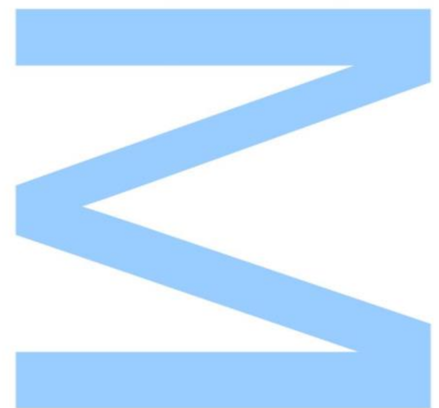
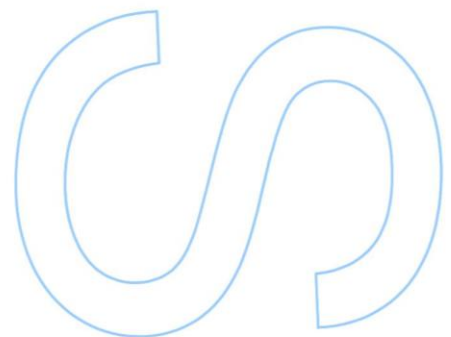
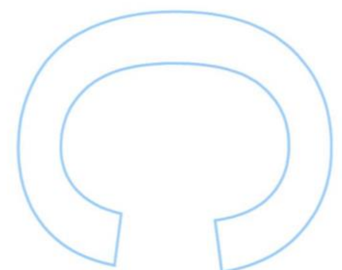
Influence of the Glucocorticoid Receptor on microRNA Profile in Triple–Negative Breast Cancer

Ricardo Daniel González da Cunha
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Departamento de Biologia
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Orientadores
Marlene Reithmair, Dr. med. vet., Institute of Human Genetics, University Hospital Ludwig–Maximilians–University Munich

Stefan Müller, PD Dr. rer. nat., Institute of Human Genetics, University Hospital Ludwig–Maximilians–University Munich

Coorientador
Luísa Azevedo, PhD, Faculdade de Ciências da Universidade do Porto/i3S–IPATIMUP

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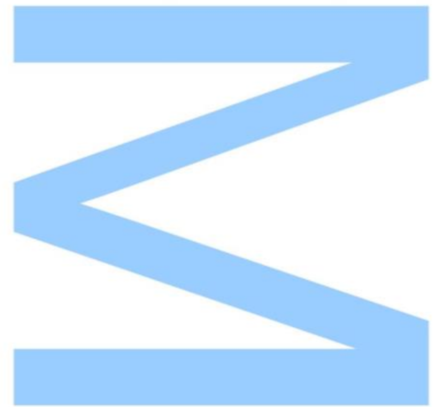

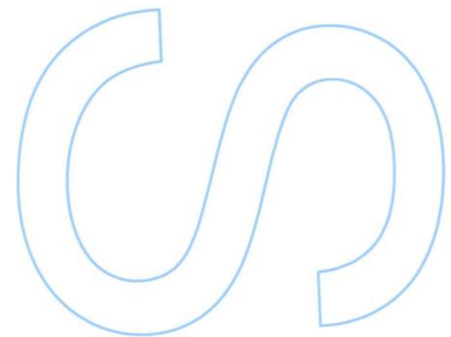

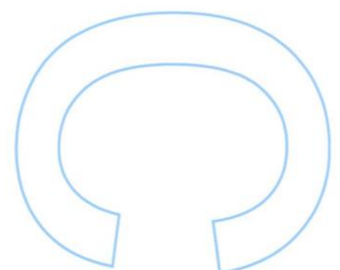

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"Vorfelan Rhinata Morie": the desire for knowledge shapes a man.

Patrick Rothfuss, in "The Wise Man's Fear"

Resumo

O cancro da mama é o tipo de cancro com maior prevalência na mulher, levando a uma elevada taxa de mortalidade (World Health Organization 2017).

Em particular, o cancro da mama triplo–negativo (TNBC) é conhecido como um subtipo de cancro da mama heterogéneo e agressivo, caracterizado pelo seu perfil negativo relativamente aos recetores de progesterona (PR), estrogénio (ER) e do recetor 2 do factor de crescimento epidermal humano (HER2), sendo estas características a razão principal pela falta de existência de um tratamento efetivo para esta patologia.

Os glucocorticoides (GCs), usados geralmente como coadjuvantes no tratamento de diversas doenças, são um grupo de hormonas corticosteroides que atuam através da ligação a recetores de glucocorticoides (GRs). GRs são factores de transcrição cruciais que estão envolvidos na regulação génica. Contudo, uma elevada expressão de GR foi recentemente associada às baixas taxas de sobrevivência em pacientes com TNBC (Chen *et al.* 2015). Para além disso, é sabido que os GRs não são apenas capazes de influenciar a expressão de genes codificantes de proteínas, mas também de modular a expressão de microRNAs (miRNAs), que são pequenos elementos não codificantes que regulam a expressão génica. A iniciação e a progressão de cancro da mama estão associadas à desregulação de miRNAs, que podem atuar quer como factores oncogénicos, quer como supressores tumorais (Andorfer *et al.* 2011).

De modo a aumentar a compreensão nesta área de investigação, este projeto teve como objectivo a identificação de miRNAs celulares regulados por GR em TNBC.

Os procedimentos experimentais incluíram: cultura celular de três linhas celulares de TNBC em três condições distintas (expressão de GR endógena; transfectadas com um plasmídeo de *NR3C1*, codificando o GR; transfectadas com RNA silenciador (siRNA), silenciando a expressão génica endógena do *NR3C1*); isolamento de RNA, incluindo controlo de qualidade e quantificação; preparação de uma biblioteca para “Next–Generation Sequencing” (NGS) e análise bioinformática de dados de NGS.

Foram encontrados sete miRNAs regulados significativamente por GR em TNBC, dos quais alguns corroboram estudos anteriores sobre associações destes com a ativação de vias oncogénicas. Os nossos resultados apontam, ainda, para que a expressão de miRNAs associados a GR possa ser específica do subtipo de TNBC.

À luz dos nossos resultados, os miRNAs poderão ser biomarcadores efetivos para o diagnóstico e prognóstico de TNBC. Mais investigação é, contudo, necessária para

descrever a sua função e apontar em que vias das subclasses de TNBC poderão estar envolvidos, de modo a que, eventualmente, os pacientes com TNBC possam ter acesso a melhores previsões de tratamento e resultados aos mesmos.

Abstract

Breast cancer (BC) is the most prevalent type of cancer in women and leads to high mortality rates (World Health Organization 2017).

In particular, triple–negative breast cancer (TNBC) is known as a heterogeneous and very aggressive BC subtype, characterized by its negative profile of progesterone receptor (PR), estrogen receptor (ER), and human epidermal growth factor receptor 2 (HER2). These features are the main reason why there is still no effective treatment available.

Glucocorticoids (GCs), which are usually used as coadjuvants in the treatment of several malignancies, are a group of corticosteroid hormones that act by binding to glucocorticoid receptors (GRs). GRs are crucial transcriptional factors involved in gene regulation. However, high GR expression in TNBC was recently linked to poorer survival rates in TNBC patients (Chen *et al.* 2015). Furthermore, it is known that GRs are not only capable of influencing the expression of protein coding genes but also modulate microRNA (miRNAs) expression, which are small noncoding elements that likewise regulate gene expression. The initiation and progression of BC are associated with miRNA dysregulation, which can either act as oncogenic or tumor suppressor factors (Andorfer *et al.* 2011).

To broaden the knowledge in this research field, the project aimed to identify cellular miRNAs regulated by GR in TNBC.

Experimental procedures included: cell culture of three TNBC cell lines in three different conditions (endogenous GR expression; transfected with a *NR3C1* plasmid, encoding the GR; transfected with silencing RNA (siRNA), silencing endogenous *NR3C1* gene expression); isolation of RNA, including quality control, and quantification; preparation of a library for Next–Generation Sequencing (NGS), and bioinformatics analysis of NGS data.

Seven miRNAs were found to be significantly regulated by GR in TNBC, of which some corroborate previous findings of associations with activation of oncogenic pathways. Our results further indicate that GR–regulated miRNA expression may be TNBC subclass specific.

In light of our findings, miRNAs may be effective biomarkers for the diagnosis and prognosis of TNBC. Further research is necessary to describe their function and to assess in which TNBC's subclass pathways they may be involved, so that TNBC patients may eventually have access to better predicted outcomes and treatment.

Keywords

triple–negative breast cancer, TNBC, glucocorticoid receptor, microRNA, next–generation sequencing

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Acronyms and Abbreviations

°C	celsius degree
µl	microliter
3’–UTR	3’–untranslated region
5’–UTR	5’–untranslated region
abs.	absolute
AF	activation function
AGO	argonaute
AR	androgen receptor
BC	breast cancer
BL1/BL2	basal–like 1/basal–like 2
BLIA	basal–like immune–activated
BLIS	basal–like immunosuppressed
bp	base pair
BRCA1/BRCA2	breast cancer 1/breast cancer 2
C1	luminal androgen receptor
C2	basal–like with low immune response
C3	basal–enriched with high immune response
cDNA	complementary deoxyribonucleic acid
DBD	deoxyribonucleic acid binding domain
Dex	dexamethasone
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ER	estrogen receptor
FBS	fetal bovine serum
FU	fluorescence units
GAPDH	glyceraldehyde 3–phosphate dehydrogenase
GC	glucocorticoid
gDNA	genomic deoxyribonucleic acid
GR	glucocorticoid receptor
GRE	glucocorticoid response element
h	hour
HBSS	Hank’s balanced salt solution
HER2	human epidermal growth factor receptor 2

HPA	hypothalamic–pituitary–adrenal
HR	hormone receptor
IHC	immunohistochemical
IM	immunomodulatory
LAR	luminal androgen receptor
LBD	ligand–binding domain
M	mesenchymal
Mio	millions
miRNA/miR	micro ribonucleic acid
ml	milliliter
MSL	mesenchymal stem–like
mRNA	messenger ribonucleic acid
NF–κB	nuclear factor κB
ng	nanogram
NGS	next–generation sequencing
NR	nuclear receptor
NR3C1	nuclear receptor, subfamily 3, group C, member 1
NRT	no reverse transcription
nt	nucleotide
NTD	N–terminal domain
pcDNA6/V5–HisA	plasmid cytomegalovirus deoxyribonucleic acid, epitope tag V5, histidine tag (6x)
pH	potential of hydrogen
PIK3CA	phosphatidylinositol–4,5–bisphosphate 3–kinase catalytic subunit
PIK6/Brk	protein tyrosine kinase 6
pmol	picomole
PR	progesterone receptor
pre–miRNA	precursor micro ribonucleic acid
pri–miRNA	primary micro ribonucleic acid
PTEN	phosphatase and tensin homolog
RISC/miRNP	ribonucleic acid–induced silencing complex/micro ribonucleoprotein particle
RPM	revolutions per minute
RNA	ribonucleic acid
RT	reverse transcriptase

RT–qPCR	reverse transcription quantitative polymerase chain reaction
siRNA	small interfering ribonucleic acid
SNP	single nucleotide polymorphism
TAE	tris–acetate–ethylenediamine tetraacetic acid
TF	transcription factor
TNBC	triple–negative breast cancer
TP53/p53	tumor protein 53
UV	ultraviolet
V	Volt
x g	times gravity

1. Introduction

1.1. Clinical Data

1.1.1. Prevalence and Risk Factors

Breast cancer (BC) is the most prevalent type of cancer in women, both in developed and developing countries, although a higher rate is observed in developing countries due to the increase of medical care, and consequent increase of the average age in the populations over the last years (World Health Organization 2017).

Estimates from 2016 show that in the USA approximately 15 % of the cancers affecting women were BC, while in men this percentage was around 1.5 %. This corroborates the fact that BC is mostly a women–affecting disease, inducing men to avoid BC diagnostics, ultimately leading to an increase of male BC cases annually, mostly due to lifestyle reasons. Worldwide, men BC cases account for less than 1 % of all diagnosed BCs cases (Korde *et al.* 2010; American Cancer Society 2016; Siegel *et al.* 2016).

In 2011 more than 508 000 women around the globe died from BC. In the United States 2015's estimated values indicated almost 300 000 cases of BC in women, from which about 40 000 resulted in death, whereas estimation for 2016 predicted approximately 250 000 cases in women, and 2 600 in males, with a death estimate of about 40 500 and 440, respectively. Data from 2014 for BC mortality and incidence in women from USA, Germany, and Portugal can be found in **Table 1**. Estimated values for 2017 in the USA, have been reported by Siegel *et al.* (2017), estimating around 255 000 cases in both genders (2 470 cases in men, and 252 710 in women), with a death estimate of 41 070 (460 in men, and 40 610 in women) (World Health Organization 2014; American Cancer Society 2015; Zeichner *et al.* 2016; Siegel *et al.* 2017; World Health Organization 2017).

Table 1 Breast cancer mortality and incidence rates in women in 2014. Adapted from World Health Organization (2014).

	USA	Germany	Portugal
Mortality	16.1 %	18.8 %	16.9 %
Incidence/year	232 714	71 623	6 088

Even though BC can be found both in developed and developing countries, survival rates vary across different geographic locations. North America, Japan, and Sweden present circa 80 % survival rate, while middle–income countries show around 60 %. Low–income countries show less than 40 % survival rate, which is less than half of North America's rate. This data can be explained due to the lack of early diagnostics, and a poorly informed population, in which women will only recur to medical care when the disease is already at a late phase (World Health Organization 2017).

Human breast suffers diverse modifications during its development. These alterations are related to characteristics as size, form and/or function, and are highly correlated with women development phases, such as puberty, pregnancy, lactation, and menopause. The different development phases are all strongly associated with BC tumorigenesis (Ling & Kumar 2012; Russo *et al.* 2013; American Cancer Society 2015; World Health Organization 2017).

BC has been associated with multiple factors, some with endogenic origin, as early menarche, or advanced age on first pregnancy, others with exogenic hormonal influences, such as oral contraceptives and hormone replacement therapies (Chen 2008; Hunter *et al.* 2010; Russo *et al.* 2013).

For basal–like BC subtypes, being basal–like BC characterized by the lack of hormone receptors (HR) and human epidermal growth factor receptor 2 (HER2), it has been found that the risk of this type of BC decreases with the increase of parity, young age at first full–term pregnancy, lactation time, and number of lactated progeny. Consequently, women who did not breastfeed their child, and those who used medication to suppress lactation, show a higher risk of basal–like BC (Millikan *et al.* 2008; Badve *et al.* 2011; Russo *et al.* 2013; Zeichner *et al.* 2016).

Familial history of BC is also considered a risk factor, as well as some mutations, for example in the tumor suppressor genes breast cancer 1 (*BRCA1*), *BRCA2*, and tumor protein 53 (*TP53*). *BRCA1* and *BRCA2* are genes that produce tumor suppressor proteins that help repairing damaged DNA. 20 to 25 % of hereditary BCs are due to a mutation in these two genes, accounting moreover for 5 to 10 % of all BC types (Campeau *et al.* 2008).

Besides, ethnic/geographical groups present different predisposition for BC. Ashkenazi Jews have the highest rate of *BRCA1* associated BC, followed by Hispanic women. When carrying a *BRCA1* mutation, Ashkenazi women will have a 50–80 % lifetime risk of developing BC (Janavičius 2010; Rosenthal *et al.* 2015).

Although this hereditary factor does exist, around 70 % of BC cases in women are not familial–mutation related, meaning that sporadic somatic mutations and environmental factors may have a key function in BC development. Ling and Kumar (2012) concluded that single–nucleotide polymorphisms (SNPs) in several key genes are likewise related to BC susceptibility, and that telomere shortening may similarly be associated with familial BC (Ling & Kumar 2012).

1.1.2. Prevention

BC does not have any specific regulations that determine a proper and efficient prevention, but its control is mainly focused on early detection, treatments and, if required, palliative care (Zeichner *et al.* 2016; World Health Organization 2017).

A correctly informed and advertised population is also a type of control. By allowing and providing the essential data about the disease, e.g. dietary choices, the implementation of physical activity and consequently weight control as well as the decrease of alcohol ingestion, a long–term lower BC incidence is expected. As even implementing all of these factors cannot guaranty the elimination of risk factors, populations should be taught and be able to screen routinely, either by self or clinical breast examination, or by mammography exams (World Health Organization 2017).

The European Union has a set of screening program regulations that 25 countries follow, Portugal and Germany included.

Portugal follows the European Guidelines affirming that women older than 49 years old must pursue a mammography screening every two years. Also women with familial BC cases are recommended to annually screen for BC with a combination of mammography screening and magnetic resonance imaging. The latter is suggested to be prescribed with mammography exams, or by alternating each one every 6 months. Women with familial cases should begin the screening 10 years earlier than the earliest case in the family. Since screening exams are not fully funded by public funds, Portugal also has an association, *Liga Portuguesa contra o cancro* (Portuguese league against cancer), founded in 1986 with the main purpose to offer people free screenings. It works with mobile units that ran initially in the central area of the country, but that slowly expanded to other cities, from north to south of Portugal. They stop in the cities every 2 years, sending letters in advance to women from 49 to 69 years old so that they know when and where they will be. The radiologic exam is examined by two

radiologists who suggest a final diagnostic investigation at the hospital if indicated (LPCC 2015; Senkus *et al.* 2015; Ponti *et al.* 2017).

Germany also follows the guidelines of the European Union, but its system is fully funded. Women from 50 to 69 years old are offered a free mammography every two years. The exams can take place in clinics, hospitals or, similarly to Portugal, in mobile units. Unless stated otherwise, every woman will receive an invitation letter every two years. If the radiologic exam studied by two radiologists registers an unusual feature, they will be assigned to a specialist. In the case of familial BC cases, German women are prescribed mammographies and ultrasound exams once a year (Diekmann & Diekmann 2008; IQWiG 2016; Ponti *et al.* 2017).

