker Laboratories, Fairfield, NJ, USA) was applied between the two parallel plate electrodes, as represented in Fig. 11, to ensure a uniform electric field [188]. A custom made generator was programmed to deliver trains of ten pulses with a duration of 100 μs, a repetition frequency of 1 Hz and an electric field strength of 1000 V/cm or 2000 V/cm, according to the group. Ten of those pulse trains were applied (100 pulses in total). A pause of 10 s was performed between the pulse trains. The subset of sham operated animals were exposed to the conductive gel and positioned within the electrodes but were not electroporated.

The plate electrodes were ellipsoidal (maximum diameter = 25 mm, minimum diameter = 15 mm) and were made of gold coated copper on a custom made fiber glass printed circuit board (PCB) built using conventional PCB technology. The separation distance between the two plates ranged from 2 mm to 5 mm depending on the size of the tumor. Such separation was measured with a digital caliper in each animal before treatment. The voltage of the pulses was set so that the ratio voltage/distance matched the desired field (either 1000 V/cm or 2000 V/cm). During treatment, voltage and current were monitored for ensuring successful delivery with an oscilloscope (Agilent DSO1014A) through a high voltage probe (Tektronix P5100) and a current probe (Tektronix A622). The generator was recharged in between trains of pulses so that the voltage was kept constant during the whole treatment; relative voltage drop during each train of ten pulses was less than 10%. Currents of up to 20 A were recorded when pulses of 2000 V/cm were delivered. As a wide range of field magnitudes has been reported for complete ablation by IRE [119,136], we evaluated two different magnitudes: 1000 V/cm and 2000 V/cm and a subset of animals were sham operated.

During the first 72h after surgery all the animals were given two additional doses of buprenorphine (0.05-1 mg/kg SC) and meloxicam (1-3 mg/kg SC), spaced out over 12h increments.

We used for our work a novel IRE approach in which the IRE ablation volume was unusually large [189]: we delivered pulses across two parallel plates that comprise two segments of the left liver which corresponds to nearly 40% of its volume (see figure 11) [184,186,190].

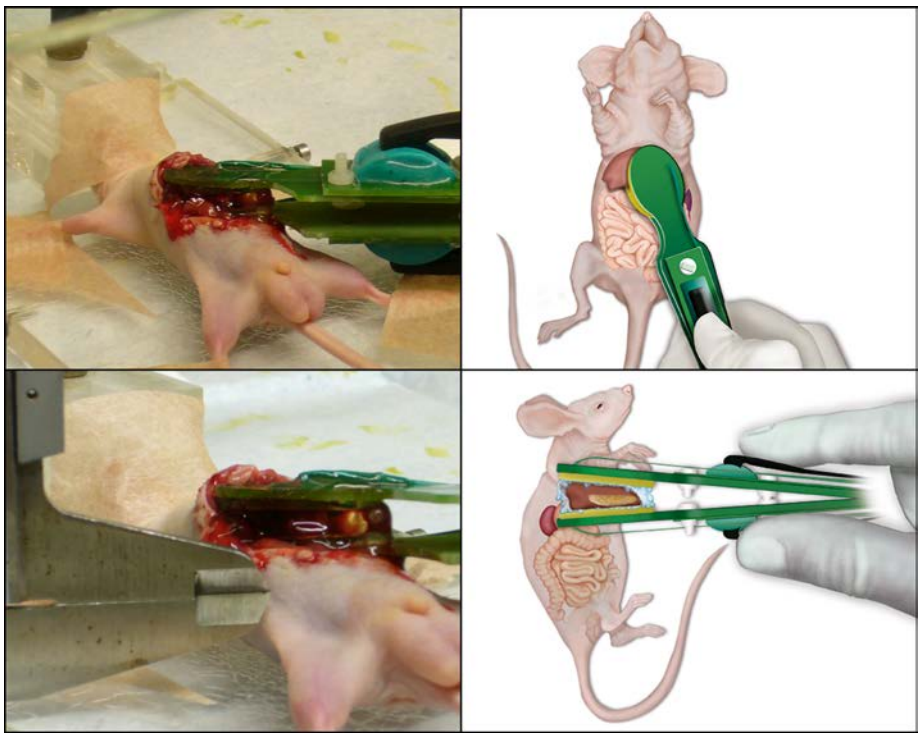


Fig. 25: IRE application using the paradigm of the plate electrodes. Right columns show the images corresponding to the real animal model. In the left columns it is shown a representative illustration of the process.

