eman ta zabal zazu

Universidad del País Vasco
Euskal Herriko Unibertsitatea

BIOLOGIA ZELULARRA ETA HISTOLOGIAKO SAILA
MEDIKUNTZA ETA ERIZAINTZA FAKULTATEA

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**Iera Hernández Unzueta**

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**Lan hau Catalysis S.L. enpresak finantziatu du.**
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# AURKIBIDEA
# INDEX

# EDUKIEN AURKIBIDEA / CONTENT INDEX

# IRUDIEN AURKIBIDEA / FIGURE INDEX

# TAULEN AURKIBIDEA / TABLE INDEX

# LABURPENA
# SUMMARY

# LABURPENA

Minbizia gaur egun heriotza eragiten duen kausa nagusienetariko da. Kirurgia eta radioterapia zein kimioterapia bezalako tratamenduek gaixotasun hau murrizten laguntzen duten arren, pazienteek zehar-efektuak jasaten dituzte eta kasu askotan, ez da behin betiko sendabiderik posible izaten, batez ere kimioerresistentzia dela eta. Kolon-ondesteko minbizia (CRC) minbizi mota guztien artean hirugarren ugariena da, zeinak urtero heriotza asko eragiten dituen, batez ere gibeleko metastasiaren ondorioz. Bestalde, hain ohikoa ez den arren, pankreako minbiziak (PCA) ere garrantzia du minbizi hilgarrienetarikoa baita. Hori dela eta, tratamendu berriak frogatzen ari dira egungo terapiak hobetzeko. Horien artean, estrategietako bat, oinarrizko terapiei landareetatik erauzitako konposatu bioaktibo naturalak gehitzea da, hala nola, polifenolak, espeziak edota bitaminak, izan ere, emaitza onak lortu baitira. Zentzu honetan, Ocoxin, besteak beste, te berdez, kanelaz, erregaliz-estraktuz, bitaminez eta mineralez osaturiko konposatu naturala da zeinak minbiziaren tratamendurako onuragarriak izan daitezkeen propietate antioxidatzaileak, antiinflamatorioak eta immunoerregulatzaileak dituen. Aipatutakoa kontuan izanik, lan honetan konposatuaren eragina aztertu da PCAn eta CRCaren gibeleko metastasian, bai bakarrik, tumorearen aurkako agente bezala, eta baita kimioterapiarekin batera erabiltzeko ere tratamendu hauen eragina hobetzeko asmoarekin. Gure ikerketen arabera, alde batetik, Ocoxinek PCA eta CRC-ko zelulen bideragarritasuna murriztu zuen *in vitro,* zelulen proliferazioa gutxituz, apoptosia handituz eta ziklo zelularraren gelditzea eraginez G2/M fasean. Gainera, Ocoxinek PCAko tumoreak ez ezik, CRCaren gibeleko tumore metastasikoak ere txikiagotu egin zituen eta metastasi zelulen migrazioa ere inhibitu zuen. Horrez gain, minbizia inflamazio kronikoarekin, batez ere pankreatitisa eta hepatitisarekin, hertsiki loturik dagoela jakinda, Ocoxinek gaixotasunaren garapenean eragin handia duten tumorearen mikroingurumeneko (TME) inflamazio-bitartekarien kontzentrazioa modulatu zuen. Are gehiago, osagarri naturalak minbiziarekin zenbait generen espresioa erregulatu zuen,

haien artean minbiziarekin erlazionatuta daudenena ere bai. Beste alde batetik, Ocoxin kimioterapiaren osagarri bezala erabili zen, tratamenduak sarritan ez baitira eraginkorrak izaten TMEak tumoreari eskaintzen dion sostenguaren ondorioz. Beraz, Ocoxinek irinotekan, paklitaxel eta genzitabinaren eragina hobetzeko, TMEak eragindako kimioerresistentzia deuseztatzeko eta zehar-efektuak arintzeko gaitasunik zuen aztertu zen PCAn eta CRCaren metastasi hepatikoan. Ocoxinek kimioterapiaren efektua indartu egin zuen zelulen proliferazioa are gehiago murriztuz eta kimioerresistentzia ezereztu egin zuen. Are gehiago, Ocoxin minbizi zeluletan estromako zeluletan baino kaltegarriagoa izan zen eta orobat, tumorearen garapena faboratzen duten fibroblastoengandik eratorritako faktore soluigarrien efektua inhibitzeko gaitasuna duela frogatu zen *in vitro*. Halaber, osagarri nutrizionalak fibroblasto eta makrofagoen migratzeko gaitasuna eta hauen infiltrazioa murriztu zituen gibeleko tumore metastasikoetan. Amaitzeko, kimioterapia eta Ocoxinen konbinazioarekin tratatuak izan ziren saguek, kimioterapia bakarrik jaso zuten animaliek baino ongizate hobea aurkeztu zuten. Hortaz, Ocoxin farmako kimioterapikoen eragina areagotzeko eta kimioerresistentziari aurre egiteko erabili litekeen konposatua da, terapia hauek eragindako zehar-efektuak arintzen dituen bitartean.

# SUMMARY

Cancer is one of the principal causes of death nowadays. Although surgery or treatments like radiotherapy or chemotherapy help to reduce this disease, patients suffer undesired side-effects and, in many cases, there is still not any definitive cure due to chemoresistance. In this regard, colorectal cancer (CRC) is the third most common cancer causing a great amount of deaths every year principally due to the liver metastasis, and moreover, although it is not that common, pancreatic cancer (PCA) is one of the most mortal cancers. Thus, new treatments are being tested in order to improve current therapies. One of the strategies adopted currently is the addition of natural bioactive compounds obtained from plants like polyphenols, spice extracts, or vitamins among others to the first line therapies, which have showed promising results. In this regard, Ocoxin is a nutrient mixture composed, among others, by green tea, cinnamon and licorice extract, vitamins and minerals with proven antioxidant, anti-inflammatory and immunoregulatory properties that could be useful to treat cancer. Hence, its effects were studied alone as an anti-tumor agent and also as a coadjuvant treatment to improve chemotherapy in PCA and liver metastasis of CRC. On the one hand, Ocoxin decreased the viability of PCA and CRC cells *in vitro* by enhancing apoptosis by reducing cell proliferation and by causing a cell cycle arrest in G2/M phase. Furthermore, it reduced PCA tumors *in vivo* and together with an inhibition of the migratory capacity of CRC cells, decreased metastatic tumor foci in mice. Lastly, Ocoxin modulated the presence of inflammatory mediators in the tumor microenvironment (TME), which are a key factor in tumor development since chronic inflammation, especially pancreatitis and hepatitis, has been tightly associated with cancer and regulated the expression of certain genes, some of them altered in cancer. On the other hand, Ocoxin was used to complement chemotherapy. Frequently, this kind of treatment results ineffective owing to the contribution of the TME which supports tumor development. Thereupon, we analyzed the effect of Ocoxin in order to improve the cytotoxic effect of irinotecan, paclitaxel and gemcitabine

in liver metastasis of CRC and PCA respectively, as well as, to revert chemoresistance and to relieve the adverse-effects. In this regard, the nutritional mixture enhanced the anti-proliferative effect of chemotherapy in PCA and CRC cells and also showed to revert chemoresistance. Moreover, we proved that the supplement inhibited the pro-tumoral effect of fibroblast-derived soluble factors *in vitro* but it did not affect to healthy stromal cells as much as to cancer cells. Lastly, we also observed that the compound impaired fibroblast and macrophage migration and that decreased infiltration of both into metastatic liver tumors. Finally, we also detected that mice treated with the coadjuvant therapy showed better welfare than the animals that received the chemotherapeutic drug alone. In conclusion, Ocoxin could be a suitable compound to enhance the action of chemotherapeutic drugs and also to revert chemoresistance together with a relief of the side-effects caused by this therapies.

# LABURDURAK
# ABBREVIATIONS

**Laburdurak / Abbreviations**

| | |
|---|---|
| **5-FU** | **5-Fluorouracil**<br>5-Fluorouraziloa |
| **α-SMA** | **α-Smooth Muscle Actin**<br>Muskulu leunaren α-aktina |
| **AEEB** | **Ethics Comitee on Animal Testing**<br>Animaliekin egiten den Esperimentaziorako Etika Batzordea |
| **AML** | **Acute Myeloid Leukemia**<br>Leuzemia Mieloide Akutua |
| **CAF** | **Cancer Associated Fibroblast**<br>Minbiziari loturiko fibroblastoak |
| **cDNA** | **Complementary DNA**<br>DNA osagarria |
| **CFSE** | **Carboxyfluorescein succinimidyl ester**<br>Karboxifluoreszein sukzinimidil esterra |
| **CM** | **Conditioned Medium**<br>Medio baldintzatua |
| **COX** | **Cyclooxigenase**<br>Ziklooxigenasa |
| **CRC** | **Colorectal Cancer**<br>Kolon-ondesteko minbizia |
| **cRNA** | **Complementary RNA**<br>RNA osagarria |
| **Ct** | **Cycle Threshold**<br>Ziklo atalasea |
| **Cy3-CTP** | **Cyanine 3 – Cytidine triphosplate**<br>Zianina 3 - zitidina trifosfatoa |
| **DAB** | **3,3'-Diaminobenzidine**<br>3,3'-Diaminobenzidina |
| **DAPI** | **4',6-diamidino-2-phenylindole**<br>4',6-diamidino-2-fenilindola |
| **DC** | **Dendritic cell**<br>Zelula dendritikoa |
| **DMSO** | **Dimethyl sulfoxide**<br>Dimetilsulfoxidoa |
| **dNTP** | **Deoxyribonucleotide triphosphate**<br>Deoxierribonukleotido trifosfatoa |
| **ECM** | **Extracellular Matrix**<br>Matrize estrazelularra |

**Laburdurak / Abbreviations**

| | |
|---|---|
| **EDTA** | **Ethylenediaminetetraacetic acid**<br>Etilenodiaminatetraazetiko azidoa |
| **EGCG** | **Epigallocatechin-3-gallate**<br>Epigalokatekina-3-galatoa |
| **EGFR** | **Epidermal Growth Factor Receptor**<br>Hakuntza Faktore Epidermalaren Errezeptorea |
| **ELISA** | **Enzyme-Linked ImmunSorbent Assay**<br>Entzimei loturiko immunoabsortzio entsegua |
| **EMT** | **Epithelial-to-Mesenchimal Transition**<br>Epiteliotik mesenkimalerako trantsizioa |
| **F(ab)** | **Fragment antigen-binding**<br>Antigenoari lotzen zaion zatia |
| **FBS** | **Fetal Bovine Serum**<br>Behi Serum Fetala |
| **F(c)** | **Fragment crystallizable**<br>Zati kristalizagarria |
| **FELASA** | **Federation for Laboratory Animal Science Associations**<br>Zientziarako laborategi animalien elkarteen federazioa |
| **FITC** | **Fluorescein-5-isothiocyanate**<br>Fluoreszeina-5-isotiozianatoa |
| **FOLFIRI** | **Folinic acid, fluorouracil and irinotecan**<br>Azido folinikoa, fluorouraziloa eta irinotekana |
| **FOLFIRINOX** | **Folinic acid, fluorouracil irinotecan and oxaliplatin**<br>Azido folinikoa, fluorouraziloa, irinotekana eta oxaliplatinoa |
| **FOLFOX** | **Folinic acid, fluorouracil and oxaliplatin**<br>Azido folinikoa, fluorouraziloa eta oxaliplatinoa |
| **GLOBOCAN** | **Global Cancer Observatory**<br>Minbizi Globalaren Behatokia |
| **H+L** | **Heavy and light**<br>Astun eta arina |
| **H&E** | **Hematoxylin and eosin**<br>Hematoxilina eta eosina |
| **HCC** | **Hepatocellular carcinoma**<br>Kartzionoma hepatozelularra |
| **HIF** | **Hypoxia Inducible Factor**<br>Hipoxiaren bidezko faktore induzitzailea |
| **HRP** | **Horseradish Peroxidase**<br>Basaerrefauaren peroxidasa |

**Laburdurak / Abbreviations**

| | |
|---|---|
| **HSC** | **Hepatic Stellate Cell** |
| | Gibeleko Zelula Izartsua |
| **IFN** | **Interferon** |
| | Interferoia |
| **IgG** | **G immunoglobulin** |
| | G immunoglobulina |
| **IL** | **Interleukin** |
| | Interleukina |
| **KC** | **Kupffer Cell** |
| | Kupffer zelula |
| **LSEC** | **Liver Sinusoidal Endothelial Cell** |
| | Gibel Endotelioko Zelula Sinusoidala |
| **MDSC** | **Myeloid derived Stem Cell** |
| | Mieloidetik eratorritako zelula ama |
| **MHC** | **Major Histocomplatibility Complex** |
| | Histobateragarritasun konplexu nagusia |
| **MMP** | **Matrix Metalloprotease** |
| | Matrizeko Metraloproteasa |
| **mRNA** | **Messenger RNA** |
| | RNA mezularia |
| **NAB** | **130-nanometer albumin-bound** |
| | 130-nanometroko albuminari loturik |
| **NK** | **Natural Killer Cell** |
| | Zelula hiltzaile naturalak |
| **NKT** | **Natural Killer T cell** |
| | T zelula hiltzaile naturala |
| **PBS** | **Phosphate buffered saline** |
| | Gatz fosfato tanpoia |
| **PCA** | **Pancreatic Cancer** |
| | Pankreako minbizia |
| **PI** | **Propidium Iodide** |
| | Propidio ioduroa |
| **PSC** | **Pancreatic Stellate Cell** |
| | Pankreako Zelula Izartsua |
| **qPCR** | **Quantitative polymerase chain reation** |
| | Polimerasaren kate-erreakzio kuantitatiboa |
| **ROS** | **Reactive Oxygen Species** |
| | Oxigenoaren espezie erreaktiboak |

**Laburdurak / Abbreviations**

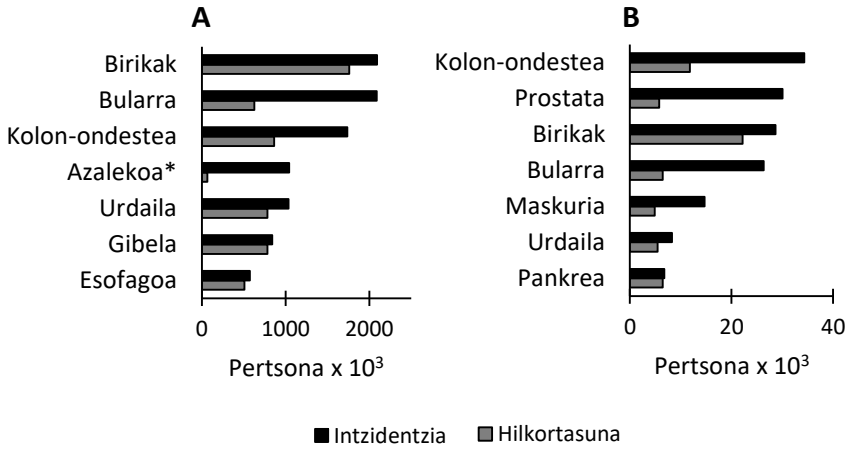
| | |
|---|---|
| **RT** | **Reverse transkription**<br>Alderantzizko transkripzioa |
| **SCLC** | **Small Cell Lung Cancer**<br>Biriketako zelula txikien minbizia |
| **SEOM** | **Sociedad Española de Oncología Médica**<br>Espainiako Onkologia Medikoaren Sozietatea |
| **SV40** | **Simian Virus 40**<br>40 tximu birusa |
| **TAM** | **Tumor Associated Macrophage**<br>Tumoreari loturiko makrofagoa |
| **TMB** | **3,3',5,5'-Tetramethylbenzidine**<br>3,3',5,5'-Tetrametilbenzidina |
| **$T_C$** | **Cytotoxic T cell**<br>T zelula zitotoxikoa |
| **TGF** | **Transforming Growth Factor**<br>Hazkuntza Faktore Transformatzailea |
| **$T_H$** | **T helper cell**<br>T zelula laguntzailea |
| **TIMP** | **Metalloprotease Inhibitor**<br>Metaloproteasen inhibitzailea |
| **TMB** | **3,3',5,5'-tetramethylbenzidine**<br>3,3',5,5'-tetrametilbentzidina |
| **TME** | **Tumor Microenvironment**<br>Tumore mikroingurumena |
| **TNF** | **Tumor Necrosis Factor**<br>Tumorearen Nekrosi Faktorea |
| **TRAIL** | **Tumor Necrosis Factor-related apoptosis-inducing ligand**<br>Tumorearen nekrosi faktoreari loturiko apoptosiaren ligando induzitzailea |
| **$T_{REG}$** | **T regulatory cell**<br>T zelula erregulatzailea |
| **VEGF** | **Vascular Endothelial Growth Factor**<br>Endotelio baskularraren hazkuntza faktoria |
| WHO | **World Health Organization**<br>Munduko Osasun Erakundea |

# I. SARRERA
# I. INTRODUCTION

# 1. MINBIZIA

## 1.1. MINBIZIAREN OROKORTASUNAK

Gaur egun heriotza dakarren gaixotasun nagusienetariko bat minbizia da, herrialde garatuetan hirugarren kausa hain zuzen ere, gaixotasun kardiobaskularren eta dementzien ostean (World Health Organization, WHO, 2016). Hori dela eta, ikertzaileak ahalegin handia egiten ari dira minbiziak nola diharduen ulertu ahal izateko eta bere aurkako terapia berriak garatzen saiatzeko, izan ere, egun erabiltzen diren farmakoak askotan ez dira gaixotasunari aurre egiteko gai izaten.




**1. Irudia. Minbizi kasu desberdinen intzidentzia eta hilkortasuna.** A) Minbizi kasu ugarienen datuak mundu mailan 2018. urtean. B) Minbizi kasu ohikoenen datuak Espainian 2017an. *Global Cancer Observatory, GLOBOCAN, 2018; Sociedad Española de Oncología Médica, SEOM, 2018*. *Azaleko minbizien artean melanoma ez da kontuan hartu.

Mundu mailan, 2018. urtean intzidentzia handiena izan zuten minbiziak biriketakoa, bularrekoa, kolon-ondestekoa (ingelesez *Colorectal Cancer*, CRC), melanoma ez den azaleko minbizia, urdailekoa, gibelekoa eta esofagokoa izan ziren besteak beste (Bray et al., 2018) (1. irudia). Espainia mailan, ordea, CRC izan zen gehien diagnostikatu zena 2017. urtean, atzetik prostatakoa, bularrekoa eta biriketakoa zeudelarik (Sociedad Española de Oncología Médica, SEOM, 2018). Hala ere, hilkortasunari dagokionez munduan bularreko minbizia da lehen postuan dagoena, jarraian CRC, prostatakoa, biriketakoa eta

tiroidekoa daudelarik (Bray et al., 2018). Espainian, aldiz, minbizi ohikoenak biriketakoa eta CRC diren arren, heriotza eragiten duen hirugarren minbizi mota pankreakoa da (ingelesez *Pancreatic Cancer*, PCA) (SEOM, 2018) (1. Irudia). Horiek horrela, nahiz eta intzidentzia baxua izan, minbizi batzuk oso agresiboak dira eta momentuz ez dago bizi-itxaropena luzatzeko aukerarik.

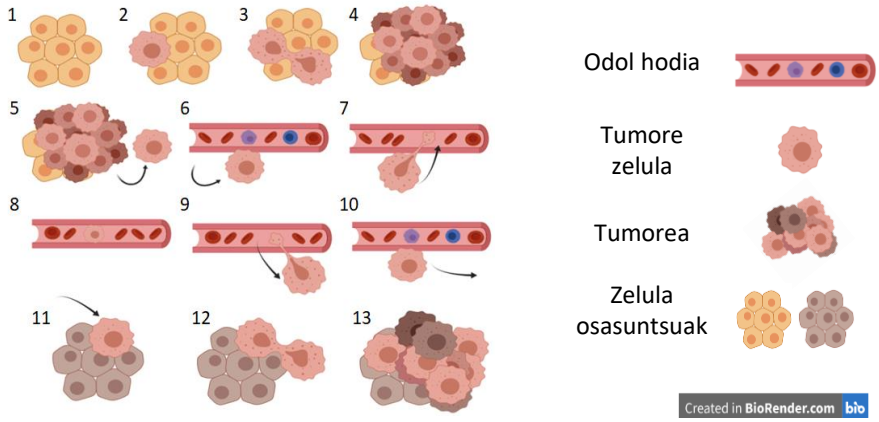
## 1.2. TUMOREAREN GARAPENA ETA METASTASIA

Minbizia gaixotasun heterogeneoa da, zelulen alterazioaren ondorioz beraien etengabeko hazkuntza eragiten duena, azkenean tumore bat eratzen delarik. Masa hori toki bakar batean eta kapsulaturik hazten bada organoan kalterik eragin gabe, onbera izan daiteke. Edozein modutan, tumorea etengabe haziz gero, ondoko ehunak inbaditzen baditu edo gorputzaren funtzioren bat asaldatzen badu, orduan minbizi izena hartzen du.

Ohikoena, tumorea zelula bakan batetik sortzea da, mutazioen metaketa pairatu duen zelula batetik zehazki. Ingurumen-faktoreek, faktore hereditarioek edota zelularen erreplikazio prozesuan sortutako akatsek mutazio genetikoak zein aldaketa epigenetikoak eragin ditzakete, hala nola, proteina kaltegarri berrien agerpena sortuz, proteinen funtzionamendu egokia galaraziz tumore-supresoreen inhibizioa eta onkogeneen aktibazioa suspertuz esaterako, edota geneen deregulazioa bultzatuz. Ondorioz, tumore zelulak bizirik jarraitzeko seinaleak jasotzen ditu eta hortaz, mekanismo desberdinak aktibatzen dira ziklo zelularraren kontrolik gabe zatitzen hasteko edota apoptosi prozesuak saihesteko. Horrez gain, zelula hauek organismoaren immunitate-sistema gainditzea lortzen badute azkenean tumore primario bat eratuko da. Are gehiago, eratutako tumore primariotik abiatuz ondoko ehunak inbaditzen hasi daitezke metastasia sortuz.

Metastasia minbizi zelulak tumore edo foku primariotik askatu, odol hodietara bidaiatu eta bertatik, beste organo berri bat kolonizatzen dutenean gertatzen da (2. Irudia). Horretarako, lehenengo eta behin minbizi zelulek ondoko zelulekin duten atxikidura loturak apurtu behar dituzte. Gero, matrize estrazelularra (ingelesez *Extracellular Matrix*, ECM) degradatu behar dute eta ondoren, intrabasazio bidez endotelioa zeharkatu behar dute odol hodietan barneratzeko. Behin odolean egonik, korronteak bultzatuta organismoan zehar bidaiatuko dute non, momentu zehatz batean, zelula endotelialei berriro atxikituko zaizkien. Orduan, tumore zelulek endotelioa estrabasatu egingo dute organo berri baten kolonizazioari hasiera emanez (Chambers et

al., 2002). Normalean, organismoa ECMren degradazioa, intrabasazioa, migrazioa, endotelioarekiko itsaspena, estrabasazioa, kolonizazioa eta inbasioa ekiditeko gai da barrera anatomiko, metaboliko eta immunitate-sistemari esker. Nolanahi ere, tumore-zelula gutxi batzuek oztopo horiei aurre egin diezakete mikro-mestastasia sortuz eta batzuetan minbizia gehiago garatu daiteke makro-metastasiak eratuz) (Chambers et al., 2002).




**2. Irudia. Metastasi prozesuaren eskema.** Minbizia ehun osasuntsu batean zelula arrotz bat etengabe zatitzen hasi eta tumorea osatzen duenean sortzen da (1-4). Kasu batzuetan tumore horretako zelula batek bere lotura zelularrak apurtu eta odo hodietaraino migratu dezake, odol hodi bati atxikitzen zaion arte (5-6). Behin bertan, intrabasazio bidez, tumore-zelula odolera pasatzen da (7-8) eta honek gorputzean zehar garraiatzen du, azkenean estrabasazio bidez odol hoditik ateratzen delarik (9-10). Beste organo batera heldu den zelula hori zatitzen hazten bada eta organo berria kolonizatzen badu metastasia gertatu dela esaten da (11-13). *Irudia BioRender.com bidez sortutakoa da.*

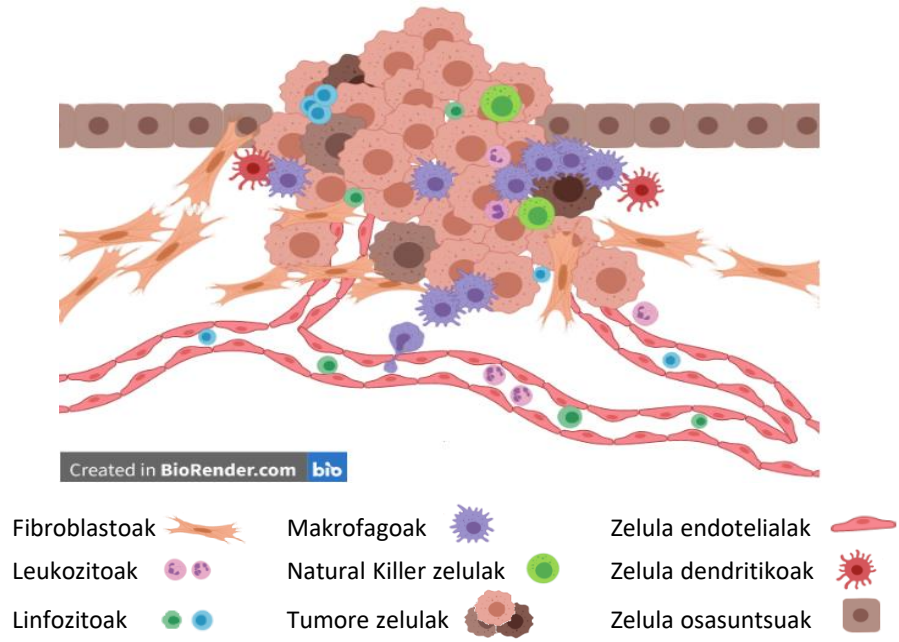
Zoritxarrez, minbizia gero eta arruntagoa da, eta etorkizunari begira, gaixotasun honen kasuak igotzen jarraituko dutela estimatzen da (Global Cancer Observatory, GLOBOCAN, 2018). Nahiz eta askotan egungo terapia eta farmakoek tumorearen aurka ondo jarduten duten, batzuetan pronostikoak ez dira onak izaten minbizi zelulak gorputzean zehar barreiatu direlako metastasi gune berriak sortuz, edota tumore zelulak kimioterapiarekiko erresistenteak direlako. Azken urteotako ikerketen arabera, kimioerresistentzia hori tumore-zelulen mutazioak direla eta, edota tumorea bera inguratzen duten zelulen laguntzaren ondorioz sortzen da (Phi et al., 2018; Yeldag et al., 2018). Alegia, minbizi zelulek bere inguruan dauden bestelako zelulen gain eragiten dute, zelula osasuntsu horiek beraien fenotipoa aldatzen dutelarik tumorearen alde joka dezaten (Sounni & Noel, 2013). Tumorearen inguruan aurkitzen diren elementu guztien multzoari

tumore mikroingurumena (ingelesez *Tumor Microenvironment*, TME) deritzo eta tumore zelulak ez ezik, fibroblastoak, makrofagoak, immunitate-sistemako zelulak, zelula endotelialak eta hauek guztiek jariatzen dituzten faktoreek osatzen dute.

# 2. TUMOREAREN MIKROINGURUMENA

Ezaguna da TMEak eragina duela minbiziaren garapenean. TMEa tumore zelulez gain, haiek inguratuz ageri diren osagaiek eratzen dute, hala nola, ECMak, zelula estromalek, linfa- eta odol-sistemak eta bestelako molekulek.

Tumore zelulak, hortaz, ECM-z inguraturik ageri dira, zeinak sostengua eskaintzen dion bai ehun osasuntsuari eta baita tumore, zein gainerako zelula estromalei ere. Estromako zelulen artean fibroblastoak, immunitate-sistemako zelulak eta zelula endotelialak aurki daitezke besteak beste (Wang, M. et al., 2017) (3. Irudia).




**3. Irudia. Tumorearen mikroingurumeneko osagai zelularren eskema.** Tumore zelulez gain, tumorearen mikroingurumenean beste hainbat zelula mota aurkitzen dira. Hauen artean fibroblastoak, odol hodiak eratzen dituzten zelula endotelialak eta immunitate-sisteman parte hartzen duten zelula desberdinak daude, hala nola, zelula dendritikoak, makrofagoak, natural killer zelulak, linfozitoak eta leukozitoak besteak beste. *Irudia BioRender.com bidez sortutakoa da.*

Hauek guztiak, egoera osasuntsuan tumorigenesi eta inbasioaren aurrean organismoari babesa eskaintzen dioten arren, tumorearekin elkarrekintzan daudenean beraien portaera aldatzeko gaitasuna dute. Tumore zelulekin batera, estromako zelulek hantura, desmoplasia (ehun konektiboaren gehiegizko ekoizpena, batez ere kolagenoarena), immunosupresioa eta geneen deregulazioa eragin dezakete, minbiziaren garapena areagotu egiten delarik (Coulouarn et al., 2012; Feig et al., 2012; Rupaimoole et al., 2016). Azken finean, tumore baten patua zehaztuko duena osagai hauen guztien konbinazio eta interakzioa izango da.

## 2.1. TUMOREAREN MIKROINGURUMENEKO ZELULAK

### 2.1.1. Tumore-zelulak

TMEan osagai nagusia tumore-zelulak dira. Aurrerago aipatu dugun bezala, tumore-zelulak mutazio desberdinen metaketaren ondorioz sortzen dira. Berez, organismoa zelula anomalo hauek detektatzeko eta deuseztatzeko gai da, baina tumore-zelula batzuek oztopo guztiak gainditzen dituzte eta hazteari ekiten diote minbizia sortuz. Hazkuntza hori errazteko tumore-zelulek zitokina eta hazkuntza faktore desberdinak ekoizten dituzte beraien zatiketa mantentzeko, heriotza zelularra ekiditeko, minbiziaren garapenean laguntza eskainiko dieten zelulak erreklutatzeko eta azken finean minbiziaren sakabanapena errazteko (Bussard et al., 2016; Pickup et al., 2014).

Tumorea hazten doan heinean tumore-masaren barneko aldean geratzen diren zelulek nutrienteak eta oxigenoa lortzeko zailtasunak izaten dituzte, hortaz, hipoxia egoera sortzen da. Ondorioz, tumore zelulek masa horren inguruan odol-baso berriak sortzea ahalbidetuko duten faktoreak jariatzen dtuzte, hau da angiogenesia bultzatzen dute(Arvelo & Cotte, 2009). Honi esker, sortu berri diren odol-basoek, tumore zelulak beharrezko dituzten osagaiez hornitzeaz gain, beste organo batera garraiatzeko aukera gehiago ematen dizkiete (Arvelo & Cotte, 2009; Chambers et al., 2002). Bestalde, minbizi-zelulen hazkuntza etengabeak eragindako hipoxia dela eta, tumore guneetan oxigeno espezie erreaktiboen (ingelesez *Reactive Oxygen Species*, ROS) metaketa gertatzen da zelula osasuntsuak kaltetu egiten dituelarik tumore zelulekiko egoera onuragarriagoa sortzen duen bitartean (Tafani et al., 2016).

### 2.1.2. Minbiziari loturiko fibroblastoak

Gaur egun nabarmenki aztertzen ari diren TMEko osagai bat minbiziari loturiko fibroblastoak dira (ingelesez *Cancer-Associated Fibroblast*, CAF). Fibroblasto arruntak ECMan egoera latentean aurkitzen dira eta ez dute uzkurtzeko gaitasunik izaten. Zauria edo ehunaren kalteren bat somatutakoan, ordea, inflamazio prozesuari hasiera ematen zaio eta fibroblasto batzuek aktibazioa jasaten dute miofibroblasto bilakatzen direlarik (Haqq et al., 2014; Sherman, 2018). Hauen zeregin nagusia zauriak itxi eta ECMa berrantolatzea izanik, α-muskulu leuneko aktina (ingelesez *α-Smooth Muscle Actin*, α-SMA) espresatzen dute zeinak uzkurtzeko eta beraz migratzeko gaitasuna ematen dien. Gainera, miofibroblastoek ECMa birmoldatzeko ahalmena ere lortzen dute, matrizea degradatzen duten metaloproteinasak (ingelesez *Matrix Metalloproteinase*, MMP), beraien inhibitzaileak (ingelesez *Tissue Inhibitor of Metalloproteinase*, TIMP) eta kolagenoa, elastina eta fibronektina bezalako proteinak ekoiztuz (Attieh & Vignjevic, 2016; Liu, T. et al., 2019).

Fibroblastoek egoera osasuntsuan zauriaren orbaintzean laguntzen dute arren, jasaten duten aktibazioa eteten ez bada, ECM gehiegi jariatzen hasiko dira, azkenean hantura prozesu jarraitua eta fibrosia sortu delarik. Minbiziaren kasuan hain zuzen ere, prozesu berdina behatu izan da eta horregatik minbizia “osatzen ez den orbain” bezala izendatu izan zen (Dvorak, 1986). Hortaz, lehenago azaldu dugun moduan, minbiziarekin erlazionatuta azaltzen diren fibroblastoak CAF izenez ezagutzen dira.

Pankrean, gibelean, biriketan eta giltzurrunetan fibroblasto berezi batzuk aurkitu izan dira, aurkezten duten forma bereizgarriarengatik zelula izartsu deritzenak (Haqq et al., 2014; Ireland et al., 2016; Sherman, 2018). Zelula hauek normalean egoera latentean egoten dira eta A bitamina metatzen dute retinil ester moduan lipido tantetan. Alabaina, aktibazioa jasaten dutenean, aurrerago aipatutako miofibroblastoen fenotipoa erakusten dute. Pankreako zelula izartsuak (ingelesez *Pancreatic Stellate Cell*, PSC) eta gibeleko zelula izartsuak (ingelesez *Hepatic Stellate Cell*, HSC) fibrosia eragiteaz gain minbiziaren progresioa errazten dutela frogatu da bai tumorearen hazkuntza areagotuz, bai tumorearen kontrako organismoaren mekanismoen edota terapia desberdinen aurka jokatuz (Liu, R. X. et al., 2017; McCarroll et al., 2014; Tommelein et al., 2015; von Ahrens et al., 2017). Are gehiago, PSC eta HSCek metastasi prozesuan laguntzen dutela ere behatu da (Huang et al., 2019; Xu, Z. et al., 2014).

PSC eta HSCen aktibazioa tumore zelulek edota tumorea infiltratu duten immunitate zelulek jariatutako hazkuntza faktoreek, zein TMEko gainerako zelulek jariatutako zitokinek eragindakoa izaten da (Allam et al., 2017; Kalluri, 2016; Tsuchida & Friedman, 2017). Ondorioz, PSC eta HSCak matrizea birmoldatzen hasten dira eta ECM zurrunagoa eratzen dute (Attieh & Vignjevic, 2016). Horregatik, CRC-ko gibeleko metastasiaren eta batez ere PCAren ezaugarrietako bat fibrosia da (Sherman, 2018). Modu honetan, bai PSC, bai HSCek kimioterapikoekiko erresistentzian garrantzia dutela frogatu dute jariatutako kolageno ugariaren ondorioz sortutako ECM zurrunak, kimioterapia tumore zeluletaraino heltzea galarazten baitute (Liang, C. et al., 2018). Are gehiago, ECMaren zurruntasunak tumoreetan eratzen den hipoxia maila ere handitu egiten du angiogenesia areagotuz (Couvelard et al., 2005; Valfre di Bonzo et al., 2009) eta ROSen metaketa eraginez (Arvelo & Cotte, 2009) minbizi desberdinetan eta baita pankrea eta gibelean ere. Bestalde, ECMa birmoldatzeaz gain, CAFek endotelio baskularraren hazkuntza faktorea (ingelesez *Vascular Endothelial Growth Factor*, VEGF) ekoizten dute angiogenesia are gehiago bultzatzeko (Valfre di Bonzo et al., 2009). Molekula honek zelula endotelialen erreklutamendua eragiten du odol baso berriak eratzeko (Olaso et al., 2003) eta horrekin batera tumorearen hazkuntza areagotzeko.

Azkenik, aipagarria da CAFek immunitate zelulak erreklutatzeko gaitasuna daukatela (Kalluri, 2016; Sherman, 2018) eta, aldi berean, immunosupresioa bultzatu dezaketela makrofagoen metaketa bultzatuz (Takahashi et al., 2017) edota linfozito zitotoxikoen hazkuntza geldituz (Aras & Zaidi, 2017).

### 2.1.3. Immunitate-sistemako zelulak

Immunitate-sistema organismoan sartzen diren gorputz arrotzak deuseztatzeaz arduratzen diren zelula mota desberdinez osaturik dago. Minbiziaren hasierako faseetan immunitate-sistemako zelulek, tumore-zelulak hautematen dituzte eta beraien aurka jotzen dute organismoa osasuntsu mantentzeko. Hala ere, zoritxarrez, gaixotasunak aurrera egitea lortzen badu, tumorea garatu ahala immunitate zelulek aldaketa fenotipikoa jasaten dute eta tumorearen aldeko funtzioak betetzen hasten dira (Henze & Mazzone, 2016). Tumorearen barnean infiltratzen diren immunitate zelulak linfozitoak, Natural Killer zelulak (NK), makrofagoak, neutrofiloak eta zelula dendritikoak dira besteak beste (Ben-Baruch, 2006; Gonzalez et al., 2018).

Hasteko, minbiziaren aurkako terapia berrien ikerketetan linfozitoek garrantzia handia dutela behatu da; haien artean, T linfozito zitotoxikoek (ingelesez *Cytotoxic T* cell, $T_c$), T linfozito laguntzaileek (ingelesez *T helper cell*, $T_h$) eta T linfozito erregulatzaileek (ingelesez *Regulatory T cell*, $T_{reg}$)(Ruffell et al., 2010) dute eginkizun nagusia. Lehenengoek, zelula arrotzak ezagutu eta apoptosira bultzatzen dituzte, bigarrenek, bestelako immunitate zelulak estimulatzen dituzte eta erantzun immunea koordinatzen dute eta hirugarrenek, erantzun immunologikoa murriztu egiten dute edota prozesuari amaiera ematen diote. Bestalde, TMEan NK zelula izeneko linfozito berezi batzuk ere aurki daitezke. NK zelulen eta T linfozitoen arteko desberdintasun nagusia espezifikotasuna da; T linfozitoek antigeno zehatz bat ezagutzen duten bitartean, NK zelulek transformaturik edo infektatuta dagoen edozein zelula lisatzeko gaitasuna dute perforina edota granzima izeneko entzima litikoak jariatuz (Gonzalez et al., 2018). Linfozitoen kasuan, aktibazio errezeptoreek antigeno desberdinak ezagutzen dituzte, zeintzuek zelula arrotz baten epitopoekin bat egitean, erantzun immunea pizten duten. Zehazki, zelulek antigeno arrotzak I klaseko Histokonpatibilitate Konplexu Nagusiaren (ingelesez *Major Histocompatibility Complex,* MHC) peptido antigenikoekin batera aurkezten dituzte, bi osagaiez eratutako konplexu hau izaten delarik linfozitoek ezagutzen dutena. Minbizi zelulek mutaziodun proteinak, onkogene, zein gene tumore supresoreen proteinak, gainespresaturiko genen produktuak edota organo zehatz batean espresatzen ez diren proteinak aurkezten dituzte tumore antigeno bezala. $T_c$ linfozitoek, beraien errezeptoreen bidez antigeno hauek ezagutu egiten dituzte eta aktibatu egiten dira zelula horren lisia eragiteko. NK zelulen kasuan, aldiz, aktibazio eta inhibizio errezeptoreak daude. $T_c$ linfozitoetan ez bezala, NK zeluletan I klaseko MHCa ezagutzen duen errezeptoreek erantzun immunearen inhibizioa eragiten dute, eta horregatik zelula hauek bestelako aktibazio errezeptoreak ere aurkezten dituzte. NKek I klaseko MHCaren peptido antigenikoak ezagutzen dituenean, zelula horiek berezko zelulatzat identifikatuko ditu eta ez du beraien aurka egingo, hau da, erantzun immunea inhibitu egingo da. Modu berean, zelula arrotz batek I klaseko MHC antigenoen espresioa murrizturik aurkezten badu eta aktibazio errezeptoreek ezagutzen dituzten ligandoen kopurua ugariagoa bada, inhibizio seinalea galdu egingo da eta ezohikoa den zelula hori lisatu egingo du.

Hortaz, tumorearen aurkako erantzunari dagokionez $T_c$ eta NK zelulak biziki aztertu izan dira, minbizi zelulak zuzenean hiltzeko gaitasuna dutelako (Ruffell et al., 2010). Ostera, gainerako linfozitoek ere tumorearen garapenean

garrantzia dutela behatu da. Adibidez, minbizi batzuen estroman $T_{reg}$ ugari dagoela behatu izan da, ingurumen immunosupresorea sortuz, Tc-en inaktibazioaz arduratzen baitira(Ohue & Nishikawa, 2019). Modu berean $T_h$-ek ere tumorearen hazkuntza baldintzatu dezakete, izan ere, kaltetutako zelulak hiltzeaz gain, makrofagoak eta bestelako zelula efektoreak erreklutatzeaz arduratzen baitira (Knutson & Disis, 2005). Dena dela, kontutan izan behar baita, linfozitoak ez direla beraien kabuz aritzen eta ingurunean aurkituko dituzten zitokina eta ligandoen arabera jokatuko dutela tumorearen aurka edo bere alde eginez.

TMEko immunitate zelulekin jarraituz, kalteturiko ehunetara lehenengo heltzen diren zelulen artean neutrofiloak daude. TMEko zelula estromalek CXC motako errezeptoreen bidez zelula hauek erreklutatzen dituzte eta batez ere patogenoak ezabatzeaz arduratzen dira. Minbizi batzuetan neutrofiloek tumorea eta metastasiaren aurkako funtzioak betetzen dituztela behatu da, baina beste kasu batzuetan tumorearen inbasioa, angiogenesia eta zelulen proliferazioa areagotu egiten duela ere egiaztatu da (Gonzalez et al., 2018). Horregatik, zelula hauek minbizian betetzen duten papera zein den argitzeko gehiago ikertzea beharrezkoa da.

Beste alde batetik, zelula dendritikoek (ingelesez *Dentritic Cell*, DC) ere beraien zeregina dute TMEan. Zelula hauek antigeno aurkezle bezala aritzen dira eta batez ere T linfozitoen tumorearen aurkako erantzuna bultzatzeko beharrezkoak dira. Baldintza normaletan DCak inaktibaturik badaude ere, patogeno edota kaltetutako ehunek hauen aktibazioa induzitu dezakete estimulu desberdinen bitartez. Modu honetan, zelula hauek edozein antigeno atzeman dezakete (patogeno batena edo tumorearena) eta I zein II motako MHCarekin batera aurkezten dute. Ostean, DCek nodulu linfatikoetaraino migratzen dute, non molekula koestimulatzaileekin batera T linfozitoen erantzuna bultzatzen duen. Hortaz, linfozitoak desberdindu egingo dira aurkeztutako antigenoarekiko espezifikoak diren $T_c$ linfozitoak emateko eta hedatu egingo dira organismoan zehar (Lee & Radford, 2019). Hala ere, minbizian DCek ez dute beti aipaturiko funtzioa behar bezala betetzen, adibidez, baldintza hipoxikoetan. Gainera, tumoreak DCak zelula immunosupresore bilakatu ditzakeela behatu izan da eta $T_c$-en hedapena bultzatu ordez, DCek T linfozito horien baztertzea eta $T_{reg}$-en hedapena suspertu lezakete (Veglia & Gabrilovich, 2017).

Immunitatearekin jarraituz, TMEari dagokionez gehien aztertu izan diren immunitate-sistemako zelulak makrofagoak dira, zehazki, minbiziari loturiko makrofago (ingelesez *Tumor-associated Macrophage*, TAM) deiturikoak. Nahiz eta hasiera batean minbiziaren aurka jo, makrofagoek beraien fenotipoa aldatu egin dezakete tumorearen eraginez eraldatuta dagoen ingurumenaren kausaz. Zentzu honetan, makrofagoek M1 edo M2 motako fenotipoa aurkeztu dezakete. Lehenengoek, funtzio efektorea edota antigeno aurkezle funtzioa izaten dute, hau da, patogeno eta tumore zelulen aurrean erantzun immunea bultzatzen dute organismoa defendatzeko. Bigarrenek, aldiz, hantura prozesua gelditu egiten dute eta ehunak birmoldatzen dituzte, aldi berean angiogenesia suspertzen dutelarik (Allavena et al., 2008; Mantovani, A. et al., 2002). Hau kontuan izanik, TAMek M2 motako makrofagoen antzerako funtzioak betetzen dituzte, azken finean immunosupresioa eragiten dutelarik tumore zelulen proliferazioa bultzatuz (Mantovani, A. et al., 2002). M2rako polarizazioa batez ere tumorearen inguruan eratzen den egoera inflamatorio kronikoaren ondorioa da (Ben-Baruch, 2006). Etengabeko hantura prozesua dela eta, kanpo mediora faktore pro-tumorigenikoak jariatzen dira eta azkenean TAMek minbizi zelulen proliferazioa, matrize estrazelularraren berrantolaketa proteolitikoa, angiogenesia eta inbasioa sustatu, zein metastasiaren sorreran laguntzeko gaitasuna lortzen dute (Aras & Zaidi, 2017; Prenen & Mazzone, 2019). Zentzu honetan, TAMek jariatutako granulinak HSCak aktibatzen dituztela behatu izan da, fibrosia sustatuz gibelean eta azken finean metastasirako bidea erraztuz (Nielsen et al., 2016). Gainera, aipatu dugun moduan, TAMek immunosupresioa eragin dezakete. Esaterako, TAMek linfozitoen funtzioa modulatzeko gaitasuna dute, adibidez, $T_c$en erantzuna inhibitzeko edota $T_{reg}$en erreklutamendua eragiteko (Aras & Zaidi, 2017; Chen et al., 2019). Are gehiago, CAFek jariatutako faktoreek TAMetan eragiten dutela behatu da, zeintzuek T linfozitoen proliferazioa gelditu egiten duen (Takahashi et al., 2017). Laburbilduz, nahiz eta immunitate-sistemako zelulek organismoa osasuntsu mantentzeko diharduten TMEko elementu desberdinen arabera beraien portaera aldatu eta tumorearen garapena sustatu dezakete.

### 2.1.4. Zelula endotelialak

Zelula endotelialak odol hodiak barrukaldetik estaltzen dituzten zelulak dira, endotelioa osatuz. Endotelio jarraietan, zelula endotelialak bata bestearekin hertsiki loturik daude. Aldiz, digestio-hodiak, gibelak, pankreak, giltzurrunetako glomeruluek eta guruinek endotelio fenestratua aurkezten

dute, non zelula endotelialen jarraitasuna eten egiten den zenbait puntutan. Endotelio mota honek poroak dauzka organoetako zelulen eta odolaren arteko komunikazioa bermatzeko.

Ehun osasuntsuetan, zelula endotelialek odolaren fluxua erregulatzeaz gain, odol hodien iragazkortasuna eta leukozitoen kieszentzia kontrolatzen dute. Aldiz, egoera patologikoetan edo zauriren bat gertatutakoak zelula hauek aktibatu egiten dira eta inflamazio prozesuaren parte bihurtzen dira. Horretarako, zelula endotelialek odol fluxua handiagotu egiten dute leukozitoak kaltea dagoen gunera hel daitezen eta beraien aktibazioa bultzatzen dute (Pober & Sessa, 2014). Era berean, aktibatutako zelula endotelialek zitokina proinflamatorioak eta adhesio molekulak espresatzen dituzte immunitate zelulen erreklutamendu eta atxikidura errazteko (Hsu et al., 2019). Honekin erlazionatuta eta inflamazio prozesuek minbiziaren sorrera erraztu dezaketela kontuan izanik, zelula endotelialek ere eragina izan dezakete tumorearen garapenean.

Bestalde, zelula endotelialak angiogenesiaz ere arduratzen dira. Ehun osasuntsu batean angiogenesia beharrezkoa da enbrioien garapenean eta zaurien sendaketa prozesuetan. Dena dela, nahitaezko prozesua bada ere, angiogenesiak minbiziaren hazkuntza bultzatu dezake. Tumorea eratzen hasten denean odol basoak honen periferian kokaturik egoten dira. Modu honetan, tumorea garatuz doan heinean, hau zenbat eta handiagoa izan, oxigenoa eta nutrienteak tumorearen erdialdeko zeluletara heltzea zailagoa da. Hori dela eta, hipoxiak eta nutrienteen gabeziak odol baso berrien beharrizana areagotzen dute eta ondorioz, angiogenesi prozesua aktibatu egiten da. Horretarako, tumore zelulek VEGF ekoizten dute, zeinak aurretik bertan zeuden odol basoetatik abiatuz, kapilar berriak sortaraztea eragiten duen. Hazkuntza faktore hau gehiegi jariatuz gero, odol basoak iragazkorragoak bilakatzen dira eta honek azken finean metastasia erraztu dezake, minbizi zelulei odolera pasatzeko aukera errazten zaielako (Maishi & Hida, 2017).

Amaitzeko, zelula endotelialek hazkuntza faktore transformatzailea (ingelesez *Transforming Growth Factor*, TGF) TGF-β1 and TGF-β2 ekoizten dituztela ere behatu da zeinak epitelialetik mesenkimalerako trantsizioa (ingelesez *Epithelial-to-Mesenchimal Transition*, EMT) eragiten duen adibidez PCAn edota gibeleko minbizian (Rawal et al., 2019; Shenoy & Lu, 2016). Prozesu honen bidez, zelula epitelialak zelula mesenkimal bihurtzen dira, hau da,

desberdintzapena galtzen dute beste zelula mota bat bihurtzeko gaitasuna lortzen dutelarik.

## 2.2. INGURUMEN INFLAMATORIOA

Inflamazioa edo hantura infekzio, zauri edota trauma baten aurrean immunitate-sistemak ematen duen erantzuna da organismoa babesteko helburuarekin. Hala ere, kasu batzuetan erreakzio hori, eta baita orbaintzea ere, denbora luzez mantendu egiten da eta ondorioz minbizia sortu liteke.

Beraz, ehunaren kaltea somatzean seinale kimikoen sare bat sortzen da zeinak inflamazio prozesua hasten duen ehuna birmodelatzeko. Hanturak zitokina eta kemokina desberdinen jariapena bultzatzen du, linfozito, makrofago eta fibroblasto bezalako zelulek mina dagoen lekuraino migra dezaten organismoa patogenoengandik babesteko eta azkenean zauria ixteko (Coussens & Werb, 2002). Zitokinak zelula desberdinek gainerako zelulekin komunikatzeko jariatzen dituzten molekula proteikoak dira, proliferazioa, desberdintzapena, mugimendua, biziraupena eta heriotza erregulatzen dutenak. Enbriogenesia, zelulen metabolismoa, hematopoiesia, inflamazioa, erantzun immunea eta beste hainbat prozesutan parte hartzen dute. Hasiera batean immunitate-sistemako zelulek soilik jariatzen zituztela uste zen arren, gaur egun jakina da bestelako zelulek ere zitokinak ekoizteko gai direla. Hori dela eta, TMEko zelula bakoitzak zitokina jakin batzuk ekoiztuko ditu beraien beharrizanak aurrera eraman ahal izateko. Honekin erlazionatuta, aipatzekoak dira zitokinen pleiotropia eta erredundantzia propietateak, hau da, zitokina desberdinek funtzio berdina izan dezakete edota zitokina berdinak zelula desberdinetan modu desberdinean joka dezake (Chung, 2009). Hortaz, askotan nahiko korapilatsua izaten da molekula hauen funtzioa zehaztea eta taldetan sailkatzea.

Tumorearekin erlazionaturik dauden zelulei dagokienez, funtsean, inflamazioa, immunitatea eta tumorearen garapena arautzeaz arduratzen dira. Horregatik, nahiz eta zitokinak nagusiki linfozito eta makrofagoek jariatzen dituzten, bestelako zelulek ere zitokinak ekoizteko gaitasuna dute, esaterako, zelula endotelialek, fibroblasto aktibatuek eta tumore zelulek (Allam et al., 2017; Dehne et al., 2017; Ino et al., 2013; Komohara & Takeya, 2017; Singh, R. et al., 2017; Tsuchida & Friedman, 2017).

Zitokina hauek guztiak etengabe daude elkarrekintzan ehunen funtzionamendu egokia bermatzeko. Linfozitoek, esaterako, interleukinak (IL)

ekoizten dituzte, inflamazioaren erregulazioan parte hartzen duten molekulak hain zuzen. Batzuek hantura areagotu egiten duten bitartean, besteek molekula antiinflamatorio gisa jokatzen dute. Hala ere, maila altuagoan edo baxuagoan aurkitzen diren arabera, zenbaitek kontrako efektua izan dezakete. Adibidez, nahiz eta IL-6a beharrezkoa den hantura prozesuetan, minbizi mota gehienetan IL-6aren maila altuak antzeman dira (Kumari et al., 2016). Bestalde, IL-1βak inflamazio akutu kasuetan organismoari babesa eskaintzen dion arren, inflamazio kronikoan neoangiogenesia sustatzen du eta gainera, minbiziari loturiko fibroblastoetan ere eragina dauka seinale antiapoptotikoak suspertu ditzakeelarik (Bent et al., 2018). Era berean, IL-10a immunoerregulatzailea bada ere, tumoreari loturik agertzen diren makrofagoek jariatutako IL-10ak immunosupresioa eragiten duela behatu da (Sawa-Wejksza & Kandefer-Szerszen, 2018; Shurin et al., 2013).

Honekin erlazionatuta, alfa tumore nekrosi faktoreak (ingelesez *Tumor Necrosis Factor Alpha*, TNF-α) molekula proinflamatorio eta antiinflamatorio bezala jokatzen du bai infekzio, bai ehunen konponketan (Josephs et al., 2018). Funtsean tumorearen kontrako aktibitatea du T linfozitoen bidezko erantzun immunologikoa bultzatuz. Tumorea haziz doan heinean, ordea, TMEan TNF-α maila altua agertuz gero, minbiziaren garapena areagotu dezake (Balkwill, 2006). Modu honetan, faktore honen espresioa deregulaturik dagoenean tumorigenesia sustatzen da (Landskron et al., 2014). Besteak beste, zelulen transformazioa eragin, biziraupena handitu eta metastasia erraztu egiten dituela behatu da (Sethi et al., 2008).

Bestelako zitokina batzuk hazkuntza faktoreak dira. Fibroblastoek TMEan duten funtzioa azaltzen den atalean (2.1.2.) VEGF molekula aipatzen da. Hazkuntza faktore hau funtsezkoa da ehunak konpontzerako orduan organismo osasuntsu batean. TMEan ordea, gainespresaturik agertzen da (Costache et al., 2015) angiogenesia eta fibroblasto aktibatuen proliferazioa bultzatzen duelarik tumorearen hazkuntza errazteko (Apte et al., 2013; Tsuchida & Friedman, 2017). Hazkuntza faktoreekin jarraituz, minbiziarekin erlazionatzen den beste mokelula bat EMTan parte hartzen duen TGF-β hazkuntza faktorea da. Hantura prozesuetan TGF-β konfigurazio inaktiboarekin jariatzen da eta ondoren, gibeleko zein pankreako HSC eta PSCek aktibatu egiten dute. Horiek horrela, TGF-β fibrosiaren arduradun nagusienetarikoa da, kolagenoaren jariapena bultzatzen baitu (Sherman, 2018). Dena dela, molekula hau, tumoreak ez ezik, CAFek eta immunitate zelulek ere ekoizten dute (Kalluri, 2016; Landskron et al., 2014). Printzipioz

propietate antiinflamatorio eta immunosupresoreak ditu, alabaina, tumorigenesi fasearen eta tumore motaren arabera jokabide desberdina izan dezake. Gaixotasunaren hasieran ziklo zelularra geldituz eta apoptosia eraginez tumore supresore bezala jarduten duen bitartean, tumorea garaturik dagoenean EMTa eta inbasioa suspertzen ditu (Landskron et al., 2014). Minbiziari lotuta ere, aipagarria da gamma-interferoia (IFN-γ). Orohar linfozito aktibatuek jariatzen dute eta makrofagoen aktibazioa induzituz hantura areagotzen du. Nahiz eta inflamazioa eraginez tumorearen hazkuntza faboratzeko joera izan, bere aurka ere jokatu dezakeela behatu da bere ahalmen antiproliferatzailea eta proapoptotikoari esker (Castro et al., 2018). Hauez gain, zitokina deritzen molekula multzoaren barneko talde berezi bat kemokinak dira, TMEan funtsezkoak direnak. Kemokinak kimiotaxia induzitzen duten molekulak dira, CC edo CXC familiako polipeptidoek hain zuzen ere. Kimiotaxi horren bidez immunitate-sistemako zelulen erreklutamendua eta zelulen migrazioa eragiten dute (Guerreiro et al., 2011). Dena dela, immunitate-sistemaz gain tumorearen garapenean ere lagungarri izan daitezke beraien errezeptoreekin interakzionatzen dutenean hazkuntza faktoreen jariapena bultzatu eta bestelako zelulak erakarri ditzaketelako (Murooka et al., 2005).

Zitokinak ez ezik, bestelako molekula eta entzimak ere lanean dihardute TMEan. Aurrerago azaldu den bezala, kolagenoaren ekoizpenarekin batera ECMaren moldaketan MMPek eta TIMPak oinarrizkoak dira. Batek ECMko proteinak degradatzen dituen bitartean, besteak proteasa horiek inhibitzen ditu ehunaren homeostasia bermatzeko. Minbizian aitzitik, MMPen espresioa handiagotu egiten da, tumore zelulen migrazioa errazten dutelarik (Phillips et al., 2003; Sherman, 2018). Bestalde, inflamazioarekin erlazionaturik ziklooxigenasak (ingelesez *Cyclooxygenase*, COX) ere badaude, zehazki, COX-2aren gainespresioa minbiziarekin erlazionatu izan da izan ere inflamazioa, zelulen proliferazioa eta angiogenesia eragiten baititu (Breinig et al., 2007; Hermanova et al., 2008; Roelofs et al., 2014).

## 2.3. INGURUMEN FIBROTIKOA

ECMa makromolekula desberdinez osaturik dagoen estruktura da, ehun bateko zelulen artean dagoen espazioa betetzen duena, sostengua eskainiz. Gehienbat, kolagenoa izaten da elementu nagusia, baina glikoproteinez, elastinez, fibronektinez eta lamininez ere osaturik dago sare fibrilar bat eratzen dutelarik (Theocharis et al., 2016). Gainera, ECMa hazkuntza

faktoreen gordailua ere bada. Beraz, ECMa zelulen euskarri izanik, integrinak bezalako atxikidura proteinen bidez, zelulak matrizeko lamininetara lotzen dira.

Tumore baten garapenean ECMaren osagaiak aldatu egiten dira bere hazkuntza errazteko (Lu et al., 2011). Lehenago azaldu den moduan, prozesu honetan fibroblastoek, makrofagoek eta tumore zelulak hartzen dute parte, baina batez ere zelula izartsuak dira garrantzia handiena dutenak. Hauek kolagenoarekiko errezeptoreak espresatzen dituzte zelulen adhesioa, proliferazioa, migrazioa eta abar erregulatzeko eta horrez gain, MMPak eta kolagenoa jariatzen dituzte ECMa desegin eta berregiteko eta aldi berean zelulen migrazioa errazteko. Horrez gain, tumore zelulak indar mekanikoaz ere baliatzen dira, hau da, hazkuntza azkarra dela eta, tumore zelulak matrizearen egitura apurtzeko gai dira. Are gehiago, inbadipodio deritzen F-aktinazko egitura zelularrak ere erabiltzen dituzte ECMko egiturak bultzatzeko (Walker et al., 2018).

Aurreko atalean azaldu den bezala, tumore-zelulek bidalitako seinaleak jarraituz PSC eta HSCak matrize ugari jariatzen hasten dira. Alde batetik, zelula hauek jariaturiko kolageno zuntzen antolaketak minbiziaren inbasioa ere erraztu dezakeela behatu izan da (Walker et al., 2018). Bestetik, PSC eta HSCen joera ECM zurrunagoa eratzea dela ondorioztatu da, kolagenoa eta fibronektina gehiago ekoiztuz (Attieh & Vignjevic, 2016). Honekin batera, ikerketa desberdinetan zelula izartsuek fibrosia eragiteaz gain minbiziaren progresioa errazten dutela frogatu da bai tumorearen hazkuntza areagotuz, bai tumorearen kontrako organismoaren mekanismoen edota terapia desberdinen aurka jokatuz (Liu, R. X. et al., 2017; McCarroll et al., 2014; Tommelein et al., 2015; von Ahrens et al., 2017). ECMaren zurruntasunak TMEra oxigenoa, nutrienteak eta farmakoak heltzea oztopatu egiten du eta horrek minbiziaren hazkuntza indartu dezake (Liang, C. et al., 2018). Are gehiago, PSC eta HSCek metastasi prozesuan laguntzen dutela ere behatu da (Huang et al., 2019; Xu, L. et al., 2014).

## 2.4. INGURUMEN OXIDATIBOA

TMEan garrantzia duen beste elementu bat ROSak dira. Molekula hauek zelulen metabolismo prozesu normalaren ondorio gisa sortzen dira, baina erradiazio ultramoreak, dietako osagai batzuek, tabakoak, inguruneko poluitzaileek eta beste hainbat agentek ere sor ditzakete (de Sa Junior et al., 2017; Prasad et al., 2017). Bestalde, hantura kronikoaren ondorioz ere

inflamazio zelulek edota zelula epitelialek ROSen eraketa areagotu dezakete, zeinak minbiziaren garapena bultzatzen duen (Murata, 2018).

ROSak maila baxuan zelulen seinalizazio-bide batzuen erregulaziorako eta homeostasia mantentzeko beharrezkoak diren arren, beraien kontzentrazioa altua denean toxiko bilakatzen dira zelulentzat (Glasauer & Chandel, 2014). ROSek dauzkaten erradikal libreen ondorioz oso erreaktiboak dira, ingurune oxidatiboa eratzen duten estres oxidatibo deritzon egoera sustatzen dutelarik. Estres oxidatibo horren kausaz zelulak hil egin daitezke. Dena dela, organismoak antioxidatzaile desberdinak dauzka eskuragarri ROS hauen efektu toxikoa deuseztatzeko, esaterako entzima endogenoak (superoxido dismutasa, katalasa, glutation peroxidasa...) edota dieta bidez hartzen diren konposatu exogenoak (polifenolak, flabonoideak, bitaminak, mineralak...) (He et al., 2017). Nolanahi ere, baliabide horiek ez dira beti nahikoak izaten eta ROSen maila altuek transkripzio faktore, kinasa, hazkuntza faktore, zitokina eta beste proteina batzuen jokabide normala aldatu dezakete gaixotasun neurodegeneratibo edota minbizia sortuz (Prasad et al., 2017). Honekin erlazionatuta, ROS maila altuek mutagenesia areagotu dezaketela behatu izan da (Liao et al., 2019) eta baita EMTa eragin ere (Jiang et al., 2017).

# 3. PANKREAKO MINBIZIA

## 3.1 Pankreako minbizia

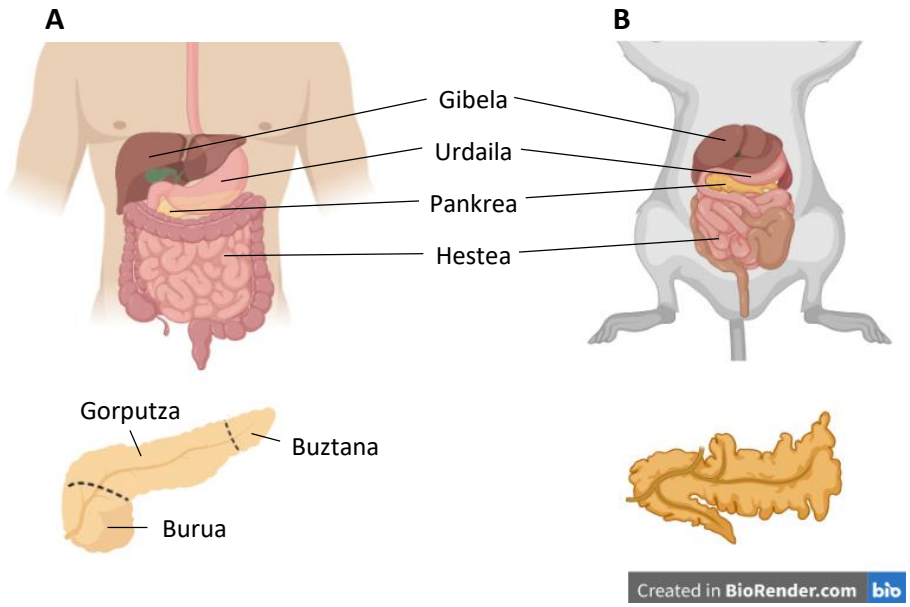
Aurrerago azaldu dugun bezala, PCA ezagutzen den minbizi agresiboenetariko bat da. PCAen % 80, bai emakume, bai gizonetan, 60 urtetik gorakoetan garatzen da (Ilic & Ilic, 2016) eta batez ere, herrialde garatuetan agertzen da. Ikerketa batzuen arabera, diabetes mellitusak PCA izatea areagotu dezakeela ondorioztatu da (Li, D., 2012). Era berean, pankreatitisa, eta batez ere pankreatitis kronikoa duten pazienteek ere PCA pairatzeko arrisku handiagoa dutela ondorioztatu dute, izan ere gaixo hauetako % 4k minbizia garatu zuela behatu baita (Kudo et al., 2011). Bestalde, herentzia genetikoak, tabakoak, elikadurak, obesitateak zein bizimodu motak ere minbizi honen agerpenean eragina izan dezakeela frogatu da (Ilic & Ilic, 2016; Lowenfels et al., 2000).

Tumorea hasierako fasean aurkituz gero kirurgia bidez erauzi daitekeen arren, normalean, gainerako organoetara hedatzen den arte ez da detektagarria izaten eta ordurako, metastasirako tratamendu bakarrak kimioterapia edota erradioterapia izaten dira (Neoptolemos et al., 2018). Edonola ere, gaur

eguneko terapiek ez dute gaixoa sendatzen, bere biziraupena handitu baizik. Hala eta guztiz ere, PCA sufritzen dutenen artean 5 urteko bizi itxaropena % 2-9 ingurukoa da soilik (Ilic & Ilic, 2016), izan ere, hilkortasun-tasa handi honen kausa garrantzitsuena gaixoek farmako kimioterapeutikoekiko garatzen duten erresistentzia izaten baita.

## 3.2. Pankrea eta bere antolaketa zelularra

Pankrea digestio-sistema eta sistema endokrinoan parte hartzen duen organoa da, hiru ataletan banatzen dena: burua, gorputza eta buztana. Gizakietan burua duodenotik hurbilen dagoen partea da eta buztana ileo eta bareraino luzatzen da. Saguen pankreak, berriz, itxura difusoa aurkezten du eta urdailak, duodenoak, jeiuno proximalak eta bareak inguratuta agertzen da (4. Irudia).

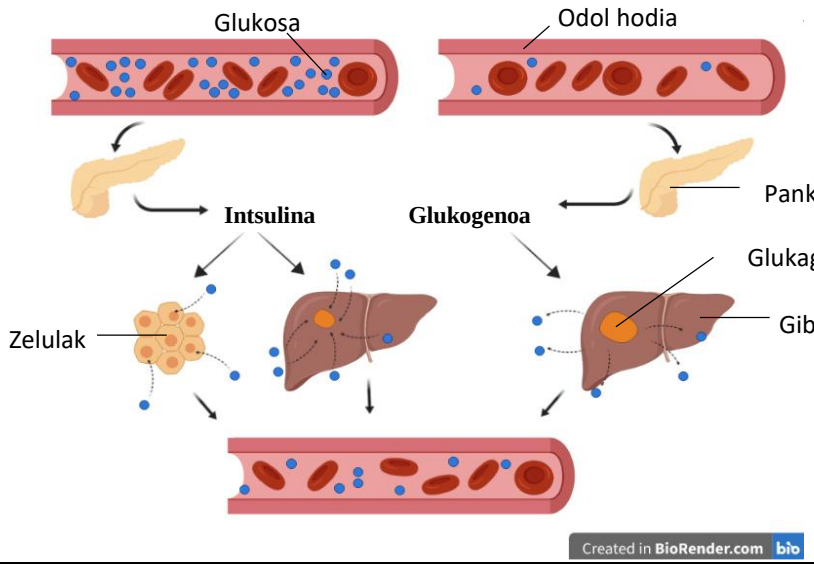



**4. Irudia. Gizakiaren eta saguaren pankrea**. Pankrea, urdaila eta hestea inguratuz ageri den arren, itxura desberdina du gizakietan eta saguetan. A) Gizakiaren pankreak forma zehatz bat du hiru zatitan bereiz daitekeena: burua, gorputza eta buztana. B) Saguaren pankreak aldiz, egitura lausoa dauka. *Irudiak BioRender.com bidez sortutakoak dira.*

Pankreak aorta abdominaletik ateratzen diren enbor zeliakotik eta goiko arteria mesenterikotik jasotzen du odola. Hauek biek adarkapen ugari dituzte organoa inguratzen dutelarik, irrigazioa bermatzeko.