1.1.3. Breast Cancer Types

BC is a heterogeneous disease with several implications to the patients. It has been differentiated into diverse types, which can be classified into four categories using standard immunohistochemistry (IHC) markers. These categories can be joined into two groups: estrogen receptor (ER)–positive and ER–negative. The first group comprises Luminal A and Luminal B BCs, while the second includes HER2–enriched and basal–like (**Figure 1**) (Dent *et al.* 2007; Ma & Ellis 2013; American Cancer Society 2015; Chang *et al.* 2015).

Most BCs are Luminal A type. This type is characterized by the positive status of ER and/or progesterone receptor (PR) (both HR), and a negative status of HER2. Luminal A BC tumors grow slower than other BC types and are also less aggressive. Since they do respond to therapy, they present the most favorable prognostics in BC (Millikan *et al.* 2008; American Cancer Society 2015).

Luminal B is also HR–positive, like Luminal A, but features HER2–positive status, representing approximately 10 % of BC cases (American Cancer Society 2015).

HER2–enriched type (HR–negative/HER2–positive) is characterized by a high growth rate and it spreads more aggressively than other BC types. Nevertheless, this subgroup can be targeted by HER2–targeted therapies (Dent *et al.* 2007; American Cancer Society 2015).

At last, basal–like is mainly composed of triple–negative breast cancer (TNBC) (circa 75 %), which is negative for ER, PR, and HER2 receptors (Hurvitz & Mead 2016).

The lack of expression in those receptors makes this BC subtype aggressive. TNBC is more frequent in African–American women, and those carrying a mutation in *BRCA1* gene. These features provide the poorest prognosis with the highest mortality rate of all BC types since there is still no effective therapy available for TNBC (Dent *et al.* 2007; Nassirpour *et al.* 2013; American Cancer Society 2015; Chang *et al.* 2015).

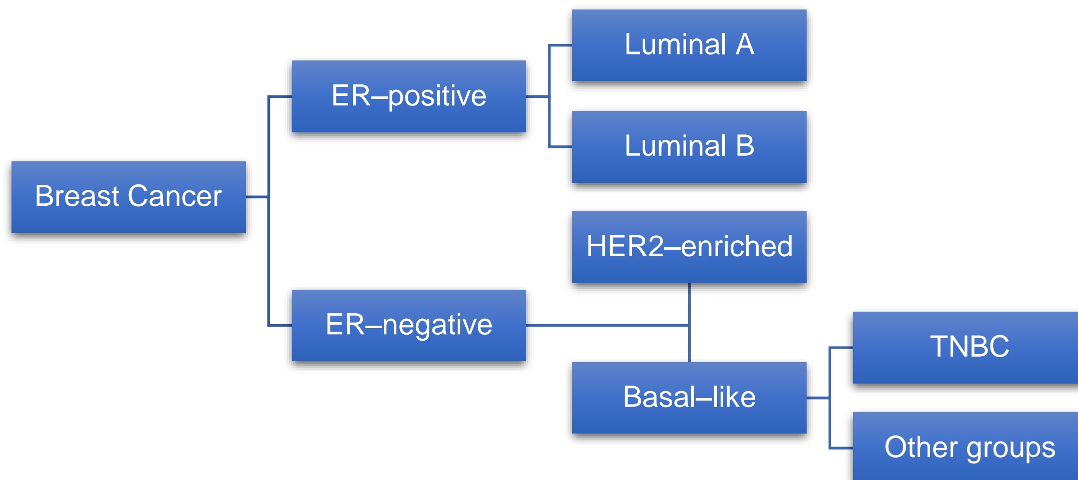


Figure 1 Breast cancer classification in two main groups: estrogen receptor (ER)–positive and negative. The first is composed of Luminal A (ER–positive, progesterone receptor (PR)–positive, and human epidermal growth factor receptor 2 (HER2)–negative), and Luminal B (ER–positive, PR–positive, HER2–positive). The latter comprises HER2–enriched (ER–negative, PR–negative, HER2–positive) and Basal–like (ER–negative, PR–negative, HER2–negative), which is mainly composed of triple–negative breast cancer (TNBC).

1.1.4. Genetics of Triple–Negative Breast Cancer

TNBC is known for its genetic instability, its ability to resist apoptosis and to have its cell cycle's checkpoints dysregulated. This can be explained by its genomic modifications due to the loss of three tumor suppressor genes: *TP53*, *BRCA1/2* and phosphatase and tensin homolog (*PTEN*) genes, while expressing cell proliferation genes, such as epidermal growth factor receptors, and stem cell factor receptor genes, which are a type of tyrosine kinase receptors binding to stem cell factors and causing the growth of some cell types (Ma & Ellis 2013; Hurvitz & Mead 2016).

BRCA1/2 genes encode essential proteins for homologous recombination–mediated repair of breaks in double–stranded DNA. Around 12 % of the mutational rate on mutation–predisposed genes, comprising circa 17 % of TNBC cases, occur in *BRCA* genes, with *BRCA1* mutations being more often found than *BRCA2* mutations.

Denkert *et al.* (2016) found that other mutations, not *BRCA*–related, involve genes with functions on homologous recombination, bringing up the suggestion that alterations in the repairing process may be important in the development of TNBC (Ma & Ellis 2013; Denkert *et al.* 2016; Hurvitz & Mead 2016).

There is no mutational pattern that can be linked to TNBC, but some mutational recurrence can be found in *TP53*, phosphatidylinositol–4,5–bisphosphate 3–kinase catalytic subunit alpha (*PIK3CA*) and *PTEN* genes. This variety in TNBC phenotypes makes it difficult to treat TNBC patients. Due to these special features TNBC is usually an exclusion diagnosis, and several TNBC classifications have been provided over the years due to its high heterogeneity at the transcriptional level. Some of these classifications are related to specific treatment response; however, no common therapy has yet been established (Denkert *et al.* 2016; Hurvitz & Mead 2016).

TNBC is mainly classified into four distinct classes:

- a basal–like class;
- a mesenchymal class;
- an immune–enriched, and;
- a luminal androgen receptor (AR) class,

each one of them expressing different features.

The basal–like class has the main characteristics of basal–like BC type and is mostly involved in pathways related to cellular cycle and damage response on DNA, which are frequently highly expressed, increasing cellular proliferation (Ahn *et al.* 2016).

The mesenchymal class shows an overexpression of biological processes concerning cell mobility clusters, as well as interaction with the extracellular matrix, or with pathways involved in growth factor signaling (Ahn *et al.* 2016).

The third class, immune–enriched, refers to tumors that overexpress genes associated with T, B, and natural killer cells, as well as tumor necrotic factors signaling (Ahn *et al.* 2016).

At last, luminal AR class is described as the one that varies the most. This class comprises patients with genes influencing hormonal regulation and the metabolism of estrogen/androgen (Yao *et al.* 2014; Ahn *et al.* 2016).

However, based on different studies and focus, other TNBC classifications were proposed (**Figure 2**). Lehmann and Pietenpol (2015) described a classification system based on gene expression analysis, dividing TNBC into 6 classes, including subclasses according to the differential response to specific treatments, denominated Vanderbilt classification.

The first one was named basal–like (BL) class, which was further subclassified into BL1 and BL2. BL1 expresses an increase in cell cycle activity and DNA damage response, while the latter shows a higher expression level of growth factor pathways.

The second one, mesenchymal class, was also divided into two, mesenchymal (M) and mesenchymal stem–like (MSL).

An immunomodulatory class and one luminal AR, characterized by androgen signaling were also classified (Lehmann & Pietenpol 2015).

Using messenger RNA (mRNA) and DNA profiling, the Baylor classification was created in a study by Burstein *et al.* (2015). This TNBC classification has 4 classes, luminal AR, mesenchymal, basal–like immunosuppressed (BLIS) and immune–activated (BLIA). BLIS shows the worst prognostics, while BLIA shows the best outcomes (Burstein *et al.* 2015).

A fourth system was created by a French group, at the Unicancer Center, with the analysis of gene expression profiling in a study by Jézéquel *et al.* (2015). In this investigation, 3 classes were pointed out: C1, referring to luminal AR; C2, comprising basal–like with low immune response, and C3, basal–enriched with high immune response (Jézéquel *et al.* 2015).

Even if these systems appear to be different, the classification system by Lehmann and Pietenpol (2015) and Burstein *et al.* (2015) have the same Luminal AR classes. Furthermore, the mesenchymal class from the second research group contains most of MSL and M subclasses from the first one (**Figure 2**) (Burstein *et al.* 2015; Lehmann & Pietenpol 2015; Ahn *et al.* 2016).

Figure 2 TNBC classifications according to different research groups. TNBC, triple–negative breast cancer; BL1, basal–like 1; BL2, basal–like 2; BLIA, basal–like immune–activated; BLIS, basal–like immune–suppressed; C1, luminal androgen receptor (AR); C2, basal–like with low immune response; C3, basal–enriched with high immune response; M, mesenchymal; MSL, mesenchymal stem–like; orange, basal–like; green, mesenchymal; yellow, luminal AR, blue, immune–related.

1.1.5. Therapy

The different molecular etiologies among the diverse types of BC result in a difficult treatment (Chang *et al.* 2015).

Generally, BC treatment depends on its type and stage. A local treatment, comprising surgery or radiation therapy, is usually used in early stage BC and has the advantage of not affecting the whole body. Systemic treatments, which are mainly used for later stages and when patients express metastasis, affect the whole body and reach cancer cells in any organismal location. Hormone therapy, chemotherapy, and targeted therapy are examples of this kind of treatments. Medication can be oral drugs or with a direct approach via bloodstream. Depending on the case both typologies, the local and systemic treatments, can be prescribed (Hurvitz & Mead 2016; World Health Organization 2017).

Nowadays there are multiple treatments for patients diagnosed with BC, especially targeted treatments, such as endocrine therapies and HER2–targeted medicine. However, for some BC subtypes such as TNBC, there is still no effective treatment available (Chang *et al.* 2015).

Tamoxifen, a selective ER modulator, was initially used to treat every BC type. It is a nonsteroidal triphenylethylene derivative that inhibits ER activity associated with tumor cell growth by competing with estrogen. Later it was pointed out that only patients whose tumors expressed hormonal receptors could benefit from its effects (Bertoli *et al.* 2015; Manna & Holz 2016).

Only after the introduction of treatments with trastuzumab (Herceptin), an antibody binding to HER2 and triggering immune cells to attack these antibody–marked cells, the importance of identifying the different tumor gene expression patterns was noted (Dent *et al.* 2007).

For TNBC, as no effective treatment has been discovered, chemotherapy remains the state–of–the–art therapy, though carboplatin treatments, which allies chemotherapy with platinum, provided some positive responses in patients with *BRCA1* gene mutation (Lehmann & Pietenpol 2015; Denkert *et al.* 2016).

1.2. Glucocorticoid Receptor

1.2.1. Function and Structure

Glucocorticoids (GCs) are a group of corticosteroids (adrenal cortical steroid) hormones secreted by the adrenal cortex, which act by binding to glucocorticoid receptors (GRs) (Skor *et al.* 2013; Abduljabbar *et al.* 2015).

The hypothalamus–pituitary–adrenal (HPA) gland axis is a neuroendocrine system that regulates GC release (**Figure 3**). Internal and external signals induce hypothalamus to release corticotropin–releasing hormone, which acts in the anterior pituitary, thus stimulating adrenocorticotropic hormone synthesis and release. The latter acts on the adrenal cortex, stimulating the production and release of cortisol. Cortisol can then act in a feedback loop manner, suppressing corticotropin–releasing hormone and/or adrenocorticotropic hormone action, influencing the pathway's function (Oakley & Cidlowski 2013).

GC/GR have an important role in physiological processes, for instance in metabolism and development, as well as in diverse systems: cardiovascular, immune, musculoskeletal, nervous, reproductive and respiratory systems. Cortisol is a natural human GC that is released as a response to circadian, stress and physiological signals. It is controlled by the HPA axis in an endocrine feedback system and released in increased quantities in the beginning of activities, this is in humans in the morning (Chung *et al.* 2011).

The physiological homeostasis is highly dependent on the continuous regulation of cortisol level in the plasma. When suffering from acute stress a high release of cortisol is observed, while a prolongated cortisol release is found under chronic stress. These stress conditions may compromise the immune system, provoke metabolic dysfunction, or even decrease thyroid function. Cortisol level may also inflict some diseases. A high concentration of cortisol, known as hypercortisolemia, is found in Cushing syndrome, while the opposite situation, hypocortisolemia, is a characteristic of Addison's disease. Plasma cortisol is mainly bound to albumin and to corticosteroid–binding globulin (>90 %). The remaining cortisol is free to get through the plasmatic membrane and bind to GRs (Sapolsky *et al.* 2000; Kadmiel & Cidlowski 2013; Grbesa & Hakim 2016).

Due to their anti–inflammatory and immunosuppressive actions, GCs are usually used for the treatment of inflammatory and autoimmune diseases (Sapolsky *et al.* 2000; Ling & Kumar 2012; Kadmiel & Cidlowski 2013; Chen *et al.* 2015).

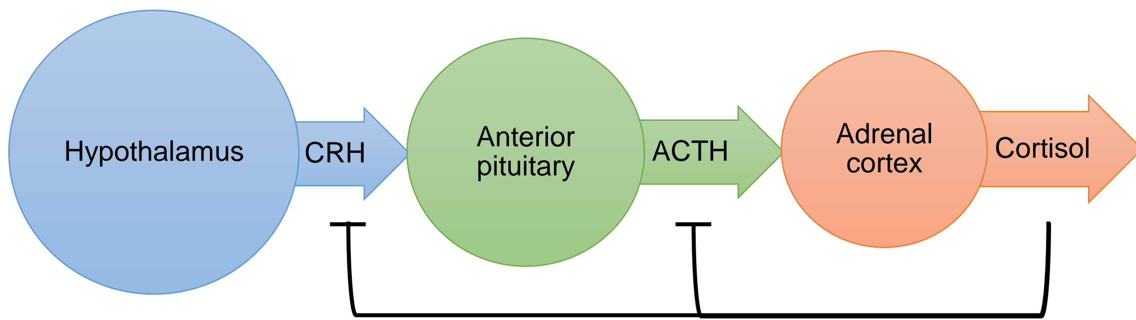


Figure 3 Hypothalamus–pituitary–adrenal (HPA) gland axis. CRH, corticotropin–releasing hormone; ACTH, adrenocorticotropic hormone; black line, feedback loops

Some synthetic GCs, such as dexamethasone (Dex), hydrocortisone or prednisone, are usually prescribed to patients for the treatment of eczema, inflammations, psoriasis and leukemia. These synthetic GCs mimic long–term exposure to a high concentration of GCs and may induce some side effects, such as hypertension, osteoporosis or diabetes mellitus (Schäcke *et al.* 2002; Kadmiel & Cidlowski 2013; Lin & Wang 2016).

GCs actions, both physiological and pharmacological, are mediated by the ligation of GCs to GRs. The GC–GR complex is then able to enhance or repress the transcription of target genes (Oakley & Cidlowski 2013).

GRs belong to the nuclear hormone receptors family and are ligand–dependent transcription factors (TFs). The human GR gene, *NR3C1* (Nuclear Receptor, Subfamily 3, Group C, Member 1, *Homo sapiens*), is localized on chromosome 5 (5q31). *NR3C1* is a zinc finger TF, which comprises 5 isoforms formed through alternative splicing of the same *NR3C1* primary transcript. However, its isoform GRα (777 amino acids residues) is the main responsible for the translational activities of GRs, being the one that is in its active form (Ling & Kumar 2012; Abduljabbar *et al.* 2015; Grbesa & Hakim 2016).

GRs are multi–domain proteins that possess three main functional components: an N–terminal domain (NTD, residue 1–420), a DNA–binding domain (DBD, residue 421–486), and a C–terminal ligand–binding domain (LBD, residue 528–777). Between the DBD and LBD there is a 42 nucleotide (nt)–long region, called hinge region, that provides flexibility to the GR (**Figure 4**). GRβ (742 amino acids residues) for instance, is not active due to the lack of the LBD (Ling & Kumar 2012; Kadmiel & Cidlowski 2013; Grbesa & Hakim 2016).

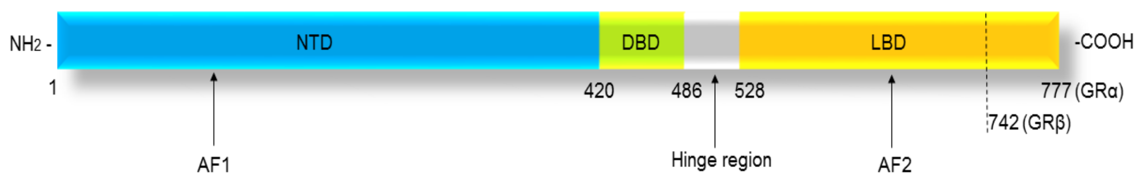


Figure 4 GR structure. The active GR (GRα) is 777 nt long, consisting of three main domains: NTD, N–terminal domain, 420 nt long, which comprises the AF1, activation function domain 1; a DBD, DNA–binding domain, of 65 nt, and a LBD domain, C–terminal ligand–binding domain (247 nt), harboring the AF2 domain, activation function domain 2.