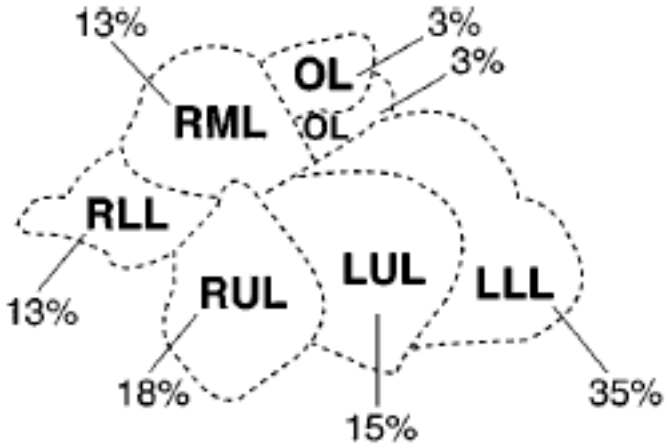


Fig. 26: Image bellow shows the division of the seven lobes and the relative contribution of each of them to total liver weigh. RUL, right upper lobe; LUL, left upper lobe; LLL, left lower lobe; RML, right middle lobe; RLL, right lower lobe; OL, omental lobe (extracted from Greene et al.[185])

4. Pharmacological therapy

Prior to surgery, the set of animals corresponding to this treatment group (n: 42) were pre-hydrated with 200 μL of 0.9% NaCl intraperitoneally (i.p) solution every 24h for two consecutive days. Calcium gluconate (GC) was injected in the preoperative care in two boluses, both intravenously (i.v.) and intraperitoneally (i.p.) at a dose of 100 mg/kg [191]. To prevent hyperkalemia, a furosemide i.v. bolus (4 mg/kg) was administered and during anaesthesia induction oxygen was mixed with inhaled salbutamol (100 mcg). After the procedure, sodium bicarbonate ($NaHCO_3$) i.p. was administered before the closure of the abdominal wall at a dose of 1 mEq/kg [191].

5. Blood samples and test

Analytical tests at 10 min post-IRE were performed to quantify blood electrolytes in all the animals with an i-STAT® device (Abbott Point of Care Inc, Princeton, NJ, USA). Blood samples obtained by a single puncture in the saphena vein. Serum levels of sodium (Na^+), potassium (K^+), and HCO_3^- were measured immediately after the extraction using the i-STAT®.

6. Estimation of electroporated volume

The following parameters were evaluated in the liver samples harvested during the in vivo experiments: total liver weight, electroporated liver weight, total liver volume and electroporated liver volume. A precision scale was used to determine the weight. Volume was measured by placing the tissue samples in a laboratory graduated cylinder filled with water and quantifying the volume displaced.

7. Histology

Following the euthanasia tumors were measured and photographed. The liver was harvested immediately after sacrifice and sectioned every 5 mm and the sections were fixed in 10% formalin and embedded in paraffin blocks. Each section was stained with

hematoxylin and eosin (H&E) for microscopic analysis. Following the euthanasia, the tumors were measured ($Dmax_1$) and photographed again. The liver was harvested immediately after sacrifice and fixed in 10% formalin. Fixed samples were embedded in paraffin, and sections of 3 µm were stained with hematoxylin and eosin (H&E) for microscopic analysis. The extent of tumor necrosis was semiquantitatively assessed and recorded as absent, focal (<10% of the tumor area), moderate (10%–30% of the tumor area) or extensive (>30% of the tumor area). Animals that worsened in BCS condition or needed to be sacrificed were also processed immediately after sacrifice.