Bai gizakietan, bai saguetan, organo honek funtzio exokrinoa eta endokrinoa burutzen ditu azinoetan eta Langerhans irletan hurrenez hurren. Eremu exokrinoak zuku pankreatiko deritzon digestio entzimez osatutako jariakina ekoizten du hainbat entzimaz osaturik dagoena, hala nola, proteasa desberdinak proteinen lotura peptidikoak apurtzeko (tripsinogenoa, kimotripsinogenoa eta prokarboxiaminopeptidasa), amilasa pankreatikoa polisakaridoak disakarido bihurtzeko eta, lipasa pankreatikoa triglizeridoak hidrolizatu eta monoglizerido eta gantz azidoak emateko. Pankreak zuku pankreatikoa ekoizten duenean, jariakina hodi pankreatikora bideratzen da, zeinak duodenoko pareta zeharkatzen duen. Azkenik, zuku pankreatikoa hestean isurtzen da entzimek bertan beraien funtzioa gauzatu dezaten.

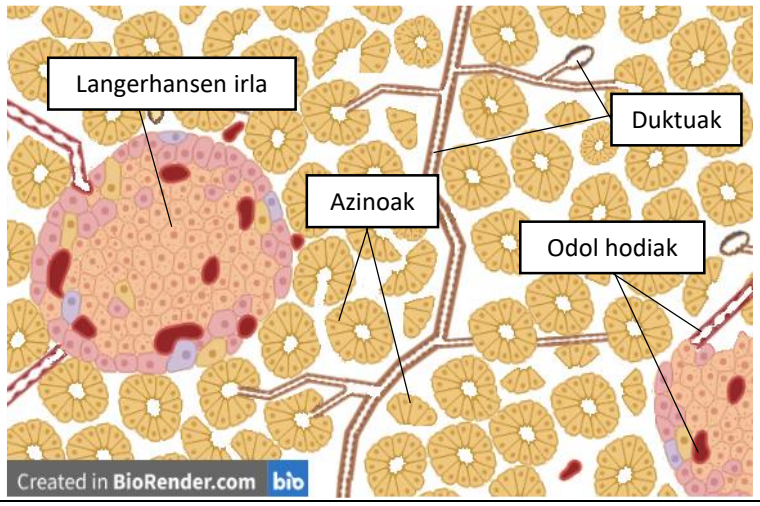
Alde endokrinoari dagokionez, pankrearen funtzio nagusietako bat odolean aurkitzen den glukosa kantitatea erregulatzea da. Horretarako, Langerhans irletako zelula pankreatikoek hormona ugari isurtzen dituzte, hala nola, intsulina, glukagoia eta somatostatina (5. Irudia). Hormona hauek pankreako zainetara isuriko dira eta ostean, porta eta bareko zainetara bideratuko dira




**5. Irudia. Pankrearen sistema endokrinoaren funtzionamendua.** Odolean dagoen glukogeno kantitatearen arabera pankreak intsulina edo glukogenoa ekoizten du. Glukosa maila altua denean, pankreak jariatutako intsulinak glukosaren metaketa eragiten du gibelean glukagoi moduan eta zelulek glukosaren bereganatzea bultzatzen dute glukosa maila jaisten den arte. Aldiz, odolean glukosa gutxi badago, pankreak glukogenoa jariatzen du, zeinak gibelean metatutako glukagoiaren metabolismoa aktibatzen duen glukosa molekulak odolera aska daitezen. *Irudia BioRender.com bidez sortutakoa da.*

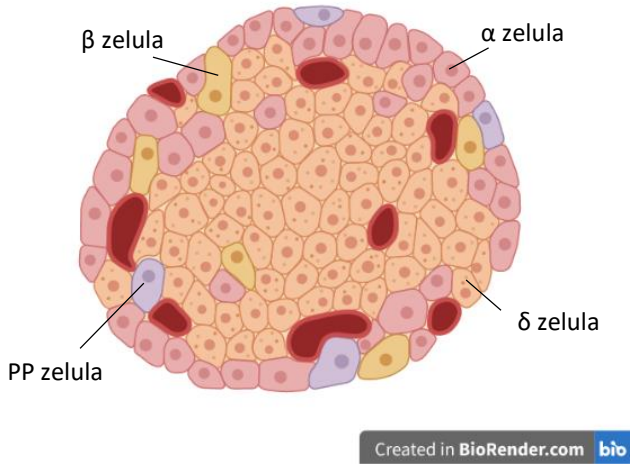
bertatik dagokion gunera hel daitezen, batez ere gibelera. Odoleko glukosa kontzentrazioa altua denean, pankreak intsulina ekoizten du, zeinak zelulek glukosa hori barneratzea eta metatzea erraztuko duen glukogenogenesi prozesuaren bidez. Bestalde, odoleko glukosa maila baxua denean, pankreak glukagoia jariatzen du, gibelean dauden glukosa erreserbak odolera aska daitezen glukogenolisiaren bitartez. Azkenik somatostatinak bai intsulina, bai glukagoiaren ekoizpena erregulatzen ditu (5. Irudia).

Horiek horrela, pankreak funtzio desberdinak betetzen dituenez gero, zelula mota ugariz osaturik dago. Gehienbat (% 95) atal exokrinoko zelula azinarrek osatzen dute, digestio entzimen sintesi, metaketa eta jariapenaren arduradunak direlarik (6. Irudia). Hauez gain, zelula zentro-azinarrak ere badaude, ura eta elektrolitoak jariatzen dituztenak urdaileko azidotasuna neutralizatzeko. Pankrearen osagai exokrinoarekin bukatzeko, azinoekin komunikatuta zelula duktalak daude. Hauek, duktu izeneko egitura tubularrak eratzen dituzte zelula exokrinoen produktuak bertara isur daitezen (6. Irudia).




**6. Irudia. Pankrea exokrino eta endokrinoaren egitura histologikoa.** A) Sagu pankrearen egitura histologikoaren eskema. B) Sagu pankrearen argazki histologikoa. Pankrea gehien bat jariapen pankreatikoa ekoizten duten azino izeneko zelula multzoez osaturik dago. Zelula hauen jariakina duktuetara bidaltzen da gero hesteraino bideratzeko. Bestalde, pankrean sakabanaturik hormonak jariatzen dituzten Langerhans islote izeneko egiturak ere bereiz daitezke, zelula desberdinez osatuak. Hauen kasuan, jariapena odol basoetara bideratzen da. *Irudia BioRender.com bidez sortutakoa da.*

Beste alde batetik, pankrean hormonak jariatzeaz arduratzen diren zelula endokrinoak aurki daitezke Langerhansen irla deritzen egiturak eratuz, organo osoaren % 1-2 hartzen dutelarik (6. Irudia). Bertan ugarienak intsulina ekoizten duten β zelulak dira (7. Irudia). Jarraian α, δ eta PP zelulak daude, zeintzuek glukagoia, somatostatina eta polipeptido pankreatikoa jariatzen duten hurrenez hurren. Are gehiago, pankrean odol basoak eratzen dituzten zelula endotelialak daude (6. Irudia). Zelula hauek T linfozitoen infiltrazioa erregulatzeaz gain, hormonak beraien helbururaino bideratzen dituzte batez ere organismoaren glukosa maila erregulatzeko. Zentzu honetan, aurretik azaldu den moduan, pankreako endotelioa fenestraduna da, hau da, zelula endotelialen artean tarteak daude aipatutako substantzien iragazkortasuna errazteko.




**7. Irudia. Saguetako ehun pankreatikoko Langerhans irlen egituraren eskema.** α zelulek glukagoia ekoizten dute eta irlen periferian kokatzen dira; β zelulek Langerhans irlen gehiengo partea betetzen dute eta intsulina ekoizteaz arduratzen dira; δ eta PP zelulak ez dira hain ugariak eta somatostatina eta polipeptido pankreatikoa jariatzen dituzte. *Irudia BioRender.com bidez sortutakoa da.*

Azkenik, pankrean, immunitate-sistemako zelulak eta PSCak ere aurki daitezke, kalteen aurrean elementu arrotzak deuseztatzeaz eta ehuna berrantolatzeaz arduratzen direnak.

## 3.3 Pankreako minbiziaren mikroingurumena

Minbizi guztietan bezala, tumore zelulekin batera, TMEko elementuek gaixotasunaren garapenean eragina dute. Zentzu honetan, PCAren ezaugarri bereizgarrienak desmoplasia eta immunosupresioa dira (Ren, B. et al., 2018) eta lehenago aipatu den moduan, faktore hauek biak TMEko zelulek baldintzatzen dituzte neurri handi batean. Desmoplasia PSCek eragiten dute, beraiek baitira ECMaren jariapenaren arduradunak. Pankreako CAFak bereziak izanik, PSCek MMPak eta TIMPak ez ezik kolagenoa ere jariatzen dute. Etengabeko ekoizpen horrek, ehunaren zurruntasuna eragiten du, zeinak odol-hodien egituraren zein antolaketaren aldaketa eragingo duen. Ondorioz, odola eta berarekin batera oxigenoa ez da modu egokian heltzen zelula guztietara eta azkenean hipoxia eta estres oxidatiboa sortzen dira. Aldi berean, honek hipoxiaren eraginez aktibatzen diren geneen espresioa suspertzen du eta tumore zelulen biziraupena eta inbasioa indartzen dute (Arvelo & Cotte, 2009; Liang, C. et al., 2018). Gainera, aurrerago aipatu dugun bezala, oxigenoa ez ezik, farmakoak ere ezin izango dira behar bezala heldu tumore zelula guztietara ECMaren egiturarengatik (Apte et al., 2013). Are gehiago, ECMaren zurruntasunak tumoreetan eratzen den hipoxia maila ere handitu egiten du angiogenesia areagotuz (Couvelard et al., 2005; Valfre di Bonzo et al., 2009). Zentzu honetan, kontuan izan behar da hipoxia baldintzapean gene espezifikoak aktibatzen direla, horien artean angiogenesia bultzatzen dutenak. Adibidez, minbizi mota askotan, PCA barne, 1-hipoxia bidezko faktore induzitzaileak (ingelesez *Hipoxia Inducible Factor*, HIF) VEGFaren transkripzioa eragiten du (Couvelard et al., 2005), zeinak aldi berean, tumorearen inbasioa erraztuko duen. Hala ere, Liang eta bere kolaboratzaileek desmoplasiak eragindako ondorioen konplexutasuna azaldu zuten, izan ere kasu batzuetan CAFen delezioak matrizeko kolageno kantitatea murriztu bazuen ere, ez zen aldaketarik behatu kimioterapikoen perfusioari dagokionez (Liang, C. et al., 2018). Honekin erlazionaturik, PSCek garraiatzaile eta seinalizazio-bide desberdinetako geneen espresioa aldatu dezaketela behatu izan da (Gnanamony & Gondi, 2017; Hesler et al., 2016) zeinak farmakoen helduera ere oztopatu dezaketen. Eta ez hori bakarrik, Hessman eta bere kolaboratzaileek burututako ikerketa batean PSCek genzitabina kimioterapikoa beraien zitoplasman gordetzeko ahalmena zutela egiaztatu zuten (Hessmann et al., 2018). Zentzu honetan, TAMek ere kimioerresistentzian garrantzia dutela behatu da, EMTa bultzatzen baitute (Kuwada et al., 2018).

Bestalde, PCAn ematen den beste ezaugarri nagusia immunosupresioa da. Tumore-zelulek, adibidez, beraien desegonkortasun genetikoaren eraginez, $T_c$-ek ezagutzen dituzten antigenoak galdu ditzakete (Martinez-Bosch et al., 2018). Eta ez hori bakarrik, ikerketa desberdinen arabera, tumore zelulez gain, hezur-muinetik eratorritako zelula supresoreak (ingelesez *Myeloid Derived Suppressor Cells*, MDSCs), $T_{reg}$-ak, TAMak eta PSCak ere immunosupresioaren arduradunak dira (Li, C. et al., 2020; Ren, B. et al., 2018; Schnurr et al., 2015). Zelula hauek jariatzen dituzten zitokinek eta hazkuntza faktoreek immunitate-sistemaren erantzuna geldiarazi dezakete eta apoptosiaren saihespena erraztu dezakete. Are gehiago, zelula endotelialek angiogenesia sustatzen duten faktoreen aurrean (VEGF esaterako) $T_c$-en tumorerako sarrera ekiditen du $T_{reg}$-en infiltrazioa ahalbidetzen duen bitartean (Motz et al., 2014).

Aurreko guztia laburbilduz, PCAren garapena aztertzerako orduan beharrezkoa da TMEko guztiak aintzakotzat hartzea ez baitute uneoro modu berdinean jokatzen.

# 4. KOLON-ONDESTEKO MINBIZIAREN METASTASI HEPATIKOA

## 4.1. Kolon-ondesteko minbiziaren metastasi hepatikoa

CRCa munduan diagnostikatzen diren minbizi guztietatik hirugarrena da prebalentziari dagokionez (GLOBOCAN, 2018). Eta are gehiago, Espainia mailan heriotza gehien eragiten dituen minbizia da (SEOM, 2018). Normalean 50 urtetik gorako pertsonetan azaltzen da, batez ere 70 urtetik gorakoetan, eta pronostikoa zelulen hedapenaren araberakoa da, izan ere gaixotasuna duten pertsonetatik erdiak metastasia jasaten baitu (Paschos et al., 2009).

Aldaketa genetiko eta epigenetikoez gain, CRCa nutrizio ohiturekin oso erlazionaturik dago. Mendebaldeko bizimodua daramatenen artean gehiago azaltzen da gantz zein karbohidrato ugaridun dietaren ondorioz. Okela gorri eta alkohol asko kontsumitzeak adibidez, minbizi modu honen agerpena areagotu dezake eta baita bizimodu sedentarioak eta tabakoa erretzeak ere (Bishehsari et al., 2014).

Kasu gehienetan tumorea heste lodian bakarrik eratzen da, zeina kirurgia bidez erauzi daitekeen. Hala ere, pazienteen % 50ak minbizia berriro pairatzen

du, baina kasu honetan metastasiarekin, batez ere gibelean. Zentzu honetan, gibela metastasia jasaten duen organorik ohikoena izaten da, odolaren % 75 iragazten baitu.

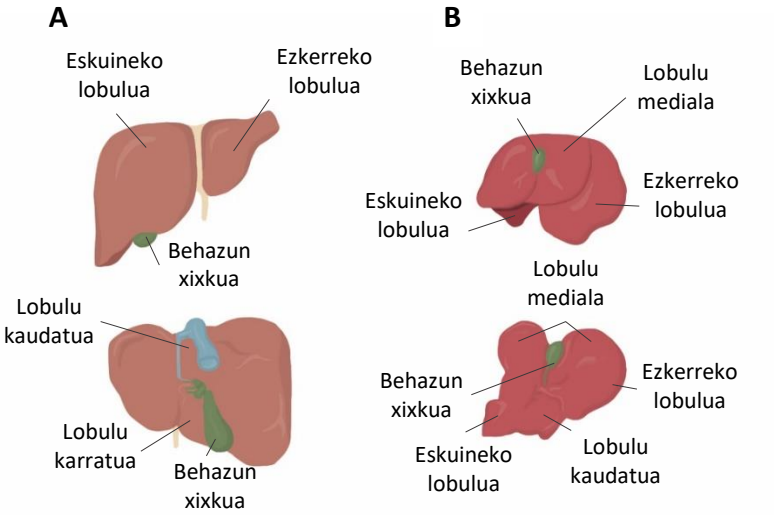
Zentzu honetan CRCaren metastasi hepatikoaren arrazoi pisutxuenetako bat, hesteetako odola bertan iragaztearen ondorio izaten da. Tumore-zelulak beraz kolon edo ondestetik askatu eta odolaren bidez gibeleko sinusoideetaraino hel daiteke, non atxikita geratu daitezkeen (Takeda et al., 2002; Van den Eynden et al., 2013). Hesteko tumoreak bezala, gibelekoak ere kirurgia bidez erauzi daitezke batzuetan. Hala era guztiz ere, gaixo hauek askoz jota 5 urteko biziraupena izaten dute (Valderrama-Trevino et al., 2017).

## 4.2. Gibela eta bere antolaketa zelularra

Gibela, organismoaren barne organorik handiena da, barrunbe abdominalaren eskuin aldeko goiko partean kokaturik dagoena. Oso organo garrantzitsua da, bizi-funtzio ugari betetzen baititu. Hauen artean aipagarria da odoleko glukosaren erregulazioaz arduratzen dela pankrearekin batera. Laburbilduz, gluzemia altua denean pankreak intsulina ekoizten du glukosa gibelean metatu dadin, eta aldiz, kontzentrazioa baxua denean, pankreak glukagoia jariatzen du, gibelean metaturik dagoen glukogenotik glukosa hondarrak askatzeko (5. irudia). Bestalde, lipidoen β-oxidazioa ere gibelean ematen da energia ATP moduan lortzeko. Are gehiago, lipidoen garraiorako proteinen eta kolesterolaren ekoizpena ez ezik, proteinen transaminazio eta desaminazioa ere gibelean gertatzen dira. Horrez gain, organo honek organismoarentzat arrotzak diren agenteak edo xenobiotikoak zein produktu toxikoak identifikatzen ditu eta prozesu kimiko eta metabolikoen bidez eraldatu egiten ditu gorputzetik kanporatu ahal izateko. Dena dela, gibelak bestelako funtzio batzuk ere betetzen ditu, hala nola, behazunaren ekoizpena, odoleko plasmako proteinen sintesia, bitamina zein glukogenoaren metaketa eta, immunitate sistemako faktoreen ekoizpena eta odolaren iragazketa besteak beste.

Bai gizaki, bai saguetan gibela lau lobuluz osaturik dago. Gizakietan eskuinekoa, ezkerrekoa, karratua eta kaudatua bereiz daitezke eta saguetan aldiz, eskuinekoa, ezkerrekoa, kaudatua eta mediala (8. irudia). Aldi berean, lobulu hauetako bakoitza, ehun konektiboz inguratutako lobuluxka izeneko segmentu poliedrikotan banaturik dago eta berezko arteria eta zain antolaketa du (9. irudia). Horrez gain, gibelari loturik behazun-xixku deritzon zakua dago non behazuna metatzen den (8. irudia). Kasu honetan ere,

segmentu guztiak behazuna garraiatzen duten konduktuen bidez konektaturik daude. Kanal horiek hepatozitoek ekoiztutako behazuna behazun-xixkura edota eta duodenora garraiatzeko erabiltzen dira. Behazuna, hondakinen garraioa eta elikagaien digestioa laguntzen duen substantzia da gantz azidoen emultsionatzaile bezala jarduten duelarik.




**8 Irudia. Giza eta sagu gibelaren arteko desberdintasunak**. A) Gizakiaren gibelean ezkerreko lobulua eta eskuinekoa bereiz daitezke, azken hau lobulu kaudatua eta lobulu karratua izeneko beste bi zatitan bereizten delarik. B) Saguetan, aldiz, ezkerreko eta eskuineko lobuluez gain, lobulu kaudatua eta mediala daude. Kasu bietan behazun xixkua ere behatu daiteke organoaren barnean. *Irudiak Amparo Hidalgo Galianak egindakoa dira (Ig: ahg_ilustracion).*

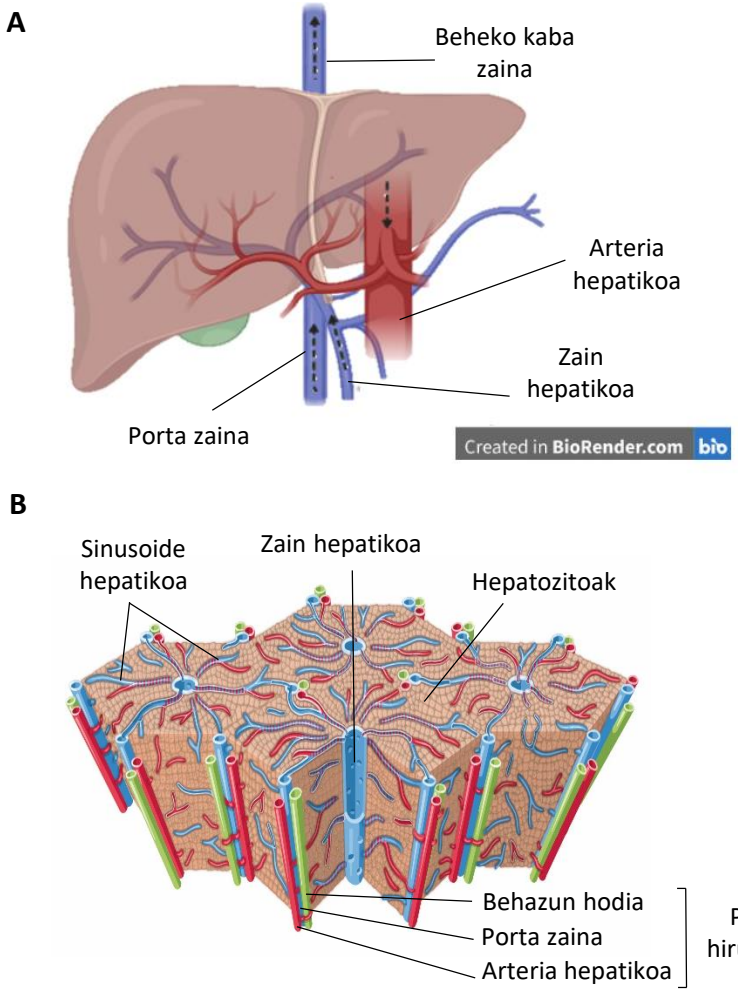
Hain organo garrantzitsua izanik, gibelak, alde batetik, arteria hepatikotik datorren odol oxigenatua jasotzen du eta bestetik, porta zain hepatikotik datorren nutrienteetan aberatsa den odol desoxigenatua ere jasotzen du, hots, urdail, pankrea, bare eta hesteetatik ateratzen den odola biltzen du (9. irudia). Behin bertan, gibela odoleko nutrienteen metabolismoaz arduratzen da. Horregatik garrantzitsua da lobuluxka hepatiko bakoitzak bere odol-sistema antolatua izatea (9. irudia). Modu horretan, lobuluxken artean arteria hepatikoaren adar bat, porta zainaren beste adar bat eta behazun-hodi bat ageri dira porta hirukotea deituriko egiturak eratuz. Porta hirukote horiek adarkatu egiten dira eta lobuluxketan barneratzen dira sinusoide hepatikoetarantz. Sinusoide horien bitartez, gibeleko zelulek kanpoko oxigenoa, nutrienteak eta substantzia toxikoak bereganatzen dituzte beraien funtzioa betetzeko. Azkenik, sinusoideetan barrena igaro den odola,

lobuluxketako erdiko zainetan jasotzen da gero zain hepatikora bideratzeko (8. irudia). Beraz, gibelera heltzen den odol guztia sinusoide hepatikoko kapilareetan zehar pasatuz iragazten da eta ostean, zain hepatikoetan biltzen da azpiproduktuak kaba zainera bideratzeko (9. irudia). Bukatzeko beheko kaba zain honen bitartez odola giltzurruneraino bideratzen da hodakinak gernuaren bidez kanporatzekoiraizteko edo bestela behazunarekin batera odola konduktu hepatikora jariatzen da, hestetik pasatu ostean gorotzen bitartez kanporatzeko.

Bere funtzioak aurrera eraman ahal izateko, gibelak hainbat zelula desberdinez osaturiko egitura konplexua dauka. Alde batetik, parenkima hepatikoa osatzen duten hepatozitoak daude. Hauek gibeleko zelula ugarienak dira (% 80) eta proteinen sintesia eta metaketa, azukre eta lipidoen metabolismoa, behazunaren ekoizpena eta agente toxikoen katabolismoa burutzen dute. Beste alde batetik, gibelaren osagai ez- parenkimala dago, gibel sinusoideetako zelula endotelialez (ingelesez *Liver Sinusoidal Endothelial Cells*, LSEC), HSC-z eta immunitate-sistemaren parte diren zelulez osaturik.

LSECak gibelaren zirkulazio baskularra gaineztatzen duten zelulak dira, hau da, sinusoide hepatikoaren endotelioa osatzen dute. Zelula hauek gainerako zelula endotelialekiko desberdinak dira: ez dute xafla basalik aurkezten eta fenestra izeneko poroak dituzte. Fenestrei esker odola eta parenkima hepatikoko zelulek jariakin, solutu eta partikulen elkartrukea gauzatu dezakete sinusoidea eta hepatozitoen artean aurkitzen den Disseren espazioan eta xafla basalaren gabeziak ere solutuak bertara zuzenean igarotzea ahalbidetzen du. Horrez gain, LSECek immunitate zelulen atxikipena bermatzen dute aurkezten dituzten adhesio molekulei esker. Bestalde, zelula hauek antigeno aurkezle bezala jokatzeko gaitasuna dute (Shetty et al., 2018), izan ere, $T_c$-en aktibazioa eragiten baitu gibelean (Knolle & Wohlleber, 2016). Dena dela, LSECek Tc linfozito aktibatuen inhibizoa eta $T_{reg}$-en indukzioa ere eragin dezakete (Eggert & Greten, 2017).

Bestalde, HSCek gibelaren % 5-8 betetzen dute. Zelula hauek Disseren espazioan aurkitzen dira eta A bitamina pilatzen dute, organismoan metatzen den erretinoideen % 95 hain zuzen. Horrez gain, ECMko proteinak jariatzen dituzte, hala nola, kolagenoa eta MMPak homeostasia mantentzeko eta sinusoide hepatikoko zelula endotelialei sostengua emateko. Gainera, sinusoideen uzkurketan parte hartzen dute odol fluxua erregulatzeko.




**9. Irudia. Gibeleko zirkulazioaren eta lobuluxka hepatikoen eskema.** A) Gibeleko zirkulazioa. Arteria hepatikoak oxigenodun odola helarazten dio gibelari eta porta zain hepatikoak urdail, pankrea, barea eta hesteetatik nutrientez beterik datorrena. Odol hori iragazi denean, hondakinak zain hepatikoetatik beheko kaba zainera edo behazunarekin batera konduktu hepatikora bideratzen dira kanporatzeko. *Irudia BioRender.com bidez sortutakoa da.* B) Lobuluxka hepatikoen eskema. Gibela lobuluxka izeneko egituratan banaturik dago. Lobuluxka hauetako bakoitzak behazun hodia, porta zainak eta arteria hepatikoak eratzen duten hirukote portalarekin komunikaturik daude, zeinak nutriente eta oxigenotan aberatsa den odola hepatozitoetaraino bideratzen duen, odola sinusoide hepatikoetara drainatuz. Behin hepatozitoek odol hori iragazi ostean, lobuluxka erdiko zain hepatikoaren adarkapen batek odola berriro biltzen du hondakinak kanporatzeko. *Irudia Amparo Hidalgo Galianak egindakoa da (Ig: ahg_ilustracion).*

Gibeleko zelulekin bukatzeko, organoaren sistema fagozitiko mononuklearraren barnean, gibeleko bertako makrofagoak edo Kupffer zelulez (ingelesez *Kupffer Cells*, KC) aurki daitzeke. Endotelioari atxikiturik daude eta beraien funtzioen artean eritrozito zaharkituen eta elementu arrotzen fagozitosia eta antigenoen aurkezpena daude. Hauez gain, gibeleko immunitatea mantentzen, linfozitoak, neutrofiloak, Pit deritzen NK motako zelulak eta T errezeptorea daramaten NK (NKT) zelulak aurki daitezke besteak beste (Peng et al., 2016; Van den Eynden et al., 2013). Pit zelulak sinusoide hepatikoan kokatzen dira, zelula endotelialekin eta KC zelulekin kontaktuan. Patogeno edo tumore-zelula bat atzematean Pit zelulek perforinak eta granzimak jariatzen dituzte zitolitikoki elementu arrotza ezabatzeko. NKT zelulak, ordea, MHCa ezagutzen duten T errezeptorea aurkezten dituztenez gero, T linfozitoak bezalakoak dira (Nakatani et al., 2004).

## 4.3. Kolon-ondesteko minbiziaren metastasi hepatikoaren mikroingurumena.

Aurreko atalean azaldu dugun bezala gibela funtzio ugari betetzen dituen organoa da, beraz, orotariko zelulak aurki daitezke gibeleko tumore baten mikroingurumenean. Gibeleko metastasiaren garapena lau fase nagusitan sailkatu daiteke: lehenengoan (fase mikrobaskularra) tumore zelula sinusoideko odol-hodietara atxikitu eta estrabasatu egiten da; bigarrenean (fase estrabaskularra), estromako zelulak mikrometastasia dagoen zonaldera hurbiltzen dira; hirugarrenean (fase angiogenikoa) zelula endotelialak erreklutatzen dira tumorea baskularizatzeko eta laugarrenean (hazkuntza fasea) tumore metastatikoaren hazkuntza gertatzen da (Van den Eynden et al., 2013). Hortaz, metastasi prozesuaren fasearen arabera, zelula batzuek edo besteek izango dute garrantzia handiagoa tumorearen garapenean.

Behin gibelera helduta, tumore zelulek aurkitzen dituzten lehenengo zelulak LSECak, makrofagoak eta, batez ere, KCak dira. Printzipioz, hauek, minbizi zelulak ezabatzeko gaitasuna dute, baina deuseztatze prozesua ondo gertatu ez bada, tumore zelulek KCak aktibatu ditzakete. Egoera berri honetan, KCek zitokina pro-inflammatorioak jariatzen hasten dira, hala nola IL-1 eta TNF-α, zeintzuek LSECen adhesio molekulen espresioa emendatu egingo duten eta beraz, tumore zelulak atxikitu egingo zaizkie, estrabasazioa ahalbidetuz (Clark, A. M. et al., 2016). Hala ere, kontuan izan behar da LSECek ere bestelako zelulekin interakzionatzen dutela eta beraz, prozesu hau beste bide batzuetatik ere aktiba daitekeela.

Ondoren, gibeleko metastasiaren fase estrabaskularrean, organoa inbaditzen hasi diren minbizi zelulek gibeleko zelula estromalen erreklutamendua bultzatzen dute. Horietatik lehendabizi heltzen direnen artean HSCak daude, zitokinak, hazkuntza faktoreak, MMPak eta I eta III motako kolagenoa ekoizten dituztenak besteak beste (Tsuchida & Friedman, 2017; Van den Eynden et al., 2013). PCAn bezala, fibrosia metastasi hepatiko batzuen ezaugarrietako bat da, kasu honetan, HSCek jariaturiko eta kolagenoaren eta HSC zein KCek ekoiztutako MMPen ondorioz gertatzen dena (Matsumura et al., 2014; Van den Eynden et al., 2013). Ekoizpen hori mikroingurumeneko gainerako zelulek bultza dezakete batez ere β Tumore Hazkuntza-Faktorearen (ingelesez *Tumor Growth Factor β*, TGF-β) bidez. Gainera, VEGFak ere HSCen jariapena sustatzen duela behatu izan da angiogenesiarekin batera (Tsuchida & Friedman, 2017). Azken hau metastasiaren hirugarren fasearen oinarria da. Aurrerago azaldu dugun bezala, tumorea hazten doan heinean inguruneko oxigenoa agortu egingo da eta odol baso berriak sortzeko beharra izango du minbiziak. Tumore zelulek, zein hipoxia egoerak zitokina eta hazkuntza-faktore pro-angiogenikoen ekoizpena bultzatuko dute zelula endotelialak erreklutatzeko (Takeda et al., 2002).

Amaitzeko, kolon-ondesteko metastasi hepatikoaren mikroinguruneko zelulek immunoerregulazioan duten papera aipatu behar da. Lehenago adierazi den bezala, makrofagoak zelula arrotzak sutsitzeaz arduratzen diren arren, minbizi zelulen presentzian, TAMek fenotipo pro-inflamatorioa eta immunosupresorea aurkeztu dezakete. Alde batetik TMEko zelula desberdinen erreklutamendua bultzatu dezakete hantura egoera mantentzeko eta bestetik, $T_c$, $T_h$ eta NK zelulak inhibitu ditzakete $T_{reg}$-ak induzitzen dituen bitartean erantzun immunea blokeatzeko (Cortese et al., 2019). Hitz gutxitan, minbiziaren garapena TMEko zelulek eta beraien jariakinen arteko elkarrekintzek zehaztuko dute.

# 5. MINBIZIAREN AURKAKO TRATAMENDUAK

Zoritxarrez, minbizia maiz sortzen den gaitza da. Kasu batzuetan gaixotasunaren agerpena goiz detektatzeko gai gara prebentzio programei esker, baina beste askotan ez daukagu gaixotasuna bera edota metastasia aurresateko markatzailerik. Are gehiago, nahiz eta tumorea aurkitu, sarritan ez daukagu gaixotasuna guztiz desagertarazteko metodorik, batez ere tumore-zelulak organismoko gainerako organoetara barreiatu badira.

Minbiziaren tratamendurako erabiltzen diren terapien artean kirurgia, erradioterapia eta kimioterapia dira metodo ezagunenak. Tumore motaren eta kokapenaren arabera, tratamendu bat edo bestea aukeratzen da gaixotasunaren aurka jotzeko. Aitzitik, askotan ez da posible izaten tumorea kirurgia bidez erauztea edota erradiazioa tumorearen kontra zuzentzea, horregatik, kimioterapia da kasu gehienetan erabiltzen dena. Edonola ere, kasu batzuetan minbiziaren aurka egiteko erabiltzen diren agente kimioterapikoek ez dute esperotako efektua izaten tumoreak aurkezten duen kimioerresistentzia dela eta. Gainera, farmako horiek organismo guztian eragiten dutenez gero, zehar-efektu ugari sortarazten dituzte eta beraz, gaixoen ongizatea bermatzea ez da posible izaten. Hori dela eta, bai kimioterapiaren eragina indartzeko, zein gaixoen bizi-kalitatea hobetzeko asmoz, gaur egun tratamendu konbinatuak ematen dira, hau da, medikamentu kimioterapikoak beste farmako batzuekin edota osagarri nutrizionalekin batera ematen dira.

Gibel eta pankreako inflamazio kronikoak minbizia sor dezakeela ezaguna da aurrerago aipatu dugun moduan. Organismo batean zauri edo kalteren bat gertatzen denean hantura prozesua aktibatzen da sendaketarako, non alde batetik, immunitate zelulak erreklutatzen diren edozein agente arrotz deuseztatzeko eta bestetik, fibroblastoak ere bai ehuna konpontzeko. Prozesua aurrera joan ahala ROSak sortzen dira, zeintzuek hantura areagotu egin dezaketen (Coussens & Werb, 2002). Horiek horrela, minbizi zelulak dauden zonaldea etengabeko hantura prozesuan badago, TMEak asaldaturik jarraituko du zelula tumoralen alde jokatuz, eta azkenean minbizia garatuko da. Horregatik, eta lehenago azaldutako faktoreak kontuan izanda, terapia berriak erabiltzen hasi dira zuzenean tumore zelulen aurka bakarrik eraso egin beharrean, TMEak minbiziari eskaintzen dion laguntza ere murrizteko eta kimioerresistentziari aurre egin ahal izateko. Adibide bezala, konposatu antioxidatzaileak daude, ROSen aurka jokatzen dutenak, eta baita farmako antiinflamatorio eta immunomodulatzaileak ere hantura egoera iraunkor hori erregulatu ahal izateko.

## 5.1. Kimioterapia

Aipatu dugunez, minbizia tratatzeko ohiko modua kimioterapia erabiltzea da kontrolik gabeko hazkuntzan dauden zelulak gelditu edota hiltzeko. Baina zoritxarrez tumore zelulak etengabe aldatzen ari dira eta kimioterapiarekiko erresistentzia garatu dezakete (Phi et al., 2018; Yeldag et al., 2018).

Pankreari dagokionez, gehien erabilitako agente kimioterapikoak FOLFIRINOX (azido folinikoa, 5-fluorouraziloa, irinotekan eta oxaliplatinoa,), paklitaxela eta genzitabina dira (Borazanci & Von Hoff, 2014). Duela urte batzuk, paklitaxela nab-paklitaxel (ingelesez *nanoparticle albumin-bound paclitaxel*) bezala erabiltzen hasi zen, lehenik bularreko metastasirako (Fu et al., 2009), eta denborarekin beste minbizi batzuetarako erabiltzen hasi da. Halaber, azken urteotan genzitabina eta paklitaxel agente bien konbinazioa erabili izan da, bata zitotoxikoa eta bestea zitostatikoa, zeinak PCA duten gaixoen bizi-itxaropena luzatzea lortu duen (Von Hoff et al., 2013). Hala ere, aurrerago aipatu dugun moduan, PCA kimioerresistentea izaten da maiz. Ikertzaile desberdinek TAMek genzitabina, paklitaxela eta beste kimioterapiko batzuen efektua murriztu egiten dutela ikusi dute (Mantovani, G. et al., 2006; Weizman et al., 2014) kimioerresistentzia areagotu egiten delarik. Honen aurka kimioterapiarekin batera erradioterapia erabiltzen ari dira, konklusio zehatzik lortu gabe (Landau & Kalnicki, 2018).

CRCaren metastasi hepatikorako, aldiz, gehien erabiltzen diren kimioterapikoak irinotekan, 5-fluorouraziloa, oxaliplatinoa eta kapezitabina dira (Grapsa et al., 2015). Hasiera batean banaka ematen ziren arren, gaurko joera beraien arteko konbinazioak erabiltzea da, FOLFOX (Azido folinikoa, 5-fluorouraziloa eta oxaliplatinoa), FOLFIRI (Azido folinikoa, 5-fluorouraziloa eta irinotekan) edo FOLFIRINOX moduan (Fujita et al., 2015). Kimioerresistentziak alabaina arazoa izaten jarraitzen du. Irinotekanek adibidez, hakuntza faktore epidermalaren errezeptorearen (ingelesez *Epidermal Growth Factor Receptor*, EGFR) aktibazioa bultzatzen du, zeinak beste funtzio batzuez gain, zelulen proliferazioa eta onkogeneen espresioa eragiten duen (Petitprez & Larsen, 2013). Bestalde, kimioterapiko hauen efektua areagotzeko gaur egun, VEGF zein EGFRen aurkako antigorputzak ematen zaizkie (bevacizumab eta zetuximab) kimioterapiarekin batera, edota baita regorafenib izeneko tirosina kinasen inhibitzailea (Vogel et al., 2017), kimioerresistentzia induzitzen duten faktoreak nolabait blokeatzeko eta minbiziaren hazkuntza geldiarazteko (Sueur et al., 2016).

Dena dela, pazienteek zeharkako-efektuak pairatzen dituzte (Borazanci & Von Hoff, 2014; Van den Eynden et al., 2013). Beraz, ikertzaileak hori ekiditeko, droga berriak bilatzen ari dira, minbizi zelulentzako toxikoak direnak, baina zelula normalentzako kalterik eragiten ez dutenak hain zuzen ere.

## 5.2. Mikronutriente naturalen efektua minbizian

Antzinatik gaixotasunak sendatzeko naturak eskaintzen dituen produktuak erabili izan dira. Gaur egun, aldiz, sendagai gehienak sintesi kimikoaren bidez sortzen ditugun arren, asko landareetatik eratorriak dira. Arrazoi horregatik, minbizia tratatzeko konposatu naturalak aztertzen hasi ziren.

Mikronutriente naturalen arloan aipatzekoak dira esaterako flabonoideak edo landareetatik datozen polifenolak, bitaminak eta mineralak. Espezia askok ere, adibidez, kurkuma, jengibrea, azafraia, piperbeltza, piperminak eta abarrek minbizia prebenitzeko eta tratatzeko gai izan daitezkeen biologikoki aktiboak diren osagaiak eskaintzen dituze (Zheng et al., 2016). Orokorrean, molekula hauek propietate immunomodulatzaileak, antioxidatzaileak eta antiinflamatorioak dituztela esan liteke eta horregatik, minbiziaren tratamenduan lagungarriak izan daitezkeela uste da. Polifenolei dagokienez, te berdearen epigalokatektina-3-galatoa (EGCG), agente antiinflamatorio, immunomodulatzaile eta antioxidatzailea da proliferazio zelularra inhibitu eta apoptosia areagotu egiten dituena minbizi desberdinetan (de Carvalho et al., 2020; Maruyama et al., 2014; Shirakami & Shimizu, 2018; Yang & Wang, 2010). Bestalde, erregalizak daukan azido glizirrizikoak propietate antiinflamatorioaz gain, minbizi zelulen apoptosia eragiten du eta biriketako minbizian ere metastasia galarazten du (Gol'dberg et al., 2008; Khazraei-Moradian et al., 2017; Wang, Z. Y. & Nixon, 2001). Metastasiarekin erlazionaturik, polifenolek, bitaminek eta bestelako molekulek minbizi zelulen migrazioa ekiditen duela ere frogatu da (AlQathama & Prieto, 2015; Davis-Yadley & Malafa, 2015; Niedzwiecki et al., 2016; Suh et al., 2018). Beste adibibe bat aipatzeko, piperminek daukaten kapsaizinak minbizian parte hartzen duten geneen espresioa aldatzen duela ikusi da CRCn, PCAn eta bularreko zein prostatako minbizietan besteak beste, tumorearen aurka egiteko gaitasuna aurkeztuz (Chapa-Oliver & Mejia-Teniente, 2016). Gainera, mikronutrienteek minbizi kasu batzuetan deregulaturik dauden geneak lehengo egoera osasuntsura itzultzeko gaitasuna daukate (Ratovitski, 2017). Aipatutako osagai naturalez gain eta bitaminetan zentratuz, B motako bitaminen gabeziak minbiziaren sorrera eragin dezakeela behatu da, elementu hauek DNAren osaketan parte hartzen dutelako (Ames & Wakimoto, 2002). Horrez gain, B6 bitaminak p53 tumoreen gene supresorea aktibatzen duela ere ezaguna da (Zhang et al., 2014). Are gehiago, pantotenako kaltzikoak (B5 bitamina) metastasia murriztu eta apoptosi maila igo egin zuen saguen obulutegiko minbizian (Penet et al., 2016). Bestalde, β-karotenoak (A bitamina) eta α-tokoferolak (E bitamina)

tumoreak berriro agertzea ekidin eta tumoreen gene supresorea aktibatzen dute (Lamson & Brignall, 1999).

Halaber, tumore zeluletan eragina izateaz gain, mikronutrienteek TMEan ere aldaketak sortzen dituztela behatu da zeina beharrezkoa den bai tumorearen garapenerako, bai metastasia sortzeko. Zentzu horretan, polifenolek immunitatea erregulatu dezakete, hala nola NK zelulak aktibatuz, TAMak suprimituz, Treg-en aktibazioa deuseztatuz eta Tc-ena suspertuz (de Carvalho et al., 2020). Bestetik, kurkuminak ere immunosupresioari aurre egiten dio immunitate-sistemako zelula zitolitikoen galera saihestuz eta erantzun immunea geldiarazten duten zelulen kantitatea murriztuz (Pan et al., 2019). Era berean, hipoxiak eragindako EMTa ere ekiditen du (Zubair et al., 2017). Horrez gain, β-karotenoa immunoerregulatzaile bezala aztertu zen NK zelulen aktibitatea suspertzen duela ikusi baitzuten (Pan et al., 2019). Jarraiki, flabonoideak ere beste adibide on bat dira, izan ere CAFen efektu pro-tumorala modu desberdinetan inhibitu dezaketela behatu baita. Esaterako, CAFek jariatzen dituzten hazkuntza faktore, zein MMPen espresioa erregulatu dezakete eta modu berean kolagenoaren ekoizpena murriztuarazi dezakete TMEaren birmodelazioa bultzatuz (Hu et al., 2017; Sartippour et al., 2002; Suh et al., 2018).

Laburbilduz, hainbat konposatuk minbiziaren aurkako propietateak dituztela frogatu denez (Lichota & Gwozdzinski, 2018), elementu hauek minbizia tratatzeko erabiltzen diren oinarrizko terapiekin batera emateak zein eragin izan dezakeen aztertzea gero eta arruntagoa da.

## 5.3. Mikronutriente naturalak kimioterapiaren osagarri bezala

Esan bezala, mikronutriente naturalek minbiziaren aurka egiteko gaitasuna dute. Gainera, substantzia naturalak diren heinean, ez dute zehar-efekturik eragiten. Horrek guztiak produktu naturalak kimioterapiarekin batera erabiltzera bultzatu ditu onkologia arloko ikertzaileak, bai kimioterapiaren eragina indartzeko, bai zehar efektuak arintzeko eta azken finean gaixoen bizi-kalitatea hobetzeko.

Horiek horrela, osagarri nutrizionalak ordura arte ezarritako terapiekin konbinatuta ematen hasi ziren, zeinak kasu askotan pazienteen pronostikoen hobekuntza eragin zuen. Azido glizirrizikoak esaterako, terapia zitostatikoen eraginkortasuna hobetu zuela frogatu zen tumore desberdinetan (Gol'dberg et al., 2008). Kurkuminak baita, ezaugarri antiinflamatorio eta

antioxidantzaileez gain, ondesteko kartzinomarako erabilitako irinotekanarekiko kimioerresistentzia murrizten duela ikusi zen (Su, X. et al., 2017). Kapsaizinak ere 5-fluorouraziloararekiko sentikortasuna areagotzen duela ondorioztatu zen saguen kolangiokartzinoman (Hong et al., 2015) eta baita leukemia mieloideko zeluletan ere (Clark, R. & Lee, 2016). Horrez gain, azido askorbikoak (C bitamina) ere genzitabina kimioterapikoarekin batera ematerakoan bere efektu antitumorala indartu egiten duela ikusi zen PCAn (Espey et al., 2011) eta baita oxaliplatino zein irinotekanena giza CRC-ko zeluletan (Pires et al., 2018).

Orain arte aipatutakoa aintzakotzat harturik, ukaezina da produktu naturalek berezko propietateak dituztela minbizi zelulen eta TMEaren elementu pro-tumoralen aurka egiteko maila desberdinetan. Horregatik, ezaugarri horiek produktu naturalak terapia osagarri bezala erabiltzeko bultzada eragin du.

## 5.4. Ocoxin

Ocoxin (Catalysis S.L.) te berdez, azido glizirrizikoz, B6, B12 eta C bitaminez, aminoazidoz eta mineralez osaturiko nutrizio osagarri bat da propietate antiinflamatorio, immunomudulatzaile eta antioxidatzaileak dituena. Beste izendapen batzuk ere izan ditu: *Oncoxin*, *Ocoxin + Viusid eta Ocoxin Oral Solution*. Jarabe honen osagai desberdinek funtzio antitumorala daukatela frogatu da eta hortaz, elementu guztien konbinazioak efektu hori indartu dezakeen aztertu nahi izan da.

Azken urteotan konposatu hau minbizi eredu desberdinetan frogatu da *in vitro* eta *in vivo*, esaterako kartzinoma hepatozelularrean (ingelesez *Hepatocellular carcinoma*, HCC), leukemia mieloide akutuan (ingelesez *Acute Myeloid Leukemia*, AML), zelula txikietako birikako minbizian (ingelesez *Small-Cell Lung Cancer*, SCLC) eta bularreko minbizian (Diaz-Rodriguez et al., 2016; Diaz-Rodriguez et al., 2017; Díaz-Rodríguez et al., 2018; Hernandez-Garcia et al., 2015). Gainera, emaitza onak lortu izan direnez, orain entsegu klinikoak egiten ari dira.

# II. HIPOTESIA ETA HELBURUAK

# II. HYPOTHESIS AND OBJECTIVES

# II.I. HIPOTESIA

Jakinik tumorearen mikroingurumeneko elementuek kimioterapiaren eraginkortasuna murrizten dutela eta konposatu antioxidatzaile, antiinflamatzaile eta immunoerregulatzaileek minbiziaren garapenaren aurka jokatzen dutela hurrengo **hipotesia** proposatzen da:

Ocoxin konposatu naturalak propietate *propietate antiinflamatorio, antioxidatzaile eta immunoerregulatzaileak dituela eta minbiziaren garapenean tumorearen mikroingurumeneko zelulek*

# II.II. HELBURUAK

Lan honen **helburu nagusia** honakoa da:

Ocoxinen efektua ikertzea pankreako minbiziaren eta kolon-ondesteko minbiziaren metastasi hepatikoaren garapenean eta tumore hauen mikroingurumenean, bai bakarrik erabilita, baita ohiko kimioterapikoekin batera konbinaturik hauen eraginkortasunean eraginik duen aztertzeko.

Horretarako hurrengo **helburu zehatzak** ezarri ziren:

1. Ocoxinek minbizi zelulen bideragarritasunean, migrazioan eta ziklo zelularrean *in vitro* nola eragiten duen ikertzea.
2. Ocoxinek PCA eta CRCaren metastasi hepatikoko zelulen espresio genikoan eragiten duen aztertzea.
3. Ocoxinek PCAren eta CRCaren metastasi hepatikoaren garapenean duen efektua *in vivo* behatzea.
4. Ocoxinek PCAko eta CRCaren metastasi hepatikoko tumoreen mikroingurumenearen elementuen gain duen eragina *in vivo* aztertzea.
5. Ocoxinek zitokinen profilean *in vivo* aldaketak sorrarazten dituen analizatzea.
6. Ocoxinek kimioterapiaren zitotoxizitatea areagotzen duen behatzea.
7. Ocoxinek estromak eragindako kimioerresistentzia *in vitro* eta *in vivo* deusezten duen aztertzea.

# III. MATERIALAK ETA METODOAK

# III. MATERIALS AND METHODS

# III.I. MATERIALAK / MATERIALS

## 1. OCOXIN

Ocoxin (Catalysis S.L., Spain) propietate antiinflamatorio, immunomudulatzaile eta antioxidatzaileak dituen mikronutriente nutrizional desberdinen konbinazioaren ondorioz sorturiko konposatua da. Bere osagaien artean elementu naturalen estraktuak, bitaminak, aminoazidoak eta mineralak daude batez ere. Konposizio zehatza eta osagai bakoitzaren kontzentrazioak hurrengo taulan bilduta daude (1. taula):

**1. Taula. Ocoxin konposatuaren konposizioa (100 ml-ko).**

| Osagaiak | Components | Kontzentrazioa |
|---|---|---|
| **Glizina** | **Glycine** | 2 g |
| **Glukosamina** | **Glucosamine** | 2 g |
| **Azido malikoa** | **Malic acid** | 1,2 g |
| **Arginina** | **Arginine** | 640 mg |
| **Zisteina** | **Cysteine** | 204 mg |
| **Glizirrizinato monoamonikoa** | **Monoammonium glycyrrhizinate** | 200 mg |
| **Azido askorbikoa** | **Ascorbic acid** | 120 mg |
| **Zink sulfatoa** | **Zink sulphate** | 80 mg |
| **Te berdearen estraktua** | **Green Tea extract** | 25 mg |
| **Kaltzio pantotenatoa** | **Calcium pantothenate** | 12 mg |
| **Piridoxina** | **Pyridoxine** | 4 mg |
| **Manganeso sulfatoa** | **Manganese sulfate** | 4 mg |
| **Kanela estraktua** | **Cinnamon extract** | 3 mg |
| **Azido folikoa** | **Folic acid** | 400 µg |
| **Zianokobalamina** | **Cyanocobalamin** | 2 µg |

## 2. FARMAKOAK

### 2.1. Barbiturikoak

#### 2.1.1. Nembutal

Nembutala edo pentobarbital sodikoa (Sigma-Aldrich, Estatu Batuak) denbora laburreko anestesikoa da. Espezie handietan sedatzaile edo hipnotiko bezala dihardu eta espezie txikietan anestesia orokor bezala erabiltzen da. Eutanasiarako ere erabili ohi da dosi altuetan. Kasu honetan, saguen kirurgia hasi aurretik animaliak anestesiatzeko erabili zen 50 mg / sagu Kg dosiarekin.

## 2.2. Kimioterapikoak

### 2.2.1. Irinotekan

Irinotekana (Sigma-Aldrich, Estatu Batuak) CRCko, PCAko edota biriketako minbizien aurka erabiltzen den konposatu zitotoxikoa da, DNA Topoisomerasa I-a inhibitzen du eta DNAren kate sinpleetan kalteak eragiten ditu, erreplikazioa blokeatuz. Lan honetan irinotekana alde batetik *in vitro* entseguetan 50 µMeko kontzentrazioarekin erabili zen eta bestetik, CRCtik eratorritako metastasi hepatikoa garatu zuten saguak *in vivo* tratatzeko ere erabili zen 20 mg / sagu Kg dosiarekin.

### 2.2.2. Genzitabina

Genzitabina (Sigma-Aldrich, Estatu Batuak) kimioterapian erabiltzen den farmakoa da. Zelula barnean metabolizatzen denean ahalmen zitotoxikoa duten 2 nukleosido sortzen dira, eta beraz DNAren sintesia galarazten du zelulen apoptosia induzituz. Ordura arte biriketako minbizia, pankreakoa, bularrekoa, obariokoa eta puxikakoa tratatzeko erabili izan da gizakietan. Lan honetan PCAko zelula lerro desberdinak genzitabina 1µM-ko dosiarekin tratatu ziren, bakarrik eta paklitaxelarekin batera.

### 2.2.3. Paklitaxel

Paklitaxela (Sigma-Aldrich, Estatu Batuak) PCA, bularreko minbizia, biriketakoa, prostatakoa, melanoma eta beste hainbat tumore tratazeko erabiltzen den kimioterapikoa da. Konposatu honek tubulina dimeroetatik abiatuz mikrotubuluen muntaia estimulatzen du, aldi berean hauen despolimerizazioa ekidituz, hau da, mikrotubuluak egonkortzen ditu. Ondorioz, paklitaxelak ardatz mitotikoaren eraketa eragozten du, zelulen zatiketa blokeatu egiten delarik. Lan honetan farmako hau PCAko zelula lerro desberdinak tratatzeko erabili zen 1µM-ko dosiarekin, bai bakarrik eta bai genzitabinarekin konbinatuta.

# 3. ZELULA LERROAK

## 3.1. Tumore zelulak

### 3.1.1 Sagu zelulak

#### *3.1.1.1 C26*

C26 zelulak (ATCC, LLGC Standards S.L.U., Espainia), CT26 edo MCA26 bezala ere ezagutzen direnak, 1,2-dimetil hidrazinaren eraginpean egondako BALB/c saguetan sortutako koloneko adenokartzinomatik erauzitakoak dira. Zelula lerro honek tumoreak sortzeko gaitasuna du bai injektatzen diren gunean eta baita metastasia eragiteko ere. BALB/c saguekiko singenikoak izanik, C26 zelulak CRCaren metastasi hepatikodun *in vivo* sagu-eredua sortzeko erabili zen. Horrez gain, Ocoxinen zitotoxizitatea aztertu zen zelula hauetan bai bera bakarrik, bai irinotekanekin konbinaturik.

#### *3.1.1.2. 266-6*

266-6 zelulak (ATCC, LLGC Standards S.L.U., Espainia) sagu transgenikoen azino pankreatikoetatik erauzita daude. Sagu transgeniko hauek 40 tximu birusaren (ingelesez *Simian Virus 40,* SV40) T antigeno txiki eta handia kodifikatzen duen gene arrotz bat daramate arratoiaren elastasa I genearen barruan, elastasa I genearen espresioa erregulatzen duten elementuekin batera. Elastasa sagu fetuaren garapenaren 14. egunean espresatzen da, pankreako zelula azinarrak desberdintzen hasten direnean hain zuzen ere. Azterketen arabera, gene hau azinoetan bakarrik esperesatzen da. Ocoxinek zelula hauetan eragiten duen zitotoxizitatea aztertzeaz aparte, 266-6 zelulak C57BL/6J saguekiko singenikoak izanik, PCAren *in vivo* eredua garatzeko erabili ziren.

### 3.1.2 Giza zelulak

Minbizia hain gaixotasun heterogeneoa izanik, PCAn Ocoxin konposatuaren zitotoxizitatea eta honek paklitaxel edota genzitabina kimioterapikoekin izan dezakeen sinergia aztertzeko, zelula-lerro desberdinak erabili ziren.

#### *3.1.2.1. BxPC-3*

61 urteko emakume baten adenokartzinoma pankreatikotik erauzitako zelula tumorigenikoak dira (ATCC, LLGC Standards S.L.U., Espainia). Zelula hauek morfologia epiteliala aurkezten dute eta ama-zelulen markatzaile ugari

espresatzen dituzte. Gainera, ez dute PCA zeluletan ohikoa den KRAS mutazioa aurkezten eta muzina, zein faktore proangiogenikoak ekoizten dituzte. Bestalde, zelula hauek, zelula osasuntsuek baino SMAD4 gene tumore supresorearen kopia gutxiago dituzte.

*3.1.2.2. Capan-2*

Duktuetako adenokartzinoma pankreatikodun 56 urteko gizon bateko pankreatik erauzitako zelulak dira (ATCC, LLGC Standards S.L.U., Espainia). Morfologia epiteliala aurkezten dute eta KRAS onkogenearen mutazioa daramate. Zehazki 12. kodoiean GGT sekuentzia GTT bihurtu da eta beraz, glizina ordez, valina daukate. Horrez gain, zelula hauek EGFR gainespresatu egiten dute, SMAD4 genearen espresioa zelula osasuntsuenaren bezalakoa den bitartean.

*3.1.2.3. CFPAC-1*

CFPAC-1 zelula lerroa (ATCC, LLGC Standards S.L.U., Espainia) 26 urteko fibrosi kistikodun paziente baten sortutako pankreako adenokartzinoma duktaletik erauzi zen. Fibrosi kistikoa duten gaixoek kasu askotan pankreatitis idiopatikoa aurkezten dute, zeinak pankreako minbizia eragin dezakeen. Horrez gain pazienteak metastasia zeukan gibelean, hots CFPAC-1 zelulek metastasia eragin dezakete. Morfologiari dagokionez, itxura epiteliala aurkezten dute eta pankreako zelula duktalen bereigazgarri diren zitokeratina zein antigeno onkofetalak espresatzen dituzte. Bestalde, zelula hauek fibrosis kistikoaren CF genearen fenilalanina-508aren delezioa, SMAD4 genearen kopien murrizketa eta Capan-2 zelulek duten KRAS genearen mutazio berdina aurkezten dituzte.

*3.1.2.4. HPAF-II*

Zelula lerro hau adenokartzinoma pankreatiko primarioa eta gibel, diafragma eta nodulu linfatikoetan metastasia zeukan 44 urteko paziente bateko fluido aszitiko peritonealetik erauzitako adenokartzinoma panreatikoko zelulak dira (ATCC, LLGC Standards S.L.U., Espainia). HPAF-IIak zelula pleomorfikoak dira eta Muc 1 eta Muc 4 muzina geneak espresatzen dituzte, hauetako lehenengoa gainespresatzen duelarik. Gainera KRAS genearen mutazioa daramate 12. kodoiean non GGT GAT bihurtzen den, hau da, glizina ordez azido aspartikoa dute eta aldi berean, genearen kopiak anplifikaturik daude.

*3.1.2.5. Panc10.05*

Pankrearen buruan adenokartzinoma pankreatikoa zeukan gizon batengandik erauzitako zelulak dira (ATCC, LLGC Standards S.L.U., Espainia). Bestalde, HPAF-II zelulen KRAS onkogenearen mutazio berbera daramate.

*3.1.2.6. SW1990*

Pankrea exokrinoan garatutako adenokartzinoma pankreatikodun gizaki baten bareko metastasitik erauzitako zelulak dira (ATCC, LLGC Standards S.L.U., Espainia). SW1990 zelulek ere HPAF-II eta Panc 10.05 zelulen KRAS onkogenearen mutazio berdina daramate eta hauek ere SMAD4 genearen kopien murrizketa aurkezten dute.

## 3.2. Zelula ez-tumoralak

### 3.2.1. Sagu zelulak

*3.2.1.1 BALB/3T3 clone A31*

BALB/3T3 clone A31 (edo 3T3) zelulak, BALB/c enbrioietatik erauzitako fibroblastoak dira (ATCC, LLGC Standards S.L.U. Bartzelona, Espainia). Alde batetik, zelula estromal osasuntsu gisa erabili ziren zitotoxizitate frogak egiteko eta bestetik, beraiek ekoiztutako faktore soluigarrietan aberatsa den medioa jaso zen estromaren eragina ikertzeko sagu tumore zelulen aurrean eta baita kimioerresistentzia aztertzeko ere.

*3.2.1.2 J774A.1*

Zelula hauek BALB/c saguen likido aszitikotik erauzitako makrofagoak dira (ATCC, LLGC Standards S.L.U. Bartzelona, Espainia). Ezaugarri bereizgarri bezala, IL-5 ekoizten dute. Zelula osasuntsu gisa erabili ziren zitotoxizitatea aztertzeko.

### 3.2.2. Giza zelulak

*3.2.2.1 MRC-5*

MRC-5 zelulak giza biriketatik erauzitako fibroblastoak dira (ATCC, LLGC Standards S.L.U. Bartzelona, Espainia). 3T3 zelulen moduan, hauek jariaturiko

faktore soluigarridun medioa batu egin zen tumore zelulengan eragiten zuen efektua eta kimioerresistentzia aztertzeko.

# 4. ZELULEN HAZKUNTZA ETA MANTENURAKO MEDIO ETA OSAGARRIAK

## 4.1. Hazkuntza medioak

### 4.1.1 RPMI-1640

RPMI-1640 hazkuntza medioa (Gibco, Estatu Batuak) zelula guztien hazkuntzarako eta tratamenduetarako erabili zen. Hazkuntza medio hau hainbat zelula mota hazteko erabilgarria dela frogatu da, hauen artean ugaztun zelulak eta minbizi zelulak. RPMI-1640 medioaren ezaugarri bereizgarriak glutation agente erreduktorea eta glukosa (2 g/L) zein bitaminen kontzentrazio altua dira (biotina, B12 bitamina eta azido folikoak duen azido para-aminobenzoikoa). Horrez gain, hazkuntza medio honek fenol gorria darama medioaren pH-a begi bistaz antzemateko. Kultiboko zelulen metabolitoen jariapenaren ondorioz medioaren pH-a azidifikatzen bada, fenol gorriak hori kolorea hartuko du.

Kultibo medio honek ez dauka proteinarik, lipidorik, ezta hazkuntza faktorerik ere. Hori dela eta, normalean gehigarri bat behar izaten du, kasu gehienetan behi serum fetala (ingelesez *Fetal Bovine Serum*, FBS) erabiltzen delarik % 10eko kontzentrazioarekin. Halaber, mikroorganismo arrotzek eragindako kutsadura ekiditeko, medioari antibiotiko eta antimikotikoak gehitzen zaizkio.

## 4.2. Zelulen mantenurako osagaiak

### 4.2.1 Antibiotiko eta antimikotikoak

#### *4.2.1.1. Antibiotiko-antimikotikoak (100X)*

Bakterio eta onddoen kutsadura ekiditeko kultibo medioari antibiotiko-antimikotiko soluzioa gehitu zitzaion (Gibco, Estatu Batuak). Honen osagarriak penizilina (100 U/ml), estreptomizina (100 µg/ml) eta anfoterizina B (0.25µg/ml) dira. Penizilina eta estreptomizinak bakterio Gram positivo eta Gram negatiboak hiltzen dituzte eta anfoterizinak onddo eta legamien hazkuntza ekiditen du.

*4.2.1.2 Gentamizina*

Gentamizina (Gibco, Estatu Batuak) espektro zabaleko antibiotikoa da mikroorganismo Gram-positibo eta Gram-negatiboen hazkuntza inhibitzen dituena, baita Pseudomonas, Proteus, Staphylococcus eta Streptococcus andui erresistenteen kasuan. Animaliengandik zuzenean lortutako zelulen kasuan, hots, kultibo primarioetan, antibiotiko hau erabili zen kultiboak babes handiagoa izan zezan. Lan honetan saguen gibeletatik erauzitako esplanteetako zelulak kultibatzerakoan erabili zen kutsadurak ekiditeko.

### 4.2.2 Behi-Serum fetala (FBS)

FBSa (Gibco, Estatu Batuak) hazkuntza faktoreak, aminoazidoak, lípidoak eta hormonak daramatzan soluzio bat da. Txahal fetuei odola erauzi ostean, odolari eritrozitoak, fibrinogenoa eta koagulazio proteinak kentzen zaizkio seruma lortzeko. FBSa erabili aurretik 30 minutuz 52 ºC-tan berotzen zen immunitate sistemako konplementuaren proteinak eta hazkuntzaren inhibitzaile ezezagunak inaktibatzeko.

FBSa RPMI-1640 hazkuntza medioaren osagarri bezala erabili zen eta baita immunomarkaketak egiteko bufferrak prestatzeko ere.

### 4.2.3 Giza intsulina birkonbinantea

Pankreak intsulina jariatzen duenez, zelula batzuk hobeto hazten dira intsulinaren presentzian. Lan honetan giza intsulina birkonbinantea (Invitrogen, Estatu Batuak) 10 U/ml kontzentrazioarekin erabili zen Panc10.05 zelulen hazkuntzarako.

### 4.2.4 Zelulen kultiborako soluzioak

*4.2.4.1 Azido etilendiaminotetraazetikodun fosfato gatz tanpoia 4mM (PBS-EDTA)*

Etilenodiaminatetraazetiko azidoaren (EDTAren) (Panreac Quimica SLU, Spain) ahalmen kelanteari esker, $Ca^{2+}$ ioiekin lotzen da eta zelulen arteko loturak apurtu egiten dira. Fosfato gatz tanpoiarekin (ingelesez *Phosphate Buffered Saline*, PBS) diluitzen da 4 mMeko kontzentraziodun etilenodiaminotetraazetikodun fosfato gatz tanpoia (PBS-EDTA) prestatzeko.

Soluzio hau zelulak plastikozko fraskoetatik desitsasteko erabili zen. PBSaren konposizioa 6.6.1. atalean aurki daiteke.

### 4.2.5 Matrize estrazelularreko proteinak

#### *4.2.5.1. I motako kolagenoa*

I motako kolagenoa (Gibco, Estatu Batuak) matrize estrazelularreko osagai nagusia da. Ehun konektiboan oso ugaria da eta organoetan ere aurki daiteke ehun desberdinei egitura emanez. Matrize estrazelularreko kolagenoak nutrienteen garraioa errazten du eta zelulei bertan finkatzeko edota migratzeko aukera ematen die. Hori dela eta, C26 zelulak kultibatzeko erabili zen 10 µl/ml kontzentrazioarekin substratura hobeto atxiki zitezen.

#### *4.2.5.2 Txerri azaleko gelatina*

Txerri azaleko gelatina (Sigma-Aldrich, Estatu Batuak) kolagenoa berotuz lortzen da. Tenperatura altuaren ondorioz, kolagenoaren helize hirukoitza destolestu egiten da eta estruktura makromolekular desordenatu bat bezala antolatzen da. Egitura berri honek disolbatzeko erresistentzia eta uzkurketa eta espantsio propietate desberdinak ditu. Gelatina % 0,1eko kontzentrazioarekin erabili zen zelulen matrize estrazelularra bailitzan 266-6 zelulak substratuarekiko hobeto lotzeko.

### 4.2.6 Entzimak

#### *4.2.6.1. Tripsina-EDTA % 0,05*

Tripsina (Gibco, Estatu Batuak) txerri pankreasetik erauzitako proteasa bat da zeinak lotura peptidikoak desegiten dituen. Entzima hau kultibo zelularretan zelulak fraskoetatik askatzeko erabiltzen da. Zehazki tripsina hau 0,53 mM EDTAtan diluituta dago eta % 0,05eko kontzentrazioa du.

# 5. ANIMALIAK

Lan honetan azalduko diren prozedurak EHU/UPV-ko animaliekin egiten den Esperimentaziorako Etika Batzordearengatik (AEEB) onartuak izan ziren eta esperimentaziorako eta beste helburu zientifikoetarako erabilitako animalien zainketa eta ostatu baldintzak ezartzen dituzten zuzendaritza-kontseiluaren 86/609/EEC direktiba, europako parlamentuaren 2003/65/EC direktiba,

Europako batzordearen 2007/526/CE gomendioa eta estatu mailako RD 53/2013 legea jarraituz burutu dira.

Animalia guztiak EHU/UPV-ko animaliategian mantendu ziren karraskarientzako pentsua eta ura *ad libitum* hartzeko aukera zutelarik.

## 5.1. BALB/c

CRC-ko metastasiko *in vivo* eredu esperimentala 8 asteko adina zuten BALB/c sagu arrekin (Charles River, Espainia) garatu zen garatzeko. Sagu andui hau Bagg albino izeneko saguetatik eratorritakoa da eta batez ere minbizi eta immunologiako ikerketetan erabiltzen da. Lan honetan andui hau erabili zen CRC-ko C26 zelulak hauekiko singenikoak direlako.

## 5.2. C57BL/6J

8 asteko adina zuten C57BL/6J sagu arrak Charles River laborategietan (Espainia) erosi ziren. Sagua andui hau ikerketarako gehien erabiltzen dena da eta bere genoma sekuentziatu diren sagu genometatik lehena izan zen. Tumore batzuekiko erresistenteak diren arren, mutazio gehienak espresatzeko gai dira animalia hauek. Lan honetan, andui hau PCAko *in vivo* eredua garatzeko erabili ziren, PCAko 266-6 minbizi zelulak animalia hauekiko singenikoak baitira.