Inactive GRs are found in the cytoplasm in a multiprotein complex. Cortisol diffuses across the cell membrane into the cytoplasm and binds to a GR, activating it. The activated GC–GR complex forms homodimers and is then transported to the nucleus via active transport. In the nucleus the complex binds directly to glucocorticoid response elements (GRE), which are degenerated DNA sequences, or indirectly by tethering to other TFs, enhancing or suppressing target gene expression (Hayashi *et al.* 2004; Grbesa & Hakim 2016).

The complex GC–GR functions as a TF and is responsible for the gene expression regulation of cellular metabolism and of other steroid receptors. However, emerging evidence suggests that GRs do not exert their TF function as homodimers, but can also bind to DNA as a monomer or tetramer. These differences in GR conformation may be due to the different types of binding locations of GRs in the genome, to different GRs concentrations, or even due to different co–activators (Ling & Kumar 2012; Skor *et al.* 2013; Abduljabbar *et al.* 2015; Sacta *et al.* 2016).

The active GR activity is maintained and controlled by two activation function (AF) domains, AF1, which is a stable part of NTD, and AF2 that is a stable part of LBD, both enhancing GR activity when in interaction with each other, and in interaction with other coregulatory proteins (Ling & Kumar 2012).

Nonetheless, the presence of GRE is not enough to the linkage of GRs, which points out that other motifs might determine GR's specificity. The binding of GRs can, therefore, be predisposed by several processes. Of interest is the fact that the ligation *loci* of GRs are accessible before GR is activated, suggesting that the accessibility to chromatin dictates where the GRs are going to attach. The factors that make chromatin accessible are, hence, important regulators of the recruitment of GR and subsequently GR–regulated cell type specific expression of target genes. This availability can be mediated by remodeling complexes that change chromatin conformation, allowing the binding of GR to GRE, which leads to specific gene expression (Li *et al.* 2007; Uhlenhaut *et al.* 2013; Grbesa & Hakim 2016).

1.2.2. Glucocorticoid Receptor and Triple–Negative Breast Cancer

In cancer GCs are used to treat lymphoid malignancies by apoptosis induction, but they are also used in cancer therapy as coadjuvant treatment together with chemotherapy in solid tumors so that apoptosis can be activated in cancer cells. They can also reduce nausea and vomits, as well as other cytotoxic side effects. It has been shown that GCs work effectively in hematopoietic diseases, such as leukemia and lymphomas, but they tend to cause adverse effects in BC cases by inhibiting programmed cell death (Mikosz *et al.* 2001; Ling & Kumar 2012; Chen *et al.* 2015).

Even though GRs are largely expressed in BC, their expression level tends to decay with BC progression. Also, GR levels are highly correlated with the expression of ER and PR (Abduljabbar *et al.* 2015).

In addition to the failure of chemotherapy and induction of tumor progression, GRs were also linked to the poor survival rate that characterizes TNBC. Around 25 % of TNBC cases are GR–positive. A high GR expression indicates poor prognostic and/or therapeutic response. Furthermore, a high GR expression was likewise correlated with early relapse in early stage TNBC. Activated GRs stimulate antiapoptotic signal pathways in breast epithelial cells by regulating the transcription of protein–coding genes from the cellular survival pathway (Skor *et al.* 2013; Chen *et al.* 2015).

Chen *et al.* (2015) studied the effects of Dex, a synthetic GC that is often used to reduce side effects throughout chemotherapy, in the BC cell line MDA–MB–231 with a p53 gene mutation, and established that Dex is associated with BC progression. Their results suggest that Dex–liganded GR binds to specific GRE, acting as an oncogene activator, thus activating proteins that inhibit apoptosis and promote proliferation, cell survival and migration in TNBC. Overall they found that GR's increased expression is associated with poor prognostics and shorter survival (Chen *et al.* 2015).

Regan Anderson *et al.* (2016) concluded that TNBC cell lines and primary TNBC tumor explants treated with Dex exhibited an elevated mRNA and protein expression of the protein tyrosine kinase 6 (*PIK6*, also known as *Brk*), which mediates the pathogenic status of cancer cells. They also found that *Brk* expression was highly associated with GR. Furthermore, GRs were phosphorylated in hypoxia state, and this also lead to an increased *Brk* upregulation, explaining the progression and metastasis in TNBC patients (Regan Anderson *et al.* 2016).

Agyeman *et al.* (2016), studying the TNBC response to inhibitors of Hsp90, a chaperone protein that modulates transcription by assisting other proteins to fold properly, found that GRs suffered a degradation process, consequently decreasing GR–mediated gene expression, making TNBC cells more susceptible to cellular death. This suggests that GR regulates antiapoptotic pathways, and that signaling pathways can be disrupted by Hsp90 inhibitors (Agyeman *et al.* 2016; Kumar 2016).

In addition, Pan *et al.* (2011), studying prognosis in BC, concluded that those with an ER–negative typology, in which TNBC is included, and those expressing higher levels of GR, had an increased risk of early relapse when compared to patients with low expression levels of GR. Additionally, it was suggested that an activation of ER status (ER–negative to ER–positive) could induce the expression of protein phosphatase 5 gene, which mediated the inactivation of GRs. This is different from the acting manner of ER–negative BC, in which GR regulate genes independently of the action of estrogen (Pan *et al.* 2011).

1.3. MicroRNA

1.3.1. Function and Structure

MicroRNAs (miRNAs) are small single–stranded RNA molecules with a length of 19 to 25 nt that control many developmental and cellular processes in eukaryotes by negatively regulate transcription and translation processes, cleaving and/or degrading target transcripts, or even by modifying chromatin. They regulate the gene expression of almost all cellular processes, such as apoptosis, cellular migration, proliferation, and angiogenesis (Nassirpour *et al.* 2013; Gyparaki *et al.* 2014; Chang *et al.* 2015).

In mammals miRNAs oversee the activity of approximately 50 % of all protein–coding genes, owning a main role in organism development, cellular differentiation, metabolism, viral infections, and oncogenesis. The alteration of miRNA expression is associated with several human pathologies (Krol *et al.* 2010; Augoff *et al.* 2012).

Numerous miRNAs are conserved in related species, and some have homologous miRNAs in distant species, suggesting that their function may be equally conserved among them. In addition, a single miRNA can regulate the translation of various target genes involved in different cellular processes, both tissue and development–specific, highly contributing to protein–expression profiles that are cell–type specific (Krol *et al.* 2010; Yang & Wang 2011; Liu *et al.* 2013; Nassirpour *et al.* 2013).

miRNAs are individual gene transcripts encompassing their own promoter, but can also be intragenic spliced portions of protein–coding genes. The first ones are often transcribed by RNA polymerase II into a primary transcript (pri–miRNA) (**Figure 5**) (Czech & Hannon 2011).

The pri–miRNA is constituted by a 7–methylguanosine cap at its 5'–end, and a 3'–poly(A) tail, and can contain some introns. The processing of long pri–miRNA shortens the molecules to 60 to 80 nt long with a secondary hairpin structure, denominated precursor miRNA (pre–miRNA). This pri– to pre–miRNA cleavage process occurs in the nucleus when the pri–miRNA is recognized by the riboendonuclease *Drosha* and by the double–stranded RNA binding protein DGCR8, a microprocessor complex unit, through the interaction with a stem–loop structure within the miRNA. When transported from the nucleus to the cytoplasm by exportin 5, the molecule is processed by *Dicer*, a cytoplasmic RNase III, together with the transactivation response RNA binding protein 2 and Argonaute (AGO) 2, denominated DICER complex, giving rise to miRNAs dimers. From those, one strand is degraded, and the other one constitutes the mature miRNA. Depending on the origin strand, mature miRNAs are denominated as "–3p" or "–5p", for the 3'– or 5'–strand source, respectively (Yi *et al.* 2003; Denli *et al.* 2004; Diederichs & Haber 2007; Krol *et al.* 2010; Camps *et al.* 2014).

The mature miRNA is then incorporated into a multiprotein complex, ribonucleoprotein particle (miRNP), also known as RNA–induced silencing complex (RISC). After that, proteins of the AGO family, present in miRNP molecules, move the target mRNA to cytoplasmic structures, *P–bodies*, where mRNA translation is either repressed and/or its degradation is enhanced. Thus, miRNAs participate in the post–transcriptional regulation of gene expression by suppressing translation and/or degradation of specific mRNAs (Czech & Hannon 2011; Andrade & Palmeirim 2014).

Generally, a small sequence (6–8 nt long) localized in the 5'–end of the miRNA is crucial for binding to target mRNAs. miRNAs can bind to many mRNAs regions, although most miRNAs bind to mRNA 3'–untranslated regions (3'–UTR) in a deficient manner, inhibiting protein synthesis by repressing the translation process or increasing mRNA decay. When they bind to mRNA in exact complementarity, it leads to mRNA degradation; if the binding is incomplete, translation is inhibited (Krol *et al.* 2010; Yang & Wang 2011).

Besides these two mechanisms, other miRNAs action's mechanisms are known. miRNAs can enhance mRNAs translation by recruiting protein complexes in the target mRNAs AU–rich regions, or by indirectly increasing the levels of target mRNAs,

interacting with repressor proteins that block mRNAs translation (Eiring *et al.* 2010; Bertoli *et al.* 2015).

Besides being regulated by their promoters, methylation, processing and RNA editing, miRNAs can also be transcriptionally self–regulated, since they can activate or shut down mRNAs that encode factors with a key role in the function and biogenesis of miRNAs themselves, using negative or positive feedback loops (Krol *et al.* 2010; Yang & Wang 2011).

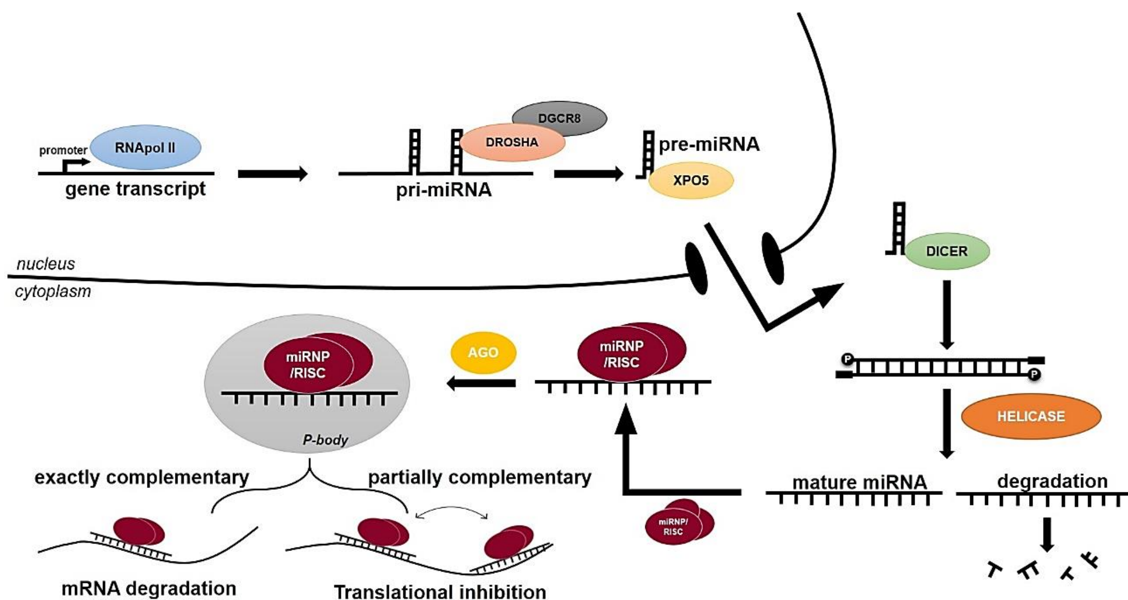


Figure 5 miRNA biosynthesis and function. RNApol II, RNA polymerase II; pri–miRNA, primary miRNA; DCGR8, microprocessor complex unit; pre–miRNA, precursor miRNA; XPO5, exportin 5; miRNP, micro–ribonucleoprotein; RISC, RNA–induced silencing complex; AGO, argonaute.

1.3.2. MicroRNAs and Breast Cancer

The initiation and progression of several human cancers, including BC, are associated with the dysregulation of miRNAs, which can either act as oncogenes or tumor suppressor genes (Andorfer *et al.* 2011).

This modification of miRNA expression can be caused by different mechanisms: (1) defects of the miRNAs biogenesis pathways; (2) gene modifications; (3) epigenetic mechanisms, or (4) transcriptional inhibition by other proteins.

(1) BC features are highly associated with reduced *Dicer* and *Drosha* expression (Yan *et al.* 2012);

(2) Frameshift mutations originate microsatellite instability. Some miRNAs that suffer these alterations have been identified, such as members of the let–7 family, miR–125b, miR–100, and miR–34a. All of those are localized in fragile sites of human chromosomes (11q23–q240), resulting in an abnormal expression of miRNAs (Calin *et al.* 2004).
(3) Many miRNAs are related to CpG islands, which provide the conditions for miRNA methylation. miR–200 was found to enhance the metastatic potential of tumors when methylated. Also, the genomic region encoding miRNA let–7e–3p, which is correlated with poor BC prognosis, is localized in a hypomethylated chromosome (Castilla *et al.* 2012; Aure *et al.* 2013).
(4) Some TFs, including the members of the tumor–related TFs family of p53 protein (p53, p63, and p73) and Myc are known to influence miRNA expression (Suzuki *et al.* 2009; Jiang *et al.* 2014).

As diagnostics and outcome prediction are a difficult task in BC, miRNAs have been broadly suggested as possible BC biomarkers, since they are easily detected in tumor biopsies (non–circulating miRNAs), but can also be found in a stable status in body fluids, such as saliva, serum, plasma, and blood (circulating miRNAs) (Chan *et al.* 2013; Ashby *et al.* 2014).

Some miRNAs have been characterized and indicated as specific targets for diagnostics in BC (miR–9, miR–10b, and miR–17–5p), outcome prediction (miR–30c, miR–187, and miR–339–5p), prognostics (miR–148a, and miR–335), and therapy (miR–21, miR34a, miR–145, and miR–150), while others have clinical interest because they can be easily screened in body fluids analysis (miR–155, and miR–210) (Ma *et al.* 2010; Corcoran *et al.* 2011; Ozgun *et al.* 2011; Rodriguez-Gonzalez *et al.* 2011; Lyng *et al.* 2012; Biagioni *et al.* 2013; Huang *et al.* 2013; Dong *et al.* 2014; Sandhu *et al.* 2014; Sochor *et al.* 2014; Kleivi Sahlberg *et al.* 2015).

In addition, some of the studied miRNAs are not specific targets for BC treatment, but they do may enhance BC therapies response by increasing the efficacy of conventional therapies (Bertoli *et al.* 2015).

An extreme expression of miRNAs has a crucial role in the tumorigenesis process of many BC types. Solely in breast tissues, there are almost 3 000 expressed miRNAs, from which some have oncogenic characteristics, promoting the malignancy of cancers, while some are known as tumor suppressors, for they reduce the production of oncogenic proteins (Chang *et al.* 2015; Panwar *et al.* 2017).

miR–10b, miR–21, miR–29a, miR–96, miR–146a, miR–181, miR–373, miR–375, miR–520c, miR–589, and the cluster miR–221/222 were found to be upregulated in

BC, which improved oncogenic assets. miR–221, for example, targets cell cycle inhibitors, increasing cell progression and reducing apoptosis (Nassirpour *et al.* 2013; Piva *et al.* 2013; Christodoulatos & Dalamaga 2014).

On the other hand, miR–30a, miR–31, miR–34, miR–93, miR–125, miR–126, miR–146a, miR–195, miR–200, miR–205, miR–206, miR–503, and let–7 are downregulated in BC. They lose their tumoral suppressor properties, consequently affecting cellular cycle and proliferation. For instance, let–7 regulates numerous oncogenes and genes that are involved in maintaining the stem cell phenotype. When those genes are not regulated by let–7 anymore, they acquire stem–like features, and cancer growth and proliferation are triggered (Kim *et al.* 2012; Jiang *et al.* 2014; Bertoli *et al.* 2015).

Wang *et al.* (2012) reported that miR–203 suppressed the expression of specific proto–oncogenes, thus decreasing cell proliferation and migration (Wang *et al.* 2012).

miR–200c was also pointed out as cell proliferation and migration regulator by Ren *et al.* (2014). They affirmed that by targeting the X–linked inhibitor of apoptosis, those features were regulated. Also, this miRNA is a member of the miR–200 family, which is proposed to control the epithelial phenotype of cancer cells by regulating E–cadherin expression levels (Park *et al.* 2008; Ren *et al.* 2014).

Dang and Myers (2015) studied the effects of hypoxia in the hypoxia–induced miR–210, advocating that a high level of hypoxia activated miR–210, thus downregulating a tumor suppressor gene, von Hippel–Lindau (*VHL*), enhancing cell proliferation, modifying DNA repair mechanisms, and remodeling chromatin (Dang & Myers 2015).

Moreover, miR–31, which usually has anti–metastatic properties, is downregulated in BC, consequently increasing metastasis–cascades (Augoff *et al.* 2012).

1.3.3. Triple–Negative Breast Cancer associated microRNAs

miRNAs have been associated with TNBC and, consequently, several studies have been performed to increase the knowledge of how they influence cellular pathways and hence tumorigenesis (**Table 2**). Furthermore, it is important to find out how TNBC–associated miRNAs can be implicated in future therapies (Gyparaki *et al.* 2014).

Studying which miRNAs are dysregulated in TNBC, Thakur *et al.* (2016) found 6 dysregulated miRNA: the oncogenic miR–21, miR–210, miR–221 and, surprisingly, the

tumor suppressor let–7a, upregulated in TNBC, while tumor suppressors miR–145 and miR–195 were downregulated (Thakur *et al.* 2016).