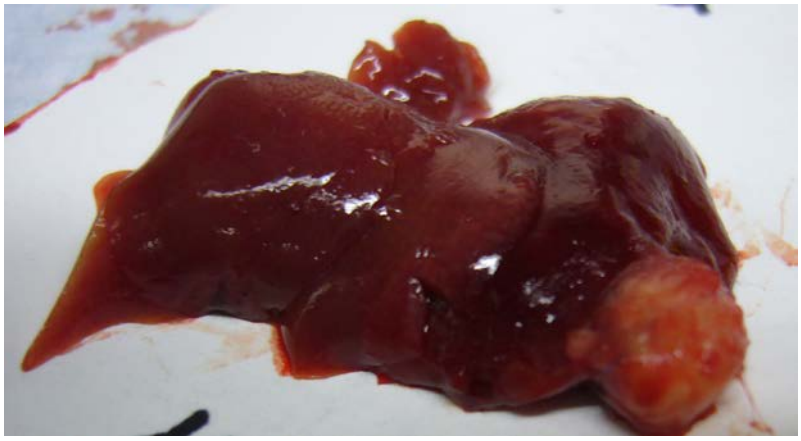


Fig. 27 It shows the harvested liver after IRE treatment. Vascular congestion can be appreciated at the left hepatic lobule**.**

8. Statistical Analysis

All the statistics were processed by the SPSS statistical software package (SPSS, version 20, IBM, Armonk, NY, USA) and expressed as mean ± standard deviation. Increases in lesion size based on one-dimensional D_max measurements were compared by a non-parametric Mann-Whitney U test. The survival curves (Kaplan–Meier curves) obtained were compared for the different treatments. A log-rank test was used to determine the statistical significance of the differences in time-to-event. The Cox regression model described how the hazard varies in response to IRE protocol. The results were expressed as Hazard Ratio (HR) with a 95% Confidence Interval (CI). Tests were considered statistically significant with a p-value <0.05.

The Kolmogorov–Smirnov test was used to determine the distribution of the variables. The Student's t-test was used to make pairwise comparisons of normally distributed parameters and the Mann-Whitney U test was used for non-parametric data. The influence of hyperkalemia and IRE protocol on mortality was determined by univariable and multivariable logistic regression analyses. Results were expressed as Odds Ratio (OR) with 95% Confidence Interval (CI). Tests were considered statistically significant with a p-value of <0.05.

RESULTS

Sánchez-Velázquez P, Castellví Q, Villanueva A, Quesada R, Pañella C, Cáceres M, et al.
Irreversible electroporation of the liver: is there a safe limit to the ablation volume?
Sci Rep. 2016 Apr 1;6(1):23781. D0I: 10.1038/srep23781

The first study addresses the first hypothesis of the thesis. We performed an ablation of around 40% of the liver's mouse with two different electrical fields in order to test how this unusual large ablation would affect the safety. We chose 87 animals from two different strains, i.e. C57-Bl6 and athymic-nude with implanted tumors as our treatment must have been tried in all the different scenarios [192]. Animals were differentiated in groups (table 1 of the manuscript) according to the applied strength field (1000V/cm, 2000V/cm or sham) and the medical treatment.

We observed that the animals of both strains that received the maximum electrical field, i.e. 2000V/cm without medical treatment, did not achieve a long survival. Concretely 88% of the animals without specific treatment died within the first 24h. Furthermore, animals that presented highest kalium concentrations in serum had statistically significant lower survival (OR 1.37 (95%CI = 1.07-1.73, p=0.01)). However, the group of animals that received the maximum electric field and were prophylactically treated with an anti-hyperkalemia therapy achieved a longer survival (OR 0.1; 95%CI = 0.02-0.32, p<0.0001). The multivariable analysis showed that the highest electrical field of the IRE protocol (2000V/cm), the anti-hyperkalemia therapy and the postoperative hyperkalemia were the three variables that significant impact in the early survival of the animals.