# 6. SOLUZIO ETA ERREAKTIBO OROKORRAK

## 6.1 Tanpoiak

### 6.1.1. Fosfato gatz tanpoia (PBS)

PBSa zelulen kultiboetarako erabiltzen den soluzio amortiguatzailea da, zelulen kultiboetan pH eta presio osmotikoa ingurune fisiologikoetan bezala manentzea ahalbidetzen duena.

PBSa zelulen garbiketak egiteko erabili zen eta baita zelulak bizirik mantentzeko animalietan inokulatu aurretik. Bestalde, immunohistokimikarako soluzioak eta blokeatzaileak prestatzeko zein garbiketak egiteko ere erabili zen. Tanpoiaren konposaketa 2. taulan ikus daiteke:

**2. Taula. PBS tanpoiaren konposizioa litroko.**

| Substantzia | Kontzentrazioa |
|---|---|
| **NaCl (Sigma-Aldrich, Estatu batuak)** | 8,0 g/l |
| **KCl (Sigma-Aldrich, Estatu batuak)** | 0,2 g/l |
| **$Na_2HPO_4 \cdot 12\ H_2O$ (Sigma-Aldrich, Estatu batuak)** | 2,9 g/l |
| **$KH_2PO_4$ (Sigma-Aldrich, Estatu batuak)** | 0,2 g/l |
| **$H_2O$** | 1 l bete arte |

*Soluzio honen pHa 7,4ra doitu zen*

### 6.1.2. Tris tanpoia

Soluzio amortiguatzaile hau prozesu askotarako erabiltzen da, hala nola, elektroforesi tekniketan edota DNAren erauzketan esaterako. Lan honetan 0,1Meko Tris tanpoia (3. taula) zink soluzio fixatzailea prestatzeko erabili zen:

**3. Taula. Tris tanpoiaren konposizioa litroko 0,1M.**

| Substantzia | Kontzentrazioa |
|---|---|
| **Tris HCl (Sigma-Aldrich, Estatu batuak)** | 15,76 g/l |
| **$H_2O$** | 1 l bete arte |

*Soluzio honen pHa 6,4ra doitu zen*

## 6.2 Detergenteak

Zelulak lisatzeko, proteinak solubilizatzeko eta garbiketa tanpoietan erabiltzen dira.

### 6.2.1 Triton X-100

Triton-X (Sigma-Aldrich, Estatu Batuak) detergente ez-ionikoa da. Zelulen mintzeko proteinak iragazkortzeko erabili zen immunohistokimika entseguetan.

### 6.2.2 Tween 20

Tween-20 (Sigma-Aldrich, Estatu Batuak) detergente ez-ionikoa da Entzimei loturiko immunoabsortzio entseguetarako (ingelesez *Enzyme-Linked ImmunoSorbent Assay*, ELISA), Western Bloterako edota immunoentseguetarako tanpoi desberdinei gehitzen zaiona emultsionatzaile moduan jarduteko. Lan honetan sodio-zitrato tanpoia prestatzeko erabili zen.

## 6.3 Erreaktibo orokorrak

### 6.3.1. Dimetilsulfoxidoa (DMSO)

Dimetil sulfoxidoa (DMSO) (Sigma-Aldrich, Estatu Batuak) substantzia organikoa da, disolbatzaile bezala, kriobabesgarri bezala edota medikamentu bezala erabiltzen dena zenbait kasutan inflamazioa eta mina murrizteko. Alde batetik liofilizaturik dauden tratamenduetarako farmakoak disolbatzeko erabili zen. Bestetik, zelulen izozketa prozesurako ere erabili zen. DMSOa zelulen bigeruza lipidikoan metatzen da eta urak zeharka ditzakeen poroak eratzen ditu. Poro horiei esker, zeluletako urak bertatik ateratzeko aukera du, izozte prozesuan sortzen diren izotz kristalak medio estrazelularrean sortuko direlarik eta ez zelula barnean.

# 7. FIXATZAILEAK

## 7.1 Formalina

Formalina (Panreac Quimica SLU, Espainia) metanolarekin estabilizatu den % 35-40ko purutasuna duen formaldehidoa da. Agente honek proteinetako hidrogeno loturen apurketa eragiten du eta sare erretikular polipeptidikoa eratzen da ehuna fixatu egiten delarik. Formalina, saguetatik erauzitako gibelak fixatzeko erabili zen.

## 7.2 Zink soluzioa

Zink soluzioa *in vivo* entseguetako saguetatik erauzitako gibelak parafinatan sartu baino lehenago organoak fixatzeko erabili zen. Antigeno batzuk (batez ere immunitate zelulekin erlazionaturi daudenak) formaldehidoarekiko sentikorrak izanik, ordezko metodo bezala hau erabili zen. Zink soluzioa prestatzeko hurrengo kantitateak erabili ziren (4. taula):

**4. Taula. Zink soluzioaren konposizioa litroko.**

| **Substantzia** | Kontzentrazioa |
|---|---|
| **$Ca(CH_3COO)_2$ (Sigma-Aldrich, Estatu Batuak)** | 0,5 g/l |
| **$Zn(O_2CCH_3)_2$ (Merck, Alemania)** | 5 g/l |
| **$ZnCl_2$ (Merck, Alemania)** | 5 g/l |
| **Tris tanpoia 0,1M pH 7,4 (Sigma-Aldrich, Estatu Batuak)** | 1 l bete harte |

# 8. ALKOHOLAK ETA DISOLBATZAILEAK

## 8.1. β-merkaptoetanola

β-merkaptoetanola edo 2-merkaptoetanola, etilenglikola eta 1,2-etanoditiolaren arteko molekula hibridoa da disulfuro zubiak erreduzitzen dituena. Hori dela eta, proteinak desnaturalizatzeko edota RNA isolatzeko protokoloetan erabiltzen da zelulen lisiaren ondorioz askatutako erribonukleasak deuseztatzeko eta beraz RNAren degradazioa ekiditeko.

## 8.2.Etanola

Etanola (Panreac Quimica SLU, Espainia) edo alkohol etilikoa prozedura askotarako erabiltzen da. Lan honetan alde batetik RNAren erauzketa gauzatzeko erabili zen. TRIzol™-a eta kloroformoaren metodoaren ostean fase akuosoan aurkitzen den RNA jasotzean etanola gehitzen zaio, zeinak azido nukleikoen prezipitazioa eragingo duen. Bestalde, histologiarako ere erabili zen laginen deshidratazio eta hidratazioa gauzatzeko.

## 8.3. Metanola

Metanolak (Panreac Quimica SLU, Espainia) edo alkohol metilikoak zelulak iragazkortu egiten dituenez, oso erabilia da oso erabilia da azido nukleikoak fixatzeko. Lan honetan alde batetik, zelulak fixatzeko erabili zen, eta bestetik izoztutako organoengandik egindako mozketak fixatzeko ere bai immunofluoreszentzia teknikekin hasi aurretik.

## 8.4. Isopropanola

Isopropanola (Panreac Quimica SLU, Espainia) edo alkohol isopropilikoa RNAren erauzketa prozesuan ere erabili zen. RNA ez da isopropanoletan soluigarria, hortaz, lagina daukan likidoari isopropanola gehituz gero, RNA soluzio horretatik atera eta prezipitatu egiten da.

## 8.5 Zitrosola

Zitrosola (Panreac Quimica SLU, Espainia) zitrikoen azaletik lortzen den disolbatzaile apolarra da, 1,8(9) P-menthadienoa hain zuzen ere. Xilenoaren ordez erabiltzen da histologian ehunen deshidrataziorako.

# 9. TINDATZAILEAK

## 9.1 Kristal bioleta

Kristal bioleta (Merck, Alemania) tindatzaile alkalinoa da zelulak tindatzeko eta mikrobiologian bakterio Gram positibo eta negatiboak bereizteko erabiltzen dena. Azido-base indikatzaile bezala ere erabiltzen da kolorea berdetik (pH 0,5) urdinera (pH 2) aldatzen duelarik. Kasu honetan, kristal bioleta migrazioa burutu zuten zelulak tindatzeko erabili zen, kuantifikazioa burutu ahal izateko.

## 9.2 Harrisen hematoxilina eta eosina

Harrisen hematoxilina (Merck, Alemania) tindatzaile basikoa da, estruktura azidoak kolore urdinez edo purpuraz tindatzen ditu. Lagin histologikoetan zelulen nukleoak tindatzen dituenez, ehunari kontrastea emateko erabili zen bai tindaketa histologikoetan, bai immunohistokimikako entseguetako laginetan. Eosina (Merck, Alemania), aldiz, tindatzaile azidoa izanik, estruktura basikoak kolore arrosaz tindatzen ditu, hala nola, zitoplasma, kolagenoa edota zuntz muskularrak. Normalean hematoxilinarekin batera erabiltzen da kontraste-tindatzaile bezala, hematoxilina-eosina (H&E) deritzon tindaketa burutzeko. Eosina ehunak tindatu eta estrukturak bereizteko erabili zen tindaketa histologikoetan.

# 10. ANTIGORPUTZAK

## 10.1. Antigorputz primarioak

### 10.1.1. Anti-Muskulu Leuneko Alfa Aktina (α-SMA) antigorputz monoklonala

Anti-α-SMA antigorputz monoklonal honek (Agilent Technologies, Estatu Batuak) muskulu leun baskularretan, miofibroblastoetan eta zelula mioepitelial zein horietatik eratorritako zeluletan espresatzen den α-SMA ezagutzen du. Lan honetan saguen gibeleko tumoreetako HSCak behatzeko erabili zen, izan ere, fibroblasto aktibatu hauek α-SMA espresatzen baitute (5. taula).

### 10.1.2. Anti-Caspasa-3 antigorputz poliklonala

Caspasa-3 apoptosiarekin erlazionaturik dagoen proteína da. Caspasa-3 32 kDa-eko proentzima inaktibo bezala sintetizatzen da, eta zelula apoptosian dagoenean, prozesatu egiten da 17 eta 12 kDa-eko bi subunitate emanez. Anti-Caspasa-3 antigorputza (Abcam, Erresuma Batua) proentzima inaktiboari lotzen zaio. Lan honetan sagu tumore zelulen apoptosi maila aztertzeko erabili zen (5. taula).

### 10.1.3. Anti-ki67 antigorputz monoklonala

Ki-67 proteina nuklearra da, ziklo zelularraren fase aktiboetan ($G_1$, S, $G_2$ eta M) espresatzen dena. Lan honetan anti-Ki-67 antigorputza (Abcam, Erresuma Batua) sagu zelulen proliferazioa detektatzeko erabili zen (5. taula).

### 10.1.4. Anti-F4/80 antigorputz monoklonala

Anti-F4/80 antigorputza (AbD Serotec, Erresuma Batua) makrofago helduen eta mikrogliaren markatzaile gisa erabiltzen da. Lan honetan saguen gibeleko tumoreetan gertatzen den makrofagoen erreklutamendua ikertzeko erabili zen (5. taula).

### 10.1.5. Anti-SV40aren T antigeno handiaren antigorputz monoklonala

Anti-SV40aren T antigeno handia (Abcam, Erresuma Batua) saguen PCAko 266-6 zelulen markatzaile gisa erabili zen, zelula hauek SV40aren T antigeno handiaren transgenea espresatzen baitute (5. taula).

**5. Taula. Sagu ehunetan analisi immunohistokimikoak egiteko erabilitako antigorputz primarioen zerrenda.**

| Antigorputza | Non ekoiztua | Zeren aurka | Klona | Etxe komertziala |
|---|---|---|---|---|
| **Anti- Caspase-3** | Untxia | Sagua | Poliklonala | Abcam |
| **Anti- α-SMA** | Sagua | Sagua | 1A4 | Agilent Technologies |
| **Anti-F4/80** | Arratoia | Sagua | A3-1 | AbD Serotec |
| **Anti Ki-67** | Untxia | Sagua | SP6 | Abcam |
| **Anti-SV 40 T antigen** | Sagua | Sagua | PAb416 | Abcam |

*Antigorputz guztiak 1:100 diluituta erabili ziren*

## 10.2. Antigorputz sekundarioak

### 10.2.1 Biotinadun antigorputz sekundarioak

Immunohistokimikan antigenoen detekziorako erabiltzen den kromogenoetako bat DABa da, zeinak hidrogeno peroxidoarekin batera HRP entzimarekin erreakzionatzen duen kolorea emateko. Hori kontuan izanik, antigorputz primarioen kokapena detektatzeko, antigorputz sekundarioei estreptabidina-HRP izeneko konplexu bat gehitzen zaie, zeinak DABaren oxidazioa eragingo duen hidrogeno peroxidoaren presentzian eta beraz, antigorputza dagoen tokian kolorea emango duen. Hala ere, estreptabidina-HRP konplexua ez da berez antigorputz sekundarioarekin lotzen, eta hortaz, antigorputz hauei biotina molekula atxikitzen zaie, zeinak estreptabidinarekiko afinitate handia daukan.

*10.2.1.1 Arratoiaren IgGen (H+L) aurkako antigorputz sekundario biotinilatua*

Biotinadun arratoiaren aurkako antigorputz sekundarioa (Invitrogen, Estatu Batuak) espezifikoa da arratoien G immunoglobulinen (IgG) kate astun eta arinekiko (ingelesez *Heavy and Light*, H+L). Antigorputz honek sagu ehunari loturiko arratoi antigorputz primarioak ezagutuko ditu. Zehazki, lan honetan anti-F4/80 antigorputz primarioa detektatzeko erabili zen (6. taula).

*10.2.1.2. Saguaren IgGen (H+L) aurkako antigorputz sekundario biotinilatua*

Saguaren aurkako biotinadun antigorputz sekundarioa (Invitrogen, Estatu Batuak) espezifikoa da saguaren IgGen H+L kateekiko, hortaz sagu antigorputz primarioei lotuko zaie. Lan honetan anti-α-SMA antigorputz primarioa detektatzeko erabili zen (6. taula).

*10.2.1.3. Untxiaren IgGen (H+L) aurkako antigorputz sekundario biotinilatua*

Biotinadun untxiaren aurkako (Invitrogen, Estatu Batuak) antigorputz sekundarioa espezifikoa da untxiaren IgGen H+L kateekiko. Antigorputz honek sagu ehunari loturiko untxi antigorputz primarioak ezagutuko ditu. Lan honetan anti-Caspasa-3 eta anti-Ki67 antigorputz primarioak detektatzeko erabili ziren (6. taula).

### 10.2.2 Fluoreszentziadun antigorputz sekundarioak

*10.2.2.1. Saguaren aurkako Alexa Fluor® 488dun antigorputz sekundarioa.*

Saguaren aurkako Alexa Fluor® 488dun (Abcam, Erresuma Batua) antigorputza espezifikoa da saguaren IgGen H+L kateekiko. Gainera, Alexa Fluor® 488 izeneko fluorokromoa darama, zeinak 495 nm-ko uhin luzeerako argirekin kitzikatuz, kolore berdea ematen duen 519 nm-tan. Zehatzago, lan honetan anti-SV40aren T antigeno handiaren antigorputz primarioa detektatzeko erabili zen (6. taula).

**6. Taula. Analisi immunohistokimikoak egiteko erabilitako antigorputz sekundarioen zerrenda.**

| Antigorputza | Non ekoiztua | Diluzioa | Etxe komertziala |
|---|---|---|---|
| **Anti-arratoia (biotinaduna)** | Ahuntza | 1:2000 | Invitrogen |
| **Anti-sagua (biotinaduna)** | Ahuntza | 1:1000 | Invitrogen |
| **Anti-untxia (biotinaduna)** | Ahuntza | 1:8000 | Invitrogen |
| **Anti-sagua (Alexa Fluor® 488duna)** | Ahuntza | 1:2000 | Abcam |

## 10.3. Antigorputz blokeatzaileak

### 10.3.1. Sagu IgGen F(ab)$_2$ fragmentuaren aurkako antigorputza

IgGa organismoak berak sortzen duen antigorputz bat da, ehunetan oso ugaria dena. Immunoglobulina guztiek bezala, antigorputzek bi gune dituzte: antigenoa ezagutzen duen F(ab) (ingelesez *Fragment antigen binding*) guneez gain, muturra eta immunitate sistemako zelulekin lotzen den F(c) (ingelesez *Fragment crystallizable*) muturra hain zuzen. Immunomarkaketa entseguetan sagu-antigorputz sekundarioak sagu-ehuneko laginetan erabiltzen direnean lotura inezpezifikoak gertatzen dira. Antigorputz sekundarioak antigorputz primarioa ezagutuko duen arren, saguaren ehunak dituen IgG endogenoak ere ezagutuko ditu eta beraz, antigorputz primarioarekin ez-ezik ehunarekin ere zuzenean lotuko da. Hori saihesteko, antigorputz primarioa erabiltzen hasi aurretik, saguen antigorputzak ezagutzen dituzten IgG endogenoak blokeatu egin ziren sagu IgGen F(ab)$_2$ fragmentuaren aurkako antigorputzak erabiliz (Invitrogen, Estatu Batuak). Informazio gehiago aurki daiteke 7. taulan.

**7. Taula. Analisi immunohistokimikoak egiteko erabilitako antigorputzen zerrenda.**

| Antigorputza | Non ekoiztua | Zeren aurka | Diluzioa | Etxe komertziala |
|---|---|---|---|---|
| **Anti-sagu IgGen F(ab)$_2$ fragmentua** | **Untxia** | Sagua | 1:500 | Invitrogen |

# 11. HISTOLOGIA ETA IMMUNOHISTOKIMIKARAKO MATERIALAK

## 11.1. Immunohistokimikarako tanpoiak eta blokeatzaileak

### 11.1.1. Peroxidasa endogenoaren blokeatzailea

Immunomarkaketa prozesuan antigorputz sekundarioari Estreptabidina-Peroxidasa sustratoa gehitzen zaio, zeinak geroago DABarekin erreakzionatuko duen. DABa peroxidasaren presentzian oxidatu egiten denez, antigorputz sekundarioa dagoen lekuan kolore marroidun prezipitatua sortuko da. Hala ere, laginak berak peroxidasa endogenoa duenez, immunomarkateta prozesua hasi baino lehen laginari hidrogeno peroxidoa gehitu zitzaion peroxidasa aktibitatea blokeatzeko. Horretarako % 3ko $H_2O_2$ (Panreac Quimica SLU, Espainia) duen PBSa prestatu zen. Ondorioz, ehunean dagoen peroxidasa endogeno horrek, guk gehitutako $H_2O_2$-arekin erreakzionatuko du eta beraz, peroxidasa agortu egingo da.

### 11.1.2. Proteina inespezifikoen blokeatzailea

Antigorputzak erabiltzen diren edozein teknikatan, erabilitako antigorputza gure interesekoa den proteinara bakarrik lotzeko, laginaren gune inespezifikoak blokeatzea beharrezkoa da. Antigorputzek, antigenoarekin lotzen den F(ab)$_2$ guneez gain, Fc guneak ere badituzte, zeinak laginetako immunitate zelulen Fc errezeptoreekin lotzen diren. Hori ekiditeko % 5 FBS-dun PBSa erabiltzen da, FBSak gune inespezifiko horietan atxikituko diren antigorputz ugari baititu.

### 11.1.3 Antigenoen aurkezpenerako Sodio-zitrato Tanpoia 10 mM

Aldehidoen bidezko organoen fixapena dela eta, ehunetan proteinen lotura gurutzatuak eratzen dira eta askotan gune antigenikoak ezkutatu egiten dira.

Ondorioz, immunohistokimikarako erabilitako antigorputzak ez dira antigenoarekin lotzen eta emaitza negativo faltsuak lortzen dira. Hori saihesteko, antigenoen berreskurapenerako soluzioa erabiltzen da. Kasu honetan pH 6dun 10 mMeko sodio-zitrato tanpoia erabili zen (8. taula), zeinak proteinen arteko lotura gurutzatuak desegiten dituen, antigenoen epitopoak agerian utziz.

**8. Taula. Sodio zitrato tanpoiaren konposizioa litroko (10mM).**

| **Substantzia** | Kontzentrazioa |
|---|---|
| **$C_6H_5Na_3O_7$ . $2H_2O$ (Sigma-Aldrich, Estatu Batuak)** | 2,94 g/l |
| **Tween 20 (% 0,05) (Sigma-Aldrich, Estatu Batuak)** | 0,5 µl |
| **$H_2O$** | 1 litro bete arte |

*Soluzio honen pHa 6ra doitu zen*

## 11.2. Antigorputzen detekziorako sistemak eta blokeatzaileak

### 11.2.1. Estreptabidina eta basaerrefau-peroxidasa konplexua (Estreptabidina - HRP)

Estreptabidina eta basaerrefau-peroxidasa konplexua (ingelesez *Horseradish Peroxidase*, HRP) (Estreptabidina-HRP) (Thermo Scientific, Estatu Batuak) basaerrefautik erauzten den entzima bat da, immunomarkaketetan antigorputzak detektatzeko erabiltzen dena. Estreptabidinak biotinarekiko afinitatea duenez, konplexu hau biotinilaturik dagoen antigorputz sekundarioekin lotuko da. Bestalde, konplexu honen peroxidasak substratu kromogenikoekin erreakzionatzen duenez, laginari DAB bezalako substratuak gehitzen zaizkio zeinak hidrogeno peroxidoaren presentzian prezipitatu marroia sortuko duen eta horrek antigorputza kolore bidez detektatu ahal izateko aukera ematen du.

### 11.2.2. 3,3’-diaminobenzidine substratua (DAB)

3,3'-diaminobenzidine substratua (DAB) (Thermo Scientific, Estatu Batuak) bentzenoaren eratorri bat da immunohistokimikan kromogeno bezala erabiltzen dena. Hidrogeno peroxidoaren presentzian, peroxidasak DABa oxidatu egiten du prezipitatu marroia eratzen delarik.

Immunohistokimikan antigorputz sekundarioak estreptabidina-HRP konplexuarekin lotu ostean, laginari DAB gehitzen zaio, zeinak HRParekin

erreakzionatuko duen kolore marroia emanez. Kolore hori mikroskopio bidez behatu daiteke.

## 11.3. Muntai medioak

### 11.3.1 DPX muntai medioa

DPX muntai medioa (Merck, Alemania) distireno, plastifikatzailez eta xilenoz osaturiko erretxina tankerako soluzioa da tindaturik dagoen edota immunomarkaketa duen lagina mantentzeko erabiltzen dena. Porta eta estalkiaren artean jartzen de lagina kanpo-mediotik isolatzeko eta kontserbatzeko.

### 11.3.2. 4',6-diamino-2-fenilindoldun (DAPI) Fluoromount™ muntai medioa

4',6-diamino-2-fenilindoldun (DAPI) Fluoromount™-a (Thermo Fisher, Estatu Batuak) fluoreszentziarekin markaturik dauden laginak muntatzeko erabiltzen da. DAPIa koloratzaile fluoreszentea da DNAren adenina eta timina base nitrogenatuetan aberatsa den zonaldeetan itsasten dena. Nahiz eta kontzentrazio altutan zelula biziak tindatu ahal dituen, normalean fixatutako zelulen edo ehunetako zelulen nukleoak markatzeko erabiltzen da, hortaz kontraste tindatzaile bezala erabili ohi da fluoreszenteki markaturik dauden ehunetan. Kate bikoitzeko DNAri lotzen zaionean absorbantzia maximoa 358 nm-tan dauka (ultramorea) eta 461nm-tan igortzen du kolore urdinarekin. RNArekin lotzen denean, ordea, 500 nm-tan emititzen du argia. Lan honetan, muntai medio hau pankreako lagin batzuk muntatzeko erabili zen non DAPIak kontraste tindatzaile bezala jardun zuen ehunean CFSEarekin markaturik zeuden zelulak desberdintzeko.

## 11.4. Histologiarako erabilitako bestelako materialak

### 11.4.1 Parafina

Parafina (Thermo Scientific, Estatu Batuak) hidrokarburo saturatuz osarturiko argizari itxurako substantzia da, giro tenperaturan solidoa dena. Histologian, ehunak parafinatan inkluditzen dira denbora luzez mantendu ahal izateko. Horretarako ehunak parafina berotan sartzen dira parafinak zirrikitu guztiak

bete ditzan eta behin parafina hoztean solido bihurtzen denez, ehun fixatuak bloke batean geratzen dira ondo kontserbatuta.

#### 11.4.2 Poli-L-lisina

Poli-L-Lisina edo Polilisina (Sigma, Estatu Batuak) bakterioen hartzidura prosezuan sortzen den homopolimeroa da. Zelulen adhesioa hobetzen laguntzen du kultibo-sustratoaren azalerako kargak aldatuz. Lan honetan immunomarkaketarako erabilitako portak gaineztatzeko erabili zen, ehunak hobeto itsas zitezen.

#### 11.4.3. Tissue-Tek® OCT™ konposatua

Tissue-Tek® OCT™ konposatua (Sakura Finetek Europe B.V, Herbehereak) erretxina bat da laginak izozturik eta mozketarako prest egonik gordetzea ahalbidetzen duena. Alkohol polibinilikoz, polietilenglikolez eta bestelako osagai ez-erreaktiboz osaturik dago. Agente kriobabesgarri moduan dihardu izotz kristalen eraketa saihestuz ehunetan. Kasu honetan konposatu hau pankreak izozturik mantentzeko erabili zen, organoan bertan fluoreszentzia bidez CFSEarekin markaturik zeuden zelulak mikroskopioan zuzenean behatu ahal izateko.

# 12. ZELULEN ENTSEGUETARAKO MATERIALAK

## 12.1. Zelulen entseguetarako erreaktiboak

#### 12.1.1. Karboxifluoreszein sukzinimidil esterra (CFSE)

Karboxifluoreszein sukzinimidil esterra (CFSE) zunda fluoreszentea da, hainbat entsegutan erabili daitekeena, hala nola zelulen bideragarritasuna aztertzeko, bizirik dauden zelulak fluoreszenteki markatzeko, zelulen zikloan zatiketa maila aztertzeko eta abar. CFSEa (Invitrogen, Estatu Batuak) zelulen gainazaleko zein barnean dagoen DNAren amina libreekin kobalenteki lotzen da zelulari kalterik eragin gabe. Erreaktiboak daraman fluoreszeinari esker, CFSEaren presentzia behatu dezakegu zelula barnean fluoreszentzia bidez. Fluoroforo honek 520 nm-tan emititzen du argi maximoa. Aipatutako lotura kobalenteari esker, CFSEa beti DNAn itsatsita geratuko da, beraz, markatutako zelula bat zatitu egiten denean, zelula alaba bakoitzak CFSE horren karga erdia eramango du. Modu berean, zelula alaba horiek zatitzean, berriro ere zelula amak zeraman CFSEaren erdia izango dute. Fluoreszentzia bidez 8

belaunaldirarte bereiz daitezke, hortik aurrera ezin baitira zelulen autofluoreszentzia eta fluoreszeinarena desberdindu. Hori dela eta, lan honetan CFSEa Ocoxinekin trataturiko zelulen zatiketa maila aztertzeko erabili zen eta baita PCAko 266-6 zelulak fluoreszenteki markatzeko ere, gero hauek saguei inokulatu ostean histologikoki detektatu ahal izateko.

### 12.1.2. PrestoBlue™

PrestoBlue™ (Molecular probes, Estatu Batuak) agentea zelulen biabilitatea ezagutzeko erabiltzen da. Konposatu honek resazurina darama, kolore urdineko koloratzaile bat, zeina zelulen barnean sartzen den inolako kalterik sortu gabe. Behin zelulen barnean egonik, zelulen ahalmen erreduktorearen eta erreduzkioa eragiten duten bestelako erreazkio kimikoen ondorioz, substantziaren pHa aldatu egiten da PrestoBlue™-k kolore arrosa hartzen duelarik. Kolore aldaketa hori absorbantzia bidez neurtu daiteke 570 nm and 620 nm-tan.

## 12.2. Zelulen entseguetarako kitak

### 12.2.1. DNA FxCycle™ PI/RNasa soluzio kita

DNA FxCycle™ PI/RNasa soluzio kita (Invitrogen, Estatu Batuak) zelulen zikloa fluxu zitometria bidez aztertzeko prestaturik dago eta alde batetik propidio ioduroa (PI) dauka eta bestetik A RNasa. PIa zelula barnera sartu eta DNAren base pareen artean interkalatzen den agente fluoreszentea da, argia 617 nm-tan igortzen duelarik. Horretarako, kitak daraman DNasa gabeko A RNasak zelulak iragazkortu egiten ditu gero PIa barnera sar dadin. Metodo honen bidez ziklo zelularraren faseak aztertu daitezke; zelulak barnean daukan DNA kantitatearen arabera PI gehiago edo gutxiago atxikituko da eta hortaz, fase desberdinak bereiztu ahal izango dira.

### 12.2.2. Annexina V-FITC apoptosiaren detekziorako kita.

Annexin V-FITC kita (Invitrogen, Estatu Batuak) zelulen apoptosia aztertzeko erabiltzen da. Anexina V proteina bat da, zelulen mintzeko fosfatidilserinekin lotzen dena. Aldi berean, proteina hau fluoreszeina-5-Isotiozianato (FITC) fluorokromoarekin markaturik daude, zeinak argi berde-horia igortzen duen, emisio maximoa 518 nm-tan daukalarik. Zelula osasuntsuetan fosfatidilserina mintzeko alde zitosolikoan aurkitzen den arren, apoptosi prozesua hasten denean, zelulen mintza bere egitura galtzen hasten da eta fosfatidilserinak

alde estrazelularrera translokatzen dira. Hortaz, molekula hauek zelulen kanpoko aldean egonik, annexina bertara lotu egingo da eta fluoreszeinari esker, fluoreszentzia bidez, zelulak apoptosi prozesuan dauden ala ez jakin ahal izango dugu. Bestalde, kit honek PI ere badarama, aurreko kitean azaldu dugun moduan, DNAren base pareen artean interkalatzen dena. Apoptosiaren azken faseetan, hots, nekrosian, zelulen mintza guztiz iragazkortzen denez, PIak zelula nekrotikoen DNA markatu egiten du, apoptosia eta nekrosia desberdintzea ahalbidetuz.

# 13. POLIMERASAREN KATE-ERREAKZIO KUANTITATIBO ETA MIKROARRAY ENTSEGUETARAKO MATERIALAK

## 13.1. RNAren erauzketarako materialak

### 13.1.1. RNAren erauzketarako erreaktiboak

#### *13.1.1.1. TRIzol™*

TRIzol™-a (Invitrogen, Estatu Batuak) zelulak lisatzeko erabili zen. Bere printzipio aktiboen artean guanidina tiozianatoa eta fenola aurki daitezke besteak beste. Guanidina tiozianatoak proteinak desnaturalizatzen ditu, RNasak eta proteína erribosomalak barne. Bestalde, fenolak azido nukleikoak disolbatzen laguntzen du. TRIzol™-arekin tratatu ostean, laginari kloroformoa gehitzen zaio RNA erauzi ahal izateko.

#### *13.1.1.2. Kloroformoa*

Kloroformoa (Sigma-Aldrich, Estatu batuak) TRIzol™-aren bidezko zelulen lisiaren ondorioz jasotako DNAren homogeneizatua edota proteinak RNArengandik bereizteko erabili zen. TRIzol™-arekin tratatu ostean, laginari kloroformoa gehitzen zaio, zeinak homogeneizatua hiru fasetan banatzen duen. Fase akuosoan azido nukleikoak geratuko dira, eta fase organikoan proteinak zein lipidoak egongo dira.

### 13.1.2. RNAren erauzketarako entzimak

#### *13.1.2.1. Erribonukleasa inhibitzailea*

Erribonukleasa edo RNasa (Invitrogen, Estatu Batuak), RNAren hidrolisia katalizatzen duen entzima da. RNArekin lan egitean RNasa inhibitzailea

erabiltzen da saiodietan, pipeta puntetan edota beste agente batzuetan egon daitekeen RNAsak inaktibatzeko eta beraz, RNAren egitura kontsebatzeko bere degradazioa saihestuz.

### 13.1.3. RNAren erauzketarako kitak

#### *13.1.3.1. Purelink™ RNA mini kita:*

Purelink™ RNA mini kita (Invitrogen, Estatu Batuak) kalitate altuko RNA isolatzeko erabiltzen da. Gibeleko tumore esplanteetatik RNA TRIzol™ eta kloroformoaren bidez erauzi ostean, kit hau lortutako RNA purifikatzeko erabili zen. Kit honek β-merkaptoetanoldun soluzioa erabiltzen du laginak lisatzeko eta proteinak desnaturalizatzeko zeinak aldi berean guanidinio isotiozianato agente kaotropikoa daukan, RNA RNAsa endogenoengandik babesten duena. Ondoren, kitak zutabe batzuk dakartza lisatutakoa bertatik pasatu eta homogenizatzeko. Ostean, DNA genomikoa kentzeko DNAsa bidezko tratamendua erabili beharrean, kitak silizez osatutako mintza daukaten zutabeak dauzka, non zentrifugazio bidez lagina bertatik igaro eta RNA itsatsita geratzen den. Bukatzeko kitak RNasa gabeko ura ere badakar mintzari atxikiturik dagoen RNA eluitzeko.

#### *13.1.3.2. Norgen FFPE RNA purification kita*

FFPE RNA Purification Kita (Norgen, Canada) parafinatan sartutako laginetatik RNA erauzteko erabili zen. Fixapen prozesuak eta laginak parafinatan sartzeak ehunetan RNA eta proteinen arteko elkargurutzamenduak sortzea eragiten du. Ondorioz, RNA fragmentatu egiten da, batez ere denborak aurrera egin ahala. Kit honi esker, fixapen prozesuak eragindako aldaketak partzialki lehengora bihurtu ditzake kalitate oneko RNA erauztea ahalbidetuz. Kit hau zutabeen bidezko kromatografian oinarriturik dago; zutabeetan aurkitzen den erretsina bereziari esker RNA gainerako osagaietatik banatzen da kontzentrazio ionikoen arabera eta zutabean atxikituta geratzen da. Zutabeez gain, kit honek laginak desparafinatzeko xileno eta etanola dakartza, eta baita laginaren digestiorako K proteinasa. Entzima honek aminoazido aromatikoen arteko talde karboxilikoen lotura peptidikoak apurtzen ditu, azido nukleikoak proteinengandik aske uztziz. Gainera, kitak proteinen lisirako eta garbiketak gauzatzeko soluzio eta tanpoiak ere badauzka. Bukatzeko, DNA genomikoaren kutsapena saihesteko RNasarik gabeko DNasa eta RNA zutabetik askatzeko eluzio soluzioa ere badakar kit honek.

## 13.2. Alderantzizko Transkripzio eta Polimerasaren Kate-erreakzio kuantitatiborako materialak

### 13.2.1. Alderantzizko Transkripzio eta Polimerasaren Kate-erreakzio kuantitatiborako erreaktiboak

*13.2.3.1. SYBR® Green Master Mix kita*

SYBR® Green Master Mixa (BioRad, Estatu Batuak) RNAtik abiatuta DNAren anplifikazioa monitorizatzeko erabiltzen da alderantzizko transkripziodun (ingelesez *retrotranscription*, RT) polimerasaren kate-erreakzio kuantitatiboko (ingelesez Quantitative P*olymerase Chain Reaction*, qPCR) entseguetan edo RT-qPCRetan. SYBR® Green koloratzaile fluoreszentea da, kate bikoitzeko DNAri lotzen zaiona. PCR prozesuko ziklo bakoitzean DNA desnaturatu, primerrekin hibridatu eta luzatu egiten da. Behin luzapena amaituta kate bikoitzeko DNA lortzen denez, SYBR® Green konposatuak anplifikatu den DNA hori markatu egingo du. Fluoreszentziaren intentsitatea DNA kantitatearen araberakoa izango da.

### 13.2.2. Alderantzizko Transkripzio eta Polimerasaren Kate-erreakzio kuantitatiborako kitak

*13.2.1.1. iScript™ cDNA Synthesis kita*

iScript™ cDNA synthesis kita (BioRad, Estatu Batuak) RNAtik abiatuz kate bakarreko DNA osagarria (ingelesez *complementary DNA*, cDNA) sintetizatzeko erabili zen qPCR analisietarako. Hasteko, kit honek alderantzizko transkriptasa dauka, zeinak mRNA oinarritzat hartuz cDNA sortzeko gaitasuna duen desoxinukleotidoak (dNTPak) lotuz. Horrez gain, RNasa inhibitzailea ere ba dauka RNA mantentzeko eta gainera oligo(dT) deritzen deoxitiminaz osaturiko oligonukleotidoak (primerrak) ere badaramatza RTa egingo duen entzimak zuzenean mRNAtik abiatuz cDNA sintetizatzeko.

*13.2.1.2. Platinum™ Multiplex PCR Master Mix 2x*

Platinum™ Multiplex PCR Master Mix 2x kita (Applied Biosystems, Estatu Batuak) aldi berean DNA sekuentzia desberdinak anplifikatzeko erabiltzen da. Zehazki, 50 - 2500 base paretako DNA zatiak anplifikatzeko optimizaturik dago. Horretarako, kit honek Taq DNA polimerasa dakar gure lagina primer

desberdinekin batera termozikladorean anplifikatzeko. Bestalde, DNAren anplifikazio prozesuan guanina eta zitosina kantitate altua duten DNA fragmentuak ez dira beti ondo erreplikatzen. Adenina eta timinarekin erkatuz, guanina eta zitosinen arteko loturak engonkorragoak dira, eta hortaz, harizpiak banatzeko tenperatura altuagoak behar dituzte. Arazo horri aurre egiteko kitak guanina eta zitosina ugari duten DNA moldeen anplifikazioa errazteko soluzio bat dakar.

### 13.2.3 Primerrak

Primerrak 20 nukleotido inguruz osaturiko molekulak dira, DNAren gune ezagunen erreplikaziorako abiapuntu bezala jokatzen dutenak. Horretarako, primerra molde bezala erabiltzen duen katean kokatzen da eta DNA polimerasari esker desoxinukleotido askeak beraien artean lotuz DNA moldearen kate osagarria sortzen da. Erreplikazioa DNAren kate bietan gertatzeko "forward" eta "reverse" primerrak (Thermo Scientific, Estatu batuak) erabiltzen dira gene bakoitzerako (9. taula):

**9. Taula. qPCRa burutzeko erabilitako primerren sekuentziak.**

| Izena | Sekuentzia (5'-3') |
|---|---|
| **COX2** | (forward) TGCACTATGGTTACAAAAGCTGG<br>(reverse) TCGGAAGCTCCTTATTTCCCTT |
| **GAPDH** | (forward) GTATGACTCCACTCACGGCAA<br>(reverse) CTTCCCATTCTCGGCCTTG |
| **IL1β** | (forward) CTGTGACTCATGGGATGATGATG<br>(reverse) GCCTGTAGTGCAGTTGTCTAAT |
| **IL6** | (forward) TCTATACCACTTCACAAGTCGGA<br>(reverse) GAATTGCCATTGCACAACTCTTT |
| **INFγ** | (forward) TTCTTCAGCAACAGCAAGGC<br>(reverse) TGTGGGTTGTTGACCCTCAAA |
| **S15** | (forward) TTCCGCAAGTTCACCTACC<br>(reverse) CGGGCCGGCCATGCTTTACG |
| **SV40 T** | (forward) AAGCTCCAACCCCTTTACCG<br>(reverse) ACATCAATGCTCACACGACG |
| **TNF-α** | (forward) CCAGTGTGGGAAGTGTCTT<br>(reverse) AAGCAAAAGAGGAGGCAACA |

## 13.3. Mikroarraia gauzatzeko materialak

### 13.3.1. Mikroarraia gauzatzeko entzimak

*13.3.1.1. AffinityScript™ erretrotranskriptasa*

Mikroarraiarekin hasi aurretik *AffinityScript™* alderantzizko transkriptasa (Agilent Technologies, Estatu Batuak) erabili zen RNAren RTa gauzatu eta cDNA lortzeko.

### 13.3.2. Mikroarraia gauzatzeko kitak

*13.3.2.1. Low Input Quick Amp Labeling Kit, One Color kita*

Low Input Quick Amp Labeling Kit, One Color kita (Agilent Technologies, Estatu Batuak) cDNAtik abiatuz cRNA sintetizatu eta anplifikatzeko erabiltzen da. Kit honek, alde batetik T7 RNA polimerasa darama cDNA molde gisa erabiliz RNA sortzeko. RNAren sintesi pozesuan nukleotido trifosfato arruntak (ATP, GTP eta TTP) zein trifosfato nukleotidoak zianina-3 fluoroforodun zitidinarekin (Cy3-CTP) konjugaturik erabiliko dira cRNA markatua lortzeko.

*13.3.2.2. RNeasy Mini kita*

Rneasy Mini kita (Qiagen, Alemania) zianinarekin markatutako RNA osagarria (ingelesez *complementary RNA*, cRNA) purifikatzeko erabili zen lagina zutabeetatik pasatuz. Purelink™ RNA mini kitaren kasuan bezala (ikusi Materialetako 13.1.3.1. atala) kit honek guanidina isotiozianatoaren bidezko zelulen lisia erabiltzen du 2-merkaptoetanolaren beharrik gabe eta silize mintzak dituzten zutabeak erabiltzen ditu RNAren purifikaziorako.

# 14. ELISA ENTSEGUETARAKO KITAK

Kit hauetako bakoitzaren plakak detektatu nahi dugun molekularen aurkako atzemate-antigorputza dakar aldez aurretik atxikituta. Bestalde, lotutako substratuaren detekziorako biotinadun detekzio-antigorputza ere badakar. Are gehiago, kitak detekzio-antigorputzaren biotinarekin lotuko den estreptabidina-HRP konplexua ere badauka. Horrez gain, kit hauek 3,3',5,5'-tetrametilbentzidina (TMB) kromogenoa daramate, estreptabidina-HRP konplexuarekin erreakzionatu ostean kolorea ematen duena. Bukatzeko, azken bi hauen arteko erreakzioa gelditzeko kit hauek azido sulfuriko soluzioa daukate. Lan honetan erabilitako kiten zerrenda 10. taulan ikus daiteke.

**10. Taula. ELISA kiten zerrenda**

| Izena |
| --- |
| **Mouse IL-1β CytoSet™ (Invitrogen, Estatu Batuak)** |
| **Mouse IL-10 CytoSet™ (Invitrogen, Estatu Batuak)** |
| **Mouse IFγ CytoSet™ (Invitrogen, Estatu Batuak)** |
| **Mouse IL-12 ELISA (Invitrogen, Estatu Batuak)** |

# III.II. METODOAK / METHODS

## 1. *IN VITRO* ENTSEGUAK

### 1.1. Zelula-lerroen kultiboa.

CRCko metastasi hepatikoa eta bere mikroingurumena aztertzeko sagu kolon kartzinomako C26 zelulak erabili ziren, 3T3 fibroblasto eta J774A.1 makrofagoekin batera. Bestalde, PCAri dagokionez, sagu adenokartzinoma pankreatikoko 266-6 zelulak eta giza PCAko BxPC-3, Capan-2, CFPAC-1, HPAF-II, MRC-5, Panc 10.05, SW1990 zelulak eta MRC-5 fibroblastoak erabili ziren.

Zelula guztiak % 10 FBS, penizilina (100 U/ml), anfoterizina B (0,25µg /ml) eta estreptomizinaz (100 µg/ml) osatutako RPMI-1640 hazkuntza-mediotan hazi ziren T75 flaskoetan eta 37 ºC eta % 5 $CO_2$ baldintzapetan inkubatu ziren. Hemendik aurrera kultibo medio honi hazkuntza edo kultibo medio osatua deituko zaio. Kasu guztietan flaskoa % 80 bete zenean, medio zaharra kendu eta PBSarekin garbitu zen. Ondoren, bai C26, bai 3T3 zelulak % 4ko EDTArekin altxatu ziren, 266-6ak eta giza zelulak (BxPC-3, Capan-2, CFPAc-1, HPAF-II, MRC-5, Panc 10.05, SW1990) tripsina-EDTArekin eta J774A.1ak, aldiz, arraspa baten bidez. Altxatutako zelulak zentrifugazio hodi batera pasatu ondoren, flaskoak berriro PBSarekin gabitu ziren bertan geratutako zelulak berreskuratzeko. Ostean, zentrifugazio hodiko zelulei medio osoa gehitu zitzaien FBSak tripsina-EDTAren eragina inaktiba zedin eta hodiak errotore baskulatzailedun zentrifugan sartu ziren 10 minutuz, 4 ºC-tan eta 125 G-ko azelerazioarekin zelula osasuntsuen kasuan eta 430 G-rekin minbizi zelulen kasuan. Bukatzeko zentrifugazio hodietako gainjalkina baztertu egin zen eta pelleta RPMI-1640arekin birsuspenditu zen behar zen zelula kontzentrazioa lortzeko. Zelula lerroa mantentzeko, zentrifugazioaren ondoren lortutako zelulak 1:10 diluitu eta flaskoetan hazi ziren berriro ere hazkuntza medio osatuarekin. Minbizi zelulak 10. pasera arte soilik mantentzen ziren beraien etengabeko hazkuntzaren ondoriozko kontaminazio edota mutazioak ekiditeko. Denbora hori igarotakoan, zelula horiek utzi eta berriak desizozten ziren. Aldiz, fibroblastoak eta makrofagoak 30 eta 20 pasera arte mantendu ziren gehienez hurrenez hurren.

Salbuespen bezala, Panc10.05 zelulei giza intsulina birkonbinantea (10 u/ml) gehitu zitzaien hazkuntza-medioan. Bestalde, giza PCAko zelulen hazkuntza

geldoa denez, 3-4 egunik behin medio zaharra kendu, PBSarekin garbitu eta medio berria gehitu zitzaien flaskoa % 80 bete zen arte.

Horrez gain, kasu batzuetan, flasko edo plakak aldez aurretik I motako kolagenoarekin edo gelatinarekin gaineztatu ziren fabrikatzailearen gomendioak jarraituz zelulak modu optimoan hazteko. C26en kasuan, esperimentu batzuetarako zelulak plaketan kultibatu baino lehen, hauek kolagenoz gaineztatu ziren zelulak hobeto itsas zitezen. Horretarako, plaken oinarria % 0,01 kolagenorekin gaineztatu ziren kultibo mediotan diluituta eta 30 minutuz inkubagailuan utzi ziren. Ordu erdi pasatutakoan, kolageno soberakina kendu egin zen flaskoetatik eta prozedurarekin jarraitu zen. Aldiz, 266-6 zelulen kasuan flaskoak gelatinaz gaineztatu ziren % 0,1 kontzentrazioarekin 4 ºC-tan 30 minutuz utziz.

## 1.2. Zelula-lerroen izozte eta desizoztea.

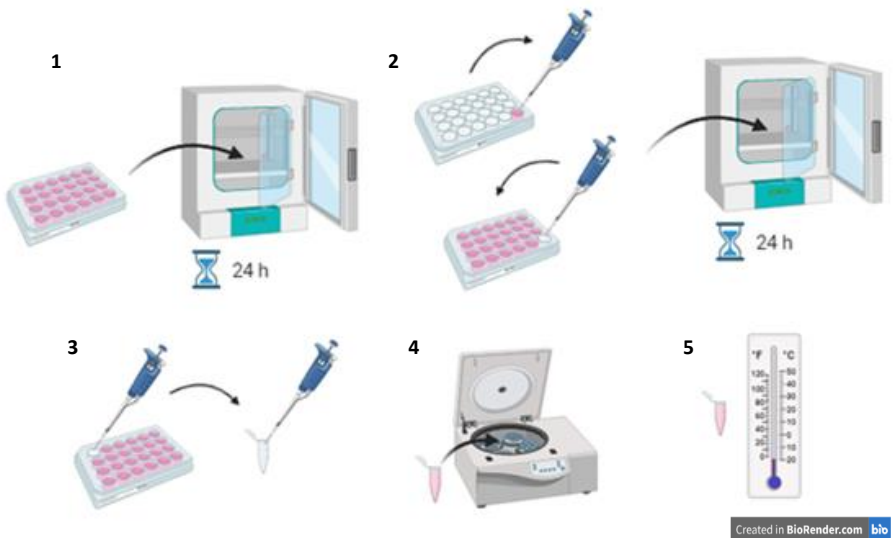
Lanean hasi baino lehen, zelula-lerro guztiak anplifikatu eta izoztu egin ziren edozein momentutan zelula berriak erabitzeko aukera izateko. Prozesu hau, zelulak erosi eta hasierako paseetan gauzatzen da, tumore zelulak izanik, mutazioen metaketa sahiesteko eta transformaziorik gabeko zelulen stocka izatea bermatzeko . Zelula erosi berriak lehenengo eta behin T25 flasko batean hazi ziren, eta gainazalaren % 80 betetakoan T75 flasko batean kultibatu ziren gehiago hazteko. Ostean, gainerako zelulekin moduan, behin T75 flaskoa % 80 beterik zegoenean zelulak altxatu, flaskoa PBSarekin garbitu eta zentrifugagailuaren bidez sendimentatu egin ziren. Gainjalkina baztertu egin zen eta hodiaren hondoko zelulak % 90 FBS eta % 10 DMSOz osaturiko mediotan eseki ziren izozte-hodietan sartzeko. Hodi horiek isopropanolez beteriko *Mr. Frostie* izeneko ontzian sartu ziren, zeinak -80 °C-tan dagoen ultraizozgailu batean sarturik, zelulen tenperatura gradualki jaisten duen kalte zelularra minimizatuz. Zehazki, hodien tenperatura minuturo 1 ºC jaisten da isopropanolari esker. Hurrengo egunean, izoztutako zelulak nitrogeno likidodun tankean sartu ziren bertan denbora luzez mantentzeko.

Bestalde, aurrerago aipatu dugun moduan, 10. pasera heldutakoan zelula berriak desizozten ziren transformazioa ekiditeko. Horretarako, nitrogeno likidotan mantendutako zelulak 37 °C-tan zegoen bainuan sartu ziren izozte-hodia etengabe mugituz eta ahal bezain laster, zelulak RPMI-1640tan diluitu ziren izozte-medioak daukan DMSOak zelulak kaltetu ez zitzan. Zentrifugatu ostean, pelleta hazkuntza medio osatuarekin eseki eta T25 flasko batean kultibatu ziren hazkuntza medio osatuarekin. Behin zelulak itsatsita

zeudenean, medio zaharra kendu eta medio osatu berria gehitu zitzaien desizozketan hildako zelulak kentzeko eta bizirik zeudenak baldintza optimoetan haz zitezen. Flaskoa % 80 beterik zegoenean T75 batean berriro kultibatu ziren aurreko atalean azaldu den bezala esperimentuekin aurrera jarraitzeko.

## 1.3. Fibroblastoek jariaturiko faktore soluigarrietan aberatsa den medioa lortzea.

Fibroblastoengandik faktore soluigarrietan aberatsa den medioa (ingelesez *Conditioned Medium*, CM) lortzeko, MRC-5 giza eta 3T3 sagu fibroblastoak lehenago deskribatu den bezala kultibatu ziren 24 orduz $2x10^5$ zelula/ml-ko kontzentrazioarekin. Ondoren, kultibo medio zaharra kendu, berriro ere medio berria gehitu eta 24 orduz inkubatu ziren. Azkenik, fibroblastoek jariatutako faktoredun kultibo-medioa jaso zen, zentrifugatu egin zen 10 minutuz 4 ºC-tan eta 125 G-ko abiadurarekin partikularik egotekotan hau baztertzeko eta -20°C-tan mantendu zen erabili arte (10. Irudia).




**10 Irudia. Fibroblastoek jariaturiko faktore soluigarrietan aberatsa den medioa lortzeko prozeduraren eskema.** Fibroblastoak plakan kultibatu eta 24 orduz inkubatu ziren (1). Ostean, kultibo medio zaharra berriztatu egin zen eta berriro beste 24 orduz inkubatu ziren (2). Denbora hori igarotakoan, fibroblastoek jariatutako faktoreak dituen kultibo medioak batu (3) eta zentrifugatu egin ziren (4). Bukatzeko -20 ºC-tan gorde ziren (5). *Irudia BioRender bidez sortutakoa da.*

## 1.4. Tratamenduak.

Ocoxinen efektuak *in vitro* aztertzeko, hasieran konposatuaren eta baita kimioterapikoen diluzio desberdinak erabili ziren (11. taula).

**11. Taula. Pankreako minbizi zelulak tratatzeko erabili ziren substantzien kontzentrazioak.**

| Substantzia | Kontzentrazioak |
|---|---|
| **Ocoxin** | 1:1000, 1:500, 1:200, 1:100, 1:50 ($V/V_f$) |
| **Paklitaxel** | Sagu zeluletan: 1, 2, 5, 10, 25, 50 (μM)<br>Giza zeluletan: 1, 5, 15, 30 (μM) |
| **Genzitabina** | Sagu zeluletan: 25, 50, 100, 200, 1000 (nM)<br>Giza zeluletan: 1, 2, 5 (μM) |
| **Aukeratutako dosiak** | **Ocoxin** 1:50 ($V/V_f$)<br>**Paklitaxe**l 1 μM<br>**Genzitabina** 1 μM |

Dosi horiek guztiak frogatu ostean, gainerako entseguetarako kimioterapiko zein Ocoxin diluzio bakarra aukeratu zen, independenteki edota konbinaturik erabiltzeko (12. taula).

**12. Taula. Kolon-ondesteko minbizi zelulak tratatzeko erabili diren substantzien kontzentrazioak.**

| Substantzia | Kontzentrazioak |
|---|---|
| **Ocoxin** | 1:500, 1:200; 1:150, 1:100, 1:75, 1:50, 1:10 ($V/V_f$) |
| **Irinotekan** | 5, 10, 25, 50, 75, 100 (μM) |
| **Aukeratutako dosiak** | **Ocoxin** 1:50 ($V/V_f$) eta 1:100 ($V/V_f$) entseguaren arabera<br>**Irinotekan** 50 μM |

Modu berean, esperimentu bakoitzerako tratamendu denbora desberdinak erabili ziren (13. Taula).

**13. Taula. *In vitro* entseguetarako erabilitako tratamendu denborak.**

| Entsegua | PCA | CRC | Zelula osasuntsuak |
|---|---|---|---|
| **Bideragarritasuna** | 48 ordu | 24 eta 48 ordu* | 24 ordu |
| **Apoptosia** | 48 ordu | - | - |
| **Migrazioa** | - | 18 ordu | 18 ordu |
| **Zatiketa zelularra** | 48 ordu | 48 ordu | - |
| **Ziklo zelularra** | 72 ordu | 72 ordu | - |

**CRC-ko zelulak Ocoxinekin bakarrik tratatu zirenean inkubazioa 24 ordukoa izan zen, aldiz, Ocoxin kimioterapiarekin konbinatuta erabili zenean 48 ordukoa.*

## 1.5. Zelulen bideragarritasun entseguak.

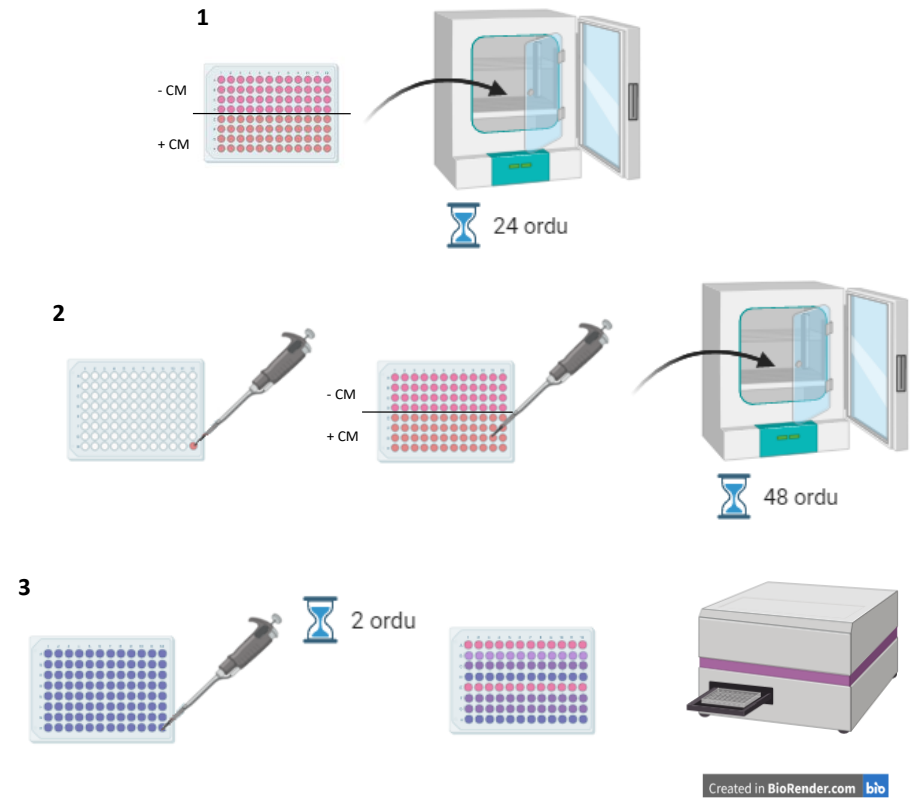
Hasteko, bideragarritasun entseguak egin ziren Ocoxinek zelula osasuntsuetan, zein minbizi zeluletan duen efektua aztertzeko. PCAko sagu zein giza-zelulak eta J774A.1ak mililitro bakoitzeko $5x10^4$ko konzentrazioarekin kultibatu ziren % 5 FBS, antibiotiko eta antimikotikoekin osatutako RPMI-1640 mediotan. Aldiz, 3T3 eta CRCko C26 zelulen kasuan serumaren kontzentrazioa % 0,5ekoa izan zen.

Beraz, Ocoxinek zelula osasuntsuetan zeukan eragina aztertzeko, 3T3 eta J774A.1 zelulen bideragarritasuna kuantifikatu zen Ocoxinen diluzio desberdinen presentzian (ikusi Metodoetako 1.4 atala). 24 ordu igaro ostean, zelulei *PrestoBlue™* gehitu zitzaien eta 2 ordu pasatu eta gero bideragarritasuna kuantifikatu zen.

Minbizi zelulen kasuan bestalde, dosi optimoa aukeratzeko asmoarekin, zelulak 24 orduz inkubatu ondoren, Ocoxin eta kimioterapiko kontzentrazio desberdinak (ikusi Metodoetako 1.4. atala) gehitu zitzaizkien bakoitza bere aldetik. Tratamendua bukatutakoan (24 edo 48 ordu ondoren) aurreko kasuan bezala, zelulei *PrestoBlue*™ gehitu zitzaien eta bideragarritasuna kuantifikatu zen. Dosi optimoak hautatu ostean (11. eta 12. taulak), entsegua errepikatu egin zen PCAko eta CRC-ko zeluletan Ocoxin eta kimioterapikoen (paklitaxel, genzitabina eta irinotekan) konbinazioa erabilita.

### 1.5.1 Zelulen bideragarritasuna fibroblastoengandik lortutako faktore soluigarrien presentzian hazitako minbizi zeluletan.

Fibroblastoek PCAko giza zelulengan duten eragina aztertzeko, zelula guztiak 96 putzuko plaketan kultibatu ziren baldintza normaletan edo fibroblastoengandik lortutako CMaren presentzian 1:2 proportzioan diluituta 24 orduz. Ostean, aurretik azaldutako prozedura berdinarekin jarraitu zen, hau da, zelulak konposatu eta konbinazio berdinekin tratatu ziren (berriro ere plaka erdia 1:2 CMrekin) eta 48 ordu igaro ostean *PrestoBlue*™ saiakuntzaren bidez absorbantzia neurtu zen (11. Irudia).

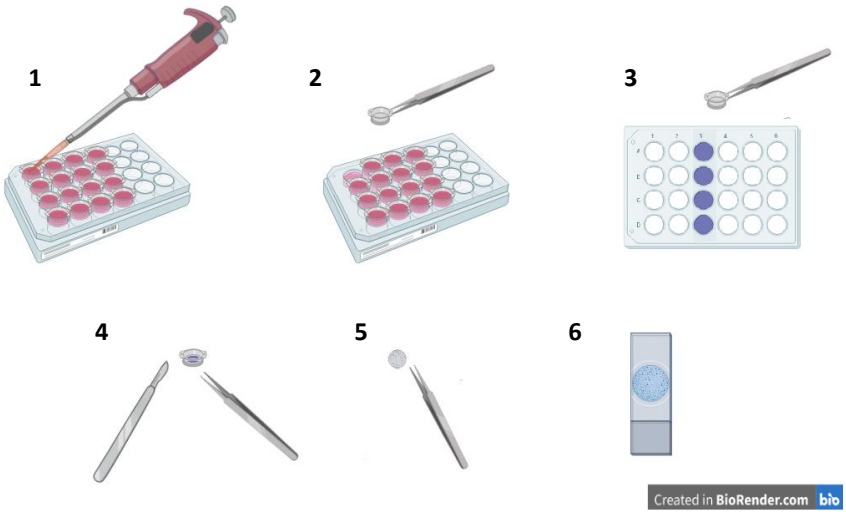



**11. Irudia. Zelulen bideragarritasun entseguaren eskema fibroblastoengandik lortutako faktore soluigarrien presentzian hazitako zeluletan.** Plaka batean kultibatutako zelulen erdia kultibo medio arruntarekin eta beste erdia fibroblastoen CMarekin 1:2 diluituta inkubatu zen 24 orduz (1). Ondoren, zelulei medio zaharra kendu eta aurreko egunekoa bezalako medio berria gehitu zitzaien tratamenduekin batera eta berriro ere beste 48 orduz kultibatu ziren. Azkenik zelulei PrestoBlue™ gehitu zitzaien eta 2 ordu igarotakoan absorbantzia neurtu zen (3). *Irudia BioRender bidez sortutakoa da.*

## 1.6. Zelulen migrazio entsegua.

Ocoxinek zelulen migrazio prozesuan duen eragina aztertzeko C26 minbizi zelulen, zein 3T3 fibroblasto, zein J774A.1 makrofagoen migrazio entsegua gauzatu zen. Horretarako, zelulak $2x10^5$ zelula/ml kontzentrazioarekin kultibatu ziren I motako kolagenoz estalitako 8µm-ko poro diametroko *Transwell* insertetan (Greiner Bio-One, Austria) 1:100 ($V/V_f$) Ocoxindun RPMI-1640 medioarekin eta 18 orduz kultibatu ziren. Ondoren, inserteko zelulak % 4 formalinarekin fixatu eta kristal bioletarekin tindatu ziren. Ostean, insertaren mintza moztu eta porta batean jarri zen migratutako zelulak zituen

mintzaren aldea gora begira egonik eta DPXarekin muntatu zen. Azkenik, inserteko mintz osoari argazkiak atera zitzaizkion eta zeharkatu zuten zelula guztien kopurua kuantifikatu zen *ImageJ* softwarea erabiliz (12. Irudia).




***12.*** **Irudia. Migrazio entseguaren eskema.** Zelulak aurretik kolagenoz estalitako *Transwell* insertetan kultibatu ziren Ocoxinen diluzio desberdinak zituzten medioekin (1). 18 ordu igaro ondoren insertak plakatik atera (2) eta % 4 formalinatan murgildu ziren zelulak fixatzeko eta ostean, kristal bioletarekin tindatu ziren (3). Bukatzeko, inserteko mintza moztu egin zen (4-5) eta porta batean muntatu zen (6). *Irudia BioRender bidez sortutakoa da.*

## 1.7. Zatiketa zelularraren analisia CFSE bidez

Ocoxinek zatiketa zelularrean aldaketarik eragiten zuen aztertzeko, zelulen zatiketa kopurua CFSE erabiliz aztertu zen. Horretarako, zelulak azaldutako prozesuaren bidez altxatu ostean, 10 mM CFSE gehitu zitzaien RPMI-1640tan diluituta eta 30 minutuz 37 °C-tan inkubatu ziren. Ondoren, zelulei FBS-dun kultibo medio osatua gehitu zitzaien CFSEa inaktibatzeko eta zentrifugatu egin ziren erreaktibo soberakinak deuseztatzeko. Azkenik CFSErekin markatutako zelula horietako alikuota bat baztertu eta CFSEak igorritako seinalea neurtu zen *Beckman Coulter Gallios*$^{TM}$ (Beckman Coulter, Estatu Batuak) zitometroaren bidez, oraindik zatitu ez diren zelulen fluoreszentzia maila ezagutzeko.

Gainerako zelulei dagokionez birsuspenditu egin ziren eta berriro medio osatuarekin kultibatu ziren flasko desberdinetan. 24 orduz hazten utzi ondoren, zelula horietako flasko bat hartu, zelulak altxatu, zentrifugatu, garbitu eta CFSEak igorritako seinalea neurtu zen, tratamendurik gabe zelulek duten zatiketa-maila zein den jakiteko. Ostean, gainerako zelulei Ocoxinen diluzio desberdinak gehitu zitzaizkien. 266-6 zelulen kasuan zelulak 48 orduz inkubatu ziren eta C26 zelulak aldiz 24 orduz bakarrik. Denbora hori igarotakoan, zelulak altxatu, zentrifugatu eta garbitu egin ziren CFSEaren fluoreszentzia neurtzeko.

Fluoreszentzia horren bidez, zelulen zatiketa maila aztertu zen kontuan hartuz zelula batek pairatzen duen zatiketa bakoitzeko CFSE kantitate erdia aurkeztuko duela.

## 1.8 Ziklo zelularraren analisia propidio ioduro bidez.

Ocoxinek minbizi zelulen ziklo zelularren gain duen efektua aztertzeko, zelulak 72 orduz tratatu ziren. Hauek PBSarekin garbitu ondoren, 4 °C-tan 15 minutuz % 70eko etanolarekin fixatu ziren. Gero, zelulen *DNA FxCycle™ PI/RNAse Solution kit*eko PI/RNAsa soluzioa erabiliz tindatu ziren ordu erdiz eta *Beckman Coulter Gallios*$^{TM}$ (Beckman Coulter, Estatu Batuak) zitometroaren bidez zelulek igorritako fluoreszentzia neurtu zen.

Fluoreszentziaren arabera ziklo zelularraren faseak 4 taldetan banatu ziren: Sub $G_1$, $G_0/G_1$, S eta $G_2$/M. Sub $G_1$ faseak apoptosia bezalako prozesuen bidez DNA kantitate murriztua eta beraz, PI fluoreszentzia gutxi aurkezten duten zelulen multzoa hartzen du. Bigarrenez, $G_0/G_1$ faseak hazkuntzan dauden zelulak hartzen ditu bere barnean, DNA kantitate arrunta azaltzen dutelarik. Hirugarrenez, S faseko edo sintesi faseko zelulen multzoa DNAren duplikazioarekin hasten diren zelulez osaturik dago, beraz hauen DNAren fluoreszentzia-maila aurrekoena baino altuagoa izango da. Azkenik, $G_2$/M faseak DNA kantitate bikoitza duten eta mitosirako prestatzen ari diren zelulak barne hartzen ditu, hortaz, fluoreszentzia maila altuena igorriko dutenak zelula hauek izango dira.

Baldintza hauek kontuan izanik *Weasel Software*a erabiliz (Weasel Software Ltd, Finlandia) ziklo zelularreko fase bakoitzean zeuden zelula kopurua zehaztu zen.

## 1.9. Apoptosiaren analisia fluxu-zitometria bidez.

Ocoxinek zikloa gelditu edo apoptosia areagotzen duen ikertzeko, hasteko zelulak hazkuntza medio osatuarekin 48 orduz kultibatu ziren $3x10^5$ zelula/ml kontzentrazioarekin. Ostean, kultibo medio zaharra deuseztatu ondoren, kultibo medio osatu berriari tratamendu desberdinak gehitu zitzaizkien (ikusi Metodoetako 1.4 atala) CMarekin eta CM-rik gabe eta zelulak beste 48 orduz inkubatu ziren. Denbora hori igarotakoan, zelulak tripsinarekin altxatu, PBSarekin garbitu eta tindatu egin ziren *Annexin V-FITC Apoptosis Detection kit*arekin fabrikatzailearen jarraibideen arabera. Horretarako zelulak $5x10^5$ zelula/ml kontzentrazioa lortu arte diluitu ziren eta Anexina V-FITC eta PIarekin inkubatu ziren 10 minutuz. Zelula hauek garbitu ostean *Beckman Coulter Gallios*$^{TM}$ (Beckman Coulter, Estatu Batuak) zitometroa erabiliz fluoreszentziaren igorpena neurtu zen 530 eta 575 nm-tan kitzikapen moduan 480 nm-ko uhin luzerako izpia erabiliz, eta *Weasel Softwarea*ren bidez (Weasel Software Ltd, Finlandia) fluoreszentzia mailak aztertu ziren. Modu honetan, guztira, lau zelula talde bereiztu ziren: Zelula biziak (fluoreszentzia igorpenik gabe), apoptosi goiztiarreko zelulak (FITC-rako positiboak), apoptosi berantiarreko zelulak (FITC eta PIrako positiboak) eta zelula nekrotikoak zein hildakoak (PIrako positiboak).