In conformity with their results, miR–21 was linked to the inhibition of two tumor suppressor genes named *programmed cell death 4* (*PDCD4*) and *PTEN*. Studying the TNBC MDA–MB–231 cell line, Dong *et al.* (2014) found that miR–21 targeted *PTEN* gene, inducing apoptosis. Furthermore, the activity of the apoptosis-associated enzymes caspases 3 and 9 was inhibited by this miRNA (Frankel *et al.* 2008; Qi *et al.* 2009; Dong *et al.* 2014).

Regarding miR–221, Miller *et al.* (2008) suggested that this miRNA functioned as an oncogene by targeting a cell cycle inhibitor. Nassirpour *et al.* (2013) knocked down miR–221 and observed that cellular cycle progression was inhibited and apoptosis was induced (Miller *et al.* 2008; Nassirpour *et al.* 2013).

Also, studying miR–221 and miR–222, Falkenberg *et al.* (2015) associated these two miRNAs with the invasive and aggressive behavior of TNBC. When upregulated these miRNAs upregulated an urokinase receptor. This urokinase receptor was reported as important in tissue reorganization and wound healing, but when upregulated, it enhanced cell invasion and metastasis. Besides, Falkenberg *et al.* (2013 and 2015) also found that these miRNAs targeted *PTEN* gene, which regulated epithelial–to–mesenchymal transition (EMT) processes. EMT promotes invasion and metastasis by the acquisition of mobility features that are characteristic of mesenchymal cells. Upregulation of these miRNAs lead to an E–cadherin expression decrease, promoting EMT and thus contributing to tumor development and malignancy (Falkenberg *et al.* 2013; Falkenberg *et al.* 2015).

Kong *et al.* (2014) found that miR–155 was overexpressed in TNBC, and suggested that it had an oncogenic role in TNBC by downregulating *VHL* gene, promoting angiogenesis (Kong *et al.* 2014).

Hu *et al.* (2015) studied migration and invasion of TNBC's miRNAs. They found a high miR–93 expression, which promoted proliferation, migration and invasion of tumor cells (Hu *et al.* 2015).

miR–18b, miR–103, miR–107, and miR–652 were found to be involved in chemotherapy resistance and metastasis in TNBC serum samples in a study by Kleivi Sahlberg *et al.* (2015). Focusing more specifically on miR–103 and miR–107, this research group confirmed the results by Neijenhuis *et al.* (2013), who associated miR–107 with EMT and DNA repair pathways, resulting in the poor prognostics and high aggressiveness of TNBC. Supporting this data, Martello *et al.* (2010) suggested that miR–103 and miR–107 were necessary to inhibit and control an overexpression of

Dicer. The upregulation of these miRNAs lead to an overexpression of miR–200, which increased EMT pathways, thus leading to the aggressiveness feature of TNBC (Martello *et al.* 2010; Neijenhuis *et al.* 2013; Kleivi Sahlberg *et al.* 2015).

Passon *et al.* (2012) investigated Drosha and Dicer expression in TNBC and reported that in most cases these two components of miRNA processing were overexpressed, and that this overexpression was associated with TNBC aggressiveness (Passon *et al.* 2012).

miR–182, overexpressed in TNBC, stimulated cellular migration. Its expression was higher in MDA–MB–231 cell line and TNBC tissues when compared with adjacent breast tissues (Liu *et al.* 2013).

Table 2 TNBC associated miRNAs and their function.

miRNA	**Function/Effect**	**Reference(s)**
let–7a	Tumor suppressor	Thakur *et al.* (2016)
miR–18b	Chemotherapy resistance and metastasis	Kleivi Sahlberg *et al.* (2015)
miR–21	Oncogenic; inhibition of tumor suppressor genes	Frankel *et al.* (2008); Dong *et al.* (2014); Thakur *et al.* (2016)
miR–93	Promotion of proliferation, migration and invasion of tumor cells	Hu *et al.* (2015)
miR–103	Chemotherapy resistance, metastasis, EMT and DNA repair pathways	Martello *et al.* (2010); Neijenhuis *et al.* (2013); Kleivi Sahlberg *et al.* (2015)
miR–107	Chemotherapy resistance, metastasis, EMT and DNA repair pathways	Martello *et al.* (2010); Neijenhuis *et al.* (2013); Kleivi Sahlberg *et al.* (2015)
miR–145	Tumor suppressor	Thakur *et al.* (2016)
miR–155	Oncogenic; promotor of angiogenesis	Kong *et al.* (2014)
miR–182	Stimulates cellular migration	Lu *et al.* (2013)
miR–195	Tumor suppressor	Thakur *et al.* (2016)
miR–200	EMT pathway	Martello *et.* (2010)
miR–210	Oncogenic	Thakur *et al.* (2016)
miR–221	Oncogenic; induction of apoptosis; poor outcome in TNBC patients	Stinson *et al.* (2011); Falkenberg *et al.* (2013); Nassirpour *et al.* (2013); Gyparaki *et al.* (2014); Falkenberg *et al.* (2015); Thakur *et al.* (2016)

miR–222	Oncogenic; invasive and aggressive behavior of TNBC	Falkenberg *et al.* (2013); Gyparaki *et al.* (2014)
miR–652	Chemotherapy resistance and metastasis	Kleivi Sahlberg *et al.* (2015)

2. Aim of the study

Previous studies have shown that TNBC is an aggressive subtype of BC that requires careful attention from clinicians and researchers alike, due to its varied features, difficult treatment, and poor overall prognostics.

GCs are often used for the treatment of diseases and cancer malignancies, but its role in TNBC is controverse since when its expression levels are high, it induces antiapoptotic effects. GR expression is also known to predict poor prognostics in TNBC patients.

To broaden the knowledge and insights in this research field, the present project aimed at identifying cellular miRNAs regulated by GR in TNBC.

The experimental workflow included the following methodological steps:

(1) Cellular culture of TNBC cell lines: three TNBC cell lines, MDA–MB–231, MDA–MB–436, and MDA–MB–468, were cultured in three different conditions: parental cell line, transfected with the vector pnDNA6/V5–HisA harboring the *NR3C1* gene, encoding the GR, or with siRNA silencing endogenous *NR3C1* gene expression;

(2) Isolation of total cellular RNA from the three different conditions of each cell line, RNA quality control and quantification;

(3) Complementary DNA (cDNA) synthesis and quantitative polymerase chain reaction (qPCR) to evaluate the transfection efficiency;

(4) Preparation of a library for Next–Generation Sequencing (NGS), including adaptor ligation, cDNA synthesis, and PCR amplification. After preparing the barcoded cDNA library, a gel electrophoresis was run for miRNA band size–selection and extraction. The extracted bands were analyzed on the Bioanalyzer, followed by sequencing of the samples. NGS was performed of all TNBC cell lines in all the three studied settings to obtain miRNA expression profiles from all three conditions;

(5) Processing and interpretation of data obtained by NGS: bioinformatics analysis, encompassing statistics and R software with DESeq package, was executed to identify GR–regulated miRNAs in TNBC.

3. Material and Methods

3.1. Material

Concerning biological material, three different TNBC cell lines were used: MDA–MB–231 (DSMZ no. ACC 732), MDA–MB–436 (CLS no. 300278), and MDA–MB–468 (DSMZ no. ACC 738). The first, MDA–MB–231, being one of the most studied cell lines, shows a stellate growth pattern when cultured. The second, MDA–MB–436 presents with a pleomorphic shape, while the third, MDA–MB–468 exhibits a rounder pattern (Chang *et al.* 2015).

Besides these three adenocarcinoma cell lines the following devices, reagents, kits and consumables were required to perform the experiments (see **Tables 3–6** below).

Table 3 List of devices.

Allegra™ 25R Centrifuge (Beckman Coulter)	Bdk® Laminar flow cabinet (Weiss Technik)
Eppendorf Research® Plus Pipets (Eppendorf)	Heracell 150i CO2 Incubator (Thermo Fisher Scientific™)
NanoPhotometer™ Pearl (Implen)	Bioanalyzer 2100 (Agilent Technologies)
Heraeus™ Fresco™ 17 Microcentrifuge (Thermo Fisher Scientific™)	CFX Real–Time PCR Detection System (Bio–Rad)
MasterCycler Gradient Thermal Cycler (Eppendorf)	MiniOpticon Real Time PCR System (Bio–Rad)

Table 4 List of reagents.

Penicillin Streptomycin (Pen–Strep) (Gibco™)	rDNAse (miRCURY™ RNA Isolation Kit) (Exiqon)
RPMI 1640 (1X) (Gibco™)	Trypan Blue staining (Gibco™)
HBSS (Gibco™)	Ethanol abs. (VWR)
Opti–MEM® medium (Gibco™)	Exo–FBS™ (System Biosciences)
Lipofectamine® 2000 (Invitrogen™)	Trypsin–EDTA 0.05% (1X) (Gibco™)
NR3C1 NM_000176.2 (MWG Eurofins)	Buffer RDD DNA Digest Buffer (Qiagen)
siRNA s6187 5 nmol (Thermo Fisher Scientific™)	pcDNA™6/V5–HisA (Thermo Fisher Scientific™)
GelRed™ staining (Biotium)	MetaPhor™ Agarose (Lonza)

UltraPure DNA Typing Grade 50X TAE Buffer (Thermo Fisher Scientific™)	GeneRuler Ultra Low Range (Thermo Fisher Scientific™)
O'RangeRuler™ 20 bp (Thermo Fisher Scientific™)	Sso Advanced Universal SYBR Green Supermix (Bio–Rad)
PrimePCR assays (Bio–Rad)	DMSO (Thermo Fisher Scientific™)
Orange Loading Dye (Thermo Fisher Scientific™)	

Table 5 List of kits.

Bioanalyzer DNA 1000 Chip Kit (Agilent Technologies)
Bioanalyzer DNA High Sensitivity Kit (Agilent Technologies)
Bioanalyzer RNA 6000 Nano Kit (Agilent Technologies)
HISeq Rapid SBS Cluster Kit V2 (Illumina)
HISeq Rapid SR Cluster Kit V2 (Illumina)
miRCURY™ RNA Isolation Kit – Cell & Plant (Exiqon)
miScript® II RT Kit (Qiagen)
miScript® miRNA PCR Kit (Qiagen)
miScript® Primer Assay (Qiagen)
Monarch Gel Extraction Kit (New England BioLabs Inc.)
Monarch PCR and DNA Cleanup Kit (New England BioLabs Inc.)
NEBNext Multiplex Small RNA Library Prep Set for Illumina [Index Primers 1–48] (New England BioLabs Inc.)
QIAquick PCR Purification Kit (Qiagen)
QuantiTect® Reverse Transcription (Qiagen)

Table 6 List of consumables.

Cell culture flasks, 25 cm^2 (TPP)	Pipet tips (Sarstedt, Peqlab)
Cell culture flasks, 75 cm^2 (TPP)	24–well plates (Greiner Bio–One)
Serological pipets (Sarstedt)	Microcentrifuge tubes (Sarstedt)
PCR tube strips (Bio–Rad)	

3.2. Methods

3.2.1. Cell Culture

For the cell culture experiments, TNBC cell lines MDA–MB–231, MDA–MB–436, and MDA–MB–468 were chosen. After collecting the three cell lines MDA–MB–231, MDA–MB–436, and MDA–MB–468 from liquid nitrogen, they were defrosted in a water bath, at 37 °C. To remove the cryoprotectant DMSO the cells were centrifuged and resuspended in a new medium. Next, the cells were placed in 25 cm^2 or 75 cm^2 culture flasks, previously filled with pre–warmed growth medium consisting of RPMI 1640 (1X), Exo–FBS (10 %), and Pen–Strep (1 %), and incubated at 37 °C and 5 % CO_2. When the medium color began to alter from pink–reddish (pH=7.4) to lemon–yellow (pH below 6.5), the medium was replaced with new one to enable the cells to continue to grow. When the adherent cells were more than 80 % confluent the cells were split and sub–cultured.

To split the cells, the medium was removed from the flask, and the cells' monolayer was washed with HBSS, removing the serum. HBSS was then removed before adding trypsin to the flask and incubating the cells for three minutes. After detaching the cells from the flask's surface, which can be seen under microscopic observation, a new medium was applied to the flask. After that, cells were resuspended, placed in a centrifuge tube, and centrifuged for five minutes at 100 *x g* (times gravity).

When centrifuged the supernatant was removed and new medium was added to the cell pellet and resuspended. New growth medium was applied to new culture flasks, and the resuspended cells were pipetted dropwise to this/these new(s) flask(s) and placed in the incubator.

3.2.2. Counting of Cells with the Neubauer Chamber

Cell number determination is an essential step to standardize and pursue accurate quantitation experiments.

The counting step was performed after trypsinization with resuspended cells. The Neubauer Chamber was prepared, and 10 µl of the cell sample were diluted in 10 µl of Trypan Blue staining. The same volume was then placed to the edge of the Neubauer chamber, and the sample was drawn under the coverslip by capillary action.

Under the microscope the grid was localized, and cells were counted in 4x4 squares. Nonviable cells stained in dark blue were not taken into account. After cell counting, the cell number per milliliter was calculated by the following algorithm:

$$cell\ number\ per\ ml = average\ count\ per\ square * 2 * 10^4 ,$$

with 2 being the dilution factor, and 10^4 being the chamber constant.

Knowing the cell number per milliliter, one can calculate the specific volume of cell suspension needed to have the required cell number.

3.2.3. Freezing Cells

The culture medium was removed from the flask, and the cells were washed with HBSS, trypsinized and centrifuged to remove the medium.

Next, the cells were resuspended in freezing medium (composed of RPMI 1640 (1X) (70%), FBS (20%) and DMSO (10%)), followed by the transference of approximately 1 Mio cells in 1 ml of the suspension to labeled cryovials. The vials were put in a freezing container at –20 °C for about 4–6 hours, followed by an overnight storage at –80 °C, before being long–term stored in liquid nitrogen.

3.2.4. Cell Experiments

The three TNBC cell lines MDA–MB–231, MDA–MB–436 and MDA–MB–468 were transfected with the vector pcDNA6/V5–HisA harboring the *NR3C1* gene (Nuclear Receptor, Subfamily 3, Group C, Member 1, *Homo sapiens*; transcript ID: NM_000176.2), which codes for the GR, or with siRNA silencing endogenous *NR3C1* gene expression. Untransfected, parental cells, served as controls, and are designated as endogenous throughout this manuscript. For the experiments, cells were counted and a total of 100 000 cells per well were placed in a 24–well plate in a total volume of 0.5 ml and incubated for 4 hours. For each cell line one untransfected cell well and two transfected cell wells (one transfected with *NR3C1* and one with siRNA) were prepared. Each experiment was performed in triplicates. Lipofectamine 2000 was used as transfection reagent. The concentration of GC for GR activation in the experimental setting was 0.16 µg/l.

Following the incubation, a transfection mix was prepared according to **Table 7**. The mix was incubated for five minutes at room temperature.

After incubation, the same amount of volume in *NR3C1* and siRNA tubes was transferred from the Lipofectamine tube to those two tubes. After mixing, the reaction was left at room temperature for 20 minutes.

Table 7 Transfection mix preparation for 1 reaction.

Components	**Tube NR3C1**	**Tube siRNA**	**Tube Lipofectamine**
OptiMEM	50 µl	50 µl	112 µl
Lipofectamine	–	–	4 µl
NR3C1 0.4 µg	4 µl	–	–
siRNA 5 pmol/ µl	–	4 µl	–
Total volume	54 µl	54 µl	116 µl

After 4 h of incubation, 108 µl of the medium was removed from each well, and the transfection mix was pipetted dropwise to each respective well. Transfection was performed for 24 h in a final volume of 0.5 ml (**Figure 6**).

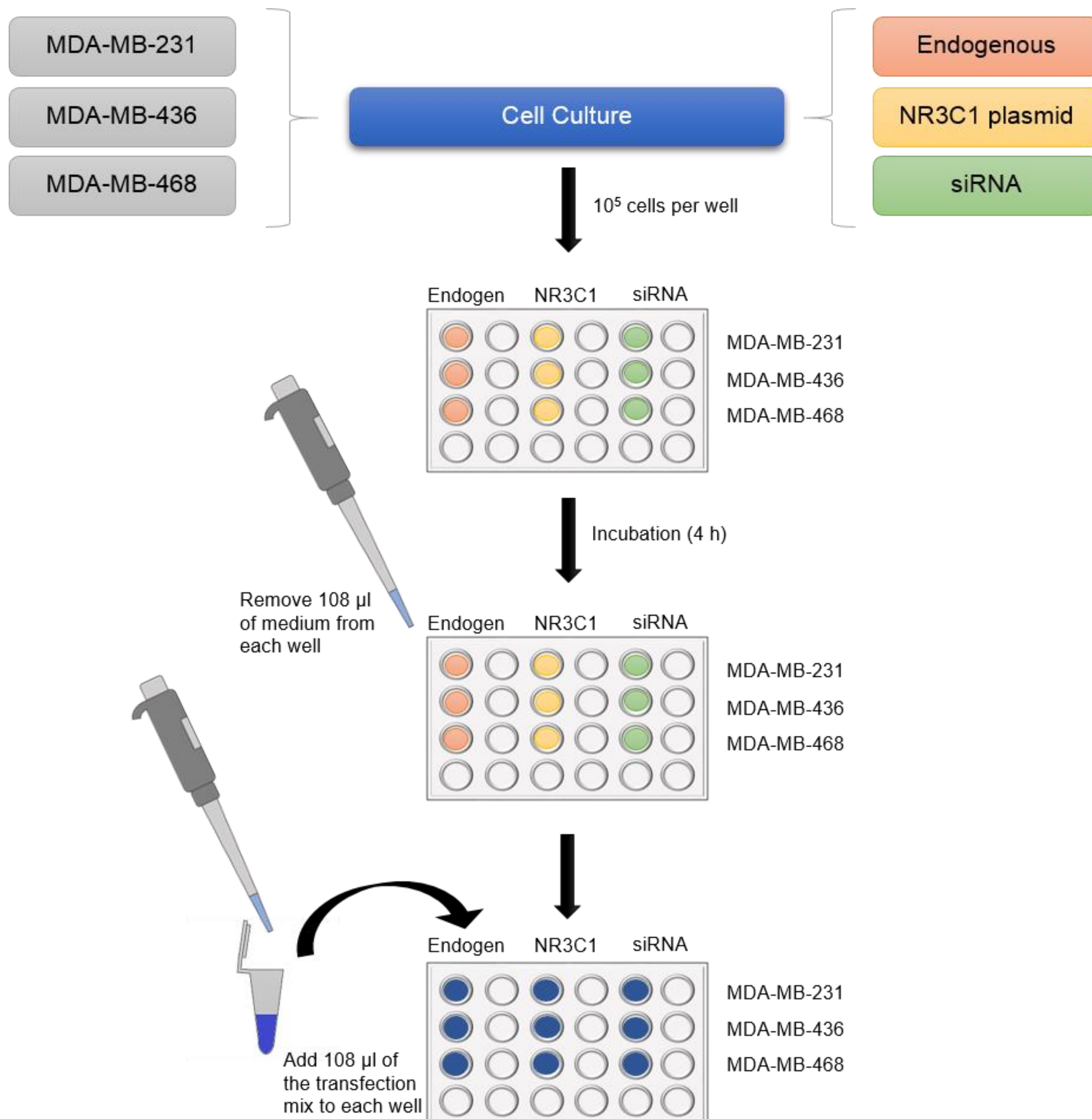


Figure 6 Transfection reaction workflow.

