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"New Strategies to Identify Susceptibility to Cardiotoxicity by Anthracyclines and Proteasome Inhibitors"

INTERNATIONAL DOCTORATE MENTION

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INTRODUCTION

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1. Cardio-oncology

1.1. Susceptibility to cardiotoxicity

Cardiotoxicity induced by antineoplastic drugs is becoming a significant health problem for patients who have received or receiving cancer treatment. Nowadays, it is the major leading cause of long-term morbidity and mortality among cancer survivors[1]. It is not known who is going to be more susceptible to develop cardiotoxicity under the same treatment regimen. Individual susceptibility to therapy-related cardiotoxicity, as well as other complex diseases, is a consequence of interactions between genetic and environmental factors. Also, currently approved biomarkers appear when the damage is already done, so, there is a necessity to identify early, more sensitive and specific novel markers as well as predict potential individuals susceptible to cardiotoxicity before the clinical manifestations appear[2]. This section focuses on what is known about in genetics and cardiac biomarkers which may help to predict cardiotoxic events in oncological patients.

1.1.1. Prediction by genetic approach

This approach offers the opportunity to identify early markers of cardiotoxicity, revealing the potential genetic variants that may contribute to drug response in each patient before starting the treatment and develop cardioprotective agents for future oncological treatment.

- Chemotherapy treatment: Anthracyclines

Single nucleotide polymorphism (SNP) associated with anthracyclines-induced cardiotoxicity include, among others, those related to NAD(P)H oxidase and doxorubicin transporter genes[3–5]. Chronic cardiotoxicity has been associated with the SNP rs1883112

in the NCF4 gene of the NAD(P)H oxidase p40phox subunit in several studies, including different types of oncological diseases and treatments[3,6] and also with cardiac fibrosis in a study developed by Cascales.et.al[4]. By contrast, acute cardiotoxicity was associated with an SNP in the CYBA gene, rs4673, corresponding to the NAD(P)H oxidase p22phox subunit[3]. There are also several genes related to free radical generation, in particular, the P450 oxidoreductase gene (POR) whose SNPs rs2868177, rs13240755, rs4732513 were associated with the functional decrease of left ventricular ejection fraction (LVEF) in a study performed in more than 250 patients after receiving antineoplastic treatment with daunorubicin and doxorubicin[7].

Interestingly, recent studies are focused on discovering which kinases could be used as regulators of the beat rate in cardiomyocytes. Lamore. S *et al.*[8] described how inhibiting the expression of *RPS6KB gene* that encodes p70s6k protein and MAP4K2 whose expressed proteins specifically activates MAPK, is translated in short action potential duration (APD) in cardiomyocytes as well as the short duration of Ca^{2+} flux and contractility transients. By contrast, knocking down RPS6KA3 and IKBKE leads to an increase in the amplitude of the Ca^{2+} transient and thus reduced the beat rate[8].

In pediatric patients has been identify several genes with significant SNPs implied in DNA damage, drug transport, oxidative stress, sarcomere dysfunction and iron metabolism[9]. Retinoid acid receptor gamma (*RARG*) variant (rs2229774) confers susceptibility to CDA through decreasing the repression to *(TOP)2B.* That leads to increase the levels of this protein which added to the effect of doxorubicin rise the double strand breaks in DNA[10]. UDP-glucuronosyltransferase *(UGT)1A6* (rs17863783) variant makes the anthracycline metabolites glucuronidate and reduct, increasing the levels of toxic metabolites in the cytoplasm of the cardiomyocytes predispose them to CDA[11]. The variant of the gene G-protein-coupled receptor (*GPR)35* (rs12468485) described by Ruiz-Pinto *et al*.[12] was related to a decrease in cell viability and oxidative stress after exposure to anthracyclines treatment. CUGBP Elav-like family member (*CELF*)*4* variant (rs12468485) regulates the alternative splicing of Troponin T gene (*TNNT2*) leading to an expression of embryonic forms of cardiac troponin T which are developmentally innapropiate[13]. Mutations in the metalloproteinase-disintegrin-like

HF3 increases the iron concentration in the cell producing higher concentrations of hydroxyl radicals causing DNA damage, among others[9].

- Proteasome inhibitors

In recent years there is an increasing interest in the genes that participate in CDA induced by new oncological treatments such as proteasome inhibitors. Until now, the studies seem to be focus on processes related to cell survival and apotosis but, not so many studies have examined genes related to susceptibility to suffer cardiotoxicity so far. There are only a couple interesting studies:

The exact mechanism by Bortezomib interacts with the cardiac activity is unknown and there are not accurate studies in the genes related to this cardiac side effects but it is described that this drug impaired the activation of transcription factor Nuclear Factor Kappa βeta (NF Kβ) important in apoptosis genes and cell survival[14].

Furthermore, in 2017, Iman *F et al*.[15] described that an imbalance expression of genes such as Mayor Histocompatibility Complex α (α-MHC), Mayor Histocompatibility Complex β (β-MHC) and Brain Natriuretic Peptide (BNP) cause disorganization in the myocytes and cardiac hypertrophy under Carfilzomib treatment in mice models. The same study describe how expression levels of NF Kβ increases and p53 gene expression significantly decrease in mice treated with this compound[15].

1.1.2. Prediction by cardiac biomarkers approach

There is a growing interest in biomarkers as an early detection indicator of cardiotoxicity during oncological treatment, as a tool for risk prediction before the treatment and as a late detection signal of cardiac events in survivor patients. Biomarkers are a cheaper alternative method to echocardiography or cardiac magnetic imaging and less time consuming to cancer patients, only a blood draw which can be arranged by the physician at the same time as the routine blood tests[16].

- Troponin I

Troponin I (TnI) and troponin T (TnT) are part of the Troponin protein complex involved in the actin-myosin interaction in cardiomyocytes. It is mostly found in the sarcomeres. When cardiac damage occurs, there is a significant release of troponins into circulation after the sarcomere breakdown. Historically Tns were used as a diagnostic tool in different clinical settings as cardiac hypertrophy, acute coronary syndromes, or heart failure among others but in the last two decades, the attention is focused on the evaluation of oncological settings as detection of cardiac damage due to oncological treatment[17]. Although high levels of troponin are detected in other scenarios such as renal failure, several studies prove the utility of these tests. Cardinale *et al.* conducted a temporal study of 240 breast cancer patients who received high doses of anthracyclines[18]. It shows how for seven months, patients with elevated levels of TnI had a reduction of LVEF. TnT elevation together with decrease of LVEF, was also observed in hematological patients after chemotherapy treatment[19]. Nowadays, high sensitivity troponin measurements help us to detect lower concentrations than 16ng/L, as explained in Shah *et al.*[20] The flaw in these studies is that the damage is already done when troponins are detected, so nowadays, its predictive power is questioned.

- Brain-type Natriuretic Peptide (BNP) and N-Terminal pro Brain natriuretic peptide (NT-pro BNP)

Brain natriuretic peptide and its inactive N-terminal amino acid fragment NT-pro BNP are the second most reached in the biomarker list. Both derived from the prohormone proBNP synthesize in the cardiomyocytes upon ventricular wall overload or stress[21], once released in the circulation is cleaved into BNP and NT-pro BNP in a 1:1 ratio by the Corin protease[22] (**Figure 1**).

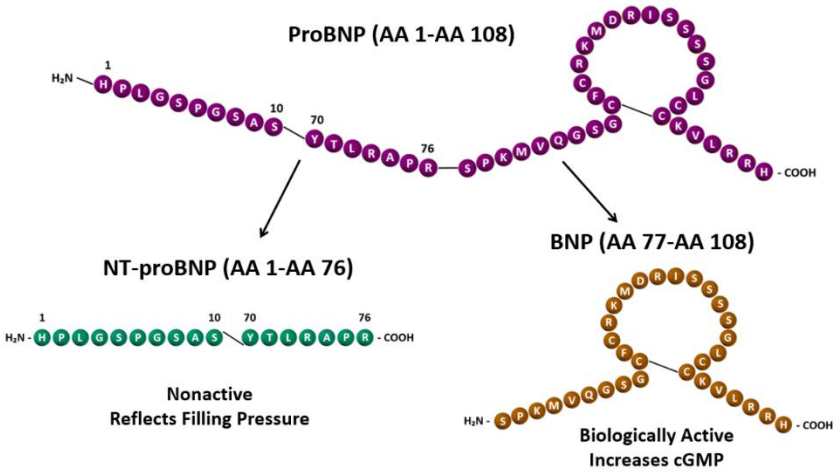

Figure 1. Pathway of NT-proBNP and BNP synthesis from proBNP.
proBNP is cleved by corin enzyme resulting in two residues. The average time in circulation is 120 minutes for NT-proBNP (dark green) and 20 min for BNP (orange).

Current guidelines recommend using these two well-known parameters as reliable markers of cardiac damage[23], and several studies have shown more sensitivity than echocardiography studies[24]. In an exciting study with 204 pediatric patients suffering from acute leukemia and treated with anthracyclines, the detection of high NT-pro BNP levels in the first three months was a predictor of cardiac dysfunction four years later[25]. Another study shows that BNP measurements in the entire course of the treatment helped to predict cardiotoxicity in 104 lymphoma and sarcoma patients[26].

Nevertheless, natriuretic peptides have limitations, as with troponin detection, small sample size, and lack of ranges references[16]. Furthermore, the elderly and woman population seems to have naturally higher levels of these biomarkers[27,28], and recent evidence suggests that oncological patients with metastatic complications present an increase of BNP levels compared with those without this complication[29].

- miRNAs

Non-coding RNA molecules play an essential role in regulating gene expression and other important mechanisms in cardiovascular system[30]. After the discovery of

circulating miRNAs in body fluids, including serum or plasma, they were quickly introduced as potential non-invasive diagnostic and prognostic biomarkers in many diseases, including cancer[31]. Despite the initial promising results, miRNA are not still validated and approved as clinical biomarkers[32], their detection in serum or plasma is more complicated than expected, in part due to the lack of standard methods to detection and normalization[33], and the no consensus on endogenous controls.

Even though it is still unclear that miRNAs can predict cardiovascular effects, several studies carry out on this topic. A recent study shows how the let-7 family is implied in several cardiovascular diseases and let-7g is downregulated in doxorubicin-induced cardiomyopathy[34]. The miR-125b seems to be a *so-called* cardio-miR that is upregulated in patients suffering from heart failure together with miR-195 and miR-214[35]. Higher levels of miR-30 are protective against anthracycline toxicity and in a study developed in murine heart exposed to doxorubicin showed that is downregulated through GATA-6 which plays a crucial role in heart development[36]. miR-21 is mainly expressed in adult cardiomyocytes where regulates the ERK/MAP kinases pathway in cardiac fibroblast but also plays a vital role in cardiac development and regulating cell proliferation. Its overexpression after doxorubicin treatment attenuated doxorubicin-induced apoptosis and when the miRNA is inhibited the apoptosis increased under the same treatment regime[37]. In concrete, its 5'arm, miR-21-5p, is well characterized for its contribution to myocardial disease through MAP Kinases signaling in fibroblast[38] and, more importantly, has been recently described as being upregulated by the oncogene HER2, which opens new options for therapies[39].

Therefore, miRNAs seem to be a useful tool in protection, diagnosis, and regulation of different mechanisms in cardiomyocytes against oncological treatments. There are hundreds of them illustrating their essential regulatory roles in the pathogenesis of human diseases. Even though some may have a specific function, others do not have a definite pattern, and some studies seem contradictory. The identification and characterization of novel dysregulated miRNAs as a potential markers of drug-induced cardiac injury could be useful in the susceptibility understanding struggle.

1.2. Classification of the cardiotoxicity

For over a decade (2005), the chemotherapeutic agents, which can cause cardiotoxicity, have been divided into two main groups[40]:

Type I group causes irreversible damage by a cumulative dose in the heart. It can also be classified as acute, subacute and chronic depending on the time from the initiation of the treatment; acute and subacute when develops up to 2 weeks, and it is characterized by arrhythmias, abnormalities in ventricular repolarization and QT intervals, and chronic, when appearing, one year after completing the treatment, is a severe asymptomatic condition which leads to an irreversible heart failure. Drugs included in this group are: anthracyclines (Doxorubicin, Daunorubicin, Epirubicin and Idarubicin), alkaline agents, the oldest type of chemotherapy (Cyclosphosphamide, Melphalan or cisplatin derivates), taxanes (docetaxel and paclitaxel) used very frequently with anthracyclines in combined treatment, topoisomerase inhibitors, that are used in combination with other antineoplastic drugs (tretinoin and vinca alkaloids) and antimetabolites, classified as pyrimidine (5-Fluorouracil, Capecitabine or Cytarabine) or purine analogs (6-Mercaptopurine).

Type II or reversible cardiotoxicity include monoclonal antibodies: Trastuzumab, Pertuzumab, Bevacizumab and small molecule inhibitors lapatinib and sunitinib. characterized by non-cumulative effect and often independent of the administered dose[41]. Symptoms are associated with cardiac dysfunction but no structural damage and are reversible in up to 76%[42] of the cases.

According to Witteles *et al.* [43], this classification has two main flaws; the first one is the incorrect term “reversible” in the type II group. After prospective studies about the cardiotoxicity of trastuzumab in HER2 breast cancer patients, the long-term effect seemed to have as hard effects as anthracyclines. The second flaw is related to the kind of cardiotoxicities produced by the oncologic treatments which should be at least nine based on the mechanism of action and distint patterns of cardiotoxicity as described in his paper.

1.3. Stages of heart failure during chemotherapy

According to the American College of Cardiology, there are four stages of heart failure[44]:

-Stage A: pre-heart failure. The patient has a high risk of developing heart failure but does not present structural heart disease symptoms.

-Stage B: pre-heart failure. The patient presents structural heart disease; echocardiography shows an ejection fraction of less than 40% but no signs of heart failure.

-Stage C: Structural heart disease with symptoms of heart failure.

-Stages D and E: refractory heart failure.

Currently, cardiotoxicity induced by oncological drugs is diagnosed in stages B to C, but new clinical outcomes offer diagnosed cardiotoxicity much earlier in Stage A[45].

1.4. Proposed diagnostic tools for detection of the cardiotoxicity

In breast cancer survivors older than 65 years old, cardiovascular diseases are the primary cause of death[46]. This fact is due to 2 main factors, first, the direct impact of the cancer treatment against the cardiovascular system and second, the lack of attention in cardiac care until the first symptoms appear. Furthermore, some studies suggest that once the cardiac damage appears, particularly with anthracyclines treatment, the complete recovery of left ventricular function only occurs in a minority of the patients[47]. This scenario makes the cancer therapy-related heart failure emerged as one of the fields of great interest in cardiology. Now the preferred strategy of physicians is prevention rather than rescue therapy[16].

1.4.1. Echocardiography

Echocardiography was started to use in 1970 as a technique to measure the left ventricular stroke volumes[48]. Nowadays, it has emerged as the first method to detect cardiotoxicity in oncological patients. There are two different variations:

- 3D based LVEF

The use of 3D echocardiography-based myocardial strain is one of the methods of choice for diagnosis of cancer therapy-related to heart failure. The reason is the wide-spread availability, excellent reproducibility, and lack of radiation. In 2018 Zang *et al*.[49] proved it in a study with 142 women treated with anthracyclines. This study is the most extensive to date describing temporal changes in breast cancer patients. One of the critical conclusions its reaches are the decrease of myocardial strain (circumferential, longitudinal, and principal strain) and 3D LVEF after anthracycline treatment when compared with a control group. Unfortunately, there is no universal threshold to define cancer therapy-related cardiac dysfunction. The European Society of Cardiology in 2016 established that a decline of 50% of normal left ventricular ejection fraction (LVEF) is considered a high reduction in functionality. The disadvantage is the reduced availability, is not always clinically accesible[50].

- 2D Simpsons LVEF

Although the American Society of Echocardiography recommended this method for measuring LVEF[51] several studies have shown shortcomings in terms of inter and intra variability[52]. This method is based on a modified biplane Simpson's technique that is based on dividing the LV cavity into a predetermined number of discs, usually 20[53]. This geometric assumption, the inability to detect subtle variation in the heart wall motion, and the comparative studies prove that 3D echocardiography is more accurate, makes the 3D image acquisition the recommended technique when possible[52].

1.4.2. Left Ventricular Global Longitudinal Strain (GLS)

This assessment measures the cardiac deformation based on three different parameters, longitudinal, radial, and circumferential in two points at the end of the systole and the end of diastole. The data can be obtained from echocardiography or cardiac magnetic resonance imaging (CMR). GLS has become popular as a useful tool for sub-clinical LV dysfunction in oncological patients[45] and is considered very accurate when used the same processing software across the study. Nowadays, and increase of GLS, more than 15% compared with control patients, is considered an abnormal value.

1.4.3. Nuclear Cardiac Imaging (MUGA)

The non-invasive technique, Multiple Gated Acquisition Scan or ERNA, has been used since the 1980s to detect left ventricular dysfunction in patients treated with anthracyclines[54]. This method is used to evaluate the ejection fraction of the heart by measuring how much blood is pumped into the ventricles of the heart in each heartbeat. A small amount of radioactive solution is administered intravenously. The substance has an affinity for blood cells and will be detected by the camera as they travel through the heart and then derive the LVEF. This technique is not advantageous for patients receiving chemotherapy to detect the loss of strength in the heart. Although it is ideal in serial exams, the main disadvantage is the exposure of patients to ionizing radiation[50].

1.4.4. Cardiac Magnetic Resonance CMR

Sometimes known as a cardiac Magnetic Resonance Imaging (MRI), CMR is non-invasive cardiac imaging developed in the 21st century. This method is the gold standard quantification of LVEF, but its also used for the evaluation of different cancer therapy-related cardiac toxicity[50] as changes in cardiac distorting, vascular assessment, pericardial disease, and myocardial inflammation or fibrosis. The technique is based on the same basic principles as MRI but with optimization in the use of the cardiovascular

system. MRI is a non-invasive tool that is suitable for long monitoring of cancer patients and early cardiotoxic detection, as demonstrated in several recent studies[55–57].