Sánchez-Velázquez P, Castellví Q, Villanueva A, Iglesias M, Quesada R, Pañella C, et al. Long-term effectiveness of irreversible electroporation in a murine model of colorectal liver metastasis. Sci Rep. 2017 Mar 22;7:44821. DOI: 10.1038/srep44821

Our second study demonstrated what histological changes experiment the tumors after a 6 months follow-up. Forty-six athymic-nude mice were implanted with the KM12C tumor and underwent different electrical fields (i.e. 1000V/cm, 2000V/cm and Sham) depending on the group.
We allowed the mice to continue to grow till they achieve the end-point criteria (death or high BCS) in order to compare the different effects of the IRE in the biological tissue in vivo.

We demonstrated a significant prolonged survival in animals belonging to the group 2000V/cm compared to the 1000V/cm and Sham. In the histological analyses, the 2000V/cm group showed also a significant grade of tumoral necrosis in comparison with the other groups. Our results suggest that the electrical field strength has a fundamental role in the oncological outcomes of the ablation and show for the first time a histological assessment of the tumors 6 months after the ablation.

DISCUSSION

Irreversible electroporation is a promising technique and its advantages over other ablative techniques have been demonstrated in animal models and human clinical trials [107,136,194]. With the high demand for alternative treatments for primary, as well as metastatic, hepatic malignancies, many newer focal ablative methods that complement conventional oncologic therapies have been developed and investigated. The great benefit of the IRE relies on its peculiar action mechanism, which for instance is not yet fully understood, however our study provides interesting insights of the cellular death after the delivery of electrical pulses.

Cellular death is achieved by alteration of the shape of the plasmatic membrane of the cells, which conducts to disturbances in its homeostasis. These alterations cannot be compensated by the cell repair mechanisms so that it leads to cellular death by following an apoptosis pathway. However, as the target of the pulses is specifically the plasmatic membrane, the extracellular matrix (ECM) remains unharmed, maintains the structural integrity of the tissue and potentially its ability to regenerate. For the foregoing reasons we hypothesized that irreversible electroporation of a large amount of liver parenchyma would be feasible and useful to ablate large unresectable tumors or perform several ablations at the same time. With improved surgical techniques and chemotherapy, surgically unresectable tumors are limited to large, multifocal tumors or to those located in difficult situations [195]. In that regard we assume that plate electrodes, which are known to produce a more uniform electrical field (see fig. 11) would be optimal to ablate a large volume or several lesions at the same time.
Furthermore, it has been described that the success rate of IRE rapidly decreases with the tumor size [172]. In a recent systematic review, Scheffer *et al*. showed that, while from 67 to 100% primary efficacy was obtained in small lesions, efficacy dropped dramatically to 45% in large tumors [176]. Although in current clinical practice such ablation volume is improbable, large liver ablations by IRE are being assayed in animal models and, presumably, will be tried in humans once the generators are able to apply the required electric fields and currents.

We decided to arbitrarily establish a 40% of the liver as an amount of tissue that could be potentially ablated in one single intervention. By the way that is the figure of healthy liver parenchyma, which could be safely resected in humans without a

compromise of the future liver function [33]. A future liver remnant of 60% should be enough to take over the whole liver function. Our major concern was that because of the increase in cell membrane permeability caused by electroporation certain potentially dangerous intracellular species freely diffuse from the intracellular medium to the extracellular medium. This phenomenon would be comparable to that of tumor lysis syndrome that occurs at the beginning of treatment with chemotherapeutics, in which tumor cells release intracellular products to bloodstream that overwhelm the excretory system [196,197]. Potassium is one of the ions found in greater intracellular concentration[198] (98% of the body's potassium is in the intracellular fluid in a concentration of about 140 mmol/L). Cellular potassium levels are regulated by Na-K-ATPase and a passive leak mechanism driven by the electrochemical gradient favours potassium leaving the cell [199]. We presumed that the larger the ablation target volume, the greater the disturbances expected in the homeostasis, and hence the higher the risk of early mortality. Ball *et al.* demonstrated slight metabolic acidosis and hyperkalemia in 4 subjects from a group of 28 patients (14.3%) in which IRE was applied for ablation [165]. It was suggested that diselectrolytemia occurred in individuals who underwent ablation of very large tumors.