3.2.5. RNA Extraction and Quantification

After 24 h of transfection incubation, total RNA was extracted from each of the three wells using miRCURY RNA Isolation Kit – Cell & Plant.

Cells were washed with HBSS, 350 µl of Lysis Solution was added to the wells, and incubated at room temperature for 5 minutes. Next, the cells were scraped and transferred to 1.5 ml microcentrifuge tubes. 200 µl of 96–100% Ethanol abs. were

added to each tube and mixed by pipetting, before transferring 600 µl of the lysate to a collection tube with a column. After centrifugation for 1 minute at 3 500 *x g*, the flow through was discarded.

The cells were washed with 400 µl of Wash Solution by centrifuging for another minute at 3 500 *x g*. To remove any remaining genomic DNA (gDNA) 10 µl per sample of DNAse and 70 µl per sample of RDD Buffer were mixed and the solution was added to each tube, and let stand at room temperature for 15 minutes. Three more washing steps were performed as described before. To dry the membrane, tubes were centrifuged for 2 minutes at 14 000 *x g*, after which the collector tube was discarded, and the column was placed in a new microcentrifuge tube for RNA elution. 50 µl of Elution Buffer were pipetted into each tube, and the samples were submitted to two centrifugation steps: 2 minutes at 200 *x g* followed by 1 minute at 14 000 *x g*.

Total extracted RNA was quantified and quality–checked using a nanophotometer and Bioanalyzer 2100, using RNA 6000 Nano Kit, according to the manufacturer's instructions.

For the Bioanalyzer analysis: first, the ladder aliquot was denatured at 70 °C for 2 minutes. To prepare the gel–dye mix 550 µl of RNA gel matrix was filtrated and centrifuged for 10 minutes at 1 500 *x g* at room temperature, aliquoted 65 µl in 0.5 ml tubes, and stored at 4 °C. Next, 1 µl of dye was added to the 65 µl gel aliquot. The solution was vortexed and centrifuged at 1 300 *x g* for 10 minutes. With the gel–dye mix prepared an RNA chip was placed on the chip priming station, and 9 µl of the gel–dye was added to the corresponding well. The chip priming station was closed, and the syringe plunger was pressed down for 30 seconds. After that, the plunger was released, the priming station opened, and 9 µl of the gel–dye mix added to two more wells.

5 µl of the marker was pipetted in all 12 sample wells of the chip, and in the ladder well. 1 µl of denatured ladder reagent was added to its corresponding well, and 1 µl of each RNA sample was added to the wells. Following 1 minute at 2 400 rpm (revolutions per minute) in the vortex mixer, the chip was placed in the Agilent 2100 Bioanalyzer and was left to run. The Bioanalyzer provides RNA concentration and the RNA integrity number (RIN), which is an algorithm that assigns integrity values to RNA measurements. A RIN value of 10 means that the RNA is intact, while a RIN value of 1 signifies complete degradation of the RNA. RIN analysis on miRNA molecules displays higher values when compared to other RNA molecules because they are not as susceptible to degradation by RNAse as the latter are (Schroeder *et al.* 2006; Becker *et al.* 2010).

The quality investigation has an important role in subsequent analysis. It has been demonstrated that the decrease of RNA quality was associated with an increase of Cq values by SYBR Green–based reverse transcription quantitative polymerase chain reaction (RT–qPCR) (Becker *et al.* 2010).

Quality analysis by Bioanalyzer 2100 Small RNA assay is a method based on fluorescence dyes that attach to specific RNA sequences, converting the signal to standard curves (Buschmann *et al.* 2016).

3.2.6. cDNA Synthesis

To evaluate the transfection efficiency of the *NR3C1* plasmid and siRNA by quantitative polymerase chain reaction (qPCR), template RNA was reverse–transcribed into complementary DNA (cDNA).

The QuantiTect™ Reverse Transcription kit was used according to the manufacturer's instructions. This kit provides a fast and convenient protocol for an efficient reverse transcription and elimination of gDNA in two main steps. gDNA elimination reaction was prepared on ice according to **Table 8**. Reagents were mixed in a PCR tube and incubated for 2 minutes at 42 °C (**Figure 7**).

Table 8 gDNA elimination reaction components.

Component	Volume/reaction
gDNA Wipeout Buffer, 7x	2 µl
Template RNA (400 ng)	Variable
RNAse–free water	Variable
Total volume	14 µl

Next, reverse transcription of the template RNA samples was performed (**Table 9**).

Table 9 Reverse transcription components.

Component	Volume/reaction
Quantiscript RT	1 µl
Quantiscript RT Buffer, 5x	4 µl
RT Primer Mix	1 µl

Reaction from table 6 (containing the template RNA)	14 µl
Total volume	20 µl

The transcription mix was incubated in a thermocycler for 15 minutes at 42 °C, followed by 3 minutes at 95 °C to inactivate Quantiscript reverse transcriptase. Aliquots were stored at –20 °C (**Figure 7**).

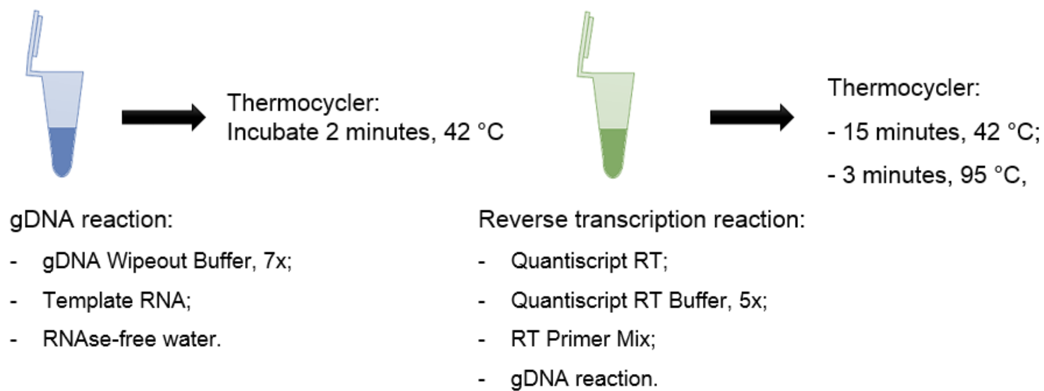


Figure 7 cDNA synthesis reaction.

3.2.7. Quantitative PCR for Glucocorticoid Receptor Expression

To verify if the transfection protocol for GR overexpression and silencing was successful, a RT–qPCR was performed.

19 µl of the master mix were pipetted for each reaction in a PCR tube and 20 ng of each template cDNA was added (**Table 10**).

As positive control a previously validated *NR3C1* positive sample was used, and water for the negative control. Amplification was performed on MiniOpticon Real Time PCR System in 33 cycles according to protocol depicted in **Figure 8**.

Table 10 Master mix reagents for qPCR reaction.

Component	Volume/reaction
PrimePCR assay	1 µl
SsoAdvanced Universal SYBR Green Supermix	10 µl
cDNA (20 ng/µl)	1 µl
H_2O	8 µl
Total volume	20 µl

The CFX Manager Software was used for data analysis and normalization, using *GAPDH* gene as a reference gene.

The mathematical model ΔΔCq (p–value < 0.05) was applied. By normalizing the targeted genes with the treatment conditions to the reference gene, *GAPDH*, which yields a ubiquitous expression, normalized, relative gene expression values for the studied cells were obtained. These values were then normalized to the expression of targeted genes in a separate control sample.

The formula for the ΔΔCq calculation is as follows:

$$\Delta Cq = Cq_{(target)} - Cq_{(reference)}$$

$$\Delta Cq \text{ exponential expression} = 2^{-\Delta Cq}$$

Calculate mean of the replicates and standard deviation

$$\Delta\Delta Cq = \Delta Cq - \Delta Cq_{(control)}.$$

For our experiments, the modified formula was:

$$\Delta Cq = Cq_{(miRNA)} - Cq_{(reference)}$$

$$\Delta Cq \text{ exponential expression} = 2^{-\Delta Cq}$$

Calculate mean of the replicates and standard deviation

$$\Delta\Delta Cq = \Delta Cq - \Delta Cq_{(reference)}.$$

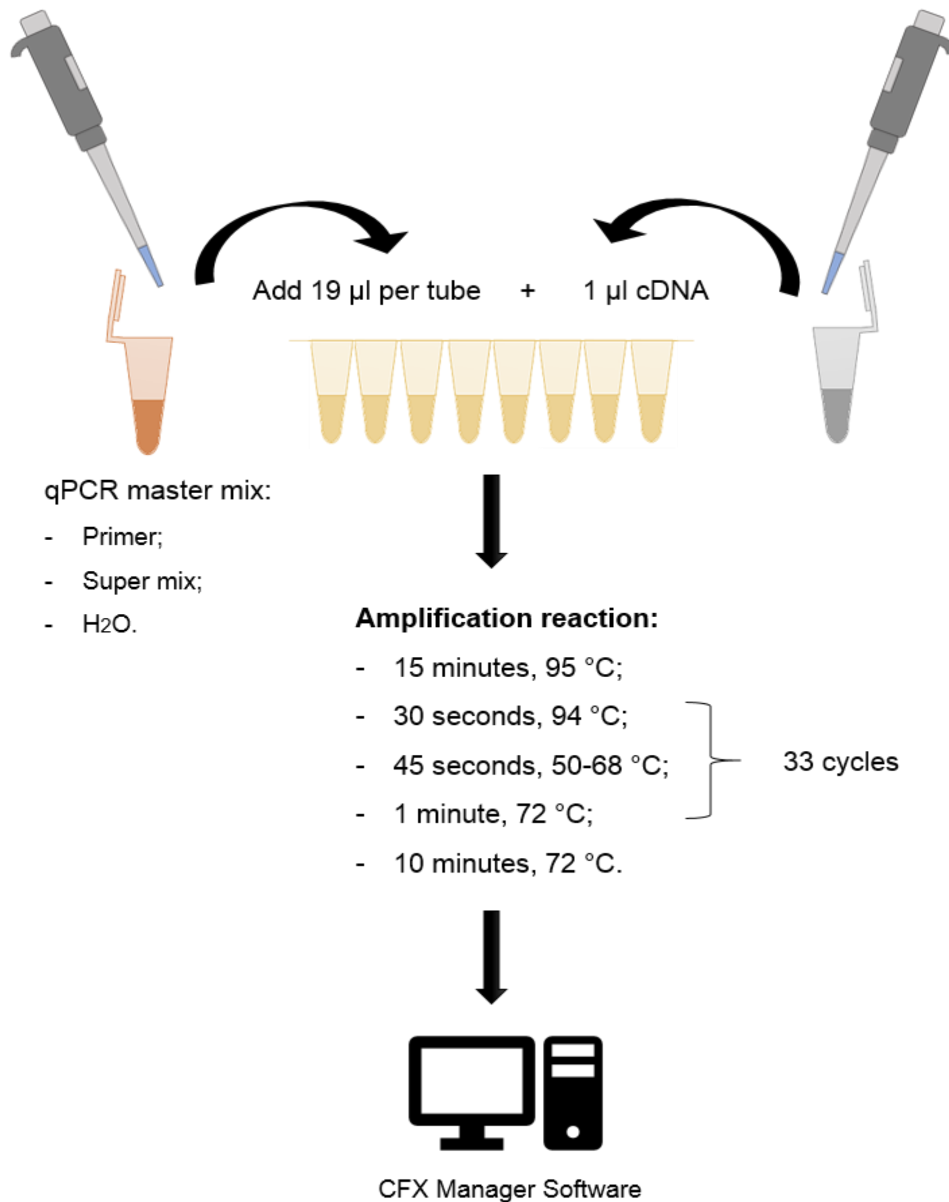


Figure 8 qPCR workflow for GR expression .

3.2.8. Library Preparation for Next–Generation Sequencing

To obtain expression profiles of all miRNAs present in the three treated and untreated TNBC cell lines, NGS was performed.

NEBNext Multiplex Small RNA Library Prep Set for Illumina kit, with some modification of the protocol by Spornraft *et al.* (2014), was used (**Figure 9**). All reagents and reaction mixes were kept on ice during pipetting. 190 ng RNA of each sample was used as starting material.

Barcoded cDNA libraries were obtained from the previous transcripts following the steps:

(1) Adaptor ligation to 3' strand;
(2) Primer hybridization;
(3) Adaptor ligation to 5' strand;
(4) First strand synthesis;
(5) PCR amplification;
(6) PCR cleanup;
(7) Library quantification;
(8) Gel electrophoresis;
(9) Size selection and gel extraction.

(1) The first step, 3' Adaptor Ligation, was performed by adding to each RNA sample the 3' Ligation Adaptor and nuclease–free water (**Table 11**).

Table 11 Components for 3' adaptor ligation reaction.

Component	Volume/reaction
3' Adaptor Ligation (step 1)	
Input RNA 190 ng	1–6 µl
3' Ligation Adaptor	1 µl
Nuclease–free water	Variable
Total volume	7 µl
3' Adaptor Ligation (step 2)	
3' Ligation Reaction Buffer (2x)	10 µl
3' Ligation Enzyme Mix	3 µl
Total volume	20 µl

The reactions were incubated for 2 minutes at 70 °C. The 3' Ligation Reaction Buffer (2x) and 3' Ligation Enzyme Mix were then added to the mix and incubated for 1 h at 25 °C.

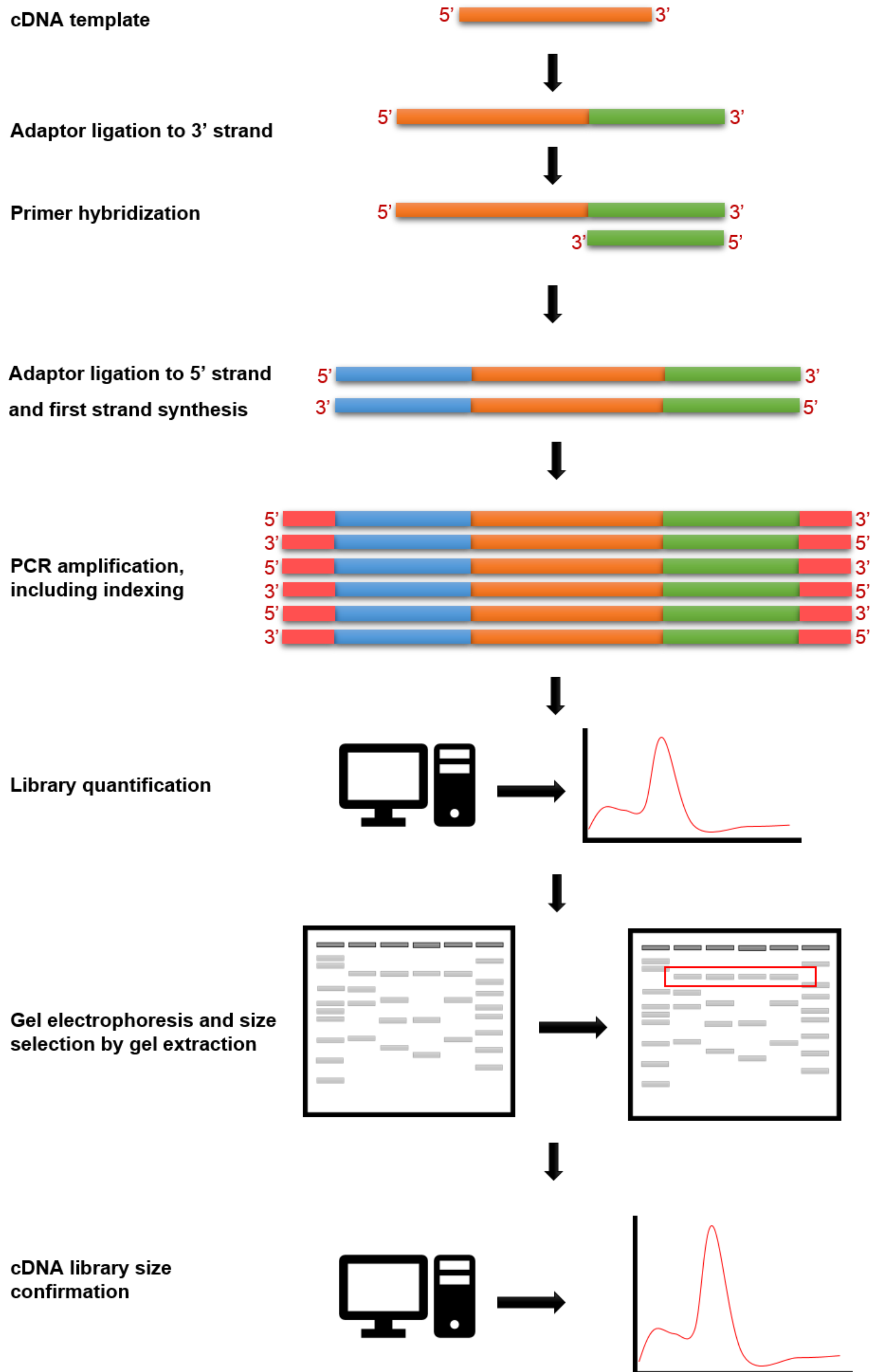


Figure 9 Library preparation workflow for NGS.