The University Hospital of Salamanca has proposed 16 definitions of functional cardiac damage based on different degrees. The list of definitions was as follows (**Figure 2**):

Definition 1	LVEF decrease ≥ 10% during the first six months of therapy
Definition 2	LVEF decrease reduce ≥ 10% during the complete follow-up.
Definition 3	LVEF decrease reduce ≥ 5% during the first six months of therapy.
Definition 4	LVEF decrease ≥ 5% during the complete follow-up.
Definition 5	BASAL systolic dysfunction according to CMR Guidelines.
Definition 6	BASAL systolic dysfunction during the follow up according to CMR Guidelines
Definition 7	Systolic dysfunction during the first six months of therapy according to the CMR Guidelines
Definition 8	De NOVO systolic dysfunction (not basal) during follow-up according to CMR Guidelines.
Definition 9	De NOVO systolic dysfunction (not basal) during the first six months of therapy according to CMR guidelines.
Definition 10	De NOVO dysfunction during follow-up and LVEF decreased ≥ 5%.
Definition 11	De NOVO dysfunction during follow-up and LVEF decreased by ≥ 10%.
Definition 12	BASAL LVEF lower than 55%.
Definition 13	LVEF lower than 55% during the first 6 months of therapy.
Definition 14	LVEF lower than 55% during the complete follow up
Definition 15	De NOVO (not basal) LVEF lower than 55% during the first six months
Definition 16	De NOVO (not basal) LVEF lower than 55% during the complete follow-up .

Figure 2. List of the 16 definitions of the functional cardiac damage.

1.5. Cardiac testing in hiPSCs-CMs

1.5.1. Personalized medicine

As explained previously, although some of the effects of the oncological treatments can be predicted, unexpected cardiotoxicity still occurs. Animals models have shed light on this issue and are an essential step before going to a clinical trial, but differences in physiology, metabolism, or gene expression sometimes make the translational interpretation difficult. It is necessary to accurate human in vitro models to study the mechanism in detail and desirably in the future to be able to predict the susceptibility of the patients before starting the treatment and scale the dose based on this data.

The discovery of the induced pluripotent stem cells has opened new options to save drug screening for cell-based safety and toxicity and allow the pharmaceutical companies to move safely from the bench to the clinical trials[58]. One of the significant benefits of using this technology is that it can be produced in vitro in limitless quantities and develop the trials in real human models.

In the concrete case of human induced pluripotent stem cells-derived cardiomyocytes (hiPSCs-CMs), with the advances in genome editing, we can create in vitro genetic cardiovascular diseases introducing mutations mimicking the human disease. This feature is particularly exciting in the pharmacogenomic field in early-stage drug candidates[59]

However, there are some limitations to the use of hiPSCs-CMs. Functional immaturity, homogeneity in the cell culture and lack of 3D architecture are the main concerns among researchers[60], and over the past few years they have put different efforts in developing strategies: the T-tubule development to mature the excitation-contraction coupling[61], the use of 3D tissue engineering[62]or computational platforms to asses in a quantitative way this problem[63] to produce more mature like adult-cells.

But in the near term is the most suitable technology to model diseases, especially in the context of the earliest stages or drug discovery processes.

1.5.2. Physiological drug screening

New high throughput methods to quantify the kinetics of excitation and contraction make it possible to conduct physiological screening experiments on a large scale[59]. This section is focused mainly on two treatments.

- Anthracyclines

Because of their extensive use in oncological treatment, several research groups focus their attention on chemotherapy mediated cardiotoxicity. hiPSCs-CMs have

revolutionized the study in this field, getting relevant information that can be applied in the clinic. One recent study showed that hiPSCs-CMs from breast cancer patients who suffered cardiotoxic events during the chemotherapy treatment are more sensitive in terms of cell viability, impairment of calcium handling or metabolic function than breast cancer patients that after the chemotherapy did not present cardiotoxicity[64]. In the study of Kitani *et al.*,[65] they made an in-depth description about the cardiotoxicity produced by Trastuzumab and its seriously effect in the contractility and the use of calcium without cardiac death or sarcomere disorganization. This effect is different to the indiced by anthracyclines, which causes decrease in cell viability through necrosis and apoptosis within hours of administration in the IPSCs-CMs, in agreement with human biopsies studies[66], that lead to functional effect causing reduced beating rate and abnormal patterns[67].

- Proteasome inhibitors

Proteasome inhibitors (PIs) in combination with immunomodulators and glucocorticoids are currently used in combination in the treatment of multiple myeloma. These PIs inhibit the activity of the proteasome leading to the disruption of the ubiquitin proteasome system (UPS) the major mechanism of degradation of cytosolic and nuclear proteins, consequently, unfolded proteins accumulate in the cell and activate the unfolded protein response (UPR) which produce the activation of pro-apoptotic cascades and the cell death[68]. It has been shown that this mechanism also affects the cardiac proteasome in sarcomere the reason why several clinical reports have mentioned cardiotoxic events[69,70].

All three approved PIs (Bortezomib, Carfilzomib, and Ixazomib) induce cardiotoxicity in different degrees in clinic[71]. Besides, there are three new proteasome inhibitors, two of them analogs to Bortezomib and Carfilzomib (Oprozomib and Delanzomib) that still need to be studied in their cardiac effects. Testing these new compounds in hiPSCs-CMs, as have already done in this work, lead us to have preliminary information about the possible cardiac impact in terms of toxicity or protection of the treatment.

Immunomodulators, including lenalidomide, pomalidomide and thalidomide, have shown dramatically increase in thromboembolic effects in combination with PIs[72] and some research groups suggest cytokine levels or genetic factors behind these effects but the exact mechanism of action is not clear[73].

2. Breast cancer

2.1. Epidemiology

The breast cancer statistics rates situate this malignancy as the most frequent and the second leading cause of death in women worldwide after lung cancer[74]. In 2018 there were registered over 2 million new cases, and the number of fatalities were around 600.000, which is approximately 15% of all cancers among women[75]. In the past 2019 an estimated of 300,000 new cases in women and 2,670 in men according to the American Cancer Society[76].

Furthermore, the number of cases is increasing, not only in countries when the incidence is always higher as in Europe but also in countries where the rate was historically very low as in Asia, Africa, and Latin America[77]. The hypothesis is that undeveloped countries are starting to embrace a lifestyle that encourages the development of the disease. This includes sedentarism, obesity, a decrease in the number of children, delaying the start of childbearing, or exposure to carcinogenic as in developed countries.

Despite this data, survival is one the highest after melanoma of the skin. According to the American Cancer Society[78], 90% of people who are diagnosed with breast cancer will live for at least five years more, including all types and stages and the survival rate for localized and early detection staging is 99%.

2.2. Classification of breast cancer tumors

Breast cancer is a heterogeneous disease classified in multiple subtypes. In the beginning, the identification of the different subtypes was based on immunochemistry[79] and gene expression[79], but currently, several factors are included in this clinical process, such as tumor morphology, tumor size, lymph node metastasis, proliferation marker Ki-67 and expression of HER2, ER and PR receptors. Later, the pioneering studies conducted by Sørlie *et al.* started to classify the tumor in 5 main groups explained in the next section[79,80]. The rationale underlying such classification was identifying by clustering of gene expression among cancer subtypes reflecting the fundamental differences of the tumors at the molecular level[81].

- Luminal A and B

Both subtypes of breast cancer are hormone-receptor-positive (estrogen-receptor and/or progesterone-receptor positive) and express luminal cytokeratin 8 /18[82]. Luminal A tumors is the most common subtype of breast cancer tumors; are HER2 negative and have low levels of the protein Ki-67, tend to grow slowly, and are associated with better prognosis. At the genome level, these tumors have mutations in PI3KCA and MAP3K1[83]. Luminal B tumors are either HER2 positive or negative. These tumors are more proliferative (higher Ki-67 values) and the prognosis is slightly worse. These tumors show high expression levels of genes related to proliferation, such as AURKA, and lower levels of luminal markers, such as FOXA1 or PR[84]. Luminals tumors are the most common subtypes of breast cancer, thus, between60% to 70% of invasive ductal carcinomas are luminal[85].

- Triple-negative/basal-like breast cancer

These tumors are hormone-receptor negative (estrogen-receptor and progesterone-receptor negative) and HER2 negative. Their frequency is between 15% to

20% of all tumors. These tumors express basal cytokeratin 5,6,7 and 17, and genes related to ERBB2[86]. This subtype of breast cancer is more common in women with *BRCA1* gene mutations[87] and younger and African-American women[88]. The prognosis is worst than in luminal tumors but long-term survival rates show similar data as found in luminal B[84].

- HER2-enriched

Around 10% to 15% of all breast cancer tumors are HER2 subtype. They are hormone-receptor negative (estrogen-receptor and progesterone-receptor negative) and overexpressed ErbB2. Several subtypes of breast cancer tumors express HER2, but this group presents more mutations. TP53 and PIRKCA appear in 72% and 39% of the cases[89], intermediate expression of luminal genes ESR1 and PGR, and low expression of cytokeratin 5 and FOXC1. These tumors tend to grow faster than luminal cancers and have worst prognosis but are typically treated successfully with targeted therapies as Trastuzumab, Pertuzumab or novel tyrosine kinase inhibitors as Lapatinib or Neratinib[90].

- Normal like breast cancer

This subtype is similar to Luminal A, accounting for 7.8% of the breast cancer tumors. It is characterized for being hormone-receptor-positive (estrogen-receptor and/or progesterone-receptor positive), HER2 negative. It is the only subtype that displays the tumor-initiating stem-cell phenotype with low expression of CD24, high expression of CD44, and high expression of vimentin and TWIST1[91]. Its prognosis is slightly worse, which means more aggressive than luminal A.

In an analysis of human and murine breast tumors was discovered the Claudin-low breast cancer subtype[92]. This subtype is associated with low expression of cell-cell adhesion genes, high expression of epithelial-mesenchymal transition (EMT), and stem cell-like related genes expression[93]. Besides, it presents immune and stromal cell infiltration, expressing high levels of PD-L1, reduced proliferation levels. In summary,

several studies considered claudin-low tumors as a breast cancer phenotype defining it as a heterogeneous tumor, but this classification is not currently applied in clinic.

2.3. Pharmacological treatment of breast cancer

2.3.1. Hormonal therapy

This therapy is based on those tumors which express hormonal receptors. The main objective is to reduce the effects of the hormones produced in the patients by these receptors. There are three strategies:

1. Suppression of the hormones produced in the ovary. In premenopausal women, the ovary is the highest producer of estrogens, so their suppression leads to a decrease in the whole levels. One option is the oophorectomy or, as a less aggressive alternative, the use of pharmaceutical agonist of the gonadotropin-releasing hormone (GnRH) which disrupt the circulating concentration of estrogen through their action on the hypothalamus-pituitary axis. Goserelin and leuprolide are examples of these compounds[94].
2. Inhibition of the estrogen synthesis. Aromatase inhibitors are currently used in the clinics to disrupt the biosynthesis of androgens into estrogens[95]. The third-generation compounds are: anastrozole, letrozole (nonsteroidal) and exemestane(steroidal)[96].
3. Disruption of binding of a ligand to estrogen receptor: Tamoxifen binds to the estrogen receptor-blocking the proliferative effect of estrogen on the mammary epithelium. In case of those tumors that do not respond, Fulvestrant is used as an estrogen receptor antagonist[97]

2.3.2. Chemotherapy

The main feature in tumor cells is their proliferative status. Chemotherapy drugs interfere in essential cellular processes to block proliferation through cell cycle arrest or

cell death. However, they are not highly specific and affect healthy tissues specially the most proliferative as the mucous membrane in the mouth, hair follicle, intestine, bone marrow and germinal cells; that is why cause serious side effects. Some of these drugs are:

1. Alkylating agents. They were the first non-hormonal compounds used as oncological treatment. They bind to the DNA strand blocking its replication. Some examples are nitrogen mustards (cyclophosphamide), cisplatin, nitrosoureas (carmustine, lomustine, and semustine), alkyl sulfonates (busulfan), aziridines (thiotepa), and triazines (dacarbazine).
2. Antimetabolites. They are molecules similar to DNA and RNA components to shift the naturally occurring compounds and block the cell's normal metabolic status. They are classified in these groups: folic acid analogues, pyrimidine analogues, cytidine analogues and purine analogues.
3. Anti-Microtube agents. Anti-mitotic agents through the disruption of the microtubule dynamics during cell division. Taxanes and Vinca alkaloids have this mechanism of action.
4. Topoisomerase II inhibitors. These inhibitors are split into two classes: *Topo II catalytic inhibitors* which eliminate the enzymatic activity of Topo II, blocking the enzyme turnover, belong to this group ICRF 193 and genistein. The second group, the *Topo II poisons*, lead to increase levels of Topo II and are named poison because these agents generate "lesion" that includes DNA strand breaks and protein covalently bound to DNA not allowing the correct replication and transcription. Some examples are doxorubicin, epipodophyllotoxins etoposide and teposide[98].

The mechanism of action of these compounds are clearly described in Goodman & Gilman's: Pharmacological Basis of Therapeutics[99]. Anthracyclines and taxanes are among the top choices for basal-like metastatic breast cancer and those who resist hormone therapy and have never been exposed to the treatment before[100]. Doxorubicin plus docetaxel combination is an approved protocol and widely used in the clinic for the last 20 years[101].

2.3.3. Targeted therapy

These therapies include compounds which target key molecules involved in vital mechanisms in the tumor cells. That means fewer side effects in non-tumoral tissues. Some of these treatments are:

1. Receptor ER+ tumors. Apart from hormone therapy, palbociclib, an inhibitor of CDK4/6, and everolimus, an inhibitor of the mTOR pathway, are currently approved by FDA[102].
2. Receptor ERBB2+ tumors. The currently approved antibodies against this receptor are trastuzumab and pertuzumab, humanized monoclonal antibodies against different epitopes in the extracellular region in ERBB2; and lapatinib, a tyrosine kinase inhibitor against the kinase domain of EGFR and ERBB2. A new strategy is the use of immunoconjugates, which includes a toxic particle in the antibody. In ERBB2 tumors, T-DM1 is approved as a combination of trastuzumab and emtansine, a microtubule inhibitor agent[103].
3. PI3K/AKT/mTOR pathway. This pathway is active in 70% of the tumors. Apart from everolimus, promising results in clinical trials suggest pictilisib, a PI3K inhibitor, as a future therapeutic[104] as well as an inhibitor of AKT[105].

2.4. Cardiotoxicity in breast cancer

The incidence of cancer treatment-induced cardiotoxicity depends on the cancer therapy used, duration of the treatment, and the patients. Up to 48% of cancer patients treated with anthracyclines suffer from cardiovascular problems after the treatment. This percentage includes the treatment of solid tumors as breast cancer and hematologic cancer as Hodgkin/non-Hodgkin, lymphoma, leukemias, among others[106].

Regarding the monoclonal antibodies, Trastuzumab-induced cardiotoxicity alone ranges from 3% to 7%, and when administered with other chemotherapeutics, specially anthracyclines, affect 27% of the patients[107]. By inhibiting ErbB2, trastuzumab reduces the protection from mitochondrial damage in cardiomyocyte[108].

An interesting retrospective cohort study evaluated on 2011 by Patnaik J, *et al.* [109] shows how the leading cause of death in diagnosed breast cancer patients are the cardiovascular events related to chemotherapy treatment (**Figure 3**).

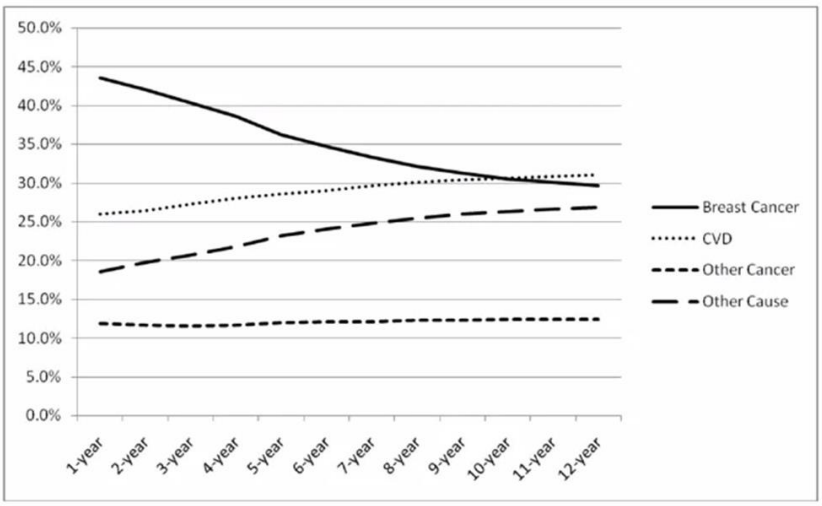

Figure 3. Distribution of leading causes of deaths in diagnosed breast cancer patients over the years. The image is taken from Patnaik J, et al 2011.

The study was a follow-up of 105 months in 63,566 women diagnosed with breast cancer. In the first ten years, breast cancer was the cumulative primary cause of death. Then cardiovascular diseases became the cumulative primary cause of death. The number of deaths due to other reasons also increased as years passed. According to the study some examples of complications are pneumonia or diabetes. The study concludes that older women diagnosed with breast cancer have the same probability of dying of cancer as of cardiovascular disease. This conclusion highlights the importance of having a follow-up in terms of cardiac side effects.

