Zinc homeostasis and disease

Impact on breast cancer progression and infection severity

Marina Vogel González

DOCTORAL THESIS UPF / 2021

Thesis supervisors

Dr. Rubén Vicente García

Dr. Miguel Ángel Valverde

Experimental and Health Sciences Department

Universitat Pompeu Fabra Barcelona

A mi abuela,

Dixoça la rama que ar tronco çale.

Acknowledgements

Lâ graçiâ çon pa reírçe.
— Mi abuela.

No mentiré, he retrasado y retrasado el momento de escribir estos agradecimientos porque sé, de sobra, que es la parte que más lectores tendrá (no os culpo, haría exactamente lo mismo). Espero no olvidarme de mucha gente y estar a la altura. De todas formas, si lees estas líneas, ya tengo algo que agradecerte. Así que gracias.

Empecemos por el principio. Hace ya cinco, ¿seis?, años tuve una entrevista con Miguel y con Chema. Esa entrevista me dio la llave para entrar en este laboratorio y hacer lo que tanto deseaba, la tesis. Quiero agradeceros a ambos la oportunidad que me brindasteis en aquel momento. Gracias, jamás olvidaré la ilusión y la alegría. Sin vosotros, esto no sería real.

Carlos, querido mentor, a ti te debo mucho. Gracias por enseñarme tanto, dentro y fuera de la poyata. Sin tu ejemplo y tu apoyo todo habría sido mucho más difícil. Sabes, como yo, que aquellos que te ayudan a dar los primeros pasos te marcan. Así que te llevo conmigo, pa' siempre.

Después llegó Rubén. Rubén, esta tesis es tan mía como tuya. Si no llegas a aparecer, esto probablemente no existiría. En este párrafo no voy a ser capaz de expresarte, jamás, lo agradecida que estoy por estos años trabajando juntos. Gracias por la confianza que me has demostrado, por tenerme siempre en cuenta. Apareciste en el momento justo y, gracias a eso, hoy este trabajo es real. Gracias por ser tan comprensivo, empático y asertivo. Eres el jefe que cualquiera desearía. Gracias por compartir conmigo la ilusión y la

desilusión. Gracias por no tirar nunca la toalla y por tener un criterio científico tan acertado. He aprendido muchísimo de ti y ha sido un enorme placer.

Durante estos cuatro o cinco años he tenido la gran suerte de conocer a maravillosos compañeros con los que aprender y crecer científicamente. En la portada vais, para no olvidarme nunca de todo lo que me habéis dado. A la “vieja” guardia, Vicky y Roberto (Carlos ya ha tenido su momento), quiero daros las gracias por sacar tiempo, cuando estabais acabando, para enseñarme. Vicky, aún me acuerdo de ti cada vez que hago un WB. Selma y Fanny, me gustaría agradeceros los consejos dados, el interés por los problemas que me han ido surgiendo y la ayuda en resolverlos. Por cierto, creo que la ciencia debería aprender de vuestro trabajo en equipo. Cris, supongo que estás acostumbrada a todo lo que te diga, aunque eso no lo hace menos verdad (al contrario). Eres la piedra angular del laboratorio, sin ti nada funcionaría. Además, eres la experiencia, tanto dentro como fuera del laboratorio. Puede sonar que te estoy llamando persona mayor, pero no (ya sabemos que tenemos la misma edad). Lo que digo es que nos escuchas y nos ayudas a relativizar, viendo las experiencias de los que nos han precedido. Por esto y por todos los cafés, gracias. Mercé, el ser de luz. Además de ser brillante, científicamente hablando, vas irradiando felicidad y ternura por donde pasas. Gracias por iluminar el laboratorio. Daniela, viniste a enseñar tanto… Sólo te diré que ojalá hubiera más gente como tú, te admiro muchísimo. Natalia y Sonia, ¡Natalia y Sonia! Cómo os echo de menos. Vosotras me habéis dado los mejores momentos en el laboratorio, y lo sabéis. Gracias por las risas, por los desahogos, por las noches de fiesta, por los “si no me acuerdo no pasó” y por vuestra amistad. Julia, se

te aplica absolutamente todo lo que les acabo de decir a Natalia a Sonia, pero tú has seguido a mi vera hasta el final. Compañera de fatigas, compañera de desahogos, compañera de tesis desde el día uno: compañera y amiga. El camino entero habría sido diferencialmente (con estrellitas) más duro sin ti. Gracias a las tres por enseñarme tanto, tanto, tanto. Por dejarme aprender de lo maravillosas que sois y por regalarme vuestra amistad. Acabo de darme cuenta he nombrado del tirón (y sin querer) a mujeres maravillosas con las que es un placer compartir espacios. Me habéis enseñado infinito, gracias.

A los más jóvenes (no necesariamente en edad, sino aquellos que llegaron después de mí): Polete, bonito mío, no dejes jamás de ser lo bueno que eres. Da gusto ver tu cara de felicidad (cuando es la tienes) porque haces que todos sonriamos. Gracias a ti también por los desahogos y los cafés. Te voy a echar mucho de menos. ¡Víctor! ¡Contigo no sé ni por dónde empezar! Mola tener a tu lado a alguien con tanta integridad científica (aquí estamos para cervezas de desilusión, siempre). Inspiras pasión: si no sale una cosa, enseguida tienes otra hipótesis en la mano, ¡incansable! Espero haber estado a la altura en aquellos momentos en los que tuve que “enseñarte”. Albert, sabes que eres mi persona favorita para que me hable catalán (lo primero lo importante). Eres asquerosamente admirable, parece que se da todo bien dentro de la poyata, eres listo y guapo. Gracias por “arreglar” tantas veces el mundo conmigo y por el Resum. De oprimida a oprimido, cuando el barco se hunda, nos vamos juntos, aunque no haya pateras de vuelta. Iván, tío, cuando te fuiste del laboratorio dejaste un gran vacío, al menos para mí. Tus ganas trabajando son impresionantes, esa energía es incansable. Me siento afortunada de haber compartido contigo espacio y, más

aún, de que mi tesis lleve tu portada. Gracias por ofrecerte, gracias por hacerla, es perfecta sólo por de dónde viene. Por último, a aquellos estudiantes que han pasado poco periodo de tiempo, tengo que agradeceros la motivación con la que llegáis, siempre renovando el aire y trayendo sonrisas nuevas. Maz, David, Francisco, Marc, Joan, Alex A., Alex B, Souhe… ha sido un auténtico placer compartir espacio diariamente con vosotros; y un gran motivo de alegría. Y gracias por enseñarme a enseñar. Dunia, a ti te agradezco la paciencia de tener que aprender de alguien que está terminando el doctorado. Gracias por estar siempre tan dispuesta a todo y perdona la gran ausencia.

Aún sin salir del edificio, quiero darle las gracias a todo el grupo del IBE por tenerme siempre tan presente y hacerme sentir una más. Clàudia, Jéssica, Marc, Marina(s), Irene, Luis, Raquel, Meritxell, Juan, David, Esther… Las cervezas con vosotros siempre me han sabido a gloria.

Me voy al IBEC para agradecerle toda su ayuda, sus ánimos y su franqueza a Juanfra. Creo que nunca te lo he dicho, pero tu acento murciano siempre me ha hecho sentirme un poco en casa.

Saliendo de la ciencia, toca mi familia. Mamá, papá, sin vosotros no habría llegado hasta aquí. Gracias por darme todas las herramientas necesarias. Me veo obligada a mencionar que mi padre, en mi tierna juventud, osó hablarme de una tal Marie Curie. Yo era pequeña, pero ese día decidí que quería ser científica (suena a carta motivacional para entrar en un laboratorio, pero os juro que es verdad). ¡La importancia de los referentes! (Ya podría haberme hablado de una ingeniera, tendría más salida). Mamá, gracias también por el Résumé, hace que esta tesis sea un poquito más

personal. Nano, a ti te agradezco tu compañía y tus cuidados cuando vuelvo a Sevilla, eres mi pilar en el sur. El orgullo que me expresas me hace conquistar metas con más fuerza. Y gracias por una vida de confidencias. Al resto de mi familia, especialmente a mi tía e incluyendo a los nuevos fichajes, quiero darle las gracias por todos los cuidados de siempre. En especial a mis primos, por ser núcleo, y a mis primas, por recordarme lo que realmente es importante en la vida cada vez que las veo.

Y, ¿qué habría hecho yo sin mis otros amigos en Barcelona? Carmen, tenerte aquí estos años ha sido la mejor de las suertes y más en el tramo final. Creo que nos hemos cuidado de una manera muy especial, sin preguntar mucho, pero presentes en los momentos clave (como a nosotras nos gusta). Gracias por dejarme aprender tanto de ti, no sabes cuánto te admiro. Dani, ¿en qué momento caíste en la trampa de Barcelona? Lo cierto es que ¡bendita trampa! Mi otra suerte. Desde vivir contigo hasta quedar en la Bodega de Barri han sido momentos que he disfrutado al máximo. Gracias por escucharme y ser un amigo incondicional. Y por elegirme para confiar en mí. Espero no tener que echarte nunca mucho de menos. Amaranta, gracias por simplificar la vida siempre que tienes oportunidad de dar un toque. Las cervezas con vosotros y los demás maravillosos andaluces (Jose, Miguelón, Helena, Jose…), me han salvado muchas veces. Pilar… has aparecido en casi la recta final, cuando pensaba que Barcelona no podía darme a nadie más me dio a una de las personas más interesantes e inspiradoras que he conocido jamás. Sea como sea, me encantaría poder seguir aprendiendo a tu lado, aunque no te gusta la Cruzcampo, siembre habrá una Alhambra para ti en mi casa. Migue, tú no estás en Barcelona, pero todos sabemos que es mentira.

Gracias por todos los momentos que hemos compartido estos años de doctorado, aquí, en Bilbao, en Sevilla y hasta en la France. Eres una bonita sorpresa que siempre llega con diversión y hace olvidar cualquier problema, tienes ese don.

Aitor, tío, tengo que agradecerte ser el mejor compañero de piso, sólo superado por el Lucky. Ha sido un verdadero placer compartir tantos años viviendo juntos. Gracias por esos momentos, por las cervezas, por todas las series y por corregirme la tesis, por supuesto. Y por ser tan genial sin saberlo. También quiero darle las gracias a Eduardo, el señor padre de Aitor, por ser tan atento, por el pan, las comidas y ser una maravillosa compañía. Aprovecho para volver a nombrar a Víctor: gracias por formar hogar con nosotros (y las cervezas). Y al Lucky quiero agradecerle todo el amor gatuno y los buenos ratos. Se quitó del medio el día que empecé a escribir la tesis, muy listo él.

A mi Bar Coyote le doy las gracias por estar siempre, siempre, siempre. Por los reencuentros, las risas y los debates interesantes. Vamos, por ser tan buenos amigos. Y, dentro del Bar Coyote, una mención especial al Sex and the City, por ser un pilar diario, por escucharme, en lo bueno y lo malo, y por no fallar jamás. Os quiero mucho.

Manolo, mi más fiel compañero. Parece increíble que hayamos llegado (¡al fin!) a este punto. Quiero agradecerte tu presencia diaria. Quiero agradecerte el haber creado hogar, tan lejos de casa. Quiero agradecerte el dar por hecho, siempre, que yo podía; sin dudar ni un segundo de que tomaría la mejor decisión en cada momento. Quiero agradecerte cada serie que hemos visto juntos, cada vez que hemos ido al cine, cada vez que me has recomendado un libro, cada

viaje y cada cerveza (que no son pocas). Todo esto me ha hecho el camino más ameno, más agradable. Gracias por darme la mano, también, en esta etapa de mi vida. Espero, si así lo queremos los dos, que los proyectos sigan creciendo y esta mano no se suelte fácilmente: por aquello de la constante, ya sabes. Gracias por ser mi sur y por retroalimentarme con tu Andalucía. Por cierto, GRACIAS, por todos los besos y abrazos, no rogados. Y por darme siempre un hueco donde descansar de todo y sentir una genuina paz, que me aleja de la ansiedad que me causan tantas situaciones. Y gracias, de nuevo, por, simplemente, estar a mi lado. ¡Ah! ¡Enhorabuena cuasi doctor!

Quizás esto sea un poco controvertido, pero quiero agradecerme a mí misma el trabajo que hay detrás de las páginas que vienen a continuación. Y, por supuesto, quiero agradecerle a esta tesis, mi tesis, todo lo que me ha dado.

Por último y lejos de ser lo menos importante, quiero agradecerle a todas aquellas mujeres que alguna vez lucharon por nuestra libertad y nuestros derechos el haberlo hecho. Hace años no podría haber pisado ni siquiera una escuela y ahora “conquisto” el máximo nivel de formación universitaria gracias a vosotras. Aún queda muchísimo trabajo por hacer. Por vosotras, por nosotras y por las que vendrán: seguimos y no os olvidamos. Gracias, de corazón.

Pd. Releyendo los agradecimientos creo que debería agradecerle algo a la cerveza también, ya que la nombro bastante. Así que nada, gracias.

Abstract

Zinc is an essential trace element with structural, catalytic and signaling roles in our body. At cellular level, zinc homeostasis participates in fundamental functions such as cell proliferation, migration, differentiation and survival. Therefore, a dysregulation of zinc concentration at systemic and cellular levels can be the cause of human disease.

The first goal of this thesis is to explore the role of zinc during the metastasis of triple negative breast cancer to the brain. For that, we used MDA-MB-231 cells and their brain metastatic derived MDA-MB-231-BrM2 cells. We modulated their internal zinc content and proved its contribution in the metastatic hallmarks. We found a relevant impact of zinc concentration in brain-specific microenvironment modulation and cancer stem cell capacity to generate a secondary tumor.

Besides, zinc homeostasis is fundamental for the proper functioning of the immune system. Since the COVID-19 pandemic outbreak, another objective of this work has been to study the potential role of zinc supplementation and zinc ionophores in the treatment of this disease. *In vitro* experiments using VeroE6 cells and an observational study with patients pointed out that low zinc level is a risk factor for the fatal outcome of the SARS-CoV2 infection. Moreover, we demonstrated that chloroquine and hydroxychloroquine do not have zinc ionophore activity, further questioning the rationale of using these drugs to treat COVID-19.

Overall, the results of this thesis highlight the importance of maintaining zinc homeostasis to keep the health of individuals.

Resumen

El zinc es un elemento traza esencial con papeles estructurales, catalíticos y de señalización en nuestro organismo. A nivel celular, la homeostasis del zinc participa en funciones fundamentales. Así, una desregulación de su concentración a nivel sistémico o celular puede causar enfermedad en humanos.

El primer objetivo de esta tesis es explorar el papel del zinc durante la metástasis del cáncer de mama triple negativo al cerebro. Para ello, usamos células MDA-MB-231 y sus derivadas MDA-MB-231-BrM2, metastásicas cerebrales. Modulamos su zinc interno y probamos su contribución en marcadores metastásicos. Encontramos un impacto relevante de la concentración de zinc en la modulación del microambiente cerebral y en la capacidad de sus células madre para generar un tumor secundario.

Además, la homeostasis del zinc es fundamental para el funcionamiento correcto del sistema inmune. Desde el brote de la pandemia de COVID-19, otro objetivo de este trabajo ha sido estudiar el potencial papel terapéutico de la suplementación de zinc y sus ionóforos esta enfermedad. Experimentos *in vitro* usando células VeroE6 y un estudio observacional con pacientes señalaron que un bajo nivel de zinc es un factor de riesgo para el fatal desenlace de la infección por SARS-CoV2. Además, demostramos que la cloroquina y la hidroxicloroquina no son ionóforos de zinc, cuestionando más el uso de estos medicamentos en el tratamiento de la COVID-19.

En general, los resultados de esta tesis destacan la importancia de mantener la homeostasis del zinc para la salud de los individuos.

Résumé

Le zinc est un élément-trace essentiel avec des rôles structurels, catalytiques et de signalisation dans notre corps. Au niveau cellulaire, l'homéostasie du zinc participe à des fonctions fondamentales. Une dérégulation de sa concentration au niveau systémique ou cellulaire peut provoquer des maladies humaines.

Le premier objectif de cette thèse est d'explorer le rôle du zinc lors de la métastase du cancer du sein triple négatif au cerveau. Pour cela, nous avons utilisé des cellules MDA-MB-231 et leurs dérivés MDA-MB-231-BrM2, métastatiques cérébrales. Modulant son zinc interne et vérifions sa contribution aux marqueurs de métastases. Nous avons trouvé un impact pertinent de la concentration de zinc sur la modulation du microenvironnement cérébral et la capacité de ses cellules souches à générer une tumeur secondaire.

De plus, l'homéostasie du zinc est essentielle au bon fonctionnement du système immunitaire. Depuis le déclenchement de la pandémie COVID-19, un autre objectif de ce travail a été d'étudier le potentiel rôle thérapeutique de la supplémentation en zinc et ses ionophores dans cette maladie. Des expériences *in vitro* utilisant des cellules VeroE6 et une étude observationnelle avec des patients ont indiqué qu'un faible taux de zinc est un facteur de risque d'une issue fatale de l'infection par le SARS-CoV2. En outre, nous montrons que la chloroquine et l'hydroxychloroquine ne sont pas ionophores de zinc, remettant en question l'utilisation de ces médicaments dans le traitement du COVID-19.

En général, les résultats de cette thèse soulignent l'importance de maintenir l'homéostasie du zinc pour garder la santé des individus.

Resum

El zinc és un element traça essencial amb papers estructurals, catalítics i de senyalització en el nostre organisme. A nivell cel·lular, l'homeòstasi del zinc participa en funcions fonamentals. Així doncs, una desregulació de la concentració de zinc a nivell sistèmic o cel·lular pot donar lloc a malalties en humans.

El primer objectiu d'aquesta tesi és explorar el paper del zinc durant la metàstasi del càncer de mama triple negatiu al cervell. Per a això, utilitzem cèl·lules MDA-MB-231 i les seves derivades MDA-MB-231-BrM2, metastàtiques cerebrals. Modulem el seu zinc intern i comprovem la seva contribució als marcadors metastàtics. Trobem un impacte rellevant de la concentració de zinc en la modulació del microambient cerebral i de la capacitat de les cèl·lules mare cancerígenes per a generar un tumor secundari.

A més, l'homeòstasi del zinc és fonamental pel correcte funcionament del sistema immunitari. Des del brot de la pandèmia de COVID-19, un altre objectiu d'aquest treball ha estat estudiar, en aquesta malaltia, el potencial paper de la suplementació de zinc i el seu tractament amb ionòfors d'aquest. Experiments *in vitro* utilitzant cèl·lules VeroE6 i un estudi observacional amb pacients van apuntar que un baix nivell de zinc és un factor de risc pel desenllaç fatal de la infecció per SARS-CoV2. A més, demostrem que la cloroquina i la hidroxicloroquina no tenen activitat ionòfora de zinc, qüestionant encara més la justificació de l'ús d'aquests medicaments en el tractament de la COVID-19.

En general, els resultats d'aquesta tesi destaquen la importància de mantenir l'homeòstasi del zinc per mantenir la salut dels individus.

Preface

Zinc biology has largely evolved since this metal was discovered to be an essential trace element for human health 60 years ago. Nowadays, it is known that zinc has functions at the structural, enzymatic and signaling level. As a secondary messenger, zinc has been demonstrated to maintain the well-balance of cellular functions such as proliferation, migration, differentiation and survival. In this context, the preservation of zinc homeostasis has been revealed as an especially important process to prevent cancer progression and for the proper functioning of the immune system against infections, the two main subjects of this thesis.

The Introduction is divided into three sections. In the first section, the reader can find historical remarks of zinc biology discoveries, its importance in human health and an explanation about its roles at the systemic and cellular level. The second section introduces breast cancer physiology with a special focus on the triple negative variant and brain metastasis. This part is followed by a background summarizing the known roles of zinc and its transporters in cancer progression. The last part of the introduction is devoted to describing zinc impact in the immune system and viral infections, with an especial focus on the SARS-CoV2 infection.

Results are divided into 2 chapters. In Chapter One, we explore the role of zinc in the brain metastasization of triple negative cancer cells. We report a difference in the zinc homeostasis between cells before and after the brain colonization. Moreover, we manipulate the cellular zinc content to study its contribution to this process. We describe how an increase in the zinc concentration helps metastatic cells in the brain microenvironment modulation and the induction of

a whole secondary tumor, boosting its cancer stem cell characteristics. In Chapter Two, through *in vitro* experiments with VeroE6 cells and an observational cohort study with 249 patients, we determine that zinc deficiency favors SARS-CoV2 infection and is associated with COVID-19 severity. Studying the potential prophylactic role of zinc supplementation and zinc ionophores against COVID-19, we found that chloroquine and hydroxychloroquine do not have this capacity. In the annex of Chapter Two it is shown that these drugs specifically increase zinc in the lysosomes and that both, zinc absence and chloroquine, lead to lysosomal basification.

Finally, these results are discussed in the context of other studies of the respective fields. Moreover, together, these works highlight the importance of zinc homeostasis for human health at both, systemic and cellular levels.

Abbreviations

ACE2: Angiotensin II Converting Enzyme.
Asn: asparagine.
Asp: aspartate.
CSCs: cancer stem cells.
CQ: chloroquine.
Cys: cysteine.
ESCC: esophageal squamous cell carcinoma.
EMT: epithelial-mesenchymal transition.
ER: endoplasmic reticulum.
EsR: estrogen receptor.
Glu: glutamate.
HCMV: human cytomegalovirus.
HCQ: hydroxychloroquine.
HCV: hepatitis C virus.
His: histidine.
HIV: human immunodeficiency virus.
HPV: papilomavirus.
IL: interleukin.
MT: metallothioneins.
NPC: nasopharyngeal carcinoma.
OSCC: oral squamous cell carcinoma.
PA: plasminogen activator.
PMNs: polymorphonuclear cells.
PR: progesterone receptor.
ROS: reactive oxygen species.
RT: retrotranscriptase.
SARS-COV2: severe acute respiratory syndrome coronavirus 2.
TMD: transmembrane domain.
TNBC: triple negative breast cancer.
TNF: tumor necrosis factor.
ZIP: zrt- and Irt-like proteins.
ZnT: zinc transporters.
WHO: World Health Organization.

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Introduction

Zinc: an essential trace element

Zinc is a chemical element whose atomic number is 30. Its symbol is Zn and it belongs to the 12th group in the periodic table, the transition metals. Zinc exhibits a normal oxidation state of +2 and it has five stable isotopes. It shows no redox activity and acts as a Lewis acid in biological reactions. At room temperature it is found as a silvery-white metal with a blue tone. According to the National Minerals Information Center of the U.S., zinc is the 23rd most abundant element in the Earth's crust[1].

There is evidence that zinc has been used by humans since the 3rd millennium B.C.[2,3] but the Greeks and Romans were the first to acknowledge its existence. During history, different civilizations have discovered independent methods to melt this metal into valuable alloys[4], which points out the importance of zinc in the development of societies. Zinc was first isolated in India in the 13th century. In Europe, the German chemist Andreas Sigismund Marggraf rediscovered zinc in 1746 and named it after the german word "*zinke*", meaning tooth, due to the shape of the metal when it crystallizes.

In the fields of biology and nutrition, zinc has gone from invisible to essential in less than 100 years. The French scientist Raulin was first to identify zinc as an essential element for microorganism growth. During his thesis, in 1869, he reported that this element was needed by *Aspergillus niger* to grow[5]. In 1905, zinc was identified as a constituent of the sea snail liver. At that time it was thought to play a respiratory role unique to this animal[6]. In 1926, Sommer and Lipman proved that zinc was also indispensable for the life and

growth of plants[7]. After that, several scientists hypothesized that zinc could be nutritionally required by higher animals[8–11]. However, it was not until 1934 that zinc was firmly proved to be essential for the normal development of rats[12]. In 1955 it was demonstrated that zinc deficiency caused the skin disorder parakeratosis in pigs[13] and, in 1958, it was also shown to be needed by growth poultry[14]. Although these findings were pointing at zinc as an essential nutrient for people's health, the scientists of this epoch thought that its deficiency was probably not a problem in humans. Finally, in 1963, Dr. Prasad established a link between zinc deficiency and illness in a population from Egypt[15]. He used the radioactive isotope ^{65}Zn to determine the deficiency. The study subjects presented dwarfism and hypogonadism and, in later research, it was proved that zinc supplementation resulted in growth of the patients and normal development of their genitalia[16].

In 1974 zinc was declared an essential micronutrient for humans by the National Research Council of the National Academy of Sciences and a recommended dietary allowance (RDA) was established[17]. Nowadays, this RDA value for adults is 8-12 mg/day, depending on the body weight[18].

Relevance of zinc in human health

Zinc is available in most of the foods we consume. Therefore, zinc deficiency can be easily avoided with a well-balanced diet. Good sources of zinc are red meat and poultry. Grains and plants also have a substantial concentration of zinc, however, the phytates contained in them avoid zinc absorption[19].

The World Health Organization (WHO) estimates that 31% of the global human population suffer from zinc deficiency. The rates of

zinc deficiency around the world can vary from 4-73% and developing countries present the highest prevalence due to their cereal-based diet[20]. On the one hand, in children, zinc deficiency is estimated to cause 4% of global morbidity and mortality, especially in the case of diarrhea and pneumonia-related deaths[21]. On the other hand, elderly people tend to suffer from zinc deficiency, even in industrialized countries[22], which can lead to problems in the immune system[23].

The normal zinc levels in serum and plasma for an adult are around 70 µg/dl. Patients with serum zinc levels below 50 µg/dl start to present clinical symptoms of severe zinc deficiency[24]. However, measuring zinc content in plasma and serum has its limitations since it does not reflect the intracellular level. In this case, it is possible that functional effects of the zinc deficiency will be observed before seeing a decrease in the plasma content[25].

Zinc deficiencies can range from severe to mild. Severe zinc deficiency is characteristic in patients suffering from acrodermatitis enteropathica, an inherited autosomal recessive skin illness caused by a defect in zinc absorption. It is a rare condition (global incidence of 1:500,000 newborns) distinguished by bullous pustular dermatitis, alopecia, ophthalmic signs, emotional instability, weight loss, growth retardation, male hypogonadism, diarrhea, etc. Superinfections are also commonly reported[26]. This condition has been treated with enteral or parenteral zinc supplementation since 1973, when it was established that symptoms cleared up after this therapy[27].

Moderate zinc deficiency is normally caused by a poor intake of zinc from the diet. Intestinal malabsorption syndromes, inflammatory bowel diseases such as Crohn’s disease, chronic urinary zinc loss,

cirrhosis, alcoholism or chronic inflammatory diseases that increase interleukin-1 (IL-1) can also cause a moderate zinc deficiency[25]. This level of deficiency is the most prevalent worldwide. In fact, the individuals that Dr. Prasad studied in 1963 had this grade of deficiency[15]. The symptoms are growth retardation, male hypogonadism in adolescents, rough skin, poor appetite, mental lethargy, delayed wound healing, cell-mediated immune dysfunctions, and abnormal neurosensory changes[28].

Mild zinc deficiency is difficult to identify. In 1997, Dr. Prasad and colleagues induced a specific mild deficiency in human volunteers in a controlled experiment. They observed decreased serum testosterone level, oligospermia, decreased natural killer (NK) cell lytic activity, decreased IL-2 activity of T helper cells, decreased serum thymulin activity, hyperammonemia, hypogeusia, decreased dark adaptation, and decreased lean body mass. They concluded that even mild deficiency of zinc has adverse effects in clinical, biochemical and immunological functions[29].

Since the establishment of zinc deficiency as a human health problem, zinc supplementation has been used to treat several diseases. In acute diarrhea in children, it decreases morbidity and mortality. In fact, the WHO recommends a daily supplementation with 10-20 mg of zinc for 10-14 days upon diarrheal onset[21]. Moreover, zinc supplementation has been proved to be effective against the common cold caused by rhino- and coronaviruses[30]. In age-related macular degeneration, which is the third cause of legal blindness in the world[31], people treated with zinc showed a decrease in cardiovascular related mortality[32].

As mentioned before, elderly people have a higher risk of developing zinc deficiency, as more than 57% of them do not have enough of this micronutrient[23]. The causes of this tendency are varied: inadequate diet, altered intestinal absorption, inadequate mastication, psychosocial factors, drug interactions, altered subcellular processes. Zinc deficiency in the elderly is associated with a decrease of immune efficiency. Therefore, it has been proposed that zinc supplementation in these subjects helps reduce or delay the appearance of diseases[23].

Zinc supplementation is considered quite safe and is used in many conditions as just described. However, an excess will lead to health problems. Acute effects of zinc toxicity are nausea, vomiting, loss of appetite, abdominal cramps, diarrhea and headaches. Chronic zinc toxicity causes gastric problems, a reduction in the immune function, a decrease in HDL cholesterol and low serum copper concentration[33].

Systemic zinc homeostasis

Zinc is the second most abundant trace element in the human body. There are 2-3 g of zinc in a 70 kg adult, and the daily diet restores around 0.1% of it[25]. Zinc is not equally distributed across tissues. Out of the total amount of zinc in the body, 60% is stored in the skeletal muscle, 30% in the bone, 5% in the liver and the remaining 2-3% in other tissues (Fig.1). Just 0.1% of the zinc is in the serum, from which 80% is loosely bound to albumin and 20% is firmly bound to α2-macroglobulin. Zinc is absorbed directly from the diet in the duodenum and jejunum thanks to the ZIP4 transporter. The absorption efficiency can be boosted to 90% if zinc is limited. In contrast, when there is an excess of zinc in the body, it is disposed

of by the gastrointestinal tract, sloughing mucosal cells and renal excretion[34].