(2) To prevent adaptor–dimer formation, a hybridization step was performed. The primer hybridizes with the excess of 3' adaptor so that the single–stranded 3' adaptor turns into a double–stranded DNA molecule. As stated in **Table 12**, nuclease–free water and RT primer, previously diluted in a 1:3 ratio, were added to the previous mix and placed in the thermocycler for:

- 5 minutes at 75 °C;
- 15 minutes at 37 °C;
- 15 minutes at 25 °C.

Table 12 Components for hybridization reaction.

Component	**Volume/reaction**
Nuclease–free water	4.5 µl
RT Primer	1 µl
Total volume	5.5 µl
Final volume	25.5 µl

(3) First, 5' adaptor was resuspended in 120 µl of nuclease–free water diluted 1:3, and then incubated for 2 minutes at 70 °C. Denatured 5' adaptor, Ligation Reaction Buffer, and Enzyme Mix were added to the reaction mix from (2) and incubated at 25 °C for 1 h (**Table 13**).

Table 13 Components for 5' adaptor ligation.

Component	**Volume/reaction**
5' Adaptor (denatured)	1 µl
5' Ligation Reaction Buffer (10x)	1 µl
5' Ligation Enzyme Mix	2.5 µl
Total volume	30 µl

(4) Reverse transcription was achieved by mixing the previous adaptor–ligated RNA samples with first strand synthesis reaction buffer, murine RNase inhibitor and reverse transcriptase. The reaction mix was then incubated at 50 °C for 1 h (**Table 14**).

Table 14 Components for reverse transcription.

Component	Volume/reaction
Adaptor–Ligated RNA	30 μl
First Strand Synthesis Reaction Buffer	8 μl
Murine RNase Inhibitor	1 μl
Reverse Transcriptase	1 μl
Total volume	40 μl

(5) To perform the PCR amplification, the components from **Table 15** were added to the RT reaction mix from (4), and followed the PCR cycling conditions:

- 30 seconds at 94 °C for the initial denaturation;
- 12 to 15 cycles:
  - 15 seconds at 94 °C for denaturation;
  - 30 seconds at 62 °C for the annealing step;
  - 15 seconds at 70 °C for extension;
- 5 minutes at 70 °C for the final extension.

Table 15 Components for PCR amplification reaction.

Component	Volume/reaction
LongAmp Taq 2x Master Mix	50 μl
Primer	2.5 μl
Index Primer	2.5 μl
Nuclease–free water	5 μl
Total volume	100 μl

(6) A PCR clean–up step was then performed with Monarch PCR and DNA Cleanup Kit.

The samples were diluted in a 5:1 ratio. 500 μl of buffer were mixed with 100 μl of the samples from (5) and then pipetted onto the columns. The columns were centrifuged for 1 minute at 16 000 *x g*, after which the flow through was discarded.

Two washing steps were then performed by adding 200 μl of DNA Wash Buffer to the columns, followed by centrifugation at 16 000 *x g* for 1 minute.

The columns were then placed in new collector tubes, and centrifuged for 5 minutes with open lids. After that columns were transferred to new 1.5 ml tubes, and 8 µl of Elution Buffer were pipetted onto the membranes of the columns, and let stand at room temperature for 1 minute. The tubes were centrifuged for 1 minute at 16 000 *x g* to collect the eluted DNA.

(7) After this step, a DNA 1000 Chip was run for each sample to assess the length distribution and concentration of the cDNA library. The chip was prepared according to the manufacturer's instructions (for details of chip preparation see chapter 3.2.5).

The Bioanalyzer analysis was performed so that the concentration of cDNAs between the range of 130–150 bp, which represents the miRNA fraction, could be assessed. A gel electrophoresis was run.

(8) For the gel electrophoresis, 12 g of agarose was dissolved in 300 ml 1X TAE and 9 µl of GelRed™. The solution was stirred and heated up until dissolved. The bottle was weighted and the evaporated volume was replaced with 1X TAE until it equaled the initial weight.

The gel was then poured into the chamber, and let to cool for 45 minutes, after which it was placed for 30 minutes at 4 °C.

For an input of 8 ng/sample, volumes for each sample were calculated according to the concentrations obtained from the DNA 1000 Chip and pooled together in a total of approximately 120 µl. As the gel slot's maximum pipetting volume is 30 µl the pool was divided into 5 gel slots. After the pooling, Orange DNA Loading Dye was added to the sample pool at a ratio 1:6. When the gel was ready, the pools were pipetted into the gel slots, each pool in between two ladders slots, GeneRuler Ultra Low Range DNA, and O'RangeRuler™ 20 bp DNA ladders (**Figure 10**), so that the 147 bp target band could be localized between the 150 bp band from the first ladder and the 140 bp band from the second one.

The gel was left running in 850 ml Buffer volume with 150 V at 4 °C for about 2.5 h. After that, the bands were visualized under the UV light and left to cool down for 30 minutes. A picture was taken and the bands with a size of around 147 bp were selected.

(9) The bands were cut out under UV light, and a cleanup step was performed with the Monarch Gel Extraction kit to purify the cDNA.

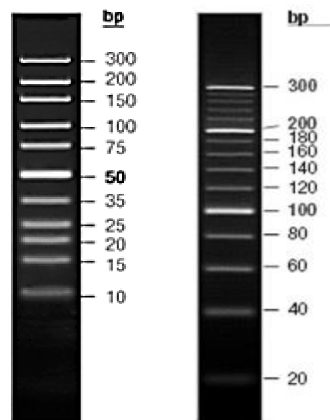


Figure 10 GeneRuler Ultra Low Range and O'RangeRuler™ ladders. Adapted from https://www.thermofisher.com/

In detail, the gel pieces were placed in tubes and Gel Dissolving Buffer was pipetted in each. The tubes were vortexed a few times and left at room temperature until the gel was dissolved. Up to 800 µl of the samples were then transferred onto columns and centrifuged at 16 000 *x g* for 1 minute. The flow through was discarded, and 200 µl of the Washing Buffer was added to each tube and centrifuged for 1 minute at 16 000 *x g*. This step was repeated one more time.

The columns were transferred to the DNA LoBind tubes, and 10 µl of water were pipetted onto the membrane and let stand for 1 minute, followed by a new centrifugation step at 16 000 *x g* for 1 minute to elute the DNA.

With the purified DNA samples a DNA High Sensitivity Chip was run on the Bioanalyzer to confirm the cDNA library size of 147 bp.

3.2.9. Next–Generation Sequencing Data Analysis

The raw NGS data files (FASTQ–files) obtained from the HiSeq run were processed in 4 steps: (1) quality control and adaptor trimming; (2) alignment of reads; (3) normalization, and (4) differential expression analysis (Buschmann *et al.* 2016).

(1) Adaptor sequences were removed using the software Btrim. Reads shorter than 15 nt were excluded. Quality control was studied by evaluating principal component analysis (PCA) and Phred results. PCA investigates the main components influencing the data, while Phred algorithm measures the quality of the identification of the nucleotide bases resulting from the sequencing. The

higher the score value, the lower is the error probability. A Phred score of 30 corresponds to an error probability of 0.1 % (Ewing & Green 1998).

(2) The reads were then mapped to RNAcentral database containing reference sequences for rRNA, tRNA, snRNA and snoRNA. Reads matching to these reference sequences were depleted. The remaining reads consisting of degraded mRNA and miRNA sequences were matched against the miRNA database (miRbase version 21), using Bowtie to identify the read counts for each known human miRNA in each sample (Griffiths-Jones 2004; Li *et al.* 2009; Li & Homer 2010; Kozomara & Griffiths-Jones 2014).

(3) To normalize data from the differences in the library, such as GC–content and batch effects, individual read counts were first divided by the library size of each sample, followed by multiplying to the arithmetic mean of the library size of all samples (Bullard *et al.* 2010; Leek *et al.* 2010; Risso *et al.* 2011).

(4) Differential expression analysis (DEA) was performed using DESeq, which models the observed mean–variance relationship for all genes via regression. To identify significantly GR–regulated miRNA, the miRNA dataset of the samples with endogenous GR expression was compared to that of GR overexpression. The following three criteria were taken into account: a p–value < 0.05; a BaseMean ≥ 50; and a $Log_2FoldChange \geq |1|$. The regulation direction of the resulting miRNAs was then checked in DEA of the dataset with endogenous GR expression compared to that of GR silencing. Only miRNAs that showed significant regulation in the first DEA and no significant regulation in the same direction in the second DEA were considered valid. To correct for false discovery rate the Benjamini–Hochberg method was applied (Anders & Huber 2010; Love *et al.* 2014).

3.2.10. Validation of Significant microRNAs by Quantitative PCR

A quantitative real–time PCR allows the detection and measurement of amplified products as the reaction progresses, this is, in real time. This detection is possible due to the inclusion of a fluorescence molecule that signals the increase of molecular material, proportional with fluorescence signal (DeCaire *et al.* 2015).

To validate the miRNAs that have been found to be regulated in the NGS dataset, a RT–qPCR was performed using miScript® II RT and miScript® miRNA PCR kits.

The protocol is divided into two steps: reverse transcription and real–time PCR. For each sample reverse transcription was performed in triplicates.

The RT master mix for the samples and for the no reverse transcription (NRT) control were prepared as described in **Table 16** with a sample input of 111 ng.

Table 16 Components for reverse transcription master mix.

Component	**Volume/Reaction**	**NRT**
5x miScript HiSpec Buffer	2 µl	2 µl
10x miScript Nucleics Mix	1 µl	1 µl
RNAse–free water	Variable	1 µl
miScript RT Mix	1	–
Template RNA 111 ng	Variable	Variable
Total volume	10 µl	10 µl

For NRT 2 µl RNA of each condition and cell line studied were pooled, which resulted in 9 NRT pool groups (parental condition: MDA–MB–231, MDA–MB–436, and MDA–MB–468; overexpression: MDA–MB–231, MDA–MB–436, and MDA–MB–468; and siRNA: MDA–MB–231, MDA–MB–436, and MDA–MB–468).

The master mix was mixed and spinned down before pipetting 4 µl to each well of a 96–well RT plate. 6 µl of template RNA and NRT were then added to each well containing the master mix. The plate was sealed, vortexed and spinned down, followed by the RT reaction cycle in the thermocycler:

- 60 minutes at 37 °C;
- 5 minutes at 95 °C;

The resulting cDNA was diluted for real–time PCR by adding 100 µl of RNAse–free water to the samples and 10 µl to the NRT.

For the real–time PCR, the following sequential steps were completed:

First, lyophilized primers were reconstituted through the addition of 550 µl of TE (pH 8.0), mixing and spinning the tube, and stored on ice. Reagents were thawed at room temperature before usage, mixed and spinned down, and stored on ice.

The PCR master mix was prepared for the template cDNAs, as well for no template controls (NTC), in which RNAse–free water was pipetted instead of template cDNA according to **Table 17.**

Table 17 Components for the PCR master mix.

Component	Volume/Reaction	NTC
2x QuantiTect SYBR Mix	5 µl	5 µl
10x miScript Universal Primer	1 µl	1 µl
10x miScript Primer	1 µl	1 µl
RNAse–free water	Variable	Variable
Template RNA	1 µl	1 µl H_20
Total volume	10 µl	10 µl

The master mix was mixed and spinned down before pipetting 9 µl to each well of the plate. 1 µl of diluted cDNA and NTC were then added to each well containing the master mix, and mixed by pipetting. The plate was sealed and set to the qPCR reaction in the CFX Real–Time PCR Detection System, following the program:

- 15 minutes at 95 °C for the activation of polymerase;
- 45 cycles:
  - 15 seconds at 94 °C for denaturation;
  - 30 seconds at 55 °C for the annealing step;
  - 30 seconds at 70 °C for extension;
- 60 to 95 seconds, 0.5 °C/s, for the melting step.

The data obtained was then processed and analyzed in Microsoft Office's Excel®. Raw data from the miRNAs and reference miRNAs were used for the calculation. To evaluate which genes could be pointed out as normalizing genes, the software GenEx Professional was used. The program runs two distinct algorithms, GeNorm and NormFinder. The first one expresses the results in M–values (average expression stability), where the smallest values are the best results, and the second one expresses its results in standard deviation values. Final results derived from the comparison of the values given by both algorithms (Buschmann *et al.* 2016).

After the selection of the reference miRNAs, the mathematical ΔΔCt value for each miRNA was calculated by substracting the reference miRNAs' value to that of the studied miRNA. The same formula was used for the average values of studied and reference miRNAs, and the result of this calculation was subtracted to the first one:

$$\Delta\Delta Ct = (miRNA - referece\ miRNA) - \left(mean_{miRNA} - mean_{reference\ miRNA}\right).$$

Then the exponential value of the result is calculated, $2^{-\Delta\Delta Ct}$, and a Student's *t*–test was performed to evaluate if there were any significant differences between the data.

4. Results and Analysis

In this project, the main goal was to identify cellular miRNAs regulated by GR in TNBC. This study was performed using three different cell lines, MDA–MB–231, MDA–MB–436, and MDA–MB–468, which have been cultured and transfected with the vector pcDNA6/V5–HisA harboring the *NR3C1* gene encoding the GR, or with siRNA silencing endogenous *NR3C1* gene expression.

4.1. Total RNA Isolation

To perform the necessary cell experiments in this project, total RNA had to be extracted from the cultured cells. RNA samples were then quantified and quality–checked using Nanophotometer and Bioanalyzer 2100 (RNA 6000 Nano Kit). RNA concentration was assessed, as well as RIN values. These results are listed in **Table 18**.

Table 18 RNA concentration and RIN values.

Cell line and condition	RNA Concentration (ng/µl)		RIN
	Nanophotometer	Bioanalyzer	
MDA–MB–231 R1 parental	110	68	9.4
MDA–MB–231 R2 parental	72	58	9.3
MDA–MB–231 R3 parental	50.8	39	9.9
MDA–MB–231 R1 NR3C1	55.2	49	9.3
MDA–MB–231 R2 NR3C1	46.8	44	9.2
MDA–MB–231 R3 NR3C1	38	29	9.3
MDA–MB–231 R1 siRNA	76.4	64	9.6
MDA–MB–231 R2 siRNA	55.2	53	9.2
MDA–MB–231 R3 siRNA	39.2	42	9.4
MDA–MB–436 R1 parental	104	118	9.4
MDA–MB–436 R2 parental	76.8	110	9
MDA–MB–436 R3 parental	71.2	93	9.2
MDA–MB–436 R1 NR3C1	59.6	60	9.3
MDA–MB–436 R2 NR3C1	47.6	54	9
MDA–MB–436 R3 NR3C1	31.6	37	9.1
MDA–MB–436 R1 siRNA	67.2	66	9.1

MDA–MB–436 R2 siRNA	62.8	87	8.7
MDA–MB–436 R3 siRNA	44	74	9
MDA–MB–468 R1 parental	58.4	50	9.7
MDA–MB–468 R2 parental	58.8	50	9.7
MDA–MB–468 R3 parental	124	130	9.5
MDA–MB–468 R1 NR3C1	41.6	25	9.1
MDA–MB–468 R2 NR3C1	33.6	24	9
MDA–MB–468 R3 NR3C1	58.4	59	9.2
MDA–MB–468 R1 siRNA	49.2	51	9.2
MDA–MB–468 R2 siRNA	47.2	44	9.3
MDA–MB–468 R3 siRNA	78.4	97	9.4

RNA concentrations ranged from 29 ng/µl to 110 ng/µl in the cell line MDA–MB–231, from 31.6 ng/µl to 118 ng/µl in MDA–MB–436 and from 24 ng/µl to 130 ng/µl in MDA–MB–468. Both quantification methods depicted similar concentration values for each sample.

RIN values ranged from 8.7 to 9.9, meaning that the extracted RNA was not degraded.

4.2. *NR3C1* Transfection Efficiency

The transfection efficiency of the *NR3C1* plasmid and siRNA was evaluated by RT–qPCR in all three cell lines, using *GAPDH* as reference gene.

Figure 11 shows the quantification results of the normalized gene expression of the three conditions. The data clearly show that the transfection was efficient for the three cell lines. All of them display an increase of expression in the *NR3C1* plasmid condition, and a decrease of expression when treated with siRNA. For the cell line MDA–MB–231, an overexpression of approximately 134–fold and a knockdown of 60 % was observed. The same panorama was found in the cell lines MDA–MB–436 and MDA–MB–468, for *NR3C1* overexpression and knockdown (172–fold and 338.5–fold overexpression, 93 % and 92 % knockdown, correspondingly, in comparison with the parental cells' state).

