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TFEB, SIRT1, CARM1 EXPRESSION AND PITX2 METHYLATION IN BREAST CANCER CHEMORESISTANCE

Dottoranda
Dott.ssa Serena Bertozzi

Supervisore
Prof. Umberto Baccarani

Co-supervisori
Prof.ssa Laura Mariuzzi
Prof.ssa Bruna Corradetti
Dott. Ambrogio P Londero

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Talent is universal. Opportunity is not. (M.Ferrari)

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Abstract

Objective. Breast cancer chemoresistance is attributed to a wide variety of mechanisms, including autophagy. Transcription factor EB (TFEB) has been recently identified and characterized as one major regulator of autophagy and lysosomal genesis. The objective of this thesis is to evaluate the prognostic impact of TFEB and its pathway in breast cancer chemoresistance.

Materials and methods. This retrospective study analyzes the expression of TFEB, CARM1, and SIRT1 and the methylation of PITX2 in breast neoplasia. A group of breast cancer patients treated with chemotherapy, who relapsed within 12 months from treatment initiation, were compared to a sub-cohort of chemotreated patients who did not recur within 12 months of follow up. The expression of TFEB, CARM1, and SIRT1 was analyzed by both immunohistochemistry and RT-PRC on formalin-fixed paraffin-embedded samples. PITX2 methylation was tested with the diagnostic CE-marked kit Therascreen PITX2 RGQ PCR. In the final model, 136 cases of chemotreated breast cancer were included.

Results. A higher TFEB expression correlates with shorter survival in patients with chemotreated invasive breast cancer. Furthermore, the protein expression of SIRT1 is significantly associated with that of TFEB and CARM1, so that a very low SIRT1 expression (lower than the first quartile of the H-score distribution) correlates with a low expression of TFEB and CARM1 and with longer survival. Finally, in the basal-like and Her-enriched tumors, TFEB and SIRT1 seem to have a lower H-score in comparison with the luminal subtypes. PITX2 methylation analysis was feasible only in 65% of the selected samples, but no significant differences between the cases and the controls was found, as well as no correlation with the expression of the TFEB pathway.

Conclusions. TFEB and SIRT1 seem to have a potential prognostic significance in patients with chemotreated breast cancer, likely because of their role in the regulation of autophagy. In addition, no correlation between TFEB and PITX2 methylation was found, likely because they act two different roles within the autophagy process.

Keywords. TFEB; SIRT1; CARM1; PITX2; breast cancer; chemoresistance.

Riassunto

Obiettivo. La chemioresistenza del carcinoma mammario viene attribuita a una vasta gamma di meccanismi cellulari, tra cui l'autofagia. Il fattore di trascrizione EB (TFEB) è stato identificato e caratterizzato come uno dei principali regolatori dell'autofagia e della genesi lisosomiale. L'obiettivo di questa tesi è valutare il ruolo prognostico della pathway di TFEB nella chemioresistenza del carcinoma mammario.

Materiali e metodi. Questo studio retrospettivo analizza l'espressione di TFEB, CARM1 e SIRT1 e la metilazione di PITX2 nel carcinoma mammario. Le pazienti trattate con chemioterapia che hanno recidivato entro 12 mesi dall'inizio del trattamento sono state confrontate con una sub-coorte di pazienti con carcinoma mammario chemiotrattato che non sono state colpite da recidiva entro i primi 12 mesi di follow-up. L'espressione di TFEB, CARM1 e SIRT1 è stata analizzata sia mediante immunohistochimica che RT-PRC su campioni fissati in formalina ed inclusi in paraffina. La metilazione di PITX2 è stata testata con il kit diagnostico marchiato CE Therascreen PITX2 RGQ PCR. Nel modello finale sono stati inclusi 136 casi di carcinoma mammario chemiotrattato.

Risultati. L'aumentata espressione di TFEB è associata a una minore sopravvivenza nelle pazienti con carcinoma mammario invasivo chemiotreatato. Inoltre, l'espressione proteica di SIRT1 è significativamente correlata a quella di TFEB e CARM1, quindi un'espressione molto bassa di SIRT1 (inferiore al primo quartile della distribuzione del H-score) è associata a una bassa espressione di TFEB e CARM1 e ad una sopravvivenza più lunga. Infine, nei tumori triplo-negativi ed Her2-positivi, TFEB e SIRT1 tendono ad avere un H-score più basso rispetto ai sottotipi luminali. L'analisi della metilazione di PITX2 è stata possibile solo nel 65% dei campioni selezionati, ma non si sono evidenziate differenze statisticamente significative tra i casi e i controlli, nonché nessuna correlazione con l'espressione della pathway di TFEB.

Conclusioni. TFEB e SIRT1 sembrano avere un potenziale significato prognostico nelle pazienti con carcinoma mammario chemiotrattato, probabilmente a causa del loro ruolo nella regolazione dell'autofagia. Non è stata trovata invece alcuna correlazione tra la metilazione di TFEB e PITX2, probabilmente perché le due proteine svolgono ruoli differenti nel processo di autofagia.

Parole chiave. TFEB; SIRT1; CARM1; PITX2; carcinoma mammario; chemioresistenza.

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Chapter 1

Introduction

1.1 Breast cancer incidence

Approximately 53'000 new breast cancer cases have been diagnosed in Italy during 2018. Not considering skin cancer, breast cancer represents the most prevalent malignancy in the female gender (about one tumor every three is a breast one). The incidence of breast carcinoma presented a reduction in many areas of the world around the year 2000, due to both a reduction of hormone replacement therapy prescriptions and the effect of incidence saturation determined by the first rounds of mammography screening programs.

Breast cancer incidence shows also a wide geographical variability, with higher rates in the most economically advanced countries. The differences between macro-areas observed in Italy during the period 2010-2014 confirm greater incidence in the North (162.9 cases / 100'000 women) compared to the Center (141.5 cases / 100'000 women) and the South and Islands (127.1 cases / 100'000 women). These differences express the sum of the various factors in play, from the different diffusion of mammography screening to inhomogeneities in risk factors exposure.

At the moment, the trend in breast cancer incidence in Italy appears to be slightly increased (+ 0.3% per year), while mortality continues to significantly fall dawn (-0.8% per year) [1]. However, despite the remarkable progress in both diagnosis and treatment, breast carcinoma continues to be a leading cause of cancer-related death among women worldwide. In fact, it is the second biggest killer of all malignancies in the female gender after lung and bronchial neoplasia [2, 3].

Metastatic (stage IV) breast carcinoma remains a major challenge for the breast spe-

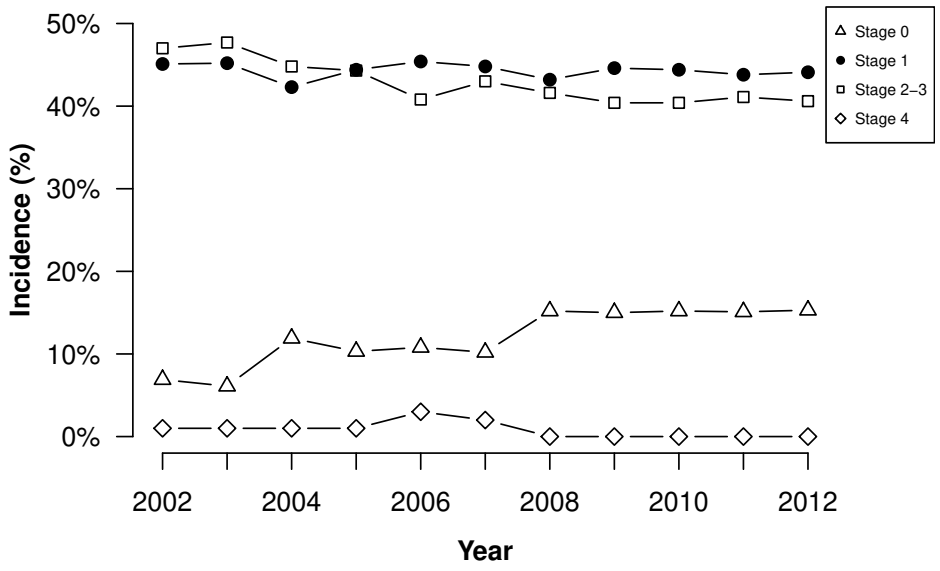

Figure 1.1: Incidence of TNM stage between 2002 and 2012 (Breast Unit, Department of Surgery, University Hospital of Udine).

cialist, and unfortunately its incidence has not experienced great changes during the last years. In the same period, on the other hand, we have assisted to a progressive decline of the number of locally advanced breast carcinomas and a concurrent increase of the number of early breast malignancies, due to the systematic introduction of a scheduled mammographic screening [4, 5] (Figure 1.1). The regional screening in Friuli-Venezia Giulia is offered every two years and covers the age group between 50 and 69 years, in which about half of all operated tumors of our Breast Unit are included (Figure 1.2).

The 5-year overall survival of breast cancer patients in Italy results 87% with little heterogeneity between age groups (91% in young women (15-44 years), 92% among women aged 45-54, 91% among women aged 55-64, 89% among women aged 65-74 years, 79% among older women aged >75). Altogether in Italy there are about 800'000 women who have been diagnosed of breast cancer, equal to 43% of all women living with a previous cancer diagnosis and 24% of all cancer survivors, including men and women.

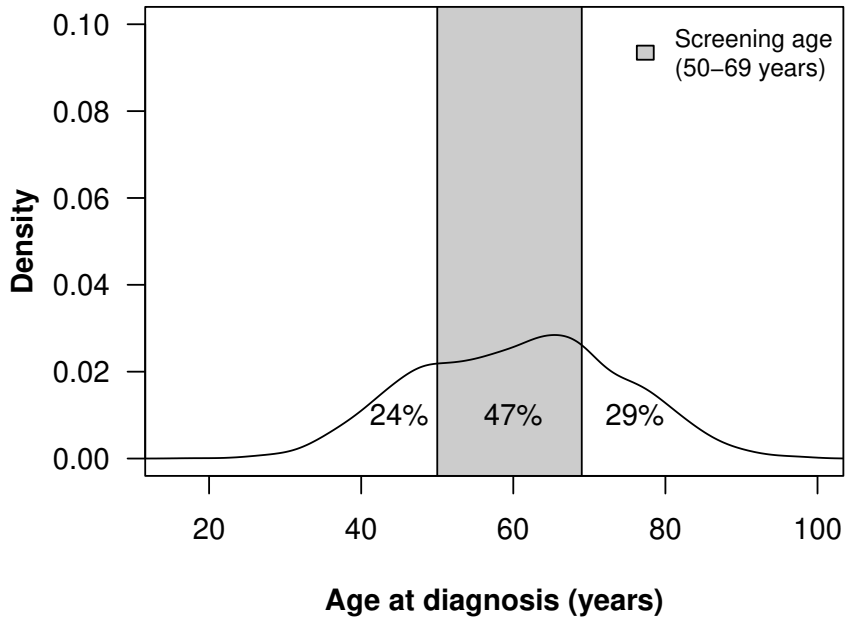

Figure 1.2: Breast cancer age at diagnosis (Breast Unit, Department of Surgery, University Hospital of Udine).

1.2 Breast cancer prognosis

Breast cancer prognosis is historically based on some clinical evidences. In particular, the TNM classification predicts the prognosis by considering the combination of tumor major size (T), number of involved lymph nodes (N) and eventual distant metastases (M) [6]. However, this instrument results many times inaccurate. As a consequence, breast malignancies have been classified into several subgroups according to their immunophenotype. In particular, based on the expression positivity for estrogen receptor (ER), progesteron receptor (PR) and human epidermal growth factor receptor 2 (Her2/neu), they can be divided into the following molecular subtypes [7]:

- luminal A (high ER and low proliferation index Mib1/Ki-67);
- luminal B (low ER and high proliferation index Mib1/Ki-67);
- Her-2 enriched (Her2/neu overexpression independently by ER expression);
- triple negative (no expression of ER, PR or Her2/neu).

This kind of classification seems to be more useful in the therapeutic decision-making process, as it is more predictive of tumor responsiveness to chemotherapy and/or hor-

monal therapy. However, tumor resistance to systemic therapies remains a major challenge for the breast specialist.

Considering the hormonal therapy, many breast cancer patients who are administered Tamoxifen, a selective ER modulator, are included in the luminal subtypes, and ERα works as a predictive marker for the determination of sensitivity to tamoxifen. ER status may be assessed by the estradiol-binding assay, which detects both ERα and ERβ, or by other immunoassays sensitive only to ERα. Interestingly, about 5 to 10% of the ERα-negative breast carcinomas seem to show a somehow unexpected sensitivity to Tamoxifen. Moreover, great interest in Tamoxifen action mechanism has been triggered by the fact that many ER-positive breast cancer patients become resistant to tamoxifen during the course of therapy.

For what concerns chemotherapy, it seems to be very useful in the case of unfavorable molecular subtypes with lower differentiation grade and higher proliferation index, while its indication in the case of more favourable molecular subtypes has been questioned for long time. Anyway, nowadays it is recognized that genetic factors exist, which are able to accurately predict the efficacy of chemotherapy among luminal tumors independently by their proliferation index or other prognostic factors traditionally related to the tumor.

For instance, Oncotype DX is a 21-Gene recurrence score prognostic assay, which is both prognostic and predictive. In fact, it helps to determine if a patient has a risk of metastases and if she would benefit from chemotherapy or if hormone therapy is sufficient treatment. This tool is particularly useful in the case of luminal breast carcinomas, which are not usually considered for chemotherapy, because in the high-risk molecular subtypes the choice to eventually omit chemotherapy is guided by other factors related to the patient, such the age or the presence of important comorbidities. Considering the cost-benefit issue, this kind of test addresses the breast specialist to offer chemotherapy to patients who will really benefit from it, saving money and unpleasant complications to those who would not.

Different other types of tests are on the market (i.e. Endopredict, Prosigna, Mammaprint, Breast Cancer Index), which are based on different technologies and analyze different groups of genes [8]. However, at the moment, the Oncotype DX test is the only one indicated for its prognostic and predictive ability towards the benefits of chemotherapy in the most important international guidelines (including those of St Gallen and Asco).