## 1.10. 266-6 zelulen SV40 T antigeno handiaren espresioaren egiaztatzea fluxu-zitometriaren bidez.

Adenokartzinoma pankreatikoaren *in vivo* eredu berriarekin hasi aurretik, 266-6 zelulek SV40aren T antigeno handia espresatzen zutela ziurtatu zen fluxu zitometroaren bidez. Hortaz, lehenengo eta behin zelulak metanol hotzarekin fixatu ziren 5 minutuz giro tenperaturan. Garbitu ostean, zelulak % 5eko FBS-dun PBS-tan inkubatu ziren 40 minutuz antigorputzen lotura inespezifikoa ekiditeko. Ondoren, zelulak SV40aren T antigeno handiaren aurkako antigorputzarekin inkubatu ziren 1:100 diluituta giro tenperaturan ordu batez. Jarraian garbiketak egin ziren eta saguaren aurkako Alexa Fluor 488 fluorokromodun antigorputz sekundarioarekin inkubatu ziren berriro ere beste ordu batez. Bukatzeko PBSarekin 266-6 zelulak garbitu eta zitometrotik pasatu ziren SV40aren T antigeno handiaren espresiorik zegoen frogatzeko. Markatzailearen presentzia baieztatzean, *in vivo* eredua gauzatu zen.

# 2. *IN VIVO* ENTSEGUAK

Jarraian azalduko diren prozedura guztiak EHU/UPV-ko animaliekin egiten den Esperimentaziorako Etika Batzordeak (AEEB) onartuak izan dira eta estatu mailako zein nazioarteko ikerketarako diren animalien zainketa eta babesa bermatzen duten jarraibideen arabera burutu ziren. Halaber, animaliak EHU/UPV-ko animaliategian mantendu ziren karraskarientzako pentsua eta ura *ad libitum* hartzeko aukera zutelarik.

## 2.1. Adenokartzinoma pankreatikoaren *in vivo* eredu esperimentalaren garapena

PCAren eredu esperimentala gauzatzeko, 6-8 astetako C57Bl/6J sagu arrak eskuratu ziren. Lehenik eta behin, *in vivo* ereduarekin animalia guztiekin batera hasi aurretik, 3 saguri CFSErekin markaturiko tumore zelulak (Ikusi Metodoetako 1.7 atala) inokulatu zitzaizkien. 48 ordu pasa ondoren animaliak sakrifikatu egin ziren eta pankreak *Tissue-Tek® OCT™*-tan sartu ziren izoztuta mantentzeko, beranduago 266-6 zelulen presentzia fluoresentzia bidez histologikoki baieztatu ahal izateko. Zelulak bertan zeudela egiaztatu ondoren, gainerako animaliekin prozedura berdina jarraitu zen. Horretarako, sagu guztiak 50 mg/kg pentobarbitalekin anestesiatu ziren injekzio intraperitoneal bidez. Ondoren, ebaketa bat egin zitzaien saguaren ezkerraldean, abdominaren altueran, eta peritoneoa moztu zen. Pankreak egitura difusoa aurkezten duenez, organoa aurkitzeko, lehenengo barea atera zen eta hesteari loturik agertzen den gantza jarraituz bertaraino heltzea lortu zen. Behin pankrea aurkituta, 266-6 zelulak inokulatu zitzaizkien, guztira $2x10^5$ zelula izanik, $2x10^6$ zelula/ml kontzentrazioarekin PBS-tan diluiturik eta jarraian ziztada gunea presioa eginez estali zen minutu batez zelulak kanpora atera ez zitezen. Amaitzeko, organo guztiak berriro gorputzean sartu eta peritoneoa eta azala hariarekin itxi ziren eta saguak esnatu arte itxaron zen. Animaliek beroa galtzea ekiditeko, anestesiapean zeuden bitartean tapaki elektriko baten gainean jarri ziren.

## 2.2. Kolon-ondesteko minbiziaren metastasi hepatikoaren *in vivo* eredu esperimentalaren garapena.

Beste alde batetik, CRCko metastasi hepatikoaren *in vivo* eredu esperimentala garatzeko, 8 asteko BALB/c sagu arrak eskuratu ziren. Aurreko kasuan bezala, animaliak 50 mg/kg pentobarbitalekin anestesiatu ziren injekzio

intraperitoneal bidez eta ondoren, saguaren ezkerraldean ebaketa bat egin zen barea ateratzeko. Gero, barearen behe poloan $2x10^5$ zelula injektatu zitzaizkien $2x10^6$ zelula/ml kontzentrazioarekin eta amaitzerakoan orratzak utzitako zuloan presioa egin zen zelulak baretik ez ateratzeko. Azkenik, zauria hariarekin itxi zen eta saguak esnatu zirenean kaioletara bueltatu ziren. Kasu honetan ere, saguak tapaki elektriko baten gainean mantendu ziren esnatzen ziren arte.

## 2.3. Tratamenduen dosi eta patroien ezarpena

### 2.3.1. Ocoxinen tratamenduaren dosi eta patroiaren ezarpena

*In vivo* eredu egokiena aukeratzeko, lehenik eta behin dosi eta tratamendu patroi desberdinak frogatu ziren (13. Irudia). Horretarako, minbizi eredu bietan animaliak talde desberdinetan banatu ziren bakoitzak 7 animalia zituelarik:

Kontrol taldea: Tratatu gabeko saguez osaturiko taldea, minbiziaren garapen normala monitorizatzeko.

Halaber, tratatu gabeko hiru animalia tumore-zelulak inokulatu eta egun batzutara sakrifikatu egin ziren tumoreen garapena kontrolatzeko: PCAren *in vivo* ereduaren kasuan 17. eta 24. egunetan sakrifikatu ziren eta CRC-ko metastasiaren ereduan, aldiz, 7. egunean. Gainerako animaliak zelulak inokulatu eta 35 egun pasa ostean sakrifikatu ziren PCAko ereduan eta CRC-ko metastasiaren kasuan, aitzitik, 21 egun igarotakoan.

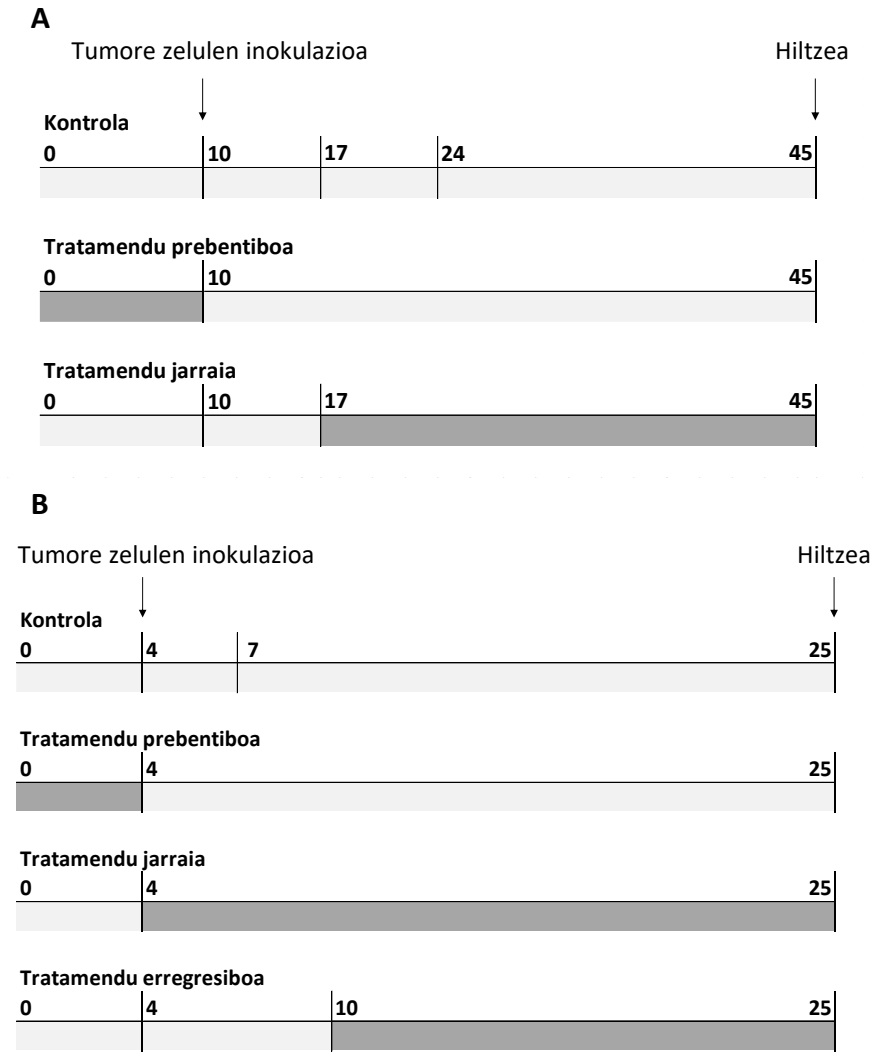
Tratamendu prenbentiboaren taldea: Taldeko honetako saguak tumore-zelulak inokulatu aurretik 10 egunez tratatu ziren bi ereduetan, Ocoxinek tumore zelulak itu-organora heldu baino lehenago eragin prebentiborik eskaintzen duen aztertzeko.

Tratamendu jarraiaren taldea: Talde honetako saguak egunero tratatu ziren tumorea inokulatu zitzaienetik sakrifikatu arte, itu-organoan estrabasatu ondoren, Ocoxinek garapen metastasikoan duen eragina aztertzeko. Hori dela eta, talde hau kolon ondesteko minbiziaren metastasiko *in vivo* ereduan bakarrik ezarri zen.

Tratamendu erregresiboaren taldea: Talde honetako saguek tumore zelulak inokulatu eta 7 egun beranduago hasi zuten tratamendua. Animaliak egunero

tratatu ziren sakrifikatu ziren arte, Ocoxinek jada ezarritako tumore-foku zein metastasiaren erregresioa eragiten duen aztertzeko.

Aldi berean, talde hauek azpitaldetan banatu ziren jasotako Ocoxin dosiaren arabera. PCAko minbizi ereduaren kasuan 100 µl eta 200 µl erabili ziren eta CRCko metastasi hepatikoaren *in vivo* ereduan 50 µl-ko dosia ere gehitu zen.




**13. Irudia.** ***In vivo*** **ereduetan erabilitako tratamenduen eskema.** Kolore argiz tratatamendurik jaso ez zuten periodoa eta kolore ilunez tratamendua jaso zuten denbora tartea. A) Adenokartzinoma pankreatikoko sagu ereduan erabilitako tratamenduen patroia. B) Kolon-ondesteko minbiziaren metastasi hepatikoko sagu ereduan erabilitako tratamenduen patroia.

Aipatzekoa da tratamendua kanula intragastrikoen bidez eman zitzaiela saguei eta Ocoxinik jaso ez zutenen kasuan, animaliak kanulatu egin zirela tratamendu prozesuak eragindako estresa simulatzeko.

Amaitzeko sagu guztiak dislokazio zerbikal bidez sakrifikatu ziren eta odola atera zitzaien kaba zainetik. Sakrifizioaren ondoren, organoak erauzi egin ziren prozesamendu histologikorako.

### 2.3.2. Ocoxin eta irinotekanez osatutako tratamendu konbinatuaren dosi eta patroiaren ezarpena.

Ocoxinen dosi eta emate-patroi efektiboena zehaztu zirenean, Ocoxinen eragina aztertu zen irinotekan agente kimioterapeutikoarekin batera konplementu gisa erabiltzerakoan. Horretarako, tratamendu erregresiboko taldearen eredua jarraituz (ikusi Metodoetako 2.3.1. atala) 4 animalia multzo berri bereiztu ziren tratamenduaren arabera (14. taula). Ocoxinekin tratatutako saguei osagarri nutrizionala egunero ematen zitzaien kanula intragrastriko bidez eta irinotekana, aldiz, injekzio intraperitonealaren bitartez ematen zitzaien 2 egunik behin 14. taulan azaltzen diren dosiak erabilita.

Gainerako prozedura aurreko atalean azaldutako berdina izan zen.

**14. taula. Kolon ondesteko minbiziaren metastasi hepatikoko *in vivo* ereduan animaliekin erabili ziren tratamendua kolon-ondesteko minbiziaren ereduan frogatu ziren tratamenduen zerrenda.**

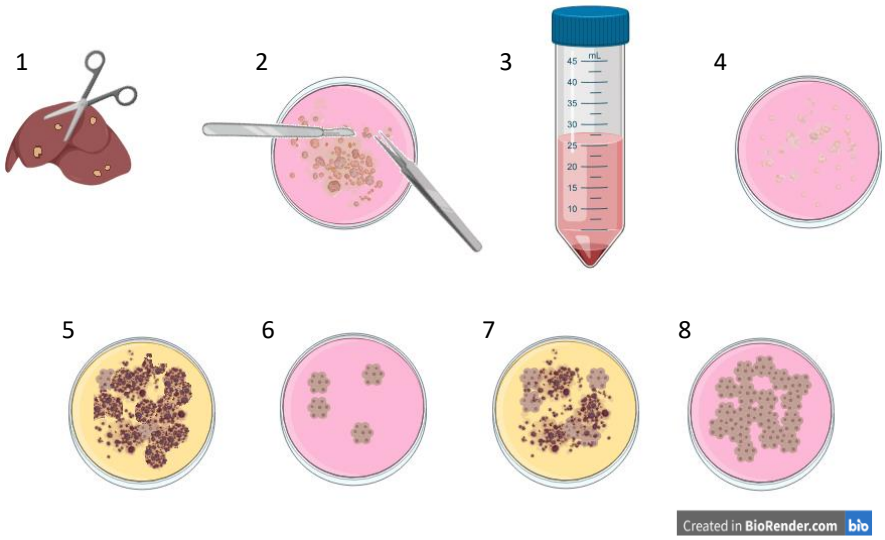
| **Tratamenduak** |
|---|
| **Kontrola (tratamendurik gabe)** |
| **Ocoxin 100 µl** |
| **Irinotekan 20 mg/Kg** |
| **Irinotekan 20 mg/Kg + Ocoxin 100 µl** |

## 2.4. Gibel esplanteetatik erauzitako tumore zelulen isolamendua.

Ocoxinek *in vivo* C26 zeluletan zuen eragina aztertzeko, tratamendu desberdinak jaso zituzten saguen gibeletako CRCaren tumore metastasikoak erauzi egin ziren (ikusi eredu esperimentala Metodoetako 2.2 atalean). Esplante horietatik banakako zelulak lortzeko tumoreak disgregazio mekanikoz zatitu ziren eta Petri plaketan % 0,05eko tripsina-EDTArekin inkubatu ziren disgregazio entzimatikorako 10 minutuz. Ondoren, 430 G eta 4 ºC-tan 10 minutuz zentrifugatu eta % 10eko FBS-dun RPMI-1640 medioarekin

eseki ziren, azkenik Petri plaketan kultibatzeko 0,1 µg/ml gentamizinarekin batera kutsadurak saihesteko (14. Irudia).

Disgregatutako lagin horietan gibel zelula primario desberdinak zeudenez, minbizi-zelulak bakarrik lortzeko asmoarekin eta gainerakoak baztertzeko kultiboa aste batez mantendu zen egun birik behin hazkuntza medioa aldatuz eta tumore zelulak hazkuntza-prozesuan zeudela egiaztatuz. Minbizi-zelulak hazten zeuden bitartean gainerako gibeleko zelula primarioak oso sentikorrak direnez, gehienak hil eta hazkuntza medioa aldatzen zen bakoitzean galdu egiten ziren. Hortaz, astebete igaro ondoren kultiboan tumore-zelulak bakarrik zeudela kontsideratu zen.




**14. Irudia. Gibel esplanteetatik tumore zelulak isolatzeko erabilitako teknikaren eskema.** Gibeletatik tumore makroskopikoak erauzi ziren eta disgregazio mekanikoz eta kimikoz zatitu ziren (1-2). Zelulak zentrifugatu ostean Petri plaka batean kultibatu ziren (3-4). Bi egunik behin medioa aldatu zitzaien hildako zelulak kentzeko (5-7) eta aste baten buruan tumore zelulen kultiboa lortu zen (8). *Irudia BioRender bidez sortutakoa da.*

# 3. ANALISI HISTOLOGIKOAK

## 3.1. Laginen prozesamendua.

Organoak saguengandik erauzi ondoren, alde batetik, pankreak % 3,7-4 v/v formaldehidotan fixatu ziren 24 orduz eta bestetik, gibelak zink soluziotan

mantendu ziren 12 orduz eta horren ostean % 50eko etanoletan murgildu ziren. Ondoren, organoak *Shandon Citadel 2000* (Thermo Scientific, Estatu Batuak) ehunen prozesatzailean sartu ziren, alkohol gradiente baten bitartez deshidratatzeko eta parafinaz betetzeko. Amaitzeko, pankrea zein gibelak parafinatan inkluditu ziren.

CRCko gibeleko metastasiaren *in vivo* eredutik lortutako parafinatan sartutako gibeletan 7 µm-ko lodieradun sei ebaketa egin ziren mikrotomoarekin beraien artean 500 µm-ko tartea zegoelarik organoaren azalera altuera desberdinetan aztertzeko asmoz. PCAko *in vivo* ereduko saguetatik erauzitako pankreetan ere, 7 µm-ko lodieradun sei ebaketa egin ziren altuera desberdinetan.

Hala ere, organo batzuk ez ziren zink soluziotan fixatu, *Tissue-Tek® OCT™*-tan inkluditu ziren izoztuta mantentzeko ondorengo analisietarako.

## 3.2. Fluoreszentziadun laginen prozesamendua.

Fluorezsentziarekin markaturiko zelulak daramatzan organoak aztertzeko, izoztutako organoen 10 µm-ko lodierako laginak moztu ziren kriostatoan eta % 70eko etanolarekin fixatu ziren. Ostean, DAPIdun *Fluoromount™*-a gehitu zitzaien zelulen nukleoak markatzeko eta lagina muntatzeko eta azkenik, *Zeiss Axioskop (Carl Zeiss Incorporated)* (Zeiss, Alemania) kameradun fluoreszentziazko mikroskopioan, *Cell*$^{A}$ programarekin 266-6 zelulen presentzia aztertu zen.

## 3.3. Hematoxilina eta Eosina tindaketa.

Hematoxilina eta eosina tindaketa egiteko, lehenengo eta behin sekzio histologikoak alkohol gradiente batetik pasatu ziren laginak desparafinatu eta berhidratatzeko eta ostean, hematoxilina eta eosinarekin tindatu ziren 15. taulako prozedura jarraituz. Ondoren eta laginak berriz ere alkohol gradientetik pasa ziren 15. taulako protokoloa alderantzizko noranzkoan jarraituz ehunak deshidratatzeko. Azkenik, sekzio histologikoak DPX muntai medioa erabiliz muntatu eta lehortutakoan *Olympus XC50* kameradun *Olympus BX50* mikroskopio optikoarekin eta Cell$^{A}$ programa erabiliz (Olympus Soft Imaging Solutions, Alemania) lagin guztiaren argazkiak atera ziren.

**15. taula. Parafinatan dauden laginen deshidratazio-berhidratazio prozedura eta hematoxilina-eosina tindaketaren protokoloak.**

| Alkohol gradientea | |
|---|---|
| **Substantzia** | **Denbora** |
| **Zitrosola** | 10 minutu (x2) |
| | 5 minutu |
| **100°ko etanola** | 10 segundu |
| **96°ko etanola** | 10 segundu (x2) |
| **70°ko etanola** | 10 segundu |
| **50°ko etanola** | 10 segundu |
| **Ur distilatua** | 5 minutu |

| H&E tindaketa | |
|---|---|
| **Substantzia** | **Denbora** |
| **Hematoxilina** | 2,5 minutu |
| **Ur distilatua** | 5 minutu (x2) |
| **% 0,5eko HCl** | 3 segundu |
| **Ur distilatua** | 5 minutu |
| **Eosina** | 25 segundu |
| **Ur distilatua** | 5 minutu |

Irudien analisiari dagokionez, *ImageJ* softwarea erabiliz lagin osoko tumore-foku kopurua eta hauek okupaturiko azalera kuantifikatu zen. Horretarako, 500 µm-ko tartea zuten hiru sekzio aztertu ziren organo bakoitzean eta batez bestekoa kalkulatu zen.

# 4. IMMUNOENTSEGUAK

## 4.1. Analisi immunohistokimikoak

Analisi immunohistokimikoekin hasi baino lehen, aurreko atalean azaldutako protokoloa jarraituz, ehunak desparafinatu eta berhidratatu egin ziren alkoholen gradientetik pasatuta. Behin laginak hidrataturik izanik, % 3ko $H_2O_2$ eta % 3ko FBS-tan inkubatu ziren, 40 minutuz bakoitza, peroxidasa endogenoa eta proteina inespezifikoak blokeatzeko. Ondoren, laginei antigorputza gehitu zitzaien % 0,1 Triton X-100dun PBStan diluituta ehuna iragazkortu eta antigorputza zelulen nukleoetaraino hel zedin. Zelulen proliferazioa aztertzeko anti-Ki-67 antigorputza erabili zen, apoptosirako anti-Caspasa-3 eta makrofagoen eta fibroblastoen infiltrazioa detektatzeko anti-F4/80 eta anti-α-SMA antigorputzak hurrenez hurren (ikusi Materialetako 10.1. atala). Salbuespen moduan anti-α-SMA antigorputza erabili aurretik α-SMAren kasuan, saguen immunoglobulina endogenoak blokeatu egin ziren immunoglobulinen F(ab) fragmentuaren aurkako antigorputzaren bidez 1:500 diluituta, beranduago erabiliko den antigorputz sekundarioa, bai ehuna saguarengandik erauzitakoak direnez, lotura inespezifikoak saihesteko. Laginak hurrengo egunera arte inkubatu ondoren garbiketak egin ziren eta bakoitzari dagokion antigorputz sekundarioa gehitu zitzaien (ikusi Materialetako 10.2. atala). Azkenik, antigenoen espresioa Estreptabidina-HRP eta 2-Solution DAB kitaren bitartez errebelatu ziren eta laginak DPXarekin muntatu ziren.

Lehortutakoan, sekzio bakoitzari argazkiak atera zitzaizkion bere osotasunean *Olympus XC50* kameradun *Olympus BX50* mikroskopio optikoarekin eta Cell$^{A}$ programa erabiliz (Olympus Soft Imaging Solutions, Alemania). Azkenik, tumoreak identifikatu egin ziren eta immunomarkaketa positiboa azaltzen zuen tumorearen azalera portzentaia kuantifikatu zen *ImageJ* softwarea erabiliz.

## 4.2. Plasmako zitokina-mailen analisia ELISA bidez

Adenokartzinoma pankreatikoaren sagu ereduari dagokionez, tratatu gabeko eta tratamendu erregresiboa jarraituz 100 µl eta 200 µl Ocoxin ($V/V_f$) jaso zuten saguetan (ikusi Metodoetako 2.3.1. atala) IL-1β, IL-10, IL-12 eta IFNγ zitokinak aztertu ziren ELISA bidez. Teknika honen oinarria antigorputzek daramatzaten entzimak dira, sustratuarekin erreakzionatzen dutenean, kolore-aldaketa bat sortzen dutenak hain zuzen. Labur esanda, entzimadun antigorputzak (atzemate-antigorputza) antigeno bat ezagutzen du (aztertu nahi den laginean) eta gero horri antigenoarekin lotuko den beste antigorputz bat (detekzio-antigorputza) gehitzen zaio, zeinak substratua daraman, azkenean entzima eta substratuaren arteko erreakzioak gertatuko delarik.

Animaliak sakrifikatzerakoan kaba zainetik odola atera zitzaien eta agente koagulatzailedun hodiak erabiliz, seruma erauzi zen zentrifugazio bitartez. Ostean, *Mouse IL-1β* and *IL-10 CytoSet*$^{TM}$ kitak eta *Mouse IFNγ* eta *IL-12 ELISA Set*ak erabiliz ELISA entseguak gauzatu ziren fabrikatzailearen arauak jarraituz. Horretarako atzemate-antigorputzez (anti-IL-1β, anti-IL-10, anti-IFNγ edo anti-IL-12) gainestalirik dauden plaketan gure lagina jarri zen eta ondoren, horri biotinadun detekzio-antigorputza (anti-IL-1β, anti-IL-10, anti-IFNγ edo anti-IL-12) gehitu zitzaion, aurretik harrapaturik geratu ziren antigenoekin lotzeko. 2 orduz inkubatu ostean, plakak garbitu eta estreptabidina-HRP konplexua gehitu zitzaien, detekzio-antigorputzaren biotinarekin lotuko dena eta ordu batez ikubatu ziren. Berriro ere garbiketak egin ostean, estreptabidina-HRP konplexuarekin erreakzionatzen duen TMB substratua gehitu zitzaien. TMBak kolore urdina aurkezten du, baina peroxidasarekin erreakzionatzean bere kolorea hori bihurtzen da. Substratu eta entzimaren arteko erreakzioa gelditzeko azido sulfurikoa gehitu zitzaien laginei eta bukatzeko, laginek igorritako argia *Multiscan Ex®* plaka irakurgailuan (Thermo Scientific, Estatu Batuak) aztertu zen 450 nm eta 620 nm-ko filtroak erabiliz.

# 5. ENTSEGU MOLEKULARRAK

## 5.1. Polimerasaren kate-erreakzio kuantitatiboa

Lan honetan, pankreako tumorearen garapena eta gibeleko zitokinen maila aztertzeko RT-qPCR teknika erabili zen, zeinak mRNA (RNA mezularia) kuantifikatzen duen. Pankrearen kasuan, PCAren hazkundea detektatzeko 266-6 zelulen SV40 birusaren T antigeno handia erabili zen PCAren markatzaile gisa (Ornitz et al., 1987). CRC-ko metastasidun gibelari dagokionez, aldiz, zitokina eta inflamazio-bitartekari desberdinak aztertu ziren: ziklooxigenasa-2 (COX2), IL-1β, IL-6, INFγ eta TNF-α.

Horretarako, RNA totala parafinatan inkluditutako sekzioetatik erauzi zen Norgen FFPE RNA Purification kitarekin, zeina zutabeen bidezko kromatografian oinarriturik dagoen. Hasteko, laginei parafina kentdu zitzaien xileno eta etanolaren bidez. Ondoren, laginak K proteinasa eta digestio tanpoiaren bidez digeritu ziren eta honen ostean, etanola eta beste tanpoi baten bidez, laginak lisatu eta zutabeetan jarri ziren. Zentrifugazio bidez lagina zutabeetatik igaro eta RNA bertan atxikiturik geratzen da gainerako osagaiak zutabearen gainean geratu edo zutabea zeharkatu eta galdu egiten direlarik. Pausu honen ostean, nahi izanez gero, DNA genomikoaren hondarrak deuseztatu daitezke RNAsarik gabeko DNAsa gehituz zutabeei. Bukatzeko, garbiketak egin ostean eluzio soluzioaren bidez, RNA zutabetik askatu zen eta RNA purifikatua lortu zen. Orduan, bere kontzentrazioa eta kalitatea *NanoDrop®ND-1000* (Thermo Fisher, Estatu Batuak) espektrofotometroaren bidez ebaluatu zen. Ondoren, erauzitako RNA horretatik 2µg hartu ziren eta *iScript™ cDNA Synthesis kit*a erabiliz laginak *C1000™ Thermal Cycler* (Bio-Rad, Estatu Batuak) termozikladorean inkubatu ziren fabrikatzaileak agindutako jarraibideak kontutan hartuz eta 16. taulako protokoloa jarraituz:

**16. taula. cDNAren sintesirako termozikladorean erabilitako protokoloa.**

| **Prozesua** | Denbora | Tenperatura |
|---|---|---|
| **Bateratzea** | 5 minutu | 25 °C |
| **RT** | 30 minutu | 42 °C |
| **RT inaktibazioa** | 5 minutu | 85 °C |

RNAren RTa gauzatutakoan, lortutako cDNA laginak anplifikatzeko *Platinum™ Multiplex PCR Master Mix 2x* kita erabili zen, hau da, laginari DNA polimerasa

eta primer desberdinak gehitu zitzaizkion lortutako cDNA anplifikatzeko eta berriro ere termozikladorean inkubatu ziren (ikusi 17 taula).

**17. taula. Multiplexerako termozikladorean erabilitako protokoloa.**

| Prozesua | Denbora | Tenperatura | Ziklo kopurua |
|---|---|---|---|
| **Aktibazioa** | 2 minutu | 95 °C | x 1 |
| **Desnaturalizazioa** | 30 segundu | 95 °C | |
| **Hibridazioa** | 1:30 minutu | 60 °C | x 14 |
| **Luzapena** | 30 segundu | 72 °C | |
| **Luzapena** | 10 minutu | 72 °C | x 1 |
| **Mantentzea** | ∞ | 4 °C | |

Behin laginak anplifikatuta zeudenean DNA horren qPCRa gauzatu zen, kasu bakoitzean detektatu nahi zen generako primer zehatza (9. Taula) eta fluoroforo bezala *SYBR® Green*-a erabiliz 96 putzuko plaketan, gure intereseko genea bakarrik anplifika zedin. RTaren kontrol positibo bezala S15 gene konstitutiboa erabili zen pankreako laginen kasuan eta GAPDHa gibelekoenean (9. taula). Plakak denbora tarte batez zentrifugatu ziren burbuilak kendu eta konposatu guztiak hondora joan zitezen eta cDNAren anplifikazioa *C1000™ Thermal Cycler* (Bio-Rad, Estau Batuak) termozikladorean gauzatu zen 18. taulan azaltzen den protokoloa jarraituz.

**18. taula. qPCRa gauzatzeko termozikladorean erabilitako protokoloa.**

| Prozesua | Denbora | Tenperatura | Ziklo kopurua |
|---|---|---|---|
| **Desnaturalizazioa** | 10 minutu | 95 °C | x 1 |
| **Hibridazioa** | 20 segundu | 95 °C | x 39 |
| **Luzapena** | 1 minutu | 59 °C | |
| **Banantzea** | 5 segundu | 65 °C | x 1 |

Azenik, cDNA *ABI7900HT Sequence Detection system*-ean kuantifikatu zen *ABI Prism, SDS2.0* softwarearekin (Thermo Fisher, Estatu Batuak) eta aztertutako gene guztien espresio erlatiboa normalizatu egin zen kontrol gene konstitutiboarekiko ΔΔCt metodoa erabiliz (Livak & Schmittgen, 2001). Hortik intereseko dugun genearen atari-zikloa edo Ct balioa (ingelesez *Cycle Threshold*) lortzen da, zeinak anplifikazio-kurba konkretu batentzat fluoreszentziaren detekzio-ataria gainditzeko behar diren zikloen kopurua adierazten duen.

## 5.2. Tumore zeluletako RNAren mikoarraia

### 5.2.1. RNAren erauzketa

Ocoxinek tumore zeluletako geneen espresioan daukan eragina aztertzeko, alde batetik *in vitro* tratatutako PCAko 266-6 zelulak analizatu ziren eta bestetik, Ocoxin hartu zuten CRC-dun gibeleko metastasia zuten saguen esplanteetatik erauzitako zelulak ere aztertu ziren. Horretarako, zelula guztiak P6 plaketan hazi ziren. RNAren erauzketarako, lehenengo eta behin zelulei hazkuntza medioa kendu eta $10^6$ zelulako 1 ml TRIzol™ gehitu zitzaien. 10 minutuz erreakzionatzen utzi ostean, plaketako laginak hodietara transferitu ziren eta 0,2 ml kloroformo gehitu zitzaizkien. 3 minutuz giro tenperaturan ikubatu ostean, hodiak zentrifugatu egin ziren 4 ºC-tan 15 minutuz eta 15.000 G-ko azelerazioarekin. Gainjalkinak beste hodi batera transferitu ziren eta 0,5 ml isopropanol gehitu zitzaien eta giro tenperaturan 10 minutu igaro ostean, laginak berriro zentrifugatu ziren 4 ºC-tan 10 minutuz eta 12.000 G-ko azelerazioarekin. Kasu honetan gainjalkina kendu egin zen eta pelletari % 75eko etanol 1 ml gehitu zitzaien berriro zentrifugatzeko 4 ºC-tan 7500 G-rekin eta 5 minututz. Amaitzeko, gainjalkina berriro ere baztertu egin zen eta pelletak lehortzen utzi ziren giro tenperaturan. Laginek alkoholik ez zeukatenean 16 µl RNasa gabeko uretan birsuspenditu ziren.

Horren ostean, erauzitako RNA purifikatu egin zen *PureLink™ RNA Mini Kit*a erabiliz. Horretarako laginari RNasa endogenoengandik babesten duen lisi soluzioa gehitu zitzaion eta zutabe batzuetatik pasatu zen homogenizatzeko. Ostean laginari etanola gehitu eta beste zutabe batzuetatik pasatu zen non RNA atxikita geratzen zen. Bertatik RNA eluitzeko, zutabeetatik RNasa gabeko ura pasarazi zen. Azkenik, RNAren osotasuna aztertu egin zen *Lab-chip*arekin *Eukaryote Total RNA Nano Assay*aren bidez *Agilent 2100 Bioanalyzer*-ean *Agilent RNA 6000 Nano Chips*ekin batera (Agilent Technologies, Estatu Batuak).

### 5.2.2. Mikroarraiaren analisia

Mikroarrairako *SurePrint G3 Mouse GE 8x60K Microarray Kit*a erabili zen (ID: 074809). Porta bakoitzak 8 azpi-arrai berdin dauzka 27.122 gene eta 4.578 RNA ez kodifikatzaile luzerentzako (ingelesez *Long non-coding RNA*, lncRNA) sondekin eta kalitate kontrol bezala sonda horietako 300 10 aldiz erreplikatuta daude. Arrayaren diseinurako datu base desberdinak erabili ziren: *Ensembl*

*Release 76, GenBank* (2014ko Abuztua), *RefSeq Build 66*, *RIKEN 3* eta *Unigene Build 236*.

Mikroarraia *Low Input Quick Amp Labeling kit*, *One-Color*-arekin kitaren protokoloa jarraituz gauzatu zen lagin bakoitzeko 100 ng RNA erabili zirelarik markaketa bakoitzean. Hasteko, RNA *AffinityScript™* alderantzizko transkriptasarekin (*AffinityScript™ RT*) erretrotranskribatu egin zen zianina-3 fluoroforodun zitidina trifosfato nukleotidoekin (Cy3-CTP) batera cRNA markatua lortzeko. Kontrol positibotzat *Spike-in* sondak gehitu zitzaizkien laginei, RNA markatu aurretik. Ondoren, markaturik dauden *laginak RNeasy Mini kit* zutabeetatik pasatuz purifikatu ziren eta *NanoDrop®ND-1000*ean (Thermo Fisher, Estatu Batuak) kuantifikatu ziren. Horren ostean, markatutako cRNAetatik 600 ng erabili ziren 40 µl-tan *SureHyb* hibridazio ganbaren bidez laginak eskuz hibridatzeko. Laginak 65 ºC-tan inkubatu ziren 19 orduz eta 10 rpm-ko azelerazioarekin Agilent *Microarray Hybridization Oven* (Agilent Technologies, Estatu Batuak) labean eta bukatutakoan garbiketak egin ziren. Azkenik portak eskaneatu egin ziren *DNA microarray scanner G2535CA* erabiliz eta en A*gilent Scan control (v. 8.5.1.)* softwarearekin (Agilent Technologies, Estatu Batuak). Eskaneatutako irudiak *Agilent Feature Extraction (v. 10.7.3.1)* softwareari esker (Agilent Technologies, Estatu Batuak) prozesatu ziren.

### 5.2.3. Mikroarraitik lortutako datuen analisia

*Agilent Feature Extraction (v. 10.7.3.1)* softwarearen bidez lortutako datu guztiak *GeneSpring GX 13.0* (Agilent Technologies, Estatu Batuak) programaren bidez prozesatu eta normalizatu ziren fitxategi bakar bat lortzeko non emaitzak guztiak laburbildurik zeuden. Analisi estatistikoak egin ostean (ikusi Metodoetako 6. atala), aldaketa esanguratsua jasan zuten geneen sailkapena *PANTHER (v.10.0)* izeneko analisi sistema erabiliz burutu zen.

Mikroarraiarekin bukatzeko lortutako emaitzak datu base desberdinekin erkatu ziren. Pankreako zelulei dagokionez, tratatu gabeko 266-6 zelulekin alderatuz espresio maila desberdina aurkeztu zuten geneak hurrengo bilduma hauetan bilatu ziren, lortutako geneak aldez aurretik minbiziarekin erlazionaturik zeuden aztertzeko:

·*Pancreatic Expression Database (PED)*
(http://www.pancreasexpression.org; 2019)

·*Pancreatic Cancer Database (PCD)*
(http://pancreaticcancerdatabase.org; 2019)

CRCaren metastasi hepatikoko ereduan, berriz, tratamendurik jaso ez zuten saguengandik erauzitako zelulen espresioarekiko desberdintasunak azaldu zituzten sagu tratatuengandik lortutako zelulen geneen izenak jarraian agertzen den datu baseko geneekin konparatu ziren, deregulazioa jasan zuten geneak minbiziarekin erlazionaturik zeuden ezagutzeko.

·*Colorectal Cancer Genome Database (CoReCG)*
(http://lms.snu.edu.in/corecg/; 2019)

·*Liver Cancer Expression Resource (CancerLivER)*
(https://webs.iiitd.edu.in/raghava/cancerliver/; 2019)

# 6. ANALISI ESTATISTIKOAK

Orokorrean lan honetan esperimentu bakoitza gutxienez 3 aldiz errepikatu zen eta emaitzetako datuak esperimentu guztien batez besteko moduan aurkeztu ziren desbideratze estandarrarekin batera. Esanguratasuna Student-en T testa erabiliz kalkulatu zen.

Salbuespen bezala, saguen *in vivo* eredu esperimentalen garapena 2 aldiz bakarrik errepikatu zen bai PCAan, bai CRCaren metastasi hepatikoan, kasu bakoitzean 30-40 sagurekin, eta microarraien kasuan analisi estatistikoa *MultiExperiment Viewer* softwarearen (Saeed et al., 2003) 4.9.0 bertsioa eta *Linear Models for Microarray Data* edo *LIMMA* metodoa erabili zen (Smyth, 2004).

# IV. RESULTS
# IV. EMAITZAK

# 1. EFFECT OF OCOXIN ON THE PROLIFERATIVE AND MIGRATORY ACTIVITY OF NON-TUMORAL CELLS *IN VITRO*.

## 1.1 Only the most concentrated dilutions affected murine fibroblasts and macrophages.

Taking into consideration the influence of the different stromal cells on tumor development, first of all, we studied the effect of Ocoxin on the viability of stable non-tumoral cell lines frequently found in the TME: fibroblasts and macrophages. To do so, 3T3 fibroblasts and J774A.1 macrophages were treated with increasing concentrations of Ocoxin from 0 to 1:200 ($V/V_f$) for 24 hours.

Results revealed that Ocoxin did not affect murine 3T3 fibroblasts up to the 1:100 ($V/V_f$) concentration, which reduced their viability by 58 % and almost by 100 % when they were treated with the 1:50 ($V/V_f$) dose (Figure 15). On the other hand, murine J774A.1 macrophages showed higher resistance towards the nutritional supplement since the showed cell viability decrease by 50 % and by 74% when treated with 1:75 ($V/V_f$) and 1:50 ($V/V_f$) dilutions respectively (Figure 15). Besides, the 1:10 ($V/V_f$) dilution caused a complete cell death in both cell lines.

## 1.2. The migratory capacity of fibroblasts and macrophages was reduced by Ocoxin.

Tumors are known to promote stromal cell recruitment so that the TME becomes favorable for the development of cancer. Among those cells, fibroblasts and macrophages are known to migrate to the tumor site and have shown to be closely related to tumor progression. Therefore, the effect of Ocoxin was analyzed on the migratory potential of healthy 3T3 fibroblasts and J774A.1 macrophages. To do so, cells were cultured in the presence of Ocoxin onto 8 µm-diameter pore transwell inserts. 18 hours later, the total number of cells that went through the insert were counted. Our results displayed a significant 68 % and 41 % decrease on 3T3 and J774A.1 cell migration respectively when the 1:50 ($V/V_f$) dilution of Ocoxin was added to the culture medium. However, no statistical differences were observed on their migratory with the 1:100 ($V/V_f$) treatment (Figure 16).

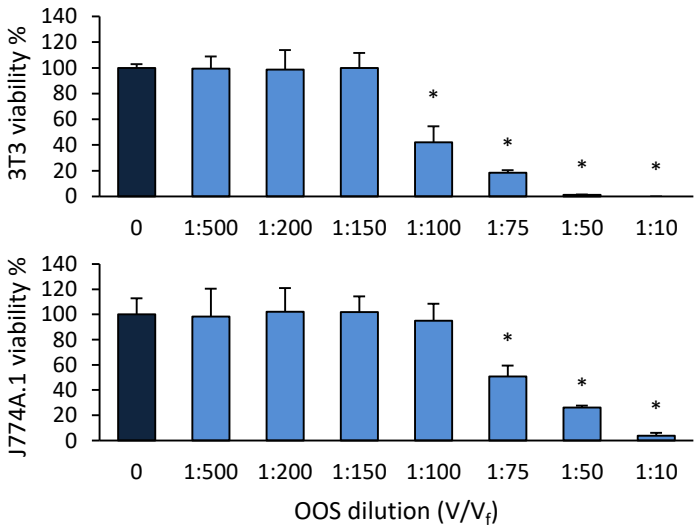



**Figure 15. Viability of 3T3 and J774A.1 cells treated with different dilutions of Ocoxin.** 3T3 murine fibroblasts and J774A.1 macrophages were grown for 24 hours in the presence of different dilutions of Ocoxin to analyze their viability. Significances were expressed as *p<0.05 comparing the different treatments versus untreated cells according to the Student's T-test.

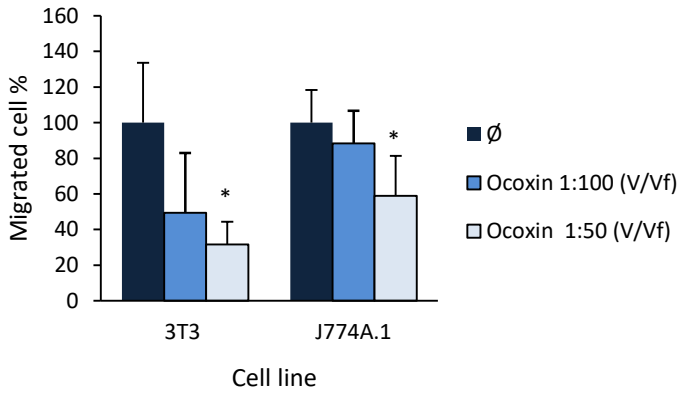



**Figure 16. Migratory capacity of 3T3 and J774A.1 cells treated with Ocoxin.** 3T3 fibroblasts and J774A.1 macrophages were let migrate for 18 hours through 8 µm-diameter pore transwell inserts under the presence of 1:50 and 1:100 ($V/V_f$) dilutions of Ocoxin. Significances were expressed as *p<0.05 comparing the different treatments versus untreated cells (Ø) according to the Student's T-test.

# 2. EFFECT OF OCOXIN ON THE PROLIFERATIVE ACTIVITY OF PANCREATIC CANCER CELLS *IN VITRO*

## 2.1. Effect of Ocoxin in murine pancreatic cancer cells.

### 2.1.1. Ocoxin reduced the viability of the 266-6 murine pancreatic cancer cells.

Murine PCA cells were used to analyze the effect of Ocoxin in pancreatic tumor cell viability. To do so, 266-6 cells were cultured with increasing concentrations of Ocoxin ranging from 1:1000 ($V/V_f$) to 1:50 ($V/V_f$). After 24 hours, cells were incubated with PrestoBlue™ for 2 hours until they metabolized the compound. Then, cell viability was quantified. Figure 17 shows that Ocoxin significantly decreased tumor cell viability in a dose-dependent manner. Only doses over the 1:200 ($V/V_f$) dilution showed significant differences, being 1:100 ($V/V_f$) and 1:50 ($V/V_f$) those that reduced cell viability more than 50 % compared to untreated cells (Figure 17).

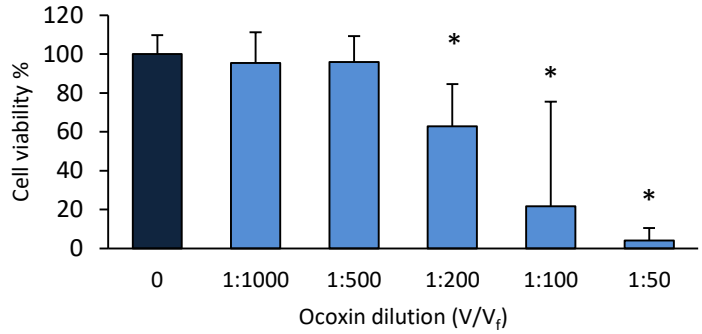



**Figure 17. Viability of 266-6 cells treated with different dilutions of Ocoxin.** 266-6 murine PCA cells were grown for 24 hours in the presence of different dilutions of Ocoxin in order to analyze their viability. Significances were expressed as *p<0.05 comparing the different treatments versus untreated cells according to the Student's T-test.

### 2.1.2. Ocoxin treatment slowed down the cell cycle of the 266-6 murine pancreatic cancer cells.

In order to know whether the reduction of 266-6 cell viability provoked by Ocoxin was due to a change in the cell cycle or cell division rate, cells were stained with PI and CFSE and they were analyzed by flow cytometry. PI staining revealed a decrease of cells gated in the $G_0/G_1$ phase and a mild

increase of those in $G_2$/M phase in cells treated with the 1:100 ($V/V_f$) dilution of Ocoxin (Figure 18 A), meaning that Ocoxin provokes changes in cell cycle but does not enhance cell death *in vitro*. Besides, compared to the cell division rate of untreated cells, CFSE labeling showed that 1:200 and 1:500 ($V/V_f$) Ocoxin dilutions reduced 266-6 cell division by 10 % while 1:100 ($V/V_f$) dilution decreased it by 35 % (Figure 18 B). Hence, taking these results into account and considering that Ocoxin did not reduce cell viability of healthy 3T3 and J774A.1 cells below 1:100 ($V/V_f$) dilution (figure 15), that dose was chosen to perform the following *in vitro* experiments. In this case data were represented as the mean value ± SD of 2 independent experiments.

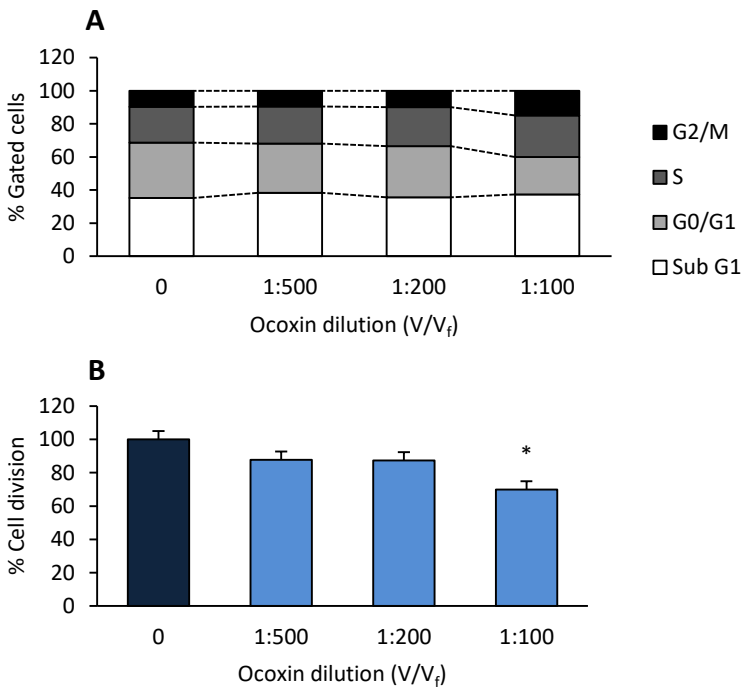



**Figure 18. Cell cycle and cell division analyses on 266-6 cells treated with Ocoxin.** 266-6 cells were treated with 1:500, 1:200, and 1:100 ($V/V_f$) dilutions of Ocoxin for 48 hours and flow cytometry analyses were carried out. A) Cells gated in each cell cycle phase according to the PI assay. B) Cell division rate according to CFSE labeling. Significances were expressed as *$p<0.05$ comparing the different treatments versus untreated cells according to the Student's T-test.

### 2.1.3. Ocoxin altered the gene signature of the 266-6 murine pancreatic cancer cells.

Previous observations confirmed that Ocoxin reduces the viability of 266-6 cells and causes changes in their cell cycle. Thereby, a comparative microarray study was performed to analyze whether this effects were produced by the alterations on gene expression. To do so, total mRNA was extracted from 266-6 cells that had been either untreated or treated with the 1:100 ($V/V_f$) dilution of Ocoxin for 48 hours. Obtained results showed that Ocoxin significantly altered the gene profile compared to that of untreated cells. In fact, 273 genes were significantly deregulated by Ocoxin with a >1.45 fold-change (Table 19).

**Table 19. Genes deregulated by Ocoxin treatment in 266-6 murine PCA cells (fold-change >1.45).**

| | Upregulated genes | Downregulated genes | Total |
|---|---|---|---|
| **Ocoxin vs Untreated** | 123 | 150 | 273 |

Interestingly, 14 of the identified genes had been previously associated with PCA which regressed to control levels after the administration of Ocoxin (Table 20). Moreover, those genes of the murine PCA 266-6 cell line deregulated by Ocoxin participate mainly in processes related to binding, catalytic and transporter activities, molecular function and transcription regulation, and molecular transducer activities (Table 21). On the other hand, these genes are also involved in biological processes such as cellular processes, metabolic processes and biological regulation, many of which are related to tumor development (Table 22).

For further information a list of all the deregulated genes can be found in Appendix I.

**Table 20. Summary of genes described to be deregulated in PCA that were significantly reverted by Ocoxin treatment.**

| Gene | PCA vs Healthy cells * | Treated vs Untreated |
|---|---|---|
| **ANGPTL4** | Downregulated (Gadaleta et al., 2011) | Upregulated |
| **ASNS** | Downregulated (Gadaleta et al., 2011) | Upregulated |
| **CRABP1** | Upregulated (Nakamura et al., 2004) | Downregulated |
| **CREB1** | Upregulated (Friess et al., 2003) | Downregulated |
| **EIF4EBP1** | Downregulated (Crnogorac-Jurcevic et al., 2001) | Upregulated |
| **FIGNL1** | Upregulated (Gadaleta et al., 2011) | Downregulated |
| **FOXF1** | Upregulated (Gadaleta et al., 2011) | Downregulated |
| **GRAP2** | Upregulated (Hustinx et al., 2004) | Downregulated |
| **HEY1** | Upregulated (Mann et al., 2012; Nakamura et al., 2004) | Downregulated |
| **NELL2** | Upregulated (Gadaleta et al., 2011; Nakamura et al., 2004) | Downregulated |
| **PAK7** | Upregulated (Capurso et al., 2006) | Downregulated |
| **RAPGEF3** | Upregulated (Weeks et al., 2008) | Downregulated |
| **SERPINF1** | Downregulated (Yu et al., 2005) | Upregulated |
| **SLC7A1** | Downregulated (Gadaleta et al., 2011) | Upregulated |
| **THY1** | Upregulated (Friess et al., 2003) | Downregulated |
| **TRPC4** | Upregulated (Capurso et al., 2006) | Downregulated |
| **WNT4** | Upregulated (Lowe et al., 2007) | Downregulated |

*Data were obtained from the Pancreatic Expression Database v3.0 (available at http://www.pancreasexpression.org; 2019) and the Pancreatic Cancer Database (available at http://pancreaticcancerdatabase.org; 2019).

**Table 21. Classification of genes deregulated by Ocoxin according to their molecular function.**

| Molecular function | Genes |
|---|---|
| Binding (GO:0005488) | 61 |
| Catalytic activity (GO:0003824) | 45 |
| Transporter activity (GO:0005215) | 18 |
| Molecular function regulator (GO:0098772) | 15 |
| Transcription regulator activity (GO:0140110) | 13 |
| Molecular transducer activity (GO:0060089) | 12 |
| Structural molecule activity (GO:0005198) | 3 |

**Table 22. Classification of genes deregulated by Ocoxin involved in biological processes.**

| Biological Process | Genes |
|---|---|
| Cellular process (GO:0009987) | 92 |
| Biological regulation (GO:0065007) | 65 |
| Metabolic process (GO:0008152) | 60 |
| Response to stimulus (GO:0050896) | 35 |
| Signaling (GO:0023052) | 28 |
| Localization (GO:0051179) | 26 |
| Multicellular organismal process (GO:0032501) | 19 |
| Cellular component organization or biogenesis (GO:0071840) | 18 |
| Developmental process (GO:0032502) | 14 |
| Multi-organism process (GO:0051704) | 3 |
| Biological adhesion (GO:0022610) | 2 |
| Immune system process (GO:0002376) | 2 |
| Locomotion (GO:0040011) | 2 |
| Behavior (GO:0007610) | 1 |
| Cell population proliferation (GO:0008283) | 1 |
| Reproduction (GO:0000003) | 1 |
| Reproductive process (GO:0022414) | 1 |

### 2.1.4. Ocoxin enhanced the effect of the chemotherapeutic agents paclitaxel and gemcitabine on murine pancreatic cancer cell viability.

As reported in the previous results, Ocoxin decreases cell division rate and viability in PCA cells. In line with this, we studied whether Ocoxin could enhance the effect of the chemotherapeutic agents paclitaxel and gemcitabine used routinely in clinical practice. To that end, first of all, the viability of the murine PCA cells was measured after the treatment with increasing concentrations of paclitaxel (1 - 25 µM) and gemcitabine (200 - 1000 nM) separately in order to select the most effective dose. As shown in figure 19, paclitaxel reduced cell viability by 20 % approximately with all the proven concentrations and those cells treated with 200 nM, 500 nM, and 1000 nM of gemcitabine showed a viability decrease by 18 %, 28 %, and 50 % respectively.

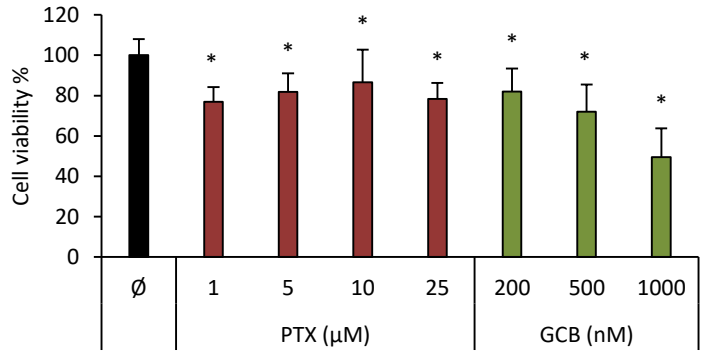



**Figure 19. Viability of 266-6 cells treated with increasing concentrations of paclitaxel and gemcitabine.** Murine PCA cells were treated with increasing doses of paclitaxel and gemcitabine and cell viability was quantified 48 hours later. Significances were expressed as *p<0.05 comparing the different treatments versus untreated cells according to the Student's T-test. *GCB: Gemcitabine; PTX: Paclitaxel; Untreated: Ø.*

Taking this results into consideration, on the one hand, the highest dose of gemcitabine was chosen for further studies and on the other hand, as paclitaxel showed the same cytotoxic effect with every dose, the lowest concentration was selected. Hence, the most effective doses, which were paclitaxel 1 µM and gemcitabine 1000 nM (1 µM) were used combined with Ocoxin to treat 266-6 cells. Although at first we used the 1:100 ($V/V_f$) dilution, here we represent the results obtained when using the 1:50 ($V/V_f$) dose of

Ocoxin, which showed higher effectiveness. The addition of the nutritional supplement as a coadjuvant to paclitaxel showed that Ocoxin increased the effect of the chemotherapeutic drug by 19 % regarding cell viability, while no differences were detected with gemcitabine or paclitaxel plus gemcitabine treatments (Figure 20). Moreover, the combination of both chemotherapeutic drugs enhanced the cytotoxic effect of paclitaxel alone but did not increase the effect of gemcitabine.

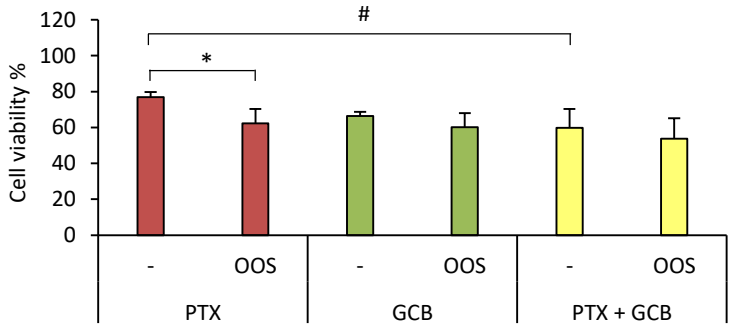



**Figure 20. Viability of 266-6 cells treated with Ocoxin as a complement to paclitaxel, gemcitabine and the combination of both chemotherapeutic drugs.** Murine PCA cells were cultured for 48 hours with 1 µM of paclitaxel, 1 µM of gemcitabine or the combination of both, alone or in the presence of 1:50 ($V/V_f$) of Ocoxin. Significances were expressed as *$p<0.05$ in comparisons between the treatment alone versus the treatment with Ocoxin or as #$p<0.05$ in comparisons between the chemotherapeutic drugs paclitaxel or gemcitabine alone versus the mixture of both according to the Student's T-test. *GCB: Gemcitabine; OOS: Ocoxin; PTX: Paclitaxel.*

## 2.2. Effect of Ocoxin in human pancreatic cancer cells.

### 2.2.1. Ocoxin reduced the viability of human pancreatic cancer cells.

After assessing the cytotoxic potential of Ocoxin in murine PCA cells, its effect on the viability of 6 different human PCA cell lines (BxPC-3, Capan-2, CFPAC-1, HPAF-II, Panc10.05 and SW1990) was studied. The assay revealed the same as in the murine cell line, Ocoxin reduced cell viability in a dose-dependent manner except in HPAF-II and SW1990 cells (Figure 21). Moreover, the compound did not affect significantly to BxPC-3 until the 1:200 ($V/V_f$) dilution, to Capan-2 cells until 1:100 ($V/V_f$) and SW1990 until the 1:50 ($V/V_f$) dose being that one the most effective dilution in all the cell lines. According to the results, the 1:50 ($V/V_f$) dose of Ocoxin decreased cell viability by 90 % in

BxPC-3 cells, 70 % in Capan-2, 60 % in CFPAC-1, 75 % in Panc 10.05 and 60 % in SW1990 cell lines but only 37% in HPAF-II cells (Figure 21).

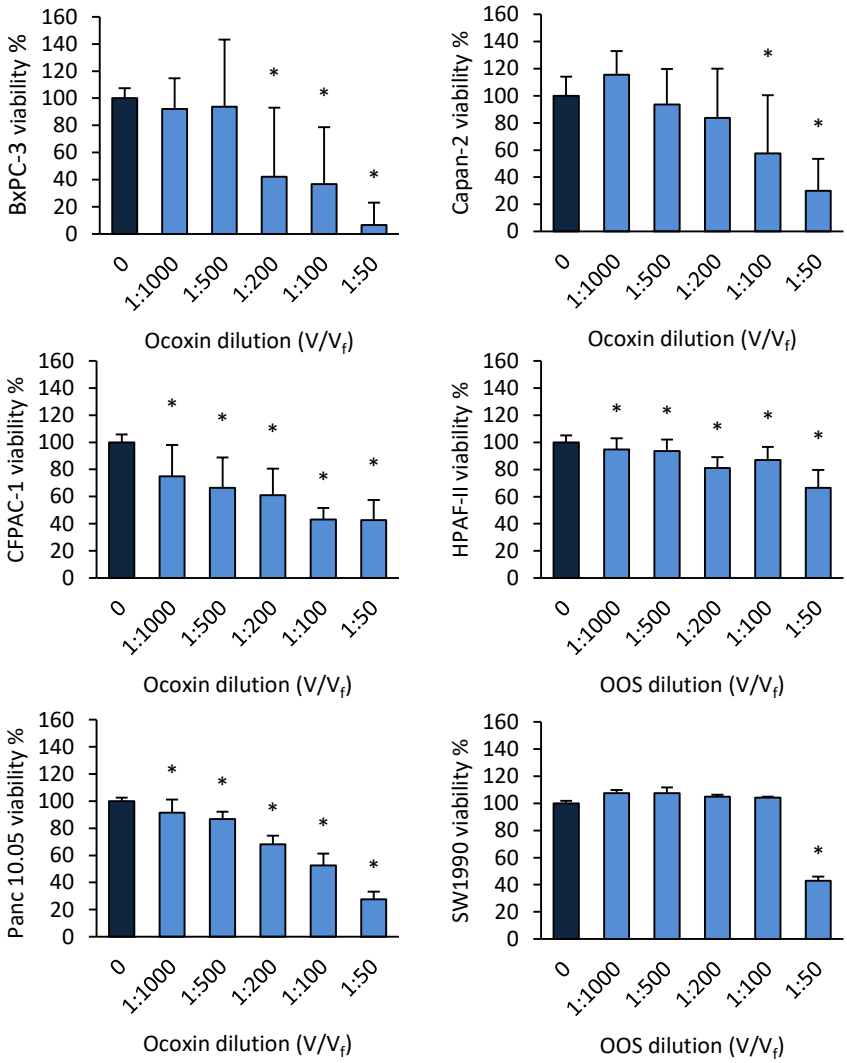



**Figure 21. Viability of human PCA cell lines treated with different dilutions of Ocoxin.** BxPC-3, Capan-2, CFPAC-1, HPAF-II, SW1990 and Panc 10.05 cell lines were treated with increasing dilutions of Ocoxin for 48 hours. Significances were expressed as *p<0.05 comparing the different treatments versus untreated cells according to the Student's T-test.

### 2.2.2. Ocoxin raised apoptotic levels of human pancreatic cancer cell lines.

Bearing in mind that Ocoxin potentiates the cytotoxicity of paclitaxel and gemcitabine against cancer cells, a cell death assay was performed by flow-cytometry using Annexin V/PI staining in order to confirm if viability reduction was accompanied by a rise of apoptotic levels after culturing cells with the 1:50 ($V/V_f$) dose of Ocoxin for 48 hours. Figure 22 shows that the compound increased apoptotic cells substantially by 60 % in BxPC-3 and Capan-2 cell lines, 35 % in CFPAC-1, 22 % in SW1990 and 6 % in Panc 10.05 cells. Apoptotic levels of the HPAF-II cell line were not analyzed due to the high mortality rate of these cells during the process. Data were represented as the mean value ± SD of at least 3 independent experiments.

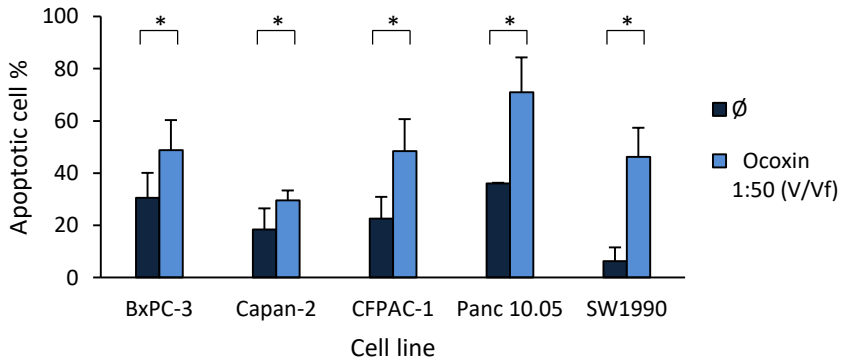



**Figure 22. Apoptosis levels in human PCA cell lines treated with Ocoxin**. Cells were cultured with 1:50 ($V/V_f$) of Ocoxin for 48 hours and cell death was quantified by flow cytometry by using an Annexin V/PI kit. Significances were expressed as *p<0.05 comparing the different treatments versus untreated cells (Ø) according to the Student's T-test.

### 2.2.3. Ocoxin enhanced the effect of the chemotherapeutic agents paclitaxel and gemcitabine on human pancreatic cancer cell viability.

As wells as in the murine 266-6 cells, the effect of Ocoxin was tested as a complement to the anticancer drugs paclitaxel and gemcitabine on the viability of various human PCA cell lines.

To do so, first of all the PCA cell lines were incubated with increasing concentrations of both chemotherapeutic drugs for the dose adjustment. Results of cells treated with 1 µM of paclitaxel disclosed a reduction on cell viability up to 57 % in BxPC-3, 62 % in Capan-2, 72 % in CFPAC-1, 50 % in SW1990 and only up to 31 % in HPAF-II and 35 % in Panc 10.05 cells (Figure

23). On the other hand, gemcitabine provoked a reduction of 70 % in CFPAC-1 cell viability while the rest of the cell lines showed lower values, such as, 40 % in BxPC-3 cell viability, 35 % in Capan-2, 10 % in HPAF-II, 33 % in Panc 10.05 and 36 % in the SW1990 cell line (Figure 23). No differences were observed with higher concentrations than 1 μM of both drugs, therefore, this dose was used for further studies.

According to the results, cell viability was again analyzed using paclitaxel, gemcitabine and paclitaxel plus gemcitabine together with Ocoxin. As shown in figure 24, the cytotoxicity of both drugs increased when they were combined with the 1:50 ($V/V_f$) dilution of Ocoxin in all the studied cell lines. Indeed, the supplement improved the antitumor activity of paclitaxel by 25 % in BxPC-3, 71 % in Capan-2, 32 % in CFPAC-1, 15 % in HPAF-II and 35 % in Panc 10.05 and SW1990 cells. On the other hand, the addition of Ocoxin to gemcitabine, reduced cell viability by 37 % in BxPC-3, by 58 % in Capan-2, by 17 % in CFPAC-1, by 40 % in Panc 10.05 and by 46 % in SW1990 cell lines while it did not show any effect in HPAF-II cells (Figure 24). Besides, Ocoxin enhanced the effect of paclitaxel and gemcitabine administered concomitantly as well in all the studied cells (Figure 24). Finally, in order to find out the mechanism involved in that viability decrease provoked by Ocoxin, the apoptotic levels of the cells were analyzed by flow cytometry. Results did not show significant differences except when Panc 10.05 cells were treated with paclitaxel supplemented with Ocoxin (Figure 25).