3. Multiple Myeloma

3.1. Etiology

Multiple Myeloma (MM) is considered a rare disease but is the second most common hematological cancer after non-Hodgkin disease. Very often MM begins with

a condition known as monoclonal gammopathy of undetermined significance (MGUS) this implies: presence of an abnormal protein antibody known as monoclonal immunoglobulin (M protein) at <3g/dl levels in the blood or urine, a small amount of abnormal plasma B cells in the bone marrow (less than 10%) and absence of damage in the target organs[110]. Not all the patients with MGUS developed MM, only a 1% progress to the active disease; some of them develop an intermediate stage between MGUS and MM called smoldering MM, is asymptomatic and present >3g/dl levels of M protein and 10%-60% of plasma cells in the bone marrow[111]. Then a 10% of these patients will develop MM in the first five years. The probability is higher than MGUS patients and after this time, the probability decreases.

It is believed that MGUS premalignant status came from a plasma cell that regained its capacity to proliferate through two possible different events. First, more than 50% of the MGUS and MM cells present hyperdiploid usually in the odd number chromosomes (3,5,7,9,11,15,19). Second, in the non-hyperdiploid chromosomes are present well-described translocations, t(11,14) in the gene *IGH* (Immunoglobulin Heavy-Chain) and t(14,22) in the *IGLL5* (Immunoglobulin Lambda Like Polypeptide 5) that confers more risk to develop the disease[112]. But as in order tumors, multiple hits take part in the disease; secondary translocations plus different genetic events drive the clonal evolution of MGUS to MM. Each newly diagnosed patient has mutations in a large number of genes but among the most repeated are *TP53, KRAS, NRAS, ATM*, *CCND1* and *FAM46C*[113] and is essential not to overlook that changes in tumor microenvironment accompany MM development.

3.2. Epidemiology

According to the American Cancer society in 2019 were diagnosed 32,110 of new MM cases: 18,130 men and 13,980 women, and about 12,960 deaths are expected to occur[74]. People diagnosed with MM are at least 65 years old, and only 1% of the cases are younger than 35 years old. Men are more likely to suffer the disease than women and some MM subtypes are up to 3 times more common in African Americans individuals as compare to European American[114].

Sometimes MM does not respond to the treatment (refractory) or return after 60 days of treatment, months or even years (relapses)[115]. That recurrence can be clinical, which implies the symptoms described in the next section or biochemical when there is a rise in monoclonal M proteins in serum or urine[116].

3.3. The common signs in MM and stages of the disease

The signs are used to stage the disease and can be described with the acronym CRAB[117]: C for calcium means hypercalcemia due to malignant plasma cells grow up into the bones. This high concentration causes renal failure. R for renal and kidney dysfunction caused by the accumulation of the M protein in the urine, as well as the induced by the hypercalcemia; advanced MM patients required dialysis. A for anemia, as a malignant plasma cells, take out space in the bone marrow and disrupt the production of blood cells. B for bone disease, particularly painful in the back and chest. Calcium is removed from the bones, so they become more fragile and thinner causing osteoporosis that increases the chance of breakage. Furthermore, the International Myeloma Working Group (IMWG) in 2014, added three new criteria in case of the patients do not present the symptoms clearly, clonal bone marrow plasma cells greater than or equal to 60%, serum-free light chain (FLC) ratio greater than or equal to 100 provided involved FLC level is 100 mg/L or higher, or more than one focal lesion on MRI[118].

Regarding the pathology of the disease, stages in MM can be classified using two different methods; the more recent and commonly used is the International Staging System[96] which evaluates four main things: concentration of Albumin, β2 microglobulin, lactate dehydrogenase and genetic changes. Based on those, there are three main stages. Stage I, it presents low levels of those molecules. In this stage, most people do not know they are suffering the disease until it is more advanced. Stage II, β2 microglobulin is a bit high but the other molecules present normal concentrations. Stage III, β2 microglobulin is very high, 5.5mg/L or more that indicates the disease is advanced. Also, albumin and lactate dehydrogenase are either elevated and high-risk of cytogenetic alterations.

3.4. Pharmacological treatment of Multiple Myeloma

3.4.1. Ubiquitin proteasome system

The ubiquitin-proteasome system (UPS) plays a critical multistep process in cellular protein homeostasis being necessary for cell physiology and survival. It is responsible for the degradation of unneeded or damaged proteins through proteolysis. The peptides resulting can be waste or recycled to produce new useful proteins. That implies processes as the adaptation to physiological conditions through the control of the proteins responsible for protein expression in response to stress.

This system starts with the coordination of three enzymes, E1, E2, and E3 ligases which ubiquitinate a specific protein to be degraded by the proteasome (**Figure 4**).

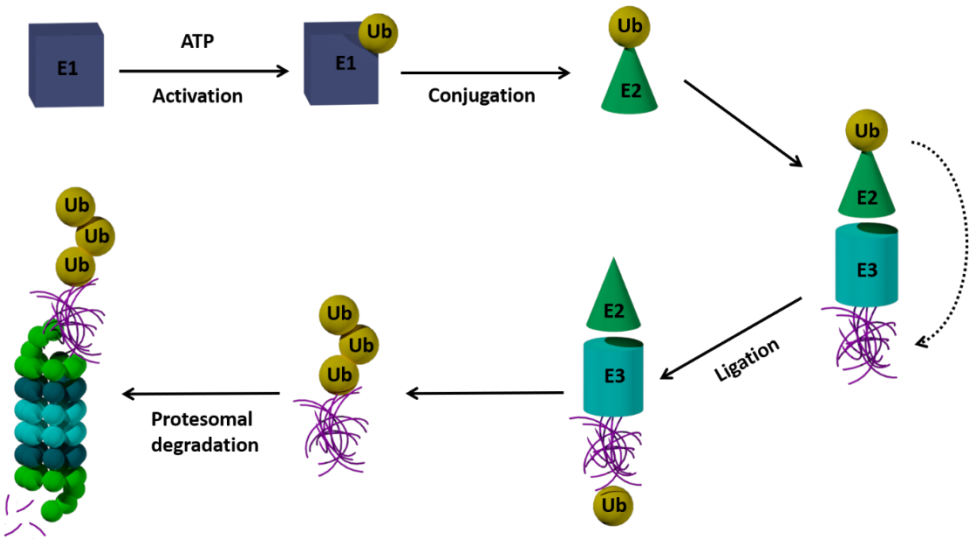

Figure 4. Overview of the Ubiquitin-Proteasome Pathway (UPP).
Dark blue, green and light blue structures are E1, E2 and E3 enzymes, respectively. The three main steps are activation, when E1 enzyme forms a thioester bond with the ubiquitin molecule in the presence of ATP; the next step, conjugation of the activated ubiquitin to the E2 enzyme, and then to the E3 enzyme. E3 transfers the ubiquitin to the lysine residue in the substrate protein. The target ubiquitinated protein is then recognized by the 19s cap of the proteasome.

The proteasome is a structure located in both places, in the cytosol, associated with the centrosomes, cytoskeleton and the outer surface of the endoplasmic reticulum, and in the nucleus along with the nucleoplasm, but not in the nucleoli. It is formed by a

conserved multicatalytic enzymes separated in two complexes: the 19S particle binds both sides end of the 20S core and its formed by the lid, responsible of selecting the proteins ready for degradation and performs the deubiquitylation process and the base, which is constituted of ATPase and non-ATPase subunits in charge of unfolded the proteins and translocation into the 20S core[119].

The 20S core is composed of 7 α and β subunits forming four heptameric rings. The outer α rings bind to both β rings and their primary function is being a gate through which proteins enter the proteasome. The β subunits are the proteolytic site, in three of the subunits rely the hydrolyzing activities that breaks the peptides bonds on the carboxyl site of acidic (Caspase-Like, C-L), basic (Trypsin-Like, T-L) and Chymotrypsin-Like (CT-L). All together complete the 26S proteasome (**Figure 5**).

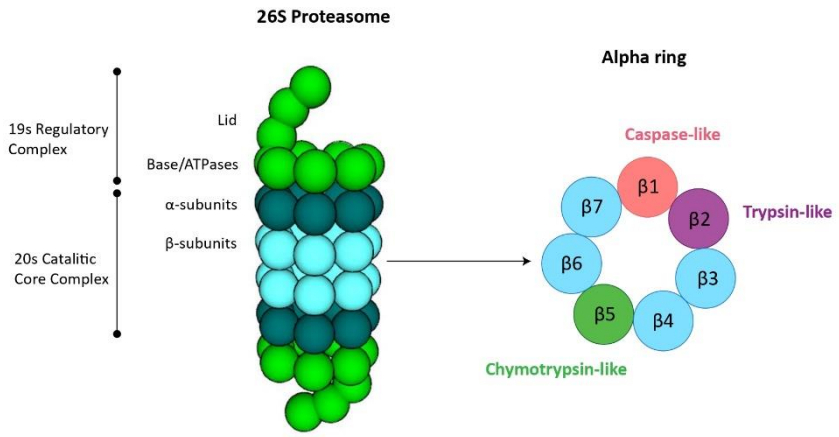

Figure 5. Proteasome 26S in detail

Green structure is the lid and the base (ATPase activity), together form the 19S Complex. Dark blue are alpha rings and light blue beta rings. On the right, the alpha ring in detail. Pink subunit; Caspase-like; Purple subunit; Trypsin-like and Green subunit: chymotrypsin-like.

- Proteasome inhibitors

The use of proteasome inhibitors has revolutionized the treatment of Multiple Myeloma disease. To date, three agents in this class have been approved by the FDA. The first was Bortezomib (PS-341, Velcade)[120] in 2003 which binds reversibly to the subunits caspase-like (β1) and chymotrypsin-like (β5), then in 2012 a second-generation agent Carfilzomib (PR-171, Kyprolis)[121] that binds irreversibly to the chymotrypsin-like subunit (β5) and finally, Ixazomib (MLN9708, Ninlaro), the first oral PI that reversibly binds to the three subunits[122]. Furthermore, three second-generation proteasome inhibitors are currently in Phase I/II investigations: Oprozomib, Delanzomib and Marizomib **(Supplementary Table 1)** with their different pharmacological properties they have demonstrated a safety profile and positive results in patients resistant to bortezomib[123–125]. These PIs in doublet o triplet regime with immunomodulators and glucocorticoids present promising results in relapsed or refractory patients (**Figure 6**).

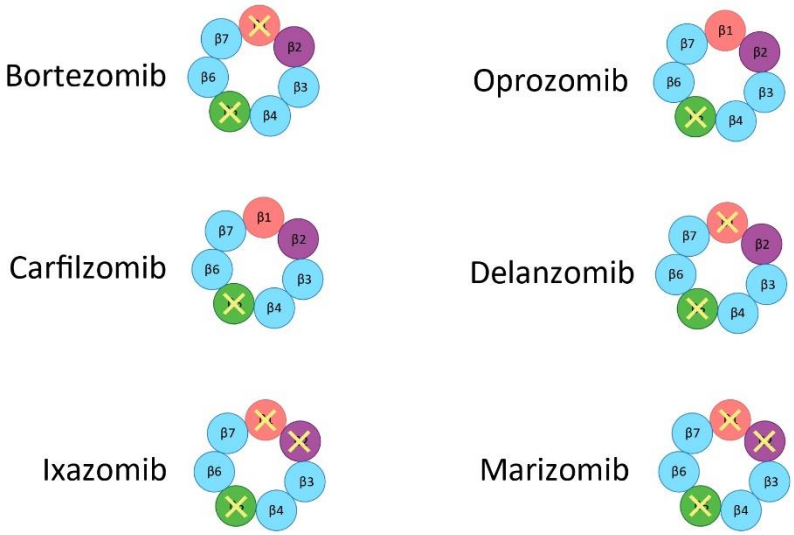

Figure 6. Six proteasome inhibitors and their target subunit
The yellow cross indicates at which subunit is bind each proteasome inhibitor. The color coded is the same as in Figure 5.

As other potential targets, the immunoproteasome, ubiquitin E3 ligases, the 19S proteasome, and deubiquitinates in pre-clinical studies represent possible directions for future generation inhibitors of the ubiquitin-proteasome system in the treatment of MM and other cancers[122].

- Glucocorticoids

Usually dexamethasone is the selected steroid for completing the combined treatment in multiple myeloma disease. It has been described several mechanisms in which the activated glucocorticoids inhibit the NFKβ pathway. In lymphocytes and monocytes have been proved that dexamethasone induces the transcription of the IkB gene that means an increase of NFKB inactive in the nucleus. However, further investigations describe how glucocorticoid response element (GRE) transactivation is entirely related to apoptosis in treated myeloma cell lines and how NFKβ activation or RAFTK phosphorylation are not required[126].

- Immunomodulators (iMiDs)

Immunomodulators work by different mechanisms in the treatment of MM. They have an anti-angiogenic effect, anti-inflammatory, anti-proliferative and immune-modulatory effects[127]. The survival success in any tumor cell is the endogenous tolerance offered by the immune system. Immunomodulators can activate T cells but only if they are previously activated by a CD3+T cell. Lenalidomide seems to be up to 2000 times more potent inducing T-cell proliferation than thalidomide and pomalidomide appears more potent than lenalidomide in terms of T-cell stimulation[128,129]. Apart from modulating the immune environment, iMiDs also present an antiproliferative effect inhibiting the cyclin-dependent kinase pathway. Their anti-angiogenic properties are not well established yet; some studies suggest that occurs via modulating some endothelial factors like VEGF, TNFα, or βFGF rather than direct inhibition of the endothelial cell proliferation[130].

3.5. Cardiotoxicity in Multiple Myeloma

The three current proteasome inhibitors approved by the FDA are a promising treatment for multiple myeloma. Their side effects in the heart were not monitoring from the beginning, and studies gradually emerge reporting every new case. Some of

the data reveal that patients treated with the combination Bortezomib, lenalidomide and dexamethasone present up to 8% arrhythmias, including atrial fibrillation and dysregulation of QT interval and up to 4% thrombosis. Regarding Carfilzomib there is an interesting parallel study[131] comparing two regimes Carfilzomib plus Lenalidomide, and Dexamethasone and Carfilzomib with Dexamethasone alone. The results showed that up to 6.4% of the patients treated with the first treatment and 4.1% treated with the second one, presented chronic heart failure; 5.9% ischemia heart disease with the first treatment and 4.6% in the second one; thromboembolic events in 10.2% with the first treatment and 6.2% in the second one and hypotension in 14.3% with the first treatment and 6.9% in second treatment. If Bortezomib plus Dexamethasone and Carfilzomib plus Dexamethasone are compared, it was found that 1.75% vs. 4.75% of the patients suffered from cardiac failure, respectively. Finally, Ixazomib is the newest proteasome inhibitor approved by the FDA, limited data are available of the cardiac effects of this drug until now only one case has been reported in the literature suggesting a potential adverse effect as other proteasome inhibitors.

Regarding the new proteasome inhibitors, they offer new advantages over the first generation in terms of via of administration (oral) and it seems to have potentially less toxicity. As a novel therapy, it forces the researches to study in-depth and developed new tools to predict and mitigate any side effect in cancer patients because the high-risk patient population remains unknown. With our study, we pretend to provide relevant information in the field.

4. Complex diseases and missing heritability

Susceptibility to suffer cardiotoxicity due to an oncological treatment is not driven by a unique gene but caused by multiple genes with a weak effect[132]. Even in cases when patients have genetic predisposition to suffer the disease, these genes have low penetrance and adjacent genes modified the risk. Regarding the anthracyclines-induced cardiotoxicity up to 28 studies have examined the association between the genetic variants and this undesirable side-effect, finding some risk variants which could

contribute to the development of the disease[133]. Furthermore, other features present in the disease as age, the oxidative stress factors, response to the treatment and the oncological disease itself are considered as complex phenotypes that are regulated by the effect of genes implied in several processes at different biological levels.

4.1. The missing heritability problem

Part of the phenotypic variability observed in a complex trait in a population has a genetic origin. It is determined by genetic differences between the individuals in the population and the influence of the environment on them. *Heritability*, broadly speaking, is defined as the proportion of phenotypic variance explained by the genotypic variance, while *heritability,* in the strict sense is the proportion of the phenotypic variability due to the additive genetic component, excluding the genetic determinants related to epistasis and gene dominance[134]. In linkage analysis, the genomic regions associated with the variability of a complex phenotype are called *quantitative trait loci* or QTL.

There are traits where the proportion of the phenotypic variance explained by the genetic component is enormous. As an example, the height of a person is explained by 80%-90% of the heritable genetic component[135]. Nevertheless, there has been identified several genetic variants associated with the variability in height in humans but together only explained 5% to 10% of the phenotypic variance. This is not a specific problem of the height. In general, there is a large discrepancy between the proportion of phenotypic variability that is believed it is due to genetic differences; and the percentage of this variability which could be directly attributable to the genetic variants previously associated with this phenotype. This discrepancy is known as *missing heritability*[136,137].

Regarding the complex diseases, two theories have been proposed to explain the genetic component and its influence. Initially, there was strong support for the so-called “common disease-common variant” hypothesis, which holds that disease susceptibility is explained by high allele frequencies in a concrete population[138]. The given examples were diseases as Alzheimer's or diabetes. Genome-wide association studies (GWAS)

were used as a powerful tool to identified genetic variants that may contribute to the disease; in fact, these studies enabled us to identify several QTL associated with complex phenotypes. However, the effect of every identified region only explained 1% to 2% of the phenotype variance and altogether no more than 30%[139]. So, GWAS studies failed to solve the problem of *missing heritability*[140].

Thus, a second hypothesis came out: the "common disease-rare variant," which argues that rare variants with relatively high penetrance in the population are the major contributors to common diseases[141]. GWAS are not adequate to detect these rare variants but massive sequence techniques allowed us to identify some of them which influence the complex phenotypes[142,143]. These studies proved that this new hypothesis is correct but still not solve the missing heritability problem. The genetic variants found do not explain a big part of expected heritability.