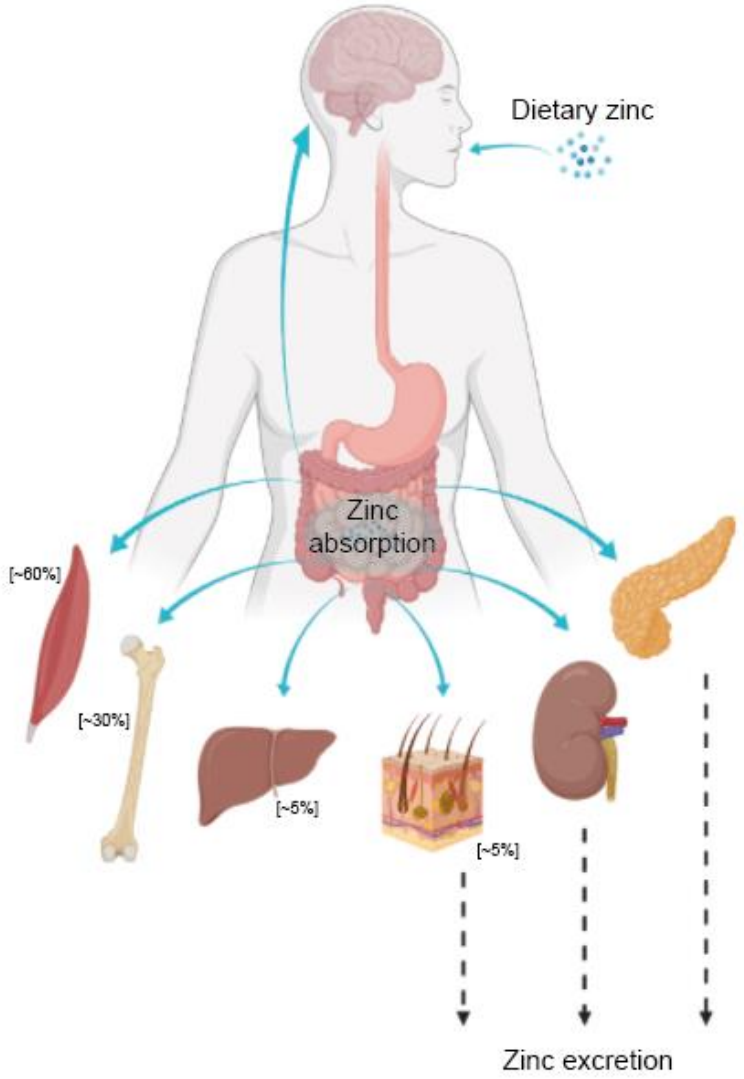

Figure 1. Systemic zinc homeostasis in a normal adult. Zinc absorption occurs in the intestine, where ZIP4 is expressed for this purpose. Zinc is distributed around the body according to each tissue necessity. Skin, kidney and pancreas have excretory functions. Image created with BioRender.com and inspired by [34].

Roles and homeostasis of zinc at a cellular level

As mentioned at the beginning of this thesis, zinc is a redox-inert ion with only the +2 valence state. It is a transition metal, so zinc belongs to d-block cations, which means that the d sublevel of electrons is in process of being filled up to ten electrons. Thanks to these chemical characteristics, zinc is able to bind proteins tightly (during its whole lifetime) or loosely (in a reversible way). When the bound is weak the dissociation rates are high, which results in a controlled concentration of free cations that cells use as secondary messengers. This behaviour turns zinc into the ideal ion to accomplish a variety of key biological processes. In fact, zinc plays structural, catalytic and signaling roles. In order to maintain all this process well balanced, it is indispensable to firmly control the zinc homeostasis. A higher concentration than optimal will let zinc bind proteins that it should not. At the same time, if the concentration of zinc is lower than desired, it will not be available for signaling[35]. This way, zinc concentration inside cells is controlled by more than 30 proteins[34].

50% of the cellular zinc is in the cytoplasm, 30-40% in the nucleus and 20% in the membrane. The normal zinc concentration in any given cell ranges between 10 and 100 µM. This zinc is distributed in four pools: tightly bound to metalloproteins or metalloenzymes, bound to proteins with low affinity, stored in organelles or free in the cytoplasm[36]. The majority of cations are linked to proteins and the free concentration in the cytoplasm is estimated to be from low nM to pM[34]. This high difference between total and free zinc in cells is a characteristic of zinc biology[37]. There is controversy about the zinc concentration in some cell compartiments. A concentration of 0.2 pM has been reported in the Golgi apparatus[38]. In the endoplasmic

reticulum (ER), a wide difference in concentrations ranging from 0.9 pM to 5 nM have been described. A similar scenario has been observed in the mitochondrion, where zinc concentration ranges of 0.14-300 pM have been measured. For the mitochondrial matrix, the measures are between 0.2-72 pM. These differences are the result of experimental limitations, as the concentrations are so low that a measurement with different probes can cause notable changes in the final values[39].

For didactic reasons, the roles and the zinc homeostasis are going to be explained separately in this thesis. However, it is very important to clarify that this whole system works as one and it is the interaction between the structural and catalytic role, the free signaling zinc and its homeostasis which makes zinc biology an extremely fine and important machinery for life.

Structural and catalytic role of the zinc

The structural role of zinc was first proved in 1938 after the crystallization of insulin[40]. Recent studies assume that around 3,000 proteins need zinc to be functional, which represents a 10% of the encoded human proteins[34,41]. The first time that zinc was demonstrated to be essential for catalysis was in 1939, when the carbonic anhydrase dependence on zinc was established[42]. Nowadays, it is known that zinc stabilizes negative charges from the substrates of some enzymes thanks to its strong Lewis acid properties[43].

Zinc cations bind metal sites with a very high affinity, just surpassed by copper[41]. The affinity of each protein to be bound by zinc is a key characteristic. Zinc has coordination numbers from 4 (forming

tetrahedral geometry) to 6, binding most frequently nitrogen from histidine (His), sulfur from cysteine (Cys) and oxygen from aspartate (Asp) or glutamate (Glu). Combinating these ligands, proteins can modulate their affinity for zinc, so they can be used for structural or catalytic functions[44]. Notably, zinc finger motifs, first identified in 1985[45], are small domains stabilized by zinc that allow a protein to interact with other proteins, DNA, RNA or lipids. They can have very diverse structures and more than 20 classes exist[34].

In order to avoid its bonds with some proteins before other metals such as the iron, free zinc concentrations have to be very low. For this reason, only the proteins that have a very high affinity for zinc are going to be tightly linked to this cation. Other proteins, with a lower affinity, will form labile complexes with a high dissociation constant. That means that these complexes will be or not formed depending on the zinc concentration surrounding the protein. Proteins like these are also fundamental because they are involved in buffer zinc processes[44].

In this context, it becomes obvious that zinc is determinant for the proper functioning of many proteins. However, an excess of zinc will lead to some undesirable bonds. For example, zinc can bind and inhibit the sodium-potassium-ATPase, the calcium-ATPse, the electron transfer ubiquinone, the cytochrome b of the bc1 complex, cytochrome c oxidase activities and many others.

Zinc signaling

The understanding of zinc as a secondary messenger began very recently[46]. Since then, the signaling role of zinc has been widely proven and even considered as the calcium of the 21st century[47].

Zinc ions are necessary in both intercellular and intracellular communication. The extracellular release of zinc has been demonstrated to be involved in endocrine, paracrine, autocrine and synaptic communication. Cells participating in these pathways have vesicles called zincosomes containing a great zinc concentration (in the order of mM), ready to be released after the triggering stimuli[48].

This type of signaling is important in several tissues. In the prostate, zinc secretion into the prostatic fluid is essential for inhibiting proteolytic enzymes. The concentration of this fluid has to be around 500 fold greater than in plasma, as lower ones activate the enzymes and result in liquefaction and release of motile sperm cells. For this reason, prostate epithelial cells accumulate high levels of zinc[49]. In the pancreas, zinc is needed for its endocrine and paracrine functions. Pancreatic β cells have the highest zinc concentration with 10-20 mM in the insulin secretory granules, where zinc helps the insulin crystallization. In addition, zinc secreted by β cells appears to inhibit glucagon secretion by the α cells, but the molecular mechanisms behind this process remain unclear[50]. In the presynaptic terminal of the hippocampal neurons, high numbers of zinc-containing vesicles are also found. When this zinc is released into the synaptic cleft, it works as a neurotransmitter or neuromodulator inhibiting, for example, calcium entry by the NMDA receptor[51]. Zinc mediated intracellular signaling is also very relevant in bone (it regulates mineralization), paneth cells (microbicidal), retina (visual synapse), mammary gland epithelial cells (milk production) and skin (defence)[48].

The intracellular signal transduction is associated with an increase in the free cytosolic zinc originated from the extracellular media or the organelles and vesicles following a stimulus. A change in the

internal zinc concentration will lead to a biological response. When the signaling occurs seconds or minutes after the stimulus, it is called fast or early and it does not involve protein transcription. In contrast, late signaling takes hours and involves changes in the gene expression. The zinc acting as secondary messenger modulates the activity of a wide variety of signaling enzymes, including tyrosine phosphatases, phosphodiesterases, calcineurin, caspases and kinases like MAPK or PKC[34].

Therefore, it is not surprising that zinc has been involved in an elevated number of vital cell processes, such as proliferation[52–54], migration[55–57], differentiation[58–60] and survival[61] in different cell types[62]. The underlying mechanisms of zinc regulation in many of these processes are not completely elucidated yet and further investigations are needed. For instance, it has been reported that zinc enhances proliferation in the intestinal epithelium[53] but it impairs it in neuronal precursor cells, inducing apoptosis[63]. What is clear is that zinc modulates these processes delicately and in a cell type dependent manner.

Zinc homeostasis machinery

The complexity of zinc regulation is evident when looking at the extensive machinery involved. More than 30 proteins are described to play a role in zinc homeostasis. As mentioned before, a tight control of zinc concentrations in the cytoplasm and in the different cell compartments fine-tunes proper zinc-protein interactions. The zinc-binding protein family of metallothioneins (MT), and two different family of zinc transporters, Zrt- and Irt-like proteins (ZIP) and Zn transporters (ZnT), are in charge of the maintenance of this homeostasis[34].

MTs are low-molecular-weight metal binding proteins. 5-15% of the total zinc in the cell is bound to these proteins with low affinity, so they act as zinc buffers. This is possible because of their lack of disulfide groups and their one-third cysteine residues. Each MT molecule is able to bind seven zinc ions. As metal-binding proteins, they can also chelate cytotoxic metals, lowering their cytosolic concentration. There are, in total, 11 functional isoforms of MTs in humans, divided into four classes. MT1 counts with eight different active genes while MT2, MT3 and MT4 are encoded only by one gene each. MT1 and MT2 are ubiquitous, their amino acid sequences have a high level of homology and it is not clear if they have functional differences. MT3 is expressed in the brain and MT4 is abundant in some epithelial tissues. In general, MT proteins release zinc when its concentration in the cytoplasm is decreased[36]. Furthermore, upon a rise in cellular ROS production, MTs release zinc ions producing a cytosolic zinc release that leads to a complex anti-ROS transcriptional program[64]. MTs have also been proposed as chaperons for zinc proteins/enzymes[36].

Zinc transporters are the most important proteins to maintain zinc homeostasis since they take up or flow out this metal through biological membranes. The first zinc transporter, Zrc1, was identified in *Saccharomyces cerevisiae* in 1989 as a gene conferring resistance to zinc and cadmium[65]. Since then, nine ZnT and 14 ZIP transporters have been identified[34].

ZnTs are the transporters that reduce the zinc concentration inside the cytoplasm, mobilizing the cations to the extracellular medium or to the intracellular organelles. The only 3D structure of a ZnT-family protein member that has been verified to date is the *Escherichia coli* homolog YiiP. ZnTs form homo- or heterodimers, where each

subunit is thought to have six transmembrane domains (TMDs) with both, the amino- and the carboxyl-termini, present in the cytosol. Moreover, each monomer has 2 His and 2 Asp, forming an intramembranous tetrahedral Zn-binding site used for zinc transport. Finally, ZnT transporters have been shown to act as Zn^{2+}/H^{+} antiporters. Within the ZnTs there are some structure variations, for example, ZnT10 changes a His residue for an asparagine, which also allows it to transport manganese. ZnT5 and ZnT6 form heterodimers in which the ZnT6 subunit may have modulatory functions, and thus, it does not have zinc-transport activity[62].

ZIP transporters are responsible for the increase of cytosolic zinc concentration by transporting zinc ions from the extracellular medium or the subcellular compartments. They are mainly located in the plasma membrane except for ZIP7, which is located in the ER membrane. They also form homo- or heterodimers. Each subunit is thought to have eight TMDs with the amino and carboxyl-termini located outside the plasma membrane or in the inner part of the organelles. There is not a complete understanding of the zinc specificity of the ZIPs yet. In fact, ZIP transporters are also involved in iron, manganese, copper and cadmium transport. A conserved His residue has been thought to be involved in an intramembranous zinc-bonding site and may play a key role in the zinc specificity. Moreover, this His is replaced by a Glu in ZIP8 and ZIP14 leading to a change in metal specificity. Some studies indicate that ZIPs are involved in a Zn/bicarbonate symport while others point at a selective electrodiffusional channel mechanism, supported by the evidence that phosphorylation can activate the transport. Depending on their sequences, ZIPs are classified into four subfamilies: I (ZIP9), II

(ZIP1, ZIP2, ZIP3), LIV-1 (ZIP4, ZIP5, ZIP6, ZIP7, ZIP8, ZIP10, ZIP12, Zi13 and ZIP14) and gufA (ZIP11)[34,62].

In Figure 2 there is a representation of how all these proteins are distributed along the cell and the concentration gradients that they aim to maintain in each cellular compartment.

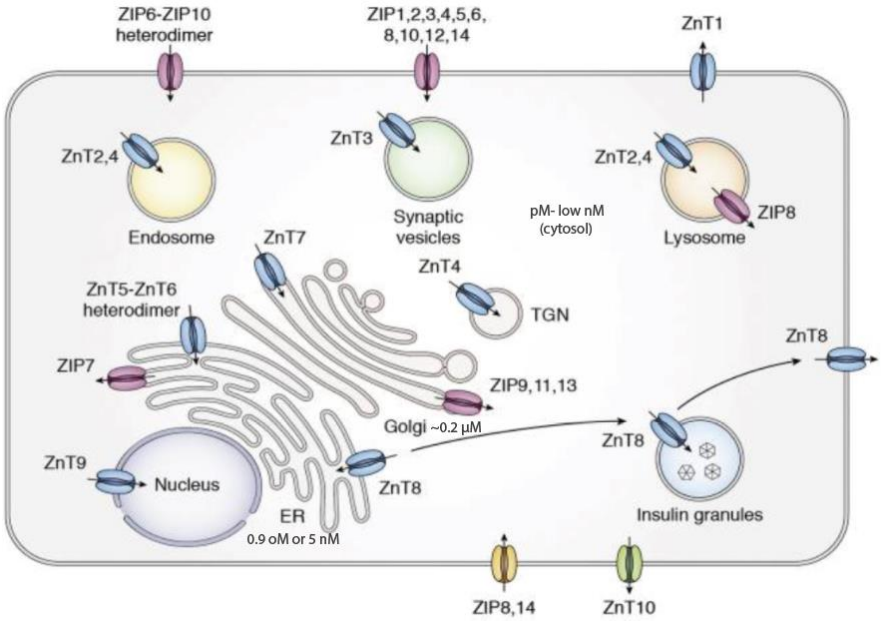

Figure 2. Zinc concentrations, zinc transporters and subcellular localization. Adapted from [66]

All this zinc homeostasis machinery is regulated by zinc itself. In vertebrates, the effector has been identified as a metal-response element-binding transcription factor called MTF-1, which is a zinc sensing protein. When cellular zinc levels increase, the cations bind the six finger domains of MTF-1 and they activate its transcriptional functions. MTF-1 increases the transcription of MTs, ZnT1 and ZnT2, and represses genes like ZIP10, in order to reduce zinc cytosolic concentration. Another zinc-responsive zinc finger transcription

factor, ZNF658, has been identified. When ZNF658 is knocked-down, the expression of ZnT5 and Znt10 is reduced[67]. The regulation of MTF-1 and ZNF658 is completely independent. It is also known that the expression of other transcription factor, such as ZEB1[68] or API1, JNK and ERK[69] can be influenced by zinc signaling pathways.

Moreover, ZIPs are usually post-translationally regulated by zinc. These transporters rapidly mobilize to the cell membrane in zinc deficiency conditions while they are endocytosed and degraded when an excess of cytosolic zinc is detected. The degradation occurs via ubiquitin-proteasome or via lysosomal pathways. It is also important to take into account that the activity of the zinc transporters affects the activity of zinc enzymes, thus, they are able to proportionate or remove zinc ions, which are needed to be coordinated to the enzyme's active site[34,62].

As mentioned before, the handling and balance of zinc concentrations is fundamental to a wide range of cellular processes. The function of these transporters has been shown to be cell or tissue specific, so it has to be studied individually. Moreover, altered expression and/or function of specific zinc transporters are associated with certain disorders summarized in Table 1.

Transporter	Tissue and cellular location	Function
ZnT1	Ubiquitous[70]: PM. Polarized epithelial cells: basolateral membrane. Ves. in some cells[34]	Zinc transport to circulation. Embryonic development[71].
ZnT2	Prostate. Ves., end. and lys. Breast cells: ER. Mammay cells: inner mitochondrial membrane. Pancreatic acinar cells: zymogen granules[34].	Zinc resistance. Provides zinc to the maternal milk[71].
ZnT3	Hippocampus and cerebral cortex: synaptic Ves. Pancreatic β cells[71].	Zinc homeostasis to regulate presynaptic MAPK signals. Insulin production. Prevention of Azheimer's disease, amyotrophic lateral sclerosis and Schizophrenia [71].
ZnT4	Ubiquitous: end., lys., ves., GA and trans-Golgi network. Higher expression in the brain and digestive tract[34].	Differentiation processes[34].
ZnT5	Ubiquous. High expression in pancreatic β cells[34,71].	Glucose resistance. Activation of alkaline phosphatase. Osteoblast maturation. Cardiovascular development[71]. Control of growth hormone[34].
ZnT6	GA and trans-Golgi network[34].	No zinc transport activity. Porpursed as modulator of the heterodimer ZnT5-ZnT6[34]. Prevention of Azheimer's disease, amyotrophic lateral sclerosis [71].

ZnT7	GA[34].	Activation of alkaline phosphatase. Control in the early secretory pathways. Insulin receptor substrate 2 and Akt phosphorylation in skeletal muscle cells[34].
ZnT8	Pancreatic β cells: insulin secretory ves. Pancreatic α cells[34].	Glucose-stimulated insulin secretion. Diabetes prevention[34].
ZnT9	Ubiquous: cytoplasm, nucleus[71] and ER[72].	Mutated in cerebro-renal syndrome[72]. Inverse proportion with the body mass and fat index[71].
ZnT10	Brain, liver and retina: end. or GA[34,71].	Downregulated by IL-6. Avoid cellular senescence in vascular smooth muscle cells. Possible manganese transport as primary function. Related to Parkinson and hepatic cirrhosis[34]. Prevent Alzheimer's diseas[71].
ZIP1	PM[34].	Differentiation into osteoblasts. Positively associated with growth hormone[71]. High in allergic airway inflammation[34].
ZIP2	Keratinocytes[71]. Monocytes and macrophages[34].	Keratinocytes differentiation. Suggested role in the chiasma formation[71]. Epidermis turnover[34]. Low in hepatitis B and C. Upregulated in pulmonary tuberculosis and asthma. Low in aged retinal pigment epithelial cells[71].

ZIP3	PM. Apical membrane in lactating mammary glands[34]. Embryonic brain and neurotube[71]	Possible reuptake of zinc from alveolar space. Differentiation of secretory mammary epithelial cells in response to prolactin. High in allergic airway inflammation[34]. Suggested role in the chiasma formation[71].
ZIP4	Apical membrane of the enterocytes in the small intestine, including duodenum and jejunum[34].	Dietary zinc absorption. Intestinal integrity[34].
ZIP5	Small intestine, pancreas, liver and kidney. Basolateral membrane of polarized cells[71].	Zinc excretion. Involved in zymophagy process[71].
ZIP6	PM[34].	Epithelial-mesenchymal transition (EMT)[34]. Activation of the T lymphocytes[73].
ZIP7	High rates in intestinal crypts[71]. ER and GA[34].	Proliferation and migration[74]. Glycemic control in skeletal muscle. My facilitated the processing and storage of insuline in pancreatic β cells[34].
ZIP8	Liver, kidneys, lungs and testes[71]. PM. Apical membrane of polarized cells. Lys.[34].	Regulates negatively proinflammatory responses. Activation of T lymphocytes. Embryonic organogenesis and hematopoiesis. Cadmium, manganese and iron transport[34].
ZIP9	PM and GA[34].	B-cell receptor signaling. Membrane androgen-receptor[34].

ZIP10	PM[71].	Cell migration[57] and mitosis[75]. Lymphocytes survival[34] and B-cell receptor signaling[71].
ZIP11	Testes and digestive system. Nucleus and GA[34].	Not reported.
ZIP12	Central nervous system. PM[34].	Neuronal differentiation[34]. Pulmonary vascular response in chronic hypoxia[76].
ZIP13	GA and cytoplasmic ves.[34].	Development of periodontal tissues. Protein degradation VCP-dependent ubiquitin proteasome pathway[71]. Low in spondylodysplastic Ehlers-Danlos syndrome[34]
ZIP14	PM. Apical localization in polarized cells[34].	Systemic growth. Cadmium, manganese and iron transport[34]. Gluconeogenesis.

Table 1. Compilation of zinc transportes, their locations and functions. PM: plasma membrane; ves.: vesicules; end.: endosomes; lys.: lysosomes; GA: Golgi apparatus; ER: endoplasmic reticulum.

Breast cancer and zinc regulation

Cancer is a collection of related genetic diseases. Mutations in certain genes result in uncontrolled growth with power to spread and invade other tissues. This invasion is called metastasis, the primary cause of death from cancer. The spreading potential differentiates a benign tumor from a cancerous tumor[77].

The oldest historical record about cancer dates from the 3000-2500 year B.C. In the Edwin Smith Surgical Papyrus, the writer describes a breast tumor and concludes that it was a grave disease with no treatment[78]. The term cancer comes from the greek word *karkinos,* which means crab. Hippocrates and his disciples named it this way because the cancerous growth reminded them of the movement of this animal. Greek physicians already connected cancer with age and they knew about the existence of skin, mouth, stomach and breast tumors and treated them with surgical procedures[79].

Cancer is the second most common death cause worldwide (10 million deaths in 2020[80]), especially in industrialized areas. Moreover, it leads the list of cause-specific Disability-Adjusted Life Years. This parameter takes into account not only the mortality but also the years lived with disability, therefore, cancer supposes the highest clinical, social and economic burden in terms of human diseases in current times. The concern about the future of this disease is increasing. The odds of developing cancer between 0-74 years of age is 20.2% and from 2030 this disease is expected to be the first death cause in the world[81].

Tumorigenesis is a multistep process. The transformation from a normal cell to a cancerous one is progressive and it is the result of

cumulative genetic alterations. Considering that these alterations are not always the same, cancer results in a group of diseases with different characteristics. However, in 2000, Hanahan and Weinberg established six hallmarks to define cancer[82], which were expanded into eight in 2011[83]. These hallmarks are the common capabilities that all cancers need to acquire and represent a great tool to understand the complexity of this disease. The first hallmarks defined were self-sufficiency in growth signals, insensitivity to anti-growth signals, limitless replicative potential, sustained angiogenesis, evading apoptosis and tissue invasion and metastasis[82]. Then, reprogramming energy metabolism and immune response evasion were added to the list. Moreover, tumor microenvironment and the different cancerous cell types were highlighted as factors responsible for tumor biology and its interactions[83].

Since then, much has been achieved in the understanding of cancer biology. Even though all cancers share the aforenamed features, the molecular strategies by which they obtain them are distinct. Researchers are focused on understanding the genetic alterations, mutations and changes in protein expression and receptors of different cancer subtypes. This knowledge is crucial because it could be used to establish ideal targets for each cancer subtype to be treated. Molecular targeted therapies block specific molecules needed by each particular tumor for growth, progression and metastasis. In addition, within a single tumor, there are usually different cell populations and if one of them is resistant to the therapy, cancer will survive[84].

In this sense, the effort of scientists to understand the specific molecular pathways of the different cancer subtypes, its cell

populations and the complexity of its adaptations is constant. Elucidating the biology of each tumorigenesis process has to be a priority in order to stop cancer mortality.

Breast cancer

Among all cancers, the most common in 2020 was breast cancer, with 2.26 million diagnoses around the world[80]. In 2018, the number of diagnosed breast cancers were 2.09 million[81], which indicates an increasing tendency in the incidence. In addition, breast cancer supposes the higher risk of dying from malignancy in women[81].

Nowadays it is well known that breast cancer is a heterogeneous disease at the molecular, histological and clinical level. According to the expression profile of estrogen receptor (EsR), progesterone receptor (PR) and epidermal growth factor 2 (HER2), there are four subtypes. Luminal A can be EsR positive, PT positive or both, however, it is always HER2 negative. This subtype is characterized by a low proliferation rate and the best prognosis[85]. Luminal B is EsR or PR positive, or both, usually with less PR expression than Luminal A tumors, and HER negative or positive with a high proliferation rate[85]. HER2-enriched tumors are ER and PR negative but HER2 positive. They are characterized by the highest number of mutations across the genome and a high proliferation, which makes them very aggressive. Fortunately, the HER2 overexpression is used as a target to successfully treat this cancer subtype with a monoclonal antibody[85]. Triple negative breast cancers (TNBC) -no EsR, no PR, no HER2- are the most aggressive and heterogeneous[86]. Confronting them is more complex than confronting other breast cancers because specific molecules are not identified to target this

disease[87]. In Figure 3 there is a schematic representation of breast cancer molecular subtypes and its features.

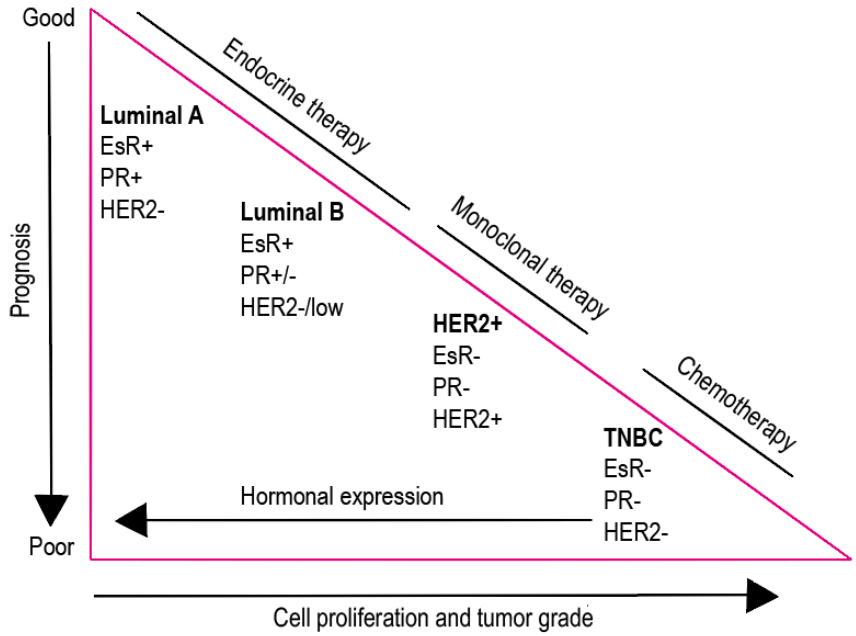

Figure 3. Schematic representation of the breast cancer subtypes according to its molecular expression. Relationship with prognosis, proliferation, tumor grade and therapy. Image inspired by[86].

Triple negative breast cancer

TNBC is a breast cancer type characterized by the lack of expression of EsR, PR and HER2+. It constitutes almost 20% of all breast cancer cases, with a special high incidence in premenopausal young women[88]. TNBCs are usually more aggressive than other breast cancer subtypes, presenting a higher oncological grade, more advanced stage at diagnosis and a lower survival time of the patients. In fact, within five years after diagnosis, the mortality rate is 40%. 46% of the patients develop distant metastasis and, after this

event, the median survival time is 13.3 month. Secondary tumors often appear in the brain and visceral organs three years after the diagnosis. After surgery, the risk of recurrence is 25% and the average time of relapse is 19-40 months, while it is 35-67 months for non-TNBC patients. In addition, the mortality rate after recurrence is 75%[89].

Due to its lack of EsR, PR and HER2, TNBC is not treatable with specific therapies used in other breast cancer subtypes that are directed against these receptors. The normal treatment involves surgery, radiotherapy and systemic chemotherapy, but the efficacy is poor[88,89]. In this scenario, it is urgent to further investigate this breast cancer type in order to find a reliable and effective therapy to treat it.

In 2011, in order to identify enriched pathways to treat pharmacologically, TNBCs were classified into six different categories according to their gene expression profile[90]:

- Basal-like 1: high expression of cell cycle and proliferation genes, low expression of DNA repair-related genes.
- Basal-like 2: high activation of growth signaling pathways, glycolysis and gluconeogenesis. It expresses myoepithelial markers.
- Mesenchymal: enrichment of cell motility and differentiation pathways. High expression of genes involved in EMT and growth factor signaling.
- Mesenchymal stem-like: enrichment of cell motility and differentiation pathways. Low proliferation and high expression of angiogenesis genes.