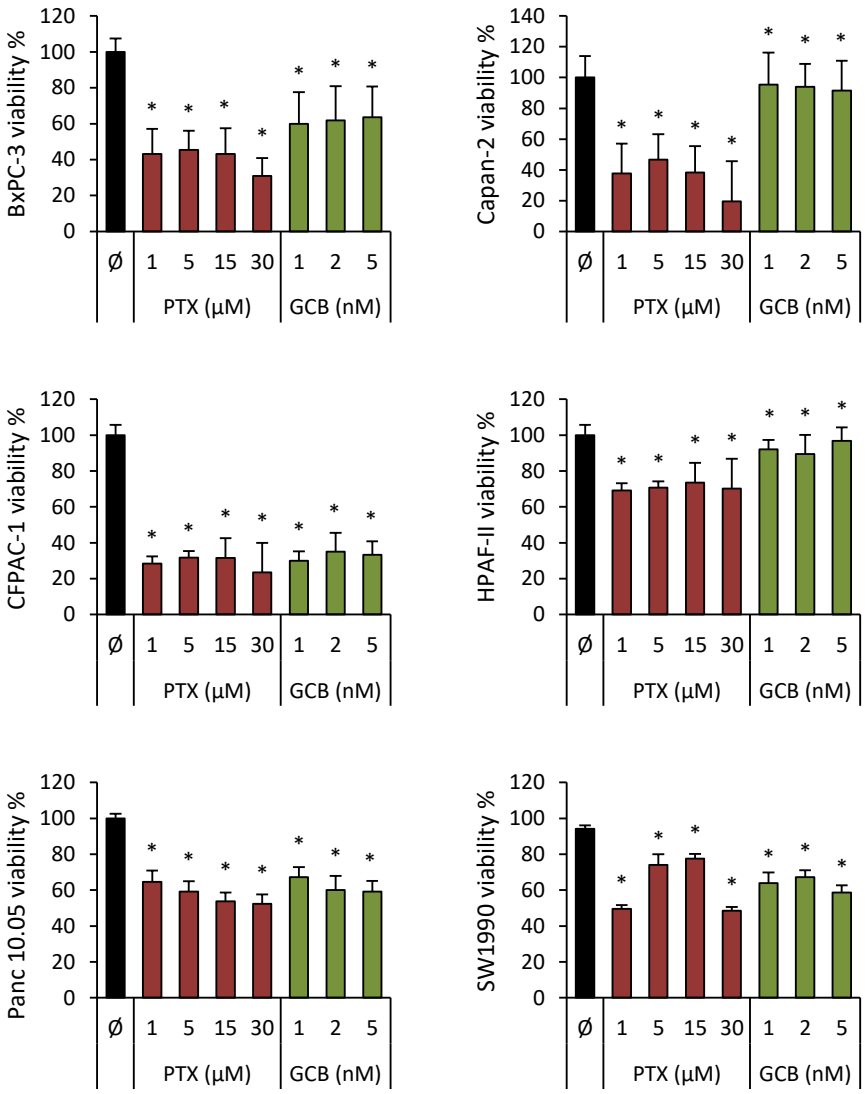



**Figure 23. Viability of several human PCA cell lines treated with increasing concentrations of paclitaxel and gemcitabine.** Cells were treated with different dilutions of chemotherapeutics for 48 hours. Significances were expressed as *p<0.05 comparing the different treatments versus untreated cells according to the Student's T-test. *GCB: Gemcitabine; PTX: Paclitaxel; Untreated: Ø.*

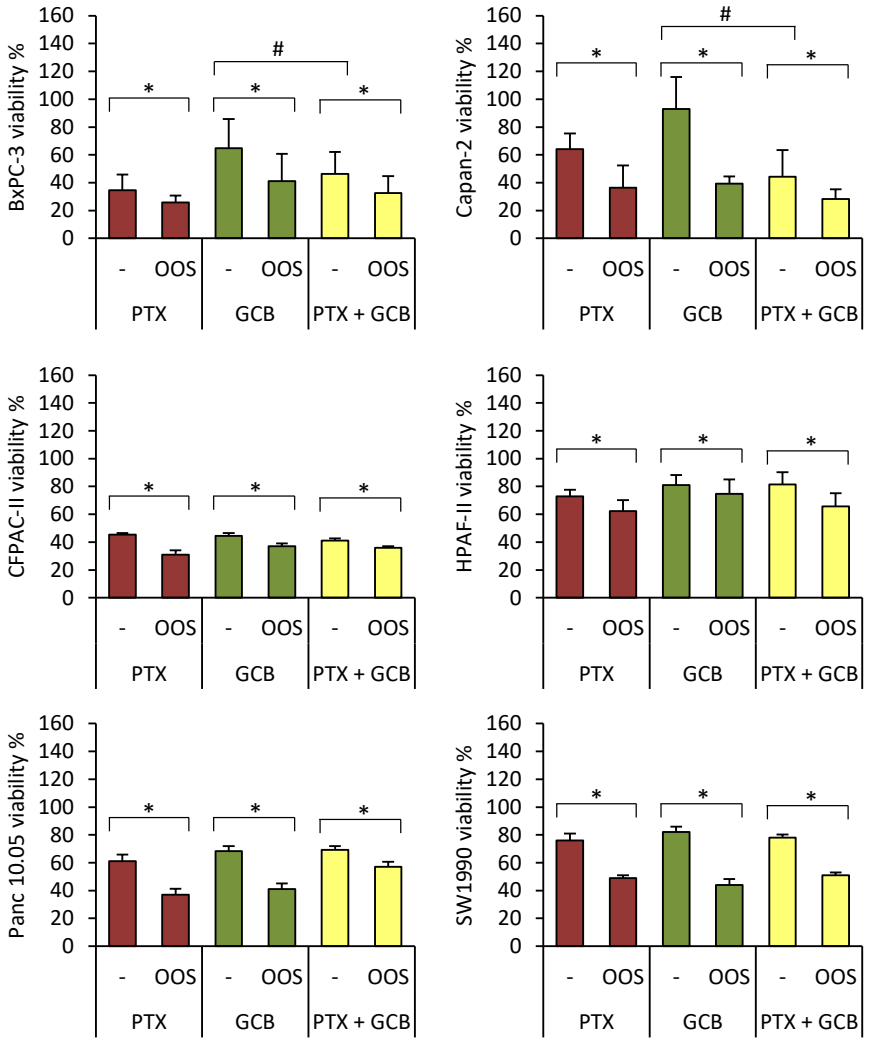



**Figure 24. Viability of different human PCA cell lines treated with Ocoxin as a complement to paclitaxel, gemcitabine or the combination of both chemotherapeutic drugs.** Cells were treated with 1 µM of paclitaxel and/or gemcitabine alone or combined with the 1:50 ($V/V_f$) dilution of Ocoxin for 48 hours. Significances were expressed as #p<0.05 comparing paclitaxel or gemcitabine with the mixture of both dugs and as *p<0.05 comparing the different treatments (paclitaxel, gemcitabine or the combination of both) with Ocoxin versus the same treatments without the nutritional supplement according to the Student's T-test. *GCB: Gemcitabine; OOS: Ocoxin; PTX: Paclitaxel.*

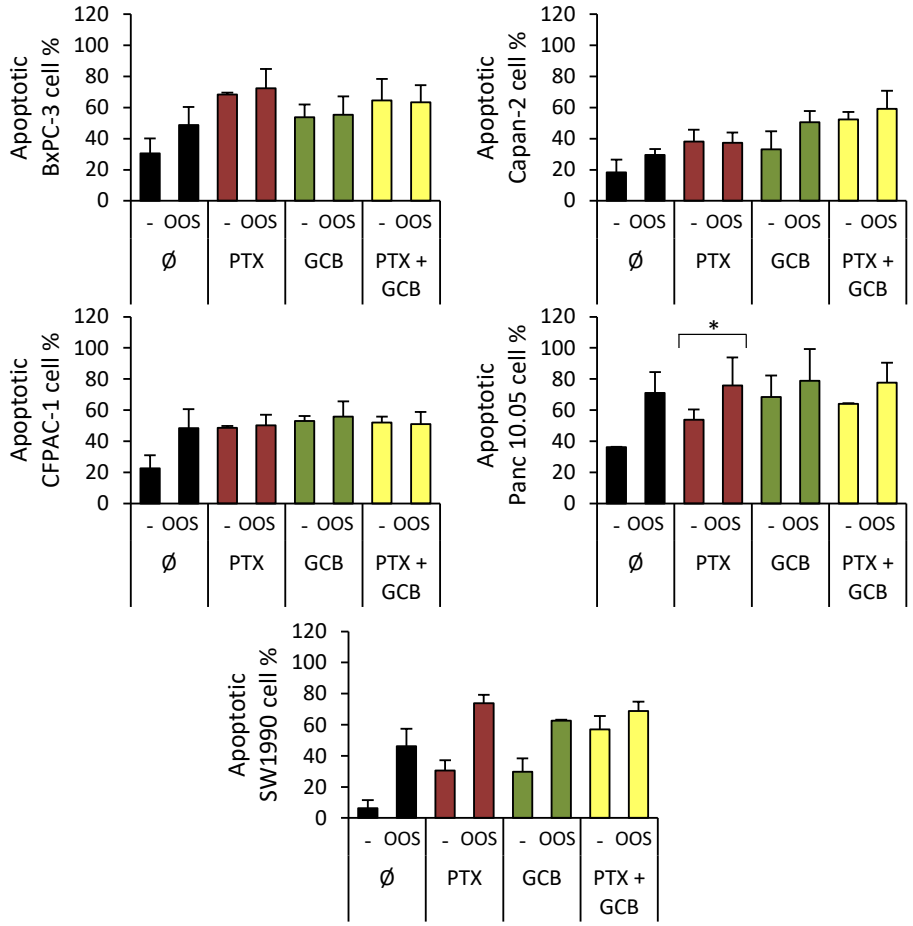



**Figure 25. Apoptotic levels of human PCA cell lines treated with paclitaxel, gemcitabine with or without Ocoxin.** Cell death was quantified by flow-cytometry after culturing different human pancreatic cancer cell lines for 48 hours with 1 µM paclitaxel, 1 µM gemcitabine or a mixture of both alone or combined with the 1:50 ($V/V_f$) dilution of Ocoxin. Significances were expressed as *p<0.05 comparing the treatments alone versus the treatments with Ocoxin according to the Student's T-test. GCB: *Gemcitabine; OOS: Ocoxin; PTX: Paclitaxel; Ø: Untreated.*

## 2.2.4. OCOXIN REVERTED THE STROMA-MEDIATED CHEMORESISTANCE IN HUMAN PANCREATIC CANCER CELLS.

Considering that Ocoxin reinforced the cytotoxic effect of paclitaxel and gemcitabine, the same combined treatments were used to test whether the 1:50 ($V/V_f$) dilution of the nutritional supplement could revert fibroblast-mediated chemoresistance in human PCA cells. First, first we tested whether fibroblast-CM affected tumor cell viability. As shown in figure 26 fibroblast-CM promoted cell viability significantly in all the studied cell lines but in HPAF-II cells.

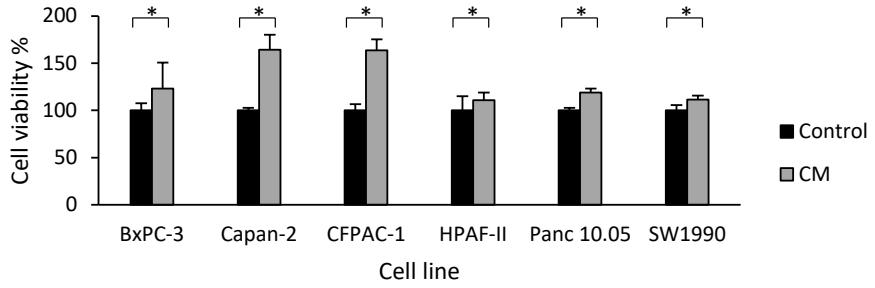



**Figure 26. Viability of several human PCA cell lines grown under the presence of fibroblast-CM.** Cells were cultured with or without fibroblast-CM for 48 hours and viability was analyzed. Fibroblast derived soluble factors significantly increased cell viability. Significances were expressed as *p<0.05 comparing control cells versus those cultured with fibroblast-CM according to the Student's T-test.

Later we analyzed if these soluble factors might interfere with the chemotherapeutic effect of paclitaxel, gemcitabine and the combination of both. As shown in figure 27 the cytotoxicity against tumor cells of the chemotherapeutic drugs was reduced in almost all the studied cell lines when they were treated in the presence of fibroblast-CM pointing out the chemoresistance produced by the soluble factors. However that loss of effectiveness was not observed in HPAF-II cells treated with gemcitabine nor in BxPC-3 cells treated with paclitaxel plus gemcitabine in the presence of fibroblast-CM. Furthermore, while both chemotherapeutic drugs reduced viability effectively in Panc 10.05 cells grown under pro-tumoral conditions, a decrease of the cytotoxic effect of the combination of paclitaxel and gemcitabine was observed in the presence of fibroblast-CM (Figure 27).

Finally, once analyzed the effect of fibroblast derived soluble factors on the effectiveness of the chemotherapeutic drugs, we added Ocoxin to the chemotherapeutic treatments to study the effect of the nutritional

supplement as a complement to chemotherapy in the presence of fibroblast-CM.

As shown in figure 28 the addition of Ocoxin to paclitaxel, gemcitabine or the combination of both reverted the chemoresistance produced by the soluble factors derived from fibroblasts. Thus, Ocoxin recovered the cytotoxic effect of paclitaxel and gemcitabine in every tested cell line that was cultured under pro-tumoral conditions (Figure 28). In that regard, Ocoxin reduced significantly the increase of cell viability provoked by fibroblast-CM in cells treated with paclitaxel up to 39 % in BxPC-3, 42 % in Capan-2, 29 % in CFPAC-1, 12 % in HPAF-II, 23 % in Panc 10.05 and 10 % in SW1990 cells. Likewise, the viability of cells that had been treated with gemcitabine under pro-tumoral conditions was reduced in the presence of Ocoxin by 12 % in BxPC-3, 38 % in CFPAC-1, 23 % in SW1990 cells and up to 70 % in Capan-2 cells. However, the reduction in HPAF-II and Panc 10.05 cells was not significant (Figure 28). Finally, the addition of Ocoxin to the combination of paclitaxel and gemcitabine also reduced significantly the viability of BxPC-3, CFPAC-1 and SW1990 cells by 64 %, 35 % and 14 % respectively and showed a trend towards a viability decrease in Capan-2, HPAF-II and Panc 10.05 cell lines.

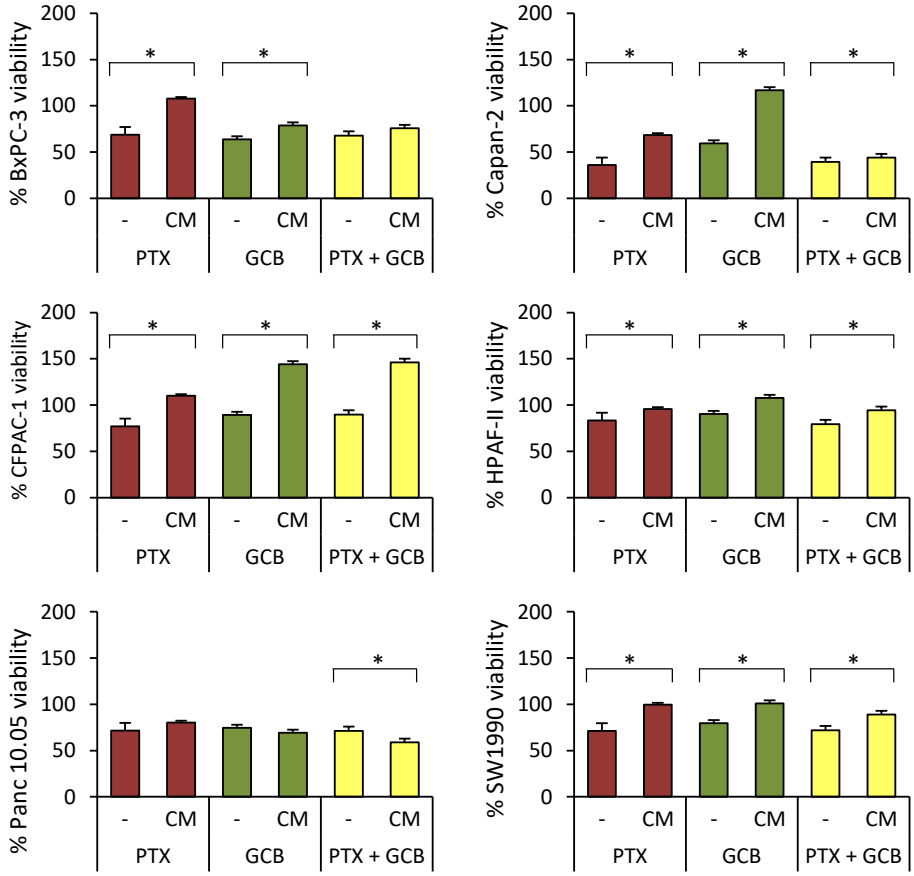



**Figure 27. Viability of different human PCA cell lines treated with paclitaxel, gemcitabine or both under normal and pro-tumoral conditions.** Cells were cultured for 24 hours with and without CM obtained from fibroblasts and then, they were treated with 1 µM of paclitaxel, 1 µM of gemcitabine or a mixture of both for another 48 hours in order to analyze cell viability. Significances were expressed according to the Student's T-test as *p<0.05 when the treatments without CM were compared to that with CM. GCB: Gemcitabine; PTX: Paclitaxel.

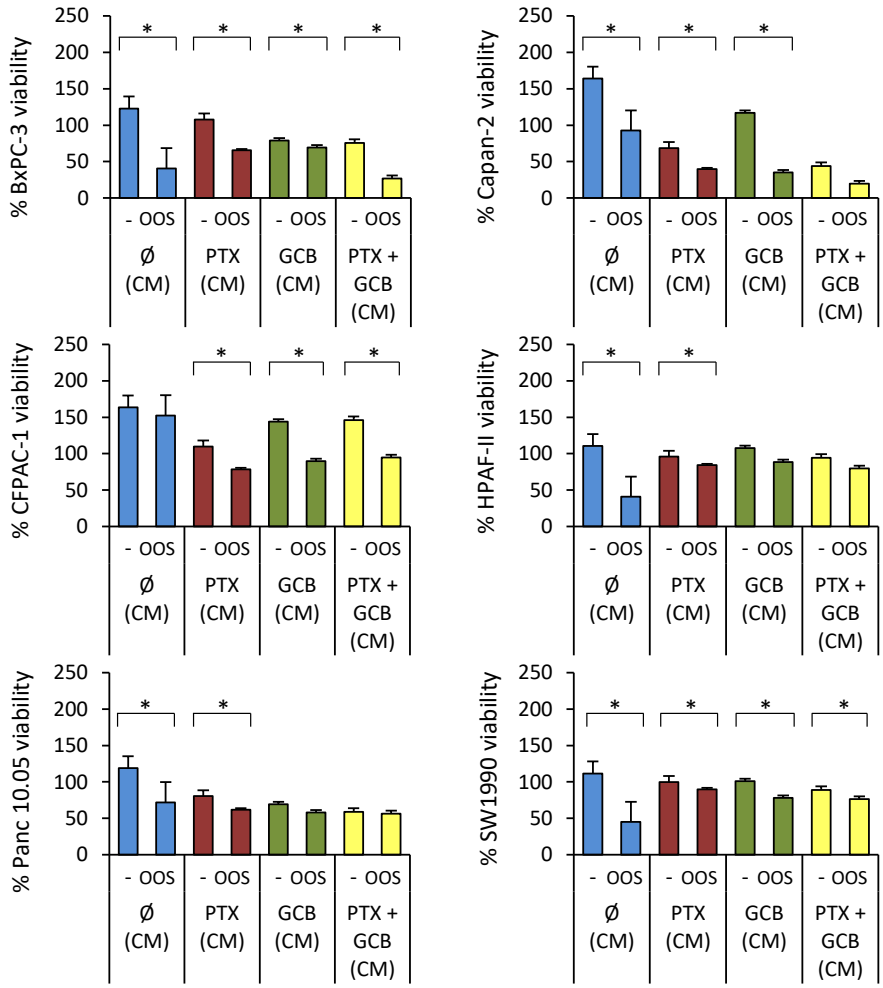



**Figure 28. Viability of different human PCA cell lines treated with Ocoxin as a complement to paclitaxel and gemcitabine to revert chemoresistance induced by fibroblast-derived soluble factors.** Cells were cultured for 24 hours with and without CM obtained from fibroblasts and then, they were treated with 1 µM of paclitaxel, 1 µM of gemcitabine or a mixture of both combined with the 1:50 ($V/V_f$) dilution of Ocoxin for another 48 hours in order to analyze cell viability. Significances were expressed according to the Student's T-test as *p<0.05 when treatments were compared to that combined with Ocoxin. *GCB: Gemcitabine; OOS: Ocoxin; Untreated: Ø.*

### 2.2.5. Ocoxin recovered fibroblast-induced chemoresistance to paclitaxel and gemcitabine of human pancreatic cancer cells by increasing apoptosis.

Previously (figure 25), we showed that Ocoxin did not change the apoptotic levels of several human PCA cell lines when administered together with chemotherapy. However, we studied if the reversion of chemoresistance provoked by Ocoxin (figure 28) was mediated also by the same cellular process.

First we aimed to analyze whether apoptosis was reduced in PCA cells grown under pro-tumoral conditions. As in the previous apoptosis assay, HPAF-II cell line apoptosis was not analyzed due to the high mortality rate of cells during the experimental process. In that regard, our results did not show any significant differences between the cells cultured with or without fibroblast-CM, probably due to data variability (figure 29). Nonetheless, a trend was observed towards apoptosis reduction in BxPC-3 and Capan-2 cells cultured with fibroblast-CM while CFPAC-1 cells showed a slight increase of apoptotic cell counts. Regarding Panc 10.05 and SW1990, they showed similar apoptotic values in both cases.

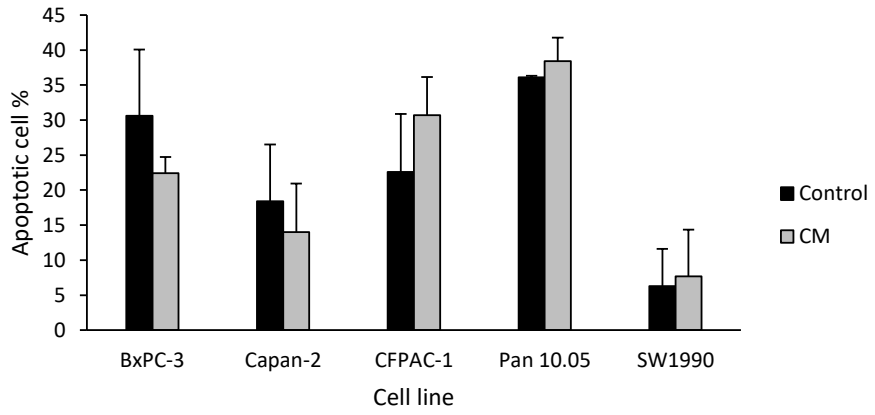



**Figure 29. Apoptotic levels of several human PCA cell lines grown under the presence of fibroblast-CM.** Cells were cultured with or without fibroblast-CM for 24 hours and apoptosis was analyzed by flow cytometry using Annexin-V/PI. No significant differences were observed according to the Student's T test.

Later we tested if Ocoxin potentiates the response of paclitaxel and gemcitabine against cancer cells grown in the presence of fibroblast-CM by enhancing apoptosis. According to our results, soluble factors derived from

fibroblasts also weakened the apoptotic effect produced by chemotherapy. Compared to cells treated with paclitaxel grown under non pro-tumoral conditions, apoptosis was reduced by 37 %, 17 %, 11 %, and 34 % in BxPC-3, Capan-2, CFPAC-1 and Panc 10.05 cells respectively in the presence of fibroblast derived soluble factors (Figure 30). Likewise, fibroblast-CM caused 28 % reduction of apoptosis in BxPC-3 and Panc 10.05 cells and 15 % and 10 % decrease in Capan-2 and CFPAC-1 cell lines treated with gemcitabine (Figure 30).

Lastly, PCA cells were treated with paclitaxel and gemcitabine but in this case combined with 1:50 ($V/V_f$) of Ocoxin in order to study whether the nutritional supplement could revert the decrease of the apoptotic levels caused by fibroblast-CM. As shown in figure 31, Ocoxin demonstrated to overcome the chemoresistance conferred by fibroblast-derived soluble factors against paclitaxel and gemcitabine by increasing apoptosis in all the human PCA cells. To start with, the addition of Ocoxin to paclitaxel to cells grown under pro-tumoral conditions increased apoptosis by 32 %, 28 %, 10 % and 88 % in BxPC-3, CFPAC-1 and Panc 10.05 respectively, and up to 88 % in SW1990 cells but did not show remarkable effects in Capan-2 cells. Besides, Ocoxin used as a coadjuvant with gemcitabine also enhanced apoptotic cell counts by 51 % in BxPC-3 cells, by 42 % in Capan-2 cells, by 26 % in CFPAC-1 cells, by 45 % in Panc 10.05 cells and by 57 % in SW1990 cells compared to those treated without Ocoxin in the presence of soluble factors (figure 31). Thus, Ocoxin acted synergically with both chemotherapeutic drugs. Lastly, the same human PCA cells were treated with the combination of paclitaxel and gemcitabine in the presence of fibroblast-CM and was supplemented with Ocoxin. As expected, the nutritional mixture enhanced apoptosis by 47% in BxPC-3, 21 % in CFPAC-1 cells, 52 % in Panc 10.05 and doubled apoptotic cell counts in SW1990 cells treated with paclitaxel and gemcitabine concomitantly. However, as we observed in Capan-2 cells treated with paclitaxel and Ocoxin, the loss of the apoptotic effect of gemcitabine caused by the addition of soluble factors was not reverted by the nutritional supplement in Capan-2 cells either (figure 31).

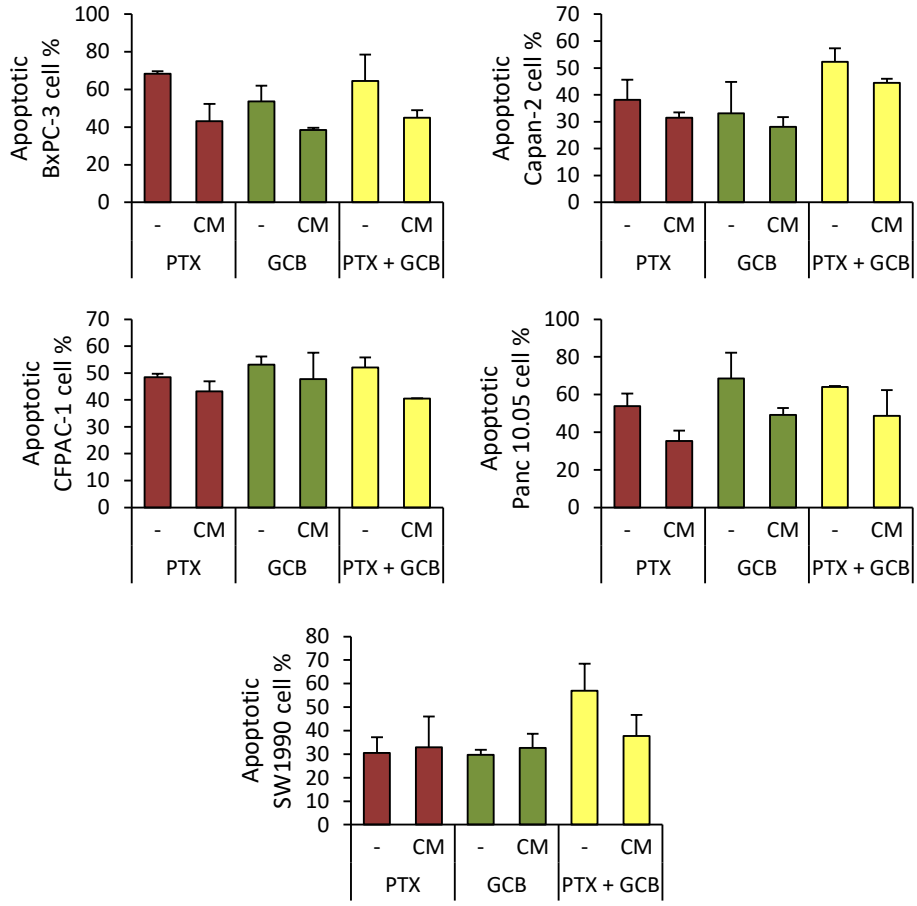



**Figure 30. Apoptotic levels of human PCA cell lines treated with paclitaxel, gemcitabine or both under normal and pro-tumoral conditions.** Cell death was quantified by flow-cytometry using Annexin-V/PI after culturing different human pancreatic cancer cell lines for 24 hours with or without fibroblast-CM and then they were treated with 1 µM paclitaxel, 1 µM gemcitabine or a mixture of both for 48 hours. No significant differences were observed according to the Student's T test. GCB: Gemcitabine; PTX: Paclitaxel.

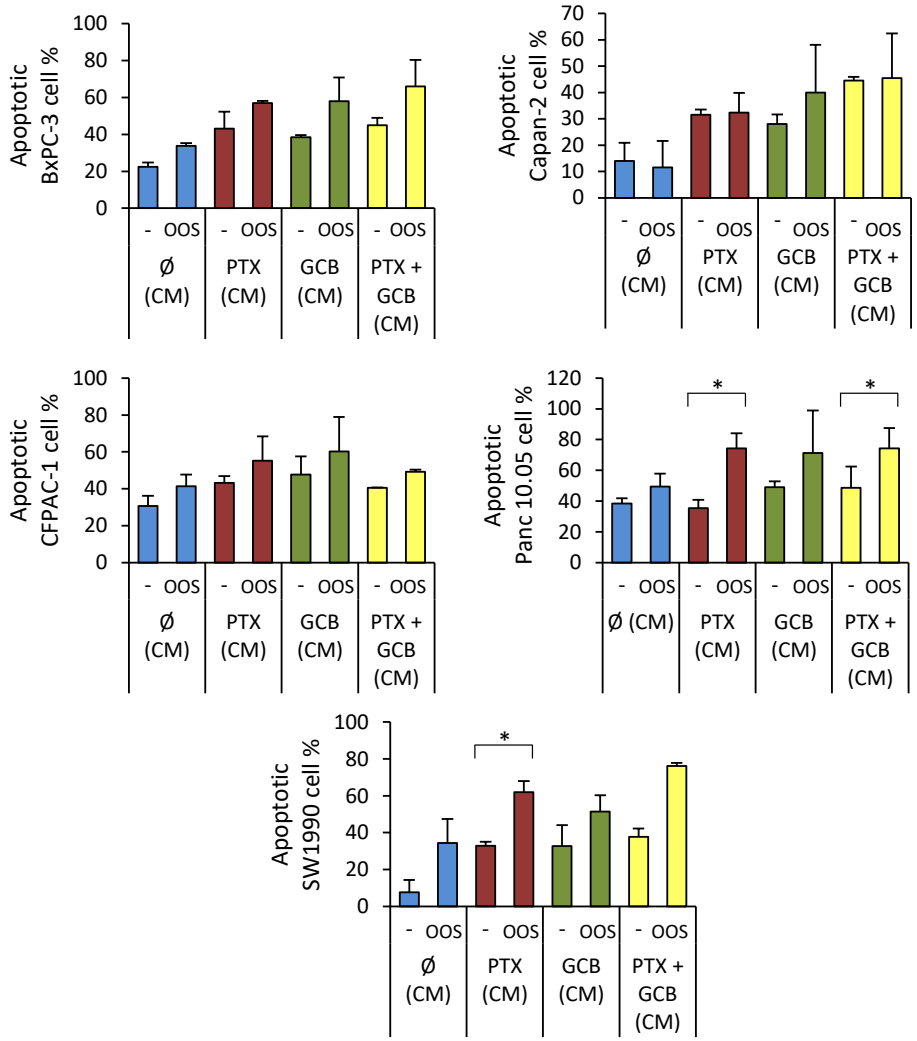



**Figure 31. Apoptotic levels in human PCA cell lines treated with Ocoxin as a complement to paclitaxel and gemcitabine to revert chemoresistance induced by fibroblast-derived soluble factors.** Cells were cultured for 24 hours with CM obtained from fibroblasts and then, they were treated with 1 µM of paclitaxel, 1 µM of gemcitabine or a mixture of both combined with the 1:50 ($V/V_f$) dilution of Ocoxin for another 48 hours in order to quantify cell death by flow-cytometry using Annexin-V/PI. Significances were expressed according to the Student's T-test as *p<0.05 when treatments were compared to that combined with Ocoxin. *GCB: Gemcitabine; OOS: Ocoxin; PTX: Paclitaxel; Ø: Untreated.*

# 3. EFFECT OF OCOXIN ON TUMOR GROWTH IN A MURINE MODEL OF PANCREATIC CANCER.

## 3.1. Ocoxin reduced the presence of pancreatic tumor cells in mice bearing pancreatic cancer.

Before starting the studies on the effect of Ocoxin on PCA development *in vivo*, we first confirmed the presence of the injected tumor cells in the pancreas. To do so, cells were labeled with CFSE before inoculation and mice were sacrificed 48 hours later. Then, the pancreas was fixed and embedded in paraffin for histological analyses. Fluorescence microscopy observations confirmed the presence of CFSE positive cells within the pancreatic tissue (Figure 32), indicating that cancer cells were located in the organ by that time. Therefore, in order to assess the effect of Ocoxin on the *in vivo* model of PCA, mice were treated as described in methods (Figure 13 A). In short, 266-6 cells were injected into the pancreas of mice that were treated with Ocoxin following different patterns and finally pancreatic tissues were collected, fixed and embedded in paraffin.

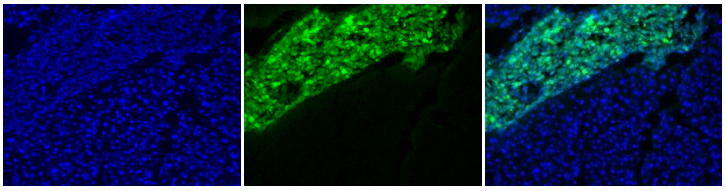



**Figure 32. Presence of CFSE labeled 266-6 cells in murine pancreatic tissue.** CFSE labelled 266-6 cells were inoculated in the pancreas of mice and 24 hours later, organs were collected. Pancreatic slides were observed under the fluorescence microscope to confirm the presence of the injected cells (green).

The effect of Ocoxin on the *in vivo* development of PCA was analyzed by means of the detection of the specific SV40 T antigen of 266-6 cells. Thus, before cell injection, the expression of the T antigen in these cells was confirmed by flow cytometry (figure 33 A). Then, total RNA was extracted from pancreatic tissue and the antigen expression was quantified by RT-qPCR. As shown in figure 33 B, mice treated with 200 µl of Ocoxin according to the preventive treatment pattern, showed a significant 82 % decrease of the T antigen expression in the pancreatic samples. However, no differences were observed in the pancreas

of the animals treated with 100 µl. On the other hand, the animals that received 100 µl and 200 µl of Ocoxin following the regressive treatment pattern, showed a reduction of the T antigen expression by 40 % and 52 % respectively (Figure 33 B) compared to that of untreated mice. Data were represented as the mean value ± SD of at least 3 independent experiments.

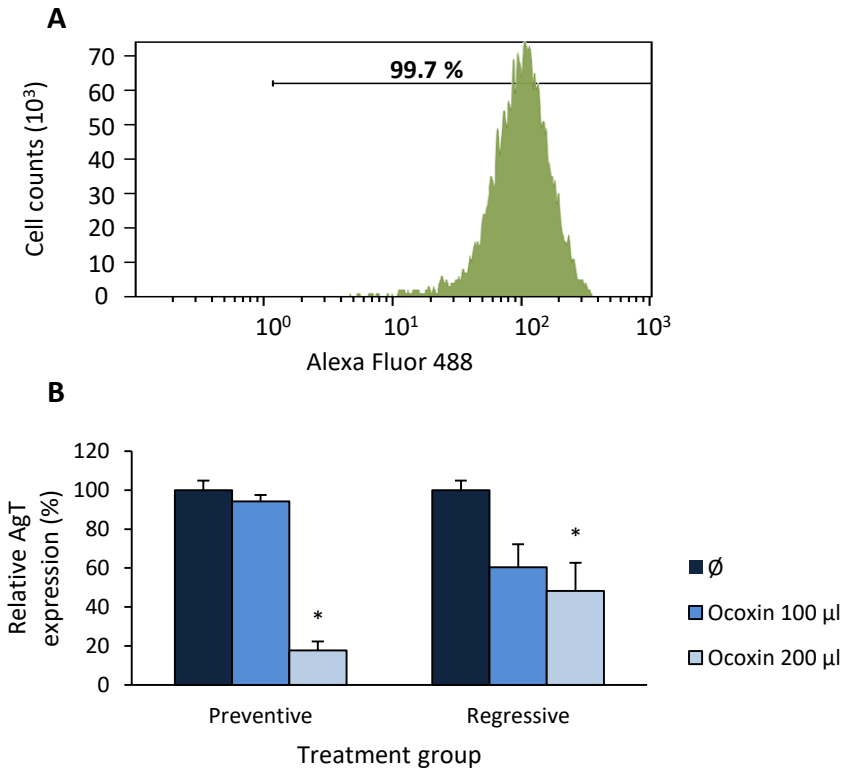



**Figure 33. Expression of the SV40 large T antigen in pancreatic tissue of mice bearing PCA treated with Ocoxin.** A) Confirmation of the expression of the SV40 large T antigen in 266-6 cells by flow cytometry. B) Relative expression of the SV40 large T antigen in the pancreas of mice treated with 100 µl and 200 µl of Ocoxin following the preventive and regressive administration patterns. Significances were expressed as *p<0.05 comparing the different treatments versus the untreated condition (Ø) according to the Student's T-test.

## 3.2. Ocoxin treatment altered serum levels of cytokines in mice bearing pancreatic cancer.

It is widely known that inflammation predisposes to the development of cancer. In that regard, we analyzed whether the anti-inflammatory properties of Ocoxin could modify serum levels of cytokines in mice bearing PCA.

According to our results, control levels of the studied inflammatory mediators were similar in the preventive and the regressive group. However, figure 34 shows that Ocoxin treatment reduced IFNγ, IL-1β and IL-12 serum levels in both groups although at different efficacies. On the one hand, while serum IFNγ levels showed a reduction by 0.8-fold in mice treated with 100 µl and 200 µl of Ocoxin in the preventive group, the regressive treatment pattern caused a complete reduction with both doses. On the other hand, IL-1β levels were reduced similarly in both groups, 0.5-fold and 0.6-fold in the preventive group with the 100 µl and 200 µl doses respectively and 0.6-fold in the regressive group. Besides, IL-12 levels were also decreased by around 0.8-fold in the preventive and regressive group with the two doses of Ocoxin.

On the contrary IL-10 levels were increased in serum of mice treated with Ocoxin. As shown in figure 34, IL-10 significantly rose 2-fold in mice belonging to the preventive group treated with 200 µl of Ocoxin and also increased 6- and 1.5-fold in animals of the regressive group treated with 100 µl or 200 µl of Ocoxin respectively (Figure 34).

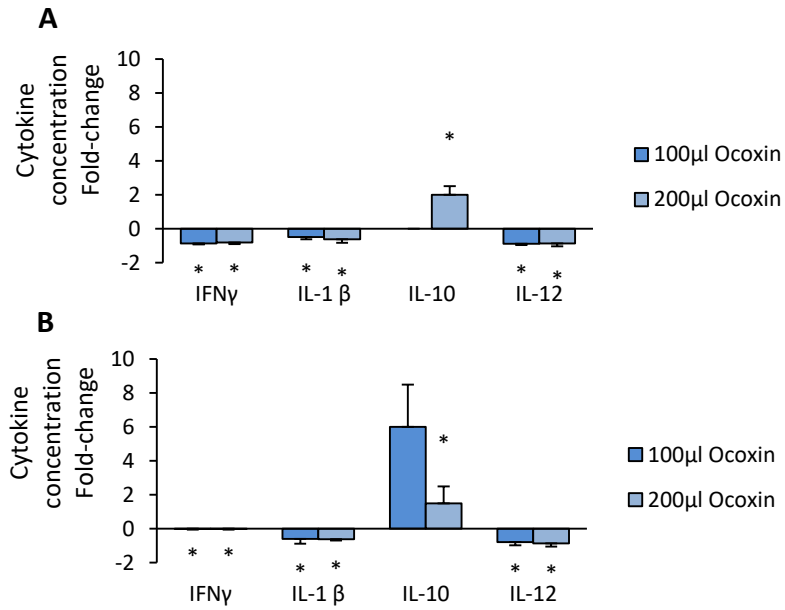



**Figure 34. Serum cytokine concentration fold-change in PCA-bearing mice treated with Ocoxin compared to untreated.** A) Cytokine concentration fold-change in serum of mice included in the preventive treatment group. B) Cytokine concentration fold-change in serum of mice treated according to the regressive group. Significances were expressed as *p<0.05 comparing the different treatments versus the untreated condition according to the Student's T-test.

# 4. EFFECT OF OCOXIN IN THE PROLIFERATIVE ACTIVITY OF METASTATIC COLORECTAL CANCER CELLS *IN VITRO*

Until now we have focused our research on PCA, one of the most lethal cancers, not due to the incidence but rather because of its qualities which hinder an early detection. Consequently, by the time PCA is diagnosed, the tumor shows and advanced stage and in many cases, it has already colonized other organs. This process, known as metastasis, is often the cause of death of many other cancers. Such is the case of CRC. Even though death rates from this cancer have declined progressively during the last decades owing to the early surgeries and effective primary treatments, sometimes CRC cells manage to survive and to escape from immune surveillance which leads to metastasis. In this sense, since the prevention of this process is a key factor in cancer development, we studied the effect of Ocoxin in the metastatic progression.

## 4.1. Ocoxin reduced C26 murine colorectal cancer cell viability in a dose dependent manner.

To begin with the analyses of the anti-tumoral effect of Ocoxin on the development of CRC liver metastasis, first, the effect of Ocoxin was studied on the viability of murine CRC C26 cells. To that end, cells were grown in the presence of increasing concentrations of Ocoxin ranging from 0 to 1:200 ($V/V_f$) for 24 hours. According to the results, the viability of C26 cells was reduced in a dose-dependent manner from 20 % with 1:200 ($V/V_f$) to more than 90 % with the 1:50 ($V/V_f$) dose compared to the untreated cells (figure 35).

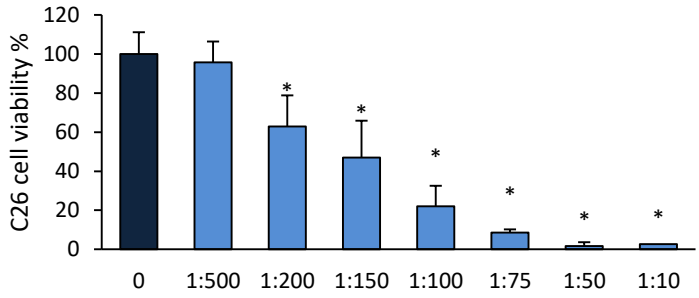



**Figure 35. Viability of C26 cells treated with different dilutions of Ocoxin. C26 murine colorectal cells were grown for 24 hours in the presence of different dilutions of Ocoxin to analyze their viability.** Significances were expressed as *p<0.05 comparing the different treatments versus untreated cells according to the Student's T-test.

Taking into account that Ocoxin did not act as a cytotoxic agent for healthy 3T3 and J774A.1 cells below 1:100 ($V/V_f$) dilution (Figure 15) and that the cytotoxic effect of Ocoxin on C26 started also at the same dilution, the 1:100 ($V/V_f$) dose was chosen to perform the following *in vitro* experiments.

## 4.2. The migratory potential of C26 murine colorectal cancer cells was reduce by Ocoxin.

Despite the efficacy of current anti-tumoral treatments for CRC, some malignant cells are able to escape from the primary tumor, migrate to another organ and give rise to the metastasis. Therefore, the effect of Ocoxin on the migratory capacity of C26 cells was analyzed by culturing them onto 8 µm-diameter pore transwell inserts in the presence of Ocoxin for 18 hours. After counting the total number of cells that went through the insert, results showed that 1:100 ($V/V_f$) and 1:50 ($V/V_f$) dilutions of Ocoxin decreased cell migration significantly by 60 % and 50 % respectively compared to the untreated cells (Figure 36).

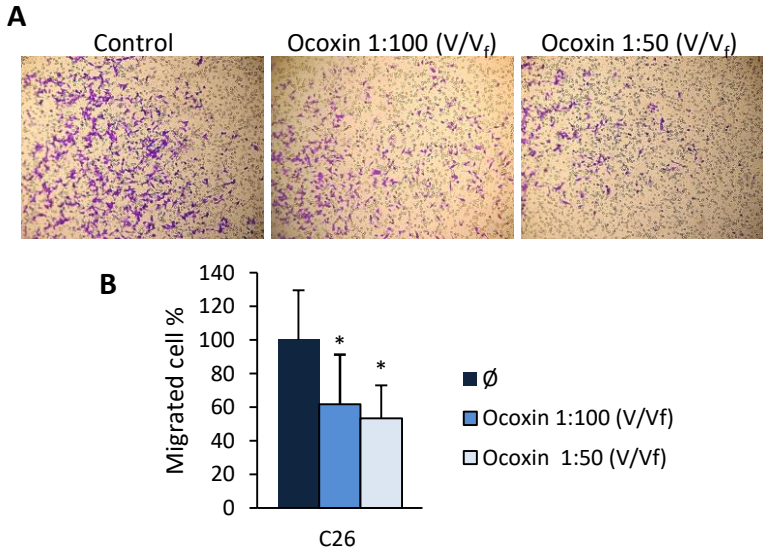



**Figure 36. Migratory capacity of C26 cells treated with Ocoxin.** C26 murine colorectal cells, were let migrate for 18 hours through 8 µm-diameter pore transwell inserts under the presence of 1:50 and 1:100 ($V/V_f$) dilutions of Ocoxin. A) Representative microphotographs of insert membranes containing stained migrated cells. B) Quantification of migrated cells. Significances were expressed as *p<0.05 comparing the different treatments versus untreated cells (Ø) according to the Student's T-test.

## 4.3. Ocoxin treatment slowed down cell cycle of the C26 murine colorectal cancer cells.

Since we have observed that Ocoxin affects C26 tumor cell viability, we afterwards analyzed if this effect could be due to a reduction or a delay in cell division. In order to know if Ocoxin has an influence on cell cycle, C26 cells were cultured for 48 hours with the 1:100 ($V/V_f$) dose of the nutritional mixture, the least concentrated dilution which inhibited C26 proliferation. On the one hand, some cells were labeled with CFSE before the treatment to analyze differences regarding cell division and on the other hand, some other cells were stained with PI after the treatment and the amount of cells in each cell cycle phase was quantified by flow cytometry.

As shown in figure 37 A, an increase of cells gated in phase $G_2$/M and Sub $G_1$ and a decrease of those in phase S was observed in cells treated with Ocoxin compared to the untreated cells. Regarding $G_0/G_1$ phase, no changes were observed (Figure 37 A). Moreover, according to the CFSE labelling, cells treated with Ocoxin presented a decrease by 29 % on cell division (Figure 37 B) compared to control cells. This reduction could be attributed to the increase of cells gathered in the apoptotic phase Sub $G_1$ and pre-mitotic phase $G_2$/M observed with the PI staining.

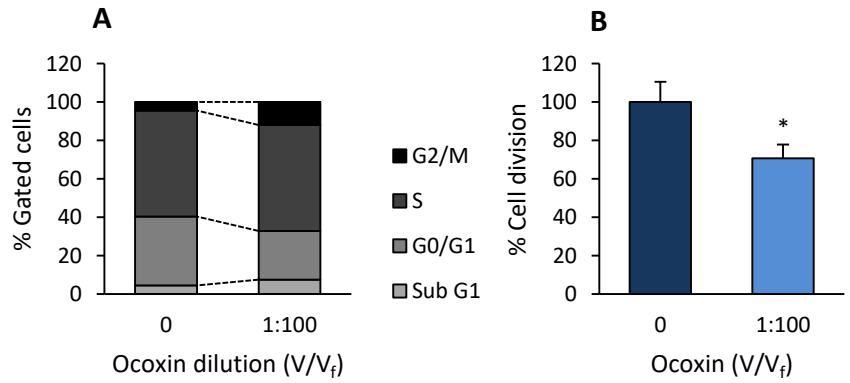



**Figure 37. Cell division and cell cycle analyses on C26 cells treated with Ocoxin.** CRC C26 cells were treated with the 1:100 ($V/V_f$) dilution of Ocoxin for 24 hours and a flow cytometry analyses were carried out. A) Cells gated in each cell cycle phase according to the PI assay. B) Cell division rate according to CFSE labeling. Significances were expressed as *$p<0.05$ comparing the different treatments versus untreated cells according to the Student's T-test.

## 4.4. Ocoxin enhanced the effect of the chemotherapeutic agent irinotecan on C26 murine colorectal cancer cell viability.

Once the anti-tumoral effect of Ocoxin was assessed on the viability, cell cycle and on the migratory potential of C26 cells, we attempted to analyze the influence of the nutritional supplement in the efficacy of the chemotherapeutic agent irinotecan. First of all, different concentrations of irinotecan were tested on C26 cells to select the most effective one. Although every dose reduced cell viability compared to the untreated cells, only the highest doses (50 µM, 75 µM and 100 µM) decreased it by more than 50 % (Figure 38). Thus, the concentration of 50 µM was used in the following experiments, since it was the lowest dose which halved cell viability.

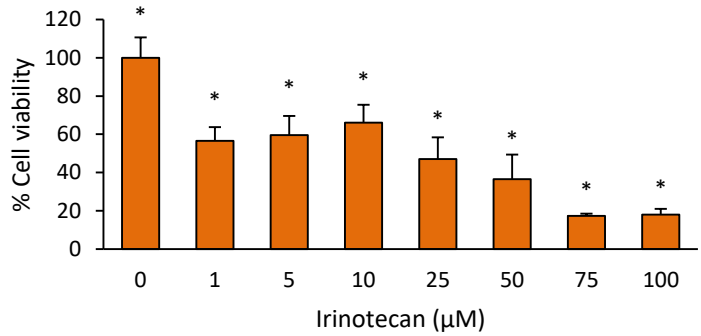



**Figure 38. Viability of C26 cells treated with increasing concentrations of irinotecan.** Cells were treated with different doses of irinotecan from 1 µM to 100 µM for 48 hours and then cell viability was quantified. Significances were expressed as *p<0.05 comparing the different treatments versus untreated cells according to the Student's T-test.

After choosing the most effective doses, in particular 50 µM of irinotecan and 1:100 ($V/V_f$) of Ocoxin, the viability of C26 cells was evaluated in presence of the combination of both compounds. Results displayed that while Ocoxin and irinotecan alone reduced C26 viability by 37 % and 32 % respectively, the combination of 50 µM of irinotecan and 1:100 ($V/V_f$) of Ocoxin acted synergically decreasing cell viability up to 65 % (Figure 39).

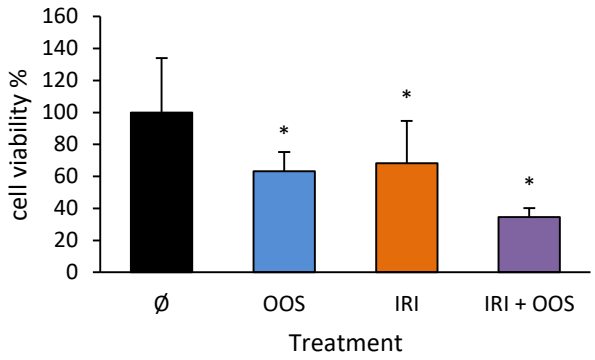



**Figure 39. Viability of C26 cells treated with Ocoxin as a complement to irinotecan.** C26 murine CRC cells were cultured for 48 hours with either Ocoxin or irinotecan alone, or combined and cell viability was quantified. Significances were expressed as *p<0.05 comparing the different treatments versus untreated cells and as # p<0.05 comparing irinotecan treatment versus the combination of irinotecan and Ocoxin according to the Student's T-test. *IRI: Irinotecan; OOS: Ocoxin; Untreated (Ø).*

# 5. EFFECT OF OCOXIN ON TUMOR DEVELOPMENT IN A MURINE MODEL OF LIVER METASTASIS OF COLORECTAL CANCER *IN VIVO*.

## 5.1. Ocoxin did not change bodyweights but improved physical sequels derived from metastatic development.

Besides the histological analyses, the external and internal physical condition of mice was evaluated right before and after sacrifice. Bodyweights were unchanged with all the three treatments compared to the untreated animals (Figure 40).

However, mice from each group showed different physical conditions and metastatic development. As seen in Table 23, untreated animals mainly showed tumor dissemination, rough fur and testicular inflammation. Besides, irinotecan treatment provoked testicular inflammation in 80 % of mice. On the contrary, these conditions were not observed in those animals treated with Ocoxin or the coadjuvant therapy.

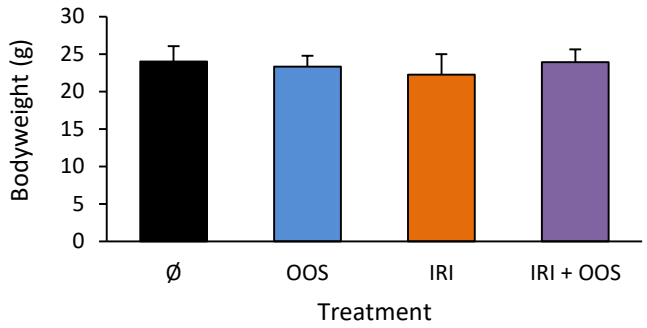



**Figure 40. Bodyweights of mice with CRC liver metastasis treated with Ocoxin, irinotecan and both compounds.** No significant differences between groups were observed according to the Student's T-test. *IRI: Irinotecan; OOS: Ocoxin; Untreated (Ø).*

**Table 23. Percentage of mice with metastatic CRC showing health impairment aspects after receiving different treatments.**

| | Ø | OOS | IRI | IRI + OOS |
|---|---|---|---|---|
| **Ascites** | 8 % | - | - | - |
| **Impaired liver blood circulation** | 8 % | 8 % | - | 9 % |
| **Peritoneal / Intestinal dissemination** | 33 % | - | - | 9 % |
| **Rough fur** | 17 % | - | - | - |
| **Testicular inflammation** | 67 % | - | 80 % | - |

*Ø: Untreated; IRI: Irinotecan; OOS: Ocoxin*

## 5.2. Ocoxin altered tumor growth differently depending on the administration pattern.

Based on our *in vitro* results, the anti-tumoral effect of Ocoxin was also studied *in vivo*. To begin with, Balb/c mice were treated according to different administration patterns and doses of Ocoxin as described in Methods (Figure 13 B). Briefly, C26 cells were injected to four groups of mice that were treated with the nutritional mixture before or after receiving the tumor cell injection. Once livers of those mice were collected and processed for histological analyses, liver slides were stained with H&E and the area occupied by tumor foci was quantified in each liver.

As shown on figure 41, the preventive treatment of Ocoxin did not affect liver tumor growth with any dose when compared to untreated mice. On the other

hand, livers obtained from the continuous group revealed an increase of the tumor mass with all the doses, although this rise was not significant due to the high variability of data. Lastly, livers collected from mice of the regressive group showed a significant reduction of the liver area occupied by tumor mass when mice were treated with either 100 µl, 200 µl or 500 µl of Ocoxin, being 100 µl the most effective dose. In this sense, metastatic area was significantly decreased by 92 % and 88 % with 100 µl and 200 µl of Ocoxin respectively (Figure 41). Hence, the regressive administration pattern and the dose of 100µl of Ocoxin were chosen for the next experiments as it showed to be the most efficient treatment.

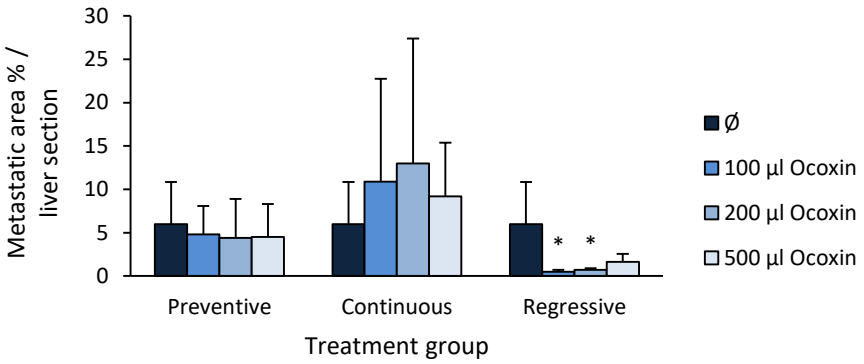



**Figure 41. Effect of different treatment administration patterns of Ocoxin on the metastatic area of liver sections.** Animals were treated with 100 µl, 200 µl or 500 µl of Ocoxin according to the preventive, continuous and regressive treatment administration patterns and livers were collected in order to quantify metastatic areas after H&E staining. Significances were expressed as *p<0.05 comparing the different treatments versus untreated mice (Ø) according to the Student's T-test.

Bearing in mind the previous results, for the next *in vivo* experiments mice were treated with 100 µl of Ocoxin following the regressive treatment administration pattern (Figure 13 B), that is, animals were treated for 2 weeks starting 7 days after tumor cell injection once metastatic foci were formed in the liver. Nonetheless, considering the promising results of the compound in the previous *in vivo* experiment a lower dose of 50 µl of Ocoxin was additionally used in the following experiments and further analyses were carried out. As described before, after the treatment livers were collected, fixed in zinc solution and embedded in paraffin.

## 5.3. Ocoxin reduced tumor foci number, size and the metastatic area in mice bearing liver metastasis of colorectal cancer.

Afterwards, liver sections were stained with H&E for histological analyses ( Figure 42). First, the number of foci was quantified in each section, which showed a decrease by 62 % in mice treated with 50 µl of Ocoxin and by 75 % when treated with 100 µl (Figure 43 A). Furthermore, the quantification of tumor foci based on their size confirmed that this treatments reduced the number of foci which were bigger than 0.4 $mm^2$ significantly by 64 % and 85 % with the 50 µl and 100 µl doses respectively compared to those observed in untreated animals (Figure 43 B). Lastly the liver section area occupied by tumor was also measured showing a significant decrease with both Ocoxin doses.

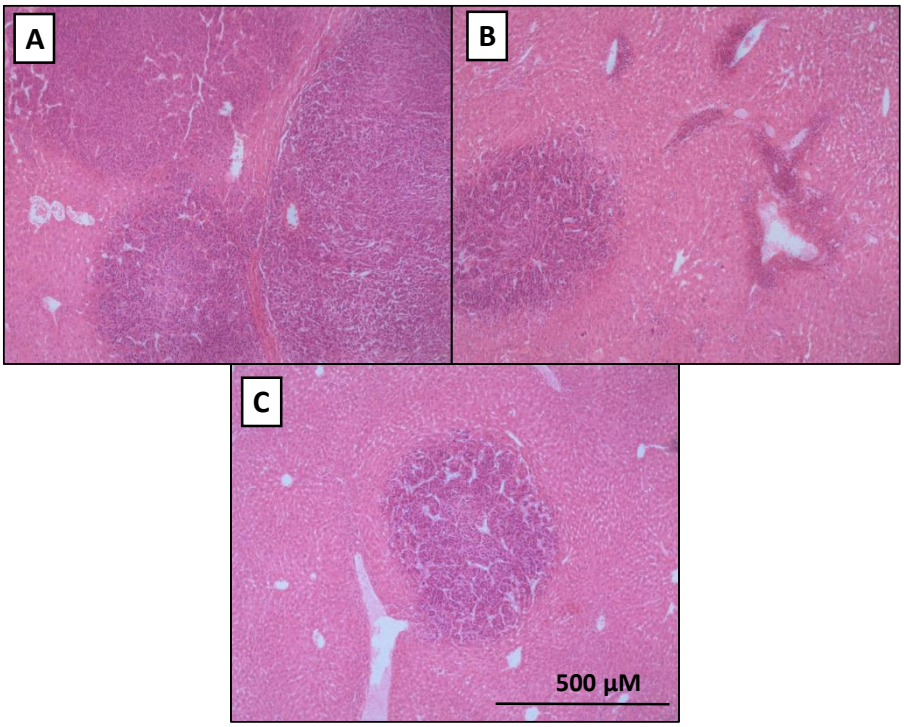



**Figure 42. Representative microphotographs of Hematoxylin/Eosin staining in liver sections of mice bearing hepatic metastasis of CRC treated with different doses of Ocoxin.** Liver sections of mice treated according to the regressive group. A) Liver of an untreated mouse. B) Liver of a mouse treated with 50 µl of Ocoxin. C) Liver of a mouse treated with 100 µl of Ocoxin.

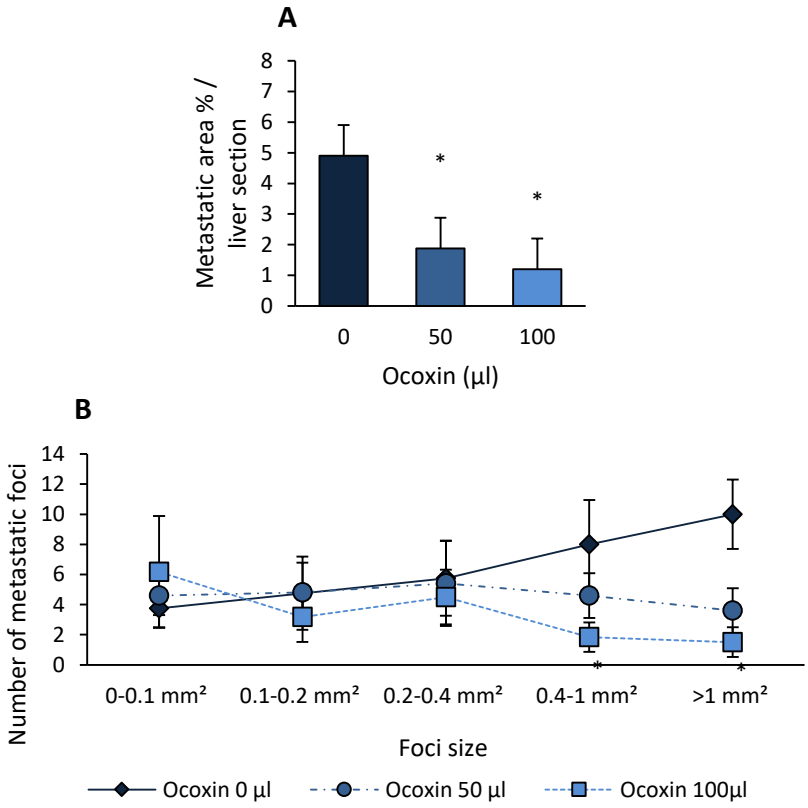



**Figure 43. Quantification of the metastatic area and tumor foci number in mice with hepatic metastasis of CRC treated with Ocoxin.** Mice were treated with 50 µl and 100 µl of Ocoxin daily beginning 7 days after tumor cell inoculation and then organs were collected and stained with H&E. A) Metastatic area of liver sections. C) Size and number of foci observed in liver slides. Significances were expressed as *p<0.05 comparing the different treatments versus untreated mice according to the Student's T-test.

## 5.4. Ocoxin reduced proliferation and increased apoptosis in metastatic liver tumors.

In order to know more about the mechanisms implicated in the reduction of the tumor growth altered by Ocoxin, cell proliferation and apoptosis levels within the tumors were analyzed (Figure 44). Therefore, liver sections were incubated with antibodies against Ki67 and Caspase-3 to determine whether Ocoxin caused a decrease in cell proliferation or an increase in apoptosis levels. As shown on figure 45 A and B no significant differences were detected on tumors of mice that were treated with 50 µl of Ocoxin compared to the

untreated mice in any of the two markers. However, while in mice treated with 100 µl of Ocoxin the proliferative Ki67 marker halved, Caspase-3 expression within tumors was increased 5-fold in mice treated with 50 µl and 10-fold with 100 µl of Ocoxin (Figure 45 A and B).

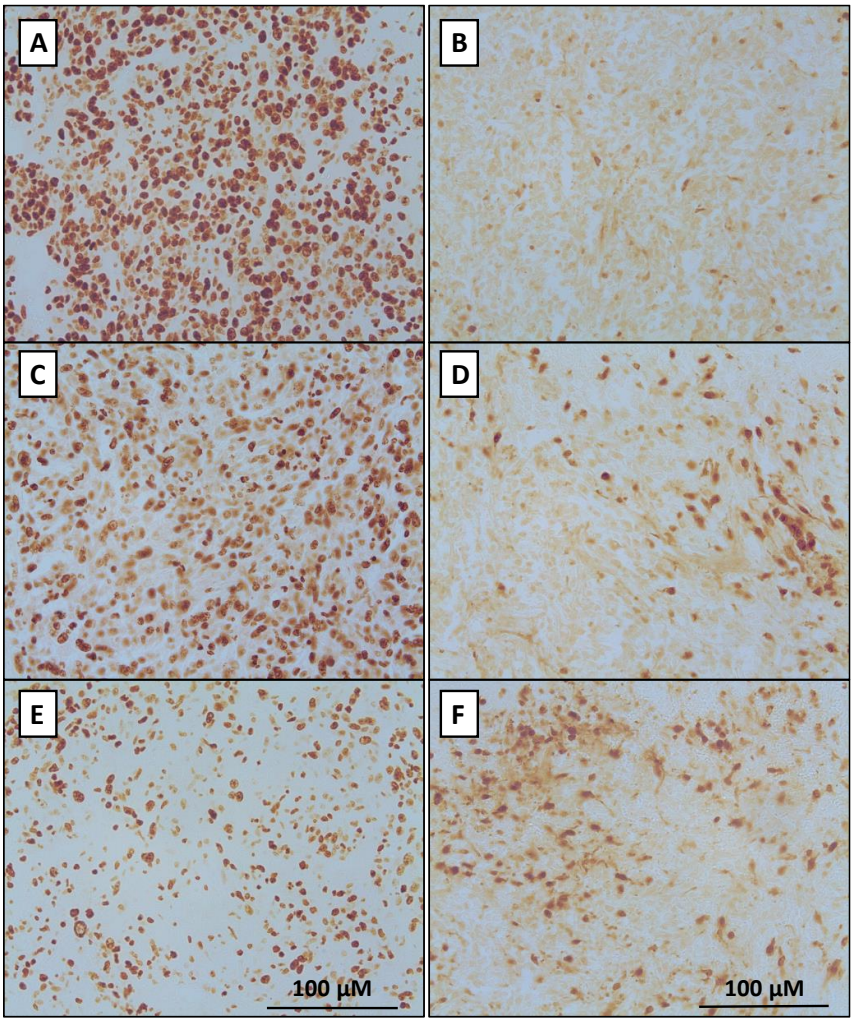



**Figure 44. Proliferation and apoptosis marker expression in liver sections of mice with hepatic metastasis of CRC.** Immunohistochemical analyses were performed in order to detect cell proliferation (Ki67) and apoptosis (Caspase-3) in liver tumors of mice treated with different doses of Ocoxin. A) Expression of Ki67 and B) expression of Caspase-3 in a liver tumor of an untreated mouse. C) Expression of Ki67 and D) expression of Caspase-3 in a liver tumor of a mouse treated with 50µl of Ocoxin. E) Expression of Ki67 and F) expression of Caspase-3 in a liver tumor of a mouse treated with 100µl of Ocoxin.

The ratio between the Ki67 and Caspase-3 expression % per tumor area was calculated so as to visualize the anti-tumoral effects of Ocoxin more clearly. As shown in figure 45 C, a reduction was observed in the Ki67/Caspase-3 expression % ratio in the livers of animals treated with the nutritional supplement, meaning that, compared to the livers of untreated animals, in this tumors there were more cells undergoing apoptosis than proliferating.

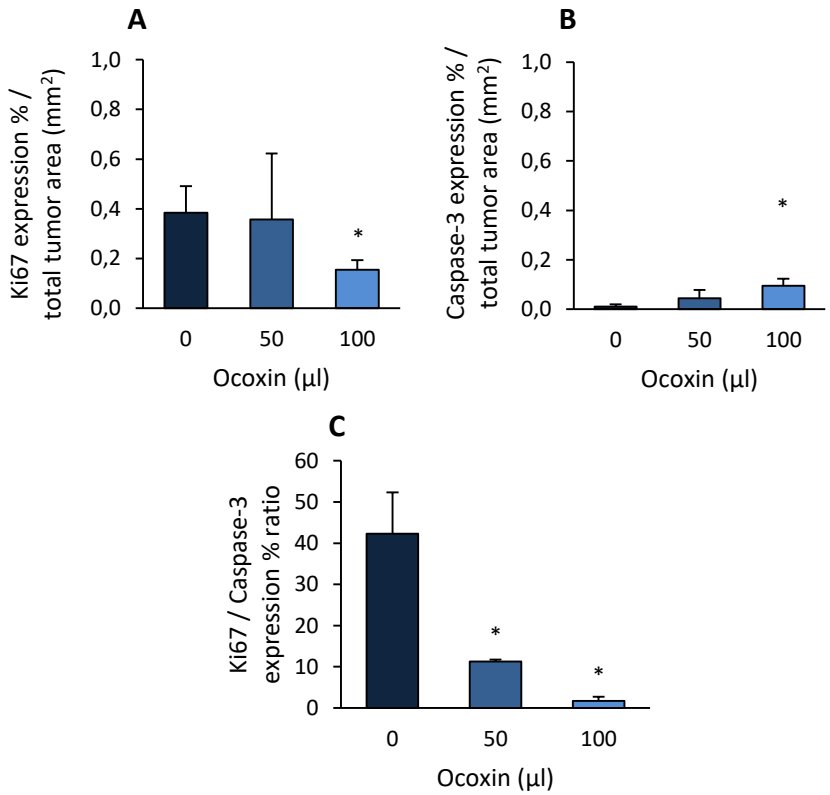



**Figure 45. Quantification of proliferation and apoptosis marker expression in liver sections of mice with hepatic metastasis of CRC.** Immunohistochemical analyses were performed in order to detect cell proliferation (Ki67) and apoptosis (Caspase-3) in liver tumors of mice treated with different doses of Ocoxin. A) Quantification of cell proliferation. B) Quantification of apoptosis. C) Ratio between cell proliferation and apoptosis. Significances were expressed as *p<0.05 comparing the different treatments versus untreated mice according to the Student's T-test.

## 5.5. Ocoxin reduced infiltration of activated fibroblasts and macrophages within metastatic liver tumors.

TME plays a key role in cancer development promoting tumor growth through different mechanisms. In this regard, fibroblasts and macrophages are known to infiltrate tumors enhancing cancer invasiveness. Hence, we analyzed by immunohistochemistry whether Ocoxin provoked any alteration in the recruitment of those cells within liver tumors in mice treated with 100 µl of the compound since that was the dose that showed the most significant differences regarding tumor cell proliferation and apoptosis. To do so, the expression of α-SMA and F4/80 was analyzed as indicators of HSC and macrophage infiltration respectively (Figure 46). The expression % of these markers was quantified in each tumor and then a mean value was calculated for the total tumor area in every liver (Figure 47).