4.2. Intermediate phenotypes in complex traits

Any complex phenotype is a result of the effects of intermediate phenotypes; in other words, phenotypes that affect a lower biological level. Complex diseases as ischemic cardiopathy, diabetes or sporadic cancer are explained by these intermediate phenotypes participating in their pathogenesis. In some instances, the intermediate phenotypes are complex phenotypes, influenced by phenotypes from lower levels generating complex interacting networks between different processes at different levels that will determine the final phenotype, the disease. Thus, the variability of every intermediate phenotype influences the variability of phenotypes in the next levels and the final phenotype is a result of each intermediate phenotype contribution (**Figure 7**).

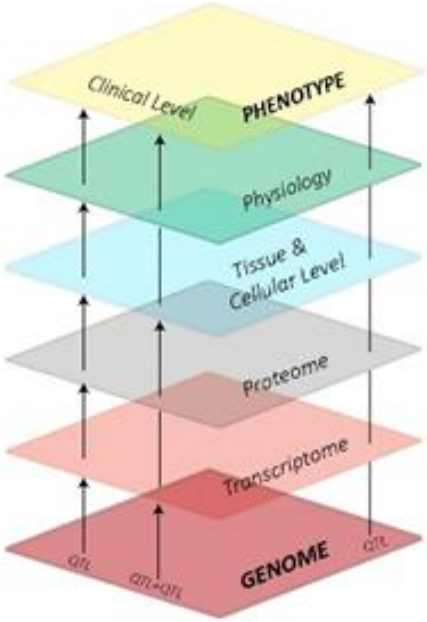

Figure 7. Intermediate phenotypes contribute to the pathogenesis of the complex disease through different levels: Systemic, organic/tissue, cellular and molecular. There are multiple interactions at each level and between levels. Besides, all are influenced by the genetic level and the environment.

The strategy of dividing a disease in different intermediate phenotypes to study the contribution of their genetic component in the genetic component of the disease was used mainly in psychiatry[144]. Psychiatric disorders are complex diseases whose diagnoses are based on features challenging to measure, so it is certainly complicated to find genetic determinants associated with this kind of neuropsychiatric disorders. That is the reason why in 2003, different intermediate phenotypes, as the neurotransmitters, were characterized as an easy way to identify the disease association parameters. And it is not the only field of study addressed, in other complex diseases as long QT syndrome[145], idiopathic hemochromatosis[146], juvenile myoclonic epilepsy[147] or familial adenomatous polyposis[148] were succesfully identified as well.

4.3. Missing heritability explained by genetic determinants and intermediate phenotypes

If we consider that complex trait is a consequence of the effect of their intermediate phenotypes, the genetic determinants associated with the principal phenotype, are contributing to the variability of this phenotype through their intermediate phenotypes. In this way, only the genetic determinants with a substantial effect on the central phenotype could be detected. For example, if we perform linkage analysis on a complex trait of interest, we will only identify those QTL with a robust active contribution in the association between these two traits. It could be possible that all the intermediate phenotypes that take part in the variability of a complex phenotype were not directly detected and related to the primary phenotype. Nevertheless, all the genetic determinants associated with the intermediate phenotypes will have an effect on the primary phenotype and those not detected could have and influence in the *missing heritability*[149].

That is the reason why we have proposed a strategy to identify a part of the missing heritability related to complex diseases studying the intermediate phenotypes which are participating in the pathogenesis of the disease, in our case, anthracycline-induced cardiotoxicity.

HYPOTHESIS AND GOALS

HYPOTHESIS AND GOALS

Oncological drug-induced cardiotoxicity is a complex disease. The susceptibility and evolution of this side effect varies between patients based on the environment influence and their genetic background[150]. As a polygenic disease their variability is due to complex interactions on the whole organism undertaken by the intermediate phenotypes. On the other hand, the use of hiPSCs-CMs has revolutionized the drug screening field giving us a safe disease human modeling to identify the possible cardiac side effects of novel drugs in the heart[151].

In this work we hypothesized that part of the susceptibility to develop the disease could be explained by differences in intermediate phenotypes. Prove this theory in humans is arduous, the population is too heterogenic and their interaction with the environment is difficult to control and quantify[152]. To accomplish this problem, we work in a heterogenic cohort of mice genetically simplify and in controlled environmental conditions. Furthermore, considering that nowadays is impossible to makes a primary culture of human heart if we use human embryonic stem cells-derived cardiomyocytes instead may help us to understand and classify new oncological drugs according to their possible toxicity.

To validate the first study, we assessed the drug-induced cardiotoxicity in mice with different genetic background, therapy regime and aging and then, trying to identify genetic determinants associated to this cardiotoxicity. We were interested in knowing to what extent the intermediate phenotype in response to treatment help to define the behaviour of the disease. First in the parental strains *so-called* F1 and FVB and then in the heterogenic cohort of mice generated through a backcross (BX2) which its genetic variability contributes to different response to treatment. To validate the second study, cardiomyocytes were differentiated form hiPSCs in order to work with a healthy human model. Different drug combinations, mimicking the first-line treatments in clinic and the current clinical trials in multiple myeloma, were tested to describe possible cardiotoxic effects as measured by contractility and voltage analysis.

Overall Goal

This works aims describe novel strategies to identified susceptibility to cardiotoxicity by anthracyclines and proteasome inhibitors both in combination with other compound as established in clinics in a context of breast cancer and multiple myeloma.

Specific goals

- Goal 1: To identify genetic and plasma markers of anthracycline-induced cardiotoxicity in patients by using intermediate phenotypes in the context of breast cancer.

- Goal 2: Screening of the specific cardiotoxicity produced by proteasome inhibitors and their combinations using hiPSCs-CMs in the context of multiple myeloma.

MATERIALS AND METHODS

MATERIALS AND METHODS

1. Animal models

1.1. Mouse strains

The inbred strains used in this study were: C57BL/6J mice that were resistant to breast cancer[153]. FVB/N strain, which is susceptible to develop breast cancer[154], both of them were collected from the *Laboratorios Charles River España*.

The FVB/N-Tg(MMTVneu)202Mul/J transgenic mice (Erbb2/Neu mice, from now), on FVB genetic background, carry the *Erbb2/Neu* protooncogene under the expression of the LTR of the Mouse Mammary Tumor Virus (MMTV) promoter. These mice were collected from Jackson Laboratories and generated in the laboratory of Dr. Muller[155]. The transgene contains the cDNA wild type version of the *Erbb2/Neu* gene from rat expressed under thee MMTV promoter; the construct also contains alternative splicings and polyadenylation sequences from the SV40 virus that gives stability to the transcript. The transgene does not induce phenotypic changes in males and is expressed at lower levels in the mammary epithelium, salivary glands, spleen, thymus, and lungs, but at high levels in tumors. In contrast, in females, induces breast adenocarcinomas surrounded by hyperplasic mammary tissue from 4 months of age with an average of 7 months. Besides, more than 70% of the mice develop lung metastases when older than eight months of age.

Animals were kept in the *Servicio de Experimentación Animal* in the *Universidad de Salamanca* under specified pathogens free (SPF) conditions. During the study the practices were under the provisions of the European Union under the Real Decreto *1201/2005* on October 10 at Ministerio de Agricultura, Pesca y Ganadería regarding the "*Protección de animales utilizados para experimentación y otros fines expecíficos*." The project was approved by the Bioethics Committee at the Universidad de Salamanca.

1.2. Mouse cohort generation with phenotypic and genetic variability by a backcross

The backcross strategy (**Figure 8**) aims to generate a mouse cohort with different behavior in a specific phenotype, cardiotoxicity induced by chemotherapy. Once the mice developed breast cancer, they were treated with chemotherapeutic agents under conditions similar to those used in clinics. The goal is to study the different cardiotoxic patterns in each mouse. As parental strains, we selected the resistant mouse strain to breast cancer C57BL/6J (F/F) and the susceptible strain FVB/NJ (B/B). After the cross, the offspring was genetically heterogeneous and carried an allele from each parental strain and were called the F1 (F/B) mice.

The F1 mice were backcrossed with the Erbb2/Neu mice under the susceptible FVB/NJ genetic background. The Erbb2/Neutransgenic mice carried the transgene in both chromosomes in homozygous status. Thus, the F1 backcross cohort (F1BX mice after that) generated carried the *MMTV-Erbb2/Neu* transgene in all the mice. Also, each mouse from the backcross cohort carried a unique combination of alleles from both strains, in a variable proportion. In this genetic background, the genetic component from the FVB strain was the majority since it was the one used to generate the backcross with the F1 mice. FVB alleles can be either in homozygous or heterozygous status, while the C57 component is reduced and is always in heterozygosity when it appears.

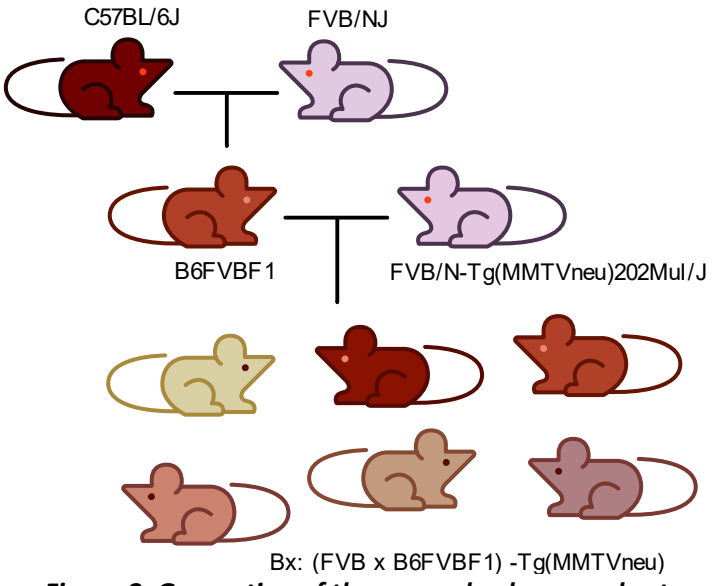

Figure 8. Generation of the mouse backcross cohort

1.3. DNA isolation and quantification

DNA was phenol-chloroform extracted using tail and heart tissues previously frozen in liquid nitrogen and stored at -80°C. Samples were incubated with 500µl lysis buffer (Tris 100mM pH 8, Etilenediaminetetraacetic acid (EDTA) 5mM, SDS 0.2%, NaCl 2mM) and 1µl of Proteinase K (100mg/µl) (Roche, #3115801001) overnight at 55˚C. The next day 500µl of phenol/chloroform/isoamyl alcohol (25:24:1) was added (AppliChem #A2493). The whole mixture was transferred to a 5PRIME Phase Lock Gel (QuantaBio #2302820) tubes, and after centrifugation for 5 minutes at 12000 rpm, the organic phase and the interphase materials are effectively trapped in or below the barrier gel, thus enabling complete and easy decanting or pipetting of the entire aqueous phase. This aqueous phase, that contains the DNA was taken into a new 1.5 mL tube (Eppendorf# 033871) with isopropanol (Fisher Reagents #W992DM). DNA is not soluble in Isopropanol, so it precipitates. Again, after 5 minutes of centrifugation at 12000 rpm, the supernatant was removed, and the DNA pellet was washed with 70% ethanol to eliminate salts and was centrifuged in the same conditions. After removing the supernatant and allowing the residual Ethanol to be evaporated completely, DNA extracted was resuspended in 100µl of buffer TE (Tris 100mM pH 8, EDTA 1mM) and was homogenized at room temperature at least one hour before use. The concentration of double-stranded DNA (dsDNA) was measured at a wavelength of 260 nm by a microspectrophotometer (Nanodrop ND-1000) in triplicate.

1.4. Detection of the MMTV-Erbb2/Neu transgene by PCR

A small piece of a tail was cut by a heating system *(Hot bead sterilizer, #18000-45, F.S.T.)* to get cauterization, hemostasis, and sterility at the same time and DNA was extracted from it (*see DNA extraction protocol*). The sequences of the primers were:

oIMR0386: 5’-TTTCCTGCAGCAGCCTACGC-3’

oIMR0387: 5’CGGAACCCACATCAGGCC-3’

The PCR program is described in the following **Table 1**. The size of the transgene was 600 pair bases (pb) and was visualized in a 1.5% agarose gel by electrophoresis.

Table 1: PCR program for the amplification of the MMTV-Erbb2/Neu transgene

Step	Temperature	Duration	Notes
1	94˚C	3 min	
2	94˚C	20 sec	x 12 cycles (-0.5˚C each cycle)
3	64˚C	30 sec	
4	72˚C	35 sec	
5	94˚C	20 sec	x 25 cycles
6	58˚C	30 sec	
7	72˚C	35 sec	
8	72˚C	2 min	
9	10˚C		Hold

1.5. Mouse Genotyping

Briefly, tail DNA concentrations were measured by the Nanodrop ND-1000 Spectrophotometer and by the PicoGreen double-stranded quantification method (Molecular Probes, Thermo Fisher Scientific Inc., Waltham, MA USA # P11495). For genotyping, the genome-wide scan was carried out at the Centro Nacional de Genotipado (CEGEN) at the Centro Nacional de Investigaciones Oncológicas (CNIO, Madrid, Spain). The Illumina Mouse Medium Density Linkage Panel Assay was used to genotype 130 F1BX mice at 1449 single nucleotide polymorphisms (SNPs). Genotypes were classified as FVB/FVB (F/F) or FVB/C57BL/6 (F/B). Ultimately, 806 SNPs were informative from the FVB and C57BL/6 mice; the average genomic distance between these SNPs was 9.9 Mb. The genotype proportion among the F1BX mice showed a normal distribution.

1.6. Chemotherapy treatment in the backcross population

The mice were treated with chemotherapy once they had developed breast cancer. Thus, we evaluated cardiotoxicity in 165 mice: the F1BX (N = 130) and in the FVB (N = 18) and F1 (N = 17) female mice.

One group (N = 88) was treated with a total of 25mg/kg of doxorubicin (Pfizer) in five intraperitoneal injections of 5mg/kg at ten days intervals[156], and another (N = 77) received a combined therapy of doxorubicin (Pfizer) (5 mg/kg) plus docetaxel (Sanofi Aventis) (25 mg/kg)[157], administered intraperitoneally every ten days under isofluorane anesthesia. The mice received at least four cycles of therapy and a maximum of five cycles when the chemotherapy was well-tolerated. Once the treatment had finished, the progress of the mice and tumor development was assessed for two months. Then, necropsies were performed, and the heart and other tissues were obtained.

1.7. Heart tissue processing

The mouse hearts were fixed in 4% paraformaldehyde (Scharlau FO) for 24 hours and then were processed in an automatic system (Shandon Excelsior, Thermo). The subsequent samples were sectioned, embedded in paraffin, and stained with hematoxylin-eosin with a standard protocol or with the Masson Trichrome Goldner kit (Bio-Optics) to evaluate for cardiac fibrosis and cardiomyocyte area.

1.8. Heart damage quantification

To evaluate the histopathological damage of the heart tissue from mice following chemotherapy, we quantified the area of heart fibrosis and the average area of the cardiac fibers as pathophenotypes of CDA using the ARIOL slide scanner. These pathophenotypes of cardiotoxicity were quantified in both the subendocardial and the subepicardial zones. Five areas were randomly chosen from each zone.

1.9. Protein isolation and quantification from heart mice

Approximately 10-15 mg of cardiac tissue previously-stored at -80˚C were homogenized using the FastPrep Homogenizer system (FP120, Bio 101 Thermo Savant) and ceramic beads (Precellys Lysing Kit CkMix, Precellys #10409) in Lysis buffer (Lysis Buffer 1X, Milliplex #43-040) through 2 simultaneous 10 seconds beats, 5.5 intensity (m/s) at 4 ˚C. The pulverized samples were incubated on ice for 20 minutes and then centrifuged at high speed at 4˚C, 5 minutes to allow solids not dissolved in the buffer formed a pellet. The supernatant was removed and filtered through 0.65µm pores, which is an essential step for the Luminex assay (see below). A 1:10 dilution was made, and protein quantification was carried out using the BCA Protein Assay test (BCA protein assay reagent A, 500ml, #23228, and BCA protein assay reagent B 25mL #23224), according to the manufacturer's instructions. Each sample was analyzed in triplicate. Absorbance was measured using the Ultra-Evolution TECAN and the Xfluor program at 570nm. Data were analyzed on Excel Office 365 software, and protein concentration was calculated by comparison between the standard curve made with known concentration levels of Albumin, which cover the expected range of the samples tested (Thermo Fisher Albumin standard #11811345) regarding their unknown protein concentrations.

1.10. Quantification of signaling proteins in heart samples: Luminex technology

Quantification of signaling proteins in protein lysates of hearts of the F1BX cohort was performed by multiplex assays with Millipore Luminex xMAP® technology (Milliplex®) following the manufacturer's instructions, and the Bioplex 200 device (BIO-RAD), borrowed from the Biochemistry Service of the Salamanca University Hospital and The Proteomic Unit in our Centre. Measurements were taken from distinct samples in 96-well plates. These multiplex assays have the advantage over ELISA that they allow quantifying the levels of various proteins in a single assay. They are based on the use of 6.45 µm diameter magnetic microspheres internally stained with a mixture of two

fluorescent dyes, and which have a capture antibody attached to them (**Figure 9**). The levels of total ATR, pCHK1(S345), pCHK2(T68), ɤH2AX, p-P53(S15), total MDM2 and total P21 were quantified using the 7-plex DNA Damage/Genotoxicity Magnetic Bead Kit (Milliplex Map Kit #48-621MAG, Millipore), to which the buffer-compatible MAPmates assays were added to quantify the levels of activated caspase-3, and β-tubulin (#46-713MAG). Another test used was the 9 Plex Multi-Pathway Magnetic Bead Kit (Milliplex Map Kit #48-680MAG), which included the reagents needed to quantify pERK1/2(T185/Y187), p-P38MAPK(T180/Y182), p-NFκB(S536), pJNK(T183/Y185), pAKT(S473), p-P70S6K(T412), pCREB(S133), pSTAT3(S727) and pSTAT5(Y694/699). Finally, we quantified the levels of TGFβ-1, TGFβ-2, and TGFβ-3 with the Milliplex MAP TGFβ Magnetic-Bead 3 Plex Kit (#TGFMAG64K-03, Millipore).