In the USA, for example, it is the only test strongly recommended for decisions about the use of chemotherapy in women with invasive breast cancer in the early stages and negative lymph nodes in the 2018 guidelines of the National Comprehensive Cancer Network (NCCN). The decision is based on the results of the large prospective TAILORx study (conducted on over 10'000 women), presented at Asco 2018 and published simultaneously in the New England Journal of Medicine [9].

But the research for new markers that predict the response to breast cancer therapies does not end here, because numerous other markers are being studied that can help us choose therapies in a more targeted and effective way.

1.3 Autophagy steps and regulation

Many risk factors, including both genetic mutations and epigenetic changes, are recognized to promote malignancies development and to influence their natural history. And the oncological research, during the last decades, has tried to shed light on the complex mechanisms involved in breast carcinogenesis. Among the mechanisms underlying malignant transformation, autophagy seems to play a crucial role in the switch from normal to neoplastic breast cells [10–13].

Autophagy is a catabolic process which is physiologically adopted by the normal cell. In normal conditions, it allows to assure cellular homeostasis whereas, in the case of tissue damage, it represents a strategic survival mechanism that recycles energy and nutrients under special conditions. In detail, autophagy consists in a sort of cellular self-digestion through the removal of excessive, long-lived or dysfunctional organelles and proteins. Many stress conditions, such as hypoxia and nutrient deprivation, may be trigger of autophagy as a critical adaptive response against starvation. We can distinguish three different forms of autophagy from a morphological point of view:

- macroautophagy;
- microautophagy;
- and chaperone-mediated autophagy (Figure 1.3).

In particular, macroautophagy consists in the engulfment of both cytoplasm and intracellular organelles in the so called autophagosome (Figure 1.3). This structure then fuses

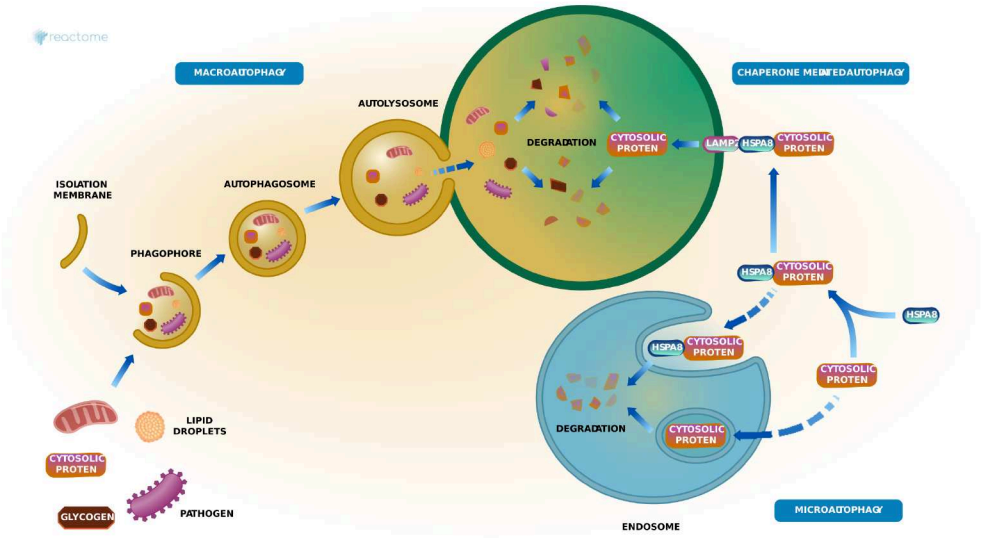

Figure 1.3: Schematic representation of autophagy. It is a natural, regulated cell system that disassembles unnecessary or dysfunctional cell components and is subdivided in different mechanisms: macroautophagy that could be a bulk process or selective autophagy as the autophagy of oragenelles (i.e. mitophagy - mitocondria); microautophagy (i.e. involves the direct engulfment of cytoplasmic material into the lysosome); or chaperone mediated autophagy that is a complex and specific pathway (source www.reactome.org).

with a lysosome in order to create the autolysosome, which thanks to its lysosomal hydrolases is responsible for the degradation of the segregated cellular material.

The development of autophagic vacuoles makes use of several members of the ATG gene family, involving also the regulator protein beciclin 1, which we will meet in the next paragraphs as a possible target for antiblastic therapies. Other activated members of the ATG family may also recruit the protein LC3, which consent the final formation of the autophagosome vescile through the conjugation with phosphatidylethanolamine.

Another major regulator of the macroautophagy process has been identified in the serine-threonine kinase mTOR (mammalian target of rapamycin). In particular, mTORC1 complex has been observed to negatively regulate macroautophagy, whereas mTORC2 complex plays a role in cellular survival regulation and cytoskeletal formation.

For what concerns microautophagy, usually a direct invagination of the cellular membrane occurs to sequestrate proteins to unmake. Finally, the chaperone-mediated autophagy involves the translocation across lysosomal membrane of damaged soluble cytosolic proteins by chaperone-dependent selection [10, 14–19] (Figure 1.3).

1.4 Autophagy role in cancer pathogenesis

The autophagy process has been confirmed to have a fundamental pathogenetic role in various human disorders, including malignancies, neurodegenerative, infective, cardiovascular, metabolic, and pulmonary diseases, as well as aging. For what regards cancer development, there is evidence of a likely dual role of autophagy, which seems to act both as tumor suppressor and tumor promoter, repsectively.

In fact, on one side, autophagy serves to survey the normal cells and to protect them from malignant transformation by eliminating malfunctioning organelles or proteins, removing reactive oxygen species (ROS), preventing DNA damage and mitochondrial abnormalities. On the other side, by supporting the access to fundamental nutrients, inhibiting cellular death and increasing antiblastic resistance, autophagy seems to have a crucial function also in malignancy development, metabolism and growth. In addition, the autophagy mechanism plays a crucial role also in many other processes which may promote cancer development, such as flogosis, immune response regulation and genome stability mantainance [15–17, 19].

1.5 Autophagy role in cancer drug resistance

Resistance to antiblastic agents is one of the most challenging issues in cancer treatment. Drug resistance may be intrinsic or acquired. Among possible explanations for therapy resistance the most common are tumor cell genetic heterogeneity, drug metabolism, and environmental exposure-induced epigenetic alterations. Also for what concerns anticancer drug activity, autophagy seems to have a somehow paradoxical function in either inducing and inhibiting anticancer therapy response. Therefore, if on one side autophagy may promote tumor cells death, on the other side its inhibition is reported to re-sensitize previously resistant tumor cells and increase cytotoxicity of chemotherapeutic drugs.

The role of autophagy mechanisms in chemotherapy sensitization has been determined by various preclinical studies which tested chemical inhibitors of autophagy or siRNA to knockdown different autophagy genes. However, the majority of autophagy chemical inhibitors have poor specificity and frequent off-target effects. Therefore, this kind of studies about chemical inhibitors may benefit from the concurrent autophagy genes knockout through the use of si-RNA, in order to verify the effective role of autophagy inhibition. For example, in glioblastoma, lung cancer, cervical cancer, prostate cancer leukemia

and breast cancer, the autophagy process inhibition is demonstrated to resesitized cancer cells to different antiblastic drugs. For example, autophagy upregulation can protect tumor cells against many therapies including temozolomide, resveratrol, vitamin D3, anthocyanins, radiotherapy and tamoxifen. And these results support the active function of autophagy in modulating cancer resistance to antiblastic agents [10, 16, 17, 19–22].

1.6 Autophagy and breast cancer

The exact function carried out by autophagy in breast cancer, like in other malignancies, remains argument of great debate and investigation. For what concerns in particular the development of the autophagosome, clinicopathological studies on breast cancer tissue indicate three discernible LC3-positive patterns:

- diffuse cytoplasmic;
- cytoplasmic/juxtanuclear;
- dense round 5 μm 'stone-like' structures.

Both the diffuse cytoplasmic and cytoplasmic/juxtanuclear LC3 staining are associated with estrogen and progesterone receptor expression in the breast. Moreover, the 'stone-like' LC3 stained correlates with high grade tumors with poorer prognosis, supporting a direct autophagy correlation with tumor biological aggressiveness [10, 23].

Preclinical studies also reveal a role of the autophagy process in antiblastic agents resistance. In fact, autophagy is observed to protect MCF-7 breast cancer cells from epirubicin, whereas inhibition of autophagy through beclin-1 siRNA is demonstrated to restore the sensitivity to epirubicin. Moreover, when treated with DNA-damaging antiblastic agents, such as Camptothecin or Etoposide, autophagy can delay the apoptosis onset in mammary tumors cells. And this effect can be reversed by the selective knockdown of Atg7 and beclin-1 genes.

In addition, administration of the proteasome-inhibitor Bortezomib to MCF-7 breast tumor cells is reported to potentially stimulate the autophagy process, as well as the unfolded protein response (UPR). This latter process initially promotes the restoration of normal cell functions by interrupting the protein synthesis and increasing the chaperon-mediated protein folding, but thereafter induces apoptosis if the normal protein folding

has not been successfully restored. The authors of these studies suggest then that both autophagy and UPR activation help the neoplastic cells survive, giving also a likely explanation for the scarse response to Bortezomib in vivo [10, 24].

Breast cancer sensitivity to drugs targeting HER2 (i.e. Trastuzumab) and the EGF receptor tyrosine kinase inhibitors (i.e. Lapatinib) seem also to be influenced by the autophagy process. For instance, Trastuzumab induces a sort of cytoprotective response promoting LC3-positive punctate formation in SKBr3 cells, which represents an HER2-amplified breast cancer cell line. Moreover, autophagy inhibition through 3-MA and LY294002 restores tumor cells response to Trastuzumab, as well as to Lapatinib in BT-474 cells, which are another HER2-amplified breast cancer cell line commonly resistant to Lapatinib. These results support a role of autophagy modulation in the resensitivization of breast tumor cells to antiblastic agents [10, 25, 26].

Interesting evidences exist also about the outcome of autophagy stimulation in the case of breast cancer tumor cells subjected to antiestrogen therapy. For example, among estrogen-positive MCF-7 breast tumor cell population, treated with antiestrogen drugs such as Tamoxifen and ICI 182780, the cells who do not survive the therapy show an active autophagosome formation, suggesting that antiestrogen-stimulated autophagy results in effective cell death [27]. However, more recent studies support that this observation is more likely to be the result of cells failure in survival attempt. In particular, they demonstrate that, after antiestrogen treatment administration, autophagosomes are more actively formed in those MCF-7 cells which survive the therapy, suggesting that the effective count of autophagosomes in each tumor cell is directly correlated with cell survival [28]. Moreover, they observed that the inhibition of autophagosomes formation through beclin-1 siRNA enhances antiestrogen effectiveness and consequently MCF-7 tumor cell death, supporting the pro-survival role of autophagy during antiestrogen treatment.

Other authors tried to inhibit various mechanisms of the autophagy process, in different cell line populations, through siRNA with Atg5, beclin-1 and Atg7. In particular, they observed an augmented mitochondrial-mediated apoptotis rate, and a concurrent decrease cell viability rate, in the case of autophagy knockdown added to tamoxifen treatment. Furthermore, the inhibition of autophagy process using beclin-1 shRNA or 3-MA treatment in some particular resistant tumor cell populations (such as the ICI resistant, tamoxifen cross-resistant MCF-7/LCC9 breast cancer cell line) is described to

partially restore antiestrogens effectiveness [29]. Moreover, a good resensitivization of resistant breast tumor cells to antiestrogens is reported while concurrently inhibiting Bcl-2 (chemically through YC137) and beclin-1 (through genetic knockdown), suggesting that the combined inactivation of both autophagy and Bcl-2 is more effective than their inhibition alone, in order to restore breast tumor sensitivity to antiestrogens.

Studies about the estrogen signaling in breast tumor cells show that overexpression of beclin-1 causes a reduction in the estrogen-related tumor growth through the transcription reduction of estrogen-regulated genes such as c-myc, c-fos and egr1. Although this drop down of the estrogen-related tumor development by beclin-1 overexpression might seem antitumorigenic, unfortunately the overexpression of beclin-1 in breast tumor cell lines is also reported to cause desensitivization to antiestrogens such as raloxifen and tamoxifen. Therefore, new preclinical studies have been designed about possible drug to combine with antiestrogens in order to overcome antiestrogens resistance. For instance, some novel investigated targets are histone deacetylase inhibitors or proteasomal inhibitors. In particular, in combination with antiestrogen therapies, Bortezomib has shown beneficial effects while tested in antiestrogen-resistant T47D and MCF-7 ER-positive breast tumor cell lines. In these cell populations, in fact, Bortezomib induced an effective apoptosis and inactivation of autophagy pathways, that was determined by an accumulation of p62 and LC3.

All the reported preclinical results strongly support an active autophagy role in drug resistance of breast tumor cells and promising data about their resensitivization through autophagy inhibition. Therefore, some clinical trials are currently underway which target autophagy pathway. An easily accessible and safe drug, which has been extensively used for the treatment and prophylaxis of malaria and which has been recently tested in tumor cell lines, is hydrochloroquine. Currently, there are more than twenty trials using hydrochloroquine in patients affected by cancer worldwide, and the majority of them provide preliminar evidence of its antiblastic potential. For example, a study conducted on patients affected by ER-positive ductal carcinoma in situ, who were administered tamoxifen, chloroquine or a combination of the two for 3 months before surgical removal of the tumor, has given very promising results [10, 12, 13, 30].