- Immunomodulatory: immune cell processes enriched such as cytokine signaling or antigen processing and presentation.
- Luminal androgen receptor: expression of androgen receptor gene and its downstream pathways. It has a luminal gene expression pattern.

TNBC has a great number of cells that have undergone EMT. This EMT turns cells into more migratory, invasive and dedifferentiated cells[91]. Histopathological and gene expression analyses revealed that TNBC, compared to non-TNBC, are enriched in the CD44(+)CD24(-/low) cell population[92,93]. This expression profile in breast cancer cells is the signature of cancer stem cells (CSCs)[94].

CSCs are a small population within cancer cells with self-renewal capacity and, at the same time, the ability to induce cancer intratumor heterogeneity that confers an advantage to the tumor progression. In addition, these cells can adopt a dormant phenotype in their new niches, survive quiescence and lead to new tumors even two decades after the diagnosis[95]. Therefore, CSCs are thought to be responsible for cancer progression, metastasis and tumor initiation[96]. Moreover, this intratumor heterogeneity and dormancy leads to chemoresistance and tumor recurrence[95,97,98], both characteristic aspects of TNBCs[89]. Molecular drivers associated with this process are the core transcription factors for stemness manteinance Nanog, Oct4 and Sox2[99].

Brain metastasis

Metastasis is considered to be the ultimate manifestation of cancer. This process consists in the cancer dissemination into other body parts by blood, lymph or body cavities, and the establishment of a

secondary tumor. 90% of cancer deaths are estimated to be directly related to metastasis[100]. Getting the inspiration from the work of Hanahan and Weinberg, Welch and Hurst established the hallmarks of metastasis in 2019[101]. The idea was to facilitate the understanding of this process and the assessment of its tractability. These hallmarks are: **motility and invasion, modulation of the microenvironment, plasticity and colonization**[101].

The **motility and invasion** step is essential. Cells have to migrate in order to get emancipated from the primary tumor. While getting away from the primary location, cells have to penetrate membranes to get to a secondary niche in which the new tumor will grow if the metastatic cascade arrives at its end. The EMT is usually an important part of this step. The **modulation of the microenvironment** consists of the ability of cells to restructure their new location by changing, for example, the metabolism of the surrounding areas, altering the extracellular matrix, changing the behaviour of other cells or nullifying the anti-tumor actions of the local immune system in order to survive. **Plasticity** is the capacity of a tumor to be heterogeneous, which confers a selective advantage. During the metastasis process, cells will face adversities and the redundancy of mechanisms will let them accomplish the metastatic cascade. Also, this heterogeneity is key for the cells to adapt to any of the environments that they transit or reach[101]. CSCs are thought to be responsible for inducing this heterogeneity and, thus, they are needed in the secondary niche to re-establish the heterogeneous population of tumor cells[102,103]. **Colonization** is a *sine qua non* condition for the metastasis to succeed, without this step the metastasis is just dissemination. Cells have to proliferate in the

secondary niche in order to form a macroscopic lesion while they continue manipulating the microenvironment to survive and grow[101].

In Figure 4 it is summarized the mechanism by which a TNBC cell metastasizes successfully to the brain.

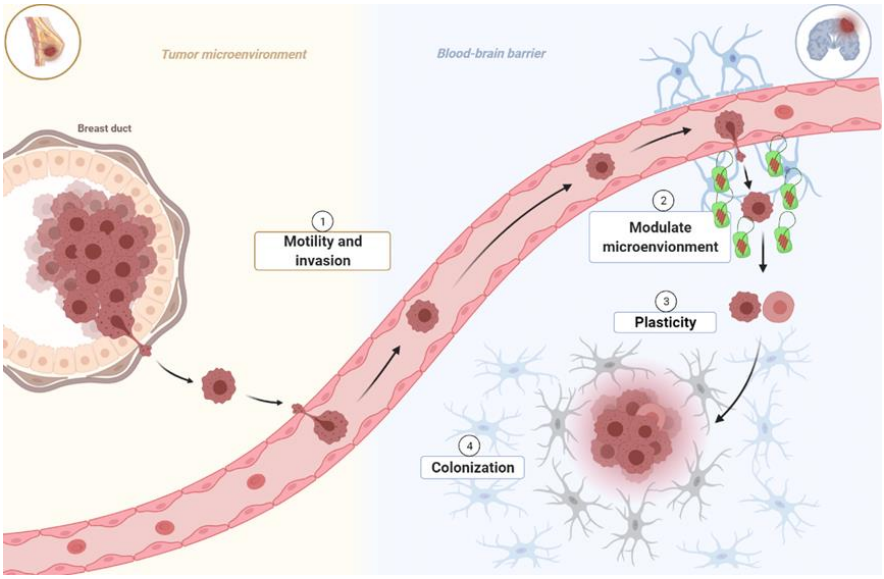

Figure 4. Sequence of hallmarks that have to present a breast metastatic cell that targets the brain in order to survive and succeed. Image created with BioRender.com.

The secondary niche election during metastasis is not random. Each primary tumor has affinities for different new environments. For example, breast cancer metastasizes often to bones, lungs, liver and brain; lung adenocarcinoma goes and colonizes the brain, bones, adrenal gland and liver; skin melanoma has affinity for the lungs, brain, skin and liver[104]. Site-specific metastases are the result of the gene expression patterns of cancer cells and how the consequent phenotype interacts with particular host-tissues, determining the organ-specificity. Tumor cells, then, have to adapt their expression

programs to their host environment. Cells from different primary tumor subtypes will adapt differently to metastasis, expressing different gene sets, thus, they will have different affinity for the secondary niches[105].

Brain metastases are the most common brain tumors. The prevalence, according to epidemiologic data, is 8.5-9.6% between the cancer patients, but it is estimated to be higher. In addition, as people suffering from malignancies tend to live longer than before due to the medical advances, it is believed that the incidence of brain metastasis is increasing[106]. People that develop this disease have an estimated survival time of less than one year[107]. Breast cancer is the second most common cause of brain metastasis, just after lung cancer, contributing with 15% of total cases[108]. Lung cancers develop brain metastases quickly, some months after the diagnosis, while breast cancers achieve brain metastases after a long period of remission, suggesting different pathways[109]. Up to 30% of patients with invasive breast cancer will develop brain metastasis, according to autopsy studies[110]. Not all molecular breast cancer subtypes metastasize to the brain with the same frequency because, just as explained, this affinity will depend on the gene expression profile of the cells[105]. 50% of the cases come from HER2+ tumors, while up to 40% are TNBC[108], in which case brain colonization is faster[111]. The HER2+ brain metastases are treated with specific treatments developed against HER2+ breast cancers[108] and their overall survival is 16.5 months[111]. Patients with TNBC brain metastasis have the worst prognosis due to the lack of specific treatment, with an overall survival of only 4.9 months[111].

In 2009, Bos and colleagues performed a gene expression analysis in order to identify the genes involved in the breast cancer

metastasis to the brain[109]. They identified some important extravasation genes necessary for the brain and lung brain metastases, such as COX2 or EGFR ligands. As a specific mediator from breast to brain they found ST6GALNAC5, whose expression is usually restricted to the brain and enhances cell adhesion to brain endothelial cells. Interestingly, they also found the SerpinB2 to be upregulated in the cells that specifically have metastasized to the brain[109]. Later, SerpinB2 protein was demonstrated to be key for the survival of the metastatic cell in the brain. When a tumor cell extravasates from the blood vessels to the brain matrix, it has to face its defences. Astrocytes become reactive when they detect invasive cells and start producing plasminogen activator (PA). Neurons produce plasminogen that is turned into plasmin thanks to the PA activity. This plasmin cleaves FasL, another protein which is highly expressed by astrocytes. Then sFasL is released and binds the Fas receptor of other cells, activating apoptosis. At the same time, plasmin also cleaves the adhesion protein L1CAM, inhibiting the cell adhesion capacity. In this hostile context, SerpinB2 is able to inhibit the PA and, this way, the cancer cell expressing this protein will avoid the brain defences[112], as shown in Figure 5.

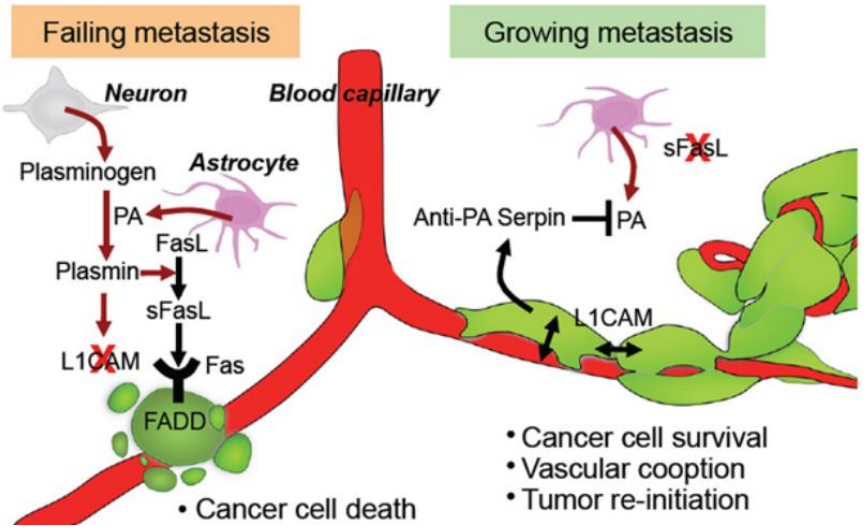

Figure 5. Model of the SerpinB2 action against brain defences. Extracted from[112].

Interestingly, SerpinB2 has recently been proposed to be an indicator of CSCs tumorigenicity in several cancer types, including TNBC[113]. This points out not only a microenvironment modulation role during breast cancer metastasis to the brain, but also an important role in plasticity and colonization.

Altogether, it is clear the need for further investigation of specific pathways by which each primary tumor develops the specificity for the secondary niche. This way, the success of the metastasis and the tragic outcome for the patient could be avoided. This work is focused on elucidating how TNBC develops brain tropisme unrevealing the role of zinc and its homeostasis machinery all along this journey. The idea rises from the new findings about the zinc role in malignancy development and aggressiveness that will be detailed in the following section.

Zinc in cancer progression

Zinc homeostasis is altered in cancer patients. Thus, a lower serum zinc concentration has been reported in patients suffering from gallbladder, prostate, endometrial, lung and breast cancer[114–118]. Interestingly, tumor prostate tissue has a lowered zinc content[115] while tumor breast cancer tissue displays increased zinc levels[118], which reveals that the alterations are tissue-specific. In this sense, zinc dyshomeostasis seems to be related with tumorigenesis. As mentioned, zinc regulates proliferation[52–54], migration[55–57], differentiation[58–60] and survival[61] in healthy cells, the same processes altered in cancer[83]. Given that zinc transporters are the gatekeepers of zinc homeostasis, the reported zinc dysregulation in cancer is largely due to their aberrant expression, especially from ZIPs. In Table 2, there is an overview of the alterations in the expression associated with the progression of different tumors. MT expression has also been found to be altered in some cancers. They are upregulated in breast, ovarian, nasopharyngeal and urinary bladder cancers, while downregulated in hepatocellular, prostate and papillary thyroid cancers[119].

Cancer	Transporter involved
Bladder	ZIP11[120], ↑ZnT1[121].
Breast	↑ZIP6[122], ↑ZIP7[123], ZIP9[124], ↑ZIP10[125], ↑ZnT2[126].
Cervix	↑ZIP7[127].
Colorectal	↑ZIP7[128].
ESCC	↑ZIP5[129], ↑ZIP6[130].
Hepatocellular	↑ZIP4[131], ↓ZIP14[132]/↑ZIP14[133], ↑ZnT9[133].
Kidney	↓ZIP1[134], ↑ZIP10[135].
Lung	↑ZIP4[136].
NPC	↑ZIP4[137].
OSCC	↑ZIP4[136].
Ovary	↑ZIP4[138].
Pancreas	↓ZIP1[139], ↓ZIP2[139], ↓ZIP3[139], ↑ZIP4[140].
Prostate	↓ZIP1[141], ↓ZIP2[142], ↓ZIP3[142], ↓ZIP4[143], ZIP9[124], ↓ZnT1[144,145], ↓ZnT4[146,147].

Table 2. Summary of the alterations in the expression of zinc transporters reported in different cancer subtypes.

ZIP4 is one of the transporters whose overexpression has been frequently associated with cancer progression. In pancreatic cancer, it was revealed to increase cell proliferation and invasion[140,148]. Its upregulation has also been involved with apoptosis resistance[149]. In addition, ZIP4 increases ZEB1 expression, an EMT transcription factor, and downregulates ZO-1 and claudin-1, two tight junction proteins, promoting metastasis through migration and invasion[68]. Recently, ZIP4 and ZEB1 overexpression have also been related to chemotherapeutic resistance because they reduce the expression of ENT1, the transporter involved in the drug uptake[150]. Moreover, ZIP4 has been identified as a stem cell marker in ovarian cancer with high grade[138].

Focusing on breast cancer patients, it has been described a decrease in their serum zinc concentration together with an increase in the tumor tissue[118]. Besides, zinc concentration correlates with the histological malignancy grade, thus, it has been proposed as a biomarker in this cancer type[151,152]. The underlying zinc transporter network dysregulation seems to be cancer subtype dependent. ZIP6 is associated with EsR positive tumors and it has been proposed as a marker for Luminal A breast cancers[122]. STAT3 has been shown to induce ZIP6 expression leading to a zinc accumulation, Snail activation and E-cadherin downregulation. As a consequence, cell migration and metastasis is promoted[153]. In a similar manner, an overexpression of ZIP10 in invasive and metastatic breast cancer cell lines has also been reported[125]. In addition, ZIP10 has been identified to form a heterodimer with ZIP6 and both stimulate the cell motility and EMT by downregulating the E-cadherin expression[57]. More recently, this same heteromer has been demonstrated to play a key role in the mitosis of breast cancer cells[75]. ZnT2 has been

reported to be overexpressed in luminal tumors but not in the triple negative ones, so it has been proposed as a key mechanism to protect cells from zinc accumulation and its contribution to an invasive phenotype[126].

Drug resistance has also been linked to zinc transporter alterations in breast cancer cells. It has been demonstrated that tamoxifen-resistant EsR positive cells have an increased zinc content and overexpress ZIP7[123]. The increased cytosolic zinc content is triggered upon ZIP7 phosphorylation by CK2[154]. This results in the activation of tyrosine kinase pathways such as EGFR, IGF-1R and Src, which mediate tumor growth[123]; or MAPK, mTOE and PI3K-AKT involved in cell survival and proliferation[74]. Other zinc homeostasis players associated with breast cancer chemoresistance are ZIP9, that mediates the apoptosis testosterone-dependent cascade through zinc signaling[124], and MTs, upregulated in invasive breast cancer[155].

Infectious disease: zinc implications and COVID-19

Infection is the result of the entrance and multiplication of pathogenic organisms into the body. When an infection becomes an illness, it is called infectious disease. The causing organisms are frequently bacteria or viruses, but they can also be protozoans, funghi, prions... Infectious diseases are transmissible directly from an infected person, animal or reservoir, or indirectly through intermediate hosts, vectors or microenvironments[156]. The immune system of the host is the defence mechanism that fights infections by means of two sequential and interconnected responses: innate and adaptive[157].

Until a good understanding of infectious diseases was achieved during the 19th century, they were the most serious health problem wordwide[156] causing a life expectancy in 1800 lower than 40 years old[158]. Infectious diseases are thought to have determined the course of human civilization, influencing political, social and theological opinion of different societies as well as the economic and military fields[159,160]. Especially in the current situation, it is not difficult to empathize with the fear, insecurity and panic that people have felt due to the lack of understanding and unpredictability of infectious diseases.

The process of learning about infectious diseases has been long. Hippocrates, back in ancient Greece, revealed a link between the environment and diseases from people living in it[161]. In 1530, the Italian physician Giloramo Fracastoro, proposed the idea of transmission or contagion of syphilis by direct contact for the first time. Later, he also described the possibility of disease transmission via indirect contact or even by air[162]. Around 100 years before understanding how infectious diseases originate, a huge therapeutic

advance was achieved: in 1796 Edward Jenner developed smallpox vaccines using observational approaches. He did not know, at that time, that he was using the immunological response of the hosts to protect them[163]. In 1854, John Snow pointed at water as the transmission source for cholera and helped stop an outbreak[164]. Finally, the germ theory was born around 1870 with the parallel works of Pasteur and Koch, who confirmed that infectious diseases were caused by living microscopic agents[165,166]. Another big step in the fight against infectious diseases was the discovery of antimicrobial molecules by Paul Enrich in 1909[167]. In 1928, Alexander Fleming discovered penicillin, the first antibiotic widely used in medicine[168].

Infectious diseases can occur in different ways regarding the human populations they affect. The endemic form is when it happens at an expected frequency in a certain location during a certain period of time. The epidemic way is when the occurrence of the disease is higher than expected in a certain region. If the epidemic affects large populations in different countries, it is called pandemic[156].

Pandemics are especially dangerous because they can substantially increase morbidity and mortality in a huge area[169]. For these same reasons they are also especially frightening. There are records about pandemics that have occurred throughout human history (Fig. 6). Pandemics are not only a health issue, they result in economic, social and political damages[169]. In a communication of 11th March 2020, the WHO director declared that the coronavirus disease (COVID-19) could be characterized as a pandemic[170]. It is a priority to find a way to control this disease because it continues to threaten the life of patients, healthcare systems, economics and whole societies around the world.

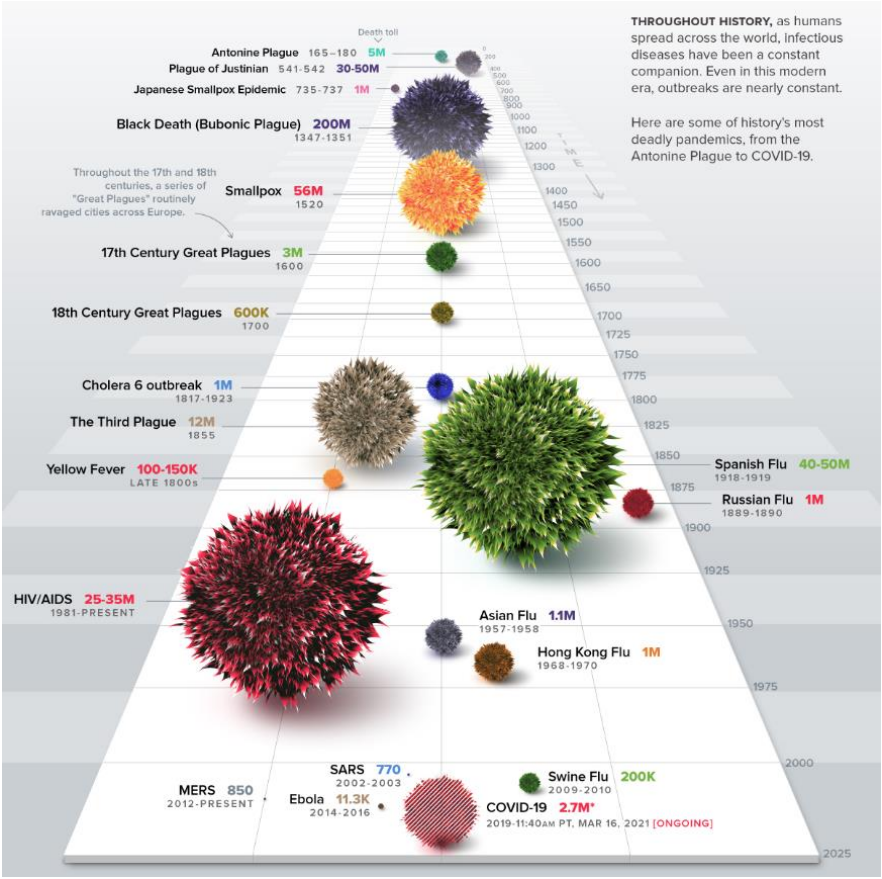

Figure 6. Timeline of pandemics during human history and number of deaths they caused. Adapted from[171].

The actualization made by the WHO on 31th May 2021 indicated that there have been 170,051,718 confirmed cases of COVID-19 worldwide, including 3,540,437 confirmed deaths[172]. This disease is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV2), which emerged at the end of 2019 in Wuhan, China[173,174]. The genome of this virus consists of a single RNA positive-strand of nearly 30 Kb. It has a spike protein, like the rest of coronaviruses, that binds the host receptor Angiotensin II Converting Enzyme (ACE2), and that is recognized by the immune system[175].

The approximate incubation time of this virus before presenting symptoms is 5.2 days[176]. From that moment to the death of the patient are stimate to pass between 6-41 days, depending on the age and medical status[177]. The clinical manifestations of COVID-19 range between no symptoms to mild, moderate or severe infections with respiratory failure[175,178]. It has not been completely elucidated why there are such huge differences between patients, therefore, it is urgent to identify the risk factors.

The SARS-CoV2 infection can be very severe for two things. First, this virus uses the highly expressed ACE2 receptor to infect the cells, which leads to a systemic infection. Second, and more decisive for the outcome, the host immune system reaction. That reaction can range between protective and dysregulated[175], which can result in the cytokine storm syndrome. This is an inflammatory syndrome proposed as the ultimate cause for the severe symptoms and fatality of COVID-19[179,180]. In this context, the urgency to find tools to control the immune system response is evident.

Zinc in an essential trace element, between other purposes, because its homeostasis is a *sine qua non* requirement for the proper functioning of the immune system[28,181]. In addition, there is evidence of the higher recurrence of infectious diseases between populations suffering from zinc deficiency[28]. Moreover, zinc has been demonstrated to have a direct antiviral activity against some virus, including coronaviruses[30,182,183]. Altogether, these facts made this thesis focus on the potential role of zinc in the fight against the COVID-19 pandemic at both levels.

Immune system defence and impact of zinc

The immune system is a network of organs, tissues, cells and molecules that interact and carry out biological processes in order to defend the body against external (such as infections) or internal (such as cancer) threats. The firsts to identify immune system elements were Elie Metchnikoff and Paul Ehrlich, both sharing the Physiology or Medicine Nobel Prize in 1908. Metchnikoff discovered phagocytes and phagocytosis, while Ehrlich described the side-chain theory (lock-and-key principle) of antibody formation. They are considered, respectively, the father of innate and adaptive immunity[184].

The innate immune system is the first line of defence, recognizing the pathogens and initiating the immune response immediately. It is mainly conformed by polymorphonuclear cells (PMNs), monocytes and macrophages. Innate immune cells release cytokines and chemokines to communicate with other innate and adaptive immune cells and, thus, coordinate the whole system. Cytokines can be pro-inflammatory or anti-inflammatory, both necessary for a balanced immune response. Dendritic cells are in between the innate and adaptive responses, helping with pathogen recognition. The adaptive immune system is based on lymphocytes T and B. These cells have receptors that recognise antigens in a very specific way. When this recognition happens, there is a selective expansion of the activated clones, resulting in a high number of cells that specifically target an antigen. B cells produce the antibodies while T cells are responsible for the cell-mediated immune response. T lymphocytes can be divided in two groups: T helpers and regulators (CD4+) and T cytotoxic (CD8+). Some of these cells remain after the infection as

memory cells, ready to act faster than the first time if it occurs again[185,186].

Zinc is an essential player in the proper functioning of both, innate and adaptive responses, due to its signaling role modulating specific enzymes, signalling cascades and transcription factors. This capacity can evoque the complexity of the effects of zinc in the immune system[187].

Zinc affects the innate immune system in many steps. PMNs, the first cells to arrive at the source of the infection, phagocyte and kill the threat generating reactive oxygen species (ROS)[185]. Zinc is a negative regulator of NADPH oxidase[188]. Therefore, zinc deficiency results in an increase of ROS production by PMNs[189]. Besides, zinc deficiency has also been reported to impair phagocytosis[185]. Importantly zinc regulates dendritic cell maturation since ZIP6 and ZIP10 are suppressed and some ZnTs are upregulated during this essential process for the activation of the adaptive response[190].

T-cells are very vulnerable to zinc deficiency. On the one hand, developmentally, this deficiency causes a thymic atrophy and T-cell lymphopenia. On the other hand, without zinc, T-cells cannot mature and be operative[191]. In this sense, it has been proven that ZIP6 is essential for T-cell activation[73]. In addition, zinc deficiency implies a dysregulation in the different T-cell subtypes, leading to a less effective cell-mediated response[192–194]. Zinc deficiency also causes a reduction in B-cells and, thus, it affects antibody production[186].

The nuclear factor NF-κB signaling pathway is one of the most important inflammatory pathways. It promotes the expression of pro-inflammatory cytokines, like IL-1β, IL-6 and tumor necrosis factor (TNF)-α. Zinc inhibits NF-κB transcription factor[195,196]. As a

consequence, zinc deficiency provokes a systemic increased NF-κB activation, leading to an increase in inflammation[197]. This state of high inflammation is similar to the provoked by the coronavirus antiviral response, which produces the cytokine storm[179,180].

In summary, individuals with zinc deficiency suffer from an increase in infection susceptibility, an impairment of the innate response, thymic atrophy, lymphopenia, a dysregulation of the adaptive response, an increase in the inflammation and, eventually, an increased risk of death[28,181,191,198].

Zinc and virus interplay

In view of the importance of zinc for life, it is not surprising to find out that zinc is as well an integral component of viral proteins like enzymes, proteases and polymerases. This opens the possibility of modulating viral replication by changing zinc homeostasis[183]. In fact, zinc is used as an antibacterial and an antifungal weapon by the host[199,200]. However, zinc-mediated responses against viruses are more complex because changes in intracellular zinc might affect both the virus and the host cell[183].

Virus influence on zinc homeostasis

There is evidence demonstrating that viruses can change zinc homeostasis to their favor. Papilloma viruses are known to alter intracellular zinc homeostasis in order to enhance their replication and persistence[201]. Thus, the viral E5 protein interacts with ZnT1 and inhibits the export of zinc from the nucleus. The subsequent intranuclear zinc accumulation activates the AP1 transcription factor, which is needed for the genome expression of these viruses[69].

Moreover, it is also established that MTs are induced by some viruses such as the coxsackievirus[202], measles virus[203], influenza virus[203,204], human immunodeficiency virus (HIV)[205] or hepatitis C virus (HCV)[206]. The mechanisms by which these viruses induce MTs remain unclear, but it is thought that they are needed to influx or redistribute zinc[207,208].

In HIV-infected monocytes, MT1 expression and intracellular zinc levels are significantly increased[205]. Both phenomena are thought to inhibit monocyte apoptosis[209,210] and, this way, they help the HIV to preserve its reservoir-cell. Moreover, zinc deficiency is common in HIV patients suffering from symptoms[211–213]. Zinc and MTs enhance NF-κB binding to the human cytomegalovirus (HCMV) promoter, which, triggers viral replication[214,215]. In this case, MTs are proposed to be zinc donors for NF-κB binding[183]. In HCV infection, knocking-down MT1 and MT2 increases both viral replication and cellular zinc content. The antiviral action of MTs is hypothesized to take place either because they sequester the zinc needed for the proper functioning of viral metalloproteins[216] or because they act as zinc chaperons to facilitate the antiviral signaling[183]. MTs have also been reported to have antiviral effects against flaviviruses and the alphavirus Venezuelan equine encephalitis but not against other viruses like the West Nile and Chikungunya[217]. These data suggest that the antiviral role of the MTs is virus-dependent, thus it will rely on the viral zinc requirements for its replication.

Zinc influence in viral life cycle

In physiological conditions zinc does not appear to have any antiviral action due to its low concentration. However, it has been demonstrated that at mM concentrations zinc can induce antiviral

effects in a virus-specific way[183]. Therefore, zinc supplementation has been postulated as a potential therapeutic tool. Zinc effects in different virus types are listed hereunder:

- Herpesvirus: several *in vitro* studies claim that zinc inhibits herpesvirus 1 and 2 viral polymerase function[218], its protein production[219] and that it inactivates the free virus[220,221]. *In vivo* studies also demonstrate a significant reduction in the duration and recurrence of infections caused by the herpesviruses when they are treated with topical zinc[222–225]. In the Varicella-Zoster virus, zinc ions are also able to inactivate the free virus[226].

- HCV: treatments with 100 µM $ZnSO_4$ showed a 50% reduction in its viral replication[206,227]. When HCV infections become chronic, the plasma zinc concentration of the patients is significantly reduced[228]. Zinc supplementation in these cases reduces hepatic inflammation markers and improves the rate of viral clearance[229–231].

- Togavirus: these viruses infect by receptor-mediated endocytosis. The fusion of membranes promotes the virus release into the cytosol[232]. It has been reported that, *in vitro,* zinc ions inhibit membrane fusion[233–235] by binding to an essential viral protein when the pH of the endosome is low[235].

- Retrovirus: zinc inhibits their retrotranscriptase (RT) antivity[236,237]. In the case of HIV, zinc displaces manganese and forms a very stable complex with less activity[238]. Zinc also has been shown to inhibit its protease[239] and the viral transcription[240]. Zinc supplementation may increase blood zinc concentration and CD4+ T count in HIV patients[241].

- Papilomavirus (HPV): these viruses are oncogenic due to their oncoproteins E6 and E7, that degrade the p53 and pRb tumosupressors. Exogenous zinc treatment results in the inhibition of these E6 and E7 proteins forcing the carcinoma cells to undergo apoptosis[242]. These viruses can cause warts and individuals suffering from them are often zinc deficient[243]. Zinc supplementation has been found to be the most effective systemic treatment against these persistent warts[244].