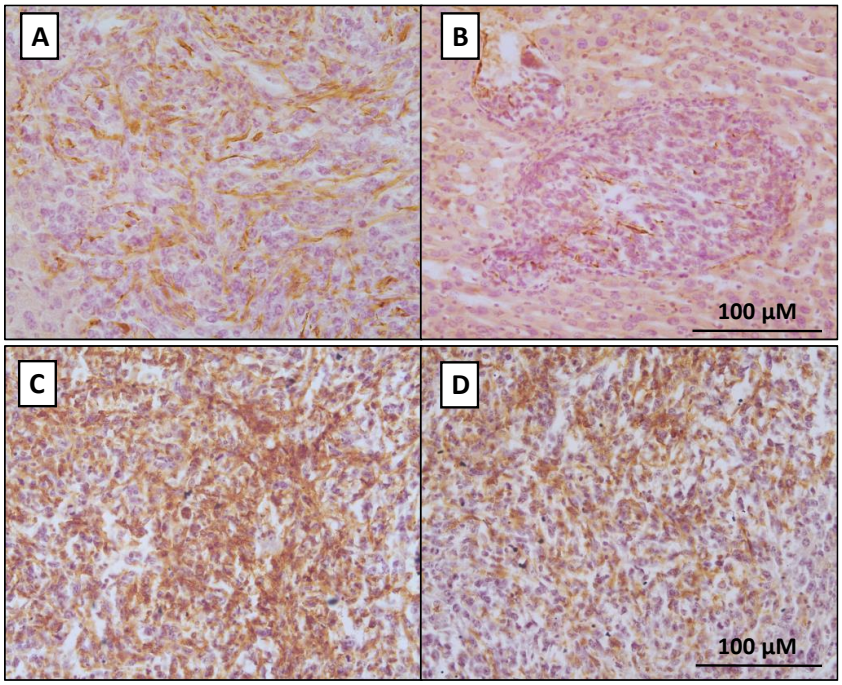



**Figure 46. HSC and macrophage marker expression in liver section of mice with hepatic metastasis of CRC.** Immunohistochemical analyses were performed in order to detect the infiltration of HSCs (α-SMA) and macrophages (F4/80) within the tumors of mice. A) Expression of α-SMA in a liver tumor of an untreated mouse. B) Expression of α-SMA in a liver tumor of a mouse treated with Ocoxin. C) Expression of F4/80 in a liver tumor of an untreated mouse. D) Expression of F4/80 in a liver tumor of a mouse treated with Ocoxin.

As shown in Figure 47 HSC and macrophage recruitment was reduced by 50 % and 60 % respectively within tumor foci developed in the livers of mice treated with 100 µl of Ocoxin compared to tumors present in livers collected from untreated mice.

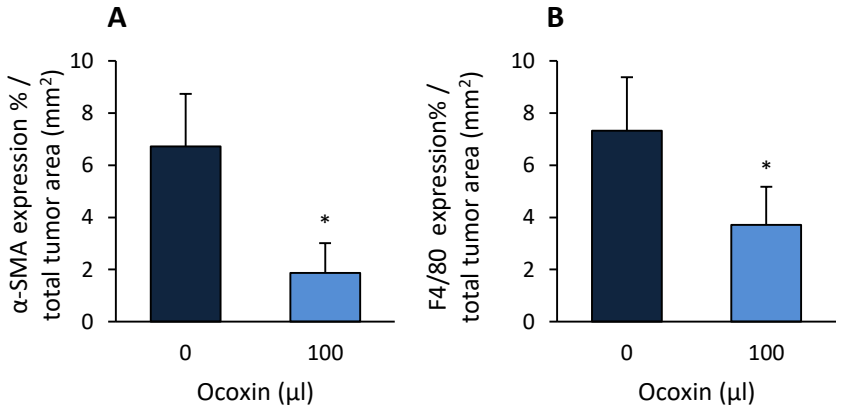



**Figure 47. Quantification of HSC and macrophage marker expression in liver section of mice with hepatic metastasis of CRC.** The expression of tumor infiltrating HSC (α-SMA) and macrophage (F4/80) markers was quantified in liver tumors of treated and untreated mice. A) Expression % of α-SMA. B) Expression % of F4/80. Significances were expressed as *p<0.05 comparing the different treatments versus untreated mice according to the Student's T-test.

## 5.6. Ocoxin modulated the expression of inflammatory mediators in livers of mice with metastatic colorectal cancer.

Cancer arises in association with inflammation in many cases. In fact, cytokines and inflammatory mediators are able to maintain the inflammatory response benefiting tumor growth. Thus, considering the anti-inflammatory properties of Ocoxin we quantified the gene expression levels of some cytokines and inflammatory mediators in livers collected from tumor bearing mice either untreated or treated with 100 µl of the compound as they represent a target for diagnostics and therapeutic strategies.

To do so, total RNA was extracted from liver tissue and the expression of COX2, IFNγ, IL-1β, IL-6, TNF-α and VEGF was quantified by qPCR. Results revealed that the administration of 100 µl of Ocoxin decreased gene expression levels of all the analyzed inflammatory mediators significantly in livers collected

from mice with CRC metastatic tumors, such as COX2, IFNγ, IL-1β, IL-6, TNF-α and VEGF (Figure 48).

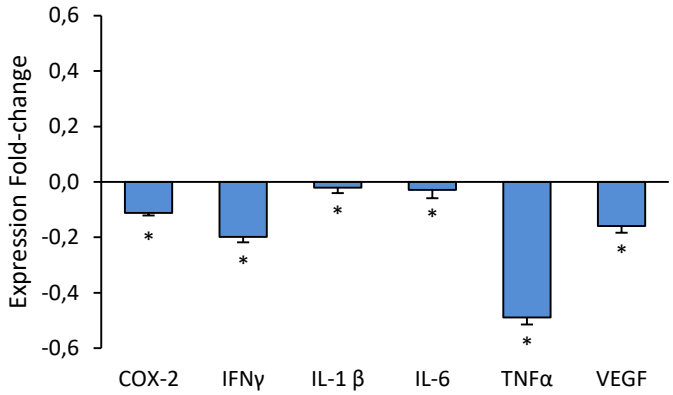



**Figure 48. Cytokine and inflammatory mediator expression fold-change in livers of mice bearing metastatic CRC treated with Ocoxin.** RNA was extracted from liver sections of mice bearing metastatic CRC that were treated with 100 µl of Ocoxin or untreated in order compare the expression levels of the inflammatory mediators in the organ by qPCR. Results were represented as fold-change values. Significances were expressed as *p<0.05 comparing the different treatments versus untreated mice according to the Student's T-test.

## 5.7. Effect of Ocoxin used as a coadjuvant to improve the anti-tumor activity of the chemotherapeutic agent irinotecan.

### 5.7.1. The addition of Ocoxin to irinotecan did not reduce the metastatic area more than the chemotherapeutic agent administered alone.

Considering that Ocoxin enhanced the effect of the chemotherapeutic agent irinotecan against C26 tumor cells *in vitro* and also bearing in mind the anti-proliferative and pro-apoptotic action of Ocoxin in liver metastatic tumors of CRC, we studied whether the anti-tumor effect of irinotecan could be enhanced by the supplementation of the compound *in vivo*. Therefore, both compounds were administered to mice following the pre-established regressive administration pattern treatment (Figure 13 B), that is, starting 7 days after tumor cell inoculation, but with a daily dose of 100 µl of Ocoxin and 20 mg/Kg of irinotecan every two days. Results showed that Ocoxin and irinotecan administered alone reduced metastatic tumor area by 52 % and 92 % respectively compared to the untreated group, while the combined treatment, reduced tumor-occupied area by 88 % (Figure 49). Nevertheless no

significant differences were observed between the liver metastatic areas of mice treated with irinotecan alone or with the combination, pointing out that no synergistic effect was produced between the two compounds.

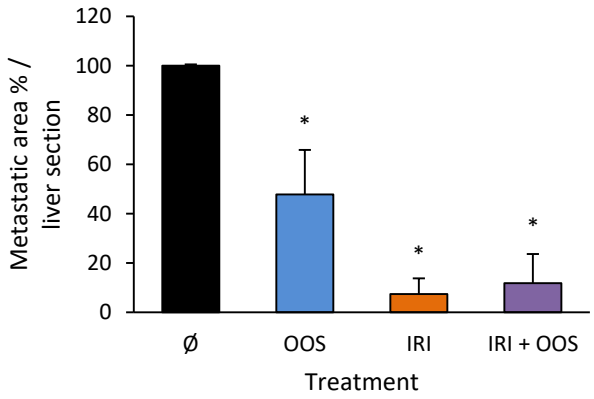



**Figure 49. Metastatic area of liver section of mice bearing hepatic metastasis of CRC treated with Ocoxin as a complement to irinotecan.** Animals were treated with 100 µl of Ocoxin alone, irinotecan 20 mg/Kg alone or both concomitantly in order to analyze whether Ocoxin could serve as a coadjuvant agent administered together with irinotecan. Significances were expressed as *p<0.05 comparing the different treatments versus untreated mice according to the Student's T-test. *IRI: Irinotecan; OOS: Ocoxin, Ø: Untreated.*

### 5.7.2. The supplementation of Ocoxin potentiated the effect of irinotecan by further decreasing the proliferative index and enhancing apoptosis levels in metastatic liver tumors.

Following the same experiments performed before to look into the mechanisms that explain the reduction of tumor areas in livers of mice treated with Ocoxin, we analyzed again if the addition of the nutritional supplement to irinotecan provoked a further reduction of cell proliferation and an increase of apoptosis. Results shown in figure 50 A revealed that Ocoxin and irinotecan administered alone decreased Ki67 expression by 80 % in both cases and the combined treatment decreased proliferation by 93 %. Nevertheless, differences between irinotecan alone and the combined treatment were not significant. On the other hand, caspase-3 quantification showed a 3-fold and 12-fold significant increase of apoptosis in livers of mice treated with Ocoxin and irinotecan alone respectively compared to livers of untreated mice (figure 50 B). Furthermore, when irinotecan was supplemented with Ocoxin a 5-fold increase was observed in the apoptotic marker compared to that of the untreated animals. However, the differences between apoptotic levels

between irinotecan and the coadjuvant treatment were not significant. Hence, the addition of Ocoxin did not improve the anti-proliferative and pro-apoptotic effect of irinotecan.

Finally, the ratios between proliferation and apoptosis marker expression % showed a reduction up to 90 % in livers obtained from mice under Ocoxin treatment and up to 99 % in those which received either irinotecan alone or the combined therapy (Figure 50 C) meaning that in treated mice there were less cells proliferating than undergoing apoptosis.

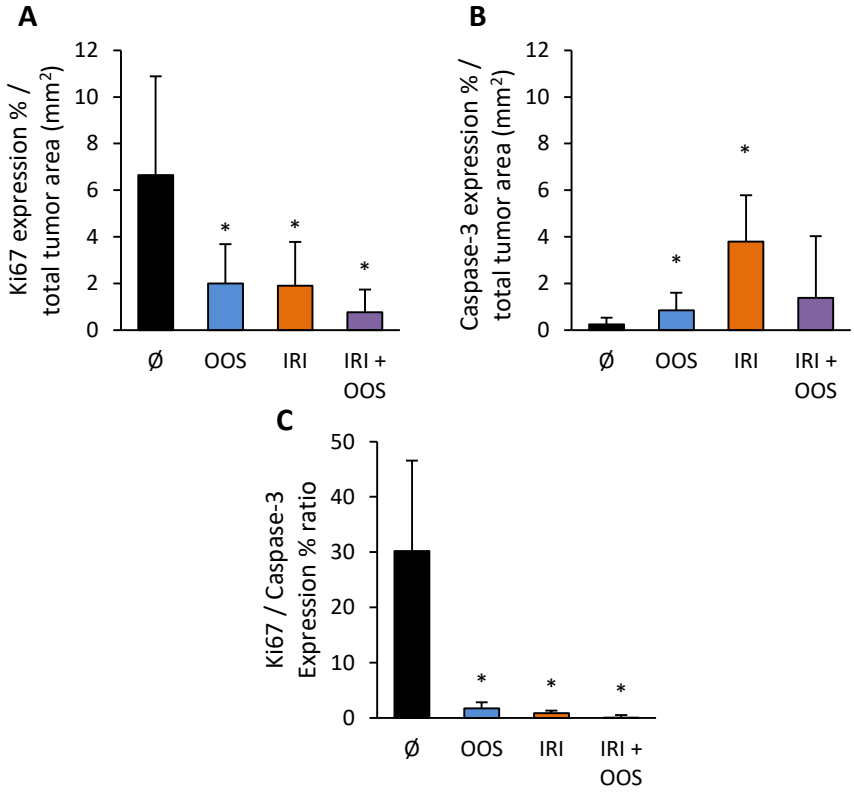



**Figure 50. Proliferation and apoptosis marker expression in liver sections of mice with hepatic metastasis of CRC treated with Ocoxin as a complement to irinotecan chemotherapy.** Proliferation and apoptosis were analyzed by immunohistochemistry in tumors of mice treated with 100 µl of Ocoxin and 20 mg/Kg of irinotecan using the markers Ki67 and Caspase-3 respectively. A) Proliferation analysis using an anti-Ki67 antibody. B) Apoptosis analysis using an anti-Caspase-3 antibody. C) Ratio between Ki67 and Caspase-3. Significances were expressed as *p<0.05 comparing the different treatments versus untreated mice according to the Student's T-test. *IRI: Irinotecan; OOS: Ocoxin; Ø: Untreated.*

## 5.8. Ocoxin alone and administered as a complement to the chemotherapeutic agent irinotecan modulated the gene signature of tumor cells extracted from livers of mice with metastatic colorectal cancer.

Cancer cells usually show an altered gene expression pattern. For that reason we aimed to compare the gene signature of tumor cells isolated from liver explants collected from tumor bearing mice that were either untreated or treated with 100 µl of Ocoxin, 20 mg/Kg of irinotecan or the combination of both After cancer cell isolation, RNA was purified for the analysis of gene expression by using microarray technology.

Results showed that Ocoxin treatment alone changed the expression of 35 genes while irinotecan altered 152 (Table 22). Moreover, the supplementation with Ocoxin resulted in the reversal of most of the deregulations observed when mice were treated with irinotecan alone; only 6 genes were altered under the combined treatment. Nevertheless, just one gene which codes for Arglu1 presented an altered expression in every group. Furthermore, when the gene expression levels were compared between the combined treatment and irinotecan alone only 14 genes showed an altered expression, all of them were upregulated. However, none of these genes was altered with irinotecan alone meaning that Ocoxin did not revert the gene deregulation caused by irinotecan treatment. Finally, it is interesting to note that 93 % of the altered genes of mice treated with irinotecan were downregulated, while Ocoxin treatment upregulated 85 % and 83 % of them when used alone or in combination with irinotecan respectively (Table 24). All the mentioned altered genes were significantly altered with a >1.45 fold-change.

**Table 24. Summary of the amount of genes deregulated by Ocoxin, irinotecan or the combination of both in liver explants obtained from mice with CRC metastasis.**

| | Upregulated | Downregulated | Total |
|---|---|---|---|
| **Ocoxin vs Untreated** | 30 | 5 | 35 |
| **Irinotecan vs Untreated** | 11 | 141 | 152 |
| **Ocoxin + Irinotecan vs Untreated** | 5 | 1 | 6 |
| **Ocoxin + Irinotecan vs Irinotecan** | 14 | 0 | 14 |

After obtaining this results, all the significantly altered genes were compared with those available in the CoReCG database in order to confirm whether they had been previously related to colorectal cancer. Surprisingly, none of our deregulated genes was recognized by the database as genes involved in colorectal cancer development.

Later, all the deregulated genes were analyzed and classified according to their molecular function using the PANTHER (v.10.0) software. Regarding the gene deregulation observed in liver explants obtained from mice treated with 100 µl of Ocoxin, results revealed that most of the of these genes act as binding proteins and catalytic agents but also as molecular function regulators, molecular transducers, transcription regulators and transporter activities (Table 25). Later, genes altered by irinotecan showed that the drug altered more genes than Ocoxin and most of them were also mainly related to catalytic and binding activities together with those mentioned before. Finally, the adjuvant treatment showed to alter less genes but they were also related to catalytic and binding functions.

Lastly, genes were also classified according to the biological process they take part in. Most of the genes deregulated by Ocoxin were involved in cellular and metabolic processes and biological regulation (Table 26).

**Table 25. Classification of genes deregulated by Ocoxin according to their molecular function.**

| Molecular function | Genes |
|---|---|
| **Binding** | 10 |
| **Catalytic activity** | 7 |
| **Molecular function regulator** | 6 |
| **Molecular transducer** | 5 |
| **Transcription regulator** | 2 |

**Table 26. Classification of genes deregulated by Ocoxin involved in biological processes.**

| Biological Process | Genes |
|---|---|
| **Cellular process** | 13 |
| **Biological regulation** | 9 |
| **Metabolic process** | 5 |
| **Multicellular organismal process** | 3 |
| **Localization** | 3 |

For further information a list of all the deregulated genes can be found in Appendix II.

# V. DISCUSSION
# V. EZTABAIDA

Cancer is one of the leading causes of mortality worldwide. As reported by the WHO, 9.6 million people died from cancer in 2018 and 18.1 million new cases were diagnosed. Among all, CRC is one of the most common cancers, taking the second position of deaths caused by this disease. Even though the overall 5-year life expectancy of patients has increased during the past years due to new therapies, people die owing to the spread of malignancy to other organs, especially the liver (Paschos et al., 2009). Other less frequent cancers, such as PCA, present an elevated mortality rate with 5-year survival rates below 9 % as a result of the difficulties to diagnose the malignancy and the lack of effective treatments (Ilic & Ilic, 2016). Over the past years, chemotherapy has become one of the most common routine treatments in clinical settings when surgery is not an option and also as an adjuvant therapy (Novotny & Szekeres, 2003). However, side effects provoked by chemotherapy and the development of chemoresistance have increased the interest of investigating new compounds in order to improve drug efficacy, to reduce the adverse effects, to increase life-quality and also to diminish or even reverse chemoresistance. In that regard, several studies support the anti-cancer properties of antioxidants, polyphenols, vitamins, minerals and many other natural compounds (Davis-Yadley & Malafa, 2015; Dutta, S. et al., 2019; Shirakami & Shimizu, 2018; Zhang et al., 2014; Zhao, Q. et al., 2015). Thereby, in this work we analyze the effect of a nutritional mixture named Ocoxin which includes biologically active compounds, mainly derived from green tea, licorice and cinnamon, as well as vitamins and oligoelements, some of which are known to exert anti-tumor activity (Dutta, A. & Chakraborty, 2018; Gloria et al., 2014; Gol'dberg et al., 2008; Park, E. Y. et al., 2008; Shirakami & Shimizu, 2018; Yang & Wang, 2010). This compound possesses anti-inflammatory, antioxidant, and immunomodulatory properties which, considering the role of ROS, inflammation and immunity in cancer development, could be useful to treat this disease. Thus, Ocoxin was studied as a possible anti-tumor agent to directly face cancer, to further improve the effectiveness of chemotherapeutic drugs, to reduce TME induced chemoresistance and also to minimize the undesired side effects in two different cancer models, PCA and liver metastasis of CRC.

# 1. OCOXIN AS A SUPPLEMENT TO REINFORCE THE CYTOTOXIC EFFECT OF CHEMOTHERAPY

The effect of Ocoxin on the viability of tumor cells reflects the anti-tumoral capacity that many natural compounds have shown previously in a great number of reports. For example, a mixture containing green tea extract, ascorbic acid, lysine and proline was cytotoxic against different cancers like hepatocellular carcinoma (HCC), glioblastoma, CRC or PCA *in vitro* (Roomi et al., 2010; Roomi et al., 2011; Roomi et al., 2014; Roomi et al., 2015b). Likewise, a seven-herb Chinese medicine formula named Qingyihuaji reduced PCA cell viability (Wei et al., 2019; Xu, Y. et al., 2015). Moreover, a polyphenol-rich extract induced cytotoxicity in human breast cancer, lung cancer, CRC and PCA cells although this compound did not affect equally each cell line showing tumor-type specific actions (Zhao, Q. et al., 2015). Similarly, we found out that even though Ocoxin reduced cell viability in all the studied cell lines, murine tumor cells were more sensitive than the human ones, and even more, HPAF-II and SW1990 PCA cells showed higher resistance to Ocoxin than the rest of the tested human PCA cells. Coincidentally, these two cell lines come from patients with metastatic cancer, thus, the aggressiveness of those tumor cells might have impaired the anti-tumoral efficacy of Ocoxin against the malignant cells. Reinforcing our findings, Ocoxin exerted anti-proliferative actions in many other cancer cells too, such as breast cancer, HCC, AML small-cell lung cancer (SCLC) and glioblastoma *in vivo* (Diaz-Rodriguez et al., 2016; Diaz-Rodriguez et al., 2017; Díaz-Rodríguez et al., 2018; Hernandez-Garcia et al., 2015; Hernandez-SanMiguel et al., 2019; Perez-Pena et al., 2019).

As we observed, the mechanism by which Ocoxin causes the decrease of cell viability after Ocoxin treatment was in part mediated by an increase of apoptosis and a reduction of cell proliferation. In this regard certain compounds included in the formulation of Ocoxin could be responsible for the observed effects. Licorice root extracts, for instance, suppress proliferation and increase apoptosis in PCA cell lines, and green tea has shown an anti-proliferative and apoptotic effect in several cancers (Shirakami & Shimizu, 2018; Yang & Wang, 2010; Zhao, C. et al., 2020). Accordingly, the human PCA cell lines treated with Ocoxin analyzed in this work showed higher cell death levels *in vitro*. Supporting those results, we also observed a reduction of tumor development in mice treated with Ocoxin. This effect of the nutritional mixture in PCA-bearing animals was identified by a lower expression of the

specific 266-6 tumor-cell marker and by a decrease in size and tumor foci number in mice bearing metastatic CRC. Moreover, those metastatic tumors showed a reduction of cell proliferation and an increase of apoptosis in livers of mice that had been treated with Ocoxin. However, such an observation could not be confirmed in the PCA model probably, due to the slow tumor development of 266-6 cells.

As reported by Niedzwiecki et al., a combination of green tea, ascorbic acid, amino acids, and oligoelements exerted an anti-cancer effect in metastatic liver tumors of CRC by causing a decrease in tumor size and the mean number of colonies through the inhibition of cell proliferation and migration, along with an increase of apoptosis *in vitro* (Niedzwiecki et al., 2016). Also, Ocoxin reduced tumor progression *in vivo* in a breast cancer model by an rise of apoptosis with a concomitant decrease of tumor cell proliferation in SCLC (Díaz-Rodríguez et al., 2018; Hernandez-Garcia et al., 2015). In contrast, Ocoxin reduced tumor cell proliferation without an augment of apoptosis in HCC and AML indicating, once more, that the compound acts differently in each tumor type (Diaz-Rodriguez et al., 2016; Diaz-Rodriguez et al., 2017). In relation to this, numerous reports about some elements that are part of Ocoxin have showed similar results when administered independently. In a rat gastrointestinal cancer model, Xu et al. observed a pro-apoptotic and anti-proliferative effect of EGCG and folic acid (Xu, Q. et al., 2011) and Dutta and Chakraborty detected an increase of caspase-3 activity after cinnamon intake together with the reduction of tumor cell proliferation in melanoma and skin cancer models (Dutta, A. & Chakraborty, 2018). Surprisingly, while PCA cells did not show any alteration in the expression of genes related to proliferation and apoptosis after Ocoxin treatment, CRC cells showed many deregulated genes involved in this process. Contrary to what we expected, the nutritional supplement provoked an upregulation of several positive cell proliferation regulators and apoptotic repressors, such as CDK13, IL1B, IL11, MARCKSL1, NOL3, NTRK1, and PGGT1B. On the contrary, the compound only downregulated one gene known to promote cell proliferation in CRC, KIF14 (Wang, J. et al., 2018). This suggests that other processes might be counteracting the effect of the upregulated genes. Moreover, a substantial amount of the genes altered by Ocoxin were involved in catalytic activities such as oxidoreductases or transferases. Oxidoreductases, known for their anti-tumor action in different cancers (Baryla et al., 2015; Krupenko & Krupenko, 2018; Kung et al., 2014), are downregulated in HCC compared to healthy liver tissue (Ngoka, 2008). Transferases are also fundamental to

control cancer development, since, for instance, aberrant glycosylation and hypermethylation could affect the interaction between ligands and receptors favoring cell proliferation, migration, and invasion (Sheta et al., 2016). Additionally, some ingredients of Ocoxin like resveratrol, curcumin, EGCG, vitamin B12 and folate revert hypermethylation of tumor suppressor genes in prostate, colon and breast cancers among others (Dammann et al., 2017; Pirouzpanah et al., 2015). Following the discussion about gene deregulation, Ocoxin altered the expression of many genes in mice bearing either PCA or CRC. Accumulating evidence reveals the capacity of natural compounds to modulate gene expression. For example, green tea polyphenols inhibited the expression of MMPs in breast cancer (Deb et al., 2015) and resveratrol changed gene-expression in prostate cancer (Jones et al., 2005). Here, on the one hand Ocoxin showed to affect the expression of 351 genes in PCA cells. Among them, 14 are known to be deregulated in PCA which interestingly got back to those levels observed in healthy pancreatic cells in the presence of Ocoxin. On the other hand the compound only altered 35 genes in CRC which are not known to be associated with colorectal or liver cancer at the moment. The reason for the difference between the amount of genes altered in each type of cancer could reside in the origin of the cells; whereas the effect of Ocoxin in PCA gene deregulation was studied in a stable cell line, gene alteration in metastatic CRC was analyzed in cells isolated from metastatic liver explants, which, unlike the pancreatic tumor cells, grew in the presence of many TME factors. Nonetheless, it is noteworthy that most of the genes deregulated by Ocoxin in both cancers were involved in binding and catalytic activities, which was also observed in SCLC and in AML (Diaz-Rodriguez et al., 2016; Díaz-Rodríguez et al., 2018). To our surprise, just Bmp7 and Cyth4, were altered by Ocoxin in both PCA and liver metastasis of CRC (upregulated) and only Elmo1, was altered in our PCA cells (downregulated) and in the model of SCLC of Pérez-Peña (upregulated) (Perez-Pena et al., 2019). Although Bmp7 expression has been associated with many cancers, different authors found that this gene is, as well, upregulated by bioactive compounds like honokiol, oridonin or resveratrol, (Liu, R. X. et al., 2017; Ren, C. M. et al., 2016; Zeng et al., 2017). In our favor, Yi et al. reported that the upregulation of Elmo1 mediated by a miRNA promoted the proliferation of HCC cells (Yi et al., 2019) and Liang et al. observed that the downregulation of Elmo1 expression reduced invasion in some breast cancer cells (Liang, Y. et al., 2018). Lastly, we have not found evidence about the involvement of Cyth4 in cancer. Taking all this into account and knowing that Ocoxin increased the expression of enzymes involved in catalytic activities that are found to be downregulated in

cancer, we speculate that part of the anti-cancer effect of the nutritional complement is related with gene regulation. Hence, more studies are needed to identify the causal relationship between the genes altered by Ocoxin and their role in cancer so as to identify the mechanism by which the compound contributes to tumor reduction.

Considering the effects of Ocoxin on tumor cell viability, the nutritional mixture was tested as a coadjuvant agent to enhance the antineoplasic action of chemotherapy. The *in vitro* administration of the first-line treatments irinotecan, paclitaxel and gemcitabine in combination with Ocoxin provoked a further increase in efficacy of the drugs in every tested cancer cell line, including those that were not affected by the supplement alone (HPAF-II and SW1900). Along with our results, Pires et al. observed a steeper decrease in human CRC cell viability when ascorbic acid was added to irinotecan, oxaliplatin or 5-FU, while, cinnamon and EGCG increased the cytotoxic action of gemcitabine in prostate cancer and PCA (Dutta, A. & Chakraborty, 2018; Pires et al., 2018; Wei et al., 2019). Furthermore, Ocoxin reduced cell viability in combination with lapatinib too in breast cancer cells and also administered with cytarabine, doxorubicin, or fludarabine in AML (Diaz-Rodriguez et al., 2016; Hernandez-Garcia et al., 2015). Moreover, it was observed that cinnamon improved the efficacy of oxaliplatin and 5-FU by increasing apoptosis in CRC (Sadeghi et al., 2019), that vitamin B enhanced the antineoplastic action of cisplatin by increasing cell death in different cell lines including the murine C26 cells (Aranda et al., 2015), and that resveratrol potentiated the effect of irinotecan reducing cell viability by activating p53 tumor suppressor and by inducing apoptosis (Aires et al., 2013). In the present study, although the addition of Ocoxin to the chemotherapeutic drugs further reduced cell viability, the increase of the apoptotic levels was not that evident neither in PCA cells nor in metastatic CRC cells, indicating that Ocoxin might enhance the cytostatic capacity of drugs rather than the cytotoxicity. Nevertheless, although the unchanged caspase-3 levels suggest that the addition of Ocoxin to irinotecan does not improve its cytotoxic capacity, this could not be confirmed due to the high variability of data between mice. Besides, instead of the intrinsic pathway that leads to the activation of caspase-3, apoptosis could have been mediated by caspase-8 which follows the extrinsic cell death pathway started by extracellular ligands such as TNF, Fas-ligand, or TNF-related apoptosis-inducing ligand (TRAIL) expressed by stromal cells (Goldar et al., 2015). In fact, in breast cancer, Ocoxin triggered apoptosis through both caspase-3 and caspase-8 (Hernandez-Garcia et al.,

2015). Thus, further studies at post-transcriptional levels or by targeting other cell death markers would be necessary so as to reveal the exact mechanisms by which Ocoxin exerts its functions. Additionally, we already mentioned that some of the genes altered by Ocoxin were related to catalytic activities. Besides transferases and oxidoreductases, the nutritional supplement modulated the expression of hydrolases, whose downregulation has been involved in chemoresistance in melanoma, CRC and breast and prostate cancers (Feferman et al., 2013). In fact, their upregulation has showed to enhance chemosensitivity (Brinkmann et al., 2013). Here we observed that irinotecan deregulated 152 genes in explant cells obtained from mice, while the combination of irinotecan and Ocoxin only displayed a number of 14 deregulated genes. This may denote that the nutritional mixture could avoid the alteration of some genes induced by irinotecan. However, none of the genes affected by the coadjuvant treatment were deregulated by the compounds independently. Hence, we consider that it would be more important to analyze the effect of the combination itself rather than analyzing the action of both agents on their own, since the treatments modify pathways instead of individual gene expression. The only difference between irinotecan treatment and the combined treatment was the alteration of enzyme regulators, suggesting that Ocoxin may possess an additional role in the regulation of the enzymatic activity regardless of its anti-inflammatory, immunomodulatory and antioxidant capacities. In this sense, the implication of Ocoxin on enzyme regulation deserves to be explored, since, instead of modifying enzyme expression directly, it could have modulated their activity.

Another mechanism to break down cancer progression is cell cycle arrest. In this way, cinnamon, licorice root, green tea and β-carotene among others, have showed to alter cell cycle (Dutta, A. & Chakraborty, 2018; Farooqui et al., 2018; Gloria et al., 2014; Singh, B. N. et al., 2011). Along with that, Ocoxin also affected the cell cycle in different cancers such as breast cancer, SCLC and AML. In this tumors, cell arrest occurred in the $G_0/G_1$ phase together with an upregulation of the cell cycle inhibitor p27 expression (Diaz-Rodriguez et al., 2016; Díaz-Rodríguez et al., 2018; Hernandez-Garcia et al., 2015). On the contrary, in HCC and in our models of CRC and PCA, cells were stacked in the G2/M phase. Besides, Pérez-Peña et al. found out that Ocoxin deregulated cell cycle checkpoint genes (Perez-Pena et al., 2019). In this work we detected the upregulation of NOL3 and PGGT1B in tumor cells obtained from livers of CRC-bearing mice, which are involved in the activation of the cell cycle. Additionally, natural compounds have also showed to synergize the effect of

chemotherapy by arresting the cell cycle. Such is the case of resveratrol, which enhanced the anti-tumor action of the active metabolite of irinotecan SN-38 and oxaliplatin by arresting cell cycle (Aires et al., 2013). In this regard, it is interesting to note that when Ocoxin was administered as a supplement to irinotecan, the expression of the genes mentioned before (NOL3 and PGGT1B) was unchanged compared to control mice. However, the upregulation of another couple of genes involved in cell cycle and division, ECT2 and NUF2, was observed indicating once more that Ocoxin regulates whole pathways instead of individual genes.

Apart from the mentioned mechanisms another factor involved in cancer aggressiveness is the migration of tumor cells. Related to this, natural nutrients have also shown to affect cell migration in addition to their cytostatic and cytotoxic properties. By using a specific nutrient mixture, Niedzwiecki et al. reported that their compound provoked a decrease on tumor cell migration apart from a reduction of tumor proliferation and an increase of apoptosis (Niedzwiecki et al., 2016),. Supporting those observations, Roomi et al. also observed that the same compound also affected cell proliferation and apoptosis equally in different cancers including CRC, HCC and PCA (Roomi et al., 2005a; Roomi et al., 2010; Roomi et al., 2015a). Furthermore, Ocoxin, as well as vitamin A and EGCG (Park, E. Y. et al., 2008; Wei et al., 2019), showed inhibitory effects on cell migration in CRC and PCA respectively. According to our *in vitro* studies Ocoxin inhibited murine C26 CRC cell migration, which could account for the decrease in the number of metastatic foci we observed *in vivo*. Nonetheless, this metastatic tumor reduction was only detected in mice that had been treated with the nutritional mixture once after the tumors were already stablished in the liver, while in animals treated before the metastatic spreading results were not significant. Thus, the anti-tumoral effect of Ocoxin could not be explained by the changes in the migratory capacity of cancer cells. However, Ocoxin also affected the migratory capacity of stromal cells, key elements on the promotion of cancer development.

# 2. OCOXIN AS A COMPLEMENT TO CHEMOTHERAPY TO REVERT CHEMORESISTANCE BY MODULATING TUMOR MICROENVIRONMENT

One of the biggest concerns during the treatment of cancer patients is the ability of tumor cells to develop *de novo* resistance to chemotherapy. This phenomenon depends not only on the phenotype of cancer cells, but also on the development of a hypoxic state, angiogenesis and the accompanying factors such as ECM composition and stiffness and growth factors, MMPs and cytokines (Yeldag et al., 2018) together with the participation of all tumor and host cells which produce them (Ferdek & Jakubowska, 2017; Komohara & Takeya, 2017; Rupaimoole et al., 2016; Wang, M. et al., 2017). In this regard, the concomitant administration of chemotherapy and some natural elements or nutritional mixtures have showed to improve chemoresistance in many cancers (Espey et al., 2011; Hong et al., 2015; Pires et al., 2018; Su, P. et al., 2018).

Ocoxin is intended for oral administration. Through this route, the compound reaches not only tumor cells but also host organs. Thus, we first analyzed the effect of the supplement on healthy cell lines that make up the TME. Interestingly, fibroblasts and macrophages were only affected by the highest doses of Ocoxin, pointing out that PCA and CRC cells were more sensitive to the compound than the healthy stromal cells. This correlates with the study of Khazraei-Moradian et al., where they found out that a specific concentration of licorice proteins increased apoptosis in murine C26 and human HT-29 CRC cancer cell lines but not in noncancerous cells (Khazraei-Moradian et al., 2017). However, Arriazu et al. observed that ocoxin reduced the viability of activated HSCs (Arriazu et al., 2012). The different origin of these cells and the specific characteristic of TME in each organs could be an explanation for the mentioned contradictory results. Nevertheless, these findings also suggests that cancer cells and activated cells could be more sensitive to Ocoxin than healthy cells, which is an interesting aspect to take into consideration for an anti-cancer compound, as it might only affect cancer and tumor-associated cells without provoking side effects. Subsequently, we investigated the effect of Ocoxin on the TME *in vivo* to get a better understanding of its global influence on cancer development. The liver tumors of mice treated with the nutritional supplement presented lower macrophage and HSC infiltration, indicating a significant reduction of stromal cell recruitment. This result is in

accordance with the *in vitro* outcome showing the inhibition of the migratory potential of fibroblasts and macrophages after the treatment with the nutritional mixture. Thus, it is tempting to hypothesize that this decrease in stromal cell recruitment would reduce the secretion of pro-tumoral factors. Among those factors present in the TME, cytokines constitute a key element. Whereas in healthy conditions they suppress tumor formation, in such a pro-tumoral environment, stromal and cancer cells can release cytokines to promote tumor growth by reducing apoptosis, inducing proliferation and by disrupting the communication between cancer cells and TME (Dranoff, 2004). In fact, cancer cells interact with immune cells, fibroblasts and substances such as chemokines, cytokines and pro-inflammatory mediators (Singh, R. et al., 2017) produced by these cells, which causes a subsequent recruitment of stromal cells that infiltrate the tumor triggering a further release of cytokines that favors a pro-tumoral microenvironment, promoting, therefore, cancer progression (Allavena et al., 2008; Bussard et al., 2016; Dehne et al., 2017; Ino et al., 2013; Kalluri, 2016; Komohara & Takeya, 2017; Prenen & Mazzone, 2019; Santoiemma & Powell, 2015). This link between cancer an inflammation has been showed repeatedly. Just as an example, the chronification of hepatitis and pancreatitis can lead to cancer (Ahn et al., 2018; Bishayee, 2014; Chai et al., 2015). Sustained inflammation originates an increase of hypoxia and oxidative stress and can also cause an immune deregulation due to the uninterrupted recruitment of inflammatory and immune cells (Grivennikov et al., 2010; Ida et al., 2015; Tafani et al., 2016). Here we demonstrate that Ocoxin reduces the expression and serum levels of inflammatory mediators in mice bearing PCA and metastatic CRC, crucial elements in tumor progression. In this context, COX-2 has been extensively studied regarding inflammation and cancer. Several authors studied the tumor-promoting effect of COX-2, concluding that the molecule induces angiogenesis and metastasis in CRC and inhibits the apoptotic susceptibility in CRC cells (Arteta et al., 2010; Sun et al., 2002; Tsujii et al., 1997). Along with this molecule, IL-1β is frequently found overexpressed in cancer and it is known to upregulate the expression of COX-2 in CRC (Liu, W. et al., 2003). Indeed, Hou et al. reported that as a consequence of IL-1β-mediated stimulation of ROS production, macrophages induce the expression of COX-2 in breast cancer (Hou et al., 2011). Here we show that Ocoxin decreased liver COX-2 expression in our mice with metastatic CRC, possibly, as a result of the antioxidant and antiinflammatory capacity of the nutritional mixture, which could have reduced ROS and the inflammatory mediators themselves. Furthermore, Ocoxin also decreased IL-1β levels in total liver and in plasma of mice with CRC metastasis and PCA

respectively. Nonetheless, tumor cells isolated from liver explants of mice bearing metastatic CRC that were treated with ocoxin showed an upregulation of IL-1β expression. This observations may indicate that ocoxin acts differently in the presence of microenvironmental factors and in stable laboratory culture conditions and furthermore, keeping in mind that tumor cells isolated from the explants were cultured under normal conditions and in the absence of the nutritional mixture for some days, this could have permitted a phenotypic reversion of these cells, which can explain the discordant result.

In any case, there are many other factors secreted by macrophages that play a pro-tumoral role by stimulating ECM remodeling, by suppressing T-cell mediated anti-tumor response, by sustaining inflammation, enhancing tumor stem cell proliferation and by promoting new vasculature formation and metastasis among others (Ruffell & Coussens, 2015; Sawa-Wejksza & Kandefer-Szerszen, 2018; Sica et al., 2006). TAMs are recruited into the tumor tissue, where they release growth factors and cytokines like TNF-α whose levels are known to be upregulated in cancer, and as a consequence of the hypoxic environment they also increase ROS production (Balkwill, 2006). Interestingly ocoxin reduced TNF-α expression in hepatic lesions of metastatic CRC. In fact, a study carried by Jiao et al. concluded that the inhibition of this molecule decreased the growth of hepatic metastasis of CRC and a preclinical study developed by Josephs and his colleagues demonstrated that the removal of soluble TNF-α receptors from plasma stabilized sarcomas (Jiao et al., 2014; Josephs et al., 2018). TNF-α is often found in association with IL-1β and IL-6, cytokines that prompt tumor cell growth and metastasis in many cancers (Allavena et al., 2008; Balkwill & Mantovani, 2001; Bent et al., 2018). Indeed, Cui et al. found out that IL-1β stimulates IL-6 in CRC, which at the same time further enhances IL-1β secretion, suggesting that tumor progression could be regulated by the IL-1β/IL-6 axis (Cui et al., 2018). In this regard, the IL-1β/IL-6 network may be a mechanism affected by Ocoxin, since, besides COX-2, TNF-α and IL-1β, the nutritional supplement decreased IL-6 expression too in livers of mice bearing metastatic CRC. On the contrary, although IL-1β was reduced, we did not observe any differences in the plasmatic levels of IL-6, and TNF-α these inflammatory mediators in mice bearing PCA. This could indicate that Ocoxin might act differently in each type of cancer or also that the effect of the supplement might not be equal at plasma and tissue levels. Regardless of that, Zhang et al. observed that, vitamin B6 inhibited IL-1β and TNF-α through the activation of the tumor suppressor p53, which increases the expression of a macrophage activation inhibitor p21 (Zhang et al., 2014).

Moreover, another element of our nutritional mixture, cinnamon, inhibited the production of several inflammatory molecules that stimulate macrophage differentiation and proliferation (Sadeghi et al., 2019). Furthermore, it has been also observed that resveratrol, curcuminoids and many other plant-derived substances like EGCG which are present in Ocoxin, suppress TNF-α expression from macrophages (Sethi et al., 2008). In agreement with those conclusions, Hernández-SanMiguel and her collaborators discovered that ocoxin inhibited the differentiation of macrophages to M2 immunosuppressive cells (Hernandez-SanMiguel et al., 2019). These cells are the main producers of the pro-inflammatory mediators we mentioned previously (COX-2, IL-1β, IL-6, TNF-α) thus, the inhibition of macrophage differentiation caused by Ocoxin could have diminished the secretion of those factors.

Following with the cytokines, IL-12 levels were also altered by Ocoxin. This molecule is a double edged sword since it acts as an anti-tumor element by stimulating immune cells like NKs or T cells, which at the same time, promote the activation of tumor-suppressing pathways, mainly supporting IFNγ production (Berraondo et al., 2018; Tugues et al., 2015). In turn, IFNγ is considered a central player in anti-tumor immunity, which makes tumor cells more susceptible to the recognition by the immune cells and enhances the activity of cytotoxic cells, as well as inhibiting tumor cell proliferation and inducing apoptosis in several cancers (Zaidi, 2019). In our case, we observed that Ocoxin reduced plasmatic concentration of both, IL-12 and IFNγ in mice with PCA. Even if this outcome does not seem beneficial, a study carried out by D'Andrea et al. demonstrated that IL-12 production and the subsequent IFNγ synthesis can be inhibited by IL-10, which curiously was increased in our mice bearing PCA that received Ocoxin (D'Andrea et al., 1993). This rise was also observed in humans taking a natural product containing vitamin C and flavonoids from echinacea and propolis (Barak et al., 2002). In this regard, it is important to note that the role of IL-10 has been questionable. Whereas some studies support its pro-tumoral effects like the promotion of immune escape or metastasis, some others, sustain that IL-10 enhances antitumor activity like for example in breast cancer or melanoma (Mannino et al., 2015). Focusing in PCA, we also found controversial publications defending that serum levels of IL-10 were associated with bad prognosis in humans, but also suggesting that a vaccinia virus with IL-10 reduces tumors in mice (Chard et al., 2015; Feng et al., 2018). Moreover, the role of IFNγ is also contentious. While a study concluded that the deficiency of IFNγ promotes CRC (Wang, L. et al., 2015),

prolonged exposure to this molecule has also showed to cause immunosuppression in different cancers including CRC (Zaidi, 2019) and even more, Detjen et al. observed that IFNγ promotes apoptosis of PCA cells (Detjen et al., 2001). Thus, probably, more than the concentration of the inflammatory mediators themselves, many other factors should be considered, such as the ratio between them or the phase of the disease when the treatment is administered, so as to understand whether ocoxin exerted a convenient immunoregulatory effect during cancer progression.

Lastly, bearing in mind that one of the purposes of this work is to study whether ocoxin could be a useful supplement for chemotherapy, the implication of macrophages in chemoresistance should be mentioned. In relation to this, Ruffell and Coussens described in their review that co-culture studies with cancer cells and macrophages impaired the antineoplasic action of chemotherapeutic drugs such as paclitaxel or gemcitabine (Ruffell & Coussens, 2015). Besides, they reported that blocking macrophage recruitment, activation, or polarization to M2 macrophage type, caused a reduction of metastasis in breast cancer and PCA and enhanced the effect of chemotherapy. We already mentioned that Ocoxin inhibited the differentiation to immunosuppressive macrophages (Hernandez-SanMiguel et al., 2019) and that it is plausible to think that the nutritional mixture could have also reduced the presence of M2 type macrophages in our models by depleting macrophage recruitment into the tumors of mice bearing metastatic CRC with the consequent diminution of the pro-tumoral factors secreted by them. As stated by De Nardo and his collaborators, the response to chemotherapeutic agents is partly regulated by the immune microenvironment, which induces the production of macrophage recruitment factors and consequently enhances macrophage infiltration into the tumor, leading to tumor progression and impairing drug effectiveness (DeNardo et al., 2011). Indeed, it has been proved that the inhibition of some inflammatory mediators such as COX-2 or TNF-α, could potentiate chemotherapy (Xu, Z. et al., 2014; Zhao, X. et al., 2016). In this regard, as discussed beforehand, the decrease of some factors probably inhibited macrophage recruitment and differentiation and therefore, we could presume that Ocoxin could likely be useful to face macrophage-related chemoresistance. Indeed, although we did not show this data here, we observed that Ocoxin acted synergically with irinotecan, causing a reduction of TAM infiltration in liver tumors of CRC metastasis. Nonetheless, further investigations should be performed about the influence of Ocoxin in TAMs *in vivo*.

In addition to their role on promoting cell proliferation, migration and survival, cytokines can also induce a modification of the tumor stroma architecture by changing the ECM composition (Hui & Chen, 2015; Rupaimoole et al., 2016; Wang, M. et al., 2017). In cancer, ECM structure is modulated by many elements like CAFs that, in pro-tumoral conditions, secrete great amounts of proteins like collagen or fibronectin along with MMPs and TIMPs leading to a full remodeling of the stroma and favoring cancer cell spreading (Bussard et al., 2016; Sahai et al., 2020). Under repeated inflammatory stimuli, HSCs and PSCs turn into activated myofibroblasts that gain migratory capacity in order to infiltrate the tumor, and also stimulate cancer cell invasion by liberating cytokines and growth factors to the extracellular compartment (Allam et al., 2017; Kalluri, 2016; Tsuchida & Friedman, 2017). Therefore, there is an active research on how to revert HSCs and PSCs phenotypes to fight cancer. Some of the attempted strategies included the reversion of these cells to a quiescent state, the induction of ECM degradation, the reduction of other cell populations in the microenvironment and the blockade of tumor-supporting soluble factors or the inhibition of cancer-promoting signaling pathways (Carapuca et al., 2016; Chiaravalli et al., 2017; Coulouarn & Clement, 2014; Schnittert et al., 2018). In this respect, Roomi et al. observed that their nutrient mixture modulated cell migration via MMP regulation, but, to date, the implication of ocoxin on cell migration related to MMP levels has not been studied (Roomi et al., 2005a; Roomi et al., 2010; Roomi et al., 2015b). Nevertheless, as we mentioned before, ocoxin reduced fibroblast migration *in vitro* and HSC infiltration *in vivo* and therefore we could assume that the nutritional mixture could have contributed to lower intratumoral collagen deposition and thus, to the modulation of CAF-mediated pro-tumoral signals. However, we still ignore whether ocoxin hindered stromal cell recruitment with the resultant cytokine level reduction or vice versa. Therefore, this relation between the inflammatory mediators and stromal cells needs to be studied in more detail.

Among the pro-inflammatory factors secreted by TAMs, VEGF worths a mention since CAFs are known to precede and to prompt angiogenesis. In response to hypoxia, cytokines stimulate HSC and PSC migration so that they can infiltrate tumors, where there they produce VEGF. In turn, secreted VEGF promotes endothelial cell recruitment to form new blood vessels that will facilitate the oxygen and nutrient input into the tumor (Di Maggio et al., 2016; Hui & Chen, 2015; Olaso et al., 2003; Rupaimoole et al., 2016). In the present study, a decrease of the liver VEGF levels was detected in mice with CRC

metastasis that received ocoxin which correlates with the diminution of HSC infiltration. Along with this observation, Roomi et al. also noticed an angiogenic decrease together with VEGF reduction when they used a nutrient mixture containing similar components as Ocoxin (Roomi et al., 2005b). Supporting our findings, cinnamon inhibited angiogenesis (Bansode et al., 2013), Qingyihuaji suppressed liver metastatic progression from PCA by reducing VEGF expression (Yin et al., 2012) and EGCG inhibited the expression of VEGF in human vascular smooth muscle cells and in gastric cancer (Park, J. S. et al., 2006; Zhu et al., 2011) while it exerted an anti-angiogenic action in vascular tumors (Fassina et al., 2004). Besides, there are more cytokines that also possess angiogenic roles. For instance, IL-6, is known to promote blood vessel formation by inducing the transformation of fibroblasts into CAFs and by stimulating their proliferation (Bussard et al., 2016), while TNF-α and IL-1β have shown induce the production of angiogenic factors prompting endothelial cell recruitment (Bani et al., 1991; Bussard et al., 2016; Greco et al., 2005; Leibovich et al., 1987; Szlosarek et al., 2006; Voronov et al., 2003). Thus, the lower expression of these cytokines in mice treated with Ocoxin could have impaired the metastatic development of CRC by reducing angiogenesis.

In addition to their protumoral role through the remodelation of the TME, CAFs are known as key inducers of chemoresistance. In fact, both liver HSCs and pancreatic PSCs have showed to give rise to chemoresistance in many reports (Huang et al., 2019; Liu, Q. et al., 2017; McCarroll et al., 2014; Tommelein et al., 2015; von Ahrens et al., 2017). Since one of the most characteristic feature of PCA and liver metastasis of CRC is the highly desmoplasic stroma, we investigated whether ocoxin could revert the chemoresistance mediated by fibroblasts *in vitro*. Predictably, fibroblast-derived soluble factors reduced the effectiveness of paclitaxel and gemcitabine in PCA cells and consistent with our previous observations, the addition of Ocoxin to the chemotherapeutic drugs recovered the cytotoxic effect of chemotherapy. Hence, we postulate that the nutritional supplement might have reverted the chemoresistance conferred by fibroblasts by interfering directly with certain factors secreted by these cells as a result of its anti-oxidant, anti-inflammatory and immunoregulatory properties.

Lastly, another reason for using natural compounds as chemotherapy adjuvants is the relief of the undesired effects caused by synthetic drugs. It is known that the intake of nutritional supplements helps to ameliorate

chemotherapy-induced side effects and to improve the health status of cancer patients. Green tea, for example, reduced the adverse effects of chemotherapy in the small intestine of mice (Wessner et al., 2007). Moreover, the supplementation of glucosamine sulfate attenuated colitis in humans which is one of the side effects caused by some chemotherapeutic drugs (Bak et al., 2014). Similarly, Mochamat et al. mentioned the advantages of administrating several elements like vitamin C, amino acids or polyphenols to patients with cachexia, a multifactorial syndrome characterized by an extreme weight loss and skeletal muscle wasting due to cancer (Mochamat et al., 2017). Taking into account the beneficial properties of the natural compounds, we monitored the health status of mice according to FELASA's (Federation for Laboratory Animal Science Associations) guidelines concluding that the animals that received ocoxin showed a better health condition than the ones which were only treated with irinotecan. Likewise, different clinical researchers observed that ocoxin decreased the deleterious side effects in terms of toxicity caused by chemotherapy and radiotherapy in patients with HCC, head and neck cancer, cervical cancer and endometrial adenocarcinoma (Al-Mahtab et al., 2015; Lorente et al., 2020; Rivas et al., 2018) together with a reduction of oral mucositis and an improvement of appetite and well-being (Kaidarova et al., 2019; Shumsky et al., 2019). Nevertheless, a bigger number of patients should be monitorized in the future in order to know if Ocoxin really improves life quality in humans.

# VI. CONCLUSIONS
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Taking into consideration all the results of this doctoral thesis, we conclude that:

1- Ocoxin **decreases PCA and metastatic CRC cell viability** by reducing proliferation, increasing apoptosis and by inducing a cell cycle arrest, while healthy cell viability is not that affected.

2- Ocoxin **reduces the migratory potential** of metastatic cancer cells, macrophages and fibroblasts *in vitro* and inhibits **infiltration of stromal cells** into the tumor *in vivo*.

3- Ocoxin **enhances the cytotoxic effect of the chemotherapeutic drugs** irinotecan, paclitaxel and gemcitabine *in vitro* and **reverts fibroblast-mediated chemoresistance** in human PCA cells treated with paclitaxel and gemcitabine *in vitro*.

4- Ocoxin **modulates inflammatory mediator levels** by reducing pro-inflammatory and enhancing antiinflammatory molecules and **regresses the expression of genes** involved in cancer to healthy values

5- Ocoxin **does not cause adverse side effects** and **improves the welfare** of mice.

"Ocoxin is a natural nutritional supplement that could be a suitable adjuvant to reinforce chemotherapy in PCA and liver CRC metastasis by enhancing its cytotoxic effect and by reducing stroma-mediated chemoresistance while contributes to the relief of adverse effects."

# VII. BIBLIOGRAPHY
# VII. BIBLIOGRAFIA

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# VIII. APPENDIXES
# VIII. ERANSKINAK

# APPENDIX I. List of genes deregulated by Ocoxin in pancreatic cancer cells.

**Table I. List of genes upregulated by ocoxin in pancreatic cancer cells.**

| Gene Symbol | OOS vs C | Gene Symbol | OOSvs C |
|---|---|---|---|
| 1700008O03Rik | ↑ | Ccr8 | ↑ |
| 1700027H10Rik | ↑ | Cel | ↑ |
| 2210408F21Rik | ↑ | Cela2a | ↑ |
| 4933406K04Rik | ↑ | Cela3a | ↑ |
| 9930105H17Rik | ↑ | Chac1 | ↑ |
| Aars | ↑ | Cox6a2 | ↑ |
| Adcy8 | ↑ | Cpa1 | ↑ |
| Adh1 | ↑ | Cpa2 | ↑ |
| Ahsg | ↑ | Creb3l2 | ↑ |
| Akr1b7 | ↑ | Creg1 | ↑ |
| Aldh18a1 | ↑ | Ctrc | ↑ |
| Aldh1l2 | ↑ | Cxcl10 | ↑ |
| Alox5 | ↑ | Cyb5rl | ↑ |
| Aloxe3 | ↑ | D7Wsu130e | ↑ |
| Amhr2 | ↑ | Daf2 | ↑ |
| Ang4 | ↑ | Derl3 | ↑ |
| Angptl4 | ↑ | Dnajc25 | ↑ |
| Anxa9 | ↑ | Dpy19l4 | ↑ |
| Apoe | ↑ | Drd3 | ↑ |
| Apof | ↑ | Edem2 | ↑ |
| Apol7a | ↑ | Eef2k | ↑ |
| Aqp8 | ↑ | Eif4ebp1 | ↑ |
| As3mt | ↑ | Entpd4 | ↑ |
| Asns | ↑ | Esrrg | ↑ |
| Atf4 | ↑ | Fetub | ↑ |
| Atf5 | ↑ | Folr2 | ↑ |
| Atic | ↑ | Gaa | ↑ |
| Avil | ↑↑ | Gdf15 | ↑ |
| Bcat1 | ↑ | Gjb2 | ↑ |
| Bmp7 | ↑ | Gm10639 | ↑ |
| Cars | ↑ | Gm11744 | ↑ |

| Gene Symbol | OOS vs C | Gene Symbol | OOS vs C |
|---|---|---|---|
| Gm20559 | ↑ | Mycl | ↑ |
| Gm6484 | ↑ | Nars | ↑ |
| Gm9925 | ↑ | Noxa1 | ↑ |
| Gsta2 | ↑ | Nupr1 | ↑ |
| Gsta3 | ↑↑ | Parm1 | ↑ |
| Gstm1 | ↑ | Pawr | ↑ |
| Gstm2 | ↑ | Pdia5 | ↑ |
| Gzmm | ↑ | Pgd | ↑ |
| H13 | ↑ | Pip5k1a | ↑ |
| Hamp2 | ↑ | Pir | ↑ |
| Hapln4 | ↑ | Pld2 | ↑ |
| Hax1 | ↑ | Psat1 | ↑ |
| Herpud1 | ↑ | Psen2 | ↑ |
| Hhip | ↑ | Psmd14 | ↑ |
| Hsd3b7 | ↑ | Psph | ↑ |
| Iars | ↑ | Ptpn14 | ↑ |
| Igsf5 | ↑ | Pycr1 | ↑ |
| Kcnk16 | ↑ | Rcan3 | ↑ |
| Kng1 | ↑ | Sec61a1 | ↑ |
| Krt8 | ↑ | Serpinb6a | ↑ |
| Lcp1 | ↑ | Serpinb6c | ↑ |
| Lgals3bp | ↑ | Serpinf1 | ↑ |
| Lyz1 | ↑ | Shmt2 | ↑ |
| Lyz2 | ↑ | Slc16a7 | ↑ |
| Mars | ↑ | Slc17a9 | ↑ |
| Marveld1 | ↑ | Slc30a1 | ↑ |
| Masp1 | ↑ | Slc3a2 | ↑ |
| Matn2 | ↑ | Slc6a9 | ↑ |
| Me1 | ↑ | Slc7a1 | ↑ |
| Mest | ↑ | Slc7a11 | ↑ |
| Mogat2 | ↑ | Ssr2 | ↑ |
| Mt1 | ↑ | St3gal6 | ↑ |
| Mt2 | ↑ | Stt3a | ↑ |
| Mthfd2 | ↑ | Sycn | ↑ |

| Gene Symbol | OOS vs C | Gene Symbol | OOS vs C |
|---|---|---|---|
| Tex2 | ↑ | Wdpcp | ↑ |
| Tmem37 | ↑ | Xkrx | ↑ |
| Tmem8 | ↑ | Yars | ↑ |
| Tnni3 | ↑ | Zfp709 | ↑ |
| Ugt1a6b | ↑ | Zp3 | ↑ |
| Ugt2b34 | ↑ | | |
| Vimp | ↑ | | |

Each arrow represents upregulation fold-change:

↑ < 2 fold-change

↑↑ > 2 fold-change.

*C: Control; IRI: Irinotecan; OOS: Ocoxin.*

**Table II. List of genes downregulated by ocoxin in pancreatic cancer cells.**

| Gene Symbol | OOS vs C | Gene Symbol | OOS vs C |
|---|---|---|---|
| 1700049E15Rik | ↓ | Cntfr | ↓ |
| 1810011O10Rik | ↓ | Cox16 | ↓ |
| 2610016A17Rik | ↓ | Cplx1 | ↓ |
| 4930522L14Rik | ↓ | Crabp1 | ↓ |
| Add2 | ↓ | Creb1 | ↓ |
| Ahcyl2 | ↓ | Crmp1 | ↓ |
| Ap1s2 | ↓ | Crtac1 | ↓ |
| Ap2b1 | ↓ | Cspg4 | ↓ |
| Arhgef3 | ↓ | Cyth4 | ↓ |
| Atp2b2 | ↓ | D430019H16Rik | ↓ |
| BC049987 | ↓ | D830024N08Rik | ↓ |
| Brinp1 | ↓ | Dennd5b | ↓ |
| Btbd17 | ↓ | Dync1i2 | ↓ |
| Cacna1g | ↓ | Dzip3 | ↓ |
| Cacna1i | ↓ | E130309D14Rik | ↓ |
| Cacna2d2 | ↓ | Edil3 | ↓ |
| Cacng4 | ↓ | Elavl3 | ↓ |
| Ccdc37 | ↓ | Elmo1 | ↓ |
| Ccny | ↓ | Eln | ↓ |
| Cdh22 | ↓ | Esyt3 | ↓ |
| Cdx2 | ↓ | Eya2 | ↓ |
| Celf3 | ↓ | Fam159b | ↓ |
| Celf6 | ↓ | Fam163b | ↓ |
| Chgb | ↓ | Fam84a | ↓ |
| Chn1 | ↓ | Fgfr3 | ↓ |
| Chn1 | ↓ | Fignl1 | ↓ |
| Chst8 | ↓ | Foxf1 | ↓ |
| Cidea | ↓ | Frmd4a | ↓ |
| Ckmt1 | ↓ | Gabpb1 | ↓ |
| Cnn3 | ↓ | Gabrb3 | ↓ |
| Cnrip1 | ↓ | Gck | ↓ |

| Gene Symbol | OOS vs C | Gene Symbol | OOS vs C |
|---|---|---|---|
| Gdap1l1 | ↓ | Htra4 | ↓ |
| Gjc1 | ↓ | Inhbe | ↓ |
| Glrb | ↓ | Iqsec3 | ↓ |
| Gm10334 | ↓ | Kcnmb4 | ↓ |
| Gm27197 | ↓ | Kif21b | ↓ |
| Gm2824 | ↓ | Kif5c | ↓ |
| Gm30938 | ↓ | Klhdc8a | ↓ |
| Gm31319 | ↓ | Klhl12 | ↓ |
| Gm31796 | ↓ | Lat | ↓ |
| Gm5065 | ↓ | Lhfpl3 | ↓ |
| Gm9926 | ↓ | Lhx3 | ↓ |
| Gng2 | ↓ | LOC102637409 | ↓ |
| Golga7b | ↓ | LOC102642140 | ↓ |
| Gp1bb | ↓ | LOC497255 | ↓ |
| Gpr20 | ↓ | Lrrc2 | ↓ |
| Gprc5b | ↓ | Lrrn2 | ↓ |
| Grap2 | ↓ | Lypd1 | ↓ |
| Grb14 | ↓ | Maff | ↓ |
| Grik3 | ↓ | Mmel1 | ↓ |
| Gskip | ↓ | Mpp2 | ↓ |
| Gucy1a3 | ↓ | Mpp3 | ↓ |
| H2afy | ↓ | Mturn | ↓ |
| Hapln1 | ↓ | Necab2 | ↓ |
| Hepacam2 | ↓ | Nell2 | ↓ |
| Hey1 | ↓ | Ninl | ↓ |
| Hist1h2ai | ↓ | Nmnat2 | ↓ |
| Hist1h2ba | ↓ | Nrgn | ↓ |
| Hist1h2bb | ↓ | Nrsn1 | ↓ |
| Hist1h3a | ↓ | Nsg2 | ↓ |
| Hist1h3f | ↓ | Nts | ↓ |
| Hist1h3i | ↓ | Nup133 | ↓ |
| Hist3h2a | ↓ | Olfr1280 | ↓ |
| Hmgb1 | ↓ | Oprd1 | ↓ |
| Hpca | ↓ | Orc1 | ↓ |

| Gene Symbol | OOS vs C | Gene Symbol | OOS vs C |
|---|---|---|---|
| Pak7 | ↓ | Shc2 | ↓ |
| Pcdh8 | ↓ | Six2 | ↓ |
| Pcdha9 | ↓ | Slc39a10 | ↓ |
| Pcdhb10 | ↓ | Slc6a11 | ↓↓ |
| Pcsk2 | ↓ | Snap25 | ↓ |
| Pcsk2os1 | ↓ | Sncb | ↓ |
| Pde9a | ↓ | Sox11 | ↓ |
| Phactr3 | ↓ | Sp4 | ↓ |
| Pianp | ↓ | Spata6 | ↓ |
| Pip4k2a | ↓ | Spem1 | ↓ |
| Pnma2 | ↓ | Srrm3 | ↓ |
| Ppm1n | ↓ | St8sia3 | ↓ |
| Prdm6 | ↓ | Stmn3 | ↓ |
| Prss1 | ↓ | Thy1 | ↓ |
| Pthlh | ↓ | Timmdc1 | ↓ |
| R3hdm1 | ↓ | Tmem158 | ↓ |
| Rab3c | ↓ | Tmem74b | ↓ |
| Rapgef3 | ↓ | Tmx1 | ↓ |
| Rasa3 | ↓ | Tnfaip8 | ↓ |
| Rasd1 | ↓ | Trp53i11 | ↓ |
| Trpc3 | ↓ | Trp63 | ↓ |
| Trpc4 | ↓ | Tubb2b | ↓ |
| Rasgrf1 | ↓ | Ttc9b | ↓ |
| Reg3d | ↓ | Tspan18 | ↓ |
| Resp18 | ↓ | Tubb3 | ↓ |
| Rfx2 | ↓ | Upp1 | ↓ |
| Rgs8 | ↓ | Vgf | ↓ |
| Ric3 | ↓ | Vstm2l | ↓ |
| Rnf170 | ↓ | Vwa5b2 | ↓ |
| Rprm | ↓ | Wnt4 | ↓ |
| Rtn2 | ↓ | Wscd1 | ↓ |
| Rxrg | ↓ | Zfp292 | ↓ |
| Scn3b | ↓ | Zcchc18 | ↓ |
| Scrt1 | ↓ | Zfp600 | ↓ |

| Gene Symbol | OOS vs C | Gene Symbol | OOS vs C |
|---|---|---|---|
| Zfp667 | ↓ | Zfp961 | ↓ |
| Zfp7 | ↓ | | |

Each arrow represents downregulation fold-change:

↓ < 2 fold-change

↓↓ > 2 fold-change.

*C: Control; IRI: Irinotecan; OOS: Ocoxin.*

# APPENDIX II. List of genes deregulated by Ocoxin, irinotecan and the combination of both in metastatic colorectal cancer cells.

**Table I. List of genes upregulated by different treatments in CRC cells isolated from liver metastatic tumors.**

| Gene Symbol | OOS vs C | OOS + IRI vs C | IRI vs C | OOS + IRI vs OOS |
|---|---|---|---|---|
| 2700081O15Rik | | ↑ | | ↑ |
| Ankk1 | ↑↑ | | | |
| B230334C09Rik | ↑↑ | | | |
| BC030308 | ↑ | | | |
| Bmp7 | ↑↑ | | | |
| Cabp1 | ↑↑ | | | |
| Cdh9 | ↑ | | | |
| Cdk13 | ↑↑↑ | | | |
| Cfd | | | ↑↑ | |
| Chil3 | | ↑↑↑ | | |
| Cypt15 | ↑↑↑ | | | |
| Cyth4 | ↑↑↑↑ | | | |
| Dzip1 | | | ↑ | |
| Ect2 | | ↑↑↑ | | |
| Fam71f2 | ↑↑↑ | | | |
| Gm38485 | ↑ | | | |
| Gm9979 | ↑ | | | |
| Hmcn1 | | | ↑ | |
| Il11 | ↑↑↑↑ | | | |
| Il1b | ↑↑ | | | |
| Kif14 | | | | ↑↑↑ |
| Krtap19-2 | | | ↑↑↑ | |
| LOC102641211 | | | ↑ | |
| Marcksl1 | ↑ | | | |
| Marcksl1-ps4 | ↑ | | | |
| Nctc1 | | | ↑↑ | |
| Nol3 | ↑↑ | | | |
| Ntrk1 | ↑ | | | |
| Nuf2 | | ↑↑↑↑ | | |
| Pggt1b | ↑↑↑ | | | |
| Pir | | | ↑ | |

| Gene Symbol | OOS vs C | OOS + IRI vs C | IRI vs C | OOS + IRI vs OOS |
|---|---|---|---|---|
| Pyroxd2 | | | ↑↑ | |
| Rgs16 | ↑↑ | | | |
| Shc3 | ↑ | | | |
| Slc14a1 | ↑↑ | | | |
| Snx14 | ↑↑ | | | |
| Ssh1 | ↑↑ | | | |
| Tstd3 | ↑↑↑↑ | | | |
| Ttl | | | ↑↑ | |
| Usp2 | | | ↑↑ | |
| Usp42 | ↑ | | | |
| Zrsr2 | | | ↑↑ | |

Each arrow represents upregulation fold-change:

↑ for a 2-4 fold-change

↑↑ for a 4-6 fold-change

↑↑↑ for a 6-8 fold-change

↑↑↑↑ for a 8-10 fold-change

*C: Control; IRI: Irinotecan; OOS: Ocoxin*.