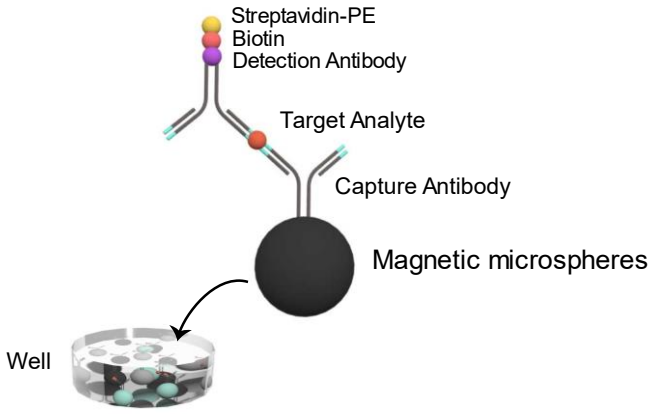

Figure 9: Luminex technology. The assay is performed in a 96-well plate with 25µg of protein. Each color-coded magnetic microsphere has a specific antibody on the surface (capture antibody), which binds to the protein of interest (target analyte). A second antibody is added (detection antibody-StreptavidinPhycoerythrin SAPE), and then with the Luminex®200™ system is analyzed.

1.11. Quantification of telomere length by QPCR

DNA from heart tissue was extracted and quantified following the protocol previously described (*see DNA isolation and quantification*). QPCR was performed following the conditions published elsewhere[158]. Briefly, PCR reactions were carried out in a total volume of 12 µl in Twin-Tec real-time PCR plates 96 (#0030132513, Eppendorf) with the following reagents: 6 µl of PerfeCTa SYBR® Green SuperMix ROX (#733-1188, VWR) (1X) and 1.6 µl of 150 mM forward and reverse telomeric primers (*5'-*

CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3' and 5'-GCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3', respectively). Also, the acidic ribosomal phosphoprotein PO (36B4) gene was used to normalize the amount of DNA. Forward and reverse primers for the 36B4 gene were *5'- ACTGGTCTAGGACCCGAGAAG-3' and 5'-TCAATGGTGCCTCTGGAGATT-3',* respectively, to which 1 µl of DNA (10 ng) and 3.4 µl double-distilled H2O were added. QPCR reactions were performed in triplicate following the conditions indicated. An automated thermocycler (Mastercycler ep Realplex2, Eppendorf) was used to carry out the PCR reactions, and data analysis was done using the $2^{-\Delta\Delta Ct}$ method[159] (*see Analysis of the Results section*).

1.12. Total RNA isolation

Total RNA from heart tissues was isolated using the QIAGEN®kit (RNeasy®, CAT#74104) following the manufacturer's instructions. Briefly, the heart tissues were disrupted and homogenized using the TissueRuptor at 4°C. The RNAs were extracted with a mix of buffers, followed by precipitation in ethanol, and purified using RNase-free columns. The RNA concentrations were determined using a spectrophotometer (Nanodrop) and microfluidic chips (Agilent).

1.13. cDNA synthesis from miRNA in the heart

The protocol chosen was obtained from the TaqMan Advanced miRNA cDNA synthesis kit (Thermo Scientific #A28007) to modify and amplify the small miRNAs in the sample. The protocol was based on four main sections:

- Poly(A) tailing reaction (55 minutes)

This step extended the 3' end with a polyadenylated (Poly A) tail to increase the stability and enhanced the translational process. The reagent's cocktail for each 2µl sample is shown in **Table 2**:

Table 2. Poly A tailing reaction. Amount of reagent in each sample.

Reagents	x1 sample(2µl)
10x Poly (A) Buffer	0.5 µl
ATP	0.5 µl
Poly (A) Enzyme	0.3 µl
Rnase-Free Water	1.7 µl
Total Volume	**3 µl**

The thermocycling settings are shown in **Table 3**:

Table 3. PCR program for the Poly A tailing reaction.

Step	Temperature	Duration
Polyadenylation	37˚C	45 min
Stop reaction	65˚C	10 min
Hold	4˚C	Hold

After this step, the ligation reaction was processed immediately.

- Ligation reaction (60 minutes)

Ligation reagents were thawed on ice during the Poly (A) tailing reaction was run in the thermocycler. In this step, the 5' ends of the miRNA were lengthened by an adaptor ligation. This step helped to enhance the expression profiles and the reproducibility between samples. The cocktail of reagents was prepared based on the following **Table 4**:

Table 4. Amount of reagents per sample for each ligation reaction.

Reagents	x 1 sample (2µl)
5x DNA Ligase Buffer	3 µl
50% PEG 8000	4.5 µl
25% Ligation Adaptor	0.6 µl
RNA Ligase	1.5 µl
RNase-Free Water	0.4 µl
Total Volume	**10 µl**

Polyethylene glycol (PEG) is extremely viscous, essential steps when performing this part are: working with the PEG at room temperature, when aspirating wait for 10

seconds before removing the pipette from the tube ,and, when dispensing in the cocktail tube keep the plunger 10 seconds more than needed, in other to allow all the PEG to be entirely dispensed.

The tube was incubated with the following settings (**Table 5**):

Table 5. PCR program for the ligation reaction.

Step	Temperature	Duration
Ligation	16˚C	60 min
Hold	4˚C	Hold

After the ligation part, the reverse transcription process was performed immediately.

- Reverse transcription reaction (20 minutes)

In this section, cDNAs were obtained from the modified miRNAs. The reaction mix was prepared based on the following table:

Table 6. Amount of reagent in each sample for the ligation reaction.

Reagents	x 1 sample (2µl)
5x RT Buffer	6 µl
dNTPs Mix (25mM)	1.2 µl
20x Universal RT Primer	1.5 µl
10x RT Enzyme Mix	3 µl
RNase-free water	3.3 µl
Total Volume	**15 µl**

And then was incubated following the next settings:

Table 7: PCR program for the reverse transcription reaction.

Step	Temperature	Duration
Reverse Transcription	42˚C	15 min
Stop reaction	85˚C	5 min
Hold	4˚C	Hold

At this point, the samples could be stored at -20˚C or proceeded to the amplification step.

- miRs amplification reaction (30 min)

This step was performed to increase the concentration of miRNAs homogeneously. The reaction mix is shown in the following **Table 8**:

Table 8: Amplification reaction. Reagent for each sample for this step.

Reagents	x 1 sample (2µl)
2x miR-Amp Master Mix	25 µl
20x miR-Amp Primer Mix	2.5 µl
RNase-Free Water	17.5 µl
Total Volume	**45 µl**

The thermocycler program was:

Table 9: PCR program for the amplification reaction

Step	Temperature	Duration	Cycles
Enzyme Activation	95˚C	5 min	1
Denature	95˚C	3 min	14
Anneal	60˚C	30 min	14
Stop Reaction	99˚C	10 min	1
Hold	4˚C	Hold	1

At this point, the samples were stored at -20˚C.

1.14. miRNAs quantification by QPCR in heart tissue

The miRNAs were quantified by Fluidigm BioMark HD technology and screened in 96 Dynamic arrays (96-96 Dynamic Array IFC for Genotyping #BMK-M-96.96 GT) (**Figure 10**). This tool allows us to perform 9,216 reactions and test 96 samples and probes simultaneously (**Table 10**). For the reaction, 1µl of each sample and 0.49µl of the

master mix were required to complete the assay. Samples were loaded in triplicates. The reaction occurred in the middle of the chip.

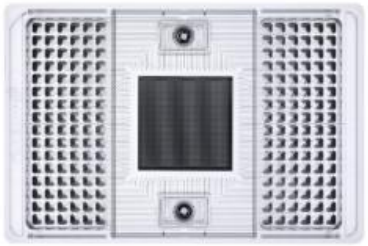

Figure 10. Chip Juno 96.96 Genotyping IFC. *The image was taken from the Fluidigm website:* **https://www.fluidigm.com/reagents/genomics/100-6499-juno-96-96-genotyping-ifc--1-ifc**

Table 10. List of the miRNAs studied. First, the name of the miRNA, then the TaqMan's probes name and finally the catalog number. In probes, hsa indicates the probes recognizes the homo sapiens sequence, but the mouse sequences for these miRNAs are the same.

miRNA	Probes	ID
miR-21a-5p	hsa-miR-21a-5p	477975_mir
let-7a-5p	hsa-let-7a-5p	478575_mir
let-7f-5p	hsa-let-7f-5p	478578_mir
let-7d-5p	hsa-let-7d-5p	478439_mir
	hsa-let-7d-3p	477848_mir
let-7i-5p	hsa-let-7i-5p	478375_mir
let-7c-5p	hsa-let-7c-5p	478577_mir
let-7e-5p	hsa-let-7e-5p	478579_mir
	hsa-let-7e-3p	479281_mir
let-7b-5p	hsa-let-7b-5p	478576_mir
let-7g-5p	hsa-let-7g-5p	478580_mir
miR-374-5p	hsa-miR-374b-5p	478389_mir
hsa-miR-497-5p	hsa-miR-497-5p	478138_mir
miR-210-3p	hsa-miR-210-3p	477970_mir
miR-100	hsa-miR-100-5p	478224_mir
miR-130a	hsa-miR-130a-3p	477851_mir
miR-152	hsa-miR-152-3p	477921_mir
miR-214	hsa-miR-214-3p	477974_mir
	hsa-miR-214-5p	478768_mir
miR-29b	hsa-miR-29b-3p	478369_mir
miR-34a	hsa-miR-34a-5p	478048_mir
miR-125b	hsa-miR-125b-5p	477885_mir
	hsa-miR-125b-1-3p	478665_mir
miR-128	hsa-miR-128-3p	477892_mir
miR-200b	hsa-miR-200b-5p	478753_mir
	hsa-miR-200b-3p	477963_mir
miR-451	hsa-miR-451a	478107_mir
miR-361-5p	hsa-miR-361-5p	478056_mir

The qPCR program used was: 50 °C (2 min), 70 °C (30 min) and 25 °C (10 min), followed by 50 °C (2 min) and 96.5 °C (10 min) for 40 cycles and then 96 °C (15 s) and 60 °C (1 min). Data were analyzed, and the cycle threshold (Ct) values were determined by the BioMark real-time PCR analysis software (Fluidigm Corp.).

DISCUSSION

DISCUSSION

Goal 1. To identify genetic and plasma markers of anthracycline-induced cardiotoxicity in patients by using intermediate phenotypes in the context of breast cancer

Cardiotoxicity due to chemotherapy is a common side effect that can be very grave and can modify the continuity of chemotherapy treatment[160,161]. Nowadays the cardio-oncology field try to predict the possible cardiac effects of every new treatment before being given into the clinical and also elucidate the susceptibility of each patient to suffer CDA which is very variable condition among the oncological patients[162,163]. Animal models and the novel human in vitro studies with the hiPSC-CMs are being used actively to reach this purpose increasingly tangible, the results that are being achieved are very close to what happens in the clinic[164]. Luckily in the future its reliability will be successfully corroborated in patients.

In the first part of the work there has been a great deal of effort to identify those patients susceptible to developing CDA using genetic markers[133] and the identification of plasma biomarkers[165] the so-called subphenotypes. As previously described along this work, susceptibility to chronic CDA has a significant genetic component, and as a complex disease or complex trait, indicates polygenic inheritance[166]. Moreover, the genetic component of complex characters is difficult to identify, and there is a much discrepancy between the proportion of phenotypic variance expected to be explained by genetic influences, known as the "expected heritability," and the heritability explained by the DNA Sequencing Variants (DSVs) identified. This difference is known as the "missing heritability"[137]. Thus, CDA, as a complex trait, has an unknown component of missing heritability and identify most of the genetic component is challenging.

Complex traits are influenced in their pathogenesis by a multitude of subphenotypes of lower rank. These subphenotypes, in turn, can be, to a greater or lesser extent, complex phenotypes. For example, myocardial infarction is a disease of complex origin whose susceptibility is determined by subphenotypes such as hypertension, hypercholesterolemia, or susceptibility to tobacco among others. All of

these subphenotypes are also complex traits influenced by lower rank subphenotypes. Below this multidirectional network of subphenotypes, there are molecular subphenotypes on which the genetic determinants act[149]. The use of subphenotypes to identify a part of the genetic component of complex traits has been previously proposed in the field of psychiatry[144] and later on applied to other areas[149]. We propose that part of the genetic component of the "missing heritability" could be explained by the QTLs associated with subphenotypes that participate in the pathogenesis of the complex trait. These QTLs linked to subphenotypes would not be strong enough to be detected by linkage analysis at the complex trait level. Thus, we suggest that the identification of QTLs associated with essential subphenotypes in the pathogenesis of a complex trait could be used to identify a part of the "missing heritability"[149].

Here, we have identified a part of the genetic component linked to molecular subphenotypes associated with CDA. This genetic component helps to identify a part of the genetic elements associated with CDA susceptibility itself. To identify putative molecular subphenotypes of CDA, we considered that anthracyclines exert their toxicity through DNA damage. Thus, CDA subphenotypes would be molecular pathways involved in DNA damage responses such as AKT[167] or ɣH2AX[168] related to protection and repairing of the DNA; molecules implicated in cell cycle arresting as chk1[169] and chk2[170] which are activated by genome instability and DNA damage; cell death through apoptosis mechanisms leading by MDM2, p53 and Caspase-3[171] and p21 which is also related to cellular senescence and cycle arrest[172–174].

But they are not the only, we also considered protein expression levels in intracellular signaling pathways related to several processes as cardiac hypertrophy where mechanical overload comprises the expression of signal-regulated kinases ERK1/2[175], JNK[176] , P38 MAPK[177,178],NFKβ[179], AKT[167]; STAT3 with a pivotal role upregulating genes related to heart protection, cardiac stress adaptor and pathological remodeling[180]; and p70S6K as part of the PI3K pathway, involved in cell death, fate and survival[181] among others[182]. Also, we hypothesized that other CDA subphenotypes could be molecules implicated in heart diseases and cardiotoxicity such as miRNAs[183] some of them tissue based-specific and recognized as a potential circulation biomarker in humans due to its stability and resistance to RNAase degradation[184]; and telomere which

length may be a predictor of cardiac events so because its deterioration is associated with cell ageing and oxidative stress, one of the common mechanism in DOX-induced cardiovascular diseases[185], although in the end was not correlated with damage response in our study.

Multiple regression models have allowed us to decipher which of these molecular subphenotypes determined in the myocardium of mice would have higher explanatory power on the phenotypic variability of the CDA. Then, we determined to which extent the QTLs associated with these molecular subphenotypes could help to explain chronic CDA. Indeed, QTLs linked to myocardium levels of p70S6K(pT412), JNK (pT183/pY185), pCREB(S133), or ɣH2AX(Ser139) increased the phenotypic variability explained that is associated with heart damage in mice treated with doxorubicin (**Figure12 and Figure13**).

Transferred to patients, allelic forms of the *RPS6KB1*, which encodes for p70S6K protein, *CREB,* and *TUBB* genes, also were associated with CDA (**Figure15**). We found p70S6K associated with CDA in four cohorts of different characteristics, two of patient ones and two of mice. This fact highlights the putative importance of allelic forms of p70S6K in the CDA susceptibility and pathogeny, and also in heart physiology. Indeed, it has been recently reported that after evaluating 65 protein kinases by an ex vivo test, the one that best predicted cardiotoxicity by these TK inhibitors was p70S6K[8], and also other drugs, including doxorubicin[186]. Also, expression of *RPS6KB1* gene resulted in a shortened action potential duration, and a shortened duration of the Ca^{+2} and contractility transients[8]. The protective heart effect of p70S6K has been suggested because its blocking has been associated with a greater area of necrosis in myocardial infarction models[187]. Also, p70S6K has been directly associated with heart fibrosis[181] and heart diseases[188,189]. Based on that It might be interesting in future experiments to test the expression levels of this protein in other oncological treatments which also have reported cardiotoxic events to confirm if they follow the same expression patterns.

Another attempt to identify patients susceptible to CDA has been through the determination of plasma biomarkers. Multiple biomarkers have been proposed, but there is not a good one that can adequately define the risk of CDA[165]. In this study, we have proposed that the quantification of molecules in plasma, whose levels in

myocardium were associated with cardiac damage in the backcross mice, could serve as plasma biomarkers of CDA. Indeed, high levels of oxidized DNA have been described in both the serum and myocardium of patients with heart failure[190]. Here, we observed an association between the plasma levels of some of these molecular subphenotypes associated with histopathological heart damage in mice and the degree of functional deterioration in humans by CMR (**Table 2**). In agreement with this, other studies have found a correlation between pathological damage and functional deterioration of the heart[191]. In any case, new studies in new patient cohorts would be needed to validate these results.

Goal 2: Screening of the specific cardiotoxicity produced by proteasome inhibitors and their combinations using hiPSCs-CMs in the context of multiple myeloma.