- Picornavirus: zinc has a clear inhibitory effect on picornavirus polyprotein processing[245–250]. Rhinovirus is one of the viruses in this family and one of the causes for the common colds. Clinical studies using zinc supplementation in these cases revealed a correlation between the zinc levels at the infection site and a better prognosis[251]. In studies where high doses of zinc were used, a 42% reduction in colds was achieved[252].

- Influenza virus: its *in vitro* replication is significantly inhibited after adding the zinc ionophore pyrrolidine dithiocarbamate[253].

- Respiratory syncytial virus: zinc salts inhibit viral replication, even when the incubation of the cells with zinc was performed before the infection[254].

- Coronavirus: before the pandemic, in 2010, *in vitro* assays established that treating with zinc SARS-CoV infected cells inhibited the RNA polymerase binding to its template. At the same time, treatment of the infection with pyrithione, a zinc ionophore, resulted in the blockage of the viral replication[182].

Role of the zinc during the SARS-CoV2 pandemic

Recapitulating, the host immune system reaction defines the progress of COVID-19. Research to reduce the inflammation and cytokine production is a priority in the scientific community. Zinc has an anti-inflammatory role and an antiviral effect, which makes it a good candidate for treating the SARS-CoV2 infection. As such, clinical trials focused on zinc treatment have been developed[255].

As of the 31st of May 2021, there are 47 different studies registered in the clinical trials website of the National Institutes of Health matching with zinc and COVID-19. 12 have been completed but only three have published results. None uses direct zinc supplementation[256]. However, two retrospective studies (there are studies based on information already produced) using zinc supplementation have revealed its benefits. The first evidence was given by Carlucci et al in October 2020. In this study, patients were treated with zinc sulfate, hydroxychloroquine (HCQ) and azithromycin or just HCQ and azitromycine. A decrease in the mortality of the patients was found using the triple treatment[257]. Two months later, a second retrospective study was published supporting the benefits of the same therapy[258]. The use of azithromycin was based on its potential role in the virus elimination[258]. HCQ was used as a safer analogue for chloroquine (CQ), which was already proven effective against SARS-CoV2 *in vitro*[259]. Recently, a multicenter cohort study in which HCQ and zinc were also used finished. Its results have not officially been published, however, in the preprint, they reported a 24% of death reduction with the double treatment but no improvements using zinc supplementation or HCQ alone[260]. The idea of using these drugs originated from the defined CQ zinc ionophore activity[261]. Interestingly, one of the published clinical trials

employed only hydroxychloroquine to treat the patients and showed no benefits[262]. More recently, two studies proving zinc supplementation effects in COVID-19 patients were published. First, a retrospective study saw no differences between individuals treated and no tretaed. However, given its retrospective nature, the trial duration, and its sample size, authors encouraged further investigations in this direction[263]. Then, a randomized clinical trial performed in ambulatory patients, that does not appear in the NIH database[256], concluded that zinc supplementation did not significantly decrease the duration of symptoms[264].

Further investigations have to be performed to properly establish the potential benefits of zinc supplementation and to deeply comprehend the mechanisms involved. Soon, new insights will be revealed from the ongoing clinical trials that will lead the next steps in the rush to overcome this pandemic.

Objectives

Zinc has recently emerged as an important player in cancer progression. However, cancer cannot be approached as a single disease because it is as diverse as the wide variety of cell types in our body. Therefore, in this work we focus on a specific type of cancer, triple negative breast cancer (TNBC), on one of its worst stages, the brain metastasis. The main objective of this thesis is to study the involvement of zinc homeostasis in the brain metastasis of TNBC. The specific objectives are:

- To characterize zinc homeostasis in a brain metastasis cellular model of TNBC.
- To study the impact of cellular zinc content in the hallmarks of the TNBC metastasis: motility and invasion, modulation of the microenvironment, plasticity and colonization.

During the course of the present thesis, the world suffered the COVID-19 outbreak. Considering the existing literature associating zinc nutritional status and infection, we decided immediately to contribute searching for therapeutic strategies against SARS-CoV-2. Therefore, the general objective of this thesis is complemented with the additional:

- To evaluate the potential therapeutic role of zinc supplementation against SARS-CoV2 infection *in vitro* and *in vivo*.

Results

Chapter One

Zinc favours triple negative breast cancer's microenvironment modulation and cell plasticity

Marina Vogel-González, Dunia Musa-Afaneh, Miguel A. Valverde and Rubén Vicente

Work in progress.

ABSTRACT

Triple negative breast cancer (TNBC), a cancer subtype without specific treatment, tends to metastasize to the brain, a step that worsens the patient's prognosis. The specific hallmarks that determine a successful metastasis are: motility and invasion, microenvironment modulation, plasticity and colonization. Zinc, an essential trace element, has been shown to be involved in all these processes, both in healthy and cancerous cells. Several connections between zinc dyshomeostasis and breast cancer malignancy have been already established including migration, proliferation and drug resistance. In this work, we focus our attention on the potential role of zinc during the TNBC metastasis. We have used MDA-MB-BrM2 (BrM2) cells, a brain metastasis model derived from the parental TNBC cell line MDA-MB-231. We discovered that BrM2 doubled MDA-MB-231 cells' zinc content. Exploring the different metastatic hallmarks, we found that zinc concentration is especially important in the microenvironment modulation of the brain metastatic cells, as it enhances the expression of the SerpinB2, an essential protein in the tumor escape from brain defences. Moreover, we show that zinc modulates the breast cancer stem cells tumorigenicity capacity and increases the number of these cells within the breast cancer population. In addition, causing a disturbance in MDA-MB-231 zinc

homeostasis by overexpressing the ZIP4 transporter, we were able to increase tumorigenicity. Nevertheless, this strategy did not recapitulate completely the BrM2 metastatic phenotype. Altogether, our work suggests that zinc signaling might play an important role in the transformative steps that tumoral cells acquire to increase tumorigenic potential and niche specificity.

INTRODUCTION

Triple negative breast cancer (TNBC) accounts for almost 20% of breast cancers and tends to be more aggressive than non-TNBCs[1]. TNBC has an increased risk for early metastasis and lower 5-year survivals than other breast cancers[2]. Given that TNBC lacks the expression of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), it cannot be targeted by specific treatments. For this reason, TNBC tumors have a higher rate of distant recurrence[3]. Breast cancer is the second cause of brain metastasis, with TNBC responsible for around 40% of the cases[4] and the worst prognosis with an overall survival of 4.9 month[5].

Metastasis is considered the ultimate manifestation of cancer. Cells, after acquiring all the characteristics needed to be tumorous, have to present the so-called hallmarks of metastasis: motility and invasion, microenvironment modulation, plasticity and colonization[6]. The motility and invasion step is essential for the cells to arrive at the secondary niche, where the metastasis is going to be developed. Once there, it is crucial for the cells to modulate the microenvironment so they can survive and progress in their new niche[7]. In the brain metastasis, overexpression and secretion by cancer cells of a molecule called SerpinB2 has been reported to play

a key role in the tumor escape from the brain defences[8]. Tumor plasticity is also needed for generating the heterogeneity responsible for the survival and the progression. Cancer stem cells (CSCs) provide this plasticity. CSCs are a small population of dormant dedifferentiated cancer cells with the capacities of self-renewal and re-establish a heterogeneous population of tumor cells, guaranteeing the tumor diversity[9,10]. In addition, CSCs are resistant to therapy and their presence leads to relapses[11], which are known characteristics of the TNBC. Finally, to achieve the colonization of the secondary niche, the tumor has to proliferate *in situ*, thanks to the interaction between the microenvironment and cells derived from these CSCs[6].

Zinc is the second most abundant trace element in the human body. Its homeostasis is essential, playing key roles as a structural, catalytic and signaling element[12]. This way, zinc is involved in different cell processes such as proliferation[13–15], migration[16–18], differentiation[19–21] and survival[22] in different cell types. Zinc dysregulation has been reported to appear in several cancers, in both serum concentration and tumor content[23–25]. This scenario is caused by alterations in the most important proteins that maintain zinc homeostasis: zinc transporters. Thus, the modificacion of the physiological expression of certain ZIPs and some ZnTs transporters has been linked to the progression of different kinds of cancers such as ZIP1 downregulation to postratic cancer or ZIP4 upregulation to pancreatic cancer[26].

In breast cancer, it has been reported that patients have a lowered zinc concentration in the serum and an abnormal elevated zinc content in the tumors[27]. Besides, zinc concentration correlates with the histological malignancy grade, thus, it has been proposed as a

biomarker for breast cancer[28]. Imbalances in the expression of some ZIPs have been proven to be involved in different ways with the progression and aggressiveness of this condition. ZIP6 appears downregulated in high-grade primary estrogen-receptor positive tumors[29,30]. ZIP10 has been associated with the invasion and metastasis[31]. Both, ZIP6 and ZIP10, increase cell migration and help with the epithelial–mesenchymal transition (EMT)[18,32]. ZIP7 has also been shown to be high in estrogen-receptor positive breast cancer tamoxifen-resistant[33].

Although it is now well established that zinc imbalance is involved in the tumorous process in many cancers, including breast cancer, studying its role in specific models is still crucial due to the heterogeneity of this condition. In this context, our work focuses on studying the role of zinc homeostasis in TNBC metastasis. For this purpose, we use MDA-MB-231 cells as the model of TNBC and MDA-MB-231-BrM2 cells (BrM2 for short) as the model of TNBC that specifically metastasized to the brain.

We have characterized the impact of zinc content on the different hallmarks of the metastasis in both cell lines. Our results show that brain metastatic breast cancer cells acquire an abnormal high zinc concentration. This phenomenon favors CSCs tumorigenicity and microenvironment modulation of their new niche.

MATERIAL AND METHODS

Cell culture

MDA-MB-231-BrM2 cells were kindly provided by Joan Massagué, Memorial Sloan Kettering Cancer Center, NY, NY. BrM2 and the MDA-MB-231 parental cell line were grown in DMEM supplemented

with 10% FBS, 1 % of L-Glutamine and 1% of penicillin and streptomycin at 37°C in a humidified 5% CO_2 atmosphere. When indicated, FBS was incubated according to the manufacturer's instructions with Chelex 100 resin (Bio-Rad Laboratories) to generate Zn^{2+}-free growth medium. $ZnSO_4$ was added as needed to the final medium to generate specific Zn^{2+} concentration conditions.

The MDA-MB-231 constitutively overexpressing ZIP4 cell line was generated using a pMSCVpuro-hZIP4 plasmid and selected with 0.25 µg/ml of puromycin in the growth medium. The selection was removed to carry out the experiments.

Zinc measurements

Cells were seeded and grown in 24-well plates until reaching 80% of confluence. Cells were incubated with 25 µM of Zinquin (Sigma-Aldrich) for 30 minutes at 37ºC (5% CO_2) in an isotonic solution containing (in millimoles) 140 NaCl, 5 KCl, 1.2 $CaCl_2$, 0.5 $MgCl_2$, 5 glucose, and 10 Hepes (300 milliosmoles/liter, pH 7.4) plus different concentrations of zinc. Cells were then dissociated with Trypsin 0.05% in 0.53 mM EDTA and were washed with PBS. Fluorescence was quantified using a LSRII flow cytometer. Further analysis was performed using Flowing Software (Perttu Terho).

Cell viability and proliferation assays

Cells were exposed to different zinc for 48h, reaching 80% of confluence in 24-well plates. Then, MTT reagent was added to obtain a final concentration of 0.5 mg/ml. Cells were incubated 2-3 h at 37ºC. After that, supernatant was removed and cells were resuspended in 100 µl of DMSO. The absorbance was read at 590 nm.

In order to study cell proliferation, 5,000 cells per well in 12-well plates were seeded. Cells were counted at 3, 5, and 7 days with a Neubauer chamber.

Real-Time RT PCR

Cells were seeded and grown in 6-well plates until reaching 80% of confluence. When mentioned, we incubated the cells at the time and zinc concentrations indicated. Total RNA from cells seeded in 6-well plates was extracted using NucleoSpin RNA isolation kit (Macherey-Nagel). RNA was measured using the NanoDrop 1000 spectrophotometer (Thermo Scientific). cDNA generation was conducted using the SuperScript Reverse Transcriptase system (Invitrogen). Quantitative PCR was performed using SYBR Green (Applied Biosystems)in the QuantStudio 12K (Applied Biosystems). Primers are listed in Table 1.

Primers	Forward sequence (5'→3')	Reverse sequence (3'→5')
ZIP1	GATTGGGGAAGACACTTGACTGCT	GAAAGAGGAAGGGGATTTGTTTGG
ZIP2	CCCTTGTCCTCTTGCTGTCACTCT	AGCTCCCGTGGAAGAATTTCTAGG
ZIP3	GTGGAGATATGAGGACCCCCTGTT	GATGAACTCAGCGCTAACCGATCT
ZIP4	AGACTGAGCCCAGAGTTGAGGCTA	TGTCGCAGAGTGCTACGTAGAGGA
ZIP5	GAGCAGGAGCAGAACCATTACCTG	CAATGAGTGGTCCAGCAACAGAAG
ZIP6	CATAGCCATGAAGAACCAGCAATG	GAGAATCAAAGTGGGAGGGCTCTT
ZIP7	ACTGAAGGAGGAGCAGTGGACAGT	AGGCCCTAATGCCAAAGTAACCAT
ZIP8	CCTCGGATTGATTTTGACTCCACT	AGCAGGATTTGCATAGCATGTCAC
ZIP9	GCCTAAAGAACTGGAAAGCCCACT	GTGTTTCACTTGCTTGGTGGTGTT
ZIP10	TAGCCGTCTTCTGTCATGACTGC	TCATAGAGGGCAATCACCAGCATA

ZIP11	TCTCCTAAGCATTTTGGTGGCCTA	TCTCTTCTTTCCACAGGGCTCACT
ZIP12	CAACCACTCAAGAAGCCTCATCAA	AAGTACTGCCTGGTGAAAGCCAAG
ZIP13	AAGAAGATCGGGCTCCTGACAAC	GAGAACAGCACCATTACCACGATG
ZIP14	CATTTGGTTTCAACCCTCTGGAAG	TTTCAGCCAGTAGCAAGCACTCTG
SerpinB2	GTTCATGCAGCAGATCCAGA	CGCAGACTTCTCACCAAACA
MT1	ATCTGCAAAGGGACGTCAGA	ACGGGTCAGGGTTGTACAAA
MT2	TCCTGCAAGAAAAGCTGCTG	TCTTTACATCTGGGAGCGGG
MT3	ACACACAGTCCTTGGCACAC	AAGTGCGAGGGATGCAAAT′.
MT2	GTGTCTGCATGTCTGGAGGA	TCTGAGCCTCCTTTGCAGAT
ZEB1	GATGATGAATGCGAGTCAGATGC	ACAGCAGTGTCTTGTTGTTGT
ZEB1-AS1	CCGTGGGCACTGCTGAAT	CTGCTGGCAAGCGGAACT
Oct4	ACATCAAAGCTCTGCAGAAAGAACT	CTGAATACCTTCCCAAATAGAACCC
Sox2	AAATGGGAGGGGTGCAAAAGAGGAG	CAGCTGTCATTTGCTGTGGGTGATG
Nanog	ACATGCAACCTGAAGACGTGTG	CATGGAAACCAGAACACGTGG
GAPDH	GGAGTCCACTGGCGTCTTC	TGGCTCCCCCCTGCAAATG

Table 1. List of used primers.

Relative mRNA abundance was calculated using the ΔΔCT method and plotted as indicated.

Western blotting

Cells were seeded in 6-well plates. When mentioned, we incubated the cells at the time and at the zinc concentrations indicated. Lysis was done with 30 µl of lysis buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40 and EDTA-free protease inhibition cocktail (ROCHE). Lysates were vortex for 30 minutes at 4ºC and

centrifuged at 10000 x *g* to remove aggregates. Lysates were boiled 5min at 95 ºC and placed 1 minute in ice. 20 µl of each sample were loaded onto a 12% polyacrylamide gel. After electrophoresis, proteins were transferred to nitro-cellulose membranes using the iBlot system (Invitrogen). Membranes were blocked with 5% of milk in TBS-Tween 0.1% for 1 h at room temperature. Primary antibodies were diluted in blocking solution: anti-SERPINB2 (ab47742, Abcam) at 1:500; anti-ZIP4 (20625-1-AP, Proteintech) at 1:500; anti-GAPDH (ab8245, Abcam) at 1:1000; and incubated overnight at 4ºC. Anti-rabbit or anti-mouse HRP secondary antibodies (1:1000; GE Healthcare) were used. The ChemiDoc XRS+ system (Bio-Rad) was used to obtain a high-quality image. Quantity One Software (Bio-Rad) was used to analyse the results.

Tumorsphere formation and analyses

10,000 MDA-MB-231 or BrM2 cells were plated onto 6-well ultra-low attachment plates (Corning). They were incubated for 21 days at 37°C in a humidified 5% CO_2 atmosphere with a serum-free medium (Gibco) containing B27 (Gibco), 20 ng/mL EGF (Sigma), 20 ng/mL bFGF (Gibco) and 4 g/mL heparin (Sigma) supplemented with zinc as needed. 50 randomized pictures per well were taken using the Zeiss Cell Observer HS. The formed tumorspheres ≥ 100 µm from each replicate were counted with the image processing software ImageJ (developed by NIH, USA). The percentage of cells with the ability to form spheres, termed the ‘tumorsphere formation efficiency (TSFE), was calculated as follows: (number of spheres that formed/number of single cells that were plated) x100.

In order to analyse markers of stemness by flow cytometry, tumorspheres were centrifuged for 5 mins at 300 G and washed

twice with PBS1x. 110 ul of PBS were used to resuspend the cells. Cells were counted using a Newbauer chamber. Cells were stained with PE Mouse Anti-Human CD24 antibody and APC Mouse Anti-Human CD44 antibody (BD Bioscience), according to the manufacturer instructions. Cells were centrifuge 5 mins at 300 G and resuspended in 250 µl of PBS. 1/1000 of Dapi (Thermo) was added. Results were obtained using a LSRII flow cytometer. Further analysis was performed using Flowing Software (Perttu Terho).

Traction forces microscopy

12-well plates with glass bottom Petri dishes (MatTek) were used. Polyacrylamide gels were manufactured on coverslips functionalized incubating with 1:1 AcCOOH and bind-silane (Sigma-Aldrich) diluted in EtOH for 10 minutes. The 12-kPa gels were prepared mixing ultrapure water, 7.5% of acrylamide solution (Bio-Rad), 0.16% of N-N′-methylene-bis-acrylamide (Bio-Rad), 1% 200-nm-diameter dark-red fluorescence carboxylate-modified beads (Fluospheres, Invitrogen), tetramethylethylenediamine (1/2000; Bio-Rad) and free radical ammonium persulfate (10% solution, 1/100 vol/vol; Bio-Rad).

Small droplets of the solution were dispensed onto the treated glass-bottomed Petri dishes, flattened using another cover-slip with a smaller diameter, and incubated at room temperature for 15 min for gel polymerization. Then, top coverslips were removed. Gels were activated with 0.5 mg/mL sulfo-SANPAH (Fisher) and introduced in the UV chamber for 5 min. After washing gels, three times with PBS1x, the gel was coated with collagen (10 µg/ml) for 1 h at 37ºC. Finally, gels were treated for 30 min with UV and rinsed three times with sterile PBS before addition of the culture medium with the corresponding zinc concentration.

5,000 cells were seeded and allowed to grow for 24 hours. 33 pictures in different fields per sample were taken using a microscope with simultaneous phase and epifluorescence illumination. Cells were detached using 0.5% trypsin and another epifluorescence image was taken. With MATLAB we measured the displacement of each bead with a program based in the Butler method. The results quantified are whole-cell traction force average stress measurements, assuming that all of the tractions outside the area of the cell must be zero.

Migration assay

5.000 cells were seeded in a multi well 6 plate and let grow for 24h. With a 10 x objective in a Zeiss Cell Observer HS we took several pictures per well each 10 minutes for 24 hours. We analysed cell migration with the FiJi software using the TrackMate Extension plugging.

Invasion assay

SPLInsert™ Hanging 24-well plates with a pore size of 8 µm (Lab Clinics) were used. The membrane was coated with 60 µl of matrigel (Corning) diluted 1:3 in serum-free medium for 1 hour at 37ºC. 30,000 cells were seeded and let grow and invade for 72 hours. The medium of the upper chamber was cointaining, in each case, the indicated zinc concentration. The bottom chamber medium was the one used for normal cell culture. The media of the bottom chamber was recollected and 500 µl of trypsin were used to detach the cells in this chamber during 1 h at 37 ºC. This trypsin was also recollected and we quantified the number of cells obtained from the bottom chamber with the LSRII flow cytometer.

Immunostaining

Cells were incubated in serum-free media with the primary antibody ZIP4 (20625-1-AP, Proteintech) at 1:200 for 1 h at 37 ºC. After washing with PBS, cells were fixed with 4% PFA for 10 minutes at RT. Then, cells were incubated with blocking solution (PBS1%, 2% BSA) 1 h at RT. And netx with 1:1000 dilution of the secondary antibody in blocking solution. Coverslips were mounted with Fluoromont-G (SothernBiotech). Pictures were taken with a Leica TCS-SP8 confocal microscope with a 63 × 1.40 immersion oil objective.

Statistics

All data are means ± SEM. In all cases a D'Agostino– Pearson omnibus normality test was performed before any hypothesis contrast test. Statistical analysis and graphics was performed using GraphPad. For data that followed normal distributions, we applied either Student's t test (when comparing MDA-MB-231 and BrM2 cells in basal conditions) and one-way analysis of variance (ANOVA) followed by Tukey's post hoc test (when comparing the one cell line in different zinc concentrations) or Dunnett's multiple comparison (when comparing against a control condition) . For data that did not fit a normal distribution, we used Mann–Whitney's unpaired t test and nonparametric ANOVA (Kruskal–Wallis) followed by Dunn's post hoc test. The criterion for a significant difference was a final value of $p < 0.05$.

RESULTS

Characterization of the cellular zinc content in TNBC cell lines

In order to delineate the relevance of intracellular zinc in TNBC brain metastasis, first, we compared the zinc concentration at basal condition between MDA-MB-231 cells with BrM2 cells, our TNBC model for brain metastasis. Using Zinquin as a zinc content reporter, we observed that BrM2 cells doubled MDA-MB-231 cells zinc content (Fig. 1A). We then characterized the expression of several zinc homeostasis molecular players such as ZIP transporters and metallothioneins (MTs)[12]. Our mRNA expression analysis showed an upregulation of ZIP4, ZIP7, ZIP8, ZIP9 and ZIP13 expression in the BrM2 cells compared to the MDA-MB-231 cells (Fig. 1B). Regarding the MTs, no significant changes were observed (Fig. 1C). Altogether, our results showed an enrichment of zinc content and altered zinc homeostasis in the brain metastatic BrM2 linage.

Then, we set up our experimental conditions with different extracellular zinc concentrations in order to study how zinc might modulate metastatic and niche specificity characteristics. We treated both cell lines with 0, 10, and 50 μM $ZnSO_4$ to reproduce zinc deficiency, physiological zinc, and zinc supplementation, respectively. After 24 h we observed significant differences in all conditions (Fig. 1D). Under these conditions for 48h, our MTTs assays showed no alteration in cell viability (Fig. 1E, 1F).

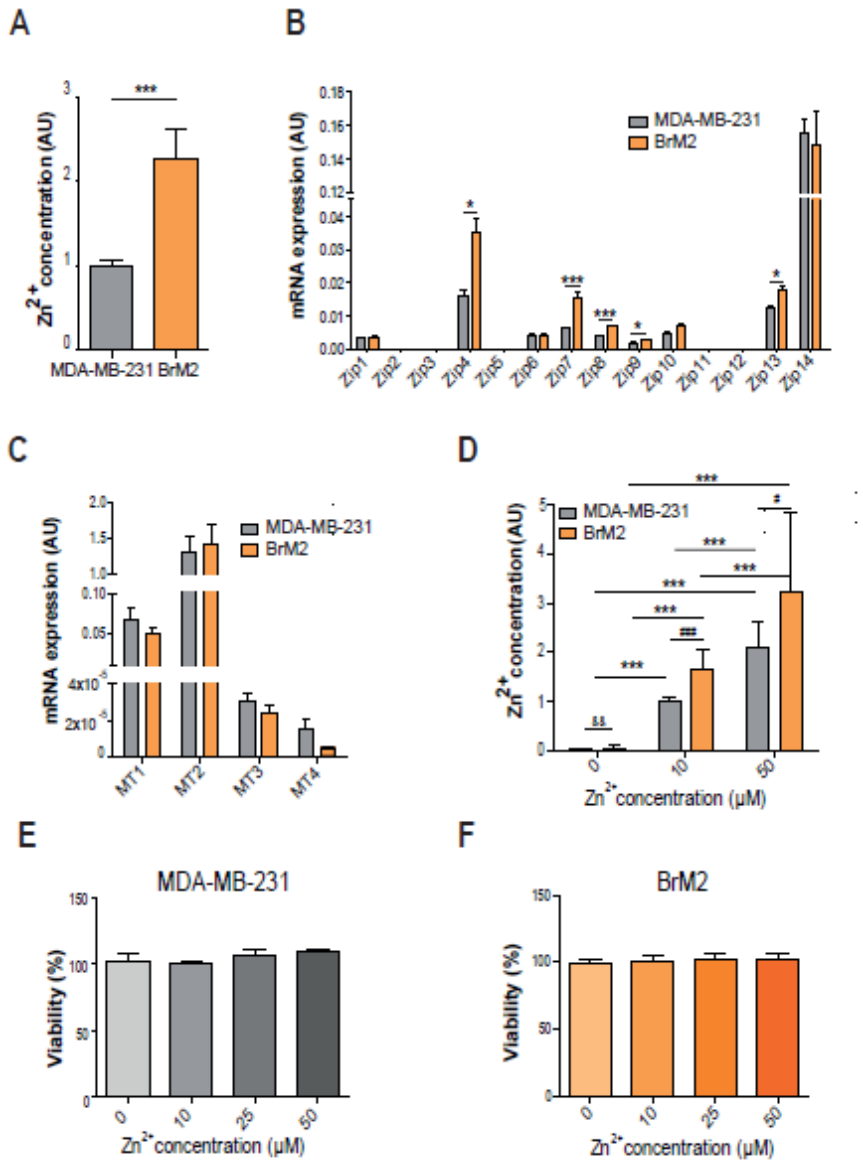

Figure 1. Characterization of zinc homeostasis of MDA-MB-231 and BrM2 cells. **A**. Evaluation of zinc content by flow cytometry using Zinquin in basal conditions. (n=12) ***p< 0.005 using the Mann-Whitney test **B-C**. Real-Time RT PCRs comparing the expression of the ZIP transporters family (B; n=3) and MTs (C; n=6-9) in MDA-MB-231 and BrM2 cells in basal conditions. 2^{-DCT} plotted using GAPDH as a housekeeping gene. *p<0.05,

***p<0.005 using t-test. **D**. Evaluation of zinc content by flow cytometry using Zinquin after 24 h of treatment with 0, 10 and 50 µM of $ZnSO_4$. (n=6-9) ***p<0.005 using the ANOVA Tukey's multiple comparison test. &&p<0.01 using the Mann-Whitney test. #p<0.05, ###p<0.005 using t-test . **E-F**. Evaluation of the cell viability by MTT assay inMDA-MB-231 cells (E) and BrM2 cells (F) grown at different zinc concentrations for 48 h. (n=3).

TNBC zinc modulation in cell migration, motility and invasion

Cell migration, an essential aspect of metastasis[6], is known to be affected by zinc signaling through different transporters[16–18]. We recorded random migration of our TNBC cell lines for 24 h in different zinc concentrations. Both cell lines, independently of the zinc concentration, showed no differences in the majority of measured parameters: total distance travelled (Fig. 2A), mean velocity speed, max distance travelled and mean straight line seed (data not shown). Besides, our data revealed a similar pattern of the migration in both MDA-MB-231 and BrM2 cells. However, in the absence of zinc two parameters were significantly lower in the MDA-MB-231 cells compared to the BrM2 cells: the confinement ratio (Fig. 2B) and the linearity of forward progression (Fig. 2C). They measure the efficiency in the displacement from its initial position and how linear is the progression, respectively. This difference was not observed in the presence of zinc.

Traction forces are required for cell movement[34]. Using hydrogels of an intermediate rigidity (12 kPa) we measured them in our TNBC cell models using different zinc concentrations. BrM2 cells showed the capacity to exert higher forces against the substrate than MDA-MB-231 cells in an independent manner of the condition studied. However, MDA-MB-231 cells appeared to have lowerer force when being in zinc supplementation (Fig. 2D). Our analyses revealed that

BrM2 cells have smaller area than MDA-MB-231 cells. Moreover, in both lines zinc supplementation increased the area compared to the absence or physiological zinc (Fig. 2F).

We finally studied whether these phenomena might impact cell invasion capacity, another crucial step during metastasis[6]. In our assay, TNBC cells were seeded in transwells at different zinc concentrations to invade through a physiological matrix. However, we did not observe any significant differences between cell types or zinc concentrations (Fig. 2F).