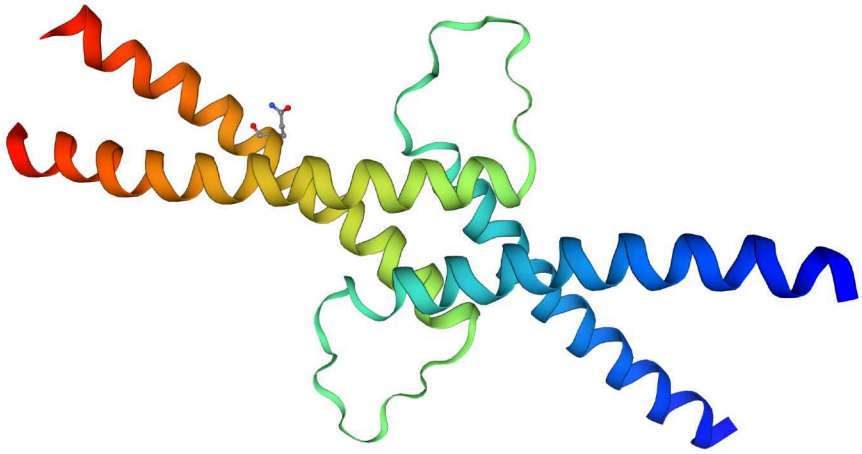

Figure 1.4: The TFEB 3D protein structure model generated by the automated SWISS-MODEL pipeline [35].

1.7 TFEB and autophagy

Among transcription factors regulating autophagy, the Transcription Factor EB (TFEB) has been detected and characterized in 2009. It is a member of the microphthalmia family (MiT family) of basic helix-loop-helix - leucine-zipper (bHLH-Zip) transcription factors, and has been recognized as a master modulator of autophagy, lysosomal biogenesis and function, and downstream of mTORC1, able to moderate the autophagic process by coordinating the expression of genes with functions at all stages of the autophagy process [31–34] (Figure 1.4).

In resting cells, TFEB has a cytosolic localization and interacts with mTORC1 and the lysosomal vacuolar-type ATPase complex. The inhibition of mTORC1 function results in TFEB dephosphorylation and its consequent translocation into the nucleus and bind-

ing with the lysosome-related genes of the so-called Coordinated Lysosomal Expression And Regulation (CLEAR) network, activating a de novo gene transcription. Accordingly, the overexpression of TFEB causes a numeric growth of lysosomes and of the lysosomal enzymes, with the consequent enhancement of lysosomal catabolic activity [32].

Furthermore, TFEB has been demonstrated to modulate a great number of genes involved not only in the lysosomal activity but also in autophagy and lysosomal exocytosis. In detail, TFEB can bind to the promoter regions of various genes of the autophagy pathway and activate autophagosome formation and autophagosome-lysosome fusion [32].

The broad and crucial role of TFEB in promoting and linking the different forms of autophagy determine its great attractivity as therapeutic target, suggesting that strategies aimed at inhibiting lysosomal and autophagic function may impact drug resistance in breast cancer [31–34].

1.8 CARM1, SIRT1 and autophagy

The co-activator-associated arginine methyltransferase 1 (CARM1) has a trascriptional coactivator role in autophagy and lysosomal genes through TFEB [36]. Furthermore, the CARM1 dependent histone arginine methylation is a fundamental nuclear event in autophagy [36] (Figure 1.5).

In parallel with the transcription factor function of TFEB that controls autophagy related genes, SIRT1 (a deacetylase involved in epigenetic changes) also influences the expression of autophagy-related genes [37, 38]. Furthermore, SIRT1 is known to be a significant prognostic factor in many cancers [39] and an high SIRT1 expression is known to be an unfavorable prognostic factor in breast cancer [40, 41] (Figure 1.6).

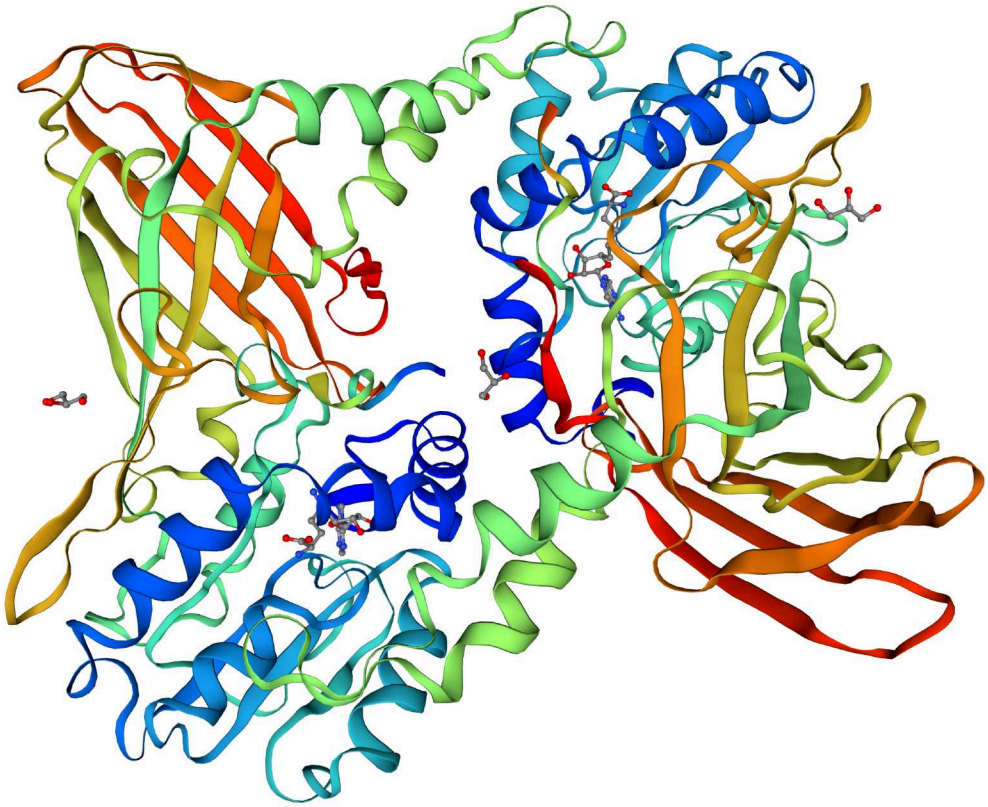

Figure 1.5: The CARM1 3D protein structure model generated by the automated SWISS-MODEL pipeline [35].

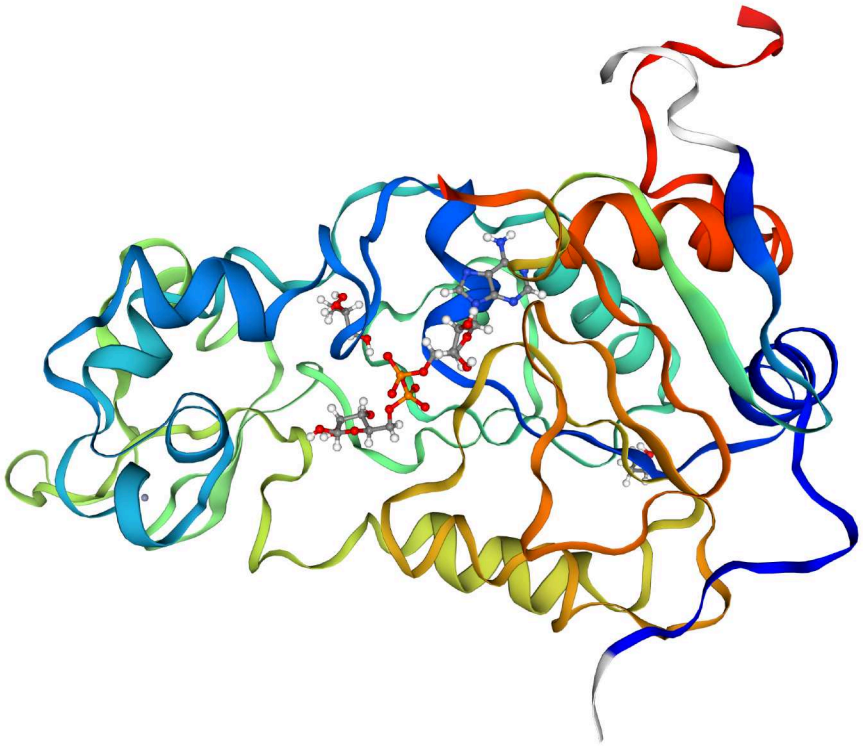

Figure 1.6: The SIRT1 3D protein structure model generated by the automated SWISS-MODEL pipeline [35].

1.9 PITX2 methylation and autophagy

PITX2 (Pituitary homeobox 2) is a DNA-binding transcription factor that regulates tissue-specific cell division and morphogenesis (Figure 1.7).

The kit for the evaluation of the PITX2 methylation is described to be a reliable prognostic factor for drug resistance and it results particularly accurate when applied to formalin-fixed paraffin-embedded tissue. This kit is available on the market and is CE-marked as Therascreen PITX2 RGQ PCR.

Methylation of PITX2 is predictive for anthracycline chemotherapy sensitivity in breast cancer [42–47]. Furthermore, PITX2 regulates the expression of DIRAS3 in lung cancer [48], and the re-expression of DIRAS3 promotes autophagy in breast tumor cells, and this mechanism favors the effect of paclitaxel on tumor cells inhibition [49].

Although both PITX2 and TFEB play a crucial role in autophagy, their functions seem to be different so that we assume that these pathways will be independently predictive of survival and drug resistance in breast cancer. Therefore, a prediction test based on these two proteins would be useful to better target antineoplastic therapies and allow a more personalized treatment strategy with the most effective drugs.

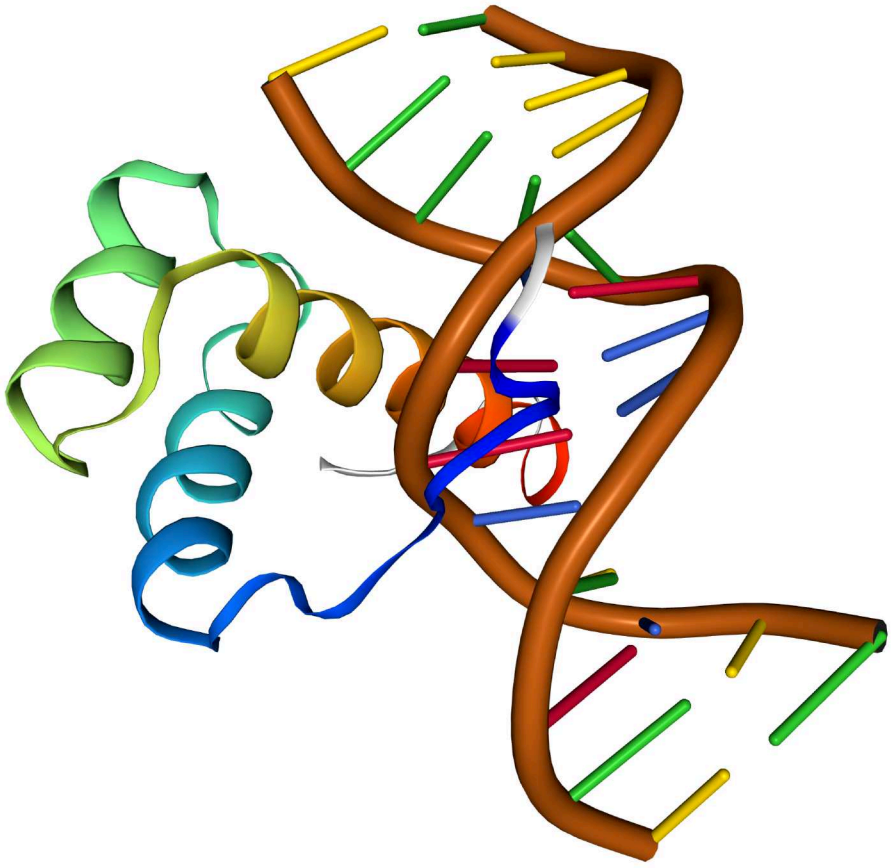

Figure 1.7: The PITX2 3D protein structure model generated by the automated SWISS-MODEL pipeline [35].

Chapter 2

The study

2.1 Objective

The main objective of this study is to assess the prognostic role of TFEB, CARM1, and SIRT1 in chemotreated breast carcinoma and to compare the TFEB, CARM1, and SIRT1 expression with the methylation of PITX2 in breast cancer treated with chemotherapy.

2.2 Materials and methods

Study population

The study population is made up of women affected by invasive breast carcinoma, treated by adjuvant and/or neoadjuvant chemotherapy, and with archived tissue specimen of the primary tumor. Tissue samples (from standard archived, formalin-fixed, paraffin-embedded tissue) and clinical data have been retrospectively collected at the Institute of Pathology of the University of Udine and at the Breast Unit of the Azienda Sanitaria Universitaria Integrata (ASUI) "Santa Maria della Misericordia" of Udine, respectively.

In this study all breast cancer cases treated by adjuvant and/or neoadjuvant chemotherapy between January 2002 and December 2016 have been included, in order to have at least twelve months of follow up. All cases were included which have enough tumor tissue stored in paraffin blocks at the time of surgery. As a consequence, all cases of pathological complete response have been excluded, as the tissue amount from preoperative biopsy did not provide enough material for the current study, as well as all cases of bad conserved or quantitatively insufficient tissues. Moreover, we excluded from the study all cases without documented follow up, breast cancer patients who did not undergo adjuvant or neoadjuvant treatments, and male patients.

Study design

This is a retrospective observational study which reviews both pathological archives and medical records for the identification of breast cancer cases treated by adjuvant and/or neoadjuvant chemotherapy. For the purpose of the study, two different types of sample selection from the original cohort have been performed:

- case-cohort study;
- case-control study.

Case-cohort study

The case-cohort study design model is a sampling methodology to randomly select a sample (called subcohort) from an assembled epidemiologic cohort, and to use this subcohort as a comparison group for the selected cases that occur in the cohort (in this specific case the breast cancer recurrence group within 12 months of follow up) [50]. This kind of study is particularly suitable for very numerous cohorts, which are too expensive to be completely followed up for disease outcomes or for peculiar information on the whole cohort [50–52].

Of the 4504 women in the cohort treated for breast pathology, 894 were eligible for the present study as they have been treated by chemotherapy and fulfilled the required inclusion criteria. The study sample was selected among these last 894 women. It was composed of a random group of 163 of the 894 eligible women (hereafter called the subcohort) together with all eligible women diagnosed with a breast cancer recurrence within 12 months of follow up after treatment initiation. We considered all recurrences (loco-regional recurrences and/or distant metastases) and unfavorable events (breast cancer related death) occurred between the baseline attendance and April 30, 2017.