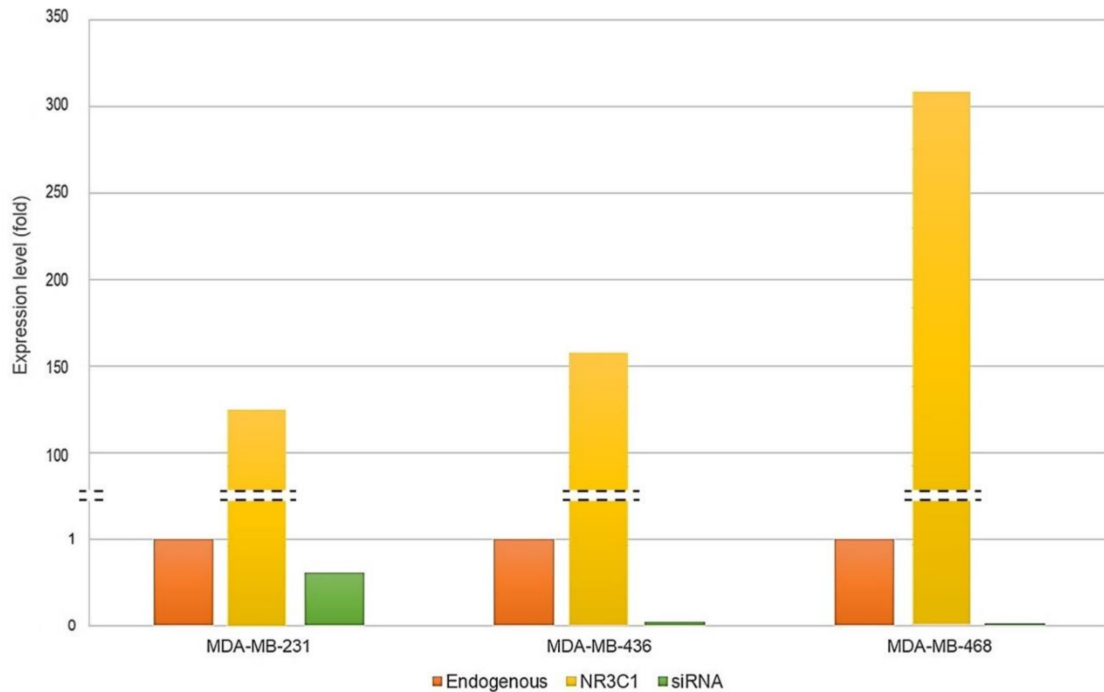


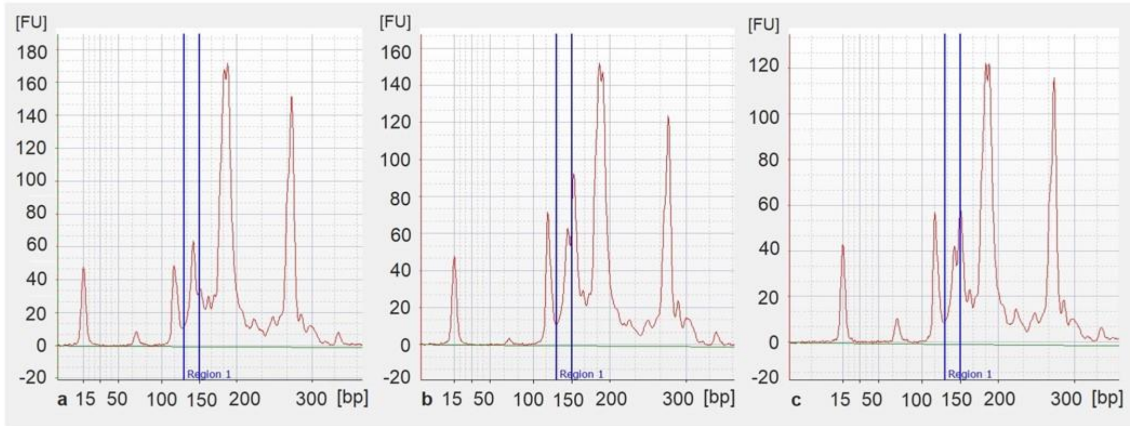
Figure 11 Gene expression quantification of *NR3C1.*

4.3. Library Preparation

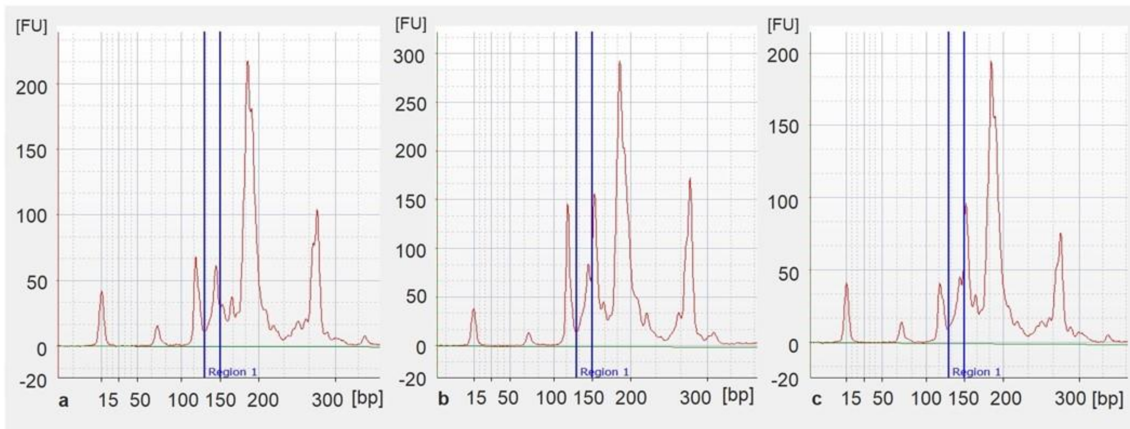
4.3.1. Length Distribution of cDNA Library before Size Selection

The library preparation, explained in chapter 3.2.8., had the main objective to prepare and quantify a valid cDNA library suitable for small RNA–NGS. To achieve this it was necessary to verify the presence of cDNA in the range of 130–150 bp and assess the concentration. In **Figure 12** the length distribution of the cDNA library before size selection with Bioanalyzer's DNA 1000 chip is shown. The blue region marks the desired cDNA fragments corresponding to miRNAs in length, and was used to calculate their concentrations (see chapter 8.1).

MDA-MB-231



MDA-MB-436



MDA-MB-468

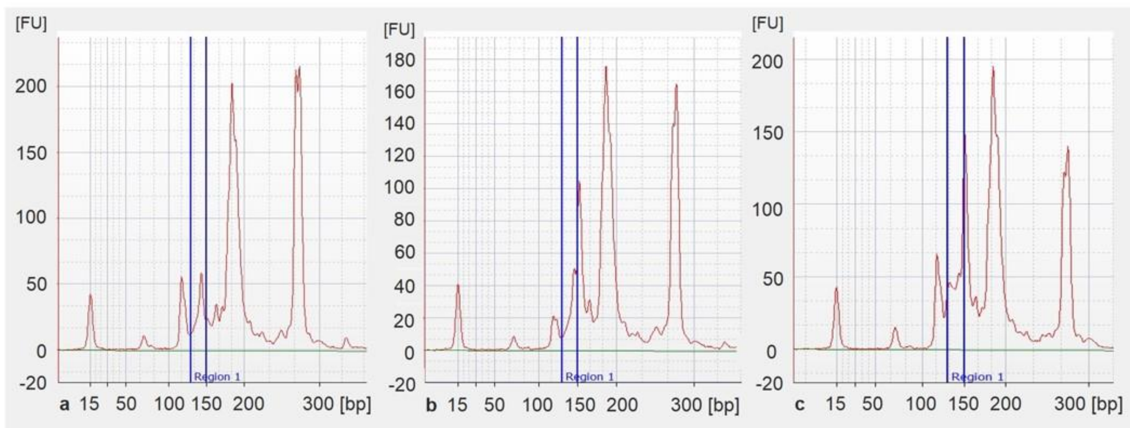


Figure 12 Library quantification for the three different cell conditions (a) endogenous, (b) GR overexpression, and (c) GR–silencing. FU, fluorescence units; bp, base pair.

4.3.2. Size–Selection of microRNAs for Sequencing

An electrophoretic gel was run to efficiently separate the cDNA fragments present in the samples (for details see chapter 3.2.8).

Since we were looking for fragment lengths corresponding to miRNAs (135 bp – 145 bp), candidate bands were selected by means of two ladders to compare and localize the targeted ones. Based on the 150 bp band from the first ladder and the 140 bp band present in the latter, the small cDNA bands corresponding to miRNAs were identified and excised with a scalpel under UV light.

The gel, before and after cutting out the identified bands, can be seen in **Figures 13a and b**.

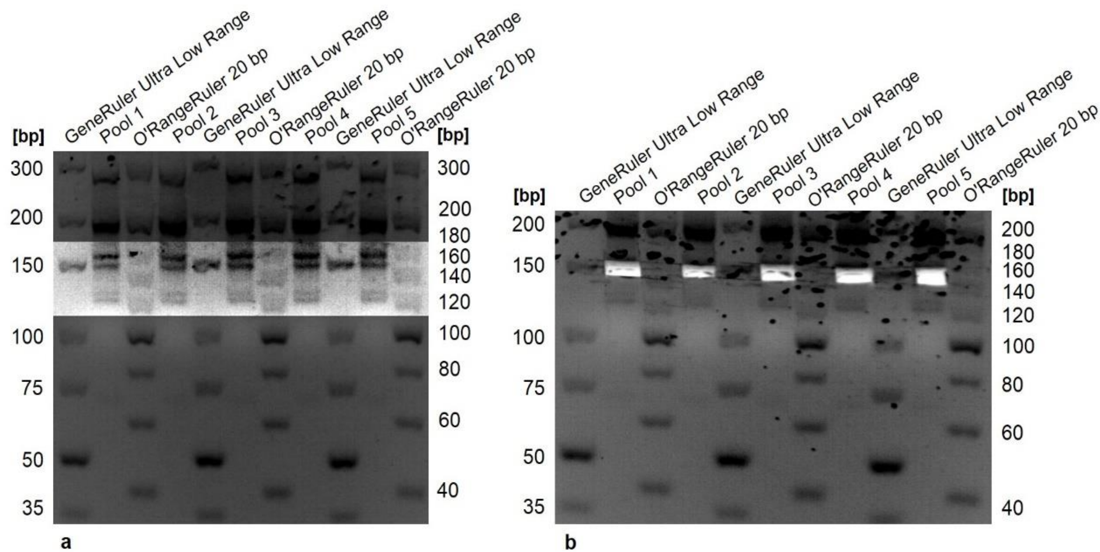


Figure 13 Electrophoretic gel results. a) before, and b) after band excision. Y–axis: bp, base pair.

The results of the chip analysis are shown in **Figure 14** and **Table 19**. The graphic displaying the resulting peak of the pooled samples confirmed the existence of cDNA fragments with the desired length.

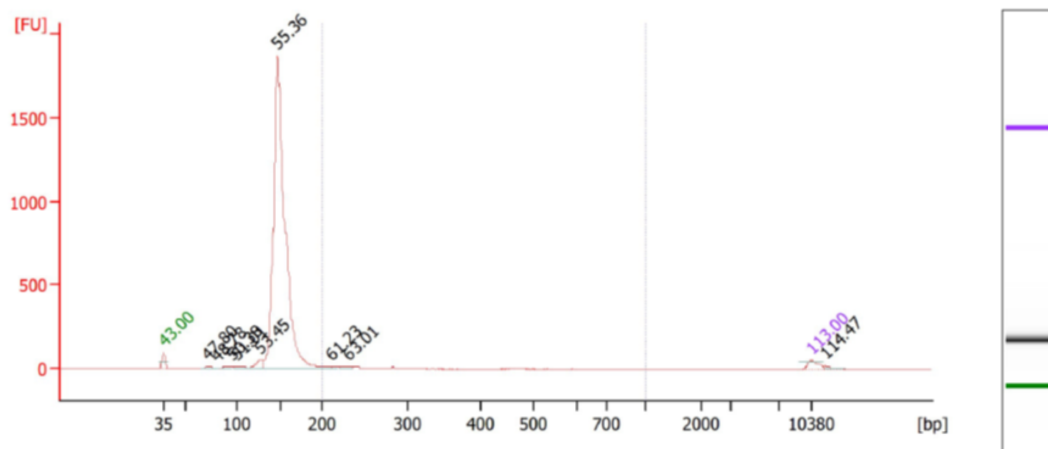


Figure 14 Bioanalyzer chip result after size selection showing the desired peak at around 148 bp. The software also displays a virtual electrophoretic gel run, on the right side. FU, fluorescence units.

The highest concentration with an approximate concentration of 10 600 pg/µl could be found for the desired band size of 148 bp (**Table 19**).

Table 19 Bioanalyser size and concentration results. The software shows the peaks produced by the samples, their respective size and concentration.

Peak		Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	◀	35	125,00	5.411,3	Lower Marker
2		72	45,64	957,9	
3		81	20,19	377,8	
4		95	54,19	861,3	
5		101	76,93	1.153,1	
6		127	190,00	2.260,5	
7		148	10.626,70	108.869,8	
8		211	17,19	123,3	
9		231	47,79	314,0	
10	▶	10.380	75,00	10,9	Upper Marker
11		11.792	0,00	0,0	

4.4. Next–Generation Sequencing Data

4.4.1. Technical Next–Generation Sequencing Quality

To assess the technical quality of the NGS run the raw data was processed and the mapping statistics and length distribution were assessed for all three cell lines.

The mapping statistics, shown in **Figure 15**, present the relative contributions of each RNA type on the samples. For the cell line MDA–MB–231, 29 % of the reads were miRNA; 27 % for MDA–MB–436, and 20 % for MDA–MB–468.

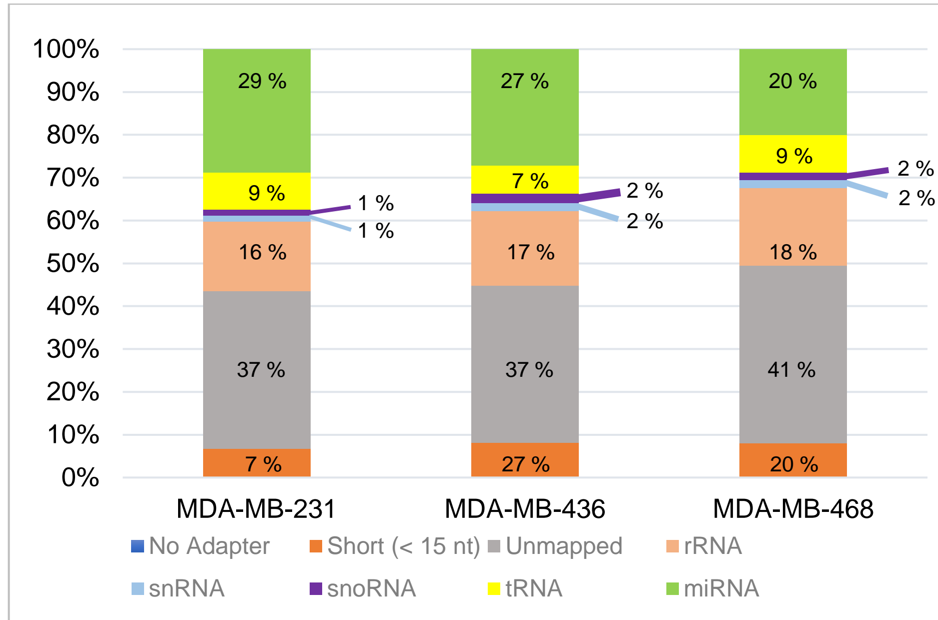


Figure 15 Relative distribution of RNA types of the NGS data.

Length distribution for the three cell lines were then evaluated to determine the number of reads present corresponding to miRNA in size (**Figure 16).**

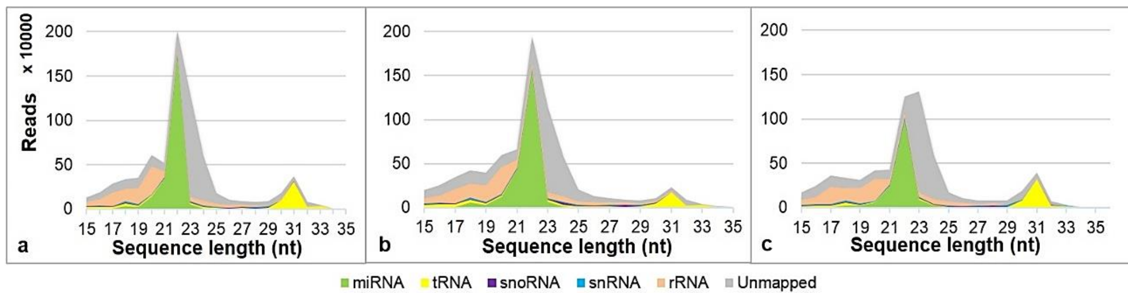


Figure 16 Relative length distribution of RNA types in cell line (a) MDA–MB–231, (b) MDA–MB–436, and (c) MDA–MB–468.

Phred score was calculated to determine the quality of the generated reads. A value of 30 represents an error probability of 0.1 % of having a false base call. The higher the Phred score, the lower is the error probability. In **Figure 17** the per base Phred scores for all three cell lines with the respective standard deviation are shown. Since miRNA varies from 19 to 25 nt in length, it was sufficient to verify the Phred score for the first 30 nt. The graphic clearly depicts that all the samples showed a score of 35 or higher for the first 30 base positions.