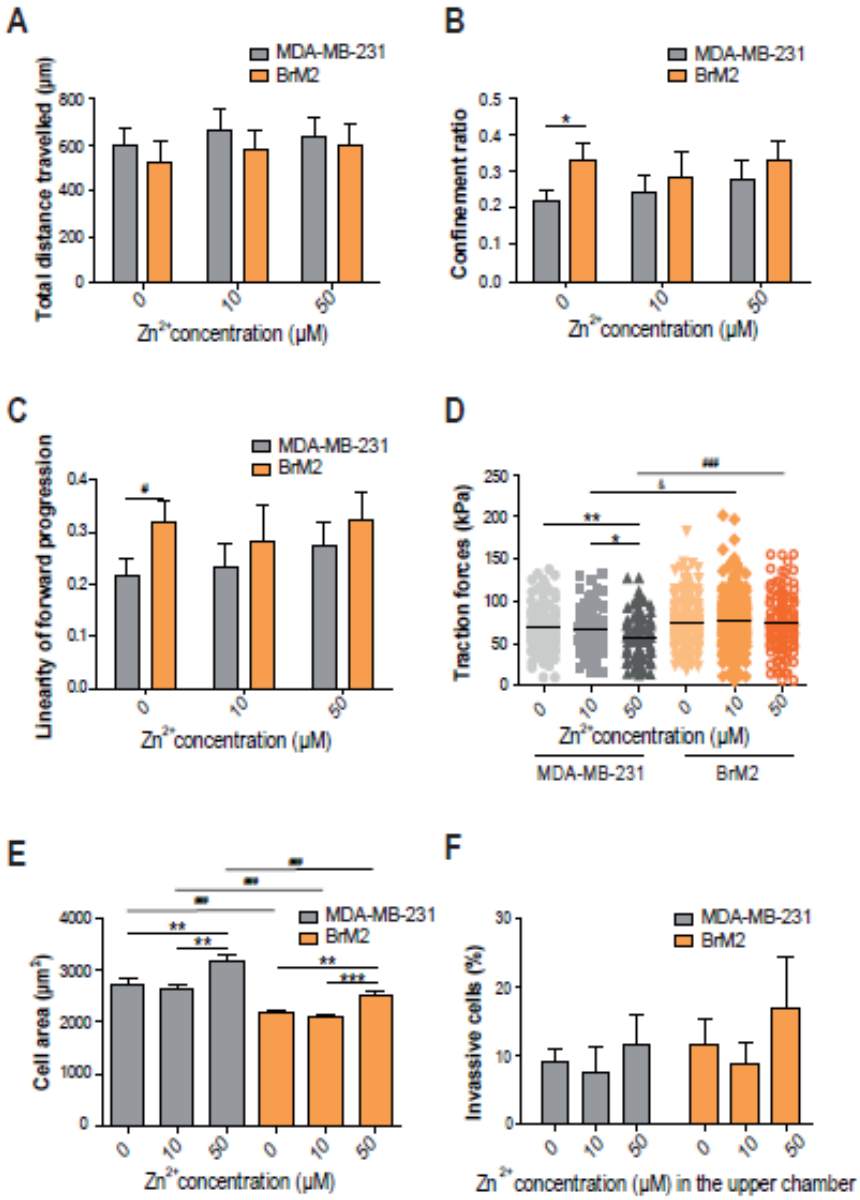

Figure 2. Characterization of the migration, motility and invasion of MDA-MB-231 and BrM2 cells in different zinc concentrations. **A**-**C**. Comparison of representative cell migration parameters including total distance travelled (A), confinement ratio (net distance/total distance travelled) (B) and linearity of forward progression [(net distance/total track time)/mean speed] (C).

(n=17-26). *p<0.05 using t-test. #p<0.05 using the Mann-Whitney test **D**. Comparison of the traction forces measured in 12 kPa hydrogels. (n=88-185). *p<0.05, **p<0.01 using the ANOVA Tukey's multiple comparison test. &p<0.05 using the Mann-Whitney test. ###p<0.005 using t-test. **E**. Comparison of the area of cells measured in hydrogels of 12 kPa. **p<0.01,***p<0.005 using Dunn's multiple comparison test. (n=88-185). ###p<0.005 using the Mann-Whitney test. **F**. Percentage of cells, incubated at different zinc concentration, that invaded the bottom chamber, with normal media. (n=4).

Implication of zinc homeostasis in the TNBC brain microenvironment modulation

Metastatic cells in the brain must confront a highly complex tissue and escape from the local defences. In this context, the secretion of the protease SerpinB2 by breast cancer cells has been reported to be a key mechanism to modulate the brain microenvironment[8]. As previously described[35,36], BrM2 cells showed increased expression of SerpinB2 at the mRNA and the protein level compared to MDA-MB-231 cells (Fig. 2A, 2D). We decided to further explore whether cellular zinc content might contribute to SerpinB2 expression. Both cell lines showed a rise in the mRNA expression levels of the SerpinB2 when supplementing with zinc (Fig 2B, 2C). However, independently of the cellular zinc content, SerpinB2 is not detectable by western blot in the MDA-MB-231 cells (Fig 2D). In the BrM2 cells, on the other hand, we observed that the expression of this protease was strongly induced by cellular zinc concentration (Fig. 2D, 2E).

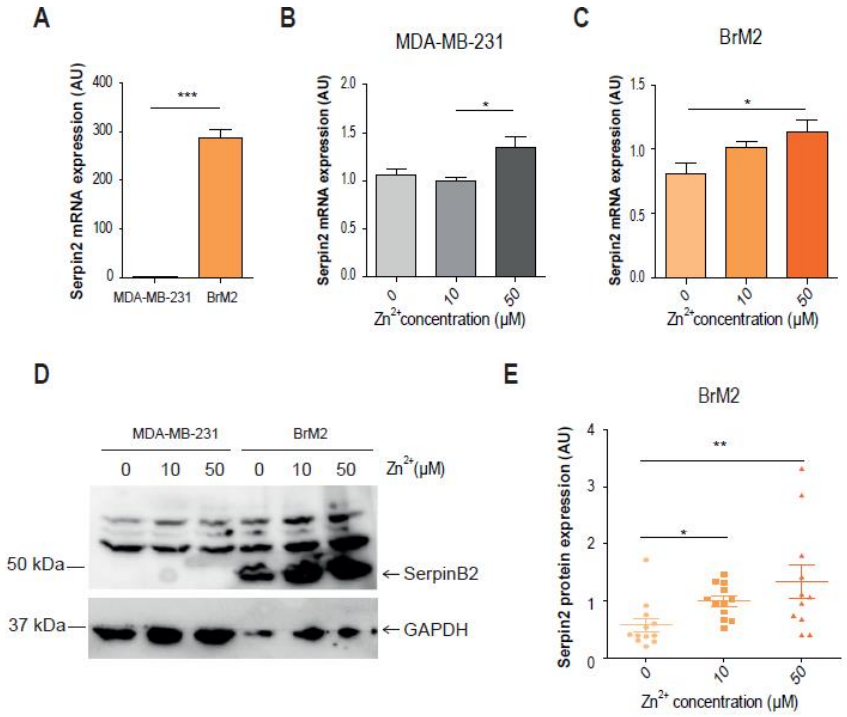

Figure 3. Characterization of SerpinB2 expression at mRNA and protein level in MDA-MB-231 and BrM2 cells **A**. Real-Time RT PCR comparing the expression of SerpinB2 in MDA-MB-231 and BrM2 cells in basal conditions. 2^{-DDCT} plotted using GAPDH as housekeeping gene and MDA-MB-231 as the control line. (n=9) ***p<0.001 using t-test. **B-C**.Real-Time RT PCRs comparing the expression of SerpinB2 in 0, 10 and 50 μM of $ZnSO_4$ after 24 h of treatment. 2^{-DDCT} plotted using GAPDH as housekeeping gene and normalized to 10 μM condition of each cell line. (n=6) *p<0.05 using the ANOVA Tukey's multiple comparison test. **D**. Representative Western blot against SerpinB2 and GAPDH in MDA-MB-231 and BrM2 after 24 h of treatment with 0, 10 and 50 μM of $ZnSO_4$. **E**. Quantification of SerpinB2 protein expression normalized by GAPDH protein expression in BrM2 cells after 24 of treatment with 0, 10 and 50 μM of $ZnSO_4$. (n=11-12) *p<0.05, **p<0.01 using Dunn's multiple comparison test.

Role of zinc in cell proliferation.

The success of metastasis involves cell proliferation and colonization of the new niche[6]. Zinc and zinc transporters have been reported to modulate the cell proliferation in several cell lines[13–15,37]. In our TNBC model, we found a lower proliferation rate in the BrM2 cells compared with the MDA-MB-231 cells (Fig. 4A). Considering the existing differences in zinc homeostasis between cell lines (Fig. 1A), we studied the effect that zinc content might have in proliferation. Consistently, when supplementing with zinc, the proliferation rate of MDA-MB-231 and BrM2 cells decreased compared to the 0 zinc condition (Fig. 4B, 4C).

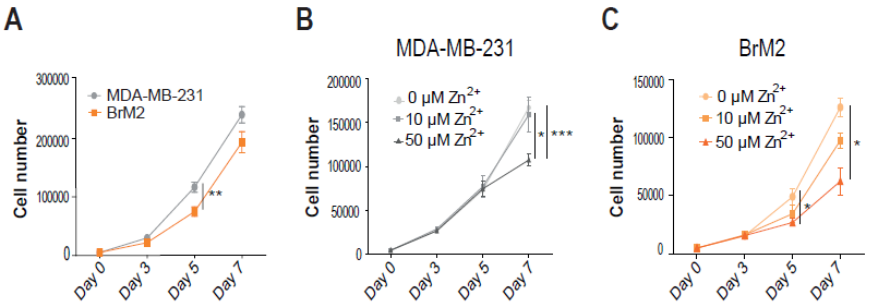

Figure 4. Proliferation assays in basal conditions and changing the zinc concentration. **A**. Proliferation analysis comparing MDA-MB-231 and BrM2 cells. Cells were counted on days 3, 5 and 7. (n=15-18). **p<0.01 using t-test. **B**. Proliferation analysis comparing MDA-MB-231 cells growing in 0, 10 and 50 μM of $ZnSO_4$. Cells were counted on days 3, 5 and 7. (n=9-15). *p<0.05, ***p<0.001 using t-test. **C**. Proliferation analysis comparing BrM2 cells growing in 0, 10 and 50 μM of $ZnSO_4$. Cells were counted on days 3, 5 and 7. (n=6) *p<0.05 using t-test.

Zinc influence in the CSCs tumorigenicity

Low proliferation is a common feature of dormant CSCs[38]. These cells, and specifically in TNBC[39], are essential for the success of the metastasis due to their capacity for generating a whole new tumor, with the needed heterogeneity[9,10]. Interestingly, SerpinB2 has been proposed as an indicator of its tumorigenicity[40]. Therefore, we wondered whether zinc exposure could enhance the population of CSCs and its tumorigenicity in our model. It has been established that stem-cell like properties are enriched in non-adhesive sphere culture conditions, in which cells form tumorspheres[40,41]. We used this system and we manipulated the zinc concentrations to be 0, 10 or 50 µM. In all conditions, tumorsphere formation efficiency (TSFE) was higher in BrM2 cells compared to the MDA-MB-231 cells (Fig. 5A-B). Moreover, our experiments showed that in MDA-MB-231 cells TSFE was positively dependent on the presence of zinc in the growth medium (Fig. 5A). In BrM2 cells, TSFE numbers were around ten fold higher than MDA-MB-231 cells and did not show significant differences between zinc concentrations (Fig. 5B)

In parallel, we characterized the cellular composition of the tumorospheres obtained by flow cytometry. Breast CSCs are known to be CD44(+)CD24(-/low)[42]. When comparing the two cell lines for each zinc concentration, we found that the CSCs population was significantly higher in the BrM2 than in the MDA-MB-231 cells at 10 and 50 µM zinc concentrations (Fig. 5C)). No major differences in CSCs content were found when modulating the zinc concentration, suggesting that zinc promotes the ability of CSC to form tumorspheres but does not influence their final composition (Fig. 5C). In addition, we found a correlation between cellular zinc content and CSCs features in the tumorspheres (Fig. 5D). Thus, the

histogram of Zinquin fluorescence intensity in MDA-MB-231 cells shifted towards higher intensity values in CD44(+)CD24(-/low) cells compared to total cells. This correlation was maintained when comparing CD44(+) cells but not with CD24(-/low) cells alone.

We wanted to further study stemness in our cell model. Therefore, we examined whether there was a correlation between zinc content and the expression levels of the well-known stem-cell markers Oct4, Sox2 and Nanog[43], as well as other tumorigenic zinc dependent markers like ZEB1[44] and SerpinB2[40]. Despite the fact that we observed some interesting tendencies in MDA-MB-231 cells, no significant differences were obtained for any marker (Supplementary Fig. 1).

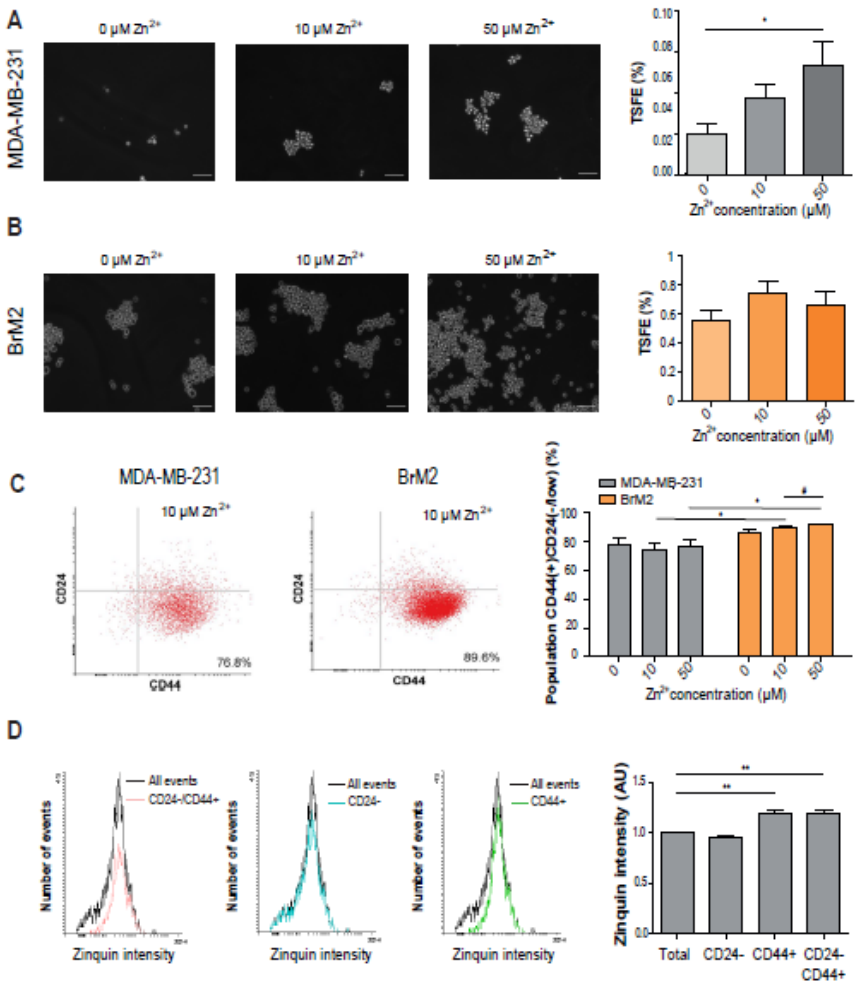

Figure 5. Characterization of the tumorspheres in different zinc concentrations. **A-B** Left, representative pictures of the MDA-MB-231 (A) and BrM2 (B) tumorspheres formed at 0, 10 and 50 μM of $ZnSO_4$ after 21 days of differentiation. Scale bar=100 μm. Right, bar graph showing the mean of TSFE for each condition. MDA-MB-231 cells (A) (n=24) *p<0.05 using Dunn's multiple comparison test. BrM2 cells (B) (n=22-27). **C**. Representative flow cytometry dot blots of MDA-MB-231 and BrM2 tumorsphere stained with CD24-PE and CD44-APC . Right, percentage of CD44(+)CD24(-/low) cells after generating the tumorspheres. (n=4-6) *p<0.05 using t-test; #p<0.05 using the ANOVA Tukey's multiple

comparison test. **E**. Evaluation of the zinc content with Zinquin by flow cytometry in MDA-MB-231 cells showing total events and, from left to right,CD44(+)CD24(-/low), CD24(-/low) and CD44(+) populations. Representative histograms. Right, analysis of these populations normalizing by the Zinquin intensity of the total cell population from each experiment. (n=3) **<0.01 using Dunnett's multiple comparison.

ZIP*4 role in brain metastatic phenotype*

Our results comparing both TNBC models revealed that zinc induces some malignancy traits of brain metastatic cancer cells. In order to dissect specific molecular players involved in this process, we focused our attention on ZIP4, a transporter differentially expressed between lines (Fig. 1B) that has been previously linked to tumor progression. Thus, in pancreatic cancer ZIP4 overexpression involves an increase in cell proliferation, survival, migration, and invasion[45–48]. Moreover, according to cBioPortal for cancer genomics[49,50], 20% of breast cancer patients have an amplification in the ZIP4 coding gene, Slc39a4. Importantly, these patients have a reduced survival compared with the rest (Supplementary Fig. 2). Therefore, we generated a MDA-MB-231 cell line with ZIP4 constitutive overexpression (MDA-ZIP4 for short). We confirmed the upregulation of ZIP4 in the MDA-ZIP4 cells by qPCR, immunostaining and western blot (Fig. 6A-C). Our zinc content measurements using Zinquin showed an increased cytosolic zinc concentration in MDA-ZIP4 cells compared to MDA-MB-231 cells (Fig. 6D). Then, we explored whether ZIP4 overexpression was sufficient to promote SerpinB2. However, similarly to the MDA-MB-231 cells, SerpinB2 protein is not detectable in the MDA-ZIP4 cells at any zinc concentration studied (Fig. 6E). Regarding cell proliferation, we saw no difference between cell lines (Fig. 6F). On

the contrary, our studies showed that ZIP4 was affecting the tumorosphere generation of MDA-MB-231 cells. Thus, we observed that MDA-ZIP4 cells had a higher number of tumorosphers than the MDA-MDA-231 control condition (Fig. 6G).

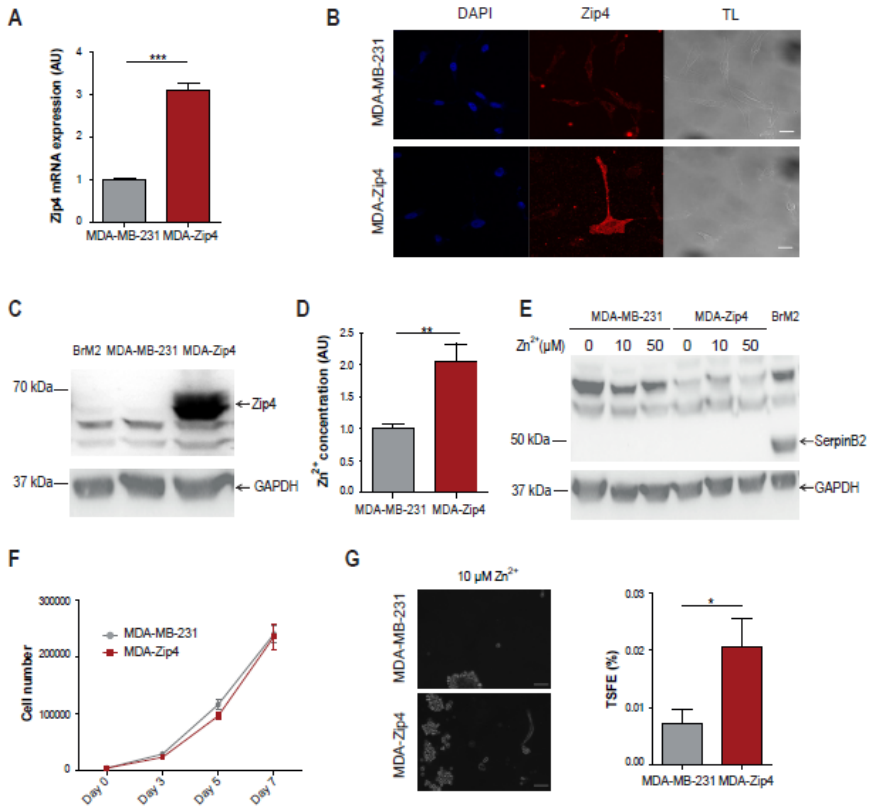

Figure 6. Characterization of MDA-ZIP4 cell line and ZIP4 overexpression in the brain metastatic processes. **A**. Real-Time RT PCR comparing the expression of SerpinB2 in MDA-MB-231 and MDA-ZIP4 cells in basal conditions. 2^{-DCT} plotted using GAPDH as housekeeping gene (n=4). ***p<0.005 using t-test. **B**. Representative pictures of the MDA-MB-231 and MDA-ZIP4 DAPI (blue), ZIP4 expression (red) and transmitted light (TL). Scale bar=20 μm. **C**. Representative Western blot against ZIP4 and GAPDH in MDA-MB-231, MDA-ZIP4 and BrM2 cells. **D**. Evaluation of zinc content by flow cytometry using Zinquin in 10 μM of $ZnSO_4$. (n=9) **p< 0.01

using t-test. **E**. Representative Western blot against SerpinB2 and GAPDH after 24 of treatment with 0, 10 and 50 µM of $ZnSO_4$ in MDA-MB-231 and MDA-ZIP4; and in BrM2 cells in basal conditions. **F**. Proliferation analysis comparing MDA-MB-231 and MDA-ZIP4 cells. Cells were counted on days 3, 5 and 7. (n=12-15). **G**. Left, representative pictures of the MDA-MB-231 (top) and MDA-ZIP4 (botton) tumorspheres formed at 10 µM of $ZnSO_4$ after 21 days of differentiation. Scale bar=100 µm. Right, bar graph showing the mean of TSFE for MDA-MB-231 and MDA-ZIP4 cells. (n=18). *<0.05 using t-test.

DISCUSSION

The fight against cancer aims to find novel treatments by identifying unique molecular features of each tumor subtype and understanding the different tumorigenesis pathways. TNBC contributes to 40% of brain metastasis coming from the breast[4], in a very quick process against which there are no specific tools[3]. In this work, we used BrM2 cells, a brain metastasis model obtained by seeding twice MDA-MB-231 cells into a mouse and recollecting those that specifically established a secondary tumor in the brain[35]. After two brain metastases, BrM2 cells have suffered a high selective process that confers them a specific phenotype. As expected, our work shows that BrM2 cells have metastatic capacities to successfully colonize the brain than the parental line. Interestingly, BrM2 cells showed higher zinc content than the MDA-MB-231 parental line. The results obtained from this study highlight the impact of cellular zinc content on specific hallmarks for brain metastasis.

An initial step during metastasis is the cellular emancipation from the primary tumor, increasing its motility, migration and invasion[6]. Zinc has been previously involved in cell migration in breast cancer cells[18,31,51,52]. In our TNBC model, MDA-MB-231 cells showed a zinc

dependency for the efficiency and linearity of displacement that was absent in BrM2 cells. We also found that BrM2 cells exert higher forces in the matrix than MDA-MB-231 cells. Interestingly, in MDA-MB-231 an excess of zinc results in a lower force exerted. Remarkably, both cell lines have a significantly increased area when grown in zinc excess. To our knowledge, this is the first time that a relationship between the cell area and cell zinc content is established. Last, we saw no major alteration in the invasion capacity using transwells when altering zinc concentration in TNBC cells. Altogether, our data show a milder phenotype in migration and invasion dependending on extracellular zinc compared to the results obtained by others using the zinc chelator TPEN or knocking down ZIP10[31]. Probably our experimental conditions have a milder impact on cellular homeostasis.

SerpinB2 protease is an inhibitor of the urokinase plasminogen activator that promotes cancer cell survival by blocking the niche defences[8]. Its expression favors brain and lung metastasis of TNBC[53]. As previously reported, BrM2 cells have higher expression of the protein SerpinB2 compared to the MDA-MB-231 cells[8,35,36]. Importantly, we found that SerpinB2 mRNA expression is promoted by zinc cellular content. However, independently of the zinc condition studied, SerpinB2 mRNA expression levels in MDA-MB-231 cells did not reach BrM2 levels. Thus, SerpinB2 protein regulation was only observed in BrM2 cells. In a more general view, SerpinB2 expression has been shown to be more frequently overexpressed in TNBC cells than in other breast cancer subtypes. It has been proven to be a poor prognostic factor linked with lymph nodes metastasis. The miRNA-200c/141 has been shown to promote SerpinB2 expression specifically in TNBC cells[53]. The

possible regulation of this microRNA by zinc is not established yet. However, there is a negative feedback loop between miRNA-200c/141 and the zinc dependent transcription factor ZEB1 that deserves further studies.

In the metastatic process, cells often undergo dormant before generating a new tumor in the secondary niche[54]. Zinc homeostasis has been shown to modulate cell proliferation and its dysregulation is involved in the growth of some cancer subtypes[37,45,47,51,52,55–57]. Interestingly, we show that BrM2 cells proliferate slower than MDA-MB-231 cells. Moreover, in both cell lines, the proliferation rate is negatively affected by zinc without an impact on cell survival. These results were unexpected considering the role recently described for ZIP6 and ZIP10 in MCF-7 cells' mitosis[37]. Cancer subtype and metabotropic actions of these transporters could be behind this discrepancy. Besides, dormancy is a characteristic of CSCs[58]. This subpopulation is able to stay dormant for years in the secondary tumor, escaping from treatment and leading to relapses after this time. In TNBC, cancer stem cells have been reported as especially important for its classical progression[58,59]. Moreover, recently, SerpinB2 has been proposed as a CSCs tumorigenicity marker[40]. In this scenario we found that zinc boosted CSCs potential to form new tumors. First, we found a higher TSFE and CD44(+)CD24(-/low) population in the BrM2, with higher zinc content, than in the MDA-MB-231 line, indicating that there are greater CSCs subpopulation within BrM2 cells and confirming their high metastatic potential. Moreover, we found that zinc induces tumorigenicity in the MDA-MB-231 line. In addition, we saw thatCD44(+)CD24(-/low) subpopulation is enriched in zinc, being CD44(+) the specific marker associated with higher zinc content. In this context, it has been reported that

ZEB1 regulates the CSCs population of prostate cancer, also characterized by the CD44 expression[44]. However, in our model of TNBCs brain metastasis we did not find zinc dependent expression neither with ZEB1 nor with the established drivers of CSCs tumorigenesis like Nanog, SOX-2 and Oct-4[43]. More research in this direction is needed.

We wanted to understand the mechanistic insights driven by zinc that increase the metastatic potential in TNBC. Therefore, we decided to modify MDA-MB-231 zinc homeostasis in order to resemble the one observed in the BrM2 line. ZIP4 is one of the proteins upregulated in BrM2 cells compared to the parental line. This transporter has been associated with cancer progression and acquisition of prometastatic features in several types of cancer[45–48,60]. In our MDA-MB-231 cell model, ZIP4 overexpression increased cellular zinc content. However, that effect, similarly to zinc supplementation conditions, was not sufficient to increase SerpinB2 protein expression. This indicates that an additional transformative process must occur in MDA-MB-231 cells to express SerpinB2 in a zinc dependent manner. Nevertheless, we confirmed the tumorigenic properties of ZIP4 expression previously described in other types of cancer. Thus, we observed an induction in the tumorsphere formation in MDA-ZIP4 cells. These results are in agreement to the reported role for ZIP4 in CSCs of ovarian cancer[60]. As a conclusion, ZIP4 overexpression facilitated tumorigenicity in TNBC but was not enough to confer MDA-MB-231 cells the whole metastatic potential and secondary niche adaptation observed in the BrM2 line.

Altogether, considering the different steps that must undergo a breast cancer tumoral cell to metastasize in the brain, our work

highlights the importance of zinc homeostasis in two of them: SerpinB2 production and cancer stemness. The transcriptional pathways involved in this regulation remain unclear and further research must be done to design novel therapeutic strategies to target TNBC metastases.