In the second section of the PhD thesis project are shown preliminary results of a small project developed through the use of hiPSC-CMs. The aim was performed a description of the responsiveness in terms of contractility and voltage of the cardiomyocytes in the presence of proteasome inhibitors and their combination therapy with immunomodulators and Dexamethasone. These compounds have revolutionized the treatment in Multiple Myeloma disease as first-line therapy and for relapsed/ refractory disease, but various clinical studies have started to document cardio toxic events in patients[192,193]. There is an added complication, the median diagnostic age of MM patients is around 70 years and at this diagnostic time, some of them present cardiovascular diseases or risk factors to develop them[194]. What makes this preliminary study attractive is the description in a human *in vitro* model the direct effect of the MM treatment in cardiomyocytes and being able to transfer this information to the clinic. Ideally in the future could contribute to distinguish between the cardiac pathology that already present some of the patients and the cardiotoxicity produced by the PI itself. As a last step being able to design personalized medicine to avoid this adverse side effects

in no-cardiac patients and improve the life quality in patients suffering from any cardiac complication.

After verifying the treatment efficiency of every drug combination on the Multiple Myeloma cell lines (always mimicking the clinic) was reproduced exactly the same treatment pattern in the human cardiomyocytes (hiPSC-CMs). These type of studies looks for any abnormalities in the action potential duration or contraction[195,196]. In our results there is a correlation between the voltage and the contractility values. We did not appreciate any indication of pro-arrythmia patterns as expected in Bortezomib[197] and Carfilzomib[131] which would have shown as an increase in the triangulation index[198]. By contrast at higher concentration levels, the effect on the cardiomyocytes was fulminant, we did not detect presence of cardiac activity at any PIs combination. This behavior is also found in their analogs Oprozomib and Delanzomib in monotherapy.

Regarding the voltage analysis, the triangulation index, the gold pro-arrythmia marker is low in some of the PIs such as Marizomib and all its treatment regime in which the values were stable but a bit low or Ixazomib and its combinations with Dexamethasone and Lenalidomide the values dropped unexpectedly. The explanation could be lower time in the cycle length (CL) consequence of action potentials which occur earlier than expected[199].

As promising exceptions: 1) Delanzomib in combination with Dexamethasone and Lenalidomide seems to be very well tolerated by cardiac cells. In the contractility and voltage analysis did not present any irregularity. These results fit with a Phase I/II study published in 2017 by Vogl DT, et al. [123] in which is described a limited cardiac toxic effect of this combination. In an attempt to explain the differences in response between Delanzomib alone and in combination was performed a proteasome activity assay that discarded the hypothesis that the three compounds were competing for the binding site. On the other hand, the option that Dexamethasone could be producing its therapeutic anti-inflammatory effect[200] and cushioning the toxic effect is not valid since our experiments are developed in an *in vitro* model, so there must be implied some surrounding mechanisms. As described in the literature, the glucocorticoid signaling in the heart is mediated by the glucocorticoid receptor (GR) and the mineralocorticoid

receptor (MR); both turnovers are regulated by the ubiquitin proteasome system and studies with the proteasome inhibitor MG132[201] showed how blocking the proteasome activity in the cells was translated in an increased on these two receptors. An increase in the glucocorticoid signaling due to GR has been described as beneficial for inhibiting cell death in DOX-induced cardiotoxicity[202] and improving the contractility performance[203] so, this could be an hypothesis on why the combination Delanzomib plus Dexamethasone and Lenalidomide did not produce an effect in the heart. This hypothesis has not being applied for explaining the rest of the PIs effects, although it is true, that long-term high-dose glucocorticoids have been related to negative effect such as abnormal conduction properties[204]. By contrast an excessive increase in MR activation in the heart has been related to inflammation leading to hypertrophy and fibrosis[205]. Studies that will demonstrate Dexamethasone binds more actively to this receptor instead of GR would explain the toxicity of certain drug combinations. Furthermore, in future experiments could be interesting test the proteasome activity in the hiPSCs-CMs and MM cell lines but only under the effect of Dexamethasone and Lenalidomide alone and see the effect on their viability without the PI influence.

2) Marizomib is presented as promising drug with high target effect on U266B1 cell line and no effect on cardiomyocytes. There are several clinical trials evaluating this compound[206,207] but briefings providing to date does not report cardiotoxic events. Also it is brain penetrant[208] so open more treatment options to oncological patients especially those with neurological affections.

These findings, although promising, are only preliminary results and need to be further validate in more cell batches.

CONCLUSIONS

CONCLUSIONS

Main Conclusion

Our studies in a backcross mice population show that genetic determinants and intermediate phenotypes can explain the susceptibility to suffer cardiotoxicity due to anthracyclines.

Furthermore, the use of hiPSCs-CMs can be tested new compounds to predict cardiotoxicity-induced by oncological treatments.

Specific conclusions

First:

The genetic background profoundly influences cardiotoxicity due to chemotherapy with doxorubicin and combined treatment with doxorubicin plus docetaxel. In this work, we have described part of the polygenic component and the intermediate phenotypes that explained part of the variability of this response in a model of genetic heterogeneity at the cardiac level.

Second:

We have proved that our strategy could help to identify susceptibility to CDA also in two different cohorts of patients, one diagnosed with breast cancer and other pediatric.

Third:

Through the use of hiPSCs, we have described how the combination with delanzomib plus lenalidomide and dexamethasone seems to be very well tolerated by the cardiac cells.

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APPENDIX

APPENDIX

1. FIGURE INDEX

2. TABLE INDEX

3. LIST OF ABREVIATIONS

ARIOL	Automated slide scanner and analysis microscope
ATR	Ataxia telangiectasia and Rad3-related protein
BCA	Bicinchoninic
cAMP	Cyclic adenosine monophosphate
CARTIER	Cardiotoxicity in elderly program
CDA	Cardiotoxicity due to anthracycline
CEGEN	Spanish National Centre of Genotyping
Chr.	Chromosome
cM	Centimorgan
CMR	Cardiac Magnetic Resonance Imaging
CNIO	Spanish National Cancer Research Center
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
EDTA	Ethylenediamine tetraacetic acid
FDA	US Food &Drug
F1	First mice generation from the cross between C57BL/6 and FVB.
F1BX	First mice cohort from the backcross.
GWAS	Genome-wide association studies.
hiPSCs	Human induced pluripotent stem cells
hiPSCs-CMs	Human induced-pluripotent stem cells derived-cardiomyocytes
hsa-miRNA	Homo sapiens micro RNA
LTR	Long terminal repeat
LVEF	Left ventricular ejection fraction.
MAP4K2	Mitogen-Activated Protein Kinase Kinase Kinase Kinase 2
Mb	Megabase
MDM2	Mouse double minute 2 homolog

miRNA	Micro RNA
MM	Multiple mieloma
p AKT	phospo Protein kinase B or PKB (activated form)
p CHK1	phospo Check point kinase 1 (activated form)
p CHK2	phospo Check point kinase 2 (activated form)
p CREB	phospo cAMP response element-binding protein (activated form)
p ERK1/2	phospo extracellular signal-regulated kinase 1/2 (activated form)
PIs	Proteasome Inhibitors
p JNK	phospo c-Jun N-terminal kinase (activated form)
p NFKß cells	phospo Nuclear factor kappa-light-chain-enhancer of activated B
p P38 form)	p38MAPK or Mitogen activated protein kinase p38 (activated
p P53	TP53 or tumor protein (activated form)
p STAT3 (activated form)	phospo Signal transducer and activator of transcription 3
p STAT5 (activated form)	phospo Signal transducer and activator of transcription 5
p P21	phospo Cyclin-dependent kinase inhibitor 1 (activated form)
p P70S6k	phospo Ribosomal protein S6 kinase beta-1 (activated form)
QPCR	Quantitative polymerase chain reaction
QTL	Quantitative trait loci
RNA	Ribonucleic acid
RPS6KA3	Ribosomal Protein S6 Kinase A3
RPS6KB1	Ribosomal Protein S6 Kinase B1
SNP	Single nucleotide polymorphisms
SV40	Simian Virus 40. Polyomavirus
TMRM	tetramethylrhodamine methyl ester
Tns	Troponines
TnI	Troponine I
TnT	Troponine T

TGFß-1	Transforming growth factor beta 1
TGFß-2	Transforming growth factor beta 2
TGFß-3	Transforming growth factor beta 3
y H2AX	gamma histone H2AX

RESUMEN

RESUMEN

Nuevas estrategias para la identificación de la susceptibilidad a la cardiotoxicidad por antraciclinas e inhibidores del proteasoma en pacientes oncológicos

Abstract

Antecedentes: La cardiotoxicidad debida a antraciclinas (CDA) es un problema frecuente en los pacientes oncológicos que limita el tratamiento quimioterapéutico y tiene repercusiones en la prognosis de la enfermedad. Además, la CDA a largo plazo tiene un impacto en la calidad de vida de los pacientes[160,161,209]. Las antraciclinas producen necrosis aguda y apoptosis en los cardiomiocitos causando fibrosis miocárdica y daño crónico funcional llegando, en ocasiones, al fallo cardiaco[210,211]. El grado de CDA crónica depende de múltiples factores entre ellos la dosis de fármaco, edad, género, enfermedades cardiacas previas y el tratamiento combinado con otras drogas, como los taxanos entre otros[162,163]. Además, debido a la dificultad de identificar pacientes susceptibles a desarrollar CDA crónica, se ha puesto mucho esfuerzo en tratar de identificar biomarcadores en plasma. Sin embargo, ensayos clínicos recientes no han demostrado hasta ahora que los biomarcadores sean una herramienta útil para la predicción de CDA en pacientes oncologicos[165,212]. Lo mismo ocurre con los marcadores genéticos, a día de hoy hay varias publicaciones que proponen candidatos para predecir la susceptibilidad a desarrollar cardiotoxicidad debido a quimioterapia[133] pero ninguna definitiva. CDA se comporta como una enfermedad de rasgo complejo con un componente poligénico[166]. Sin embargo, el problema de los rasgos complejos es cómo mejorar la identificación de su influencia genética. La proporción de variabilidad fenotípica explicada por el componente genético se conoce como heredabilidad. Las variantes genéticas identificadas asociadas con las enfermedades de rasgo complejo contribuyen en un 10-20% de la variabilidad genética atribuible a la genética[149]. El resto es considerado como heredabilidad perdida y sus causas e identificación son un tema de debate[136,213]. Además, los rasgos complejos son consecuencia de una serie de

fenotipos menores que participan en su fisiopatología y patogenia, y que se denominan subfenotipos, fenotipos intermedios o endofenotipos[144].

Hipótesis y Objetivos: En la primera parte de este estudio propusimos que diferencias en la longitud telomérica, así como diferentes niveles de expresión de miRNAs y proteínas implicadas en diferentes rutas de señalización podrían participar en la susceptibilidad a la CDA y se asociarían al daño histopatológico que se produce en la misma. Por tanto, serían subfenotipos moleculares de la CDA. Aquí, hipotetizamos que los genes que determinan dichos subfenotipos moleculares podrían contribuir a la heredabilidad perdida de la CDA; y formas alélicas de esos genes podrían ayudar a identificar a los pacientes susceptibles a la misma[149]. Además, pensamos que esos subfenotipos moleculares determinados en plasma, podrían servir como biomarcadores de la CDA en pacientes.

En una segunda parte de este trabajo de tesis doctoral, llevamos a cabo un estudio preliminar con el fin de predecir la susceptibilidad a la cardiotoxicidad in vitro de los inhibidores del proteasoma solos y en combinación con inmunomoduladores y glucocorticoides, por ser los fármacos con los que habitualmente se utilizan en la clínica. El estudio se llevó a cabo en cardiomiocitos humanos derivados de células pluripotenciales inducidas (hiPSCs-CMs). En cuanto a las combinaciones elegidas, las determinamos en base a las establecidas en la clínica o en los ensayos clínicos que se desarrollan actualmente[123,125]. En ambos estudios el objetivo fue ser capaces de identificar pacientes susceptibles a desarrollar cardiotoxicidad debido a la quimioterapia con estos fármacos.

Pacientes, material y métodos: Generamos una cohorte de 130 ratones genéticamente heterogénea a través de un retrocruce o *backcross*. Los ratones fueron tratados con doxorrubicina o doxorrubicina más docetaxel, una vez desarrollaron cáncer de mama[157]. El grado de daño histopatológico después de la quimioterapia fue evaluado mediante la cuantificación de la fibrosis cardiaca y el grosor de los cardiomiocitos. Cuantificamos en el miocardio los niveles de una serie de proteínas que participan las vías de respuesta a la genotoxicidad y de las rutas de señalización mediante Luminex; así como los niveles de miRNAs y la longitud telomérica por QPCR. Después, evaluamos su asociación con el daño cardiaco histopatológico. Después se evaluó si formas alélicas de los genes que

codifican las proteínas asociadas con CDA (subfenotipos) en ratones, se asociaron con CDA en dos cohortes de pacientes tratados con antraciclinas, una formada por pacientes pediátricos, con diferentes tipos tumorales, y la otra por pacientes adultos tratadas de cáncer de mama. Además, esas moléculas fueron cuantificadas en plasma en pacientes cuyo grado de CDA fue medido por resonancia magnética cardiaca (cRMN).

En la segunda parte del estudio, se evaluó la cardiotoxicidad in vitro de seis inhibidores del proteosoma: bortezomib, carfilzomib, ixazomib, oprozomib, delanzomib y marizomib; y su combinación con dos inmunomoduladores: lenalidomida y pomalidomida; y un glucocorticoide, Dexametasona. La elección de las concentraciones se basó en los valores de IC50 y Cmax de los fármacos; y las combinaciones de los fármacos se establecieron en función de los tratamientos que se llevan a cabo en la clínica o ensayos clínicos. La cardiotoxicidad in vitro se evaluó con la determinación de los cambios en la contractilidad y el voltaje mediante el sistema de detección IC200 (Vala Sciences, California, USA)[59].

Resultados: como ocurre en humanos, los ratones desarrollaron más CDA con la edad y con la terapia combinada. Se encontraron diferencias de CDA según su fondo genético. Identificamos 16 moléculas cuyos niveles de miocardio correlacionaron con daño histopatológico de la CDA. El análisis multivariante demostró que las moléculas con más implicación en el daño cardiaco fueron p70S6K(pT412), γH2AX(S139), P38MAPK(pT180/pY182), CREB(S133) y miRLet7b_5p. En concordancia con la naturaleza poligénica de la CDA, identificamos 80 loci de carácter cuantitativo (QTLs) ligados a la CDA. Entre ellos, como prueba de concepto, elegimos un QTL localizado en el cromosoma 4 (QTL-Chr.4) y otro en el cromosoma 11 (QTL-Chr.11) que se asociaron con daño cardiaco en diferentes condiciones. Los QTL ligados a los niveles en el miocardio de p70S6K(pT412), γH2AX(S139), y JNK(pT183/pY185) mejoraron la variabilidad fenotípica de la fibrosis cardiaca explicada por el QT-Chr.4 y QTL-Chr.11 en ratón. Los genotipos de formas alélicas de los genes *RPSKB1,* que codifica para P70S6K, *CREB5* y *β-TUBULIN-6* se asociaron con susceptibilidad a la CDA en pacientes. Además, la cuantificación en plasma de pacientes de las proteínas relacionadas con CDA en ratón, como p70S6K(pT412) y JNK (pT183/pY185), se asociaron a CDA cuantificada por cRMN en pacientes.

En la segunda parte del estudio, los resultados obtenidos permitieron clasificar a los inhibidores del proteasoma en tres grupos: primero los inhibidores de proteasoma cardiotóxicos que incluyen bortezomib y carfilzomib, que a altas concentraciones tenían un efecto fulminante en los cardios deteniendo su actividad; segundo, los inhibidores de proteasoma no cardiotóxicos: oprozomib, ixazomib y marizomib; y tercero, y más interesante, la combinación de delanzomib en combinación con inmunomoduladores y dexamethasona, que parece tener un efecto cardioprotector sobre la toxicidad producida por delanzomib en monoterapia. Esta prometedora combinación está actualmente en ensayos clínicos, pero desconocemos si en pacientes se está observando el mismo efecto cardioprotector observado in vitro.

Conclusiones: Nuestra estrategia puede servir para descubrir marcadores genéticos y de plasma de la CDA que podría ayudar a identificar pacientes más susceptibles de sufrir esta condición. Ello podría también ayudar a elegir tratamientos de quimioterapia y ajustar la dosis de forma más individualizada. La identificación del componente genético asociado a los niveles de proteínas en el miocardio asociadas con daño histopatológico después de la quimioterapia en una cohorte de ratón heterogénea ayudó identificar de parte del componente genético de la denominada “heredabilidad perdida”, en ratones y, probablemente, en humanos. Además, la cuantificación de esas moléculas en el plasma de pacientes podría servir como biomarcadores de CDA. La identificación de factores genéticos y moleculares responsables del incremento en el riesgo de CDA podría contribuir a predecir y prevenir el CDA.

Además, el uso de hiPSC-CMs podría ser una herramienta prometedora para estudiar la la susceptibilidad de los pacientes a la cardiotoxicidad por drogas oncológicas[214]. En este estudio, hemos sido capaces de clasificar los inhibidores del proteasoma en tres grupos: cardiotóxicos, no cardiotóxicos y –Delanzomib, cuyo efecto cardiotóxico se amortiguó con el tratamiento combinado.

Implicaciones traslacionales: Identificar aquellos pacientes que desarrollarán CDA es una tarea difícil. Aquí proponemos una estrategia para identificar marcadores genéticos y biomarcadores en plasma para ayudar a identificar esos pacientes.

En la segunda parte de nuestro estudio, a través de la descripción de los efectos los inhibidores de proteasoma en un modelo humano in vitro, tratar de predecir la posible cardiotoxicidad en pacientes de MM. Incluso en aquellos fármacos que aún no han sido aprobados para su uso en clínica.

INTRODUCCIÓN

1. Cardio-oncología

1.1. Susceptibilidad a la cardiotoxicidad

La cardiotoxicidad debida a la quimioterapia es un problema grave en pacientes oncológicos. Es la principal causa de morbilidad y mortalidad a largo plazo entre los supervivientes de cáncer[1].