The final sample included 203 women: 163 belonging to the subcohort and 42 breast cancer patients who experienced any type of recurrence within 12 months of follow up. Based on the approach of Cai and Zeng for power and sample size considerations, we took into account the possibility to have an unavailability of about 35% of samples for the assessment in the TMA [53]. The study had >80% power to detect HRs of 3.0, assuming alfa = 0.05 [53].

Case-control study

In the case-control sample selection, all breast cancer patients included in the tissue micro-array have been considered. The cases and the controls have been randomly selected from the above mentioned patients.

The number of the analyzed samples has been established from the maximum available resources to test PITX2 methylation. Therefore, the total number of cases was 13 and the total number of controls was 13.

Clinical information

Collected information included some patient characteristics, suche as the age at diagnosis, the body mass index (BMI = kg/mq), the eventual familial history for breast or ovarian carcinoma, the current fertility status (pre- or post-menopausal), the eventual use of estroprogestinic therapies (with contraceptive intent in the pre-menopausa or as hormone replacement therapy in the post-menopausa). Considered tumor characteristics were the histotype, grading, expression of estrogen receptor (ER), progesteron receptor (PR), HER2/neu and Mib1/Ki-67, as well as the eventual presence of multifocality/multicentricity, peri-vascular invasion (PVI), peritumoral lymphocitary infiltration, nodal extracapsular invasion or bunched axillary nodes [54]). Also surgical and non surgical treatments were took into account for data elaboration.

Pathological specimens were routinely assessed following the European guidelines [55, 56]. In particular, samples sized 30 mm or less were completely sliced and evaluated, whereas specimens sized over 30 mm underwent sampling based on the European guidelines [55, 56].

The World Health Organization criteria were used to determine the histology [57] and nodal status (TNM classification VII ed.AJCC/UICC, 2009) (Box 1) [58]. The recommendations of AFIP and Elston Ellis were considered while assessing the grading in ductal carcinoma in situ and invasive carcinoma, respectively [59, 60]. Peritumoral lymphocitary infiltration, PVI, multifocality/multicentricity, and nodal status were determined as described in previous studies [7, 61].

PR, ER, Mib1/Ki-67, and Her-2/Neu expression were evaluated by immunohistochemistry. We defined positive ER or PR in the case of positivity $\geq$1% in any nuclear staining. In addition, Her-2/Neu was deined overexpressed when staining 3+ or 2+ with FISH am-

plification, and negative if value was 0, 1+ or 2+ without FISH amplification. Through the combination of ER, PR, Her-2/Neu and Mib1/Ki-67, all invasive breast cancers have been classified in the following molecular subtypes as previously described: luminal A, luminal B, luminal Her, Her2-enriched, and basal-like [7].

Box N° 1

TNM breast cancer : Primary Tumor (T) (AJCC 7th Edition 2009) [58]

The pre-treatment clinical stage is described using the "c" prefix. The post-neoadjuvant extent of disease and response to therapy are documented prognostic factors in breast cancer and they warrant the use of the “yp” prefix. The pre-treatment clinical T is defined by clinical and radiological findings, while the post-neoadjuvant pathologic T is determined by istopathological size and extension. The ypT is measured as the largest single focus of invasive tumor, with the modifier “m” indicates multiple foci. The measurement of the largest tumor focus does not include areas of fibrosis within the tumor bed.
The T classification of the primary tumor is the same regardless of whether it is based on clinical or pathologic criteria, or both. Size is measured to the nearest millimeter. If the tumor size is slightly less than or greater than a cut-off for a given T classification, that size is recommended to be rounded to the millimeter reading that is closest to the cut-off. For example, a reported size of 0,99 mm is reported as 1 mm, or a size of 2.01 cm is reported as 2.0 cm. Designation is made with the subscript “c” or “p” modifier to indicate whether the T classification is determined by clinical (physical examination or radiological findings) or pathological measurements, respectively. In general, pathological determination takes precedence over clinical determination of T size.

- TX: Primary tumor cannot be assessed
- T0: No evidence of primary tumor
- Tis: Carcinoma in situ (DCIS, LCIS, Paget’s disease of the nipple NOT associated with invasive carcinoma and/or carcinoma in situ (DCIS and/or LCIS) in the underlying breast parenchyma)
- T1: Tumor $\leq$20 mm in greatest dimension
  - T1mi: Tumor $\leq$1 mm in greatest dimension
  - T1a: Tumor > 1 mm but $\leq$5 mm in greatest dimension
  - T1b: Tumor >5 mm but $\leq$10 mm in greatest dimension
  - T1c: Tumor > 10 mm but $\leq$20 mm in greatest dimension
- T2: Tumor >20 mm but $\leq$50 mm in greatest dimension
- T3: Tumor >50 mm in greatest dimension
- T4: Tumor of any size with direct extension to the chest wall and/or to the skin (ulceration or skin nodules) [*Note : Invasion of the dermis alone does not qualify as T4*]

- T4a: Extension to the chest wall, not including only pectoralis muscle adherence/invasion
- T4b: Ulceration and/or ipsilateral satellite nodules and/or edema (including peau d'orange) of the skin, which do not meet the criteria for inflammatory carcinoma
- T4c: Both T4a and T4b
- T4d: Inflammatory carcinoma

Immunohistochemistry and molecular biology analyses

In this study the following analyses have been performed in both the case-cohort and the case-control subjects selection. We have analyzed the presence and the quantity of mRNA and the relative protein synthesis in the selected breast cancer tumor samples. In addition (only for the subjects selected for the case-control study), the Therascreen PITX2 RGQ PCR have been used to test the PITX2 methylation.

Real time PCR (RT-PCR) to quantify mRNA of TFEB, CARM1, and SIRT1

For real-time quantitative PCR the primers have been prepared for TFEB, CARM1, and SIRT1 (Table 2.1). And glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been used as housekeeping protein (Table 2.1). The m-RNA has been extracted from formalin-fixed paraffin-embedded tumor tissue samples by manually microdissecting the tumor area that had been histologically marked by a pathologist and using a RNeasy kit (Qiagen®, Venlo, Netherlands) that was used according to the manufacturer's protocol. RNA quantity and purity were measured using the Qubit 2.0 spectrophotometer (Invitrogen®, Carlsbad, CA), and RNA integrity was quantified using the RIN (RNA integrity number) assessed by Agilent 2100 Bioanalyzer (Agilent Technologies®, Santa Clara, CA). From this m-RNA, by mean of retrotrascription (SuperScript®, III REV transcript; Life Technologies), cDNA has been obtained and quantified. Quantitative real-time PCR analysis was performed in triplicate, by three independent experiments, using the LightCycler® 480 (Roche®) and LC SYBR Green I Master (Roche®), according to the manufacturers' protocols. Quantitative data have been collected as cycle threshold (CT) values considering the meas of the triplicate runs. Finally, quantitative ΔCT expression values (2(-ΔCT)) has been calculated.

Immunohistochemical analysis (IHC)

Along with the RT-PCR analysis, TFEB, CARM1, and SIRT1 protein expressions have been investigated by immunohistochemistry among all included cases of breast cancer. Cases has been evaluated with respect to both staining percentage and intensity.

Tissue Micro Array (TMA)

The preparation and analysis of the TMA have been carried out as previously described [62–67]. Once selected blocks containing formalin-fixed paraffin-embedded tumor tissue have been selected, the hematoxylin-eosin colored sections have been analyzed, and then the tissue core samplings for the TMA have been performed being careful to include neoplastic tissue (two core biopsies per primary tumor). The receiver blocks have been assembled (which contained in the same block more cases according to the attached diagram) (Figure 2.1). Then, from the receiving blocks, 4-µm cross sections have been obtained, which have been stained with hematoxylin-eosin. At a later time other 4-µm cross sections have been obtained to prepare slides for immunohistochemical staining and subsequent analysis.

Immunohistochemical staining has been performed according to standard protocol and manufacturer instructions. For antigen retrieval, the slides were heated, after deparaffinization, for 20 minutes at 98°C in Target Retrieval Solution (low pH; Dako K8005, Glostrup, DK) with PT-link (Dako) and endogenous peroxidase activity was blocked with H2O2 for 10 minutes at environmental temperature. Slides were rinsed in PBS and then incubated with the following primary antibodies for 1 h at environmental temperature: TFEB (OriGene Technologies Inc., diluted 1:100, Rockville, MD, USA); CARM1 (OriGene Technologies Inc., diluted 1:100, Rockville, MD, USA); SIRT1 (OriGene Technologies Inc., diluted 1:200, Rockville, MD, USA). A Dako REAL™ EnVision™ Dako Rabbit/Mouse (Dako, K5007, Glostrup, DK) was used as a second antibody. HRP activity was identified utilizing Dako REAL™ DAB+Chromogen (Dako, K5007,Glostrup, DK) as substrate for 3 minutes in accordance with the manufacturer's instructions. Then, tissue sections were counterstained with hematoxylin. Negative controls included sections incubated with non-immune rabbit serum instead of the primary antibody. The immunohistochemical staining has been evaluated, independently by two pathologists, in terms of H-score (the product of actual percentage of positive-stained

cells and intensity score, evaluated as strong 3, moderate 2 and weak 1, giving a possible range of 0-300).

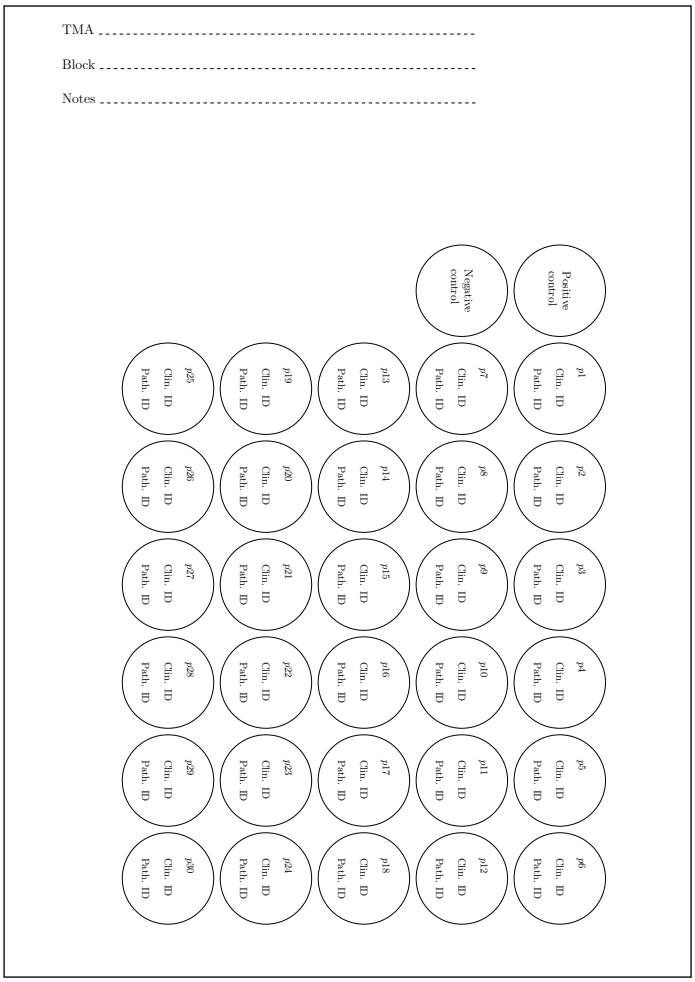

Figure 2.1: TMA recipient block map. The map of the recipient block was prepared with information for each sample to correctly identify the tumor.

PITX2 DNA methylation assay

The PITX2 promoter methylation was evaluated on DNA extracted from formalin-fixed paraffin-embedded sections (QIAmp DNA mini kit-Qiagen). In particular, the DNA was extracted from each sample starting from 5-10 sections of 10 μm thickness kept at room temperature until the time of extraction. To restrict the analysis to the DNA of the tumor area and to Therascreen® PITX2 RGQ PCR kit is a methylation specific PCR (MSP) based on real-time PCR, intended for the quantitative assessment of percent methylation ratio (PMR) in the promotor 2 (P2) of the PITX2 gene and it was validated in primary formalin-fixed paraffin-embedded breast cancer tissue [43, 68, 69] (see Box 2).

Genomic DNA was extracted from the samples using the QIAmp DNA FFPE Tissue Kit (QIAGEN Inc.). DNA was quantified using Qubit dsDNA analysis kit for QBIT 2.0 fluorimeter (Thermo Fisher Scientific). The bisulfite conversion of the DNA was performed using the EpiTect Plus DNA Bisulfite Kit (QIAGEN Inc.) and the methylated DNA was then purified using the purification module reagents provided by the same kit and quantified at QBIT 2.0. After bisulfite conversion the PMR of 3 CpG motifs of the PITX2 gene P2 was quantified by MSP using the Therascreen® PITX2 RGQ PCR kit, that contains quantitative RT-PCR reaction mix, primer, probes, positive and negative controls [68].

The quantitative real-time PCR reaction was performed using the Rotor-Gene Q MDx real-time PCR platform (QIAGEN, Inc.) and evaluated by QIAGEN Rotor-Gene Assay-Manager® (Version 2.1.0) software with Therascreen PITX2 FFPE (CE) analysis plugin for analysis and quality control [70].

Statistical analysis

Data was analyzed through R (version 3.6.1; R Core Team (2019); R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria; URL https://www.R-project.org/) and considering as significant $p<0.05$. Normality of the distribution was tested with Kolmogorov-Smirnov test. Univariate analysis was performed by Wilcoxon or t-test in cases of continuous variables, Fisher exact test or chi-square test in cases of categorical variables. Also univariate and multivariate survival analyses were performed by Kaplan-Meier curves, Log-rank test, and Cox proportional hazards regression models. OS was considered to be the main outcome. In addition, in the multivariate model all selected factors and their interactions were accommodated in a single analysis, except when the interaction term was non-significant (in which case we analyzed the no-interaction model). Correlations were tested by Spearman Rho and the relative p-value.