As suspected, early mortality (<24 hours) occurred in 47.2% of the total animals and it was shown a strong correlation between the electric field strength and this early mortality. Interestingly, 88% of the animals subjected to a 2000 V/cm electrical field died when no anti-hyperkalemia therapy was administrated. We assumed that the higher the intensity the delivered electrical field the more cellular death will be achieved and it would be reflected in the plasmatic values of the potassium released after the membrane disruption. Likewise our work shows how the tumors treated with 2000V/cm suffer an extensive tumor regression with >30% of necrosis and animals potentially achieve a cure after at 6 months. However, it was not the case in the group of animals treated with 1000V/cm that did not present severe homeostasis disturbances but they achieved a tumor necrosis not significantly higher than the Sham group.

This fact leads us to think that the irreversible electroporation is perhaps an uneven phenomenon, which is heavily dependent on the intensity of the electrical field. Current IRE devices employed in clinical practice generate a voltage of between 1500-3000V [136] and create a target electric field of 680V/cm, which is supposed to be the threshold for effective irreversible electroporation in the liver [200]. Although the electric

field applied in the present study was notably higher and sufficient to theoretically ablate the tumor, some of the animals in the 2000V/cm group still suffered a recurrence. The underlying cause of early recurrence still remains unclear, but as a very large volume was involved recurrence is not due to failure to properly treat the tumor margins.

It seems reasonable that recurrence relies on insufficient or incomplete cellular ablation. In a recent work, Golberg *et al.* [95] numerically and experimentally showed that the heterogeneity of the liver parenchyma affects the uniformity of the field distribution during IRE treatment and as a result the cellular death is uneven. Areas around the vessels in the plane perpendicular to the electrodes presented increased electric field strength, whereas the tissue surrounding the vessel on a parallel plane showed a marked decrease in electric field strength. The possible occurrence of this phenomenon had been identified previously by Ivorra *et al.*,[201] who hypothesized that the areas around the vessels could become sites of tumor recurrence.

In fact to support this hypothesis recent studies show that despite an initial good response to the IRE treatment there is a high incidence of short-term recurrences [95,172]. The initial results of on-going clinical trials in the literature reveal variable rates of response. As previously mentioned, most animal tumor models do not standardly evaluate the long-term outcomes, as animals are euthanized at most 3 weeks after treatment [90,129,174]. To the best of our knowledge, the present study is the first to evaluate the efficacy of IRE in an animal model with nearly 6 months of follow-up. In most clinical studies tumor response assessment is based on RECIST criteria, as no histopathological confirmation is routinely obtained [162,202]. However, a decrease of the tumor mass by imaging does not necessarily imply changes in the cellular architecture or tumor death. Exclusive reliance on tumor size decrease does not provide a complete assessment of tumor response and may bring to erroneous conclusions. We were concerned about the lack of histological confirmation of the tumoral regression in the clinical use and the paucity of pathological correlation with the radiological changes.

Perhaps the most crucial aspect of hypothesis is the potential of the ablated tissue to regenerate and in such a way compensate the size of the electroporated tissue. Liver is an organ with an extreme capacity of regeneration which could compensate till about

90% of removal of its organ mass. Most of studies focused in the early histology of the liver after IRE show how the tissue suffers a phenomenon of "decellularization" but preservation of the ECM. Goldberg et al. [85] showed that the preserved ECM serves as a substrate for the development of mature liver tissue. Hepatocytes suffer a double process of hypertrophy as well as progenitor cell activation. Many factors pertaining to liver regeneration have been extensively studied, among which platelets and platelet-derived serotonin appeared in the forefront of the early activation triggering hepatocyte proliferation [203]. In the work of Golberg et al. it is demonstrated how within 3 days after the ablation the ablated tissue suffers early events of cellular proliferation and by day 7 cells were found actively dividing, as detected with positive Ki67 staining, and they secreted new extracellular matrix (intensive Masson's trichrome staining). More importantly, some large, hepatocyte-like cells, near the regenerated area were found positive for Ki67, indicating that hepatocyte expansion contributed to the regeneration process. In addition they observed an upregulation of all progenitor specific markers and most progenitor cell activation factors, which supports this hypothesis.