**Table II. List of genes downregulated by different treatments in CRC cells isolated from liver metastatic tumors.**

| Gene Symbol | OOS vs C | OOS + IRI vs C | IRI vs C | OOS + IRI vs OOS |
|---|---|---|---|---|
| 1700017G19Rik | | | ↓↓ | |
| 1700086P04Rik | | | ↓ | |
| 2810454H06Rik | | | ↓↓↓ | |
| 4930401B11Rik | | | ↓ | |
| 4930556N13Rik | | | ↓↓ | |
| 4933413J09Rik | | | ↓↓ | |
| 4933415F23Rik | | | ↓ | |
| 6430550D23Rik | | | ↓↓ | |
| 9530003O04Rik | | | ↓ | |
| A730028G07Rik | | | ↓↓↓ | |
| Aak1 | | | ↓ | |
| Adcy6 | | | ↓↓ | |
| Afm | | | ↓↓↓ | |
| Agxt2 | | | ↓↓ | |
| AI481877 | | | ↓↓ | |
| Ankk1 | | | | ↓↓ |
| Apoe | | | ↓ | |
| Aqp9 | | | ↓↓ | |
| Arglu1 | ↓ | ↓ | ↓ | |
| Atf6 | | | ↓↓↓ | |
| Bfar | | | ↓ | |
| Bsph1 | | | ↓↓ | |
| C030039L03Rik | | | ↓↓ | |
| Cdh9 | | | | ↓ |
| Cela3a | | | ↓↓↓ | |
| Cfap61 | | | ↓ | |
| Cic | | | ↓↓ | |
| Clcn7 | | | ↓↓ | |
| Clec18a | | | ↓↓ | |
| Clec2e | | | ↓ | |
| Crygc | | | ↓↓ | |

| Gene Symbol | OOS vs C | OOS + IRI vs C | IRI vs C | OOS + IRI vs OOS |
|---|---|---|---|---|
| Cyp19a1 | | | ↓ | |
| Cypt2 | | | ↓ | |
| D430018E03Rik | | | ↓↓ | |
| D6Ertd527e | | | ↓↓ | |
| Dlx6os1 | | | ↓ | |
| Dnal1 | | | ↓ | |
| Dock4 | | | ↓↓ | |
| Dsc1 | | | ↓ | |
| Erich2 | | | ↓↓ | |
| Fanci | | | ↓ | |
| Fat3 | | | ↓↓ | |
| Fgr | | | ↓↓ | |
| Fkbp6 | | | ↓ | |
| Fuca2 | | | ↓↓ | |
| Gja8 | | | ↓↓ | |
| Glrx2 | | | ↓↓ | |
| Gm10760 | | | ↓↓ | |
| Gm16794 | | | ↓ | |
| Gm5126 | | | ↓↓ | |
| Gm5129 | | | ↓↓ | |
| Gm5941 | | | ↓↓ | |
| Gm8075 | | | ↓↓ | |
| Gm9548 | | | ↓↓ | |
| Gm9798 | | | ↓↓ | |
| Gpr22 | | | ↓↓ | |
| Hcrt | | | ↓↓ | |
| Hist2h3c2 | | | ↓↓ | |
| Hras | | | ↓↓ | |
| Kcnj12 | | | ↓ | |
| Kif14 | ↓↓ | | | |
| Klf11 | | | ↓↓↓ | |
| LOC105244659 | | | ↓↓ | |
| Lppr3 | | | ↓↓↓ | |
| Lrrc2 | | | ↓↓↓ | |

| Gene Symbol | OOS vs C | OOS + IRI vs C | IRI vs C | OOS + IRI vs OOS |
|---|---|---|---|---|
| Ly6c1 | ↓↓↓ | | | |
| Marveld2 | | | ↓↓ | |
| Meg3 | | | ↓ | |
| Ms4a1 | | | ↓↓ | |
| Naprt | | | ↓ | |
| Ncam1 | | | ↓↓ | |
| Nfkbid | | | ↓↓ | |
| Nkx6-1 | | | ↓↓ | |
| Nr1h3 | | | ↓ | |
| Olfr1110 | | | ↓↓↓ | |
| Olfr328 | | | ↓↓↓ | |
| Olfr549 | | | ↓↓ | |
| Olfr945 | | | ↓↓ | |
| Olfr97 | | | ↓↓ | |
| Phka2 | | | ↓↓ | |
| Raver2 | | | ↓↓ | |
| Recql5 | | | ↓ | |
| Rian | | | ↓↓ | |
| Rnf170 | | | ↓↓ | |
| Rnf220 | | | ↓ | |
| Sclt1 | | | ↓↓ | |
| Scn10a | | | ↓↓ | |
| Sel1l | | | ↓↓↓ | |
| Sept8 | | | ↓↓ | |
| Sf3a2 | | | ↓↓ | |
| Sgk3 | | | ↓ | |
| Slc1a2 | | | ↓↓ | |
| Slc26a1 | | | ↓↓ | |
| Slc5a9 | | | ↓↓ | |
| Slc8b1 | | | ↓↓ | |
| Spice1 | | | ↓↓ | |
| Stt3a | | | ↓↓ | |
| Sucla2 | ↓↓ | | ↓ | |
| Tanc1 | | | ↓ | |

| Gene Symbol | OOS vs C | OOS + IRI vs C | IRI vs C | OOS + IRI vs OOS |
|---|---|---|---|---|
| Tmem29 | | | ↓ | |
| Tmprss5 | | | ↓ | |
| Tshz2 | | | ↓↓ | |
| Ttbk1 | | | ↓ | |
| Tuba8 | | | ↓ | |
| Ube2cbp | | | ↓↓ | |
| Vmn1r19 | | | ↓↓ | |
| Vps13c | | | ↓↓ | |
| Xrcc4 | | | ↓↓ | |
| Zdhhc21 | | | ↓↓↓ | |
| Zfp318 | | | ↓↓↓ | |
| Zpbp2 | | | ↓↓ | |

Each arrow represents downregulation fold-change

↓for a 2-4 fold-change

↓↓ for a 4-6 fold-change

↓↓↓for a 6-8 fold-change

↓↓↓↓ for a 8-10 fold-change.

*C: Control; IRI: Irinotecan; OOS: Ocoxin*.

# APPENDIX III. Publications.

## Publication 1: Ocoxin® Oral Solution slows down tumor growth in an experimental modelo f colorectal cancer metastasis to the liver in Balb/c mice.

Joana Marquez, Jorge Mena, Iera Hernandez-Unzueta, Aitor Benedicto, Eduardo Sanz, Beatriz Arteta and Elvira Olaso.

Oncology Reports

DOI: 10.3892/or.2015.4486

November 2015.

ONCOLOGY REPORTS

# Ocoxin® oral solution slows down tumor growth in an experimental model of colorectal cancer metastasis to the liver in Balb/c mice

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**Abstract.** Liver metastatic disease is the main cause of death in colorectal cancer (CRC) patients. During metastatic spread of the disease an imbalance in the oxidative stress and inflammation plays a crucial role in tumor progression. In order to improve the efficacy of current therapies, new complementary therapeutic approaches are being analyzed including biologically active compounds with low side effects. The anti-inflammatory and anti-oxidant properties of Ocoxin® oral solution (OOS) prompt us to analyze its effect on the metastatic development of CRC to the liver. First, *in vitro* effect of OOS in tumor cell viability and migration was analyzed. Second, *in vivo* effect of different dosage patterns and concentrations in the development of hepatic metastasis was analyzed by intrasplenic inoculation of C26 colon carcinoma cells in Balb/c mice. Third, the expression of alpha smooth muscle actin, caspase-3 and Ki-67 expression was quantified by immunohistochemistry, then gene expression levels of inflammatory factors were measured by quantitative RT-PCR. According to our results, OOS reduced tumor cell viability and migration *in vitro*. Moreover, *in vivo* daily administration of OOS from the 7th day after tumor cell inoculation decreased the total area and size of metastatic foci in the liver. Furthermore, cell proliferation and fibroblast recruitment was decreased in tumor foci while a higher number of apoptotic cells were observed. Finally, RNA levels for the inflammatory mediators COX-2, IFNγ, IL1β, IL6 and TNFα were reduced in total liver. In conclusion, OOS reduced the metastatic development of colorectal cancer to the liver by increasing apoptosis, and decreasing tumor cell proliferation and fibroblast recruitment in the tumor foci, as well as the expression of inflammatory mediators in total liver. These results point out OOS as a potential supplement to be applied as complementary therapy for the treatment of liver metastasis from colorectal cancer.

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*Key words:* ocoxin oral solution, nutrient mixture, colorectal cancer, liver, metastasis

## Introduction

Colorectal cancer (CRC) is one of the most aggressive cancers in the western countries. Despite the development of new treatments, patients suffering from this disease die of the spread of the primary cancer to other organs, mainly the liver, rather than from the primary tumor. To date, the only effective treatment is hepatic resection, and thus, a novel and effective therapies are urgently needed to treat metastatic disease, improving life expectancy and quality in CRC patients. Several studies have included biologically active compounds due to their low toxicity and excellent potential for cancer treatment.

The relationship between inflammation and tumor development is widely accepted (1). During the progression of cancer inflammatory cells are recruited into the tumor stroma by signals derived from the tumor, as well as from the cells residing in the organ that is being colonized. This inflammatory milieu results in the trigger for oxidative stress. In this scenery, the presence of oxidative and inflammatory factors play a crucial role regulating the expression of genes involved in tumor progression and metastasis (2).

This off balance in the microenvironment of the developing tumor affects the major cell types of the host organ in the tumor, namely, the cancer associated fibroblasts (CAFs) (3). The CAFs contribute to this pro-tumoral microenvironment by further producing pro-inflammatory and pro-angiogenic factors favoring the growth of the tumor, invasion of the targeted organ, angiogenesis and finally metastasis (3).

This inflammatory and oxidative millieu has encouraged the validation of several nutritional and biological components with anti-inflammatory and anti-oxidative properties, as complements for antitumor therapies. Among others, extracts from green tea and licorice, and vitamins has been reported to possess immunomodulatory, anti-inflammatory and anti-

oxidant properties which make them excellent candidates for the validation of their anti-tumorigenic and anti-metastatic effects in different types of cancer (4-7). Moreover, the administration of several of these compounds as nutrient mixtures has shown greater efficacy than their administration as individual treatments (8,9).

OOS is a nutritional supplement with recognized anti-oxidant, anti-inflammatory and immunomodulatory properties. The solution is composed, among others, of green tea extract, glyzyrrhicic acid, vitamin C, B6 and B12, minerals and aminoacids. It was synthesized by combining two products, Viusid® and Ocoxin®. The Viusid component has been tested in different clinical trials showing beneficial effects in patients suffering from chronic hepatitis C and cirrhosis (10,11). Moreover, OOS antitumor effects have validated *in vitro* and *in vivo* preclinical breast cancer models (12). Thus, we aim to study the effects of OOS in the metastatic progression of CRC to the liver.

In the present study, we demonstrate the inhibitory effect OOS *in vitro* and an *in vivo* pre-clinical model of metastatic development of colorectal carcinoma to the liver. We show that OOS exerts its effect by reducing tumor cell proliferation and by increasing apoptosis *in vivo*. Furthermore, this nutritional supplement shows not only a reduction in the fibroblast recruitment to the tumor stroma, which has been related to angiogenesis and tumor progression (13), but also to a modulation of the inflammatory signature in the liver. Thus, OOS might be a novel and effective complementary treatment which properties might help to increase the efficacy of actual anti-metastatic therapies as well as to improve the life-quality of patients suffering from this disease.

## Materials and methods

*Animals*. Balb/c mice (male, 8-weeks old) were obtained from Charles River Laboratories España S.A. (Barcelona, Spain). The animals were fed a standard chow and had access to water *ad libitum*. All the proceedings were approved by the Basque Country University Ethics Committee for Experimental Animal with the reference number CEEA/357/2014/ARTETA RUIZ, in accordance with the institutional, national and international guidelines regarding the protection and care of animal use for scientific purposes.

*Cell line*. The murine colon cancer C26 cells, syngenic with Balb/c mice (ATCC, LGC Standards S.L.U. Barcelona, Spain) were used. Cells were grown under standard conditions in RPMI-1640 medium (Life Technologies, Madrid, Spain) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 $\mu$g/ml) and amphotericin B (0.25 $\mu$g/ml) all purchased from Life Technologies.

*Viability and cell cycle assays*. PrestoBlue® reagent (Life Technologies) was used for quantification of tumor cell viability following the manufacturer's instructions. The C26 cells were cultured on collagen (Sigma-Aldrich, St. Louis, MO, USA) precoated culture plates at a concentration of 50,000 cells/ml in RPMI-1640 supplemented with 0.5% FBS and antibiotics-antimycotics. After 24-h incubation in the presence of different OOS concentrations ranging from 0 to a maximum of 1:200 (V/Vf) PrestoBlue was added for 2 h. Finally, absorbance was measured with Fluoroskan Ascen (Thermo Labsystems, Waltham, MA, USA). Results were calculated as the average of three independent experiments. For cell cycle analyses, cells were fixed after 72-h incubation in the presence of OOS (1/100; V/Vf) and DNA stained with propidium iodide (PI; Life Technologies) for 30 min. Then, cells were analyzed by flow cytometry in a Beckman Coulter Gallios™ (Beckman Coulter Inc., Barcelona, Spain). The number of cells in each cell cycle phase was quantified by means of Weasel software.

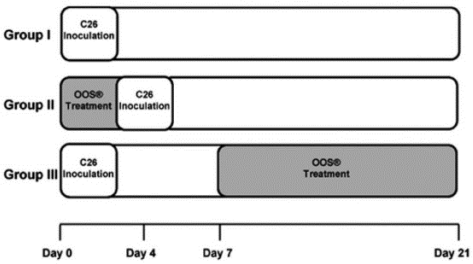


Figure 1. Diagram of the experimental animal groups according to OOS dosage pattern. Animals were divided into 3 experimental groups as follows: Group I, OOS untreated group; group II, mice treated with OOS 4 days prior to tumor cell inoculation; group III, mice treated with OOS from the 7th day after tumor cell inoculation. All treated groups were administered a daily dose during the period of treatment.

*In vitro migration assay*. The migration assay was carried out on modified Boyden chambers. Briefly, tumor cells, at a concentration of $2x10^5$ cells/ml were cultured onto 8 $\mu$m-diameter pore membrane (Greiner Bio-One, La Jolla, CA, USA) precoated with collagen type I 10 $\mu$g/ml (Sigma-Aldrich). Tumor cells were allowed to migrate for 18 h in the presence of OOS (1:100 V/Vf) in culture medium supplemented with 0.5% FBS. Migrated cells were quantified after 4% formalin fixation and crystal violet staining (Sigma-Aldrich). Results were calculated from three independent experiments and data were expressed as the mean of total migrated cells per membrane.

*Experimental development of hepatic metastasis. In vivo* metastasis assay was carried out by intrasplenical (i.s.) inoculation of tumor cells. The cells ($2x10^5$) were inoculated in the inferior pole of the spleen under anesthesia. The animals were treated with 100 $\mu$l of OOS and divided into 3 groups based on administration pattern as follows: in group I, animals received no treatment; in group II, mice were treated with OOS 4 days prior to tumor cell inoculation; and in group III, mice were treated with OOS from the 7th day after tumor cell inoculation until the day of sacrifice (Fig. 1). As control for tumor development at the time of first doses of OOS in group III three untreated animals were sacrificed the 7th day after tumor cell inoculation. Animals from groups I, II and III were sacrificed 21 days after tumor cell inoculation, and livers were collected, fixed in zinc solution (Sigma-Aldrich) and paraffin embedded for histological analyses after H&E staining. Tumor occupied area was quantified in three 7 $\mu$m-thick sections per

liver, separated 500 $\mu$m from each other and calculated as the area occupied by tumor foci per section of liver tissue. At least 6 mice per group were used per each experiment. Once the most effective administration pattern was chosen by treating mice with 100 $\mu$l of OOS, the effectiveness of a lower OOS concentration (50 $\mu$l) was tested under the same experimental conditions.

*Immunohistological analyses of liver tissue*. Liver tissue was analyzed for the expression of Ki-67 (ab16667; 1:100; Abcam, Cambridge, UK) and caspase-3 (ab4051; 1:100; Abcam) by immunohistology. Mice from group I and III were sacrificed and livers were collected for immunohistological analyses in 7 $\mu$m tissue sections by staining with specific antibodies. Additionally, the expression of α-smooth muscle actin (ASMA) (MCA5781GA; 1:100; AbD Serotec, Raeigh, NC, USA) was also quantified. Antigen retrieval was carried out in citrate buffer pH 6.0, then endogenous peroxidase and inspecific proteins were blocked by incubating for 40 min with 3% of $H_2O_2$ and 40 min with 3% FBS. Finally, liver tissue was incubated with specific antibodies and antigen expression was revealed by horseradish peroxidase (HRP)-conjugated streptavidin (Life Technologies) and 2-Solution DAB kit (Life Technologies) following the manufacturer's instructions. Antigen expression levels were quantified by ImageJ software (NIH, Bethesda, MD, USA). Results were expressed as the mean of at least 6 liver sections for each treatment.

*RT-qPCR*. Total RNA was extracted from paraffin embedded liver tissues collected from mice treated with 100 $\mu$l of OOS according to group I and III dosage pattern using Norgen FFPE RNA Purification kit (Norgen Biotek Corp., Thorold, ON, Canada) following the manufacturer's instructions. RNA concentration and quality was assessed by NanoDrop spectrophotometer (ND-1000; Thermo Fisher Scientific Inc., Rockford, IL, USA), and 2 $\mu$g RNA was reverse transcribed into cDNA with recombinant moloney murine leukemia virus reverse transcriptase and random primers (Life Technologies). Quantification of cDNA template was performed with real-time polymerase chain reaction (PCR) using SYBR-Green (Life Technologies) as a fluorophore in ABI 7900HT (Life Technologies). PCR primers (Life Technologies) were as follows: GAPDH (housekeepeing) F, GTATGACTCCACTCA CGGCAA and R, CTTCCATTCTCGGCCTTG; IL1β F, CT GTGACTCATGGGATGATGATG and R, GCCTGTAGTG CAGTTGTCTAAT; INFγ F, TTCTTCAGCAACAGCAAG GC and R, TGTGGGTTGTTGACCCTCAAA; COX2 F, TG CACTATGGTTACAAAAGCTGG and R, TCGGAAGCTC CTTATTTCCCTT; TNFα F, CCAGTGTGGGAAGTGTCTT and R, AAGCAAAAGAGGAGGCAACA; IL6 F, TCTAT ACCACTTCACAAGTCGGA and R, GAATTGCCATTGC ACAACTCTTT. Relative expression of target genes was normalized to the internal control gene GAPDH by the ΔΔCt method. Data were generated by the use of specific software (ABI Prism, SDS2.0; Life Technologies) after normalization. Results were calculated at least from 6 livers per treatment and from two independent experiments.

*Statistical analysis*. Statistical analysis was performed with the Student's two-tailed unpaired t-test. Data are expressed as the mean ± SD. The criterion for significance was $P<0.05$ for all comparisons.

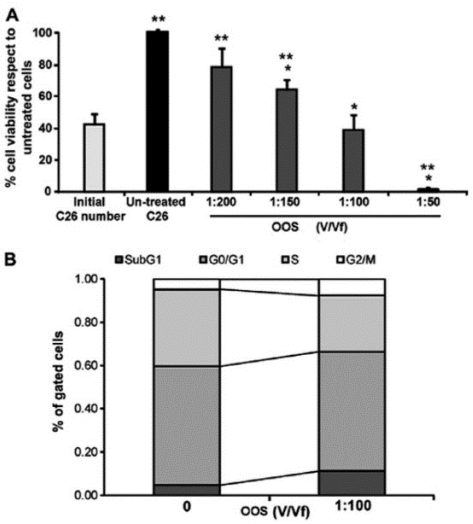


Figure 2. OOS reduces *in vitro* viability of C26 cells. (A) The viability of C26 cells was tested after 24-h incubation in the presence of OOS. C26 cells were treated with increasing concentrations of OOS ranging from 1:200 to 1:50 during 24 h before viability quantification and compared to the viability of initially cultures cells (light grey) and to that of untreated cells (black). Differences in the viability of treated cells vs. untreated cells (*) and vs. initially cultured cells (**) were considered to be statistically significant at $P<0.05$. (B) The cell number in each cell phase respect to total gated cell number was analyzed after PI staining by flow cytometry after 72-h incubation with 1:100 OOS (V/Vf). Differences between treated and untreated cells were considered statistically significant at $^*P<0.05$.

## Results

*OOS reduced the viability of C26 cell line in vitro*. In order to test the effect of OOS in the viability of colon carcinoma cells, C26 cells were treated with increasing concentrations from 0 to 1:200 (V/Vf) of OOS for 24 h. As shown in Fig. 2A, the viability of tumor cells decreased in a dose-dependent manner from 20% at 1:200 (V/Vf) of OOS to >90% at 1:50 (V/Vf) (Fig. 2A) in respect to untreated C26 cells. However, when compared to the initial cell number, control untreated cells and every treatment shows viability differences but 1:100 (V/Vf) resulted in a significant non-proliferation.

Since the only concentration which inhibited completely the proliferation of C26 cells was 1:100 OOS (V/Vf), we next, analyzed the amount of cells in each phase of the cell cycle by means of PI staining after the cuture for 72 h in the presence of OOS. Then, the cells were analyzed by flow cytometry. As seen in Fig. 2B, the number of cells in phase $G_2$/M and in the peak Sub $G_1$ were increased while the cell number ongoing DNA replication in phase S showed a significant decrease compared to those cells cultured under basal conditions.

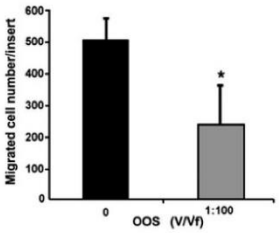


Figure 3. OOS reduces *in vitro* migration of C26 cells. C26 cells were cultured on top of 8 $\mu$m-pore membrane modified Boyden chambers. The number of migrated C26 cells were quantified after 18-h incubation in the presence of 1:100 OOS (V/Vf) Data are mean values ± SD from three different experiments. Differences were considered statistically significant at $^{*}P<0.05$.

*In vitro migratory potential of C26 cells is reduced by OOS.* To assess the effect of OOS in the migratory potential of C26 cells through a collagen type I layer, we incubated C26 cells on top of collagen type I-covered 8 $\mu$m-diameter pore membranes. The C26 cells were allowed to migrate for 18-h in the presence of 1:100 of OOS (V/Vf). The amount of migrated tumor cells was decreased by 50% in presence of 1:100 of OOS (V/Vf) (Fig. 3).

*Effects of administration pattern in OOS efficacy during the development of metastasis to the liver in vivo.* Next, the more efficient dosage pattern of OOS was assessed. The tumor cells were inoculated i.s. and 100 $\mu$l of OOS was administered once a day under the schedule described in Materials and methods (Fig. 1), until sacrifice. Under these dosage protocols, the weight of liver and spleen did not show any change (data not shown) among the experimental groups. The nutrient mixture only exerted significant effects in those animals included in group III (Fig. 4A), while no effect was detected in the preventive administration of OOS (group II) on the tumor burden in the liver when compared to untreated group I (Fig. 4A). Even though, the metastatic development was decreased at day 21 after tumor cell inoculation in mice treated with OOS from day 7, the progression was not inhibited but slowed down, since at day 7 after tumor cell inoculation only few micrometastasis could be observed (data not shown).

*Dose-dependent effect of OOS in the metastasic development to the liver in vivo.* Once the most effective pattern of administration was established, a lower dose of OOS efficacy was tested under these circumstances. Mice were treated with 50 and 100 $\mu$l of OOS following the administration pattern of group III. After the collection of livers and processing for histological analyses the quantification of liver area occupied by tumor foci were carried out. The results show, a reduction of >50% in the area of liver tissue occupied by the tumor in those livers collected from 50 and 100 $\mu$l-treated mice (Fig. 4B). Even more, after the classification and quantification of the tumor foci by their size, an decrease in the number of metastatic foci larger than 400 mm$^2$ of diameter were detect in mice treated with 50 and 100 $\mu$l OOS compared to those observed in the livers of untreated mice. Indeed, this difference was only significant in livers collected from the animals

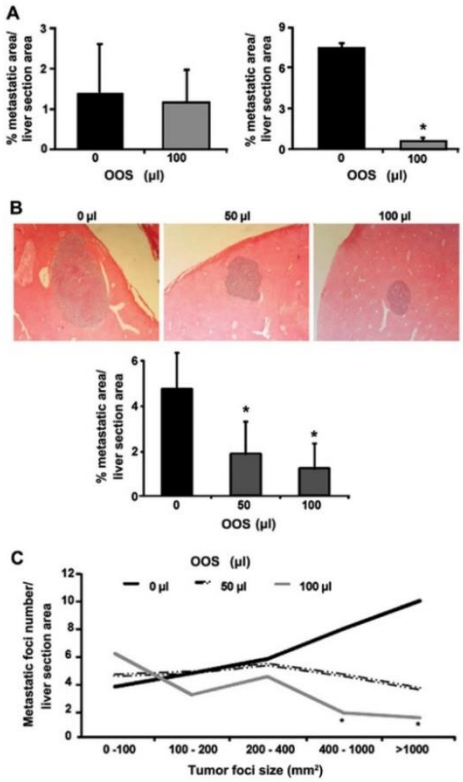


Figure 4. OOS slows down *in vivo* tumor growth. (A) C26 cells were i.s. inoculated and mice were treated with 100 $\mu$l of OOS under the administration patterns described in Materials and methods. Group I, untreated; group II, OOS administered four days prior to tumor cell inoculation; group III, OOS administered from day 7th after tumor cell inoculation until sacrifice. (B) Mice were treated with 50 and 100 $\mu$l of OOS under administration pattern of group III. (C) The amount of liver foci developed in the liver of mice untreated or treated with 50 and 100 $\mu$l of OOS was quantified in liver tissue based on tumor foci size. Image original magnification, x20. Differences were considered statistically significant at $^{*}P<0.05$.

treated with 100 $\mu$l of OOS (Fig. 4C). However, no significant difference was observed between the treatments.

*OOS affects tumor proliferation and apoptosis in vivo.* To quantify the expression levels for Ki-67 and caspase-3, livers were collected either from untreated C26-bearing mice and from C26-bearing mice treated with 50 or 100 $\mu$l of OOS from day 7 since this dosage pattern was the only one showing any kind of effectiveness. The livers were fixed and embedded in paraffin and the expression levels of Ki-67 and caspase-3 were analyzed by immunohistochemistry. As shown in Fig. 5A while the levels of caspase-3 expression were increased 10-fold after 100 $\mu$l OOS administration compared to the untreated group. No significant changes were observed in the animals treated with 50 $\mu$l of OOS. Furthermore, the levels

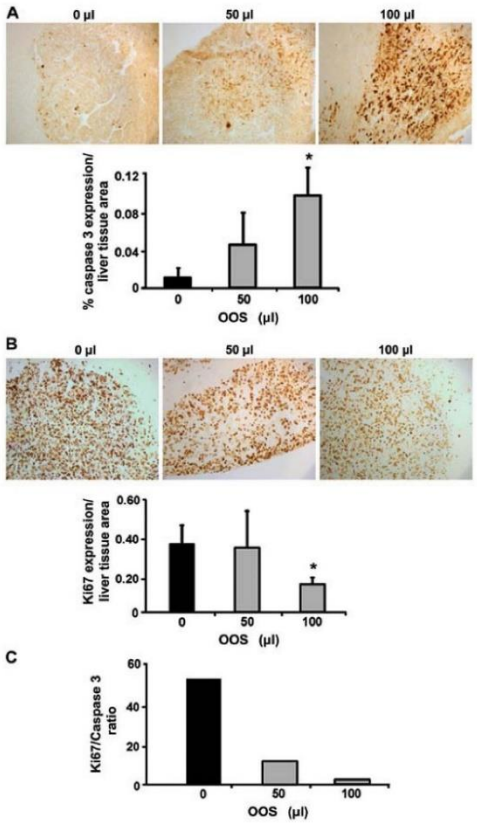


Figure 5. OOS affects proliferation and apoptosis *in vivo*. The effect of OOS on Ki-67 and caspase-3 expression in livers from tumor-bearing mice was analyzed by immunohistochemistry. (A) Caspase-3 expression (brown) was quantified in livers collected from untreated, 50 and 100 $\mu$l OOS treated C26-bearing mice as the percentage of the area positive for caspase-3 expression respect to total liver section area. (B) Ki-67 expression (brown) was quantified in livers collected from untreated, 50 and 100 $\mu$l OOS treated C26-bearing mice as the percentage of the area positive for Ki-67 expression in respect to total liver section area. (C) The ratio between Ki-67 and caspase-3 was calculated as the expression of Ki-67 relative to caspase-3 expression. Image original magnification, x20. Differences were considered statistically significant at $^{*}P<0.05$.

of Ki-67 showed a decrease in the tumor foci developed in the livers of mice treated with 100 $\mu$l OOS compared to those observed in the livers from untreated mice (Fig. 5B). However, the difference was not observed in the livers collected from mice treated with 50 $\mu$l of OOS. Even more, the ratio between Ki-67 and caspase-3 expression was reduced in mice injected with C26 cells and treated with both concentrations of OOS vs. untreated mice (Fig. 5C).

*OOS treatment limits the infiltration of myofibroblasts in the metastatic liver.* Finally, the expression of ASMA was

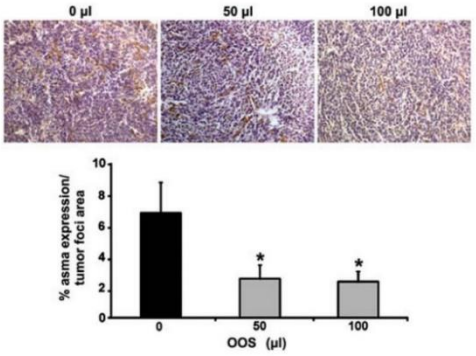


Figure 6. *In vivo* HSC infiltration in the tumor is reduced by OOS. Expression levels of ASMA were analyzed in liver tissue by immunohistochemistry. ASMA was stained with specific antibodies in liver tissue collected from untreated, 50 and 100 $\mu$l OOS treated mice. Data are calculated as % of ASMA expression per tumor foci area. Image original magnification, x20. Differences were considered statistically significant at $^{*}P<0.05$.

quantified in the metastatic liver tissue in order to analyze the infiltration by liver cancer-associated fibroblasts within the tumor foci. Sections from those livers collected either from untreated C26-bearing mice (group I) or from C26-bearing mice treated with 50 or 100 $\mu$l of OOS following the dosage pattern for group III were stained with specific antibodies for ASMA, an antigen expressed in CAFs. The intratumoral levels of ASMA expression in liver tissue collected from mice treated with 50 and 100 $\mu$l of OOS was reduced by 50% compared to the liver tumors obtained from untreated mice (Fig. 6).

*OOS modulates the expression of inflammatory genes in metastatic livers.* Since the tumor microenvironment is characterized by an inflammatory signature, the gene expression of the inflammatory molecules IFN$\gamma$, TNF$\alpha$, COX-2, IL6 and IL1$\beta$ was quantified in total liver tissue by qPCR. As shown in Fig. 7, the expression of genes coding for the aforementioned inflammatory molecules was significantly reduced in the livers collected from mice treated with 100 $\mu$l of OOS according to group III compared to those obtained from untreated mice (Fig. 7).

## Discussion

CRC is one of the leading causes of cancer-related deaths in the world, due mainly to the metastatic spread to distant organs, specially the liver. Even though great advances have been made in the development of therapies to treat CRC, they are often aggressive and with limited efficacy. Thus, new complementary therapies are being developed consisting in biological compound mixtures. Certain nutrient mixtures have been proved to be effective in several preclinical *in vivo* and *in vitro* models such as pulmonary metastasis of melanoma and cervical cancer (8,14). However, there is no report describing the efficacy of these nutrient mixtures in the *in vivo* metastatic spread of CRC to the liver. Thus, in the present study, we aimed

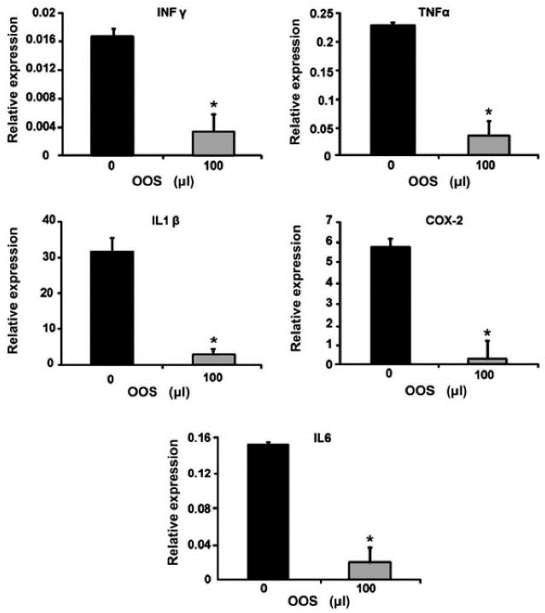


Figure 7. OOS affects inflammatory gene expression in total liver. Gene expression of IFNγ, TNFα, IL1β, IL6 and COX-2 was analyzed by RT-PCR. Total RNA was extracted from paraffin-embedded liver tissue collected from untreated or 100 µl OOS treated C26-bearing mice. Data are expressed as the mean values ± SD from six livers from two independent experiments. Differences were considered statistically significant at $^{*}P<0.05$.

to investigate the effectiveness of OOS, a complex mixture containing licorice extract, vitamins and minerals which have proven anti-oxidant and anti-inflammatory properties in other diseases (15,16) in the metastatic development of colon carcinoma cells. Here, we highlight the anti-metastatic effect of this nutrient mixture *in vivo* by means of reducing tumor cell proliferation, migration and recruitment of CAFs, along with an increase in apoptosis. Additionally, the mixture induced a decrease in the expression of RNA levels for pro-inflammatory and pro-angiogenic factors in metastasized liver provoking a slow down of the metastatic development of colorectal cancer C26 cells to the liver. Thus, these results support the need for further studies for the use of OOS as a nutritional complement during the treatment of colorectal cancer liver metastatic disease.

According to our results, the effect of OOS on the metastatic progression of C26 cells to the liver depends on the dosage pattern. Because a significant reduction in the tumor burden of the liver was observed only in the mice treated with OOS 7 days after tumor cell inoculation, but not when the solution was administered days before the tumor inoculation. Lode *et al* (17) have reported the inefficacy of a nutrient mixture against metastasis in a neuroblastoma model. However, as stated by Niedzwiecki (18) these observations might be due to several reasons. On the one hand, the excessive amount of cells inoculated by the authors or the need for longer and continuous periods of treatment may account for this lack of effect. On the other hand, antioxidants have been proved to exert their functions mainly in the presence of chronic inflammation and in a highly hypoxic environment (1). In the absence of a highly inflammatory and pro-oxidant microenvironment biologically active compounds might lack their anti-tumoral activity (19), such as the one in mice before the tumor inoculation. Thus, this could explain the lack of effect of OOS in the early stage treatment. Even so, further studies are needed to fully establish the mechanisms of OOS when it is administered at early stages.

Dietary agents are believed to suppress, among others, the hyper-proliferative processes during the development of a tumor. Several phytochemicals have been shown to exert suppressive effects on AP-1 and NF-κB interfering with growth and proliferative signals (20,21). In accordance to this, supplementation with dietary nutrient mixture OOS significantly slows down murine colon carcinoma C26 tumor growth *in vivo* in immune competent mice related to a decrease in the intratumoral expression of Ki-67, an antigen strictly associated with cell proliferation. The *in vitro* studies supported these findings since the viability of tumor cells was significantly decreased and a slight reduction in the number of cells in phase S was induced by OOS treatment. Along with these results, an increase in the amount of cells in phase $G_2$/M was observed. In fact, cathechins and licorice components cause cell cycle arrest by downregulating cyclin D1 and E, key molecules in $G_1$ to S transition and completion of the latter one (22,23). Ultimately, the cell cycle arrest will trigger cell cycle deregulation and apoptotic processes via upregulation of p21 and p27,

both well-known tumor suppressor proteins downregulated in numerous cancers (24). In addition to the effect in the cell cycle progression, OOS breaks the characteristic resistance of tumor cells to enter apoptosis. Consistent with these observations, an increase in cell number in subG1 *in vitro* and the intratumoral increase of caspase-3 *in vivo* after treatment with the complex nutrient mixture points to the induction of programmed cell death within tumor foci by this compound. The hypoxic and inflammatory tumor microenvironment induces the activation of anti-apoptotic pathways supporting the effect of anti-inflammatory or anti-oxidative drugs which have been shown to increase the sensitivity of tumor cells to pro-apoptotic signals (25). In addition to its effects in tumor cell proliferation and apoptosis, OOS reduced the migratory potential of C26 colon carcinoma cells *in vitro*. Migration of tumor cells is a key step not only for invasion of adjacent tissue in the primary organ but also for extravasation and colonization of the secondary target organ during the metastasic process. It is well-known that chronic inflammation is linked to oxidative stress and both of them are related to the invasive and migratory potential of cancer cells during tumor progression (1). Thus, the migratory capacity of tumor cells is also a potential target for nutrient mixtures with anti-inflammatory and anti-oxidant properties. Roomi *et al* (9,14) have shown that *in vitro*, a nutrient mixture consisting in lysine, ascorbic acid, proline and green tea, among others, significantly reduced the migratory potential of a selected set of different tumor cell types related to a decrease in the levels of pro-migratory and pro-angiogenic factors such as proteases and VEGF (9,14).

In the hepatic tumor microenvironment, myofibroblasts are the most prominent CAFs. In the liver, CAFs are mainly originated from transdifferentiated hepatic stellate cells in the tumor stroma, and such tumor activated-hepatic stellate cells promote tumor growth and invasiveness (26). Additionally, they contribute not only to create a pro-tumoral stroma but also to trigger the angiogenic switch allowing one step forward from an avascular to a vascular state during the metastasic tumor growth (26). Furthermore, these tumor associated fibroblasts participate actively in the production of pro-inflammatory and pro-oxidant factors taking active part in tumor expansion by inducing, among others, the trigering of an angiogenic response. Moreover, recruited fibroblasts within a tumor have been positively correlated with tumor progression and poor prognosis in colorectal cancer (27). In our model, the nutrient mixture OOS reduced the recruitment of myofibroblasts within the tumor foci, as shown by a decreased amount of ΛSMΛ expressing cells within the tumor foci which might also account for the reduced angiogenesis. This reduction in myofibroblast infiltration *in vivo* was related with an impaired migratory potential of 3T3 fibroblast *in vitro* by OOS (data not shown). The impaired recruitment of CAFs might account as an additional mechanism by which the nutrient mixture might exert its antitumoral effects. Indeed, several compounds with anti-oxidant and anti-inflammatory properties reduce the angiogenic response in different types of tumors and show a significant impact in the tumor stroma formation (28).

The antioxidant properties of several components of OOS, such as polyphenols, cannot fully explain their antitumoral activity. Cathechins modulate inflammatory pathways since they act as signaling agents by interfering with NF-κB and AP-1 which, in turn, results in inhibition of pro-inflammatory and pro-angiogenic factors, including IL6, IL-1β, TNF-α and VEGF (29). Among them, TNFα or IL-1β induce the expression of growth factors, stimulate epithelial tumor motility and tumor angiogenesis (30,31). Another proinflammatory molecule commonly upregulated in colon cancer, COX-2, has been implicated in the growth and progression of colorectal cancer via multiple pathways (32-34) including the acquisition of resistance to apoptosis and promoting angiogenesis through $VEGF$ production (35,36). Moreover, a decrease in COX-2 expression at RNA levels in total liver caused by OOS administration might be related to a reduction in VEGF levels (36). Additionally, the reduction in IL-1β, and TNFα RNA levels after OOS administration is consistent with the downregulation in the levels of COX-2 RNA which is commonly stimulated by cytokines such as IL-1β and TNFα as a result of the interaction between colon carcinoma cells and the cells of the liver microenvironment (36,37).

In summary, OOS slows down the metastatic progression of CRC to the liver. Thus, the anti-oxidant and anti-inflammatory properties of this nutrient mixture induced an inhibition of the proliferative and migratory potential of tumor cells which together with an increase in the sensitivity to apoptotic signals might modulate the metastatic development of colorectal cancer cells to the liver. Besides, OOS limits tumor infiltration by CAFs and inhibits the production of inflammatory and angiogenic factors within the tumor microenvironment. Collectively, this creates an unfavorable and non-permissive microenvironment for tumor growth suppressing the final steps of tumor progression. Therefore, OOS may constitute a pharmacologically safe complementary compound for the treatment of cancer and its metastasis slowing down the tumor growth, and, thus, increasing the life time and quality for patients suffering from CRC liver metastasis. The above justifies further characterization and validation of OOS in the metastatic development and their combination with actual therapies.

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## Publication 2: Ocoxin Oral Solution® as a complement to irinotecan chemotherapy in the metastatic progression of colorectal cancer to the liver.

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# Ocoxin oral solution® as a complement to irinotecan chemotherapy in the metastatic progression of colorectal cancer to the liver

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**Abstract.** Colorectal cancer (CRC) is an aggressive disease in which patients usually die due to its metastatic progression to the liver. Up to date, irinotecan is one of the most used chemotherapeutic agents to treat CRC metastasis with demonstrated efficacy. However, the severity of the side effects constitute the main limitation to its use in the treatment. Consequently, new complementary therapies are being developed to avoid these adverse effects while maintaining the efficacy of the antitumoral drugs. Ocoxin oral solution (OOS®) is a nutritional mixture containing biologically active compounds with demonstrated antitumoral and immunomodulatory effects. Thus, we aimed to analyze the effect of OOS® as a suitable complement to irinotecan therapy in the treatment of CRC metastasis to the liver. First, the effect of OOS®, irinotecan and the combination of both on the viability of C26 cells was tested *in vitro* and *in vivo*. Second, the expression of caspase-3, Ki67 and the macrophage infiltration by F4/80 marker was quantified in liver tissue sections by immunohistochemistry. Finally, mRNA microarray study was carried out on tumor cells isolated from tumor-bearing livers collected from mice subjected to the above treatments. Our results show that OOS® administered as a complementary therapy to irinotecan reduced tumor cell viability *in vitro*. Moreover, irinotecan administered either alone or in combination with 100 $\mu$l OOS® from the 7th day after tumor cell inoculation decreased the metastatic growth in the liver. Besides, several genes with binding and catalytic activities showed to be deregulated by RNA microarray analysis. In conclusion, OOS®, when administered as a complement to irinotecan, reduced the metastatic development of colorectal cancer to the liver. Additionally, the overall health state of the animals improved. These results point out OOS® as a potential supplement to the anti-tumoral treatments used in clinical settings in patients suffering from disseminated colorectal cancer.

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*Key words:* ocoxin oral solution, nutrient mixture, colorectal cancer, liver, metastasis

## Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer deaths in the world. However, those patients usually die to the metastatic progression, mainly to the liver, of the primary tumor. Among the various therapies used in the clinical setting to treat metastatic CRC, irinotecan is one of the most widely utilized as a chemotherapeutic agent for the treatment of this disease (1). Irinotecan is a camptothecin derivative that exerts its antitumor activity by breaking single strands of DNA inhibiting its re-ligation and, thus, blocking the DNA synthesis. As a result, cell cycle is arrested and tumor cells go into apoptosis. Irinotecan has widely demonstrated its efficacy improving the survival time of metastatic CRC patients.

Nevertheless, irinotecan exerts its effects on the tumor microenvironment as a whole, affecting not only to tumor cells but also to resident and recruited host cells. Indeed, irinotecan might also affect the recruitment of inflammatory cells including Kupffer cells, the resident macrophage population of the liver, which coordinate inflammatory networks by secreting multiple cytokines and growth factors, thereby promoting tumor cell adhesion, migration and finally, the metastatic progression (2). Additionally, the action of irinotecan on the stromal compartment might be responsible, at least in part, for the undesired side effects.

In order to diminish the adverse effects of irinotecan, different combinations of anticancer drugs or compounds with irinotecan are currently being tested (1). Several studies have shown that the use of biologically active compounds in combination with anti-tumoral drugs not only improve their efficacy, but also decrease their side effects. Gol'dberg *et al* (2008) described that glycyrrhizic acid extracted from licorice root, which has as an anti-inflammatory and immunomodulatory substance, improves the efficacy of cytostatic therapies such as cyclophosphamide that inhibits the growth and development of metastasis in lung tumor (3). Also, the slowdown of the disease progression has been observed after the addition

of certain vitamins to the diet of patients undergoing chemotherapy (4). In fact, some authors have described that melatonin and vitamin C and E decrease the extent of DNA lesions on human lymphocytes and gastric mucosa after infection with Helicobacter pylori (4,5).

In the present study, we utilized the nutritional supplement OOS® which contains green tea, licorice extract vitamins, minerals and aminoacids. This compound has probed to possess antitumoral and immunomodulatory effects (6,7) and to potentiate the antiproliferative effect of standard chemotherapeutic agents in acute myeloid leukemia (8). Márquez *et al* (2016) showed that this nutrient mixture slows down the metastatic progression of CRC to the liver in an experimental model of metastatic development to the liver (7). Thus, OOS® might be a suitable complement to tumor therapies, such as irinotecan, in the treatment of disseminated CRC. Hence, the present study aims to evaluate the benefits of OOS® as a complement to irinotecan therapy in order to improve the overall status of metastatic CRC patients by reducing the side effects, and thereby, improving their quality of life.

## Materials and methods

*Animals*. Balb/c mice (male, 8-weeks old) were obtained from Janvier Labs (Paris, France). The animals were kept in the animal facility of EHU/UPV and had access to standard chow and water *ad libitum*. All the proceedings were approved by the Ethics Committee for Animal Experimentation (CEEA) of the Basque Country University in accordance with institutional, national and international guidelines regarding the protection and care of animal use for scientific purposes.

*Cell lines*. Murine colorectal cancer C26 cells (ATCC, LGC Standards S.L.U. Barcelona, Spain) syngenic with Balb/cByJ mice were used.

Cells were cultured under standard conditions in RPMI-1640 medium (Life technologies, Madrid, Spain) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 $\mu$g/ml) and amphotericin B (0.25 $\mu$g/ml) (Life technologies). Cells were passed at a confluency of 90%.

*In vitro viability assay*. The viability was quantified by means of PrestoBlue® cell viability reagent following manufacturer's instructions (Life Technologies). To do so, $5x10^4$ cells/ml were cultured with RPMI-1640 medium (Life technologies) in collagen type I (1 mg/ml) (Sigma-Aldrich, St. Louis, MO, USA) precoated plates. After a 48 h incubation with either OOS® (1:100 (V/Vf) ), irinotecan 50 $\mu$M or a combination of both (1:100 (V/Vf) and 50 $\mu$M), a 1:100 dilution of Presto Blue® was added in RPMI-1640 to the cell cultures for 2 h. Then, the absorbance was quantified with a Fluoroskan Ascent (Thermo Labsystems, Waltham, MA, USA). Cell number was quantified respect standard line.

*Experimental development of colorectal cancer metastasis to the liver*. Balb/c mice were anesthetized with an intraperitoneal (i.p.) administration of Nembutal (50 mg/kg). Then, the spleen was exposed to carry out an intrasplenic (i.s.) inoculation of C26 cells ($1.5x10^5$ cells) in the inferior pole. Seven days later, the animals were divided into 5 treatment groups as shown in Fig. 1. Briefly, a) group I was constituted by mice receiving no treatment; b) group II comprised mice treated with an oral daily dose of 100 $\mu$l of OOS®; c) group III mice included mice treated with an i.p. dose of 20 mg/kg irinotecan once every two days; d) group IV consisted in mice receiving an oral daily dose of 100 $\mu$l of OOS® and an i.p. administration of 20 mg/kg of irinotecan once every two days; and e) group V was constituted by untreated mice sacrificed at different time points begining the 7th day after tumor cell inoculation in order to monitor tumor development. Two weeks after the initiation of the treatments, mice were sacrificed by cervical dislocation and the livers were collected and fixed in Zinc solution (Panreac, Barcelona, Spain). Finally, they were embedded in paraffin. Tumor occupied area was quantified in five 7 $\mu$m-thick sections per liver, separated 500 $\mu$m from each other and calculated as the area occupied by tumor foci per section of liver tissue. At least 6 mice per group were used per each experiment. Tumor area was quantified by ImageJ Software. Results were expressed as % liver area occupied by total tumor burden.

*Immunohistological analysis of liver tissue sections*. The expression levels of the apoptotic marker caspase-3, the proliferation marker Ki67 antigen, and the grade of macrophage infiltration by F4/80 marker was analyzed in liver tissue sections by means of immunohistological analysis. To do so, after antigens were retrieved in liver tissue, endogenous peroxidase and unspecific binding were blocked with 3% $H_2O_2$ and 5% FBS, respectively. Then, tissue sections were incubated with either specific antibodies against caspase-3 (ab4051; 1:100; Abcam, Cambridge, UK), Ki67 (ab16667; 1:100; Abcam), or F4/80 (MCA497R; 1:100; AbD Serotec, Oxford, UK). Finally, tissue was incubated with the specific biotinylated secondary antibodies and the antigen expression was revealed by horseradish peroxidase (HRP)-conjugated streptavidin (Life technologies, Carlsbad, CA, USA) and 2-Solution DAB kit (Life technologies) following the manufacturer's instructions. Antigen expression levels were quantified by ImageJ software (NIH, Bethesda, MD, USA). Results were expressed as the mean positive area per tumor foci area in at least 6 liver sections for each treatment.

*Microarray mRNA analysis from tumor explants*. Mice were inoculated with C26 cells as previously described and subjected to the same treatment protocol (Fig. 1). Then, mice were sacrificed and tumor explants were collected from livers of tumor-bearing mice and cut into small pieces in a petri dish, incubated with trypsin-EDTA 0.05% solution (Thermo Fisher Scientific, Inc., Madrid, Spain) and centrifuged. Cell pellets were resuspended in complete culture medium and supplemented with 0.1 $\mu$g/ml gentamicin (Sigma-aldrich, St. Louis, MO, USA). Once they were grown to confluency, cells were lysed and total RNA was extracted by the TRIzol® reagent (Life Technologies) and chloroform method. Total RNA was further purified by means of PureLink® RNA Mini kit (Life technologies) following manufacturer's instructions.

Afterwards, RNA integrity was analyzed by using a Eukaryote Total RNA Nano Assay with the Lab-chip in the Agilent 2100 Bioanalyzer in combination with Agilent

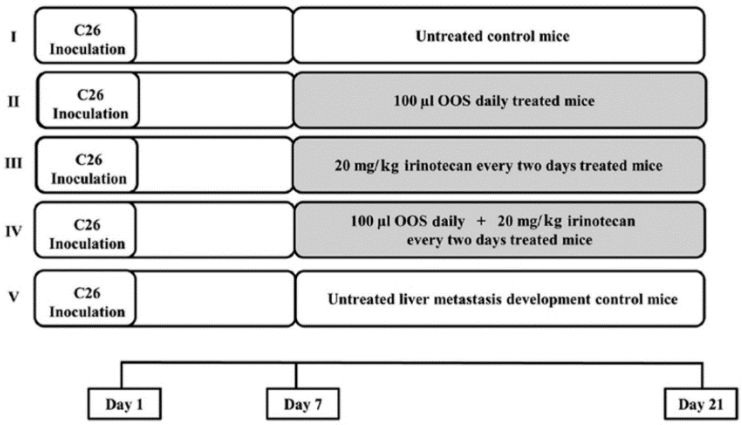


Figure 1. Experimental animal groups treatment pattern. Seven days after tumor cell inoculation, at least 6 mice per group were divided into 5 experimental groups as follows: group I, untreated mice; group II, mice treated with a daily oral administration of 100 $\mu$l of OOS®; group III, mice treated with a i.p. dose of 20 $\mu$g/ml of irinotecan every two days; group IV, mice treated with both, a daily oral dose of 100 $\mu$l of OOS® and 20 $\mu$g/ml of irinotecan every two days; group V, untreated mice used to control the metastatic development. All mice were treated for a period of 14 days.

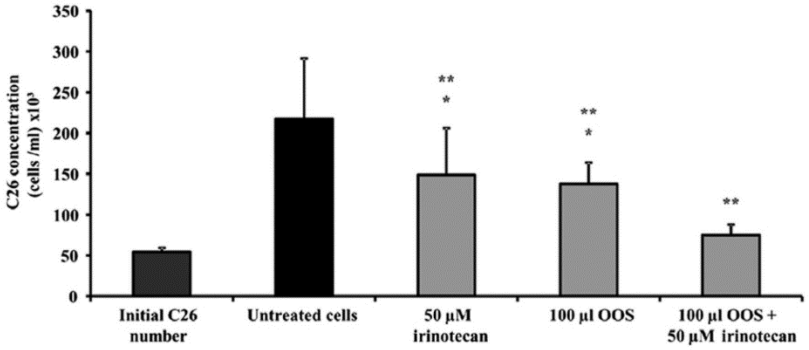


Figure 2. Effect of the combined treatment of irinotecan and OOS® in the viability of C26 cells. The viability of C26 cells was tested in the presence of OOS® (1:100), irinotecan (50 $\mu$M) or the combination of OOS® and irinotecan (1:100 and 50 $\mu$M) for 48 h. Then, the viability was quantified in untreated (black) and treated (grey) cells. Data are mean values ± SD from three different experiments. Differences in the viability of treated cells vs. untreated cells (**) and vs. initially cultured cells (*) were considered to be statistically significant at $P<0.05$.

RNA 6000 Nano Chips. Later, mRNA was labeled using the Agilent protocol 'One-Color Microarray-Based Gene Expression Analysis. Low Input Quick Amp Labeling' that uses the 'Low Input Quick Amp Labeling kit, One-Color'. In order to generate labelled cDNA, mRNA was retrotranscribed with the AffinityScript Reverse Transcriptase (AffinityScript RT) in presence of Cy3-CTP. These samples were manually hybridized using 'SureHyb' hybridization chambers (Agilent Technologies, Inc., Santa Clara, CA, USA) according to the manufacturer's guidelines for the Agilent mRNA array and were washed according to the protocol of Agilent with ozone-barrier slide covers. Then the slides were scanned using the DNA microarrays scanner G2535CA of Agilent Technologies with the Agilent Scan control Software (v. 8.5.1.) (default settings). Finally, a feature extraction of the scanned images was made by using the Agilent Feature Extraction Software (v. 10.7.3.1) (Agilent Technologies, Inc.). Gene analysis was carried out with the PANTHER (v.10.0) analysis system (9,10).

*Statistical analysis*. Each assay was repeated three times, and the results are expressed as the mean ± SD of all of them. The statistical analysis was performed with the Student's two-tailed unpaired t-test. To carry out a statistical analysis of the comparative microarray assay, the MultiExperiment Viewer (MeV) vs. 4.9.0 application was used. In order to analyze the profiles between control and treated samples, LIMMA (Lineas Models for Microarray Data) and RANKPRODUCT methods

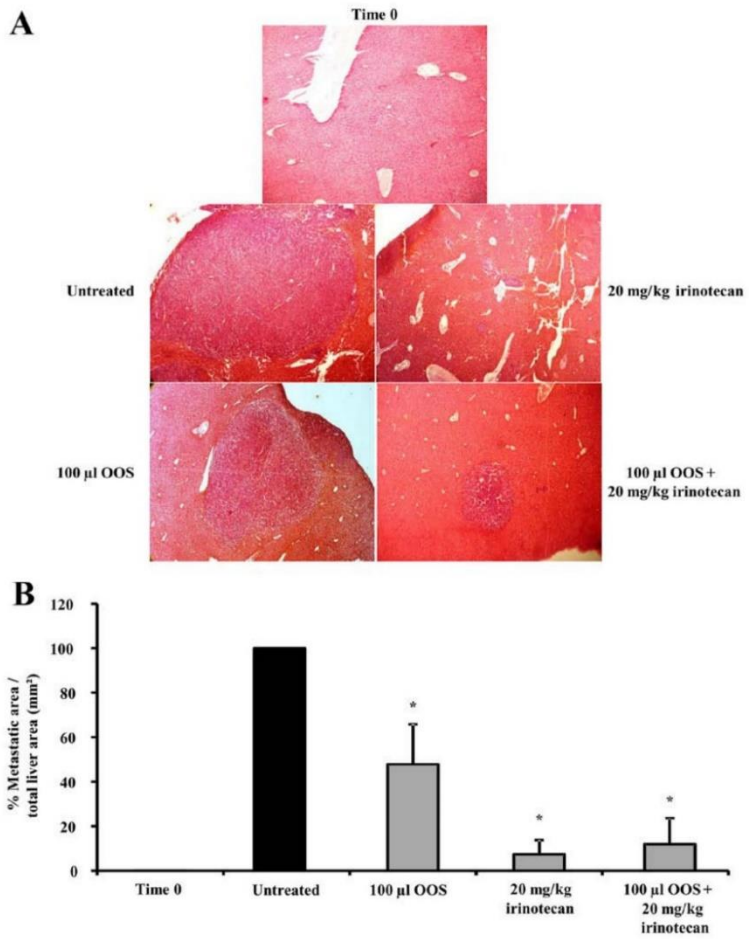


Figure 3. Effect of the combined treatment of irinotecan and OOS® in the development of CRC metastasis to the liver. C26 cells were i.s. inoculated into mice and seven days later they were divided into the five groups shown in Fig. 1 with at least 6 mice per group. Mice were either untreated or treated with OOS® (100 $\mu$l), irinotecan (20 mg/kg) or with the combined therapy (100 $\mu$l OOS® and 20 mg/kg irinotecan) as described in Material and Methods. (A) Images showing tumor foci grown in the liver of untreated and treated mice. Image original magnification was x4. (B) The total tumoral burden was quantified and represented as the percentage of liver area occupied by the tumor. Differences were considered statistically significant at *P<0.05.

were used. The criterion for significance was P<0.05 for all comparisons.

## Results

*In vitro effect of the combined therapy of OOS® and irinotecan in the viability of C26 cells.* In order to analyze the effect of OOS® as a complement to irinotecan therapy, the viability of C26 cells was evaluated in presence of OOS®, irinotecan and a combination of both. To do so, C26 cells were cultured on type I collagen for 24 h before the addition of 1:100 OOS® (V/Vf), irinotecan 50 $\mu$M alone, or the combination of both. Then, the viability was measured as described in Material and Methods. As shown in Fig. 2, irinotecan and OOS® alone reduced the viability of C26 cells by 31.66 and 36.80%, respectively. After the combined treatment, the viability of C26 cells was reduced as much as by 65%, showing a synergistic effect on tumor cell viability when both complement and irinotecan were added simultaneously (Fig. 2).

*Effect of the combined therapy of OOS® and irinotecan in the development of CRC metastasis to the liver.* To assess the effect of OOS® as a complement to irinotecan therapy in the *in vivo* metastatic progression of CRC to the liver, C26 cells were i.s.

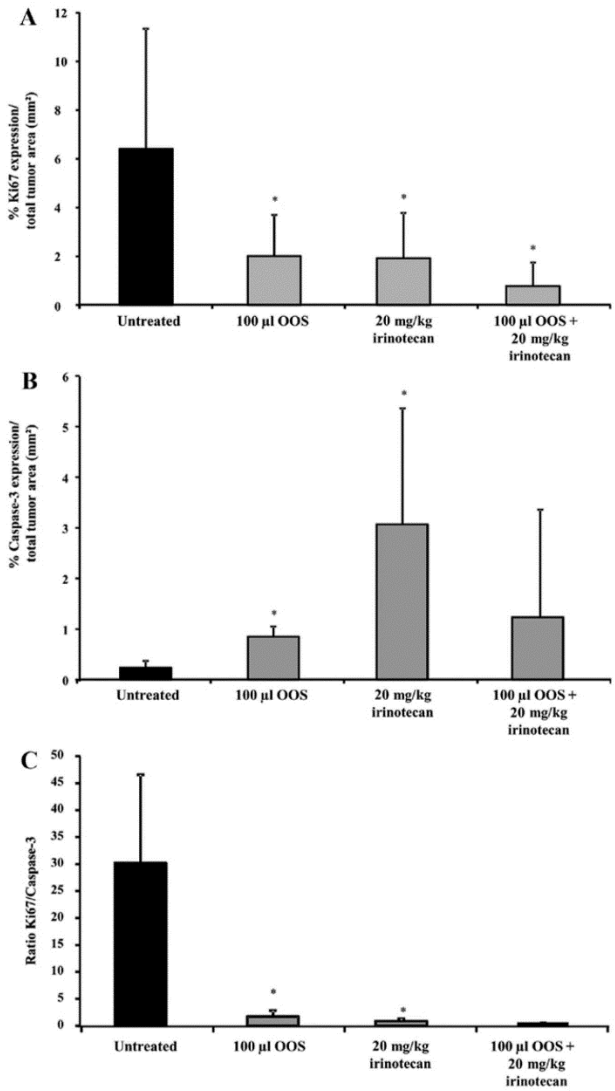


Figure 4. Effect of the combined treatment of irinotecan and OOS® in the proliferation and apoptotic markers. The expression of Ki67 and caspase-3 in livers collected from tumor-bearing mice either untreated or treated with OOS® (100 $\mu$l), irinotecan (20 mg/kg) or the complementary therapy (100 $\mu$l OOS® and 20 mg/kg irinotecan) was analyzed by immunohistochemistry with at least 6 mice per group. (A) Ki67 expression was quantified in livers collected from untreated and treated C26-bearing mice as the percentage of the area positive for Ki67 expression within tumor foci respect to the total metastatic tumor area. (B) Caspase-3 expression was quantified in livers collected from untreated and treated C26-bearing mice as the percentage of the area positive for caspase-3 expression respect to the total metastatic tumor area. (C) The ratio between Ki67 and caspase-3 in livers was calculated from results shown in A and B. Differences were considered statistically significant at $^{*}P<0.05$.

inoculated to mice. Seven days later, mice were treated with a daily oral dose of 100 $\mu$l of OOS®, an i.p. dose of 20 mg/kg of irinotecan once every 2 days, or a combination of both for 2 weeks as described in Materials and methods (Fig. 1).

Three sentinel mice were sacrificed the 7th day after tumor cell inoculation in order to establish the tumor development at the time of treatment initiation. At that point in time, micrometastasis was detected in the livers collected from tumor-bearing

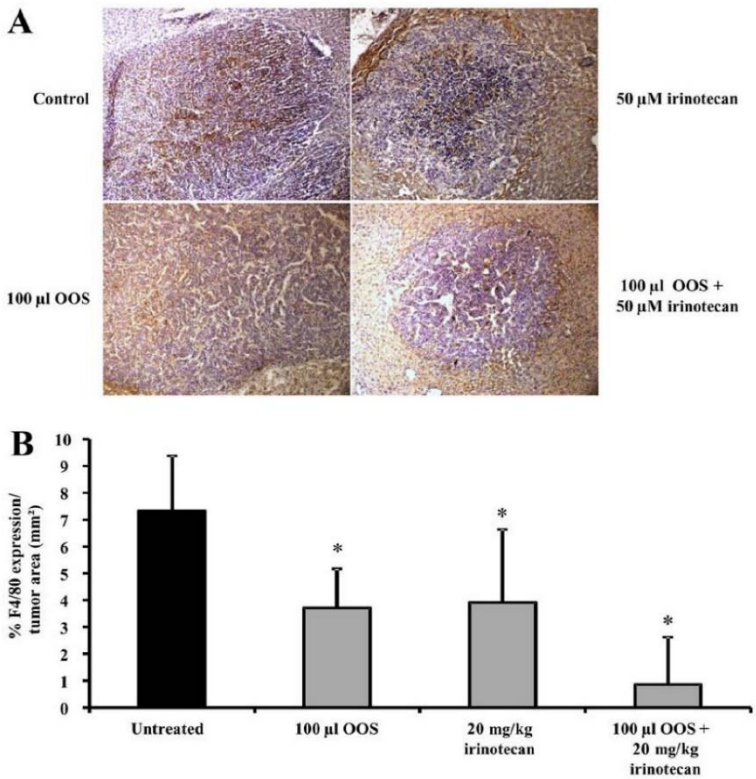


Figure 5. Effect of the combined treatment of irinotecan and OOS® on the tumor infiltration of macrophages *in vivo*. Expression level of F4/80 was analyzed in liver tissue by immunohistochemistry. (A) Images showing F4/80 expression (brown) and hematoxylin (purple) in liver tissue collected from untreated and treated mice. Image magnification was x20. (B) F4/80 expression was quantified in livers collected from untreated and treated C26-bearing mice. Data are calculated as % of F4/80 expression per tumor foci area. At least 6 mice per group were used and differences were considered statistically significant at $^{*}P<0.05$.

mice (data not shown). Tumor occupied area was quantified in three 7 $\mu$m-thick sections per liver, as described in Material and Methods. In comparison with the untreated group I, II and III showed a significant reduction of 58 and 92% in the tumor-occupied area respectively (Fig. 3A and B). Interestingly, although no synergistic effect could be observed between the irinotecan treatment alone (group III) or in combination with OOS® (group IV), the supplemented therapy reduced tumor burden by 88% (Fig. 3A and B) and furthermore, eye observations indicated an improvement in the overall fitness of the animals according to the Mouse Grimace Scale (data not shown) (11).

*Effect of the combined OOS® plus irinotecan therapy on proliferation and apoptotic markers*. The administration of either OOS® or irinotecan alone have shown to have proapoptotic and antiproliferative effects. However, the effect of the combined treatment of OOS® and irinotecan in the metastatic progression of CRC to the liver is unknown. The expression level of the proliferative marker Ki67 and the apoptotic marker caspase-3 analyzed by immunohistochemistry showed that OOS® and irinotecan administered alone decreased Ki67 expression by 80% in the tumor foci of livers collected from treated mice compared to untreated mice (Fig. 4A). Also, the combined therapy decreased Ki67 expression up to 93% (Fig. 4A). To analyze the effect on apoptosis, caspase-3 expression levels were quantified and were found to be upregulated within tumor foci of mice treated with each one of the therapies (Fig. 4B). The differences between the tumor burden in the livers collected from mice subjected to the combined therapy were not significant. This result might be due to the small tumor burden in the liver. The ratio between proliferation and apoptosis markers diminished up to 90% in livers from mice under OOS® treatment and up to 99% in livers from mice under either irinotecan treatment alone or the combined treatment (Fig. 4C).

*Effect of the combined therapy of OOS® and irinotecan on the macrophage infiltration into tumor foci in vivo*. Since macrophage infiltration into tumor foci has been related to tumor

Table I. Ocoxin oral solution (OOS®), irinotecan and the combined therapy deregulated genes.

| | Up | Down | Total |
|---|---|---|---|
| OOS® vs. control | 30 | 5 | 35 |
| Irinotecan vs. control | 11 | 141 | 152 |
| OOS®+irinotecan vs. control | 5 | 1 | 6 |
| OOS®+irinotecan vs. irinotecan | 14 | 0 | 14 |

progression and the development of chemoresistance, we analyzed the effect of OOS® and irinotecan either alone or in combination in the number of tumor-infiltrating macrophages. To do so, liver tissue sections were incubated with antibodies against F4/80, a marker for activated macrophages (Fig. 5A). As shown in Fig. 5B, the intratumoral level of F4/80 expression in the liver tissue collected from tumor bearing mice was reduced by 49 and 47% in mice treated with either 100 $\mu$l of OOS® or 20 mg/kg irinotecan alone, respectively. It is interesting to note that the infiltration of macrophages in those foci developed in livers of mice treated with OOS® and irinotecan in combination was further reduced by 88% showing a synergistic effect of both compounds (Fig. 5B).

*Metastatic tumor explants mRNA comparative microarray study.* Total RNA was extracted from the tumor explants collected from tumor-bearing mice livers as described in Material and Methods. The microarray data were subjected to two different comparisons. First, the RNA expression levels were compared between tumor cells isolated from tumor explants collected from treated mice (either treated with OOS®, or irinotecan alone or the combination of both) vs. those in the tumor cells isolated from explants collected from untreated mice. And second, a comparison was made between RNA expression levels in those tumor cells isolated from explants collected from mice subjected to the combined treatment vs. those in the tumor cells isolated from explants collected from mice treated with irinotecan alone.

The OOS® treatment alone provoked an alteration in the expression levels of 35 genes in contrast to the treatment with irinotecan alone, which altered the expression levels of 152 genes when compared to gene expression levels from untreated samples (Tables I and II). It is interesting to note that the irinotecan treatment supplemented with OOS® resulted in the reversal of most of the alterations observed when irinotecan was administered alone. That is, only 6 genes were altered in the cells collected from mice under the combined treatment (Table I). Two different patterns could be observed. 93% of the altered genes were downregulated in cells collected from mice treated with irinotecan alone when compared with control samples. In contrast, 85 and 83% of the altered genes in tumor cells isolated from mice treated with OOS® and OOS® plus irinotecan respectively showed an upregulation in their expression levels. Interestingly, only one gene presented an altered expression in every group. This gene resulted to be the one coding for Arglu1. When the gene expression levels were compared between irinotecan and combined treatment only 14 genes showed an altered expression. Interestingly all of them were upregulated (Table I).

Next, the altered genes were classified using the PANTHER (v.10.0) analysis software according to their molecular functions. In irinotecan vs. control treatment, most of the genes were classified into two principal molecular functions, catalytic activity and binding activity (Fig. 6A). Some of the genes whose expression was altered were also included in other activities, such as, receptor activity and transporter activity among others. Furthermore, the genes altered in tumor cells isolated from mice treated with the irinotecan and classified under catalytic activity were analyzed in more detail. Those genes were included in three principal catalytic activities: Oxidoreductases, hydrolases and transferases. Moreover, most of the genes altered by the combined treatment were also included in the same three catalytic activities mentioned above. Additionally, the genes with binding activity were also altered when the combined treatment was administered (Fig. 6B). In general, the genes deregulated by the action of irinotecan fall into eight of the groups classified by their molecular functions as shown in the left panel of Fig. 6A. In contrast, those genes with altered expression by the complementary therapy fall only within four of the molecular categories as shown in the left panel of Fig. 6B.

## Discussion

Colorectal cancer is one of the leading causes of cancer deaths in the world due to the spread of the primary tumor to the liver. To date, irinotecan is one of the most used chemotherapeutic drugs for the treatment of liver metastasis of CRC, which increases patient's survival. However, these treatments generate diverse side effects which influence the quality of life of patients (1). Thus, different chemotherapeutic agents are being tested in combination with biologically active compounds to avoid these effects without comprising efficacy (12,13).