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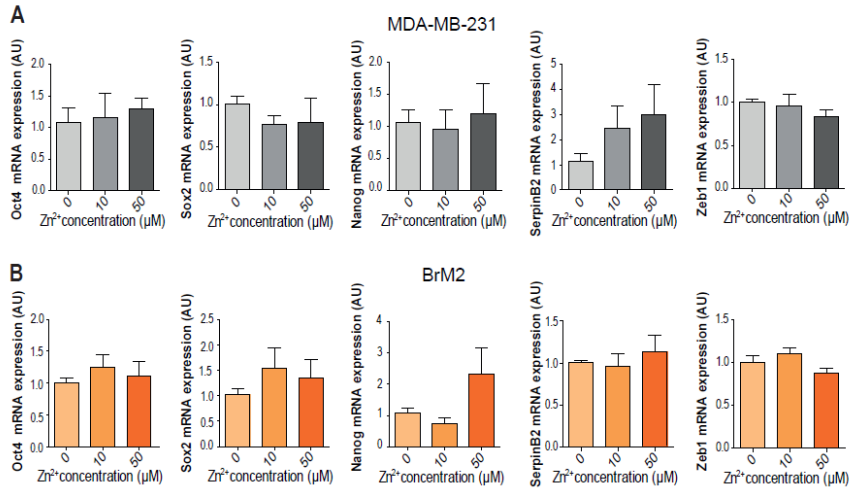
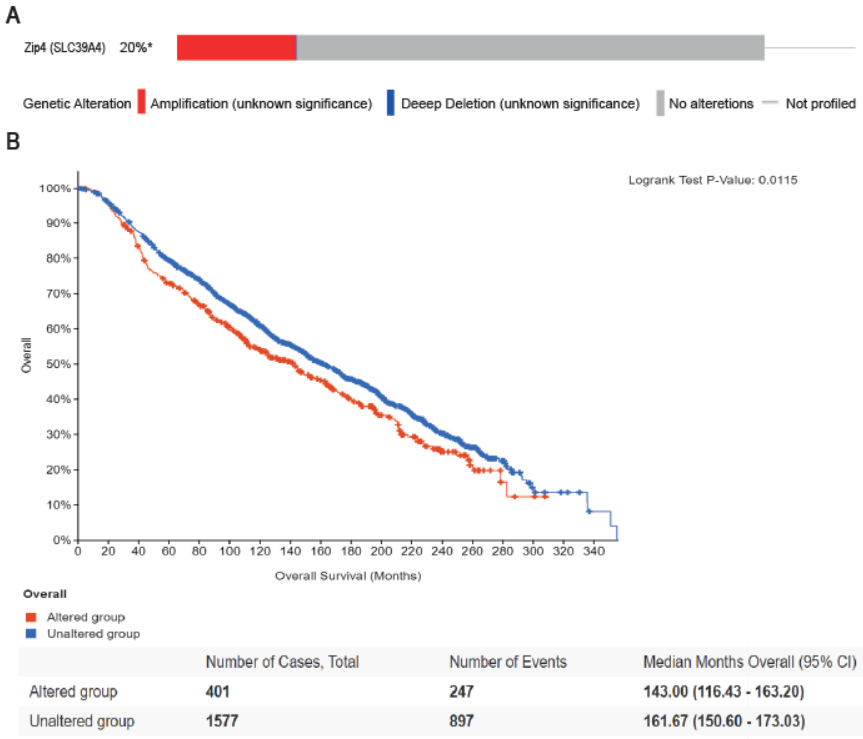
SUPPLEMENTARY DATA



Supplementary Figure 1. A. Real-Time RT PCRs comparing the MDA-MB-231 tumorspheres expression of Oct4, Sox2, Nanog, SerpinB2 and Zeb1 in 0, 10 and 50 µM of $ZnSO_4$. 2^{-DDCT} plotted using GAPDH as housekeeping gene and the 0 µM of $ZnSO_4$ condition as the control. (n=4) **B**. Real-Time RT PCRs comparing the BrM2 tumorspheres expression of Oct4, Sox2, Nanog, SerpinB2 and Zeb1 in 0, 10 and 50 µM of $ZnSO_4$. 2^{-DDCT} plotted using GAPDH as housekeeping gene and the 0 µM of $ZnSO_4$ condition as the control. (n=3-8).



	Number of Cases, Total	Number of Events	Median Months Overall (95% CI)
Altered group	**401**	**247**	**143.00 (116.43 - 163.20)**
Unaltered group	**1577**	**897**	**161.67 (150.60 - 173.03)**

Supplementary Figure 2. ZIP4 characterization in breast cancer patients. Extracted from cBioPortal for cancer genomics (METABRIC, Nature 2012 & Nat Commun 2016)[49,50].

Chapter Two

nutrients

MDPI

Article

Low Zinc Levels at Admission Associates with Poor Clinical Outcomes in SARS-CoV-2 Infection

Marina Vogel-González [1,†], Marc Talló-Parra [2,†], Víctor Herrera-Fernández [1], Gemma Pérez-Vilaró [2], Miguel Chillón [3,4,5], Xavier Nogués [6], Silvia Gómez-Zorrilla [7], Inmaculada López-Montesinos [7], Isabel Arnau-Barrés [7], Maria Luisa Sorli-Redó [7], Juan Pablo Horcajada [7], Natalia García-Giralt [6], Julio Pascual [8], Juana Díez [2,‡], Rubén Vicente [1,‡] and Robert Güerri-Fernández [7,*,‡]

1 Laboratory of Molecular Physiology, Department of Experimental and Health Sciences, Universitat Pompeu Fabra, 08003 Barcelona, Spain; marina.vogel@upf.edu (M.V.-G.); victor.herrera01@estudiant.upf.edu (V.H.-F.); ruben.vicente@upf.edu (R.V.)
2 Molecular Virology Group, Department of Experimental and Health Sciences, Universitat Pompeu Fabra, 08003 Barcelona, Spain; marc.tallo@upf.edu (M.T.-P.); gemma.perez@upf.edu (G.P.-V.); juana.diez@upf.edu (J.D.)
3 Department of Biochemistry and Molecular Biology and Institute of Neurosciences, Edifici H, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain; Miguel.Chillon@uab.cat
4 Unitat Mixta UAB-VHIR, Vall d'Hebron Institut de Recerca (VHIR), 08035 Barcelona, Spain
5 Institut Català de Recerca i Estudis Avançats (ICREA), 08010 Barcelona, Spain
6 Department of Internal Medicine, Hospital del Mar, Institut Mar d'Investigacions Mediques, 08003 Barcelona, Spain; xnogues@psmar.cat (X.N.); ngarcia@imim.es (N.G.-G.)
7 Department of Infectious Diseases, Hospital del Mar, Institut Mar d'Investigacions Mediques, 08003 Barcelona, Spain; SGomezZorrilla@parcdesalutmar.cat (S.G.-Z.); ilopezmontesinos@psmar.cat (I.L.-M.); iarnau@psmar.cat (I.A.-B.); lsorli@psmar.cat (M.L.S.-R.); jhorcajada@psmar.cat (J.P.H.)
8 Department of Nephrology, Hospital del Mar, Institut Mar d'Investigacions Mediques, Autonomous University of Barcelona, 08003 Barcelona, Spain; JPascualSantos@parcdesalutmar.cat
* Correspondence: rguerri@psmar.cat; Tel.: +34-932-483-251; Fax: +34-932-483-249
† Both authors contributed equally to the work.
‡ J.D., R.V. and R.G.-F. are joint senior authors in this work.

Abstract: Background: Zinc is an essential micronutrient that impacts host–pathogen interplay at infection. Zinc balances immune responses, and also has a proven direct antiviral action against some viruses. Importantly, zinc deficiency (ZD) is a common condition in elderly and individuals with chronic diseases, two groups with an increased risk for severe severe coronavirus disease 2019 (COVID-19) outcomes. We hypothesize that serum zinc content (SZC) influences COVID-19 disease progression, and thus might represent a useful biomarker. Methods: We ran an observational cohort study with 249 COVID-19 patients admitted in Hospital del Mar. We have studied COVID-19 severity and progression attending to SZC at admission. In parallel, we have studied severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) replication in the Vero E6 cell line modifying zinc concentrations. Findings: Our study demonstrates a correlation between serum zinc levels and COVID-19 outcome. Serum zinc levels lower than 50 µg/dL at admission correlated with worse clinical presentation, longer time to reach stability, and higher mortality. Our in vitro results indicate that low zinc levels favor viral expansion in SARS-CoV-2 infected cells. Interpretation: Low SZC is a risk factor that determines COVID-19 outcome. We encourage performing randomized clinical trials to study zinc supplementation as potential prophylaxis and treatment with people at risk of zinc deficiency.

Keywords: SARS-CoV-2; zinc; clinical outcomes

Citation: Vogel-González, M.; Talló-Parra, M.; Herrera-Fernández, V.; Pérez-Vilaró, G.; Chillón, M.; Nogués, X.; Gómez-Zorrilla, S.; López-Montesinos, I.; Arnau-Barrés, I.; Sorli-Redó, M.L.; et al. Low Zinc Levels at Admission Associates with Poor Clinical Outcomes in SARS-CoV-2 Infection. *Nutrients* **2021**, *13*, 562. https://doi.org/10.3390/nu13020562

Received: 8 January 2021
Accepted: 30 January 2021
Published: 9 February 2021

1. Introduction

Infections with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) result in a systemic disease with a variety of outcomes, from no symptoms to severe and diverse pathologies, with pneumonia and acute respiratory distress symptom being the most common. Therefore, a better knowledge of risk factors determining severity is essential for treating patients in the early stages of COVID-19.

Zinc (Zn^{2+}) is an essential trace element required for maintaining a variety of fundamental biological processes, due to its functions as a cofactor, signaling molecule, and structural element. One of the most significant roles of zinc in our body is its broad effect on the immune system [**?** **?**], as both the adaptive and the innate immunity, are affected by zinc levels. Consequently, zinc deficiency (ZD), due to low zinc intake or malabsorption, results in an immune imbalance that ultimately causes a major public health problem with high prevalence in elderly and individuals with chronic diseases [**?**]. In adaptive immunity, zinc affects T lymphocyte maturation, differentiation, and cytokine production. B cell activation and plasma cell differentiation depend on zinc signaling as well [**?** **?**]. In innate immunity, zinc has an anti-inflammatory role [**?**]. Concretely, ZD is associated with higher levels of interleukin (IL)-1beta and tumor necrosis factor (TNF)-alfa [**?**], as well as with altered activities of monocytes, neutrophils, and natural killer (NK) cells [**?** **?** **?**]. Correspondingly, ZD results in increased susceptibility to inflammatory and infectious diseases, including acquired immune deficiency syndrome, measles, malaria, tuberculosis, and pneumonia [**?**].

In this context, zinc supplementation trials have been carried out to reduce morbidity and mortality in developing countries with high ZD incidence. Importantly, zinc supplementation significantly reduced the duration of respiratory tract infections caused by rhinoviruses and coronaviruses [**?**]. Moreover, zinc has been shown to have a direct antiviral action [**?**]. Remarkably, in vitro zinc inhibited the binding of the SARS coronavirus RNA-dependent RNA polymerase to its template and the subsequent elongation, as well as viral replication in a cell culture [**?**]. Altogether, zinc status might condition COVID-19 severity, and zinc supplementation could be a useful tool to impact COVID-19 outcome.

In this work, we focus our observational study on the putative association between the zinc status of hospitalized COVID-19 patients with disease progression and clinical outcomes. Moreover, we address in cell culture the potential of zinc supplementation to directly block SARS-CoV-2 multiplication in Vero E6 infected cells.

2. Materials and Methods

2.1. Study Design and Participants

An observational cohort study was performed at Hospital del Mar in Barcelona (Spain). This is a 400-bed, tertiary university hospital in Barcelona that provides healthcare to an urban area of 500,000 people. All patients admitted with COVID-19 for $\geq$48 h between 9 March and 1 April 2020 were included. COVID-19 was defined as a SARS-CoV-2 infection, confirmed by quantitative PCR (qPCR) performed in nasopharyngeal samples obtained by trained personnel at hospital admission, and by fulfilling clinical diagnostic criteria. These include any of the following: respiratory symptoms (dyspnea, cough, sore throat, changes in taste/smell) and chest X-ray findings (uni-or bilateral interstitial infiltrates) that made the diagnosis probable in the current epidemiological situation. The Institutional Ethics Committee of Hospital del Mar of Barcelona approved the study, and due to the nature of the retrospective data review, waived the need for informed consent from individual patients (CEIm-2020/9352).

2.2. Procedures

Demographic, clinical, epidemiological, and the whole-episode (laboratory workup, vital signs, treatment) data were extracted from electronic medical records using a standardized data collection method. Comorbidity was collected individually and as the Charlson

Comorbidity Index, a clinical score that relates long term-mortality to patient comorbidity. It is considered the absence of comorbidity to be 0–1 points and high at >3 points.

Laboratory workups were systematized with an at-admission protocol that included a fasting blood draw, complete kidney and liver profile, electrolytes, blood count, coagulation profile, inflammatory markers (interleukin-6 (IL-6), serum ferritin), D-dimer, and myocardial enzymes. Zinc and selenium levels were detected by electrochemiluminescence. Follow-up laboratory workups in the clinical setting included C-reactive protein and IL-6, which made possible to determine the IL-6 peak.

2.3. Definitions

2.3.1. Time to Recovery (TR)

Time to recovery (TR) or time to clinical stability was defined as the time elapsed since the patient's admission to oxygen saturation >94% (FiO_2 = 21%), normalized level of consciousness (baseline), heart rate <100 rpm, systolic blood pressure >90 mm Hg, and body temperature <37.2 °C.

2.3.2. Clinical Severity

Clinical severity was assessed at admission with a modified early warning score (MEWS) [**?**]. The same score was used for the follow-up during the admission.

2.3.3. Low Zinc Levels

Since levels of zinc have a diurnal variation, all samples were collected through a fasting blood draw at 8 am (±2 h). In our cohort, the Q1 was 50 µg/dL (7.6 µM). According to previous publications, 50 µg/dL is a proper threshold of abnormally lower zinc concentration to predict clinical signs [**?**]. We considered this as a predictive factor the lower range (<50 µg/dL).

2.4. Cell Culture

Vero E6 cells were grown as described [**? ?**]. When indicated, fetal bovine serum (FBS) was incubated according to the manufacturer's instructions with Chelex 100 resin (Bio-Rad Laboratories, Hercules, CA, USA) to generate Zn^{2+}-free growth medium. $ZnSO_4$ was added as needed to the final medium to generate specific Zn^{2+} concentration conditions. Chloroquine (Supelco, Darmstadt, Germany) was prepared in water at 10 mM and used at the desired concentration.

2.5. Zinc Measurements

Cells were seeded and grown in multi-well 24 plates until reaching 80% of confluence. Cells were incubated with 1 µM of FluoZin-3AM (Invitrogen, Darmstadt, Germany) or 25 µM of Zinquin (Sigma-Aldrich, Darmstadt, Germany) for 30 min at 37 °C (5% CO_2) in isotonic solution containing (in mM) 140 NaCl, 5 KCl, 1.2 $CaCl_2$, 0.5 $MgCl_2$, 5 glucose, and 10 Hepes (300 milliosmoles/liter, pH 7.4), plus different concentrations of Zn^{2+} and/or chloroquine (CQ).Cells were then dissociated with Trypsin 0.05% in 0.53 mM EDTA, and were washed with PBS 1x. Fluorescence was quantified using an LSRII flow cytometer. Further analysis was performed using Flowing software (Perttu Terho, Turun yliopisto, Turku, Finland).

For in vivo confocal imaging, cells grown on 22 mm coverslips were incubated with Lysotracker, together with FluoZin-3AM or Zinquin, in a solution with 50 µM Zn^{2+} for 30 min; they were then washed twice with PBS and placed under the microscope in isotonic solution for imaging with an SP8 Leica microscope (Wetzlar, Germany).

2.6. Viability Assays

Cells were exposed to different Zn^{2+} and CQ concentrations for 48 h. Then, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent was added to obtain a final concentration of 0.5 mg/mL. Cells were incubated 2–3 h at 37 °C. After that, the

supernatant was removed, and cells were resuspended in 100 µL of DMSO. The absorbance was read at 570 nm.

2.7. Virus Infection and Quantification

Vero E6 cells were infected with SARS-CoV-2 strain hCoV-19/Spain/VH000001133/2020 (EPI_ISL_418860), and 48 h later viral RNA from the supernatant was extracted using the Quick-RNA Viral Kit (Zymo Research, Irvine, CA, USA). SARS-CoV-2 production was quantified by qPCR with the qScript XLT One-Step RT-qPCR ToughMix, ROX (Quanta Biosciences, Berverly, USA), using the specific probe 2019-nCoV_N1-P, 5′-FAM-ACCCCGCATT ACGTTTGGTGGACC-BHQ1-3′; as well as primers 2019-nCoV_N1-F, 5′-GACCCCAAAATC AGCGAAAT-3′; and 2019-nCoV_N1-R, 5′-TCTGGTTACTGCCAGTTGAATCTG-3′ (Biomers, Ulm, Germany).

2.8. Western Blot

Cells were treated with 0, 10, or 50 µM of Zn^{2+} and 10 µM of CQ for 24 h. Then, cells were washed twice with cold PBS and lysed with 35 µL of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1 mM DTT, 10 mM 13-GP, 0.1 mM Na_3VO_4, and protease inhibitors). Lysates were vortexed for 30 min at 4 °C and centrifuged at 10,000× *g* to remove aggregates. Lysates were boiled at 95 °C and placed in ice for 1 min. 20 µL of each sample were loaded onto a 12% or 14% polyacrylamide gel. After transfer, membranes were blocked with 5% milk in TBS-Tween 0.1% for 1 h at room temperature. Primary antibodies were diluted in blocking solution—microtubule-associated proteins 1A/1B light chain 3B (LC3) (L8918, Sigma-Aldrich, Darmstadt, Germany) at 1:500, p62 (ab155686, Abcam, Cambridge, UK) at 1:1000, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (ab8245, Abcam, Cambridge, UK) at 1:1000—and incubated overnight at 4 °C. Anti-rabbit or anti-mouse horseradish peroxidase (HRP) secondary antibodies (1:1000; GE Healthcare, Chicago, IL, USA) were used.

2.9. Statistical Analysis

A descriptive analysis of the main demographic characteristics of the cohort and the main clinical (laboratory, treatment and outcome) characteristics of the episode was done. Continuous and categorical variables were presented as median (interquartile range (IQR)) and absolute number (percentage), respectively. A Mann–Whitney U-test, χ^2 test, and Fisher's exact test were used to compare differences between individuals with serum zinc levels above and below 50 µg/mL. Bi-variate comparisons and a multiple logistic regression model studying the impact of at-admission serum zinc in in-hospital mortality were fitted adjusting by age, sex, Charslon comorbidity index, and severity of the episode. Taking into consideration the number of deaths observed, and to avoid overfitting the model, we selected the most significant variables and those that provided a more general characterization of the individuals. We excluded variables if they had collinearity with MEWS or age. We created exploratory alternative models, including previously well-known individual mortality risk factors, such as chronic kidney disease, lung disease, or diabetes. We selected the final model adjusted by age, sex, severity, and Charslon comorbidity index. Significance was a p-value <0.05. Stata 14 software was used.

In the in vitro assays, we applied either an unpaired Student's t-test (when comparing two conditions), or one-way analysis of variance (ANOVA) followed by a Bonferroni post-hoc test (when comparing control with any other condition) was applied. All plots comparisons and p values are described in figure legends. Analysis was performed using GraphPad software (San Diego, CA, USA).

3. Results

3.1. Influence of SZC in COVID-19 Outcome

We included 249 consecutive adults admitted to the COVID-19 unit between 9 March and 1 April 2020. The median age of participants was 65 (54–75) years, and 49% were

female. Table **??** shows the baseline characteristics of the cohort and the differences between individuals with SZC <50 μg/dL and ≥50 μg/dL at onset. Hypertension was the most prevalent comorbidity. At admission, the overall severity according to MEWS score was 2 (1–3). Fever, cough, and dyspnea were the most common symptoms, with frequencies of 203 (81%), 198 (79%), and 151 (60%), respectively. Inflammation was a hallmark of the episode, with 224 (87.5%) individuals having abnormal IL-6 at admission and with a median IL-6 of 42 pg/mL (15–89), a median C-reactive protein of 7.5 mg/dL (3.5–15.2), and a median D-Dimer of 800 UI/l (470–1450). Almost all the individuals admitted during this period received hydroxychloroquine. Other treatments were tocilizumab, dexamethasone, and methylprednisolone, each of which were prescribed in a quarter of individuals. Seventy patients (28%) were admitted to the intensive care unit (ICU), and 21 (9%) patients died during hospitalization in this period.

Table 1. Main clinical characteristics of the cohort. Differences between individuals with plasma zinc at admission at <50 mcg/dL and ≥50 mcg/dL.

	Overall		**<50 μg/dL**		**≥50 μg/dL**		***p*-Value**
Cohort Characteristics	***n* = 249**		***n* = 58**		***n* = 191**		
Median age, years (IQR)	65	(54–75)	65	(59–75)	64	(53–74)	0.363
Male sex (%)	127	−51%	30	−51%	97	−51%	0.929
Comorbidities							
Current smoker (%)	23	−9.30%	4	−7%	19	−10%	0.482
Hypertension (%)	141	−56%	33	−57%	108	−57%	0.962
Diabetes mellitus (%)	53	−21%	17	−29%	36	−19%	0.088
Chronic lung disease (%)	22	−9%	9	−16%	13	−7%	0.041
Chronic heart disease (%)	37	−14%	11	−19%	26	−14%	0.315
Chronic renal disease (%)	70	−12%	22	−38%	48	−25%	0.058
Chronic liver disease (%)	18	−7%	1	−2%	17	−9%	0.065
Dementia (%)	8	−3%	2	−3%	6	−3%	0.908
HIV infection (%)	5	−2%	1	−2%	4	−2%	0.865
Active cancer (%)	7	−3%	3	−5%	4	−2%	0.214
ACE inhibitors (%)	61	−24%	12	−21%	49	−26%	0.441
Charlson Comorbidy Index, median (IQR)	1	(0–3)	1	(0–3)	1	(0–2)	0.247
Symptoms at onset							
Median days since syntoms onset (IQR)	7	(4–9)	6	(3–7)	7	(5–10)	0.005
Dyspnoea (%)	151	−60%	42	−72%	109	−57%	0.036
Fever (%)	203	−81%	45	−77%	158	−82%	0.377
Cough (%)	198	−79%	43	−74%	155	−81%	0.246
Diarrhea (%)	68	−27%	12	−21%	56	−29%	0.196
Clinical markers at onset							
Median C-reactive protein mg/dL (IQR)	7.5	(3.5–15.2)	14.6	(5–24)	7	(3–13)	0.037
Median lymphocyte count/mL (IQR)	1.02	(0.71–1.4)	0.82	(0.57–1.18)	1.1	(0.78–1.48)	0.598
Median interleukin (IL)-6 pg/mL (IQR)	42	(15–89)	77	(39–145)	32	(11–71)	<0.001
Median lactate dehydrogenase UI/l (IQR)	288	(241–345)	356	(275–483)	274	(231–362)	0.001
Median D-dimer UI/l (IQR)	800	(470–1450)	935	(540–1700)	800	(460–1215)	0.048
Median PaO_2FiO_2 ratio (IQR)	177	(100–299)	124	(91–181)	219	(106–314)	<0.001
Median modified early warning score (MEWS) (IQR)	2	(1–3)	2	(2–3)	2	(1–3)	0.005
Median serum zinc, mcg/mL (IQR)	61	(50–71)	43	(39–48)	66	(58–74)	<0.001

Table 1. *Cont.*

	Overall		<50 μg/dL		≥50 μg/dL		*p*-Value
Treatment							
Hydroxycholoroquine (%)	248	−99.50%	58	−100%	190	−99%	0.372
Azythromicin (%)	231	−95%	57	−95%	174	−95%	0.766
Tocilizumab (%)	55	−23%	23	−40%	32	−17%	<0.001
Dexamethasone (%)	64	−26%	24	−41%	40	−21%	<0.001
Methylprednisolone (%)	59	−24%	26	−45%	33	−17%	<0.001
Clinical Outcomes							
Median Time to clinical recovery days (IQR)	10	(6–18)	25	(14–36)	8	(5–14)	<0.001
Intensive care unit (ICU) admission (%)	70	−28%	36	−62%	34	−18%	<0.001
Death (%)	21	−9%	12	−21%	9	−5%	<0.001

IQR (Interquartile Range).

SZC median at admission was 61 μg/dL (9.3 μM), with 58 (23%) of the individuals presenting SZC < 50 μg/dL. Individuals with serum zinc levels <50 μg/dL had higher prevalence of chronic kidney disease and chronic respiratory disease (9% vs. 2%, $p = 0.024$, and 16% vs. 7%, $p = 0.041$, respectively). Moreover, this group had a more severe clinical presentation MEWS score (2 (2–3) vs. 2 (1–3); $p = 0.005$) and significantly greater inflammation, measured by the nonspecific marker C-reactive protein (14.6 mg/dL vs. 7 mg/dL; $p = 0.03$) and the more specific IL-6 (77 pg/mL vs. 32 pg/mL; $p < 0.001$). At admission, SZC correlated negatively with inflammation measured by IL-6 (Pearson's $r = -0.307$; $p < 0.001$) (Figure **??**A), and interestingly, also correlated negatively with the highest value of IL-6 during the episode (Pearson's $r = -0.317$; $p < 0.001$) (Figure **??**B). This negative correlation was also observed with the nonspecific inflammatory markers, such as ferritin (Pearson's $r = -0.227$; $p = 0.001$) and C-reactive protein (Pearson's $r = -0.315$; $p < 0.001$) (Figure **??**C). We also found a correlation between SZC at admission and procoagulation factors, such as D-dimer (Pearson's $r = -0.317$; $p = 0.048$).

The median time to clinical recovery in our cohort was 9.5 days (6–18). However, subjects with SZC at admission <50 μg/dL needed longer TR compared with those with zinc ≥ 50 μg/dL: 25 days (14–36) vs. 8 (5–14) days, respectively; $p < 0.001$. Moreover, SZC at admission was correlated negatively with the TR of the episode (Pearson's $r = -0.441$; $p < 0.001$) (Figure **??**D). Two alternative multivariable regression models were created, one using zinc as a quantitative continuous variable, and by adjusting for age, sex, severity, and Charlson comorbidity index, the TR was associated with low SZC at admission (beta-coefficient = −0.21 (95% CI= 0.316 to −0.097; $p < 0.001$)) (Table S2A). In an alternative model, adjusting serum zinc as a dichotomous variable (SZC < 50 μg/dL or ≥50 μg//dL) and adjusting by age, sex severity, and Charslon comorbidity index, serum zinc levels <50 μg/dL negatively impacted the TR (beta-coefficient = 14.1 (95% CI = 4.29–23.94; $p = 0.004$) (Table S2B).

Twenty-one individuals (9%) died during this period. SZC at admission was significantly higher among individuals who survived (62 μg/dL (52–72)) compared to those who died (49 μg/dL (42–53); $p < 0.001$). Individuals with SZC at admission <50 μg/dL had a mortality of 21%, which was significantly higher compared to the 5% mortality in individuals with zinc at admission ≥50 μg/dL ($p < 0.001$). We fitted a multivariable logistic regression model, including 249 patients with data for all variables (228 survivors and 21 non-survivors). When adjusting by age, sex, Charlson comorbidity index, and severity, the model showed an odds ratio (OR) for in-hospital death of 0.94 (95% CI = 0.899 to 0.982; $p = 0.006$) per unit increase of serum zinc at admission. In an alternative age-, sex-, severity-, and Charslon comorbidity index-adjusted model with the predictive variable SZC at admission <50 μg/dL, the adjusted OR for in-hospital death was 3.2 (95% CI = 1.01 to 10.12;

$p = 0.047$). Other models, adjusting by individual mortality risk factors, along with age, sex, and severity, did not show differences with the final model for in-hospital mortality.

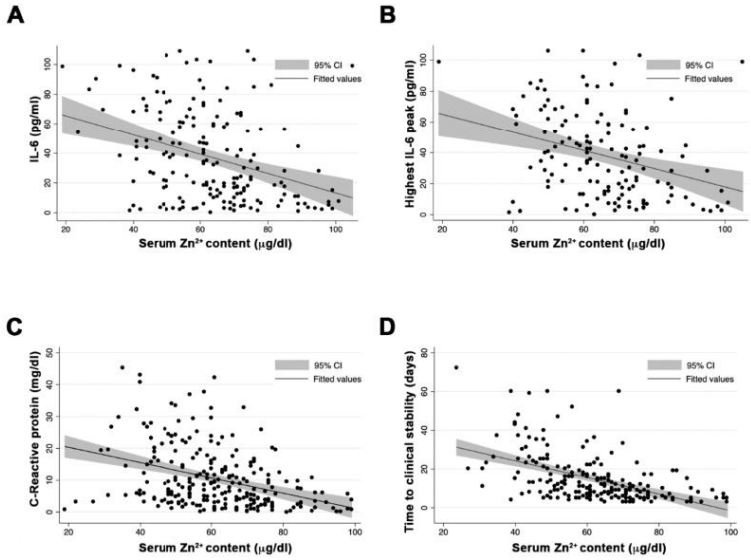


Figure 1. Correlation between serum zinc levels at admission, inflammatory markers, and clinical outcome. (**A**) Zinc and IL-6 at admission. (**B**) Zinc at admission and highest value of IL-6 during the episode. (**C**) Zinc and C-reactive protein at admission. (**D**) Serum zinc content at admission and time to clinical stability.

3.2. Impact of Cellular Zinc Content on SARS-CoV2 Expansion

Besides its impact on immune response, SZC might influence viral expansion [**?**]. Therefore, to obtain a wider picture of determinants involved in COVID-19 severity derived from SZC, we carried out assays in a cell culture to study the direct impact of cellular zinc content on SARS-CoV-2 multiplication. We used three different concentrations of zinc in the extracellular medium—0, 10, and 50 μM $ZnSO_4$—to reproduce zinc deficiency, physiological zinc, and zinc supplementation, respectively. As expected, intracellular Zn^{2+} content changed in Vero E6 cells incubated for 30 min in different extracellular Zn^{2+} concentrations and monitored with Fluozin 3AM and Zinquin fluorescence probes. Confocal images showed that the FluoZin 3AM signal was mainly localized in the lysosomal compartment, while the Zinquin signal was intracellularly spread (Figure **??**B). After 48 h incubation, different Zn^{2+} content solutions did not impact cell viability (Figure **??**C). Importantly, extracellular zinc concentrations affected SARS-CoV-2 multiplication, as measured by qPCR from the cell culture supernatant at 48 h post-infection. SARS-CoV-2 multiplication was increased at 0 μM Zn^{2+} when compared to those values at 10 and 50 μM Zn^{2+} concentrations (Figure **??**D). This indicates that zinc levels affect the SARS-CoV-2 life cycle in infected cells.