En cuanto a la CDA, aun hoy, no se puede identificar quién va a desarrollar cardiotoxicidad bajo un mismo régimen de tratamiento con antraciclinas. La susceptibilidad individual a la cardiotoxicidad es una enfermedad de rasgo complejo, consecuencia de interacciones entre factores genéticos y medioambientales. Además, se necesitan identificar nuevos biomarcadores que ayuden a predecir individuos potencialmente susceptibles de desarrollar cardiotoxicidad. La presencia de niveles elevados de ciertas moléculas se podría utilizar detectar la patología cardiaca en fases tempranas de la enfermedad de manera más específica. Esta sección de la introducción se centra en aportar información sobre los biomarcadores genéticos y cardiacos que podrían ayudar a predecir eventos cardiotóxicos en pacientes oncológicos.

1.1.1. Predicción de la cardiotoxicidad mediante factores genéticos

Esta aproximación permite identificar marcadores tempranos de cardiotoxicidad a través de potenciales variantes genéticas que podrían contribuir a predecir la cardiotoxicidad antes de empezar el tratamiento y desarrollar agentes cardioprotectores para futuros tratamientos.

- Tratamiento quimioterapéutico: antraciclinas

Actualmente, hay identificados varios SNPs directamente implicados en la cardiotoxicidad por antraciclinas. La mayoría están relacionados con daño al ADN, estrés oxidativo, metabolismo del hierro, transportadores de las antraciclinas o disfunción del sarcómero en la célula cardiaca. Algunos ejemplos podrían ser, el gene RPS6KB encargado de codificar la proteína p70s6k y MAP4K2, ambas implicadas en la ruta de las MAPK[8] o más concretamente en pacientes pediátricos el gen (*GPR)35* (rs12468485) relacionado con decremento de la actividad celular y estrés oxidativo después del tratamiento con quimioterapia[12].

- Tratamiento con inhibidores del proteosoma

No hay muchos estudios centrados en este campo, aunque cada vez despierta más interés. Caben destacar dos muy interesantes, relacionados con el inhibidor del proteasoma de primera generación, Bortezomib, y el de segunda generación, Carfilzomib. En ambos casos, se comprobó que genes implicados en la supervivencia celular y la apoptosis son los responsables de la susceptibilidad a desarrollar cardiotoxicidad con estos tratamientos. El incremento en la activación del factor de transcripción NFKβ se relacionó, en ambos casos, con cardiotoxicidad[14,15]. En el caso específico del Carfilzomib cardiotoxicidad estaría relacionada con un desequilibrio en la expresión de genes implicados en la expresión del factor natriurético tipo B (BNP) y el complejo mayor de histocompatibilidad (MHC)[15].

1.1.2. Predicción mediante biomarcadores

Existe un creciente interés en la identificación de biomarcadores útiles a la hora de detectar cardiotoxicidad de forma temprana, para predecir el riesgo de sufrirla y para identificar pacientes susceptibles a sufrir de cardiotoxicidad tardía; esto últimos en general se trata de largos supervivientes de la enfermedad oncológica. La explicación es

que son una alternativa rápida ya que mediante un análisis de sangre rutinario se podrían detectar[16].

Algunos de los biomarcadores actualmente aprobados en clínica son la troponina T[20], el péptido natriurético de tipo B (BNP)[215], la porción N-terminal del pro-péptido natriurético de tipo B (NT-proBNP)[216] y miRNAs como miR-1[183] o miR-21[217] .

1.2. Clasificación de la cardiotoxicidad por fármacos

Durante más de una década los agentes quimioterapicos causantes de cardiotoxicidad han sido divididos en dos grupos principales[40]. Los de tipo I son fármacos que provocan daño irreversible en el corazón, debido a su acumulación progresiva a medida que transcurren las dosis. Ejemplos de este tipo son la doxorrubicina, docetaxel o la Ciclosporina Los de tipo II o de cardiotoxicidad reversible incluyen anticuerpos monoclonales como trastuzumab o sunitinib, en este caso la cardiotoxicidad no es dosis-dependiente[41]. De acuerdo con un estudio reciente, esta clasificación no sería muy acertada y propone que lo correcto sería establecer una clasificación basada en los mecanismos de acción y patrones de cardiotoxicidad[43].

1.3. Herramientas de diagnóstico de la cardiotoxicidad

En los pacientes con cáncer de mama mayores de 65 años, las enfermedades cardiovasculares son la primera causa de muerte[46]. Esto se debe a dos factores fundamentales: el primero, el impacto directo de los tratamientos oncológicos sobre el sistema cardiovascular y, segundo, a la falta monitorización para prevenir daño cardiaco, la cual nunca se lleva a cabo hasta que aparecen los primeros síntomas. Además, varios estudios sugieren que una vez aparecido el daño cardiaco, particularmente en el tratamiento con antraciclinas, la recuperación de la función de la fracción de eyección del ventrículo izquierdo ocurre solo en un porcentaje muy bajo de los pacientes[47]. Este escenario hostil hace que la Cardio-oncología emerja como uno de los campos con

mayor interés futuro dentro de la Cardiología e, indirectamente, de la Oncología. A día de hoy, la estrategia llevada a cabo por los cardiólogos es la prevención[16].

Dentro de las técnicas de imagen utilizadas para llevar a cabo esta prevención encontramos el ecocardiograma, global longitudinal strain (GLS), ventriculografía nuclear isotópica (MUGA) y la resonancia magnética cardiaca (CMR).

1.4. Test en cardiomiocitos humanos derivados de células pluripotenciales inducidas (hiPSCs-CMs)

1.4.1 Medicina personalizada y screening de drogas.

El descubrimiento de las células madre pluripotenciales inducidas ha abierto nuevas opciones para testar en un modelo celular la toxicidad de nuevos fármacos y permitir a las compañías farmacéuticas moverse de forma segura desde el laboratorio hasta los ensayos clínicos[58]. Uno de los beneficios de utilizar esta tecnología es que estas células se pueden generar *in vitro* de manera ilimitada y recapitularían la cardiotoxicidad que se produciría en el corazón de los individuos de los que derivan las IPs[218].

Sin embargo, existen limitaciones a la hora de utilizar los cardiomiocitos derivados de las IPs. Son cardiomiocitos inmaduros a nivel funcional, el cultivo celular es homogéneo y carecen de estructura 3D[60]; pero, a día de hoy, son la tecnología más adecuada para estudiar enfermedades cardiacas en un modelo que recapitularía mejor la patología del paciente donante de las IPs. Por ello su uso es muy interesante para llevar a cabo una medicina más personalizada en estadios tempranos en la detección de cardiotoxicidad, así como para evaluar la posible cardiotoxicidad de nuevos fármacos antes de su evaluación en pacientes y su uso clínico.

En el caso de las antraciclinas, existen varios grupos de investigación que centran su atención en estos compuestos, pudiéndose establecer estudios comparativos de la cardiotoxicidad entre familias de fármacos. Así, se describe, por ejemplo, como el Trastuzumab[65] provoca un defecto en la contractilidad de las hiPSCs-CMs sin que haya

muerte celular, mientras que las antraciclinas afectan a seriamente la viabilidad de las células mediante procesos de apoptosis.

Si hablamos de inhibidores del proteasoma (PIs) y sus combinaciones, los tres fármacos actualmente aprobados por la FDA, Bortezomib, Carfilzomib e Ixazomib han registrado diferentes grados de cardiotoxicidad en la clínica[71]. Además, se encuentran en ensayo clínico dos análogos a los anteriores (Oprozomib y Delanzomib) y, uno nuevo, el Marizomib, de los cuales aún se necesita dilucidar si provocan cardiotoxicidad. La opción de testar estos fármacos en hiPSCs-CMs, como se ha llevado a cabo en este trabajo, nos parece una excelente alternativa para a obtener datos preliminares sobre posibles efectos cardiacos adversos.

2. Cáncer de mama

1.1. Epidemiología y tipos de cáncer de mama

Las estadísticas actuales sitúan al cáncer de mama como la enfermedad oncológica más frecuente y la segunda causa de mortalidad en mujeres de todo el mundo, después del cáncer de pulmón[74]. En 2018, se registraron alrededor de 2 millones de casos nuevos y el número de muertes fue alrededor de 600.000, lo cual supone, aproximadamente, un 15% de todos los canceres registrados en mujeres[75]. El pasado año 2019, se registraron 300.000 casos nuevos en mujeres y 2670 en hombres, de acuerdo con la *American Cancer Societ*y[76].

En cuanto a la clasificación de los tipos de cáncer de mama. Es una enfermedad heterogénea que se clasifica en diferentes subtipos basados en los estudios pioneros llevados a cabo por Sørlie *et al.*[79,80]. Así, se definen tumores Luminal A y B, Triple negativo, HER2, y *Normal-like*; además, en una clasificación reciente, no aplicada aun en la clínica, se incluye un nuevo tipo, el Claudin-low[92].

1.2. Cardiotoxicidad en el cáncer de mama

Hasta un 48% de los pacientes oncológicos tratados con antraciclinas desarrollan problemas cardiovasculares después del tratamiento[106]. En el caso de los anticuerpos monoclonales como el Trastuzumab, los porcentajes varían del 3% al 7%[107]. Un estudio retrospectivo publicado en el año 2011[109] revela como la mayor causa de muerte en pacientes diagnosticados con cáncer de mama es debido a eventos cardiovasculares consecuencia del tratamiento con quimioterapia. **Figura 1.**

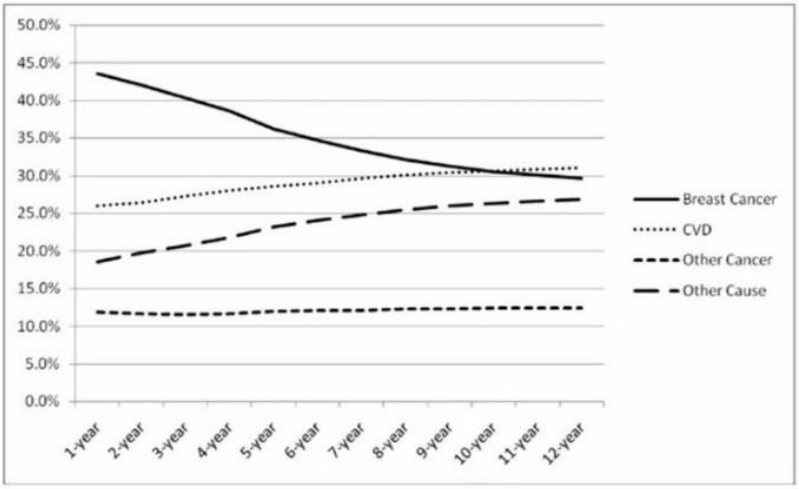

***Figura 1*. Distribución de las causas de muertes registradas en pacientes de cáncer de mama a lo largo de los años.** *CVD: eventos cardiovasculares. La imagen ha sido tomada de Patnaik J, et al. 2011.*

El estudio fue llevado a cabo en 63,566 mujeres durante 105 meses. La conclusión obtenida fue que mujeres más mayores diagnosticadas con cáncer de mama fueron igualmente propensas a morir tanto por la enfermedad oncológica como por enfermedades cardiovasculares. Esto pone de manifiesto la importancia de tener un seguimiento l cardiológico, tanto durante como a posteriori, del tratamiento con quimioterapia del cáncer.

3. Mieloma múltiple

3.1. Epidemiología y estadios de la enfermedad

De acuerdo con la *American Cancer Society,* en 2019 fueron diagnosticados 32.110 casos nuevos de mieloma multiple (MM): 18.130 hombres y 13.980 mujeres[74]. Los pacientes diagnosticados con MM, en general, fueron mayores de 65 años y solo un 1% de los casos fueron más jóvenes de 35 años. Los hombres son más propensos a sufrir la enfermedad que las mujeres[114].

En algunas ocasiones, los pacientes de MM no responden al tratamiento (se hacen refractarios al mismo) y recaen después de haber pasado 60 días de una buena respuesta r, aunque en ocasiones la recaída sucede meses o incluso años después de la buena respuesta inicial (recidiva)[115].

A la hora de describir los síntomas de la enfermedad se utiliza el acrónimo CRAB[117]. C para el calcio (hipercalcemia), R para describir la disfunción renal, A para la anemia y B para la afectación ósea (bones). En cuanto a los estadios de la enfermedad, estos son tres y están muy bien detallados en la *International Myeloma Working Group (IMWG)*[100,118]

3.2 Tratamiento y cardiotoxicidad en el mieloma múltiple

El uso de los inhibidores del proteasoma (PIs) ha revolucionado el tratamiento del Mieloma múltiple. A día de hoy, hay tres de esos fármacos aprobados por la FDA: Bortezomib (PS-341, Velcade)[120], Carfilzomib (PR-171, Kyprolis)[121] e Ixazomib (MLN9708, Ninlaro)[219].

Además, hay un grupo de tres PIs de segunda generación que se encuentran en fase II/III en ensayos clínicos; son: Oprozomib, Delanzomib y Marizomib. En la siguiente imagen se resume a que subunidad del proteasoma van dirigidos (**Figura 2).** Cabe

mencionar que estos PIs se utilizan en combinación con glucocorticoides e inmunomoduladores.

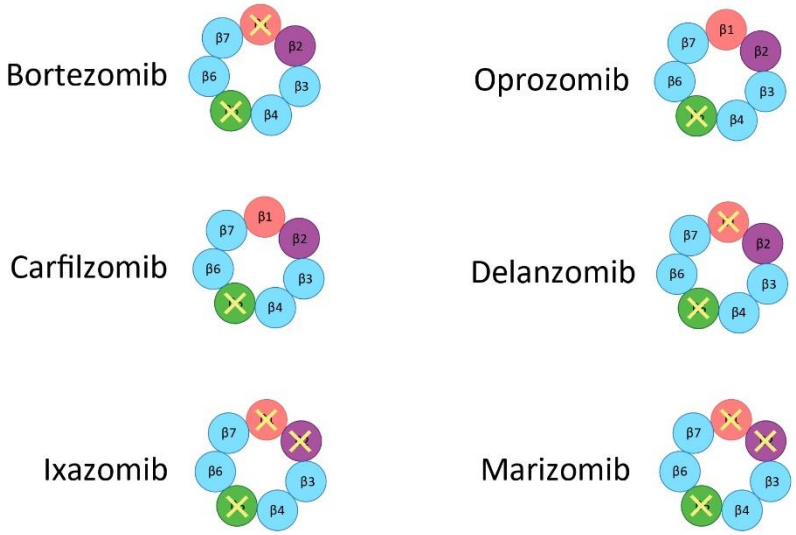

Figura 2. Representación esquemática de las subunidades que inhiben cada uno de los PIs. *El circulo rosa indica la subunidad Caspase-Like (β1), el morado indica la subunidad Trypsin-Like (β2) y el verde indica la subunidad Chymotrypsin-Like(β5).*

Los efectos secundarios que producen en el corazón no han sido bien estudiados desde el principio de su uso en la clínica. Algunos datos revelan que pacientes tratados con bortezomib, lenalidomida y dexamethasona presentan arritmias e incluso trombosis. En el caso del Carfilzomib en combinación, se ha registrado fallo cardiaco, isquemia y tromboembolismo[131].

4. Enfermedades de rasgo complejo y heredabilidad perdida

La susceptibilidad genética a desarrollar cardiotoxicidad como consecuencia de un tratamiento oncológico no es debida a un sólo gen si no, por la suma de los efectos de múltiples genes que tienen un efecto discreto[132]. Incluso en caso donde los pacientes

tienen una predisposición genética de sufrir la enfermedad, estos genes tienen baja penetrancia y el riesgo a sufrir la enfermedad se ve modificado por otros genes.

Parte de la variabilidad fenotípica observada en un rasgo complejo en la población tiene un origen genético que viene determinado por diferencias genéticas entre los individuos en la población y la influencia del medio ambiente en ellos. La *heredabilidad en sentido amplio,* está definida por la proporción de la varianza fenotípica explicada por la varianza genotípica; mientras que *heredabilidad en sentido estricto* es la proporción de la variabilidad fenotípica debido al componente genético aditivo, excluyendo los determinantes genéticos relacionados con epistasis y dominancia genética[134]. En el análisis de ligamiento, las regiones genómicas asociadas a la variabilidad de los fenotipos de rasgo complejo se denominan *locus para un carácter cuantitativo* o QTL.

Existen discrepancias entre la proporción de la variabilidad fenotípica que se cree que es debida a diferencias genéticas y el porcentaje de esa variabilidad que podría atribuirse directamente a variaciones genéticas previamente asociadas con este fenotipo. A esta discrepancia se la conoce como *heredabilidad perdida.*

Cualquier fenotipo de rasgo complejo es el resultado de los efectos de fenotipos intermedios, en otras palabras, fenotipos que participan en la fisiopatología y patogenia del fenotipo complejo principal se les denomina fenotipos intermedios o subfenotipos, los cuales van a formar redes complejas de interacción a diferentes niveles entre diferentes procesos que serán los que determinen el fenotipo final: la enfermedad, en nuestro caso la cardiotoxicidad. **Figura 3.**

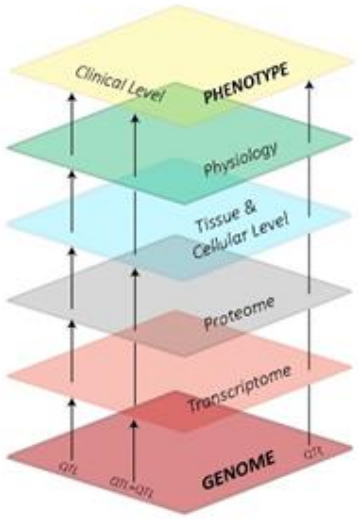

Figura 3. Los fenotipos intermedios contribuyen a la patogénesis de la enfermedad de génesis compleja a través de diferentes niveles: *sistémico, orgánico/tejido, celular y molecular. Hay múltiples interacciones en cada nivel y entre niveles. Además, todos están influenciados por el nivel genético y medioambiental.*

La estrategia de dividir la enfermedad en diferentes fenotipos intermedios para estudiar su contribución en el componente genético de la enfermedad ha sido previamente usada en psiquiatría[144].