Box N° 2

Percent methylation ratio (PMR)

$$PMR = \frac{100}{1 + 2^{CT_{FAM} - CT_{HEX}}}$$

ΔCT between FAM and HEX ($CT_{FAM} - CT_{HEX}$) is assessed by the difference between the following CT:

- Specific probe for bisDNA sequences from methylated sequences, marked by FAM dye;
- Specific probe for bisDNA sequences from unmethylated sequences, marked by fluorophore HEX.

The obtained PMR value will provide information to the treating physicians regarding the probability of the patient's response to anthracycline-based chemotherapy. If the PMR value obtained is 12 or less, the patient is likely to respond to anthracycline-based chemotherapy. If, on the other hand, the PMR value obtained is greater than 12, an alternative treatment could be proposed, since the probability that the patient responds to anthracycline-based chemotherapy is low.

Table 2.1: RT-PCR primers of TFEB, CARM1, SIRT1, and GAPDH.

TFEB	Sequence
Forward:	TGATCCCCAAGGCCAATGAC
Reverse:	TCCCTGGACTTTTGCAGGTC
CARM1	
Forward:	AGCCGGATCTAAGATGGCAG
Reverse:	GGGAACACCGACACGGTA
SIRT1	
Forward:	TGCTGGCCTAATAGAGTGGC
Reverse:	ACCTCAGCGCCATGGAAAAT
GAPDH	
Forward:	CTCTGACTTCAACAGCGACAC
Reverse:	AGCCAAATTCGTTGTCATACCAG

2.3 Results

Population description

After the identification of the forty eligible subjects, the sub-cohort of 163 patients was extrapolated. From this first procedure, it was found that four subjects were superimposed between the cases and the sub-cohort. The blocks for the creation of the TMA were then collected. During the creation of the TMA of the 199 selected samples (including 159 samples of cases who did not recur within 12 month of follow up and 40 cases who relapsed), only 109 cases who did not recur within the year and 27 cases who relapsed have been successfully included in the TMA. In fact, in the final TMA, it was not possible to include 50 samples of the cases who did not recur within 12 months and 13 samples of those who relapsed, as they were found to be unusable samples (i.e. samples used in other studies or insufficient quantity of residual neoplastic tissue or not available samples).

Table 2.2 shows the included samples description. Median age at breast cancer surgery resulted 55 years (48-65) and median BMI 25 kg/m^2 (22-29). In the 99.26% and 8.82% of cases, respectively, adjuvant and neoadjuvant chemotherapy have been administered. For what concerns the surgical treatment, in the majority of cases mastectomy has been performed (68.38%), together with complete axillary lymph node dissection (69.12%).

Lymph node macrometastases (neoplastic cell aggregates >2 micrometers) and micrometastases (neoplastic cell aggregates of 0.2-2 micrometers) have been found out respectively in the 56.62% and 5.88% of cases. Taking into account that before 2015 both macro- and micrometastases were considered an absolute indication for complete axillary lymph node dissection, and that this study includes patients operated between 2002 and 2016, likely the great majority of these cases of nodal involvement underwent complete axillary lymphoadenectomy. The remaining cases who underwent axillary lymph node dissection included patients who were clinically node-positive before neoadjuvant traetment and converted to clinically node-negative after it (Table 2.3), as these particular cases are still argument of debate worldwide, and no complete agreement is there about the best management of their axillary lymph nodes.

In Table 2.3 also other tumor characteristics are described. In the 80.88% of cases tumor histotype was invasive ductal carcinoma, followed by the invasive lobular carcinoma in the 11.03% of cases. The 55.77% of tumors presented a Mib-1/Ki-67 greater than 20%,

and 24.26% were found to be histologically multifocal or multicentric.

Table 2.4 reports breast tumors staging. The majority of tumors were classified as TNM II e III (respectively 36.03% and 24.26%) and tumor grading was classified as G2 in more than the half of patients (52.21%).

Table 2.2: Population characteristics.

Age (years)	55 (48-65)
BMI (kg/m^2)	25 (22-29)
Median follow up (months)	55 (35-94)
Tobacco smoke	12.5% (12/96)
Family history	42.22% (19/45)
Estroprogestinics use	31.82% (7/22)
Post-menopausal status	71.32% (97/136)
Definitive breast surgery	
Conservative	31.62% (43/136)
Mastectomy	68.38% (93/136)
Definitive axilla surgery	
SLNB	30.88% (42/136)
CALND	69.12% (94/136)
Non-surgical therapy	
Neoadjuvant chemotherapy	8.82% (12/136)
Radiotherapy	56.62% (77/136)
Adjuvant chemotherapy	99.26% (135/136)
Anti-hormonal therapy	74.26% (101/136)

Acronyms: BMI = body mass index; SLNB = sentinel lymph node biopsy; CALND = complete axilla lymph node dissection.

Table 2.3: Tumor characteristics.

Histology	
Invasive ductal carcinoma	80.88% (110/136)
Invasive lobular carcinoma	11.03% (15/136)
Invasive ductal and lobular carcinoma	6.62% (9/136)
Other invasive cancers	1.47% (2/136)
Molecular subtypes	
Luminal A	18.38% (25/136)
Luminal B	28.68% (39/136)
Luminal Her	8.09% (11/136)
Her enriched	11.03% (15/136)
Basal-like	13.97% (19/136)
Not described	19.85% (27/136)
Other tumor caracteristics	
Mib-1/Ki-67 (median percentage)	30 (10-70)
Mib-1/Ki-67 (>20%)	55.77% (58/104)
Comedo-like nescrosis	13.24% (18/136)
Multifacality/multicentricity	24.26% (33/136)
Extended in situ component	21.32% (29/136)
Perivascular invasion	40.44% (55/136)
Peritumoral inflammation	1.47% (2/136)
Lymph node characteristics	
Non-axillary loco-regional lymph nodes	2.21% (3/136)
ITC	0% (0/136)
Micrometastatic lymph nodes	5.88% (8/136)
Macrometastatic lymph nodes	56.62% (77/136)
Extracapsular invasion	25.74% (35/136)
Bunched axillary lymph nodes	7.35% (10/136)

Acronyms: ITC = isolated tumor cells.

Table 2.4: TNM staging and tumor grading.

Tumor size	
T1	53.68% (73/136)
T2	36.03% (49/136)
T3	5.88% (8/136)
T4	4.41% (6/136)
Nodal involvement	
N0	37.5% (51/136)
N1	36.03% (49/136)
N2	11.76% (16/136)
N3	14.71% (20/136)
TNM stage	
I	34.56% (47/136)
II	36.03% (49/136)
III	24.26% (33/136)
IV	5.15% (7/136)
Tumor grading	
G1	7.35% (10/136)
G2	52.21% (71/136)
G3	40.44% (55/136)

Acronyms: TNM = tumor node metastasis.

TFEB, CARM1 and SIRT1 expression

Median TFEB expression in terms of H-score is 185 (IQR 99-200) with an exclusive nuclear localization (Figure 2.2A and 2.2B).

Median CARM1 expression in terms of H-score is 85 (IQR 25-100) again with a nuclear localization (Figure 2.2C and 2.2D).

Finally, median SIRT1 expression is 190 (IQR 100-200) and even in this case the localization was in the nucleus (Figure 2.2E and 2.2F).

Figure 2.3 highlights the analysis of survival based on the expression of TFEB. In particular, a higher immunohistochemical expression of TFEB protein correlates with significantly shorter survival ($p < 0.05$). This difference is also present in the Cox regression analysis with a HR of 3.46 (IC.95 1.27-9.47) ($p < 0.05$). Furthermore, in the multivariate analysis this difference results independent by neoadjuvant chemotherapy (Table 2.5).

Figure 2.4 shows the association between a high expression of CARM1 with reduced survival, although this correlation is not statistically significant. And Figure 2.5 shows that a H-score of SIRT1 higher than the distribution median is not associated with significant differences in terms of survival.

Table 2.5: Survival analysis using the univariate and multivariate Cox proportional risk regression model (**), for the analyzes a H-score was considered as high, the one above the median of the distribution.

	HR (IC95%)	p	HR (IC95%) (**)	p(**)
TFEB H-score >185 (*)	3.25 (1.19 - 8.86)	<0.05	3.46 (1.27 - 9.47)	<0.05
Neoadiuvant chemotherapy	7.29 (3.1 - 17.1)	<0.05	7.88 (3.26 - 19.05)	<0.05

(*) H-score greater than the distribution median.
(**) Multivariate model.

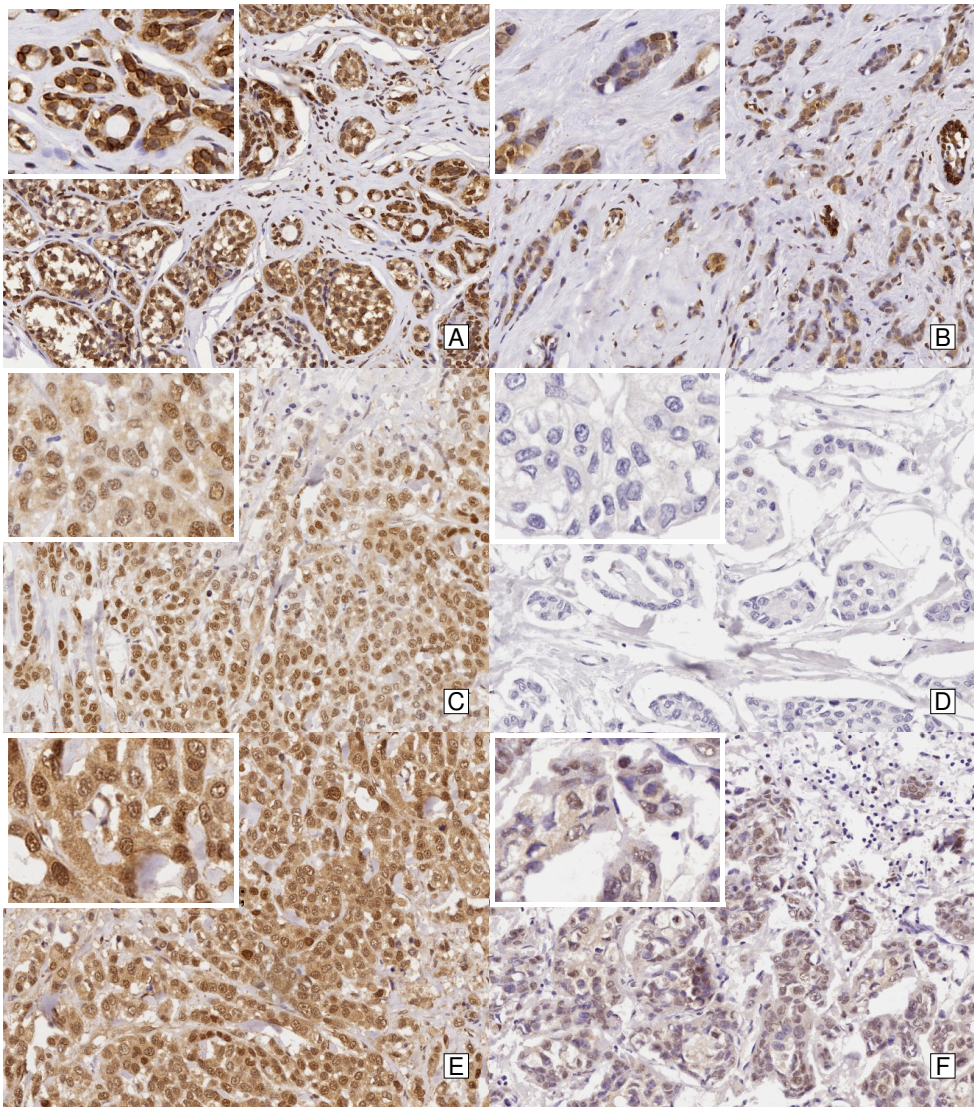

Figure 2.2: Immunohistochemical staining. Panel A: Image at 20x (and in the box at 40x) of TFEB immunohistochemical staining in the specimen of a recurrent breast cancer within 12 months of follow up. Panel B: Image at 20x (and in the box at 40x) of TFEB immunohistochemical staining in the specimen of a breast cancer which did not recur within 12 months of follow up. Panel C: 20x image (and in the 40x square) of CARM1 immunohistochemical staining in the specimen of a recurrent breast cancer within 12 months of follow up. Panel D: 20x image (and in the 40x frame) of CARM1 immunohistochemical staining in the specimen of a breast cancer which did not recur within 12 months of follow up. Panel E: Image at 20x (and in the box at 40x) of SIRT1 immunohistochemical staining in the specimen of a recurrent breast cancer within 12 months of follow up. Panel F: Image at 20x (and in the box at 40x) of SIRT1 immunohistochemical staining in the specimen of a breast cancer which did not recur within 12 months of follow up.

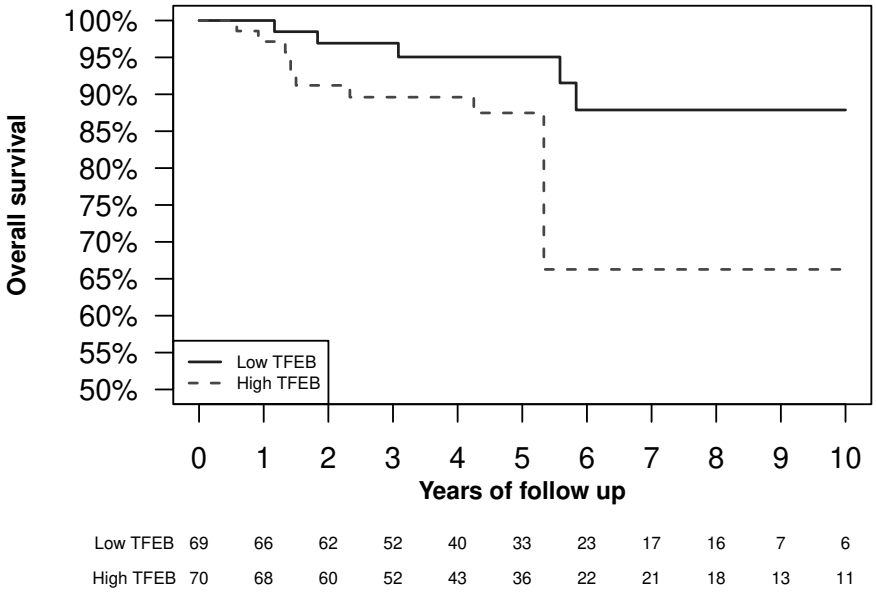

Figure 2.3: Kaplan-Meier survival curve based on TFEB expression (high expression consists in a H-score greater than the distribution median [>185] and low expression in a H-score lower or equal to the distribution median). Log-rank test $p<0.05$.