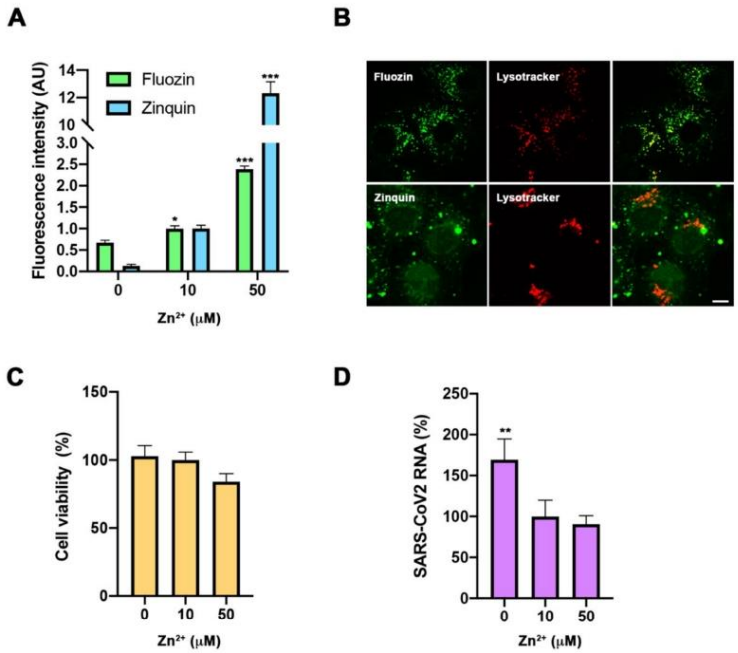


Figure 2. Evaluation of zinc homeostasis in SARS-CoV-2 infection. (**A**,**B**). Intracellular zinc content measurement using FluoZin-3AM and Zinquin probes in Vero E6 cells. (**A**). Flow cytometry in cells incubated for 30 min with 0, 10, and 50 μM extracellular Zn^{2+} content. Intensity expressed in arbitrary units (AU) ($n = 3$; * $p < 0.05$, *** $p < 0.001$; Bonferroni-corrected one-way ANOVA compared to 0 Zn^{2+}). (**B**). Confocal images of living cells incubated with FluoZin-3AM (up) or Zinquin (bottom) and Lysotyracker in 50 μM Zn^{2+} extracellular medium. Scale bar = 10 μm. (**C**). Viability MTT assay in cells incubated with 0, 10, and 50 μM extracellular Zn^{2+} content for 48 h. Data expressed in percentage compared to control condition (10 μM Zn^{2+}) ($n = 12$; Bonferroni-corrected one-way ANOVA compared to 10 μM Zn^{2+}). (**D**). Quantification in supernatant of viral RNA copies by qPCR in cells infected with SARS-CoV-2 and collected at 48 h. Data expressed in percentage compared to control condition (10 μM Zn^{2+}) ($n = 3$; ** $p < 0.01$; Bonferroni ANOVA compared to 10 μM Zn^{2+}).

3.3. Assessment of Zinc Properties as a Potentiator of Chloroquine's Antiviral Action

It has been suggested that Zn^{2+} may potentiate CQ antiviral activity, because CQ acts as a zinc ionophore [**?**]. Zinc supplementation, in combination with CQ, has been used to treat COVID-19 patients, showing benefits [**? ?**]. To test the potentiation effect against COVID-19, we carried out SARS-CoV-2 infections at different zinc concentrations in the absence or presence of 10 μM CQ; the concentration was chosen based on previously published effective concentrations [**? ?**]. Neither the CQ toxicity nor its antiviral activity were affected by zinc levels (Figure **??**A,B). CQ caused a significant reduction in SARS-CoV-2 RNA genome copies compared to control cells (59.68% ± 18.46%; $p < 0.05$) (Figure **??**B). Next, we addressed whether CQ increases the intracellular Zn^{2+} content by acting as a zinc ionophore, as was previously proposed [**?**]. For this, we evaluated the cytosolic Zn^{2+} levels in cells grown under different CQ concentrations, using flow cytometry analysis and FluoZin-3AM and Zinquin labels (Figure **??**C). Notably, an increased zinc signal was observed in a CQ dose-dependent manner using FluoZin-3AM, but not with Zinquin (Figure **??**C). This indicates that CQ modifies lysosomal Zn^{2+} content, but is not a zinc ionophore. As the described increase in lysosomal pH caused by CQ blocks autophagic

flux [?] and inhibition of autophagy impairs SARS-CoV-2 replication [?], we studied the impact on autophagy of different zinc concentrations in the presence and the absence of CQ (Figure ??D–F). As expected, at 10 μM, CQ treatment autophagy blockade resulted in an increased LC3II/I ratio and p62 expression (Figure ??E,F). However, no significant effects on these values were observed at different Zn^{2+} levels in the absence or presence of CQ. Altogether, our results indicate that CQ and zinc do not potentiate each other.

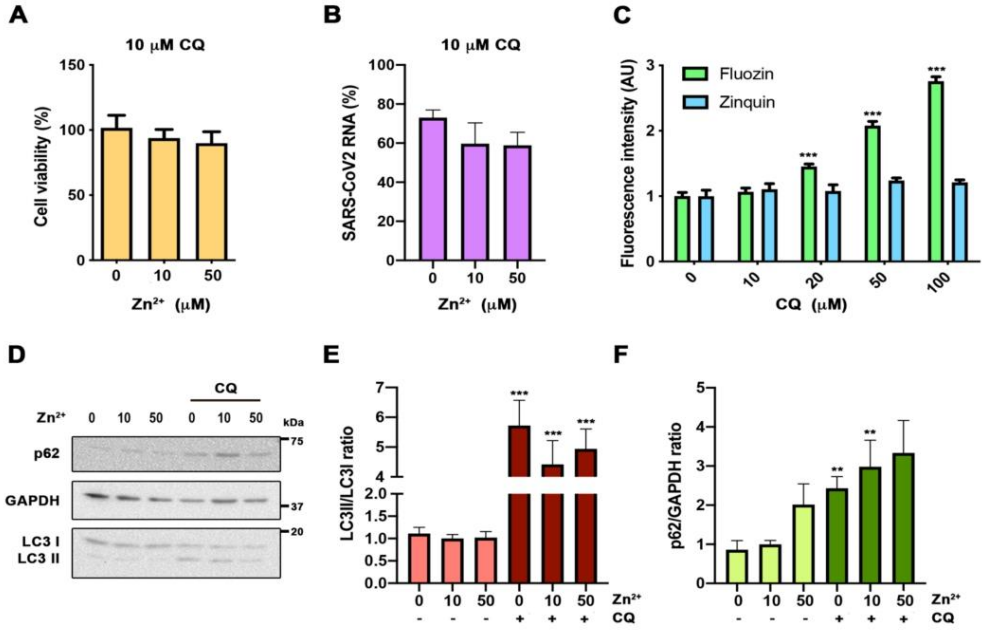


Figure 3. Evaluation of zinc potentiation of chloroquine antiviral action. (**A**) Viability MTT assay in cells incubated with 10 μM chloroquine (CQ) in 0, 10, and 50 μM Zn^{2+} content for 48 h ($n = 15$; Bonferroni-corrected one-way ANOVA compared to 10 μM Zn^{2+}). (**B**) Quantification in the supernatant of viral RNA copies by qPCR in cells infected with SARS-CoV-2 treated with 10 μM CQ in 0, 10, and 50 μM Zn^{2+}, and collected at 48 h. Data expressed in percentage compared to control condition (10 μM Zn^{2+} without CQ, Figure ??D) ($n = 3$; Bonferroni-corrected one-way ANOVA compared to 10 μM CQ in 10 μM Zn^{2+}). (**C**) Flow cytometry in cells incubated for 30 min with 0, 10, and 50 μM extracellular Zn^{2+} content using FluoZin-3AM and Zinquin probes. Intensity expressed in arbitrary units (AU) ($n \geq 4$; *** $p < 0.001$; Bonferroni-corrected one-way ANOVA compared to 0 μM Zn^{2+} from each probe). (**D**–**F**) Western blot analysis from cells incubated for 24 h in 0, 10, and 50 μM Zn^{2+} with or without 10 μM CQ. Antibodies against microtubule-associated proteins 1A/1B light chain 3B (LC3), p62, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (**D**) Representative blot. (**E**,**F**) Quantification of the bands for LC3 ((**E**) $n \geq 10$) and p62 ((**F**) $n \geq 7$) (Bonferroni-corrected one-way ANOVA compared to 10 μM Zn^{2+} or 10 μM CQ in 10 μM Zn^{2+}; ** $p < 0.01$, *** $p < 0.001$ unpaired *t*-test comparing conditions with and without CQ).

4. Discussion

Our study demonstrates a correlation between serum zinc levels and COVID-19 outcome. SZC lower than <50 μg/dL at admission correlated with worse clinical presentation, longer time to recovery, and higher mortality. These results might suggest that SZC impacts COVID-19 severity, and its adjustment might also constitute an early therapeutic intervention point. These results are in accordance with the observational studies carried out by other groups with smaller cohorts [? ?]. Moreover, we show that SZC affects the

SARS-CoV-2 life cycle in infected cells. This effect, contrary to what it has been previously suggested, does not seem to potentiate CQ activity.

The association between zinc and human health has been known for decades [**?**]. Due to poor nutrition and subsequent low zinc intake, ZD remains a major nutritional problem in multiple countries. In addition, elderly individuals are prone to ZD even in developed countries, where the incidence ranges from 15% to 31%, depending on the age and the country of study [**?**]. Older adults are the group at higher risk for severe symptoms and mortality from COVID-19 [**?**]. In our retrospective observational study, with 249 COVID-19 patients admitted to Hospital del Mar, at admission 23% of them had SZC lower than 50µg/dL, the cutoff associated with severe ZD and development of clinical signs [**?**].

At onset, higher levels of inflammatory markers, such as IL-6 and C-reactive protein, were present in low-SZC patients (Figure **??**A,C). The prognostic value of IL-6 and C-reactive protein for COVID-19 severity has already been described [**?**]. In this work, we add SCZ as a novel early predictor for COVID-19 outcome. In addition, we observed a robust negative correlation between zinc levels and the highest peak of IL-6 highest peak (Figure **??**B). These results suggest that COVID-19 patients with low SZC have an exacerbated immune response. ZD is known to be associated with proinflammatory responses at infection, showing higher reactive oxygen species production and inflammatory markers [**? ?**]. An imbalance in cytokine production by cells of both innate and adaptive immunity has also been reported [**? ?**]. Several clinical trials using zinc supplementation have been carried out to prevent and treat infections and inflammatory conditions [**? ?**]. Zinc supplementation decreases the incidence of infection in elderly and improves cytokine imbalance and oxidative stress markers [**? ?**]. Thus, the idea of supplementing low SZC COVID-19 patients with zinc in order to balance what has been called the cytokine storm caused by SARS-CoV-2 is attractive. Reassuringly, zinc supplementation for the common cold caused by rhino- and coronaviruses has been proven to reduce its duration and symptoms [**?**]. Serum zinc content is a common and recommended biomarker to assess zinc status and the risk of zinc deficiency morbidity [**?**]. Nevertheless, it is also known that upon acute infection, IL-6 promotes zinc internalization in hepatocytes [**?**]. Therefore, we cannot discard that the hypozincemia observed in COVID-19 patients might be caused and worsened by a negative IL-6 production loop. As a consequence, one limitation of our retrospective study is that our SZC measurements might not indicate the zinc nutritional status of COVID-19 patients before infection.

In addition to the impact of zinc in the modulation of the antiviral immune response, zinc has also been shown to have direct antiviral action [**?**]. We have analyzed in vitro the impact of zinc homeostasis in SARS-CoV-2 infection. Our results indicate that hypozincemia favors viral expansion in the infected cell (Figure **??**C). These results would support that the poor clinical outcome observed in low-SZC patients is caused by the effect of low zinc availability on both, inducing immune imbalance and increasing viral load by promoting viral expansion in the infected cell. However, it is noteworthy that our in vitro studies did not show a replication blockade in zinc supplementation conditions (50 µM, Figure **??**C), suggesting the need of a zinc ionophore to further increase cytosolic zinc levels in order to block viral replication, as previously shown in vitro for herpes simplex, picornavirus, arterivirus, and SARS-CoV (9,10).

During the beginning of the pandemic CQ was prescribed as a first-line treatment in the acute presentation. The rationale was that CQ has been claimed to be a novel zinc ionophore [**?**], and had shown antiviral effects against SARS-CoV-2 in vitro [**? ?**]. However, clinical trials failed to demonstrate its beneficial effects against COVID-19 [**? ?**]. Therefore, it was suggested that zinc supplementation could potentiate its antiviral activity [**?**]. In fact, there was an observational study treating COVID-19 patients with CQ supplemented with zinc that showed a reduction in mortality [**? ?**]. In our results in vitro, cellular zinc content did not modify CQ cytotoxicity, the autophagic flux blockade, or its antiviral action against SARS-CoV-2. Moreover, we monitored intracellular zinc content both with Fluozin-3AM, a probe previously done by Xue and colleagues that is retained mainly in lysosomes [**?**], and

with Zinquin, which presents more general intracellular staining. We conclude that CQ is not a zinc ionophore, as previously claimed, because its effect on cellular zinc content is restricted to the lysosomal compartment, probably by altering zinc transport at this specific organelle (Figure **??**). Thus, our study does not support the mechanistic rationale of supplementing CQ treatments with zinc. The positive results observed by Carlucci and collaborators in COVID-19 patients treated with zinc sulfate should not be attributed to a CQ potentiation effect [**?**].

This study has some limitations, since we cannot prove causality. Thus, zinc levels at admission could be impacted by other factors, such as acute phase reactants themselves [**?**]. Moreover, this is a single center study with a limited sample size. However, the study also has important strengths, since it has been conducted in a hospital with a large population area that is a representative area of an European city affected by the COVID-19 pandemic. All patients were attended in the COVID-19 unit under the same guidelines, with well-protocolized clinical procedures and with a centralized database that made the management of data uniform, reducing the weakness of the clinical data collection. This makes the quality and quantity of data coming from the COVID-19 unit reliable and homogeneous.

5. Conclusions

This work aims to focus clinical attention on serum zinc content in COVID-19 patients. Our analysis has shown a robust correlation between low SZC and COVID-19 severity and mortality. The cause is likely to be a combination of immune system imbalance and a direct benefit of viral replication. Thus, we propose SZC as a novel and additional parameter to predict COVID-19 outcome. It is then urgent to start clinical trials supplementing patients with low SZC at admission with zinc to reestablish zinc homeostasis. It should be also recommended to promote zinc supplementation programs targeted to people at risk of zinc deficiency, such as the elderly, in order to reduce COVID-19's severity.

Supplementary Materials: The following are available online at https://www.mdpi.com/2072-6643/13/2/562/s1, Table S2A: Multivariable linear regression model studying the impact in serum zinc concentration adjusted by age, gender, severity and comorbidity (Charlson Comorbidity Index) in the time to recovery, Table S2B: Multivariable linear regression model studying the impact in serum zinc concentration as a dichotomous variable (SZC <50 μg/dL or ≥50 μg/dL) adjusted by age, gender, severity and comorbidity (Charlson Comorbidity Index) in the time to recovery.

Author Contributions: R.G.-F., J.D. and R.V. designed and conducted the study, and prepared the manuscript, R.G.-F., I.L.-M. and S.G.-Z. did the analysis and supervised the results, M.V.-G., M.T.-P., V.H.-F., M.C., G.P.-V., R.V. and J.D. conducted the experiments and did the analysis of the experiments. N.G.-G., X.N., J.P., J.P.H., I.A.-B. and M.L.S.-R. contributed to the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Spanish Ministry of Science and Innovation, through grants PID2019-106755RB-I00/AEI/10.13039/501100011033 to R.V. and PID2019-106959RB-I00/AEI/10.13039/501100011033 to J.D.; an institutional "Maria de Maeztu" Programme for Units of Excellence in R&D (CEX2018-000792-M) to R.V. and J.D.; and by the 2017 SGR 909 grant from the Secretaria d'Universitats i Recerca del Departament d'Economia i Coneixement of the Generalitat de Catalunya to J.D. R.G.-F. received support and funding from Centro de Investigación Biomédica en Red de Fragilidad y Envejecimiento Saludable (CIBERFES) (grant number CB16/10/00245), FEDER funds, and the FIS Project from Instituto de Salud Carlos III, Ministerio de Ciencia e Innovación (grant number (PI19/00019)).

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and was approved by the Institutional Review Board (IRB; the Institutional Ethics Committee of the Hospital del Mar of Barcelona (CEIm-2020/9352)). Due to the nature of the retrospective data review, the IRB waived the need for informed consent from individual patients.

Informed Consent Statement: Due to the nature of the retrospective data review, the IRB waived the need for informed consent from individual patients.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to Ethics Comitee restriction for personal data protection.

Acknowledgments: The authors want to thank the students of the Medicine School at Universitat Pompeu Fabra, Barcelona, Spain, for their support and contribution to this work during these tough moments.

Conflicts of Interest: The authors declare no conflict of interest.

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Annex: Characterization of the impact of chloroquine and hydroxychloroquine in lysosomal zinc content.

INTRODUCTION

Since the outbreak of the COVID-19 pandemic, in March 2020, many treatments have been essayed in order to stop the disease progression and its fatal outcome. The strategies have been focused on drugs already used to treat other conditions, so their safety and pharmacokinetics were known. In this context, chloroquine (CQ) and hydroxychloroquine (HQC) have been widely considered for the SARS-CoV2 infection treatment.

CQ and HCQ are two drugs commonly used to prevent and treat malaria, as well as some autoimmune diseases. HCQ was synthesized in 1946 by adding a hydroxyl group into CQ, which made a safer molecule with reduced toxicity[1]. These drugs have been employed to treat SARS-CoV2 infections for several reasons. First, they have anti-inflammatory and immunosuppressive capacites[2], and it is known that the excessive host immune system reaction is the ultimate responsible for the COVID-19 mortality[3,4]. Besides, these drugs alkalinize lysosomes and impair the formation of autophagosomes and the low-pH steps of viral replication, including the membrane fusion[5]. Finally, CQ has been proposed as a zinc ionophore[6], and zinc has been proven to have direct antiviral action[7] and to inhibit, directly or through ionophores, SARS-CoV replication in cell culture[8]. *In vitro* results were promising, because they demonstrated an inhibition in the viral replication[9–11]. However, in two retrospective studies and two clinical trials it has been proved that HCQ treatment without zinc supplementation showed no benefits to improve the COVID-19 patients outcome[12–15]. Since June

15th 2020, the United State Food and Drug Administration has revoked the authorization to use CQ and HCQ in COVID-19 patients outside clinical trials[16].

Our group has recently proved that CQ is not a zinc ionophore, since it just affects zinc concentration in the lysosomes. In this context, we claimed that this would be one reason why CQ and HCQ were supposed to have benefits against SARS-CoV-2 infections but finally did not. In this annex we aim to go further and elucidate CQ and HCQ actions in the lysosomal zinc concentration and their relationship with the pH changes.

MATERIAL AND METHODS

Lysosomal pH measurements

Cells were seeded and grown in multi-well 24 plates until reaching 80% of confluence. Cells were incubated with 50 nM of lysotracker and the indicated zinc concentration for 30 min at 37ºC (5% CO_2) in isotonic conditions containing (in mM) 140 NaCl, 5 KCl, 1.2 $CaCl_2$, 0.5 $MgCl_2$, 5 glucose, and 10 Hepes (300 milliosmoles/liter, pH 7.4). When indicated, cells were previously incubated with 150 µM of TPEN for 30 min at 37ºC (5% CO_2). Cells were then dissociated with Trypsin 0.05% in 0.53 mM EDTA, and were washed with PBS 1x. Fluorescence was quantified using an LSRII flow cytometer. Further analysis was performed using Flowing software (Perttu Terho, Turun yliopisto, Turku, Finland).

Stadistical Analysis

All data are means ± SEM. In all cases a D'Agostino– Pearson omnibus normality test was performed before any hypothesis contrast test. Statistical analysis and graphics was performed using

GraphPad. For data that followed normal distributions, and one-way analysis of variance (ANOVA) followed by Tukey's post hoc test (when comparing the one cell line in different zinc concentrations) or Dunnett's multiple comparison (when comparing against a control condition). For data that did not fit a normal distribution, we used Mann–Whitney's unpaired t test and nonparametric ANOVA (Kruskal–Wallis) followed by Dunn's post hoc test. The criterion for a significant difference was a final value of $p<0.05$.

RESULTS

We first tested the CQ and HCQ toxicity to the VeroE6 cells in 10 µM zinc, the physiological condition. We confirmed, as previously published[10], that CQ is more toxic than HCQ (Fig. 1A, 1B), since it significantly reduced the cell viability at 20 µM while HCQ started to significantly reduce cell viability at 50 µM, after 48h of treatment. We also proved that 10 µM of both drugs was a safe dose to work with since it does not cause cell death. Given that we aimed to characterize the interaction between CQ/HCQ and zinc, we also evaluated the toxicity of the co-treatment with 10 µM of CQ/HCQ and different zinc concentrations. We found no difference in cell viability (Fig. 1C).

Then, we used the FluoZin-3AM (FluoZin) probe to report zinc changes in the lysosome when treating cells with 10 µM CQ or HCQ for 30 min at different zinc concentrations (Fig. 1D). At 0 µM zinc we found no differences while significant increases were registered when treating with CQ at 10 and 50 µM zinc. However, we found no differences when treating cells with HCQ at any zinc concentration. In order to see if more HCQ was needed to obtain the same effects that with CQ, we treated the cells, in zinc excess, with growing HCQ

concentrations (Fig. 1E). Again, we observed no differences in the lysosomal zinc after the treatments. Then, we explored the consequences in lysosomal zinc concentration upon long term treatments. We treated the cells with 10 µM CQ or HCQ for 24 h at physiological zinc concentration (Fig. 1F). In this case, we obtained similar increases in the lysosomal zinc content for both conditions, treated with CQ and treated with HCQ.

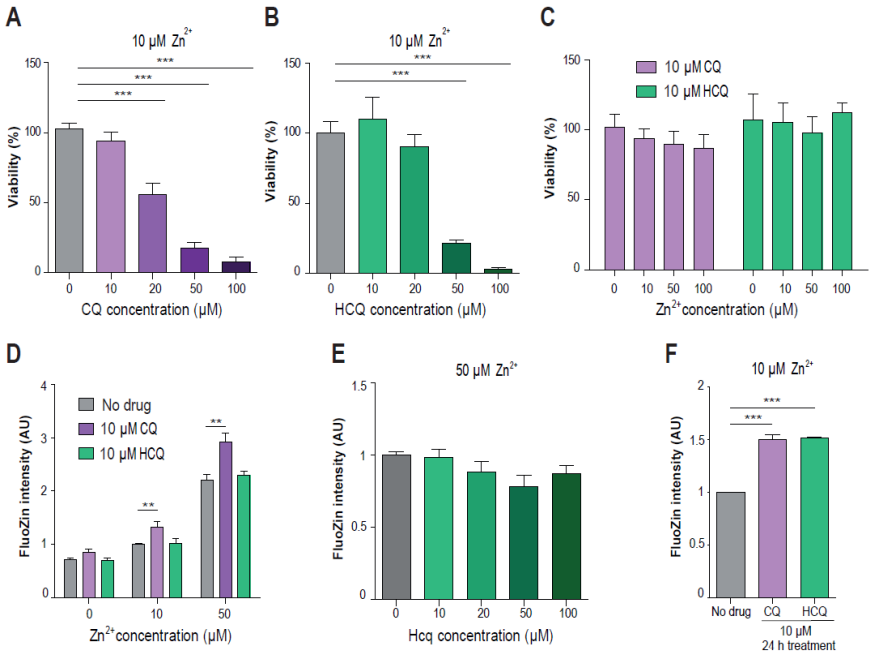

Figure 1. Characterization of VeroE6 treatment with CQ or HCQ in different zinc concentrations. **A.** Evaluation of the cell viability. MTT assay of cells growing at 10 µM of zinc and different CQ concentrations for 48 h. (n=5-24). ***p<0.005 using Dunnett's multiple comparison test. **B**. Evaluation of the cell viability. MTT assay of cells growing at 10 µM of zinc and different HCQ concentrations for 48 h. (n=6). ***p<0.005 using Dunnett's multiple comparison test. **C.** Evaluation of the cell viability. MTT assay of cells growing at 10 µM of CQ or HCQ and different zinc concentrations for 48 h. (n=7-24). **D**. Evaluation of zinc content by flow cytometry using FluoZin.

Cells treated with no drug, 10 µM of CQ or 10 µM HCQ and 0, 10 or 50 µM of zinc for 30 min. (n=9-12). **p<0.01 using Dunnett's multiple comparison test. **E**. Evaluation of zinc content by flow cytometry using FluoZin. Cells treated with 50 µM of zinc and different HCQ concentrations for 30 min. (n=9). **F**. Evaluation of zinc content by flow cytometry using FluoZin. Cells treated with 10 µM of zinc and no drug, 10 µM of CQ or 10 µM HCQ. (n=3). ***p<0.005 using Dunnett's multiple comparison test.

We performed further experiments to prove that the changes observed in the FluoZin fluorescence were the result of changes in lysosomal zinc content and not alterations of the probe fluorescence due to changes in the lysosomal pH. To answer this question, we measured FluoZin fluorescence at 0 or 50 µM of zinc and treated cells with a high CQ concentration, TPEN (a zinc quelator) or both (Fig. 2A). We found that 50 µM of CQ was able to increase FluoZin fluorescence both at 0 and 50 µM zinc concentration in the medium. That effect was abolished in the presence of TPEN. These results confirmed that the differences in the FluoZin fluorescence observed when treating cells with CQ were the consequence of zinc changes inside the lysosomes.

Then, we explored whether the known capacity of CQ to alkalinize lysosomes was zinc related. We used a fluorescent probe, lysotracker, that binds acidic vesicles. This probe binds more when the pH is lower and, as a result, more fluorescence is observed. At physiological zinc, we compared the lysosomal pH without treatment and with CQ or HCQ at 10 or 100 µM (Fig. 2B). Our results confirmed that CQ, and HCQ to a lesser extent, decreased in a dose dependent manner lysotracker fluorescence, meaning more basic lysosomes. Besides, we tested the influence of zinc in lysosomal pH (Fig. 2C). We saw that 0 µM zinc condition provoked a basification of the

lysosomes compared to the rest of the conditions with zinc. However, the presence of zinc did not alter the lysosomal pH in a dose dependent manner. Finally, we tested the influence on the lysosomal pH of the CQ treatment in absence or presence zinc, with or without TPEN (Fig. 2D). We observed that both, the CQ treatment and the absence of zinc, produced alkalinization in the lysosome. Moreover, our data showed a synergic effect of CQ and TPEN, despite being antagonic treatments, when looking at their effect on lysosomal zinc content.

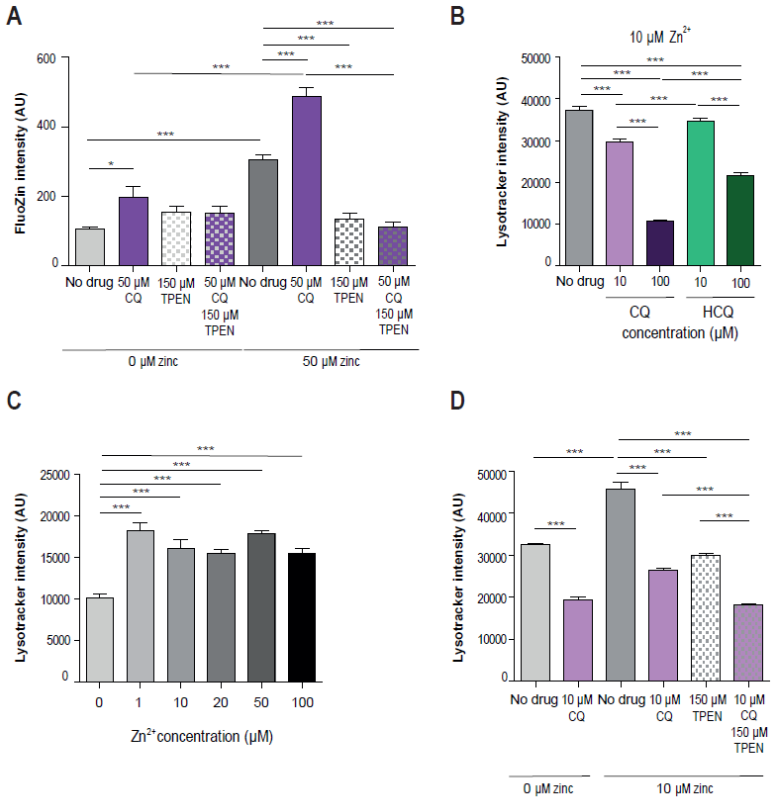

Figure 2. Characterization zinc relationship with CQ effects. **A**. Evaluation of zinc content by flow cytometry using FluoZin. Conditions with 0 µM of zinc or TPEN pre-treated for 30 minutes. Cells treated with no drug, 50 µM of CQ, 0 or 50 µM of zinc and 150 µM of TPEN for 30 min. (n=6-12).

*p<0.05, ***p<0.005 using the ANOVA Tukey's multiple comparison test. **B**. Evaluation of the lysosomal pH by flow cytometry using lysotracker. Conditions with 0 µM of zinc or TPEN pre-treated for 30 minutes. Cells treated with no drug, 10 µM of CQ, 100 µM of CQ, 10 µM of HCQ or 100 µM of HCQ in 10 µM of zinc for 30 min. (n=3). ***p<0.005 using the ANOVA Tukey's multiple comparison test. **C**. Evaluation of the lysosomal pH by flow cytometry using lysotracker. Cells treated with 0, 1, 10, 20, 50 or 100 µM of zinc for 30 minutes. (n=3). ***p<0.005 using the ANOVA Tukey's multiple comparison test. **D**. Evaluation of the lysosomal pH by flow cytometry using lysotracker. Conditions with 0 µM of zinc or TPEN pre-treated for 30 minutes. Cells treated with no drug, 10 µM of CQ, 0 or 10 µM of zinc and 150 µM of TPEN for 30 min. (n=3). ***p<0.005 using the ANOVA Tukey's multiple comparison test.