Unfortunately, we were not able to quantify in our study the degree of liver regeneration as our animals were allowed to live till the presence of end-point criteria. By the way, we did not observe at 6 months of follow-up nor macroscopically signs of parenchyma atrophy in the electroporated liver neither structural anomaly in the liver parenchyma, as shown in fig. 3g of the second manuscript. Therefore, it can be presumed that after the IRE it has been produced a massive cellular death, which is congruent with the level of diselectrolytemia achieved, but not enough to threaten the liver function of the animals in a short term. One limitation of our work would be the lack of monitoring of the liver function a few days after the IRE of 40% of the parenchyma, which undoubtedly would have given a glimpse of the extent of the hepatocyte damage.

An ideal ablation system for liver malignancies should entail a selective ablation of liver tumor while preserving the healthy hepatic tissue. Unfortunately, that is not the case with IRE despite. In fact, this technique is highly dependent on tissue conductivity [93,204] and tumors had higher dielectric properties (and higher conductivities) compared to healthy normal tissue because the higher sodium and water content of cancer cells [201]. This higher conductivity may lead to a reduced electric field in tumoral tissue than

in the surrounding healthy tissue. These differences of conductivity between healthy and tumoral tissue may be especially relevant using parallel transhepatic electrodes where tumoral tissue is around healthy tissue with less conductivity. In fact, in our second study we demonstrated that even with high electrical fields (up to 2000 V/cm) complete eradication of the tumor was rarely achieved even though when massive ionic imbalances were described (related to a massive destruction of healthy liver tissue (first study). Therefore, there are likely some current sinks as result of the inhomogeneity of the liver parenchyma and the less exposure of the tumoral tissue due to its higher conductivity. Indeed, it would be these unablated tumoral cells the ones that cause the recurrence favored by the regeneration pathways of the liver. From the foregoing it can be seen that the current electric field applied in clinical practice might not be sufficient to perform successful ablations and that could result in the under-treatment of the tumors and consequently explain the early recurrence. In order to selectively treat tumoral tissue our group is currently working on increasing healthy hepatic conductivity by hypersaline infusion through the portal vein to increase electric field in scattered tumoural nodules with IRE (given that tumoral tissue lack sinosoids are only supplied blood from the hepatic artery) [205]. Thus it would be theoretically possible to cause electroporation in tumour cells and avoid this phenomenon in healthy liver tissue.

In conclusion, as a result of our hypotheses we consider that irreversible electroporation targeted to 40% of the liver tissue could be feasible as long as the ionic imbalances are considered and therapeutic approaches envisioned. Not certainly now, because the ablated volumes are still too small, but in the future because with the annual incidence of liver cancers increasing, development of more consistent and effective treatment options is crucial.

On the other hand, the improved survival rate after a 6-month follow-up and the effective histopathological response in terms of tumoral necrosis are considered to be encouraging results. There is a need of standardize the doses and protocols used in the clinical application in order to be able to compare the outcomes of the different studies. We therefore believe that it is essential to re-evaluate the intensity and frequency of the pulses and perhaps to increase them without arriving to thermal damage or increasing the toxicity of the therapy. We firmly believe there is an important place for the irreversible electroporation in the ablative cancer therapies.

CONCLUSIONS

1. Ablation with Irreversible electroporation of 40% of the liver is technically feasible but it must be aware that potential threatening diselectrolitemias can be present. These disbalances can be easily treated with a proper medical treatment and the individuals achieve comparable survival as the untreated mice.

2. Irreversible electroporation after 6 months of follow-up achieves longer overall survival compared with the untreated individuals. This fact is reflected in a higher percentage of tumoral necrosis compared to controls (63.05% vs 25.6%) and lower tumor rate growth.

3. Both the percentage of tumoral necrosis achieved post-treatment and the overall survival was significantly dependent on the applied electrical field. Animals from the group 2000V/cm achieved an increased overall survival and histological response expressed by percentage of tumoral necrosis.

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