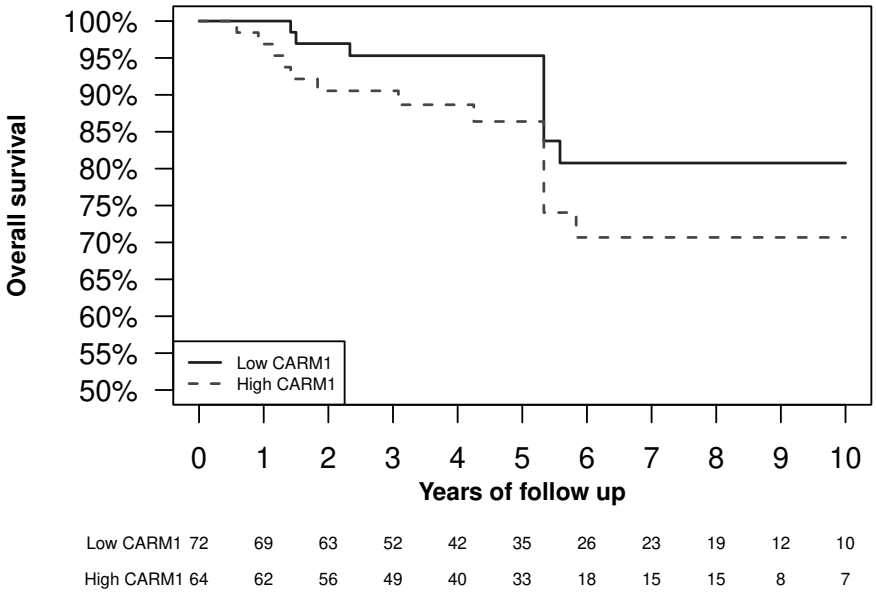

Figure 2.4: Kaplan-Meier analysis of survival based on CARM1 expression (i.e. high with a H-score higher than the median distribution [>85] or low i.e. with an H-score lower than or equal to the distribution median). Log-rank test $p = 0.156$.

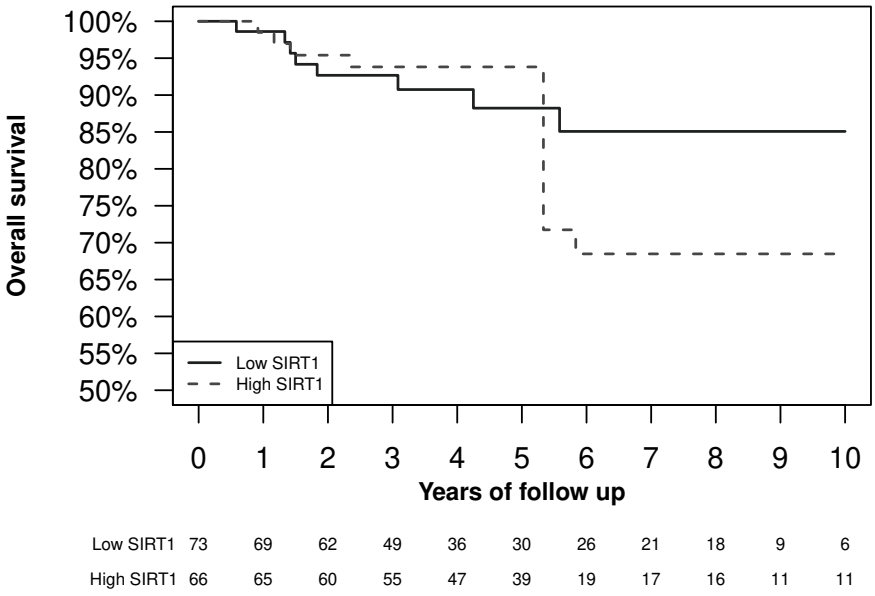

Figure 2.5: Kaplan-Meier analysis of survival based on SIRT1 expression (i.e. high with a H-score higher than the median distribution [190] or low i.e. with an H-score lower than or equal to the distribution median). Log-rank test p = 0.322.

Correlation among immunohistochemical expression of TFEB, CARM1 and SIRT1

In Figure 2.6 it is possible to observe the correlation among the expressions of the three analyzed proteins. In particular, the immunohistochemical protein expression of SIRT1 correlates, in a directly proportional and significant way, with the expression of TFEB and CARM1. In addition, also the immunohistochemical expression of TFEB significantly correlates with that of CARM1.

The graph also shows that a SIRT1 expression within the first quartile of the H-score distribution corresponds to a low expression of CARM1 and TFEB. Then, we decided to evaluate whether survival in this particular subgroup was actually longer. Thus, Figure 2.7 shows a better survival ($p < 0.05$) in the case of SIRT1 expression under the first quartile of the H-score distribution.

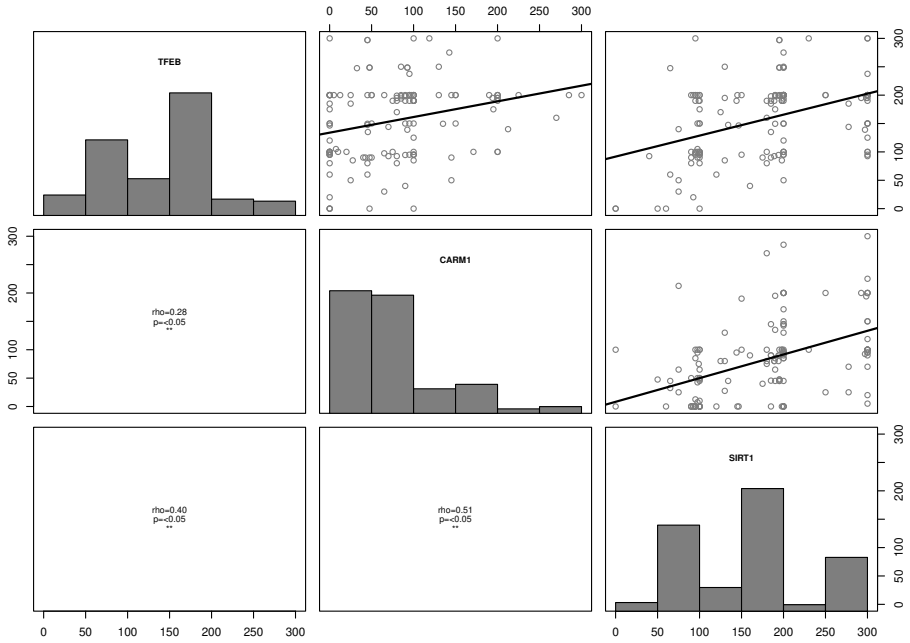

Figure 2.6: Analysis of the correlation between the immunohistochemical protein expression evaluated in H-score of the three proteins analyzed. Correlations evaluated by the Spearman test.

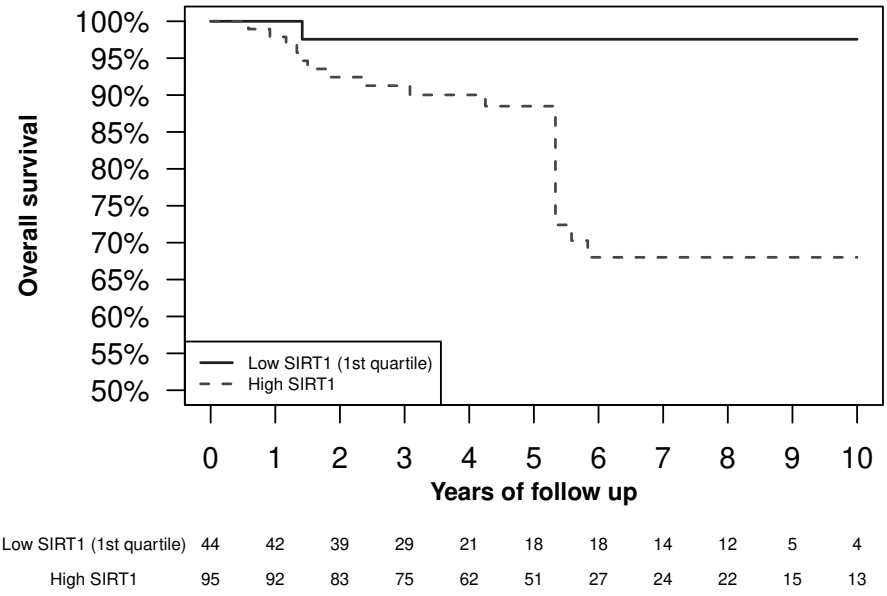

Figure 2.7: Kaplan-Meier analysis of survival based on the expression of SIRT1 (i.e. high with a H-score higher than the first quartile of the distribution [> 100] or low i.e. with a H-score lower than or equal to the first quartile of the distribution). Log-rank test $p < 0.05$.

Tumor molecular subtypes and TFEB, CARM1, and SIRT1 expression

Table 2.6 demonstrates that neither CARM1 nor SIRT1 result significantly modified while considering separately the different tumor molecular subtypes. Although not significantly, it is remarkable that the H-score of SIRT1 is lower in the basal-like and in the Her-enriched sybtypes compared to the luminal A, luminal B and luminal Her ones.

Therefore, in Figure 2.8, the Kaplan-Meier survival analysis based on SIRT1 expression has been redesigned including only the luminal A, luminal B and luminal Her molecular subtypes. And in this case no more adverse events have been recorded in the case of low SIRT1 expression (p=0.055).

Furthermore, Figure 2.9 shows that, also in the basal-like and in the Her-enriched subtypes, separately, a low SIRT1 expression results associated with a better prognosis even if not in a statistically significant way.

We also redesigned the correlations between the three molecules in Figure 2.10 and 2.11 considering the tumor molecular subtypes. In particular, in Figure 2.10 we included only the basal-like subtype, and the correlation between CARM1 and SIRT1 is maintained while the TFEB correlations with SIRT1 and CARM1 loses its statistical significance.

Finally, Figure 2.11 includes only the luminal variants (luminal A, luminal B and luminal Her), which confirm the directly proportional and significant correlations of SIRT1 with TFEB and CARM1.

TFEB, SIRT1 and CRAM1 expression in RT-PCR

The mRNA quantification was only possible in samples with high H-score and low paraffin storage duration. In particular for CARM1, which results the least expressed protein in immunohistochemistry, no mRNA was detected in the analyzed samples. As regards TFEB and SIRT1, a direct proportional correlation between immunohistochemical score and mRNA expression is shown in Figures 2.12 and 2.13, although these correlations are not statistically significant.

Table 2.6: Analysis of the expression of the considered proteins among the various molecular subtypes where the data is present. The values reported are median and range of interquartiles (IQR) while p refers to the Wilcoxon test.

	Luminal A (24)	Luminal B (38)	Luminal Her (10)	Her enriched (14)	Basal-like (18)	p
CARM1 H-score	72.50 (18.75-100.00)	87.50 (18.75-100.00)	75.00 (41.38-141.25)	90.00 (12.50-160.94)	95.00 (35.62-144.38)	NS
SIRT1 H-score	187.50 (100.00-200.00)	189.50 (100.00-200.00)	180.00 (100.00-200.00)	157.50 (100.00-198.75)	139.00 (97.88-198.50)	NS
TFEB H-score	150.00 (90.00-196.00)	112.50 (95.00-193.00)	142.50 (91.25-200.00)	192.50 (171.25-199.88)	190.00 (148.12-200.00)	6,7

Statistically significant differences ($p < 0.05$): (1) Luminal A vs Luminal B; (2) Luminal A vs Luminal Her; (3) Luminal A vs Her enriched; (4) Luminal A vs Basal-like; (5) Luminal B vs Luminal Her; (6) Luminal B vs Her enriched; (7) Luminal B vs Basal-like; (8) Luminal Her vs Her enriched; (9) Luminal Her vs Basal-like; (10) Her enriched vs Basal-like. NS = non significant differences.

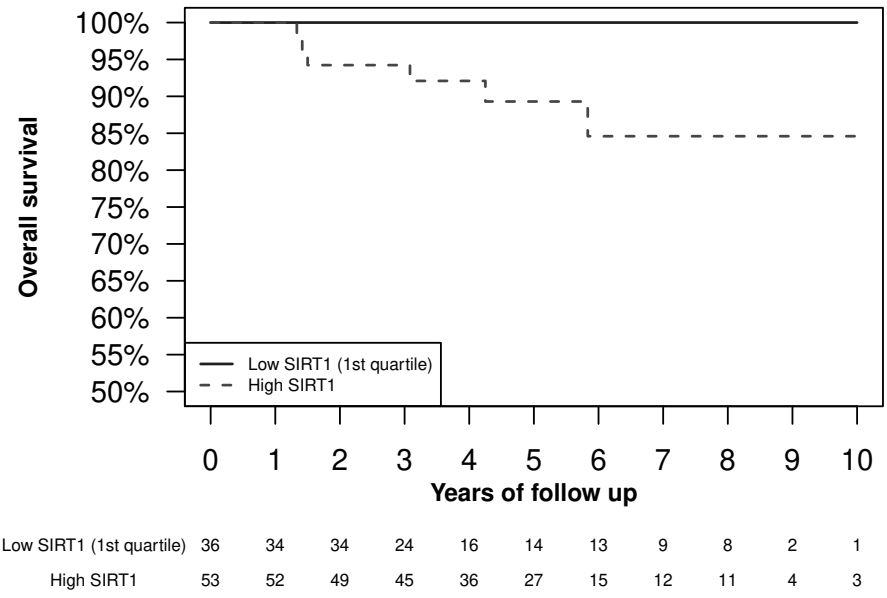

Figure 2.8: Kaplan-Meier analysis of survival based on the expression of SIRT1 (i.e. high with a H-score higher than the first quartile of the distribution [132] or low i.e with a H-score lower than or equal to the first quartile of the distribution). In this case we considered only luminal A, luminal B and luminal Her subtypes. Log-rank test p = 0.055.