DISCUSSION

The mechanisms of action by which CQ and HCQ exert their therapeutic effects are not completely understood[2]. At the beginning of the pandemic, any drug with potential benefit to treat and stop the SARS-CoV2 progression was used based on previous knowledge. In this context, COVID-19 patients were widely treated with CQ and especially HCQ, because it is a safer homologue[10]. One of the reasons why they were chosen was their zinc ionophore activity[6], given that zinc has been shown to be useful in the fight against coronaviruses[7,8]. However, we proved that this capacity is not real[17]. In this sense, the interpretation of the studies in which HCQ treatments are or are not supplemented with zinc, must be done from this new point of view[12–15].

In this regard, we think that understanding CQ and HCQ mechanisms of action is relevant for future uses against other diseases such as cancer[18]. Both drugs are lysosomotropic agents:

they are able to enter lysosomes and, once there, they get protonated and trapped. This drug's accumulation increases lysosomal pH and provokes cellular vacuolization[19]. Our results in VeroE6 cells showed that HCQ has slower effects both in the lysosomal protons and zinc content. HCQ has been previously shown to have a milder impact on lysosomal physiology[20]. In some cases this has been related with a differential therapeutic potential between CQ and HCQ. Our investigation also demonstrates that the increase of the FluoZin signal inside lysosomes produced by CQ is not an artifact. Interestingly, CQ was able to increase lysosomal zinc content in the absence of extracellular zinc. That might suggest that CQ blocks zinc transport out of the lysosome instead of promoting zinc entry. TRPML1 is a cationic channel localized in the lysosome that extrudes zinc from it. TRPML1 open conformation depends on lysosomal pH[21]. The alkalinization produced by CQ is likely responsible to inhibit TRPML1 activity and produce the accumulation of zinc in the lysosome observed with FluoZinc. Moreover, a minimal amount of zinc is needed to acidify the lysosome in our cells. It has been previously reported that ZnT transporters like ZnT2 and ZnT3 act as Zn2+/H+ antiporters and work coordinately with the vacuolar H+-ATPase[22] and the H+/K+-ATPase[23] respectively. Taken together, our results shed some light on CQ and HCQ mechanisms of action. Zinc may be essential for their effects on the lysosomal pH and this can be key to future research approaches. Better understanding the mechanisms of employed drugs will allow us to improve their use for present and future diseases.

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Discussion

60 years ago zinc's relevance for human well-being was completely unknown. Since the moment Prasad and colleagues discovered that it is an essential nutrient for the health of people[15], much has been elucidated. Nowadays zinc is considered the most important trace element, having structural, enzymatic and signaling roles. As a secondary messenger, it is involved in cell proliferation[52–54], migration[55–57], differentiation[58–60] and survival[61]. Its homeostasis is crucial to keep the balance between its functions and between the cell processes it modulates. For this reason, there are more than 30 proteins involved in zinc homeostasis maintenance[34]. Therefore, it is not surprising that zinc dyshomeostasis and these proteins' dysregulation have been reported in many pathological processes[265]. This thesis is focused, first, on zinc's alteration relationship with brain metastatic TNBC to the brain and, second, on the role of zinc in the fight against COVID-19.

Chapter One

Metastasis is the last manifestation of cancer and the main cause of death for patients suffering from this disease[100]. In order to improve the clinical tools and the overall survival, it is necessary to enlarge our knowledge about how each metastatic process happens. Thus, it is essential to dissect the molecular features and the cellular changes that are involved in the secondary niche election and adaptation. In this sense, it is important to retain that our experiments have been performed comparing MDA-MB-231 and BrM2 cells. Both cell lines are metastatic TNBC cells but they differ in the metastasization process. BrM2 cells are derived from the MDA-MB-231 cells after two specific metastasization events to the brain[109]. In this context, our results have to be understood as the consequence

of a highly selective process that confers BrM2 cells an optimal phenotype to colonize the brain. Our aim was to study this very specific process and provide new knowledge to stop it, as patients suffering from this condition have a poor survival expectancy of less than 5 months[111].

The insights about how zinc dyshomeostasis helps tumor progression are increasing, which is not surprising given that zinc regulates some of the cellular processes that are altered in cancer[82,83,101] (cell proliferation[52–54], migration[55–57], differentiation[58–60] and survival[61]). As many other molecular features, zinc and zinc transporters dysregulation are tissue-dependent[66]. An increase in the tumor cell zinc content has been reported in breast cancer[117]. Moreover, zinc was proposed as a marker of breast cancer tumor grade in a previous study, where a correlation between zinc concentration and histological malignancy was found analyzing nine samples[151]. More recently, another study in line with the previous has been published[152]. Our experiments reveal that BrM2 cells double the zinc content of MDA-MB-231 cells. This highlights the fact that zinc content may not only correlate with the aggressiveness of breast cancer, but also with its affinity for the secondary niche during metastasis. It would be interesting to further analyze zinc content in other TNBC cells that have more affinity for other secondary niches, such as lung or bones. At the same time, it would be also important to compare BrM2 zinc content with other brain-metastatic cells coming from different primary tumors, such as lung or melanoma. This way, it would be possible to know if this zinc increase is primary, secondary or both tissues-specific. Moreover, this same analysis could be done with samples from patients, an information very useful to better understand the metastasis process.

We also performed a comparison of the mRNA expression of ZIP transporters and MTs between MDA-MB-231 and BrM2 cells. Consistent with the finding that BrM2 cells have more zinc content, we describe a significant overexpression of ZIP4, ZIP7, ZIP8, ZIP9 and ZIP13 in the BrM2 but no changes in the MTs mRNA levels. In a previous study published by Massagué and colleagues[109], the authors performed a transcriptomic analysis using the HG-U133A microarray (Affymetrix) to compare the gene expression profile of these same cell lines. In this study, they did not report differences in the ZIPs that we found to be upregulated in BrM2 cells. This is probably explained because they only retained the genes with a relatively high fold-change (2.5). In our work, we used another technique to profile the gene expression, the real-time RT PCR, and we observed differences lower than 2.5 fold.

No remarkable differences were found in the migration patterns of our cell lines, revealing that the increase in the internal zinc is not helping BrM2 cells in this step, which could be in part expected because both cell lines are already metastatic. Moreover, the zinc transporters involved in the migration of breast cancer cells are ZIP6, ZIP10 and ZIP7[57,125,153], and these are not upregulated in BrM2 compared with MDA-MB-231 cells. Nevertheless, when zinc is removed, MDA-MB-231 cells suffer a decrease in the net distance migrated by the cells, revealing that zinc absence affects their normal migration. We did not obtain the high reduction in migration rate obtained by others[125], probably because they used the zinc chelator TPEN to remove zinc while we growed our cells at 0 µM zinc, a condition in which they still can use their zinc reservoir. In BrM2 cells, we did not observe any effect in the absence of zinc. This could be explained because, even after 24 h at 0 µM zinc, they still

have a significantly higher internal zinc concentration compared to MDA-MB-231 cells.

SerpinB2, whose whole biological role is still unknown, is a protein that has largely been involved in cancer. Its down or upregulation has been reported as a risk factor for different tumors, so its role seems to be subtype-specific. A decrease in the SerpinB2 expression has been associated with higher aggressivity in lung cancers[266,267] and therapy resistance in head and neck squamous cell carcinoma[268]. Similarly, its overexpression has been demonstrated to reduce metastatic characteristics in hepatocellular carcinoma[269], pancreatic ductal adenocarcinoma[270] and nasopharyngeal carcinoma cells[271]. Moreover, in 2020, it was published that double SerpinB2 knock-down mice have a high susceptibility to develop melanoma and lung cancer[272]. However, in myeloid leukemia SerpinB2 has been demonstrated as an important growth inductor[273]. In breast cancer, SerpinB2 expression has been related with both, better and worse prognosis[274]. Recent studies performed with MDA-MB-231 cells claimed, on the one hand, that SerpinB2 promotes lung metastasis in mice models and correlates with reduce patient survival[275] and, on the other hand, that SerpinB2 is involved with the MDA-MB-231 cellular senescence[276]. In this context of contradictory data, it seems that elucidating SerpinB2 functions and regulation is an important step to further understand its role in cancer and, specifically, in breast cancer. Our results support previous publication in which an overexpression of SerpinB2 in BrM2 compared with MDA-MB-231 cells was reported[109,112,277]. SerpinB2 has been identified to mediate brain metastasis[106], therefore we wondered if there was a connection between the abnormal increase of the zinc content in cells after the selective

pressure of the brain metastasis and its SerpinB2 upregulation. Our findings show a zinc-dependent modulation of the SerpinB2 expression. MDA-MB-231 cells, despite not expressing SerpinB2 protein, showed the zinc-dependent mRNA regulation in a smaller scale compared to BrM2 cells. These results highlight the role of zinc as a novel regulator of SerpinB2. In order to have better insights about this regulation, it could be interesting to further investigate the miRNA-200c/141/ZEB1 axes. In this sense, the miRNA-200c/141 has been shown to promote lung metastasis of MDA-MB-231 cells in mice by upregulating SerpinB2[275]. There is no evidence of this microRNA being regulated by zinc, however, it establishes a negative loop with the zinc-dependent transcription factor ZEB1[278]. Another interesting approach comes from the observation that BrM2 cells have an upregulation in ZIP8 and ZIP13. Both zinc transporters have been demonstrated to regulate CEBP/β activity[279,280], while this transcription factor has been found to bind SerpinB2 promoter in murine macrophages and embryonic fibroblasts, inducing its expression in inflammatory conditions[281]. It would be interesting to test if any of these pathways are responsible for the zinc-dependent expression of the SerpinB2 protein in TNBC cells.

Importantly, neither by growing MDA-MB-231 cells with a zinc excess, nor by maintaining BrM2 cells at 0 µM zinc resulted in similar SerpinB2 expression levels between cell lines. This data reveals that zinc concentration is not the main determinant of SerpinB2 expression. An additional transformative event must have occurred and selected in BrM2 cells that explain the high expression of SerpinB2. We could speculate that both, the increased zinc internal content and the SerpinB2 upregulation, are phenotypes selected to confer distinct advantages during brain metastasis.

Another aspect that we compared was cell proliferation, as has been previously demonstrated to be zinc-influenced in several cancer subtypes[74,75,123,129,131,140,141,153]. Contrary to what was expected, BrM2 cells proliferated slower than MDA-MB-231 cells and proliferation of both cell lines decreased when grown at zinc excess. Recently, Taylor et al. have demonstrated that a ZIP6/ZIP10 heterodimer is essential for mitosis in breast cancer cells, including TNBC cells[75]. There are no differences in ZIP6 or ZIP10 expression between MDA-MB-231 and BrM2 cells, so the variation observed in normal conditions should arise from the dysregulation of another pathway. Moreover, an influx of zinc in hormone positive receptor cells via these transporters leads to cell division[75]. In the absence of zinc, our cell lines have a similar proliferation to physiological zinc conditions, however proliferation is reduced after a few days under zinc excess. Maybe, chronic zinc excess induces a phenotypic change that causes cells to proliferate slower. Moreover, it would be interesting to reproduce the experiments blocking ZIP6/ZIP10 at 0 zinc conditions in order to prove whether zinc entry is the cause of mitosis triggering by these transporters or a consequence, which will explain our results at 0 µM zinc. At the same time, we could block ZIP6 and ZIP10 at physiological zinc in BrM2 cells and see if the mitosis decreases.

CSCs have been reported to be a key subpopulation for tumor initiation and metastasis success. They have been identified as especially important for classical TNBC progression[92,95]: CSCs are thought to be able to stay dormant for years in the secondary tumor, escaping treatment and leading to relapses after this time[282–285]. In the excess of zinc, we reduced proliferation, which would fit with an induction of cell dormancy. In addition, we induced SerpinB2, which

has been proposed as a CSC marker in multiple cancer types, including TNBC[113]. Therefore, we thought that zinc could be boosting CSC populations. First of all, we found a higher TSFE in BrM2 than in MDA-MB-231 cells, indicating that there are greater CSC subpopulations within BrM2 cells. This was not surprising given that CSCs can establish a whole new tumor, and BrM2 cells originate from two consecutive tumor establishments, so CSCs were probably selected. For each cell line the same number of cells were seeded at the beginning of the protocol and so were the same number of CSCs. As such, any differences after the 21 days of differentiation can be considered to be the result of the zinc concentration influence. On the one hand, in the MDA-MB-231 cell line, we saw a significant increase in the number of tumorspheres in the 50 µM zinc condition, suggesting a role in the capacity of tumorigenesis of these cells. However, no differences were found in the percentage of CD44(+)CD24(-/low) population, indicating that zinc is not changing the total number of CSCs, but their capacity to induce a new tumor. On the other hand, in BrM2 cells neither zinc absence nor zinc excess affected the number of tumorspheres obtained. We think that this is probably because zinc helps inducing a change in the phenotype that may not be reversible. Moreover, in MDA-MB-231 cells we found that the CD44(+)CD24(-/low) subpopulation has the highest zinc content and zinc concentration does not seem to correlate with the CD24 marker, but with the CD44 intensity. In prostate cancer, it has been recently reported that knocking-down ZEB1, a transcription factor controlled by zinc[68], causes a decrease of the CSCs population, also expressing CD44[286]. In this sense, we thought that maybe, zinc concentration, via ZEB1, was inducing CD44. We then measured the expression of some genes in the different conditions: stem cell classic markers[99], SerpinB2 and ZEB1.

We saw no differences in the Oct4, Sox2 or Nanog levels. We found the same scenario for the SerpinB2, so maybe, it is needed for tumorigenesis, as suggested[113], but its expression does not correlate with the power of the tumorigenesis. Finally, we showed no induction of ZEB1 expression regarding zinc concentration, indicating that zinc may be regulating other pathways in TNBCs to enhance CSCs features.

It has been previously shown that specific alterations in zinc transporters such as ZIP6, ZIP10, ZIP7 and ZIP4 are linked with tumor progression[56,57,74,75,99,123,127,128,131,133,135–138,140,143,148–150,153,287,288]. We wanted to explore whether we could induce tumorigenesis in our MDA-MB-231 cell line by altering a specific zinc transporter. We chose to overexpress ZIP4 in MDA-MB-231 cells for several reasons. First, ZIP4 is upregulated in BrM2 cells compared to MDA-MB-231 cells. Second, this zinc transporter has a large history promoting several cancer subtypes[131,136–138,140,143]. In pancreatic cancer, its role is especially well known, promoting migration and invasion[68] and therapy resistance[150]. Third, according to the BioPortal cancer genomics database (METABRIC, Nature 2012 & Nat Commun 2016), ZIP4 suffers an amplification in almost 20% of breast cancer patients and this is translated into a decrease in the overall survival of 18 month[289,290].

Once ZIP4 was overexpressed in MDA-MB-231 cells, we proved that the intracellular zinc content was increased. This increase was not sufficient to promote SerpinB2 protein expression. A result not surprising considering that 50uM concentration in MDA-MB-231 cells did not promote SerpinB2 protein either. It has been previously discussed that a prior additional transformative event must occur. On the contrary, ZIP4 expression was effective in promoting

tumorspheres formation. Our results are in agreement with a previous work that demonstrated ZIP4 to be a CSCs marker in ovarian cancer[288]. Nevertheless, it is important to remark that the change in the tumorigenesis produced by ZIP4 expression is not comparable to what is observed in BrM2 cells, indicating again that BrM2 prometastatic profile is the result of a complex transformation and cannot be exclusively explained with zinc dependent mechanisms.

The fight against cancer is focused on identifying the special molecular features of the different tumor subtypes in order to use them to establish specific treatments. This concept is really important and has changed the way to face cancer. These types of therapies allow to target the differentially expressed molecules specifically, minimizing the damage to the healthy cells during therapy and, at the same time, guaranteeing the effect of the drugs[84]. In addition, if a molecule needed for the tumor's growth, progression and metastasis is blocked, these phenomena can be avoided. In this sense, any difference between a healthy cell or tissue and a cancerous one can be a potential target. As discussed at the beginning of this section, zinc dyshomeostasis has been related with different cancer subtypes[74,75,123,129,131,140,141,153] so restoring the homeostasis can be a potential therapeutic tool. Moreover, this dyshomeostasis is usually associated with a dysregulation of zinc transporters (see Table 2), the majority located in the plasma membrane[66]. This localization makes these proteins an ideal target for drugs. Once a specific ZIP transporter is involved in the cancer progression of a tumor subtype, it can be used with different purposes: to block it and avoid the internal zinc increase that allows the cell to achieve a more aggressive phenotype or to specifically

deliver anticancer drugs, or both. That could be the case, for example, of pancreatic cancer due to its ZIP4 overexpression[140,148–150,287].

Regarding breast cancer, it has been demonstrated that there is an increase in the zinc concentration of the tumor compared to the healthy tissue[118]. Moreover, this increase correlates with tumor aggressivity[151] and has been proved to be especially high in TNBC[152]. Therefore, this increase seems to be relevant for the tumor progression and, as there are no specific tools to treat this cancer subtype[87], it would be interesting to try to restore zinc homeostasis. In order to test if this therapy would be useful in patients, first of all, *in vitro* studies with zinc chelators would be needed. TNBC cells should be incubated with these drugs in order to observe if this treatment is able to decrease the cancerous phenotype of cells. If results are positive, it would be interesting to do xenografts in mice and to prove the efficacy and safety of the potential target *in vivo*. It would also be important to use local administration strategies for the zinc chelators in order to avoid zinc deficiency of the organism and the related health problems.

In this scenario, we are aware that we should be cautious when extracting conclusions from the results of this thesis because our approach has been *in vitro*. However, this work is one more piece on top of the growing evidence that highlights the influence of zinc homeostasis in cancer progression. In this vein, we believe that this contribution could open novel strategies to fight TNBC.

Chapter Two

Since the outbreak of the coronavirus pandemic, the scientific community has been making a great effort to understand this disease and to find a treatment. COVID-19 is characterized by a wide range of symptoms, going from none to severe acute respiratory distress and death. In this context, the need to identify the risk factors that determine the disease outcome seems obvious. Given that the fatal outcome of SARS-CoV2 infection is related to a dysregulation of the immune response leading to a high inflammation and a cytokine storm[178–180], scientists have been investigating the therapeutic potential of immunomodulatory agents. In this context, our work finds a correlation between zinc deficiency and a poor COVID-19 outcome. These results are in line with other investigations[291–294]. Whether zinc deficiency is a cause or a result of the severity of the disease has not been proved yet. However, taking into account that zinc is needed to inhibit NF-κB[195,196] and, thus, to avoid the excess of inflammation[197], this correlation is not surprising. Moreover, we found an increase in the virus replication in zinc absence *in vitro*, which also might explain the worse outcome of this disease in patients with zinc deficiency. This is in agreement with the inhibition of the RNA polymerase described for the SARS-CoV outbreak [182].

Based on this previous evidence, zinc has been used as a nutraceutical in order to minimize the severe symptoms of COVID-19. A nutraceutical is defined as "a food (or part of a food) that provides medical or health benefits, including the prevention and/or treatment of a disease"[295]. Summarizing the results obtained at this moment we find two retrospective studies, one performed by Calucci

et al. and the other by Derwand et al., showed an improvement in the outcome of the patients under the treatment with HQ and azithromycin only when zinc supplementation was present[257,258]. A third study showed that daily supplementation with 50 mg of zinc and 400 mg of HCQ, same doses used by Carlucci et al. -but not zinc or HCQ alone- reduced mortality by 24%[260]. On the other side, another observational study using a daily dose of 100 mg of zinc concluded no differences in the mortality of patients receiving the supplementation, however, the authors encouraged further investigation due to its retrospective aspect and the small sample size[263]. Besides, in ambulatory care settings, with patients presenting less severe symptoms, a randomized clinical trial revealed no zinc supplementation benefits using 25 mg[264]. In all the hospital studies zinc was supplied as zinc sulfate, while in the ambulatory trial it was done using zinc gluconate. The different results between the studies are probably due to the different guidelines followed in each case (type, dosage and moment of the zinc supplementation). As demonstrated for the common cold, finding the optimal dosage and excipients is essential to see the benefits of zinc supplementation, as well as starting the supplementation fast after the onset of symptoms[252,296]. In this sense, it would be compelling to perform a meta-analysis of all the studies already conducted and the ongoing clinical trials. This way, it would be possible to really establish if zinc supplementation influences COVID-19 severity and which is the optimal administration form against the SARS-CoV2 infection.

As just discussed, the benefits of zinc supplementation in the treatment of COVID-19 patients remain unclear. However, the correlation between zinc deficiency and a poor outcome of the

disease is quite well established and is a modifiable risk factor. In a world in which 31% of the population is estimated to have zinc deficiency[20], especially among elderly people[22,23], it would be interesting to implement routinary tests measuring plasma zinc levels. This way, zinc supplementation could be provided as prophylaxis before the infection happens to prevent the associated complications related to zinc deficiency, like the immune system dysregulation. This could be useful not only for the present pandemic, but also for the proper working of immunity against other infections, as previously demonstrated[28,297].

Zinc is not the only nutraceutical that has been suggested to have potential in the prevention and treatment of COVID-19. Vitamin C has been chosen for the same reasons as zinc. It has been used against common cold and other respiratory infections[298,299] and also has known beneficial effects on the immune system[300–302]. Its deficiency seems to be common among COVID-19 patients[303,304]. Clinical trials proving its role in COVID-19 outcome have just begun. The first results have been promising, as they show a reduction in IL-6 levels and mortality[305]. Vitamin D is the other selected nutraceutical because it can modulate both the innate and adaptive immune responses[306]. Patients suffering from its deficiency have an increased susceptibility to infection[307] and are at risk of developing acute respiratory infections and respiratory distress syndrome[308]. Regarding COVID-19 patients, there is a large number of studies linking this disease with lowered levels of vitamin D, and some of them revealed a potential association with SARS-CoV2 severity[309–323]. However, clinical trials using vitamin D as treatment produced contradictory data[324–328], a similar scenario to zinc supplementation. New studies are on course to elucidate this debate. Both vitamin C

and vitamin D are in a similar situation to zinc, since they have potential roles in minimizing COVID-19 severity. More importantly, because their deficiency can be easily avoided, the overall individual health could be improved carrying out protocolary tests and supplementing individuals when needed, not only in the case of SARS-CoV2.

Given the association between zinc deficiency and COVID-19 severity, it could be thought that the higher global mortality is happening in world areas with a lower zinc access. However, as seen in Figure 7, this is not the case. A good example is Africa (Fig. 7A), the continent with the least number of deaths per million people, but, at the same time, the continent whose people have more tendency to suffer zinc deficiency (Fig. 7B). The reason is that there are more determining factors in the global SARS-CoV2 spread and impact. The case of Africa has been the cause of attention in the scientific community, as the pandemic was expected to be devastating in this continent due to its poverty, little medical resources, malnutrition and high incidence of other infections[329]. It is easy to think that these data are artefactual, the result of poor surveillance and low testing. However, the data from big cities such as Nairobi suggests differently (lower pneumonia cases than in the rest of affected hospitals)[330]. The hypotheses to explain this phenomenon are varied. First, the demography of African populations, whose median age is 18 years younger than the population of Europe and the United States. This could explain the difference in numbers, since most young people infected with SARS-CoV2 are asymptomatic or have less severe symptoms, which leads to missed cases and less mortality[330]. Another idea is that the warmer weather decreases the transmissibility of this virus, as

happens with other respiratory viruses[331]. The last hypothesis is that the people in Africa have been previously exposed to coronaviruses and, thus, they could have antibodies against conserved epitopes, such as the spike[330]. Therefore, in a context in which there is less transmission of the virus, it is not surprising that zinc does not affect severity and mortality of COVID-19.

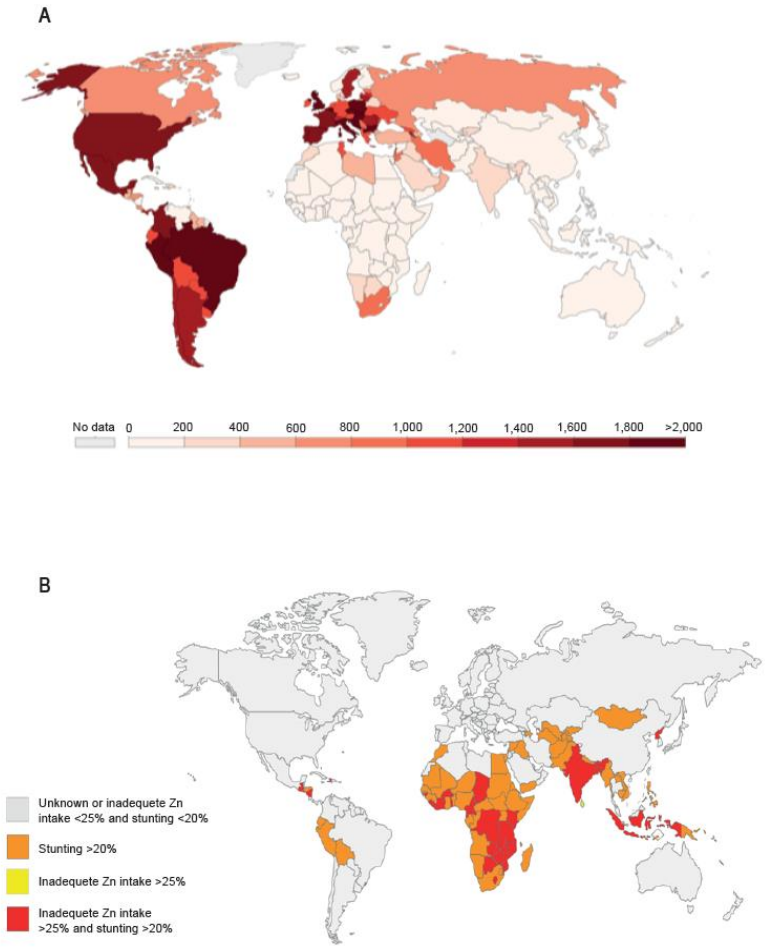

Figure 7. Maps of cumulative confirmed COVID-19 death per million people, May 18, 2021[332] (A) and national risk of zinc deficiency based on the prevalence of childhood stunting and the estimated prevalence of inadequate zinc intake[333] (B).

Finally, in our work, we also provided evidence that CQ and HCQ are not zinc ionophores as they were thought to be[261]. Observational studies and clinical trials using HCQ have already been largely discussed in this section, however, it seems important to highlight that in none of them HCQ was effective without zinc supplementation[257,258,260,262]. In this context it seems important to encourage new clinical trials using other zinc ionophores, as the prior hypothesis that this type of drug may help against SARS-CoV2 infection cannot be discarded. One ionophore option could be pyrithione, which has already proved its *in vitro* efficiency against SARS-CoV[182].

Zinc effects on human health

The two works presented in this thesis provide a good framework to understand the importance of zinc homeostasis at both systemic and cellular levels. Its roles in the proper functioning of the immune system and in cancer progression explained and discussed herein are enough to highlight its relevance for human health and well-being. However, it is also important to mention that zinc dysregulation has been involved in many other human conditions such as cardiovascular diseases, diabetes, obesity, depression or Alzheimer's disease[334]. This is not surprising given that zinc controls many important protein functions and vital signaling pathways at different levels.

At a systemic level, zinc deficiency is a spread out problem that, in developed countries, especially affects elderly people[22,23]. This deficiency is not usually lethal, however, it deteriorates the quality of life, for example increasing the susceptibility to infections[28]. Given

that our society cares not only about mortality, but also about the quality of life of their individuals, it seems necessary to point out, again, the importance of implementing routinary zinc level controls. This is especially relevant among people that are at risk of suffering this deficiency[22,23]. This way, it will be possible to prescribe zinc supplementation to these individuals, avoiding the derived health problems of this deficiency.

At a cellular level, zinc dyshomeostasis affects all of zinc's roles. If there is a zinc excess, it will bind to proteins that it should not and send incorrect signals. In contrast, if there is zinc deficiency, zinc will not be available to bind the right proteins or to be used as a secondary messenger when needed[35]. In this context, it is easy to understand why cellular zinc imbalance leads to disease. Most of the time, this dyshomeostasis originates from the dysregulation of zinc maintenance machinery expression, especially from transporters[66]. A better understanding of the function and role of these transporters in physiological conditions will lead to a better understanding of their role in disease. Moreover, from a therapeutic point of view, ZIPs and ZnT transporters can be useful molecules to target diseases, as discussed in the case of cancer, because modulating them could potentially revert zinc dyshomeostasis. During the past decades, much has been learned about these proteins, however, there are still major knowledge gaps about their structures, transport systems, subcellular localizations, metal affinities and biological functions. Therefore, there is still work to be done in this promising field.

Conclusions

1. BrM2 cells show higher expression of several ZIP transporters and have higher cytosolic zinc content than the parental MDA-MB-231 cell line.
2. Zinc promotes SerpinB2 expression in TNBC cell lines.
3. High cellular zinc content slows TNBC cells proliferation.
4. BrM2 cells have a larger CSCs population compared to MDA-MB-231 cells.
5. Zinc content correlates with CD44 expression and enhances the tumorigenesis of the MDA-MB-231 cells.
6. ZIP4 expression increases stemness in TNBC cells.
7. COVID-19 hospital patients presenting low zinc levels at admission develop poorer clinical outcomes.
8. Low cellular zinc content favours SARS-CoV2 replication in VeroE6 cells.
9. Chloroquine and hydroxychloroquine are not zinc ionophores. Both drugs modify the lysosomal zinc content.

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