Certain nutrient mixtures have shown antitumoral effects in *in vitro* and in *in vivo* preclinical models (1-3). In this context, OOS® is a mixture which has shown to have promising antitumor results in different *in vitro* and *in vivo* cancer models (6-8); thus, it might be a suitable complement for irinotecan treatment in the progression of hepatic metastasis of CRC. Previously we have shown that OOS® slows down the metastatic progression of CRC to the liver *in vivo* (7). Thus, we aimed to evaluate this nutrient mixture as a candidate for a combined therapy with irinotecan, a chemotherapeutic agent used as a common therapy to treat this malignancy (1). According to our results, OOS® and irinotecan alone reduced the viability of C26 cells *in vitro*, which was further reduced when the combined treatment was applied. However, no synergistic effect was observed *in vivo* when OOS® was administered together with irinotecan. Nevertheless, the overall fitness state of the mice treated with OOS® plus irinotecan showed an improvement according to the Mouse Grimace Scale comparing to receiving irinotecan alone (data not shown) (11). That is, untreated and mice treated with irinotecan showed a narrowing of the orbital area, a tightly closed eyelid, and/or an eye squeeze, and more unkempt fur coat. The alteration in those parameters was observed to be diminished or absent in mice treated with the combined therapy. These results are in accordance with those carried out by Dayem-Uddin *et al* (2009) showing an improvement in the quality of patient's life

Table II. Ocoxin Oral solution® (O), irinotecan (I) and the combined therapy (OI) deregulated genes.[a]

| Gene symbol | O vs. C | OI vs. C | I vs. C |
|---|---|---|---|
| 1700017G19Rik | | | ↓↓ |
| 1700086P04Rik | | | ↓ |
| 2700081O15Rik | | ↑ | |
| 2810454H06Rik | | | ↓↓↓ |
| 4930401B11Rik | | | ↓ |
| 4930556N13Rik | | | ↓↓ |
| 4933413J09Rik | | | ↓↓ |
| 4933415F23Rik | | | ↓ |
| 6430550D23Rik | | | ↓↓ |
| 9530003O04Rik | | | ↓ |
| A730028G07Rik | | | ↓↓↓ |
| Aak1 | | | ↓ |
| Adcy6 | | | ↓↓ |
| Afm | | | ↓↓↓ |
| Agxt2 | | | ↓↓ |
| AI481877 | | | ↓↓ |
| Ankk1 | ↑↑ | | |
| Apoe | | | ↓ |
| Aqp9 | | | ↓↓ |
| Arglu1 | ↓ | ↓ | ↓ |
| Atf6 | | | ↓↓↓ |
| B230334C09Rik | ↑↑ | | |
| BC030308 | ↑ | | |
| Bfar | | | ↓ |
| Bmp7 | ↑↑ | | |
| Bsph1 | | | ↓↓ |
| C030039L03Rik | | | ↓↓ |
| Cabp1 | ↑↑ | | |
| Cdh9 | ↑ | | |
| Cdk13 | ↑↑↑↑ | | |
| Cela3a | | | ↓↓↓ |
| Cfap61 | | | ↓ |
| Cfd | | | ↑↑ |
| Chil3 | | ↑↑↑ | |
| Cic | | | ↓↓ |
| Clcn7 | | | ↓↓ |
| Clec18a | | | ↓↓ |
| Clec2e | | | ↓ |
| Crygc | | | ↓↓ |
| Cyp19a1 | | | ↓ |
| Cypt15 | ↑↑↑ | | |
| Cypt2 | | | ↓ |
| Cyth4 | ↑↑↑↑ | | |
| D430018E03Rik | | | ↓↓ |
| D6Ertd527e | | | ↓↓ |
| Dlx6os1 | | | ↓ |
| Dnal1 | | | ↓ |
| Dock4 | | | ↓↓ |
| Dsc1 | | | ↓ |
| Dzip1 | | | ↑ |
| Ect2 | | ↑↑↑ | |
| Erich2 | | | ↓↓ |

Table II. Continued.

| Gene symbol | O vs. C | OI vs. C | I vs. C |
|---|---|---|---|
| Fam71f2 | ↑↑↑ | | |
| Fanci | | | ↓ |
| Fat3 | | | ↓↓ |
| Fgr | | | ↓↓ |
| Fkbp6 | | | ↓ |
| Fuca2 | | | ↓↓ |
| Gja8 | | | ↓↓ |
| Glrx2 | | | ↓↓ |
| Gm10760 | | | ↓ |
| Gm16794 | | | ↓↓ |
| Gm38485 | ↑ | | |
| Gm5126 | | | ↓↓ |
| Gm5129 | | | ↓↓ |
| Gm5941 | | | ↓↓ |
| Gm8075 | | | ↓↓ |
| Gm9548 | | | ↓↓ |
| Gm9798 | | | ↓↓ |
| Gm9979 | ↑ | | |
| Gpr22 | | | ↓↓ |
| Hcrt | | | ↓↓ |
| Hist2h3c2 | | | ↓↓ |
| Hmcn1 | | | ↑ |
| Hras | | | ↓↓ |
| Il11 | ↑↑↑↑ | | |
| Il1b | ↑↑ | | |
| Kcnj12 | | | ↓ |
| Kif14 | ↓↓ | | |
| Klf11 | | | ↓↓↓ |
| Krtap19-2 | | | ↑↑↑ |
| LOC102641211 | | | ↑ |
| LOC105244659 | | | ↓↓ |
| Lppr3 | | | ↓↓↓ |
| Lrrc2 | | | ↓↓↓ |
| Ly6c1 | ↓↓↓ | | |
| Marcksl1 | ↑ | | |
| Marcksl1-ps4 | ↑ | | |
| Marveld2 | | | ↓↓ |
| Meg3 | | | ↓ |
| Ms4a1 | | | ↓↓ |
| Naprt | | | ↓ |
| Ncam1 | | | ↓↓ |
| Nctc1 | | | ↑↑ |
| Nfkbid | | | ↓↓ |
| Nkx6-1 | | | ↓↓ |
| Nol3 | ↑↑ | | |
| Nr1h3 | | | ↓ |
| Ntrk1 | ↑ | | |
| Nuf2 | | ↑↑↑↑ | |
| Olfr1110 | | | ↓↓↓ |
| Olfr328 | | | ↓↓↓ |
| Olfr549 | | | ↓↓ |
| Olfr945 | | | ↓↓ |
| Olfr97 | | | ↓↓ |

Table II. Continued.

| Gene symbol | O vs. C | OI vs. C | I vs. C |
|---|---|---|---|
| Pggt1b | ↑↑↑ | | |
| Phka2 | | | ↓↓ |
| Pir | | | ↑ |
| Pyroxd2 | | | ↑↑ |
| Raver2 | | | ↓↓ |
| Recql5 | | | ↓ |
| Rgs16 | ↑↑ | | |
| Rian | | | ↓↓ |
| Rnf170 | | | ↓ |
| Rnf220 | | | ↓↓ |
| Sclt1 | | | ↓ |
| Scn10a | | | ↓↓ |
| Sel1l | | | ↓↓ |
| Sept8 | | | ↓↓↓ |
| Sf3a2 | | | ↓↓ |
| Sgk3 | | | ↓↓ |
| Shc3 | ↑ | | |
| Slc14a1 | ↑↑ | | |
| Slc1a2 | | | ↓ |
| Slc26a1 | | | ↓↓ |
| Slc5a9 | | | ↓↓ |
| Slc8b1 | | | ↓↓ |
| Snx14 | ↑↑ | | |
| Spice1 | | | ↓↓ |
| Ssh1 | ↑↑ | | |
| Stt3a | | | ↓↓ |
| Sucla2 | ↓↓ | | ↓↓ |
| Tanc1 | | | ↓↓ |
| Tmem29 | | | ↓↓ |
| Tmprss5 | | | ↓↓ |
| Tshz2 | | | ↓↓ |
| Tstd3 | ↑↑↑↑ | | |
| Ttbk1 | | | ↓ |
| Ttl | | | ↑↑ |
| Tuba8 | | | ↓ |
| Ube2cbp | | | ↓↓ |
| Usp2 | | | ↑↑ |
| Usp42 | ↑ | | |
| Vmn1r19 | | | ↓↓ |
| Vps13c | | | ↓↓ |
| Xrcc4 | | | ↓↓ |
| Zdhhc21 | | | ↓↓↓ |
| Zfp318 | | | ↓↓↓ |
| Zpbp2 | | | ↓↓ |
| Zrsr2 | | | ↑↑ |

[a]Each arrow represents the fold-change of the upregulated (↑) or downregulated (↓) genes. ↑↓(2-4) ↑↑↓↓(4-6) ↑↑↑↓↓↓(6-8) ↑↑↑↑↓↓↓↓(8-10).

suffering from different cancers after the addition of OOS® to chemotherapy or radiotherapy (14).

Nowadays it is known that some nutritional complements suppress, among others, the hyper-proliferative processes during the development of a tumor (15-18). In line with these studies, the combined therapy of OOS® and irinotecan reduced Ki67 expression, an antigen expressed only in proliferative cells, in the metastatic liver tissue and increased caspase-3 expression, a protease expressed during apoptosis, when compared to the expression in the liver tissue collected from mice treated with the compounds alone. Moreover, the ratio of proliferative and apoptotic cells was diminished in livers from treated mice respect to those collected from untreated mice, even though the combined treatment did not show a synergistic effect with irinotecan in the *in vivo* metastatic progression. Further studies will show if a prolonged administration of irinotecan combined with OOS® would result in a visible reduction in the development of metastatic foci and an increase in the survival rate of mice under this treatment regime.

The complex microenvironment of solid tumors, comprised not only by tumor cells but also by the surrounding stromal components, has been associated with the induction of resistance to routine chemotherapies (19). This cross reactivity might be due to the interactions taking place between cancer cells and the multiple factors existing in the tumor microenvironment such as reactive oxygen species (ROS) and cytokines. These factors induce the recruitment of macrophages within the tumor foci (2), which influence the microenvironment towards one that favors tumor development. Thus, reducing the infiltration of stromal cells, such as tumor associated macrophages, into the tumor foci might impede or slow down tumor progression. In fact, a reduction in the number of tumor-associated macrophages is correlated with a better prognosis in several types of cancer.

Here we show that OOS® alone, as well as irinotecan, reduced the recruitment of macrophages to the tumor foci in the liver of treated mice, as shown by the reduced expression of F4/80. This infiltration was even lower when OOS® was administered simoultaneoulsy with irinotecan. As shown by others (20,21), irinotecan-induced colitis might be the result of an increase in an inflammatory response which, in turn, might damage normal tissues. In fact, macrophages are responsible for many of the inflammatory factors released into the liver and a reduced activation and recruitment of these cells might account for a reduction of irinotecan-induced side effects.

Additional studies in gene expression by gene array results have shown that irinotecan treatment significantly deregulates the expression level of 152 genes in the liver CRC metastasic explants while the combined therapy deregulated the expression of 14 genes only. Furthermore, 93% of the genes identified in tumor cells isolated from mice treated with irinotecan alone were downregulated. In contrast, the 100% of the altered genes in tumor cells isolated from mice treated with irinotecan plus OOS® were upregulated. This may indicate that OOS® could revert or modify the expression of the genes altered by irinotecan. These genes, whose expression was altered, were classified according to their molecular function, which turns to be in its majority binding and catalytic activity. Besides these, another biological and molecular activities were present, but in a shorter extent.

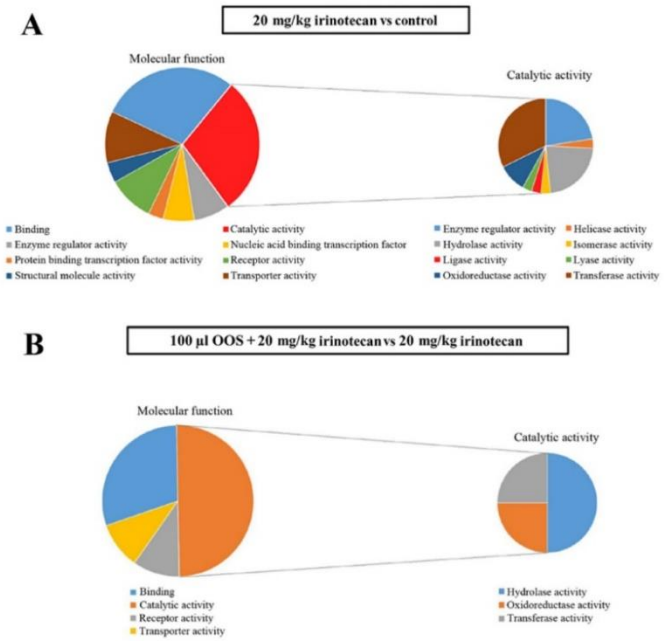


Figure 6. Metastatic tumor explant based mRNA microarray study. A microarray assay was carried out to detect differences in gene expression levels between tumor cells collected from mice treated under different protocols. Four tumor explants per experimental group were collected, each of them from one tumor bearing mouse. (A) A molecular functional gene analysis was carried out with the PANTHER (v.10.0) (9,10) analysis system for the untreated Control vs. irinotecan treatments. (B) A molecular functional gene analysis was carried out with the PANTHER (v.10.0) (9,10) analysis system for the OOS®+irinotecan vs. irinotecan treatments.

Interestingly, the genes with catalytic activities which expression was altered in the tumor cells isolated from mice subjected to the combined therapy possess enzymatic functions such as oxidoreductases, hydrolases and transferases. On the one hand, the upregulation of oxidoreductases, a group of enzymes that transfer electrons between molecules, have previously been related to the induction of apoptosis and to the increase in the cytotoxicity of several antitumoral drugs (22,23). On the other hand, transferases are the enzymes responsible for the biosynthesis of glycoprotein and glycolipid sugar chains and it is described that cancer cells show an aberrant glycosylation in their surface. This could lead to abnormal ligand-receptor interactions, and more importantly, it may favor cancer cell proliferation, migration and invasion (24,25). At last, hydrolases have also been implicated in different cancer types and their downregulation has been associated with the development of chemoresistance of melanoma and colorectal cancer to cytotoxic drugs (26,27). In this way, the OOS® added to irinotecan might counteract the action of genes downregulated by irinotecan treatment. Future studies will show if these genes are responsible for the side effects or for the resistance to irinotecan, or both.

To sum up, the combination of OOS® with irinotecan results in a reduced tumor cell proliferation and macrophage infiltration at a greater extent than OOS® or irinotecan alone do. However, a synergistic effect of the complementary therapy could not be observed in the *in vivo* metastatic progression. Nevertheless, it was observed that the combined therapy improved the animal overall status. The subjacent mechanism could be mediated in part by the reversal, induced by the combined therapy, of the downregulation exerted by the action of irinotecan in the expression of those genes with catalytic and binding activities. Further studies will be performed to validate the exact role and implication of altered genes, and to identify their exact role either in the tumor development or in the improvement of drug-induced adverse effects. Therefore, OOS® may constitute a pharmacologically safe complementary compound for the treatment of cancer and its metastasis when administered together with irinotecan by improving the quality of life in patients suffering from CRC liver metastasis.

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## Publication 3: Ocoxin Oral Solution Exerts an Antitumoral Effect in Pancreatic Cancer and Reduces the Stromal-Mediated Chemoresistance.

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ORIGINAL ARTICLE

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# Ocoxin Oral Solution Exerts an Antitumoral Effect in Pancreatic Cancer and Reduces the Stromal-Mediated Chemoresistance

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**Objectives:** Pancreatic carcinoma is one of the most aggressive cancers overcoming chemoresistance. Thus, novel compounds to complement the current antitumor agents are in need. Ocoxin oral solution (OOS) has proven antioxidant, anti-inflammatory, and antistromagenic properties. The aim of this study was to analyze the effect of OOS in an experimental pancreatic cancer model and its implication in stroma-related chemoresistance to paclitaxel and gemcitabine.

**Methods:** Murine pancreatic carcinoma 266-6 cells were treated with OOS to analyze cell cycle and to perform a mRNA comparative microarray study. Then the viability was assessed in combination with paclitaxel and/or gemcitabine. Chemoresistance induced by the medium taken from fibroblast cultures was also investigated on 6 human pancreatic carcinoma cell lines. Furthermore, an experimental model of pancreatic cancer was carried out to study the effect of OOS in vivo.

**Results:** Ocoxin oral solution enhances the cytotoxic effect of paclitaxel and gemcitabine, while it ameliorates the chemoresistance induced by fibroblast-derived soluble factors in human pancreatic cancer cells. The OOS also promotes the regulation of the expression of genes that are altered in pancreatic carcinoma and slows down 266-6 cell pancreatic tumor development in vivo.

**Conclusions:** Ocoxin oral solution could be a potential complement to the chemotherapeutic drugs for pancreatic adenocarcinoma.

**Key Words:** CAFs, nutritional supplements, OOS, pancreatic cancer, stroma, PSCs

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Pancreatic cancer is one of the most lethal diseases in the world because of its aggressiveness and the inexistence of markers for early detection and diagnosis.[1] That makes chemotherapy almost the only treatment available to cope with this disease. However, the effectiveness of the compounds used currently is far away from being ideal because this malignancy becomes resistant to the majority of the utilized drugs.[2] Gemcitabine and paclitaxel are the most used first-line chemotherapeutic agents to treat pancreatic cancer, but the effect of these compounds is feeble. Thus, a combination of both gemcitabine and paclitaxel is currently used as a second-line treatment to improve patient's response to the tumor.[3]

The local microenvironment is an active participant in the process of cancer initiation, progression, and metastasis in many tumors.[4,5] Moreover, resistance to chemotherapeutics derives from both the tumor cell response and the tumor-associated stromal microenvironment.[6–9] In pancreatic carcinoma, the stroma is especially relevant because critical players in cancer development, such as cancer-associated fibroblasts (CAFs), endothelial cells, immune cells, and extracellular matrix, comprise 80% of tumor mass.[10,11]

Among all these cell types, CAFs have recently emerged as chemoresistance promoters by secreting cytokines and growth factors that alter tumor cell response to chemotherapeutic agents.[12,13]

Nutritional supplements are sometimes used in combination with the routine therapies to overcome the development of chemoresistance.[14,15] Several studies have shown that natural compounds not only increase the efficacy of chemotherapy but also relieve the adverse effects provoked by these agents when used as complementary therapy.[16,17] Glycyrrhizic acid is a biologically active substance, extracted from licorice root with anti-inflammatory and immunomodulatory properties. Furthermore, this substance inhibits the growth of leukemia, malignant glioma, colon cancer, and lung cancer.[18] Also, several vitamins and antioxidant compounds have been widely studied as anticancer agents with reasonably good results.[19–21] Recently, the nutritional complement Ocoxin oral solution (OOS) has demonstrated to exert antitumoral effects alone and as a coadjutant of irinotecan in the development of liver metastasis from colorectal cancer.[22,23] This compound comprises a mixture of several natural compounds such as green tea extract, glycyrrhizic acid, vitamin C, vitamin $B_6$, vitamin $B_{12}$, minerals, and amino acids and possesses immunomodulatory and antioxidant properties.[22,23] The results obtained show that the nutritional supplement OOS might be a potential complementary therapy to face the proliferative effects and reduce the stroma-mediated chemoresistance of pancreatic cancer.

## MATERIALS AND METHODS

### Animals

For the in vivo experimental model of pancreatic adenocarcinoma, 6- to 8-week-old male C57BL/6 mice were obtained from Charles River (Wilmington, Mass). Mice were fed with standard chow and water ad libitum. The Ethical Committee for Experimental Animal of the Basque Country University approved all the

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proceedings with the reference number M20/2016/200, following institutional, national, and international guidelines regarding the protection and care of animal use for scientific purposes.

## Cell Lines

The murine pancreatic adenocarcinoma 266-6 cell line (ATCC, LGC Standards S.L.U., Barcelona, Spain) was used for in vitro and in vivo experiments. This cell line contains the simian virus 40 (SV40) transgene that codes for small and large T-antigen.[24] Besides, 6 different human pancreatic carcinoma cell lines, BxPC-3, Capan-2, CFPAC-1, HPAF-II, Panc10.05, and SW1990, were used for further in vitro analyses. The human MRC-5 lung fibroblast cells (all purchased from ATCC, Barcelona, Spain) were used to obtain conditioned medium enriched with soluble factors. All cells were grown in a complete medium, RPMI-1640 supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (0.25 μg/mL) (Thermo Fisher Scientific, Waltham, Mass) under standard conditions. Panc10.05 cells were supplemented with recombinant human insulin (Life Technologies, Waltham, Mass) (10 U/mL).

## OOS Solution

Ocoxin oral solution is a nutritional supplement that contains the following components (in 100 mL): 2 g of glycine, 2 g of glucosamine, 1.2 mg of malic acid, 640 mg of arginine, 204 mg of cysteine, 200 mg of monoammonium glycyrrhizinate, 120 mg of ascorbic acid, 80 mg of zinc sulfate, 25 mg of green tea extract, 12 mg of calcium pantothenate, 4 mg of pyridoxine, 4 mg of manganese sulfate, 3 mg of cinnamon extract, 400 μg of folic acid, and 2 μg of cyanocobalamin.

## Fibroblast-Derived Conditioned Medium

Fibroblast-derived conditioned medium (fibroblast CM) was obtained from MRC-5, a cell line derived from human lung fibroblasts. Briefly, cells were cultured on 6-well plates at a concentration of $2 \times 10^5$ cells/mL in a complete RPMI medium. After 24 hours, the medium was replaced for a fresh one, and after another 24 hours, the medium enriched with soluble factors produced by fibroblasts was collected, centrifuged, and stored at −20°C.

## Viability Assays

Tumor cells were cultured in 96-well plates at a concentration of $5 \times 10^4$ cells/mL in 10% FBS-supplemented RPMI medium with antibiotics-antimycotics to analyze the effect of OOS, paclitaxel, and gemcitabine on their viability. Once attached, 266-6 cells were treated separately with a concentration range of 0 to 1:50 (V/Vf) OOS (Catalysis S.L., Madrid, Spain), 1 to 10 μM paclitaxel (Sigma, St Louis, Mo), and 200 nM to 1 μM gemcitabine (Sigma). The human cells were treated with the concentration range of 0 to 1:50 (V/Vf) OOS, 1 to 15 μM for paclitaxel, and 1 to 5 μM for gemcitabine.

Next, to study the effect of OOS as a complementary therapy on the viability of cells, treatment combination assays were carried out. Cells were cultured as above and were treated with the most effective concentrations obtained in the previous tests, which was OOS 1:50 (V/Vf), combined with 1 μM paclitaxel and 1 μM gemcitabine. All the treatments were diluted in 10% FBS-supplemented RPMI medium with antibiotics-antimycotics. The controls were cultured with RPMI medium completed with 10% FBS, antibiotics, and antimycotics.

Finally, to analyze the effect of fibroblast-derived soluble factors on chemoresistance, human cancer cells were cultured at a concentration of $5 \times 10^4$ cells/mL in 96-well plates with fibroblast CM diluted 1:2 in RPMI medium supplemented with 10% FBS for 24 hours. Afterwards, cells were treated with OOS 1:50 (V/Vf), combined with 1 μM paclitaxel and 1 μM gemcitabine diluted in a complete fresh medium with fibroblast CM, whereas untreated cells were cultivated only with the whole medium. In all viabilities, after 48 hours with the treatments, PrestoBlue viability reagent (Thermo Fisher Scientific) was added to the cells for 3 hours, and the absorbance was measured with Fluoroscan Ascent (Thermo Labsystems, Waltham, Mass).

## Cell Cycle Analyses

The 266-6 cells were cultured in 6-well plates for 18 hours to study the effect of OOS in the cell cycle in vitro. Then, cells were treated with 1:100, 1:200, and 1:500 (V/Vf) dilutions of OOS in complete RPMI for 48 hours. The control cells were cultured with the control medium. Then, cells were trypsinized, washed once with phosphate-buffered saline (PBS), and fixed with 70% ethanol for 15 minutes at 4°C. Afterwards, cells were washed with PBS 3 times and incubated with propidium iodide (PI) containing FxCycle PI/RNase Solution (Thermo Fisher Scientific) following the manufacturer's indications. Finally, cell cycle changes were analyzed by flow cytometry using the Gallios cytometer (Beckman Coulter, Brea, Calif). For cell division analysis, 266-6 cells were fluorescently labeled with carboxyfluorescein succinimidyl ester (CFSE) by incubating at 37°C for 30 minutes before seeding them into 6-well plates. Then, cells were treated as described above for cell cycle analysis. After 48 hours, cells were trypsinized, washed in PBS, and resuspended for flow cytometry studies by Gallios cytometer (Beckman Coulter).

## Apoptosis Determination by Flow Cytometry

Human pancreatic cancer cells were cultured in a complete medium for 24 hours in 6-well plates at $3 \times 10^5$ cells/well concentration. Once the cells were attached, the old medium was replaced with fresh medium for control condition or with 1:2 diluted fibroblast CM for the chemoresistance studies and incubated for 24 hours. Afterward, cells were treated with OOS 1:50 (V/Vf), combined with 1 μM paclitaxel and 1 μM gemcitabine diluted in fresh medium or fibroblast CM for 48 hours. Then, cells were washed, trypsinized, pelleted by centrifugation, washed again with PBS, and double stained with the annexin V–fluorescein isothiocyanate apoptosis detection kit and PI following manufacturer's instructions (both purchased from Thermo Fisher Scientific). Finally, apoptosis was determined by the Gallios cytometer (Beckman Coulter). Note that the HPAF-II cell line was not included in this assay because of the high levels of cell death during the process.

## mRNA Comparative Microarray Study of OOS-Treated Murine Pancreatic 266-6 Cells

A microarray study was carried out to analyze whether OOS altered gene expression in the 266-6 cells. To this end, cells were cultured in 8 independent T25 flasks; 4 of them were treated with 1:50 (V/Vf) of OOS, and 4 containers as controls were cultured in complete medium for 72 hours. In both cases, the media was replaced daily. Then, the total RNA was extracted according to Purelink RNA mini kit (Invitrogen, Carlsbad, Calif) manufacturer's instructions. Afterwards, RNA integrity was analyzed by using a Eukaryote Total RNA Nano Assay with the Lab-chip in the Agilent 2100 Bioanalyzer in combination with Agilent RNA

6000 Nano Chips. Subsequently, mRNA was labeled using the Agilent protocol One-Color Microarray-Based Gene Expression Analysis (Low Input Quick Amp Labeling). Messenger RNA was retrotranscribed with the AffinityScript Reverse Transcriptase (AffinityScript RT) in the presence of Cy3-CTP to obtain cDNA. These samples were hybridized using "SureHyb" hybridization chambers (Agilent Technologies, Inc, Santa Clara, Calif) according to the manufacturer's instructions and were washed with ozone-barrier slide covers according to the protocol of Agilent. Then, the slides were scanned using the DNA microarray scanner G2535CA of Agilent Technologies with the Agilent Scan control Software (version 8.5.1.) (default settings). Finally, feature extraction of the scanned images was made by using the Agilent Feature Extraction Software (version 10.7.3.1) (Agilent Technologies, Inc).

## Experimental Development of Pancreatic Adenocarcinoma

Before the injection of 266-6 cells on mice to carry out an in vivo pancreatic cancer experimental model, a flow cytometry assay was performed to confirm the expression of the SV40 T-antigen on the tumor cells. To do so, cells were blocked in 5% FBS containing PBS for 30 minutes and labeled with the mouse anti-SV40 T-antigen antibody (1:500; Abcam, Cambridge, Mass) for 2 hours. Next, cells were washed with PBS 3 times and incubated with the corresponding secondary anti-antibody for 1 hour (1:2000, Alexa 595; Thermo Fisher Scientific). Finally, after extensive washing in PBS, the cells were processed by Gallios cytometer (Beckman Coulter).

Once the presence of the marker on cells was established, a murine orthotopic pancreatic adenocarcinoma model was developed for the in vivo analyses. Mice were anesthetized with 50 mg/kg of intraperitoneal injection of Nembutal, and a thin cut was made in the left flank to inject 100 μL of 266-6 cells directly into the pancreas at a concentration of $2 \times 10^6$ cells/mL diluted in PBS. Then, the pancreas was relocated, and the wound was sutured. The animals were divided into 3 experimental groups, consisting of 7 mice each one, according to the treatment administration pattern: group I comprised mice that received the vehicle treatment, used as control mice; group II involved mice treated daily with 100 or 200 μL of OOS, starting 10 days before tumor cell inoculation and until they were killed; group III involved mice that started treatment with 100 or 200 μL OOS 7 days after the tumor cell injection and continued the therapy until they were killed. All animals were killed 45 days after tumor cell injection (Fig. 1), and the pancreas was fixed in formaldehyde 3.7% to 4% vol/vol (Panreac AppliChem, Darmstadt, Germany) and embedded in paraffin. Four mice were inoculated with CFSE-labeled 266-6 cells, and they were killed after 48 hours to confirm the presence of the cells histologically in the organ by fluorescence.

## T-Antigen Expression in Pancreatic Cancer-Bearing Mice by Quantitative Reverse Transcription–Polymerase Chain Reaction

The 266-6 cell marker SV40 T-antigen expression was quantified by quantitative reverse transcription–polymerase chain reaction (RT-qPCR) to analyze the tumor development in the pancreas. Total RNA was purified from the paraffin-embedded pancreas of all the treated and untreated mice using the Norgen FFPE RNA Purification Kit (Norgen Biotek, Thorold, Ontario, Canada) following the manufacturer's instructions, and we assessed the RNA concentration and quality by NANODROP spectrophotometer (ND-1000; Thermo Scientific, Rockford, Ill). Then, 2 μg RNA was retrotranscribed into cDNA using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, Calif) and amplified by using the Platinum-multiplex PCR mastermix (Applied Biosystems, Foster City, Calif).

The quantification of SV40 T-antigen gene expression was performed by RT-qPCR using the Itaq Universal SYBR Green Supermix (Bio-Rad) in CFX96 Real-Time System (Bio-Rad). The following SV40 T-antigen gene primers were purchased from Invitrogen (Thermo Fisher Scientific): forward: 5′-AAGCTCCAAC CCCTTTACCG-′3, reverse: 5′-ACATCAATGCTCACACGACG-′3. Relative expression of SV40 T-antigen was normalized to the internal control gene S15 by the ΔΔCt method.

## Cytokine Levels in Mice Serum

The levels of interleukin (IL)-1β, IL-10, IL-12, and interferon γ (IFN-γ) cytokines were measured in untreated and OOS-treated tumor-bearing mice serum. A small sample of blood was extracted from the cava vein before the animals were killed, and the serum was obtained by centrifugation. Afterward, an enzyme-linked immunosorbent assay (ELISA) was carried out using Mouse IL1β and IL10 CytoSet kits (both purchased from Invitrogen) and Mouse IFN-γ and IL12 ELISA Set (both purchased from Abcam) according to the manufacturer's guidelines.

## Statistical Analysis

The statistical analysis was carried out using the Student 2-tailed unpaired *t* test. All the in vitro experiments were performed in triplicate, and the in vivo assay was carried by duplicate with at least 7 animals in each group. Data are expressed as the mean value (standard deviation [SD]). The microarray assay was performed with 4 replicates for each treatment, and the statistics were analyzed with the multiExperiment Viewer version 4.9.0 (http://www.tm4.org/mev/). The comparison of expression profiles for differential

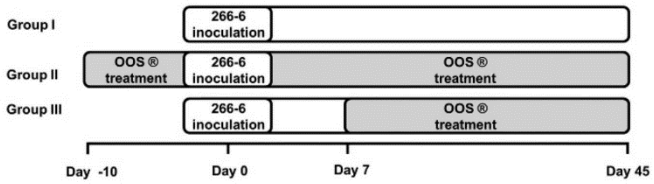



**FIGURE 1.** Scheme of experimental groups for in vivo pancreatic carcinoma development. Animals were divided into 3 groups as follows: group I: Untreated animals as control mice; group II: mice treated with 100 or 200 μL OOS starting 10 days before the tumor cell injection and continued the treatment until the animals were killed; group III: mice treated daily with 100 or 200 μL OOS from the 7 days after the tumor cell inoculation until the animals were killed.

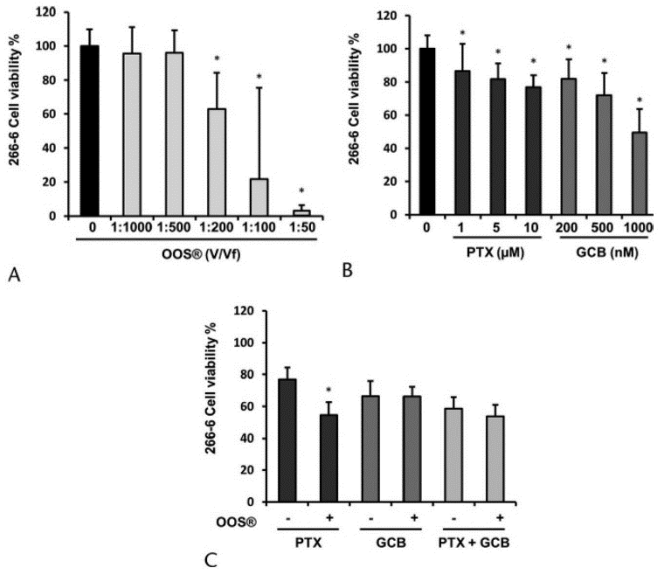



**FIGURE 2.** Ocoxin oral solution effect on the viability of the murine pancreatic adenocarcinoma 266-6 cell line. The viability of 266-6 cells was analyzed by means of the Presto Blue assay after 48 hours with different treatment combinations. A, Cell viability after OOS treatment according to 1:1000 to 1:50 (V/Vf) concentrations of (B) paclitaxel from 1 to 10 μM and gemcitabine from 200 to 1000 nM (C) combinations of all 3 of them: paclitaxel 1 μM + OOS 1:50 (V/Vf), gemcitabine 1 μM + OOS 1:50 (V/Vf), and paclitaxel 1 μM + gemcitabine 1 μM + OOS 1:50 (V/Vf). Data are expressed as the mean value (SD) of at least 3 independent experiments. Differences were considered significant for $*P < 0.05$.

expression analysis (Differential Expression) was carried out with LIMMA (Linear Models for Microarray Data) package. Results were considered statistically significant for $P < 0.05$.

## RESULTS

### Effect of OOS on the 266-6 Murine Pancreatic Adenocarcinoma Cells: Analysis of Tumor Cell Viability and Apoptosis Stage

First, the effect of OOS on the viability of the 266-6 murine pancreatic cancer cells was analyzed. The 266-6 cells were cultured with increasing concentrations of OOS. As shown in Figure 2A, OOS enhanced tumor cell death in a dose-dependent manner ranging from 4% using OOS 1:1000 (V/Vf) dilution to 95% using the OOS 1:50 (V/Vf) dose.

Then, 266-6 cells were treated as above with increasing concentrations of paclitaxel (1–10 μM) and gemcitabine (200–1000 nM) separately, to select the most effective drug dose to perform an OOS-chemotherapy combined assay. As shown in Figure 2B, paclitaxel 1, 5, and 10 μM provoked an overall 15% to 20% reduction in cell viability, and those cells treated with 200, 500, and 1000 nM of gemcitabine showed an 18%, 28%, and 50% viability decrease, respectively. Moreover, the addition of OOS as a complement to paclitaxel showed a 35% reduction in cell viability (Fig. 2C). No differences were detected when OOS was added in combination with gemcitabine or with paclitaxel and gemcitabine concomitantly.

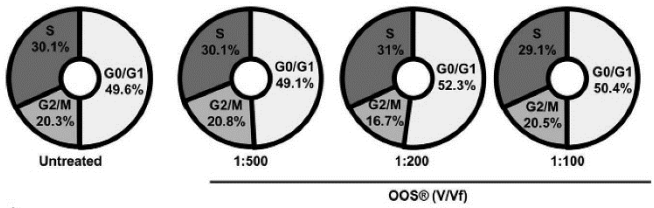
Flow cytometry analyses were carried out to analyze the effect of OOS on the 266-6 cell cycle. As shown in Figure 3A, PI incorporation was unchanged in cells treated with 1:500, 1:200, and 1:100 (V/Vf) of OOS compared with untreated cells. However, CFSE cell labeling showed that OOS 1:200 and 1:500 (V/Vf) dilutions slowed down 266-6 tumor cell division by 10% and 30% when the cells were treated with 1:100 (V/Vf) of OOS (Fig. 3B).

### Comparative Microarray Study to Determine the Effect of OOS in Tumor Gene Expression

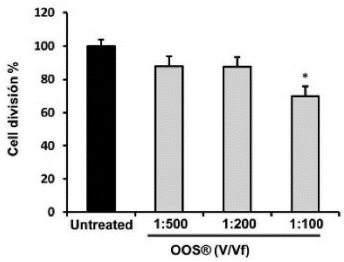
Bearing in mind that OOS treatment exerted antitumoral effects on 266-6 cells, a comparative microarray study was performed to analyze the molecular changes in gene expression promoted by OOS in 266-6 cells. The assay revealed a significantly altered gene profile compared with that of untreated cells. Fourteen of the identified genes had been previously associated with pancreatic adenocarcinoma in the Pancreatic Cancer Database[25] and Pancreatic Expression Database.[26] Interestingly, the expression levels of those genes went back to nonpathological values after OOS treatment (Table 1).

### Effect of OOS Treatment in Pancreatic Adenocarcinoma Tumor Development

The expression of T-antigen on 266-6 cells was confirmed by flow cytometry (Supplementary Figure 1, http://links.lww.com/MPA/A713). Carboxyfluorescein succinimidyl ester detection by fluorescence microscopy demonstrated that tumoral cells were




A




B

**FIGURE 3.** Cell cycle analysis of the 266-6 OOS-treated cells. 266-6 Cells were treated with 1:500, 1:200, and 1:100 OOS (V/Vf) for 48 hours the cell cycle was studied. A, Flow cytometry assay was carried out using PI (B) flow cytometry assay by labeling 266-6 cells with CFSE fluorescence probe. Data represent mean value (SD) of at least 3 independent experiments. Differences were considered significant for $*P < 0.05$.

**TABLE 1.** Comparative Microarray Study of Untreated and OOS-Treated 266-6 Cells

| Gene | Pancreatic Carcinoma | OOS Treatment | Reference |
|---|---|---|---|
| *Angptl4* | Down | Up | Gadaleta et al,[27] 2011 |
| *Asns* | Down | Up | Crnogorac-Jurcevic et al,[28] 2013<br>Gadaleta et al,[27] 2011 |
| *Crabp1* | Up | Down | Nakamura et al,[29] 2004 |
| *Creb1* | Up | Down | Friess et al,[30] 2003 |
| *Eif4ebp1* | Down | Up | Crnogorac-Jurcevic et al,[31] 2001 |
| *Fignl1* | Up | Down | Gadaleta et al,[27] 2011 |
| *Foxf1* | Up | Down | Gadaleta et al,[27] 2011 |
| *Grap2* | Up | Down | Hustinx et al,[32] 2004 |
| *Hey1* | Up | Down | Mann et al,[33] 2012<br>Nakamura et al,[29] 2004 |
| *Nell2* | Up | Down | Gadaleta et al,[27] 2011<br>Nakamura et al,[29] 2004 |
| *Pak7* | Up | Down | Capurso et al,[34] 2006 |
| *Rapgef3* | Up | Down | Weeks et al,[35] 2008 |
| *Serpinf1* | Down | Up | Yu et al,[36] 2005 |
| *Slc7a1* | Down | Up | Gadaleta et al,[27] 2011 |
| *Thy1* | Up | Down | Lowe et al,[37] 2007<br>Badea et al,[38] 2008<br>Friess et al,[30] 2003<br>Gadaleta et al,[27] 2011 |
| *Trpc4* | Up | Down | Capurso et al,[34] 2006 |
| *Wnt4* | Up | Down | Lowe et al,[37] 2007 |

Total mRNA was extracted from 266-6 cells either untreated or treated with OOS 1:50 (V/Vf) for 72 hours to carry out an RNA microarray assay to detect differences in gene expression. The genes altered by the OOS treatment were compared with the data from the Pancreatic Expression Database v3.0 (available at http://www.pancreasexpression.org:9002) and the Pancreatic Cancer Database (available at http://pancreaticcancerdatabase.org/index.php).

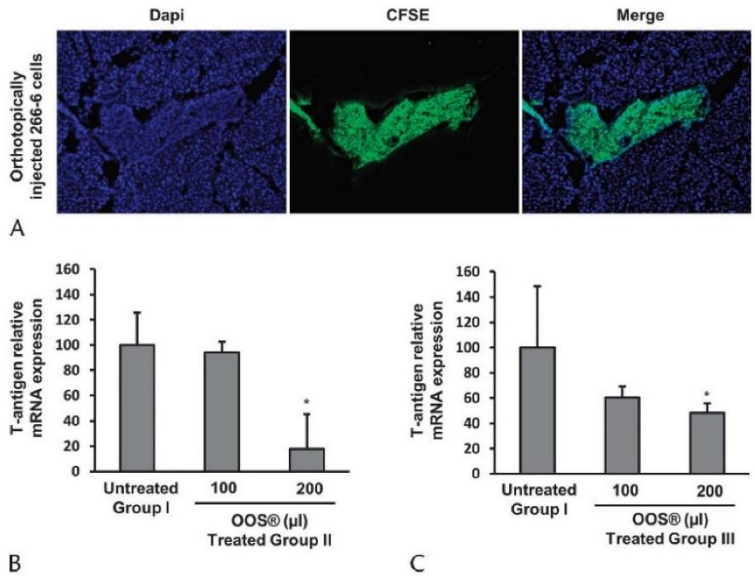



**FIGURE 4.** Ocoxin oral solution effect on T-antigen expression levels in pancreatic tissue. A, Carboxyfluorescein succinimidyl ester–labeled 266-6 cells (green) were inoculated in the pancreas of mice, and they were killed after 48 hours. The pancreas was frozen, processed, and contrasted with DAPI (4′,6-diamino-2-fenilindo) (blue) to analyze by fluorescence microscopy. B, T-antigen expression level by RT-qPCR in the pancreas of mice from groups I and II. C, T-antigen expression level by RT-qPCR in the pancreas of mice from groups I and III. Data are expressed as the mean value (SD) of at least 7 mRNA samples per each treatment and 2 independent assays. Differences were considered significant for $^*P < 0.05$.

present in the pancreas as soon as 48 hours after tumor cell injection (Fig. 4A). The effect of OOS on the development of pancreatic adenocarcinoma in vivo was studied by the detection of SV40 T-antigen by RT-qPCR on the pancreas of untreated and OOS-treated tumor-bearing mice. As shown in Figure 4B, mice from group II showed a significant reduction of 82% in the expression of the tumor marker T-antigen in the pancreas when they were treated with 200 µL of OOS from the 10th day before tumor cell inoculation and until they were killed. Mice from group III, whose treatment started 7 days after tumor cell inoculation, showed 40% reduction of T-antigen expression when they received the treatment of OOS 100 µL and a significant decrease of 52% when the 200-µL dose was used (Fig. 4C).

## Cytokine Levels in the Serum of Mice Bearing Pancreatic Adenocarcinoma and Treated With OOS

To analyze the inflammatory cytokine profile in the serum of OOS-treated mice, ELISAs were carried out. As shown in Figure 5, the same pattern of cytokine levels was observed in the serum of animals under group II or group III treatment schedules (Figs. 5A, B).

In both groups, IL-1β, IL-12, and IFN-γ were reduced in the serum, compared with serum levels in the untreated mice. In the case of group II, reductions in IL-1β, IL-12, and IFN-γ were significant in mice treated with OOS 100 µL or in the 200-µL dose (Fig. 5A). Regarding group III, the IL-1β reduction was substantial when OOS was administered at 200-µL doses and IL-12 at 100-µL doses. Either doses of OOS 100 and 200 µL significantly reduced IFN-γ in serum (Fig. 5B). On the contrary, serum concentration of anti-inflammatory IL-10 cytokine significantly rose 5-fold in the serum of mice from groups II and III, treated with 200 µL OOS.

## Effect of OOS on the Viability of Human Pancreatic Adenocarcinoma Cell Lines

As we showed in Figures 2 and 4, a cytotoxic role of OOS in mouse 266-6 pancreatic adenocarcinoma cells was probed in vivo and in vitro. Thus, we studied the potential of this compound in 6 human pancreatic adenocarcinoma cell lines. As shown in Figure 6, in vitro analyses revealed a pronounced cytotoxic effect of OOS in the BxPC-3, Capan-2, CFPAC-1, HPAF-II, Panc10.05, and SW1990 pancreatic cancer cell lines. All the cells cultured with the OOS 1:50 (V/Vf) dilution showed a strong decrease in cell viability ranging from 90% in BxPC-3 (Fig. 6A) to 70% in Capan-2 (Fig. 6B), 60% in CFPAC-1 (Fig. 6C), 37% in HPAF-II (Fig. 6D), 75% in Panc10.05 (Fig. 6E), and 60% in SW1990 (Fig. 6F).

## Cytotoxic Effect of Paclitaxel and Gemcitabine in Human Pancreatic Adenocarcinoma Cell Lines

The cytotoxic effect of increasing concentrations of paclitaxel and gemcitabine was studied in 6 pancreatic adenocarcinoma cell lines. One-micrometer paclitaxel treatment reduced cell viability up to 57% in BxPC-3 (Fig. 7A), 62% in Capan-2 (Fig. 7B), 72% in CFPAC-1 (Fig. 7C), 31% in HPAF-II (Fig. 7D), 35% in Panc10.05 cells (Fig. 7E), and 50% in SW1990 (Fig. 7F). The

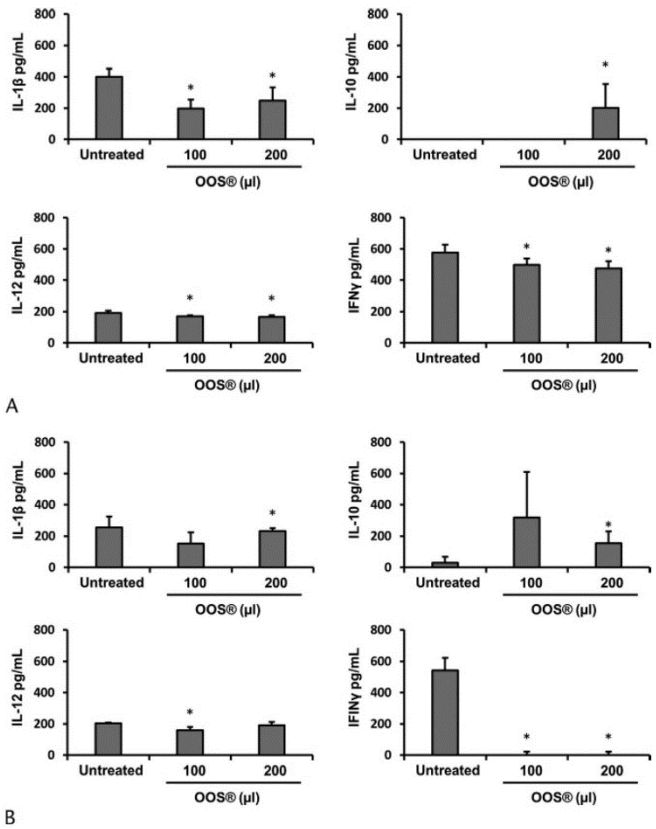



**FIGURE 5.** Cytokine levels in the serum of tumor-bearing mice. A, Cytokine levels in serum of tumor-bearing mice treated with 100 or 200 µL OOS according to group II administration pattern. B, Cytokine levels in serum of tumor-bearing mice treated with 100 or 200 µL OOS according to group III administration pattern. Data represent the mean value (SD) of at least 7 serum samples per each treatment and 2 independent assays. Differences were considered significant for $*P < 0.05$.

effect of gemcitabine is similar to that of paclitaxel, with a reduction on cell viability of 40% in BxPC-3 (Fig. 7A), 35% in Capan-2 (Fig. 7B), 70% in CFPAC-1 (Fig. 7C), 10% in HPAF-II (Fig. 7D), 33% in Panc10.05 (Fig. 7E), and 36% in SW1990 (Fig. 7F) cell lines. No differences in cell viability were observed with concentrations higher than 1 µM.

## Effect of OOS as a Complement to Paclitaxel and Gemcitabine Adjuvant Therapy on the Viability of Human Pancreatic Adenocarcinoma Cells

Paclitaxel and gemcitabine are the most used chemotherapeutic agents in pancreatic adenocarcinoma patients. Therefore, we evaluate the potential of OOS as a complement for anticancer therapies when administering these compounds. In all the studied cell lines, the cytotoxicity of both drugs increased when combined with OOS 1:50 (V/Vf) dilution. Ocoxin oral solution improved the antitumor activity of paclitaxel by 25% in BxPC-3 (Fig. 8A), 71% in Capan-2 (Fig. 8B), 32% in CFPAC-1 (Fig. 8C), 15% in HPAF-II (Fig. 8D), and 35% in Panc10.05 (Fig. 8E) and SW1990 (Fig. 8F) cells.

A similar effect was observed after treating cells with gemcitabine, since its anticancer activity was increased by 37% in BxPC-3 (Fig. 8A), 40% in Capan-2 (Fig. 8B), 17% in CFPAC-1 (Fig. 8C), 10% in HPAF-II (Fig. 8D), 39% in Panc10.05 (Fig. 8E), and 46% in SW1990 cells (Fig. 8F).

## Effect of OOS as a Complement to Overcome Stromal-Mediated Chemoresistance of Human Pancreatic Adenocarcinoma Cell Lines

The potential of OOS as a complement to counteract fibroblast CM–induced chemoresistance to paclitaxel and gemcitabine in pancreatic cancer cells was studied. As shown in Figure 9, fibroblast CM increased the viability of all the tumor cell lines examined. Moreover, fibroblast CM abrogated the effect of paclitaxel, with a 90% to 100% viability in BxPC-3, CFPAC-1, HPAF-II, and SW1990 cells (Figs. 9A, C, D, and F), and low but

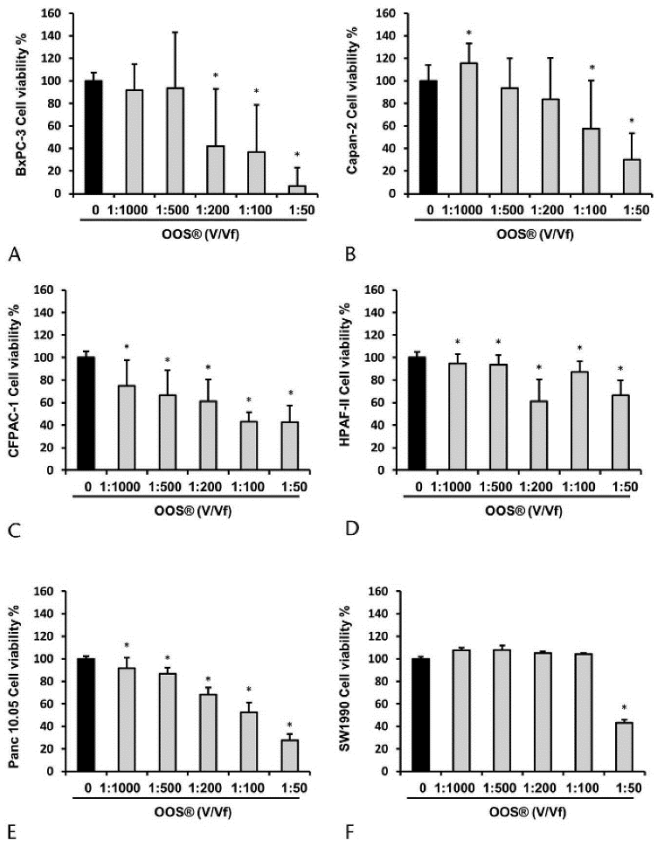



**FIGURE 6.** Cell viability of human pancreatic cancer cell lines in the presence of different concentrations of OOS. The viability of BxPC-3 (A), Capan-2 (B), CFPAC-1 (C), HPAF-II (D), Panc10.05 (E), and SW1990 (F) cells was tested by Presto Blue assay after treatment with increasing concentrations (1:1000 to 1:50 (V/Vf)) of OOS for 48 hours. Data represent the mean value (SD) of at least 3 independent experiments. Differences were considered significant for $*P < 0.05$.

sustained differences for Capan-2 and Panc10.05 cells (Figs. 9B and E). Regarding gemcitabine, fibroblast CM reverted the toxicity exerted by this drug in BxPC-3, Capan-2, CFPAC-1, HPAF-II, and SW1990 cell lines (Figs. 9A, B, C, D, and F). However, fibroblast CM did not modify gemcitabine's cytotoxicity in Panc10.05 cells (Fig. 9E).

In all the studied cell lines, the addition of the combination of gemcitabine or paclitaxel with OOS 1:50 (V/Vf) to fibroblast CM–stimulated cells reverted the chemoresistance produced by fibroblast CM and helped to recover the cytotoxic effect observed with paclitaxel and gemcitabine alone.

## Mechanism of OOS to Reduce Cell Viability

To further confirm that the reduction in cell viability was accompanied by an increase in cell apoptosis in OOS-treated cells, apoptosis levels were measured using annexin V–PI cell staining kit. As observed in Figure 10, OOS treatment increased the number of apoptotic cells in all the studied cell lines. We observed a 2-fold increase in BxPC-3 cell death (Fig. 10A), a 0.5-fold in Capan-2 (Fig. 10B), a 2-fold in CFPAC-1 (Fig. 10C), a 1.5-fold in Panc10.05 (Fig. 10D), and a 4.5-fold in SW1990 cells (Fig. 10E). Moreover, OOS acts as a synergistic agent for paclitaxel, increasing their apoptotic effect by 10% in Panc10.05 and 50% in SW1990 pancreatic cancer cell lines (Figs. 10D and E). When combined with gemcitabine, OOS promoted a further 15% apoptosis in Capan-2, 10% in Panc10.05, and 30% in SW1990 cells. Moreover, OOS administered as complement of paclitaxel and gemcitabine increased the apoptosis rate up to 25% in BxPC-3 cells (Fig. 10A) and CFPAC-1 cells (Fig. 10C) and up to 50% in SW1990 cells (Fig. 10E) and Panc10.05 cells (Fig. 10D) when cells were cultured with fibroblast CM, therefore abrogating stromal-mediated

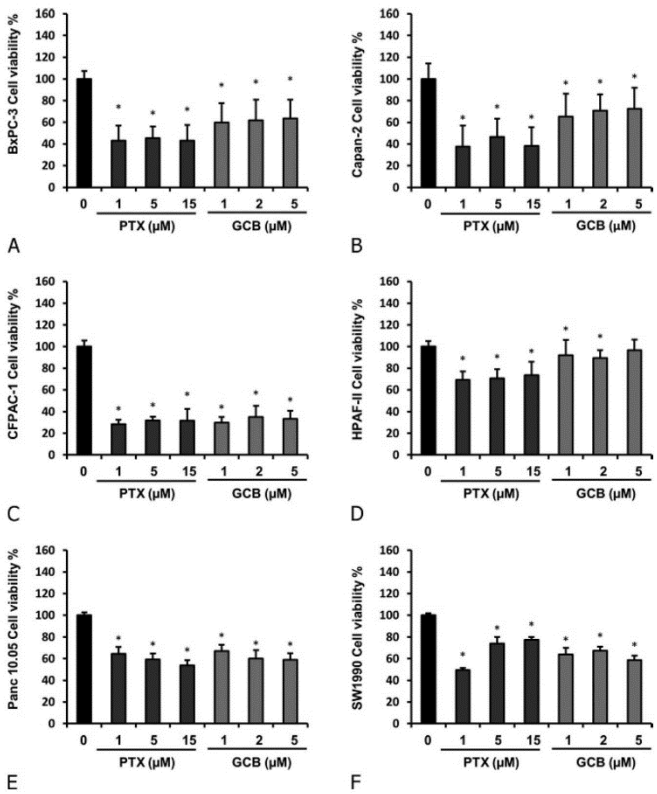



**FIGURE 7.** Cell viability of human pancreatic cancer cell lines in the presence of different concentrations of paclitaxel and gemcitabine. The viability of BxPC-3 (A), Capan-2 (B), CFPAC-1 (C), HPAF-II (D), Panc10.05 (E), and SW1990 (F) cells was tested by Presto Blue assay after treatment with paclitaxel (PTX) (1–15 μM) and/or gemcitabine (GCB) (1–5 μM) for 48 hours. Data represent mean values (SD) of at least 3 independent experiments. Differences were considered significant for $*P < 0.05$.

resistance (Fig. 10). The Capan-2 cell line only showed a 25% increased apoptosis when they were treated with gemcitabine (Fig. 10B). Thus, OOS exerted a synergistic effect with both chemotherapeutics almost diminishing fibroblast CM–mediated resistance in human pancreatic cancer cells.

## DISCUSSION

Pancreatic cancer is an aggressive disease with intense stromal reaction,[39] which remains unresponsive to conventional therapies because of both the late detection and the acquired drug resistance. Accumulated evidence has suggested that the development of the desmoplastic stroma is a significant obstacle for the actual treatments for pancreatic cancer.[12,13,40,41] Thus, the combination of nutritional supplements and chemotherapy is a new therapeutic approach to support chemotherapy itself and to reduce the produced adverse effects targeting not only the tumor but also the components of its associated stroma.[42–44] In this way, natural compounds, such as curcumin, resveratrol, flavonoids, epigallocatechin-3-gallate, sulforaphane, or nimbolide, have shown promising antitumor effects in pancreatic cancer.[20,45,46] In this context, OOS consists of a mixture of several nutrients that have been proven to exert antitumor effects alone and in combination with chemotherapy, in vitro and in vivo, in the metastatic progression of colorectal cancer to the liver.[22,23] However, to our knowledge, there is no report on the efficacy of OOS in pancreatic cancer. In this article, we unveil the antitumoral effect of OOS in the development of pancreatic adenocarcinoma through the reduction of tumor cell viability, enhancement of the antitumor effect of chemotherapeutic agents, modulation of the expression of genes known to be associated with pancreatic adenocarcinoma, and by reverting chemoresistance conferred by CAFs. Moreover, OOS treatment reduced tumor development in vivo in the pancreas of mice inoculated with 266-6 cells and decreased the level of critical proinflammatory cytokines in their serum. Thus, these results support the need for further studies to clarify whether the use of OOS as a nutritional complement could be feasible during the treatment of pancreatic adenocarcinoma.

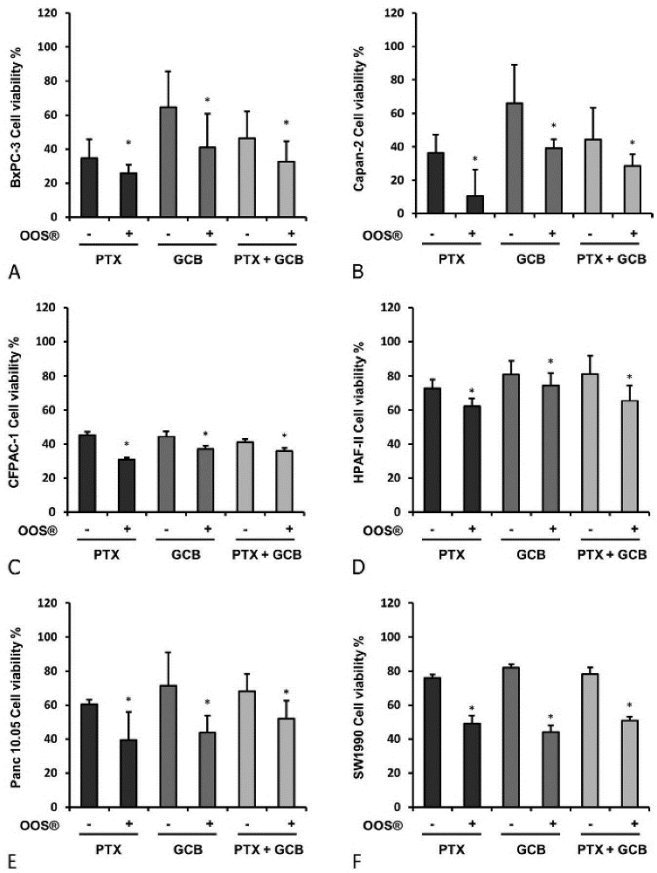



**FIGURE 8.** Cell viability of human pancreatic cancer cell lines treated with OOS as a complement to paclitaxel and gemcitabine. The most effective concentrations of paclitaxel (PTX), gemcitabine (GCB), and OOS were chosen, and viability of BxPC-3 (A), Capan-2 (B), CFPAC-1 (C), HPAF-II (D), Panc10.05 (E), and SW1990 (F) cells was tested for 48 hours in the presence of combinations of the 3 agents: paclitaxel 1 μM + OOS 1:50 (V/Vf), gemcitabine 1 μM + OOS 1:50 (V/Vf), and paclitaxel 1 μM + gemcitabine 1 μM + OOS 1:50 (V/Vf). Data are expressed as the mean value (SD) of at least 3 independent experiments. Differences were considered significant for $P < 0.05$ between the treatments alone and the treatments with OOS (*).

According to our results, OOS decreased the viability of the 266-6 murine pancreatic adenocarcinoma cells in vitro and reduced the tumor cell marker expression (SV40 T-antigen) in the pancreas in vivo, pointing out a reduction of tumor development in the pancreas. Besides, OOS also diminished significantly the in vitro viability of the human pancreatic cancer cell lines BxPC-3, Capan-2, CFPAC-1, Panc10.05, HPAF-II, and SW1990. Furthermore, the use of OOS as a complementary therapy to the agents, gemcitabine and paclitaxel, enhanced the cytotoxic effect in all the tested human cell lines.

Tumor growth and chemoresistance often correlate with the nature and the developmental degree of the microenvironment of the tumor itself.[47,48] In this context, CAFs are stromal cells associated with the developing tumor that play an essential role in tumor progression. Cancer-associated fibroblasts secrete multiple chemokines and inflammatory mediators that promote proliferation, invasion, and metastasis of cancer cells.[6] Moreover, accumulating evidence indicates the role of CAFs in the acquisition of drug resistance.[47] Following this, all the human cancer cell lines we tested showed an improvement in viability and a reduction in apoptosis levels in vitro when they were cultivated in the presence of supernatants obtained from fibroblast cultures, also becoming more resistant to paclitaxel and gemcitabine treatments.

Interestingly, when OOS was added to cancer cells cultured with fibroblast CM, the drug's effectiveness was reverted to basal conditions, whereas the number of apoptotic tumor cells increased. In our hands, OOS exerted an antioxidative and anti-inflammatory

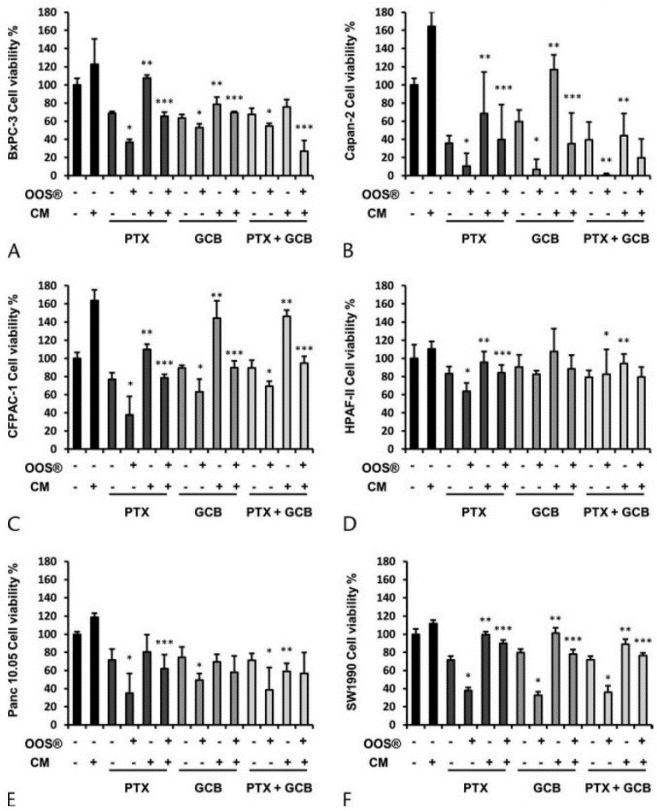



**FIGURE 9.** Cell viability of human pancreatic cancer cell lines in the presence of paclitaxel and gemcitabine alone and combined with OOS in cultures grown under normal and protumoral conditions. Human cancer cells were cultured with regular culture medium (–) and with fibroblast CM (CM). Then, the viability of BxPC-3 (A), Capan-2 (B), CFPAC-1 (C), HPAF-II (D), Panc10.05 (E), and SW1990 (F) cells was measured in the presence of paclitaxel (PTX) and gemcitabine (GCB) either alone or combined with OOS after 48 hours. Data are expressed as the mean value (SD) of at least 3 independent experiments. Differences were considered significant for $P < 0.05$ between the treatment alone and the treatment with OOS in normal conditions (*), the treatment alone and the treatment on protumoral conditions (**), the treatment on protumoral conditions and the treatment on protumoral conditions with OOS (***).

effect and reduced the in vitro viability and invasion of the murine 3T3 fibroblasts (data not shown), demonstrating that OOS exerts an impact not only on tumor cells but also on the stromal components.

Pancreatic stellate cells (PSCs) are the primary source of CAFs in the pancreas.[6,49] Pancreatic stellate cells stimulate cancer cell proliferation and inhibit cancer cell apoptosis, therefore acting as promoters of tumor growth.[50,51] Apte et al[51] utilized coculture of pancreatic cancer cell lines and PSCs or conditioned medium produced by PSCs to demonstrate that pancreatic cancer cells recruit PSCs promoting tumor growth and local invasion. Interestingly, our previous results showed that OOS reduced in vivo hepatic stellate cell infiltration into colorectal cancer liver metastasis.[22] Thus, a similar mechanism may be operating in our in vivo model of pancreatic carcinoma. Moreover, in vitro assays revealed that OOS slows down 266-6 cell cycle by 30%, therefore blocking in part, tumor cell proliferation. It is tempting to speculate that OOS may exert its antitumor effect by reducing both tumor cell proliferation and PSC infiltration into the pancreatic tumor. Further analysis is in need to clarify those results.

Inflammation and cancer progression are closely related.[52,53] In this work, OOS reduced the level of central proinflammatory cytokines, such as IL-1β, IL-12, and IFN-γ in the serum of pancreatic tumor-bearing mice, and increased anti-inflammatory IL-10. These results are in accordance with our previous results in a colorectal cancer liver metastasis model. On the other hand, the gene array performed in this study revealed that OOS reverted the expression of genes already reported as being altered in pancreatic cancer such as *Angptl4*, *Asns*, *Crabp1*, *Creb1*, *Foxf1*, *Thy1*, *Trpc4*, and *Wnt4*,[27–30,34,37,38] among others.

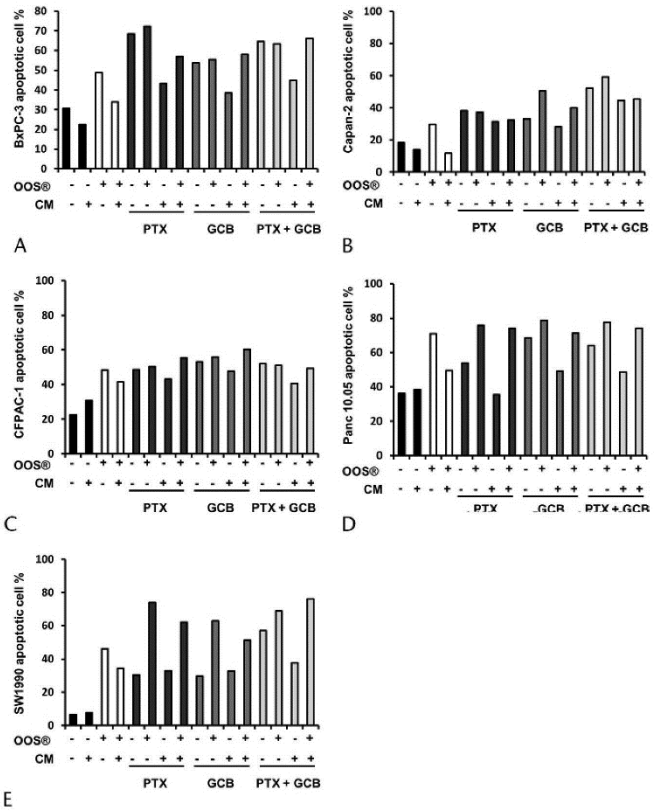



**FIGURE 10.** Effect of OOS alone or in combination with paclitaxel and gemcitabine in human pancreatic cancer cell apoptosis. Cell lines treated with paclitaxel and gemcitabine alone and in combination with OOS in both healthy and protumoral conditions. Apoptosis was analyzed by annexin V/PI to understand the mechanism of OOS that provoked the cell viability reduction. Cell death levels of BxPC-3 (A), Capan-2 (B), CFPAC-1 (C), Panc10.05 (D), and SW1990 (E) were measured in the presence of paclitaxel 1 μM and gemcitabine 1 μM either alone or combined with OOS 1:50 (V/Vf) after 48 hours. Cells were cultured with standard culture medium (–) and with fibroblast CM. Data show the most representative results.

In summary, OOS may slow down pancreatic cancer development, by decelerating cell division and increasing cell apoptosis. Besides, OOS may affect CAF infiltration into the tumor and reduce the level of inflammatory cytokines in the serum of mice. Moreover, OOS demonstrated the capacity to revert both the chemoresistance produced by the stromal cells and the expression of genes related to pancreatic cancer.

Therefore, OOS may constitute a nutritional supplement to combine with currently used chemotherapy to treat pancreatic cancer.

## ACKNOWLEDGMENTS

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