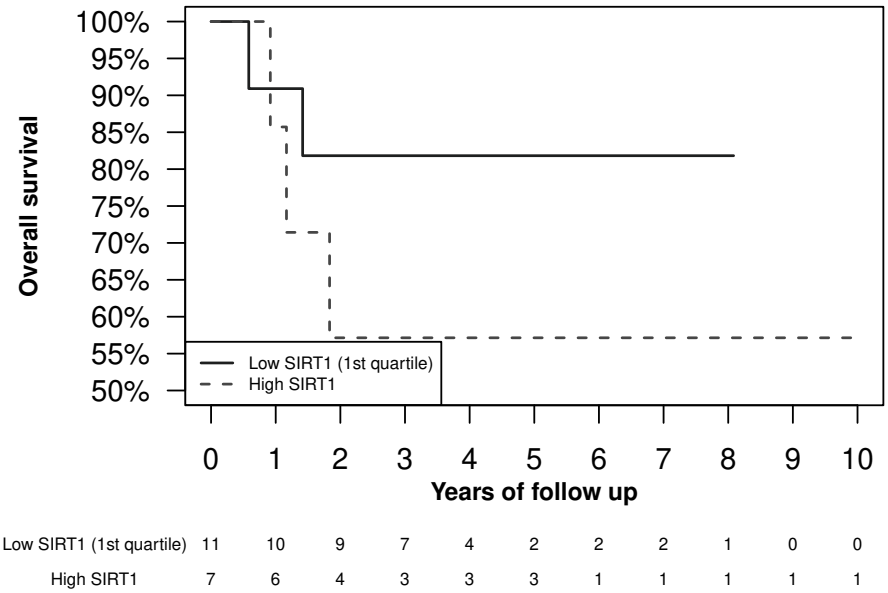

Figure 2.9: Kaplan-Meier analysis of survival based on the expression of SIRT1 (i.e. high with a H-score higher than the first quartile of the distribution [132] or low i.e. with a H-score lower than or equal to the first quartile of the distribution). In this case we considered only Her enricched and Basal-like. Log-rank test p = 0.294.

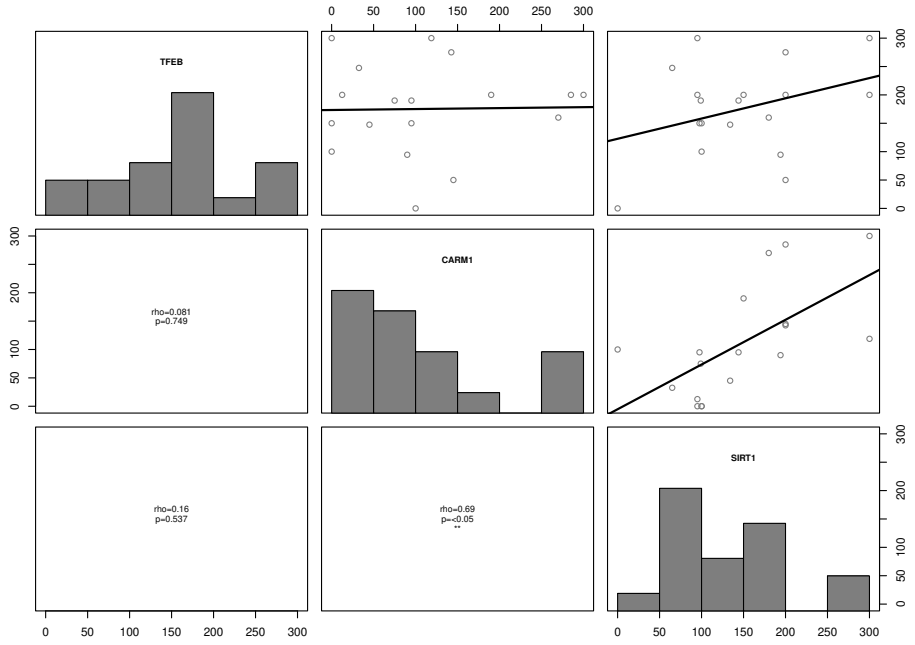

Figure 2.10: Analysis of the correlation between the immunohistochemical protein expression evaluated in H-score of the three proteins analyzed exclusively in the basal-like molecular subtype. Correlations evaluated by the Spearman test.

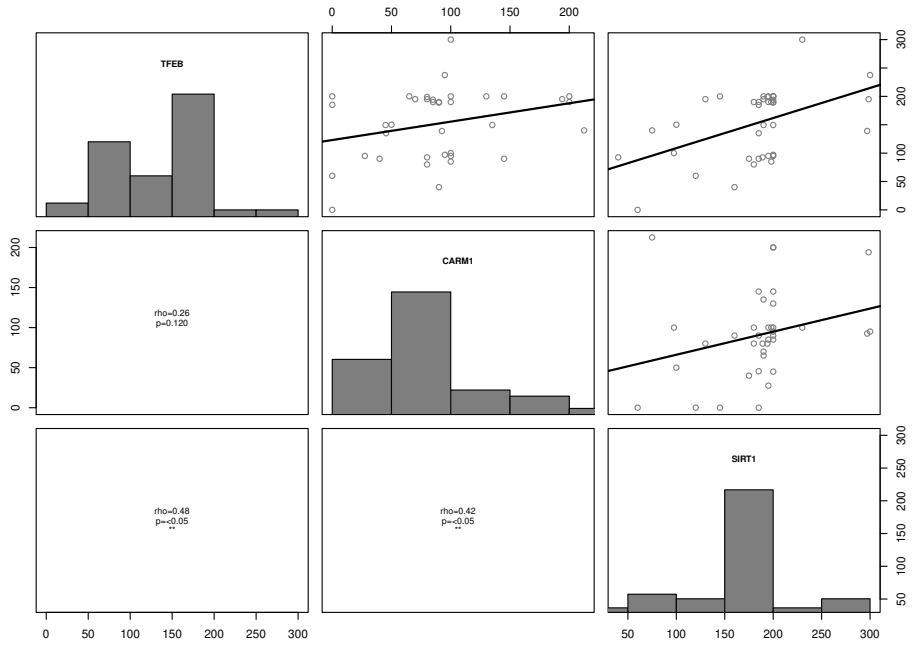

Figure 2.11: Analysis of the correlation between the immunohistochemical protein expression evaluated in H-score of the three proteins analyzed exclusively in the luminal A, luminal B and luminal Her. Correlations evaluated by the Spearman test.

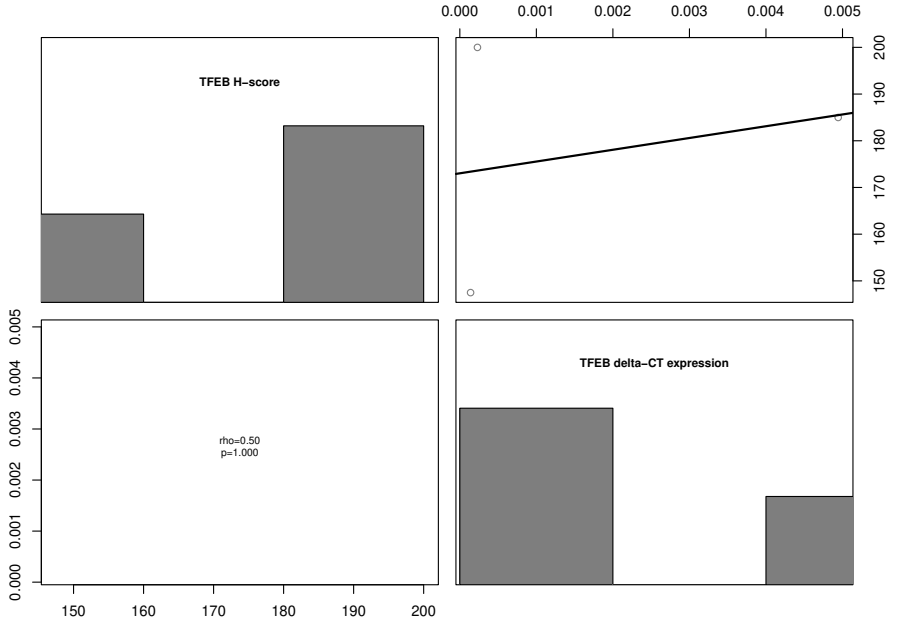

Figure 2.12: Analysis of the correlation between the immunohistochemical protein expression evaluated in H-score and mRNA expression as delta-CT expression (assessed by RT-PCR) of SIRT1. Correlations evaluated by the Spearman test.

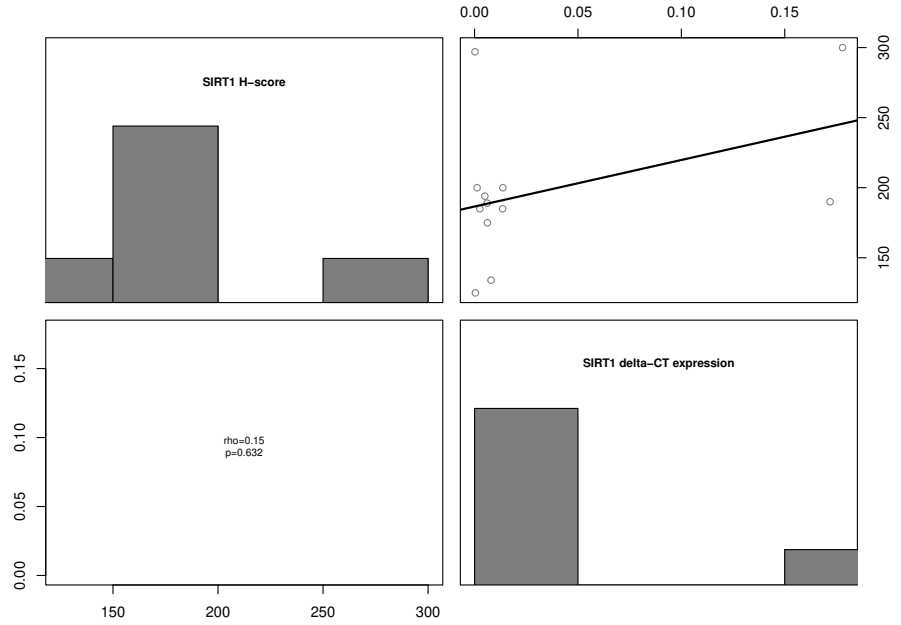

Figure 2.13: Analysis of the correlation between the immunohistochemical protein expression evaluated in H-score and mRNA expression as delta-CT expression (assessed by RT-PCR) of TFEB. Correlations evaluated by the Spearman test.

PITX2 methylation (case-control study)

The test failed in 9 samples (35%) (5 cases and 4 controls) on the total 26 tested samples. PMR was found to be >12 in the 37.50% of cases (3/8) and in 55.56% of controls (5/9) (p = 0.637).

Table 2.7 shows the characteristics of the patients in which the test was successful (8 cases and 9 controls) and no statistically significant differences were found. Although not statistically significant, the age of patients in cases with recurrence within the first year of follow up was greater than in controls, as well as the number of mastectomies, which however could be somehow linked to the greater age of patients.

Table 2.8 shows the tumor characteristics and no statistically significant differences were found except for a higher prevalence of basal-like tumor subtypes in the case of early recurrences. Table 2.9 shows the tumor stages and, also in this case, there were no statistically significant differences between the two considered groups.

In Table 2.10 the differences in the immunohistochemical expression of TFEB, CARM1 and SIRT1 are reported, as well as the PMR value of the PITX2 methylation. TFEB, SIRT1 and CARM1 were significantly more expressed in the cases that recur within the first year of follow up with respect to controls ($p < 0.05$), while no statistically significant differences are there as regards the PITX2 PMR.

In Figure 2.14 the correlations are shown between PITX2 methylation and immunohistochemical expression of TFEB, CARM1 and SIRT1, respectively. TFEB, CARM1 and SIRT1 result statistically significantly correlated, while PITX2 methylation does not correlate with the immunohistochemical expression of the TFEB pathway.

Table 2.7: Description of the population divided into the two groups (cases who recurred within 12 months from treatment initiation and controls who did not recur).

	Recurrence within 12 months (8)	No recurrence (9)	p
Age (years)	75.00 (64.00-83.25)	53.00 (44.00-67.00)	0.112
BMI (kg/m^2)	25.65 (22.57-26.85)	23.50 (22.00-25.50)	0.630
Definitive breast surgery			
Conservative	0.00% (0/8)	44.44% (4/9)	0.082
Mastectomy	100.00% (8/8)	55.56% (5/9)	0.082
Definitive axilla surgery			
SLNB	12.50% (1/8)	44.44% (4/9)	0.150
CALND	87.50% (7/8)	55.56% (5/9)	0.150
Neoadjuvant chemotherapy	37.50% (3/8)	11.11% (1/9)	0.200

Acronyms: BMI = body mass index; SLNB = sentinel lymph node biopsy; CALND = complete axillary lymph node dissection.