HIPÓTESIS Y OBJETIVOS

Nuestra hipótesis es que parte de la susceptibilidad a desarrollar cardiotoxicidad podría explicarse por diferencias en los fenotipos intermedios. Probar esta teoría en humanos es muy complicado, por tanto, desarrollamos una cohorte heterogénea de ratones a nivel genético y fenotípico, pero con grado de complejidad simplificada y también controlada a nivel medio ambiental. Evaluamos la cardiotoxicidad en nuestra esta cohorte de ratones que recibió tratamiento quimioterapéutico tras desarrollar cáncer de mama. Después, estudiamos diferencias de cardiotoxicidad en función del fondo genético, el tipo de terapia recibida y la edad, para después tratar de identificar determinantes genéticos asociados a ellos.

Para realizar nuestro segundo estudio, utilizamos cardiomiocitos diferenciados a partir de células pluripotenciales y testamos diferentes combinaciones de inhibidores del proteasoma con inmunomoduladores y glucocorticoides. Siempre imitando patrones de tratamiento establecidos en la clínica o ensayos clínicos, para describir posibles efectos cardiotóxicos en las células a través del análisis de su contractilidad y voltaje.

Objetivo general

Este trabajo pretende describir nuevas estrategias para identificar la susceptibilidad a sufrir cardiotoxicidad debida a antraciclinas e inhibidores del proteasoma.

Objetivos específicos

Objetivo 1: Identificación de marcadores genéticos y plasmáticos para identificar la susceptibilidad a la cardiotoxicidad por antraciclinas mediante la evaluación de subfenotipos moleculares asociados a la misma.

Objetivo 2: Evaluación de la cardiotoxicidad producida por inhibidores del proteasoma mediante cultivo de hiPSCs-CMs, con el fin de evaluar la cardiotoxicidad de fármacos nuevos y sentar las bases de una estrategia para predecir la cardiotoxicidad individual en el contexto del Mieloma múltiple y otras patologías en que se apliquen estos fármacos.

MATERIALES Y MÉTODOS

1. Modelo animal

Se generó una cohorte de ratones a través de una estrategia de retrocruce o backcross**. Figura 4)**. Estos ratones desarrollaron cáncer de mama y fueron tratados con quimioterapia una vez desarrollaron el tumor. Elegidos de forma estocástica y pareados por edades, N=88 ratones hembra fueron tratados con 25mg/kg de doxorrubicina (Pfizer). Los ratones recibieron cinco inyecciones intraperitoneales; y otros N=77 recibieron tratamiento combinado de doxorrubicina a la misma dosis más docetaxel 25mg/kg cada diez días, también por inyección intraperitoneal. El estudio fue evaluado positivamente por el Comité de Bioética de la Universidad de Salamanca.

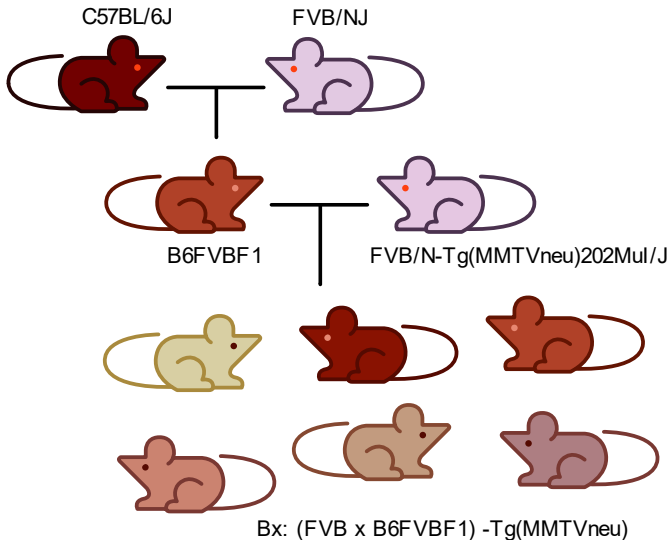

Figura 4. Generación de la cohorte de ratones *backcross*. C57BL/6J[153] ratones resistentes a desarrollar cáncer de mama se cruzaron con la cepa FVB/N[154] ratones susceptibles a desarrollar cáncer de mama. La F1 resultante se retrocruzaron con los ratones transgénicos FVB/N-Tg(MMTVneu)202Mul/J[155] para generar la cohorte de trabajo que denominamos backcross (Bx). En ella, cada uno de los ratones es genéticamente único y con unas características únicas para fenotipos complejos como son la susceptibilidad y evolución del cáncer de mama y la cardiotoxicidad por antraciclinas.

El genotipado de los ratones se llevó a cabo en el Centro Nacional de Genotipado (CEGEN) en el nodo presente en el Centro Nacional de Investigaciones Oncológicas (CNIO, Madrid), a través de la plataforma Illumina. El número de SNPs que incluye el

chip es de 1449 pero de estos, sólo 806 SNPs fueron informativos, es decir que son diferentes entre las cepas parentales FVB y C57BL/6 y por tanto pueden diferenciar a los ratones del retrocruce.

1.1. Procesado de los corazones y cuantificación del daño cardiaco.

A partir de los corazones congelados de los ratones se llevaron a cabo los siguientes estudios:

1) Cuantificación histopatológica del daño a través del sistema ARIOL que es un escáner de preparaciones de histología. Para ello se escogieron 5 regiones correspondientes a la zona subepicárdica y 5 más de la subendocárdica a un aumento de 20X, cuantificándose el área de fibrosis y el área de los cardiomiocitos, como reflejo de su grado de hipertrofia.

2) A partir de 10-15mg de proteínas extraídas de los corazones, se llevó a cabo la cuantificación de proteínas implicadas en diversas rutas de interés relacionadas con la CDA. El método utilizado fue la tecnología Luminex. Las proteínas elegidas fueron: niveles ATR total, CHK1(S345), CHK2(T68), ɤH2AX (S139), P53(S15) y niveles totales de MDM2 y P21 mediante el kit 7-plex *DNA Damage/Genotoxicity Magnetic Bead Kit* (Milliplex Map Kit #48-621MAG, Millipore). Otro test utilizado fue el 9 *Plex Multi-Pathway Magnetic Bead Kit* (Milliplex Map Kit #48-680MAG), con el que se cuantificaron ERK1/2(T185/Y187), P38MAPK(T180/Y182), NFκB(S536), JNK(T183/Y185), AKT(S473), P70S6K(T412), CREB(S133), STAT3(S727) y STAT5(Y694/699). Finalmente, cuantificamos los niveles de TGFβ-1, TGFβ-2, y TGFβ-3 con Milliplex MAP TGFβ Magnetic-Bead 3 Plex Kit (#TGFMAG64K-03, Millipore), así como caspasa 3 y tubulina.

3) Cuantificamos de la longitud telomérica a través de DNA extraído del miocardio (por el método del fenol-cloroformo[220]) mediante ensayos de qPCR mediante sondas TaqMan[158].

4) Cuantificación en los niveles de expresión de una serie de miRNAs relacionados con diversas patologías cardiacas según la bibliografía. La lista de miRNA estudiados se recoge en la **Tabla 1.**

Tabla 1: Lista de los miRNAs estudiados. ID: número de identificación de las sondas (Fisher).

miRNA	Sondas	ID
miR-21a-5p	hsa-miR-21a-5p	477975_mir
let-7a-5p	hsa-let-7a-5p	478575_mir
let-7f-5p	hsa-let-7f-5p	478578_mir
let-7d-5p	hsa-let-7d-5p	478439_mir
	hsa-let-7d-3p	477848_mir
let-7i-5p	hsa-let-7i-5p	478375_mir
let-7c-5p	hsa-let-7c-5p	478577_mir
let-7e-5p	hsa-let-7e-5p	478579_mir
	hsa-let-7e-3p	479281_mir
let-7b-5p	hsa-let-7b-5p	478576_mir
let-7g-5p	hsa-let-7g-5p	478580_mir
miR-374-5p	hsa-miR-374b-5p	478389_mir
hsa-miR-497-5p	hsa-miR-497-5p	478138_mir
miR-210-3p	hsa-miR-210-3p	477970_mir
miR-100	hsa-miR-100-5p	478224_mir
miR-130a	hsa-miR-130a-3p	477851_mir
miR-152	hsa-miR-152-3p	477921_mir
miR-214	hsa-miR-214-3p	477974_mir
	hsa-miR-214-5p	478768_mir
miR-29b	hsa-miR-29b-3p	478369_mir
miR-34a	hsa-miR-34a-5p	478048_mir
miR-125b	hsa-miR-125b-5p	477885_mir
	hsa-miR-125b-1-3p	478665_mir
miR-128	hsa-miR-128-3p	477892_mir
miR-200b	hsa-miR-200b-5p	478753_mir
	hsa-miR-200b-3p	477963_mir
miR-451	hsa-miR-451a	478107_mir
miR-361-5p	hsa-miR-361-5p	478056_mir

Los miRNA fueron cuantificados en triplicado mediante la plataforma *Fluidigm BioMark HD Technology* en un 96 Dynamic arrays (96-96 Dynamic Array IFC for Genotyping #BMK-M-96.96. Link: https://www.fluidigm.com/reagents/genomics/100-

6499-juno-96-96-genotyping-ifc--1-ifc. No es más que un Sistema para llevar a cabo la QPCR en un micro volumen de 1 microlitros.

DISCUSIÓN

Objetivo 1: Identificación de marcadores genéticos y plasmáticos para identificar la susceptibilidad a la cardiotoxicidad por antraciclinas mediante la evaluación de subfenotipos moleculares asociados a la misma

La cardiotoxicidad debida a quimioterapia es un efecto adverso común que puede ser muy grave y modificar la continuidad del tratamiento oncológico en los pacientes que la sufren[160].

En la primera parte de este trabajo se ha tratado de identificar aquellos pacientes más susceptibles de desarrollar CDA. Para ello se han tratado de identificar marcadores genéticos[161] y biomarcadores en plasma, a partir de subfenotipos que pueden participar en la fisiopatología y en la patogenia de la CDA. Como se describe previamente en este trabajo, la susceptibilidad a desarrollar CDA crónica tiene un importante componente genético,[133,166] parte de él puede ser identificado a través de la asociación a subfenotipos moleculares ligados a la susceptibilidad a CDA. Para identificar subfenotipos de DNA, consideramos que las antraciclinas provocan su cardiotoxicidad a través de daño al DNA (genotoxicidad). Por tanto, diferentes grados de CDA estarían relacionados con diferentes niveles de proteínas que participan en rutas moleculares implicadas en respuesta al daño al DNA como AKT[167] o ɣH2AX[168] o moléculas implicadas en muerte celular y apoptosis como MDM2 o p53[171].

También consideramos que diferencias en los niveles y la respuesta de moléculas que participan en rutas de señalización relacionadas con patología cardiaca como ERK1/2[175], p38MAPK[177,178] o JNK[176] y miRNAs[183] o la longitud telomérica[185] podrían ser subfenotipos moleculares de CDA. El acortamiento telomérico está asociado con estrés oxidativo y envejecimiento celular, procesos comunes en las enfermedades cardiovasculares ligadas a tratamiento con antraciclinas[170].

A través de modelos de regresión múltiple hemos identificado cuales son los subfenotipos moleculares con más poder explicativo en la variabilidad fenotípica de la CDA entre ratones. Después, determinamos hasta qué punto los QTL asociados con esos subfenotipos moleculares podría ayudar a explicar la cardiotoxicidad de algunos de los QTL más importantes asociados a CDA en nuestro modelo. De hecho, QTL ligados a niveles de expresión de las siguientes proteínas of p70S6K(pT412), JNK (pT183/pY185), pCREB(S133), y ɣH2AX(Ser139) explicada que se asociaba con daño cardiaco en ratones tratados con quimioterapia. **Figura 8.**

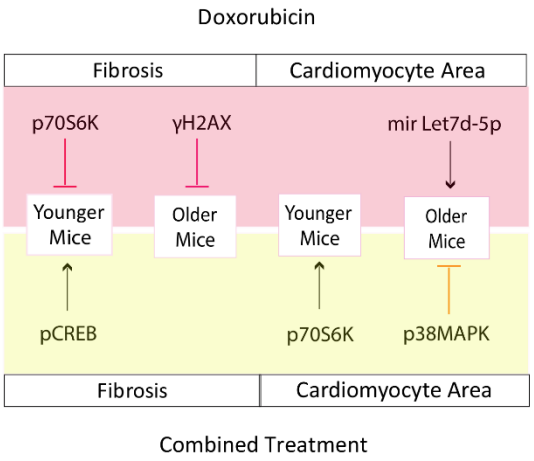

Figura 8. Resumen de los resultados obtenidos en el análisis de regresión múltiple. *Los subfenotipos en la región rosa corresponden al tratamiento con doxorrubicina y los de la región amarilla corresponden al tratamiento combinado.*

Trasladado a pacientes, formas alélicas de *RPS6KB1* que codifica P70S6K, y de los genes *CREB* y *TUBB*, se asociaron con CDA en pacientes. Es interesante señalar que P70S6K se ha propuesto como uno de los mejores predictores de cardiotoxicidad, según un estudio realizado recientemente.[186] al Además, se ha descrito que la expresión del gen *RPS6KB1* provoca potenciales de acción de menos duración en los cardiomiocitos dando lugar a una contractilidad transitoria,[8] entre otros efectos[181,187]**.** Basándonos en estos resultados podría ser interesante en futuros estudios, evaluar la expresión de estos genes en otros tratamientos oncológicos y dilucidar si su asocian es específica del daño por antraciclinas o si puede ser extrapolado a otros tratamientos.

Además, tratamos de identificar pacientes susceptibles de desarrollar CDA a través de la determinación de biomarcadores en plasma. Las moléculas evaluadas se eligieron en base a su correlación positiva con daño cardiaco en el miocardio de los ratones del backcross. En nuestro estudio detectamos en plasma marcadores que correlacionaron con daño funcional medido con resonancia magnética cardiaca en mismos pacientes. Aun así, nuevos estudios con más pacientes se requieren para validar estos resultados.

Objetivo 2: Evaluación de la cardiotoxicidad producida por inhibidores del proteasoma mediante cultivo de hiPSCs-CMs, con el fin de evaluar la cardiotoxicidad de fármacos nuevos y sentar las bases de una estrategia para predecir la cardiotoxicidad individual en el contexto del Mieloma múltiple y otras patologías en que se apliquen estos fármacos

Lo que hace este estudio preliminar atractivo es la descripción en un modelo *in vitro* que podría servir para identificar la susceptibilidad a la cardiotoxicidad por IPs u otros fármacos de forma individualizada. En este caso, nos centramos en IPs utilizados en el tratamiento del MM tratamiento contra mieloma múltiple.

Después de verificar el efecto de cada combinación de fármacos en las líneas celulares de MM, se evaluó su cardiotoxicidad in vitro en hiPSCs-CMs. Este tipo de estudios buscaba describir cualquier tipo de anormalidad en voltaje o contractilidad[195,196]. En nuestros resultados se observó una correlación entre los valores obtenidos en ambos parámetros y no apreciamos ninguna indicación de pro-arritmia como se esperaría con el Bortezomib[197] o el Carfilzomib[131], según datos de la clínica. Sin embargo, ante concentraciones altas de estos fármacos, el efecto en los cardiomiocitos fue fulminante, se detuvo su actividad; lo que también sucedió con sus análogos Oprozomib y Delanzomib.

Sobre los valores de voltaje, el marcador de pro-arritmia por excelencia es el índice de triangulación, tuvo valores muy bajos en PIs como el marizomib e ixazomib tanto en monoterapia como con sus combinaciones con Inmunomoduladores y dexametasona. La explicación puede deberse a un acortamiento en la longitud del ciclo consecuencia de potenciales de acción que ocurren entes de lo esperado[199].

Como excepciones prometedoras encontramos que el Delanzomib en combinación con lenalidomida y dexamethasona parecía ser mejor tolerados por los cardiomiocitos que en monoterapia. Este estudio coincide con uno llevado a cabo por Vogl DT, *et al*.[123] en el cual se describe el limitado efecto cardiotóxico de este PI de nueva generación. Además, se sabe que el incremento en la señalización de la ruta de los glucocorticoides (GR) es beneficiosa para inhibir la muerte celular por cardiotoxicidad debida a quimioterapia[202] y mejorar la contractilidad en los cardiomiocitos[203].

Por otro lado, encontramos el marizomib el cual pareció tener un efecto en la línea celular U266B1 y ningún efecto en los cardiomiocitos. Hay varios ensayos clínicos que están evaluando este compuesto,[125,207] pero los resultados reportados hasta la fecha no indican presencia de cardiotoxicidad en los pacientes y si efecto contra la enfermedad hematológica. Además, atraviesa la barrera hematoencefálica,[208] lo cual abre más opciones de tratamiento para otras enfermedades oncológicas que afecten al sistema nervioso central.

CONCLUSIONES

Nuestros estudios en una población backcross de ratón muestran que determinantes genéticos y fenotipos intermedios pueden explicar la susceptibilidad a sufrir cardiotoxicidad por antraciclinas.

Además, el uso de hiPSCs-CMs permite testar nuevos compuestos que ayuden a predecir la cardiotoxicidad debida a tratamientos oncológicos.

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