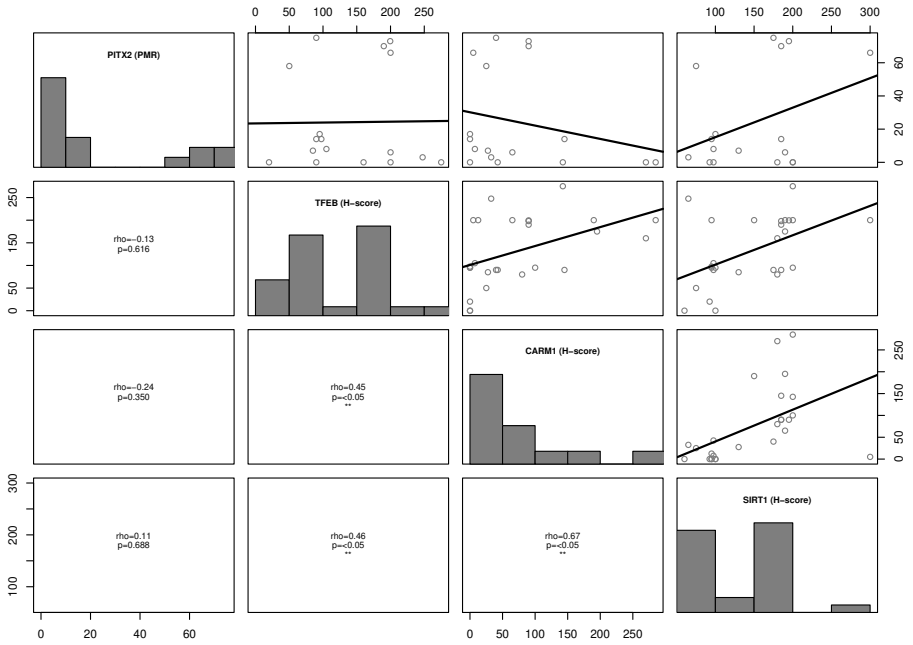

Figure 2.14: Analysis of the correlation between the PITX2 methylation (PMR) and the immunohistochemical expression (H-score) of TFEB, CARM1, and SIRT1. Correlations evaluated by the Spearman test.

Table 2.8: Tumor characteristics divided into the two groups (cases who recurred within 12 months from treatment initiation and controls who did not recur).

	Recurrence within 12 months (8)	No recurrence (9)	p
Histology			
Invasive ductal carcinoma	87.50% (7/8)	77.78% (7/9)	0.600
Invasive ductal and lobular carcinoma	12.50% (1/8)	22.22% (2/9)	0.600
Molecular types			
Luminal A	25.00% (2/8)	22.22% (2/9)	1.000
Luminal B	12.50% (1/8)	55.56% (5/9)	0.131
Luminal Her	0.00% (0/8)	22.22% (2/9)	0.471
Her enriched	12.50% (1/8)	0.00% (0/9)	0.471
Basal-like	50.00% (4/8)	0.00% (0/9)	<0.05
Other tumor caracteristics			
Mib-1/Ki-67 (>20 Comedo-like nescrosis	0.00% (0/8)	11.11% (1/9)	0.331
Multifacality/multicentricity	0.00% (0/8)	22.22% (2/9)	0.156
Perivascular invasion	25.00% (2/8)	44.44% (4/9)	0.402
Lymph node characteristics			
Micrometastatic lymph nodes	0.00% (0/8)	11.11% (1/9)	0.331
Macrometastatic lymph nodes	87.50% (7/8)	55.56% (5/9)	0.149
Extracapsular invasion	37.50% (3/8)	0.00% (0/9)	0.082

Table 2.9: Tumor staging divided into the two groups (cases who recurred within 12 months from treatment initiation and controls who did not recur).

	Recurrence within 12 months (8)	No recurrence (9)	p
Tumor size			
T1	12.50% (1/8)	44.44% (4/9)	0.294
T2	37.50% (3/8)	55.56% (5/9)	0.637
T3	12.50% (1/8)	0.00% (0/9)	0.471
T4	37.50% (3/8)	0.00% (0/9)	0.082
Nodal involvement			
N0	12.50% (1/8)	33.33% (3/9)	0.576
N1	50.00% (4/8)	66.67% (6/9)	0.637
N2	25.00% (2/8)	0.00% (0/9)	0.206
N3	12.50% (1/8)	0.00% (0/9)	0.471
TNM stage			
I	12.50% (1/8)	33.33% (3/9)	0.576
II	50.00% (4/8)	55.56% (5/9)	1.000
III	37.50% (3/8)	11.11% (1/9)	0.294
Tumor grading			
G1	0.00% (0/8)	11.11% (1/9)	1.000
G2-3	100.00% (8/8)	88.89% (8/9)	1.000

Acronyms: TNM = tumor nodes metastasis.

Table 2.10: PITX2 (PMR) and immunoistochemical expression of TFEB, CARM1 and SIRT1 divided into the two groups (cases who recurred within 12 months from treatment initiation and controls who did not recur).

	Recurrence within 12 months (8)	No recurrence (9)	p
PITX2 (PMR)	4.50 (0.00-67.00)	14.00 (7.00-17.00)	0.661
CARM1 (H-score)	90.00 (56.88-191.25)	16.25 (0.00-41.88)	<0.05
SIRT1 (H-score)	187.50 (172.50-196.25)	98.75 (95.00-163.75)	<0.05
TFEB (H-score)	200.00 (196.00-200.00)	90.00 (57.50-95.00)	<0.05

Chapter 3

Discussion

From this analysis about the role of TFEB pathway in breast cancer chemoresistance, it emerges that an increased expression of TFEB results to be associated with a reduced survival in patients suffering from invasive breast cancer who undergo chemotherapy. Our data also demonstrate a significant and directly proportional correlation of SIRT1 expression with TFEB and CARM1 expression, so that a very low expression of SIRT1 (lower than the first quartile of the H-score distribution) results associated with a low expression of TFEB and CARM1, and consequently with better survival. Furthermore, in the basal-like and Her-enriched breast cancer subtypes, TFEB and SIRT1 seem to have a lower H-score than in the luminal molecular subtypes. As far as PITX2 methylation analysis is concerned, this was feasible only in 65% of the selected cases (of the case-control study). Furthermore, the methylation of PITX2 does not present significant differences between the cases and the controls and does not correlate with the expression of the TFEB pathway analyzed by immunohistochemistry.

The ability to predict the response to antiblastic therapies and to develop new therapies in breast carcinoma is of fundamental importance. In fact, excluding tumors of the skin, breast carcinoma is the most frequent cancer affecting the female population in any age group, so that one in three cancers in women is a breast tumor [71]. Furthermore, despite the effectiveness of the screening with improved early diagnosis and the progressive advance of systemic therapies, no reduction has been observed in the prevalence of metastatic breast cancer at the time of diagnosis [2, 4, 5].

Metastatic carcinoma still represents the greatest challenge for the breast specialist, as it is mostly not susceptible to loco-regional therapies [2]. Its prevalence does not depend for the most part on the average size of tumors, which surely today are inferior to the past,

but depends above all on the intrinsic characteristics of the malignancy and therefore on its biological aggressiveness. Specifically, very small tumors with aggressive behavior are certainly more at risk of developing distant metastases than large tumors but with limited proliferation [72].

With regard to tumors with aggressive biological behavior, nowadays chemotherapy remains the most adequate tool to reduce the risk of distant metastases. Unfavorable prognostic factors, but also predictive of risk reduction in patients undergoing chemotherapy, are tumor size, loco-regional lymph node involvement, tumor histotype, hormone receptor expression, Her-2/neu overexpression, Mib-1/Ki-67 proliferation index, tumor grade, perivascular or perineural invasion, and young age of the patient [5, 54, 56, 73–75]. However, there is still no clearness about the factors which predict tumor chemoresistance, which then represents a possible target for the creation of novel drugs to be added to chemotherapy regimens in order to improve their efficiency.

As for other malignancies, autophagy seems to have a fundamental function in breast cancer chemoresistance. In particular, the autophagy process activation seems to influence breast cancer resistance against both chemotherapy and hormonal therapy [10, 13]. As a consequence, our study concentrates on some proteins which the literature already demonstrated to somehow act in the autophagy process, that is to say TFEB, SIRT1, CARM1 and PITX2.

In the literature, available data about TFEB, CARM1, and SIRT1 referred to breast cancer are currently extremely limited, especially for what concerns TFEB. Regarding SIRT1, there are reports in the current literature which describe a correlation between its increased expression and a worse prognosis in breast cancer [40, 41]. Also in our study, an expression of SIRT1 above the first quartile results associated with a diminished survival.

Furthermore, our data confirm a likely association of reduced expression of SIRT1 with the basal-like molecular subtype, association which has been reported by the previous literature [76]. Together with the disappearance of the correlation between SIRT1 and TFEB exclusively in the basal-like breast cancer subtype, our data actually suggests a different role of SIRT1 in the luminal molecular subtypes compared to that in the basal-like ones, although in both cases a low expression of SIRT1 predicts a better prognosis.

Both TFEB and CARM1 are described to have an important role in autophagy onset and regulation [36–38]. However, at the moment only one article is there which demonstrates a correlation between an increased TFEB expression in early breast cancer and a worse prognosis [77]. Our results confirm that an increased immunohistochemical TFEB expression correlates with a worse prognosis in women affected by breast cancer and treated with chemotherapy. Anyway, our study population includes not only early breast cancers but also a great number of locally advanced ones, which more commonly represent an indication for chemotherapy.

As for CARM1, its increased expression is related with a worse prognosis in breast cancer [78–80]. Furthermore, some authors found a greater expression of CARM1 in Her2-positive breast tumors [78, 80]. Also in our study, a greater expression of CARM1 correlates with a worse prognosis, but no difference was found for what concerns the expression of CARM1 among the different molecular subtypes. This may depend on a possible selection bias, as our population includes, on average, more advanced stages than previous studies. Moreover, our whole study population received chemotherapy, which reflects a marked aggressive behavior of the considered tumors.

In this study, we also evaluate the correlations among the three considered proteins. In particular, a directly proportional and statistically significant correlation is found among SIRT1, CARM1 and TFEB. Previous studies demonstrate that both TFEB and CARM1 act as effectors of the autophagy process, and their significant correlation in our results confirms this [36]. Even by selecting only the luminal molecular subtypes, it emerges that the rho coefficient of the correlation between TFEB and CARM1 approaches the significance (Figure 2.11). On the other hand, this correlation is lost in the case of the basal-like subtype. From this data, we can deduce that, in the breast cancer luminal subtypes, both TFEB and CARM1 act in the same pathway, while this may not be true in the case of the basal-like molecular subtype [36].

The correlation between SIRT1 and CARM1 can be explained by the fact that CARM1, through the methylation of HuR, stabilizes the SIRT1 mRNA and therefore promotes its production [81, 82]. Finally, while in the basal-like subtype both TFEB and CARM1 maintain the prognostic value, the correlation of TFEB with SIRT1 and CARM1 expression loses the statistical significance. A possible explanation is that probably TFEB and CRAM1 act in two different ways, which at the moment do not appear completely discernible with the data present in the current study.

Considering the case-control study, the prognostic value of TFEB and its pathway in chemotreated patients is confirmed and probably associated with its function in the autophagy mechanism. In fact, also PITX2 seems to play a role in the autophagy process. Indeed, PITX2 regulates DIRAS3 in lung cancer [48], and the re-expression of DIRAS3 promotes autophagy in breast cancer, thus enhancing the inhibitory effect of paclitaxel on breast tumor cells [49]. Although PITX2 and TFEB both have a crucial function in the autophagy process, their role seems to be different. Our data, in fact, show a significant difference regarding the TFEB pathway but no difference as regards the methylation of PITX2 between cases and controls. Furthermore, our data show no correlation between the methylation of PITX2 and the protein expression of the TFEB pathway, which would suggest the independence of these two prognostic markers.

In patients with operated breast cancer susceptible for adjuvant systemic treatment, as well as in the neoadjuvant setting, polychemotherapy should always be considered, as data derived from the meta-analyzes and clinical studies show that polychemotherapy is superior to monochemotherapy in terms of overall survival and disease-free survival [71]. It is possible to classify the available polychemotherapeutic regimens for breast cancer based on the drug administered. First-generation regimens are based on the combination of cyclophosphamide, methotrexate and fluorouracil administered for 6-12 cycles in order to decrease the relapse risk at 10 years by 30%. Second-generation regimens are those containing anthracyclines and are on average more effective than first-generation ones. However, it is clear that not all anthracycline-containing regimens are equally effective. Finally, third-generation regimens include regimens containing anthracyclines and taxanes administered sequentially or in combination. The main randomized controlled trials that compared regimens with or without taxanes in association with anthracyclines in the adjuvant therapy of patients with a high risk of relapse, show that sequential regimens generally have reduced toxicity with the same effectiveness in risk reduction[71].

Currently, adjuvant chemotherapeutic regimens are mostly based on the administration of anthracyclines and taxanes and, for this reason, our study focuses on the evaluation of PITX2, which is a molecule that can specifically influence anthracycline resistance [42–47]. All patients of our case-control study were treated with polychemotherapy including anthracyclines, but the patients tested for the methylation of PITX2 who experienced chemotherapy resistance are distributed in both groups. The use of polychemotherapy

has probably limited the prognostic effect of PITX2 in our group. However, it should be emphasized that saving the side effects of anthracyclines when there is evidence of their ineffectiveness could be an important point to ameliorate the quality of care in this group of patients. Moreover, to be more accurate, further studies should be planned taking into account in detail the chemotherapy regimen our patients received. Moreover, considering that PITX2 methylation should correlated with its reduced protein expression, it would be also interesting to determine in the future its immunoistochemical expression, in order to be sure about its absence in the tested breast cancer tissues.

The major limitations of this study are its retrospective design and the limitations linked to analysis performance on mRNA and DNA of formalin-fixed paraffin-embedded tissues that have been collected and stored for many years. At the same time, it is an important advantage to have a long follow-up that allows a better prognosis definition. Despite its limits, this study shows some interesting significant data. In fact, an increased TFEB expression in terms of H-score results to have a marked ability to identify the poor survival sub-population. In addition, it should be pointed out among the advantages of this study that the cases were treated uniformly by the same team according to the most up-to-date guidelines.

Conclusions

Our preliminary data demonstrate a potential prognostic value of TFEB and SIRT1, likely for the role that they exercise within autophagy regulation in patients affected by breast cancer and treated with antiblastic therapy. As chemotherapy resistance still represents one of the major concerns of the breast specialist, our encouraging data show the possibility to find out new therapeutic targets in order to overcome intrinsic or acquired drug resistence in breast carcinoma, and consequently to improve the prognosis of our patients.

Acknowledgments

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Alphabetical Index

Appendix A

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