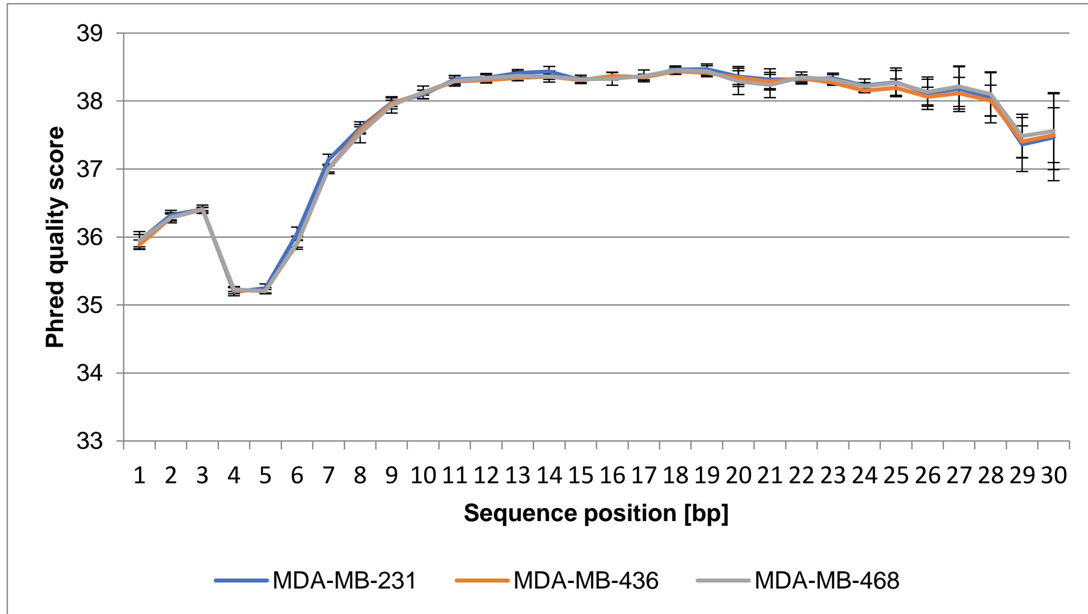


Figure 17 Phred score results. The quality of the nucleobase identification is shown. A Phred score of 30 equals an error probability of 0.1 %.

A principal component analysis (PCA) was performed to investigate the main components influencing the data (**Figures 18a and b**).

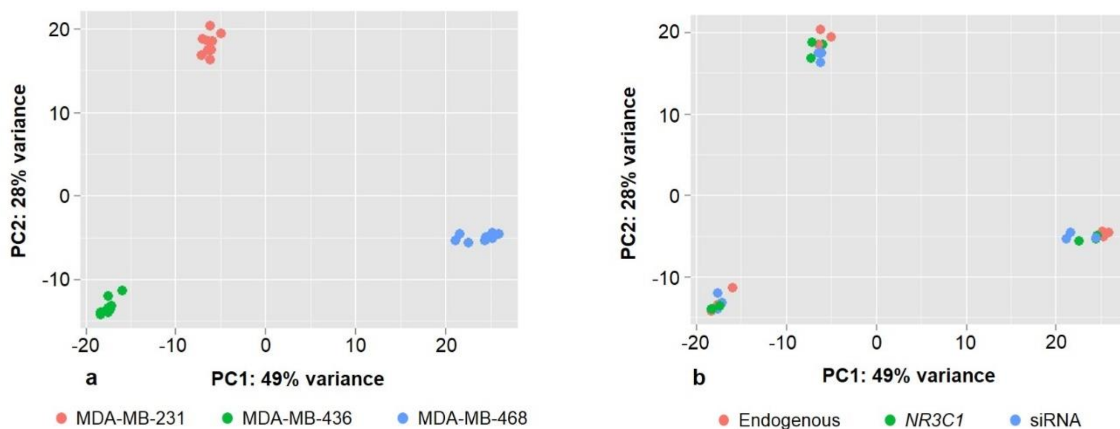


Figure 18 PCA results grouped by a) cell line, and b) experimental conditions. In both, the samples cluster according to the cell line.

In **Figure 18a)** data was organized according to the cell line, where each cell line clusters individually. In **Figure 18b)** samples were ordered by experimental conditions (parental with endogenous GR expression, GR overexpression, and siRNA silencing GR expression). The clustering was not found to be condition–related, but cell line–related. This means that the main factor influencing the NGS results was the cell line.

4.4.2. Glucocorticoid Receptor–associated microRNAs

The bioinformatic pipeline described in 3.2.9. was then applied, relying on DESeq package to give an output of miRNAs that were regulated by GR. The outcome featured 7 miRNAs regulated by GR, described in **Table 20.**

Table 20 GR–regulated miRNAs in TNBC, displaying fold changes (FC) of the *NR3C1* overexpression in comparison with the endogenous condition.

miRNA	Regulation	*p*–value	Log2FC	FC	Cell line
miR–221–5p	Upregulation	0.0009	1.130	2.189	MDA–MB–231
miR–576–3p	Upregulation	0.0071	1.107	2.154	MDA–MB–231
let–7b–3p	Downregulation	0.0118	–1.097	0.467	MDA–MB–231
miR–203a–3p	Upregulation	0.0301	1.348	2.546	MDA–MB–436
miR–4746–5p	Downregulation	0.0444	–1.074	0.475	MDA–MB–436
miR–1260a	Downregulation	0.0001	–1.535	0.345	MDA–MB–468
miR–1260b	Downregulation	0.0003	–1.535	0.345	MDA–MB–468

For the cell line MDA–MB–231, three miRNAs regulated by GR were found – two of them upregulated: miR–221–5p and miR–576–3p; one, let–7b–3p, downregulated. Both upregulated miRNAs were expressed around two times more than the endogenous condition, with a *p*–value of 0.001 and 0.007, respectively. The downregulated let–7b–3p had a *p*–value of 0.012 and a fold change value approximate of 0.5, meaning that this miRNA had half of the expression compared to the parental condition.

The cell line MDA–MB–436 exhibited two miRNAs, miR–203a–3p upregulated, and miR–4746–5p downregulated. miR–203a–3p had a *p*–value of 0.03 with a fold change value close to 2.5, while miR–4746–5p expression was reduced by more than half, displaying a *p*–value of 0.044.

For MDA–MB–468, both miRNAs were downregulated in TNBC. miR–1260a and miR–1260b exhibited equal fold change values of around 0.3, and *p*–values of 0.0001 and 0.0003, respectively.

4.5. Quantitative PCR Validation

4.5.1. Reference microRNAs

To validate the seven miRNAs from the NGS data analysis, stably expressed miRNAs serving as reference miRNAs needed to be identified.

The reference miRNAs were selected from the available NGS data, by running the GenEx Professional software. The program selects the miRNA(s) that is(are) the most stably expressed. The selected miRNAs can be different for each cell line, or the same for all of them. As described in 3.2.9. the package runs two different algorithms, GeNorm and NormFinder, and the reference miRNAs are designated by taking both algorithms into account. To verify the consistency of the results, analysis were performed for a BaseMean ≥ 50 cut off. The most stable ones from the list were selected. GeNorm and NormFinder results for identifying suitable candidate reference miRNAs can be found in chapter 8.2.

Before qPCR was performed, 4 miRNAs had been selected as candidates for normalization: let–7a–5p, miR–24–3p, miR–25–3p and miR–148b–3p.

4.5.2. Validation of microRNAs from Next–Generation Sequencing

After qPCR, GeNorm and NormFinder algorithms were re–ran, and the 3 most stably expressed reference miRNA for each cell line were selected. For cell line MDA–MB–231, miR–221–5p, miR–25–3p and let–7b–5p were selected. For MDA–MB–436, miR–203a–3p, miR–24–3p and miR–25–3p were chosen, while for MDA–MB–468 miR–148b–3p and let–7a–5p were nominated.

For the validation of the 7 miRNAs the samples from those and the reference miRNAs were subjected to a qPCR analysis according to the established protocol in 3.2.10.

After the calculation of ΔΔCt values and confirmation by a Student's *t*–test, two miRNAs could be validated: miR–203a–3p and miR–1260a, from cell lines MDA–MB–436 and MDA–MB–468, correspondingly. Fold change and *t*–test results can be found in **Table 21**. qPCR results for all the seven miRNAs can be found in chapter 8.3.

Table 21 Validated miRNAs.

miRNA	Fold change	*p*–value	Cell line
miR–203a–3p	1.5060	0.00003	MDA–MB–436
miR–1260a	0.7672	0.00044	MDA–MB–468

5. Discussion

Multiple miRNAs have already been pointed out as being TNBC–associated, and suggested to be relevant in the pathways that may produce the aggressive outcomes of this BC subtype. Chen *et al.* (2015) found that a GR overexpression was associated with poor survival rate in TNBC. Further highlighting GR importance in TNBC, it has also been reported that around 25 % of TNBC cases are GR–positive. Several studies argument that the dysregulation of miRNAs can trigger BC initiation and progression (Andorfer *et al.* 2011). Given that, the objective of this study was to investigate if and if so how GR, miRNA and TNBC are linked, by identifying GR–regulated miRNAs in TNBC.

From our NGS results, seven miRNAs were found to be regulated under GR overexpression: the upregulated miR–221–5p, miR–576–3p, and miR–203a–3p, and the downregulated let–7b–3p, miR–4746–5p, and miR–1260a/b. Two, miR–203a–3p and miR–1260a could be further validated by RT–qPCR.

Diverse biological functions have been predicted and discovered related to oncogenic pathways in BC, including in the heterogenous TNBC subtype. Pan *et al.* (2011) stated that GR signaling may trigger antiapoptotic pathways, and that those paths could be associated with poorer prognosis in ER–negative patients, which include TNBC patients. Besides, a high expression of GR was also associated with an increased risk of early relapse. In conjunction with the miRNAs influenced by GR, new insights on TNBC aggressiveness and lack of an effective treatment can be portrayed (Pan *et al.* 2011).

miR–221–5p, previously known as miR–221*, has been reported as an oncogenic factor by several research groups. When upregulated, miR–221–5p targeted cell cycle inhibitors, which have a major role in preventing the progression of cell cycle and consequently preventing tumor formation (Miller *et al.* 2008; Nassirpour *et al.* 2013; Thakur *et al.* 2016).

Its overexpression has also been found to directly regulate a protein isoform with a role in tissue organization (uPAR2), by Falkenberg *et al.* (2013 and 2015), thus increasing cell invasion and metastasis. miR–221–5p targeted this isoform, upregulating it, which caused the degradation of the extracellular matrix. Besides, the group also linked this miRNA with metastasis provoked by EMT processes. When upregulated in the cell line MDA–MB–231, miR–221–5p lead to EMT, by targeting the EMT regulator gene *PTEN*. Pan *et al.* (2016) associated this miRNA with E–cadherin

expression levels, and consequently with EMT, in the same cell line. They suggested that the upregulation of *SLUG* positively regulated miR–221–5p expression, which in turn decreased E–cadherin protein level, thus promoting cellular progression (Falkenberg *et al.* 2013; Falkenberg *et al.* 2015; Pan *et al.* 2016).

Similarly, upregulation of miR–221–5p was reported to be involved in the transformation of normal fibroblasts into myofibroblasts. Myofibroblasts, known to repair tissues during wound healing, can disrupt organ function when protein secretion is excessive (Hinz *et al.* 2007). They have been associated with poor overall survival by Liu *et al.* (2016b). Thus, the association of miR–221–5p with fibroblast transformation provides one explanation for the poor prognosis and survival rate observed in TNBC.

Therefore, several studies have already described this miRNA as important in TNBC when upregulated. This is in line with our findings. Though not validated by qPCR, miR–221–5p was upregulated in the NGS results in the cell line MDA–MB–231. These findings suggest that this miRNA might impact cell invasion, cell progression, and metastasis in MDA–MB–231 cell line, so we can infer that miR–221–5p may be responsible for those worse outcomes associated with TNBC.

Concerning miR–576–3p, in bladder cancer, Meng *et al.* (2017) linked miR–576–3p downregulation to poor clinical outcome. On the contrary, Liang *et al.* (2015) had previously stated for the same cancer type that when overexpressed, miR–576–3p inhibited repression of cell proliferation through targeting cyclin D1. These results are inconsistent, as both suggest that tumor formation can be observed equally under up– and downregulation of miR–576–3p.

When regarding BC, various studies reported its role in affecting functional pathways of cyclins, as well as in chemoresistance. It has also been shown that its expression is dysregulated in patients expressing *BRCA1* gene mutation. Lv *et al.* (2014) investigated the role of chemoresistance in BC patients, which can, among other factors, also be triggered by miRNA expression. Their results showed a downregulation of miR–576–3p in the cell line MCF–7, a luminal A BC cell line. Since they found this miRNA to be downregulated in both MCF–7 BC cells and in chemoresistant tissues, they acknowledged its downregulation might be associated with chemoresistance and thus with poor prognosis (Lv *et al.* 2014).

On the contrary, an upregulation of miR–576–3p was reported by Yan *et al.* (2015) in two TNBC (MDA–MB–231 and MDA–MB–468), and two luminal A (MDA–MB–453 and MCF–7) cell lines with *BRCA1* mutated gene. They pointed out that an upregulation of miR–576–3p suppressed cyclin D1 translation. Cyclin D1 is known to phosphorylate *BRCA1*, thus inhibiting *BRCA1* DNA–dependent activities. The observed

influence of miR–576–3p on cyclin D1 regulation is similar to that of bladder cancer found by Liang *et al.* (2015) above.

Some of the studies do not corroborate ours, as we found this miRNA to be upregulated in the NGS results in cell line MDA–MB–231. Even though this cell line has been used by other research groups, no clear association with TNBC has been described except for the work by Yan *et al.* (2015). However, we should have in mind that this miRNA could not be validated by qPCR in our study, and that those different outcomes may be due to different assays used to evaluate miRNA expression. As an example, qPCR was used for validating the miRNA results by Yan *et al.* (2015), but they studied miRNAs by beadchips technique. The different biological sources can also be an influence for the diverse outputs, since we used cell lines while some groups such as Meng *et al.* (2017) analyzed tumor tissues.

Regarding let–7b–3p, a miRNA that belongs to the let–7 family of miRNAs, its downregulation has been connected to diverse cancers, including BC. It has been linked to functions such as cellular progression, inflammation, and cancer growth (Iliopoulos *et al.* 2009; Spolverini *et al.* 2017).

Spolverini *et al.* (2017) correlated let–7b–3p to cell migration and progression in cancer–derived cells. By targeting components of the histone machinery, an overexpression of the miRNA upregulated histone H2B ubiquitylation, consequently suppressing cell progression (Spolverini *et al.* 2017).

Iliopoulos *et al.* (2009) described the role of let–7b–3p in inflammation and transformation in BC. By evaluating gene expression of a modified non–tumorigenic human breast cell line, MCF–10A, expressing a kinase oncoprotein, they found that the activation of the oncoprotein initiated the activity of NF–κB, a TF that downregulated let–7b–3p, leading to cancer growth (Iliopoulos *et al.* 2009).

Another study reported let–7b–3p downregulation in metastatic BC, as well. Further, it could be shown that let–7b–3p reduced E–cadherin expression level, thus activating EMT processes (Zhou *et al.* 2017).

Our NGS results demonstrated a downregulation of let–7b–3p in the cell line MDA–MB–231. Most of the described studies corroborate our findings. Nonetheless, if the pathway defined by those groups could likewise be observed in TNBC cell lines, they could be a reason for TNBC aggressiveness. As EMT is a well–known tumorigenic factor, this miRNA–signaling cascade may give a reasoned explanation for BC metastasis.

In terms of miR–4746–5p expression in TNBC, no report could be found in the literature. Regarding BC, Camps *et al.* (2014) described that this miRNA was

upregulated under hypoxia in the cell line MCF–7, suggesting that it may be involved in angiogenesis and apoptosis pathways, in which hypoxia plays an important role (Camps *et al.* 2014).

Our NGS results displayed a downregulation of miR–4746–5p in the cell line MDA–MB–436. This finding has not yet been associated with GR or TNBC in the literature. Despite the fact that its expression was not validated by qPCR, miR–4746–5p downregulation in our project may indicate a tumor suppressor activity of this miRNA, which is regulated by GR.

In terms of miR–1260a/b, few information related to BC has been described in the literature. Camps *et al.* (2014) found miR–1260a and miR–1260b to be downregulated in breast cancer cell line MCF–7 under hypoxia, which is associated with an increased risk of metastasis and mortality. Cascione *et al.* (2013) investigated mRNA and miRNA signatures in normal, TNBC and metastatic tumors, and concluded that downregulation of miR–1260a could contribute to the aggressiveness of TNBC by promoting metastasis via upregulation of collagen 1A1. On the other hand, Park *et al.* (2014) found an upregulation of miR–1260a in blood samples of luminal A BC patients when describing a panel of miRNA biomarkers (Cascione *et al.* 2013; Camps *et al.* 2014; Park *et al.* 2014).

The dysregulation of miR–1260a/b could be observed in other tumor entities as well. In skin cancer, for instance, Sand *et al.* (2013) found an upregulation of miR–1260a, however no functional statement was provided. An upregulation was also found in hepatocellular carcinoma cell lines, after treatment with the chemotherapeutic agent taxol. The upregulated miR–1260a targeted cyclin D1, giving rise to worse outcomes (Yan *et al.* 2013). Regarding miR–1260b, Xu *et al.* (2015) described the association of an overexpressed miR–1260b with lymph nodes metastasis in non–small cell lung cancer. Its overexpression was defined to be associated with cancer development and metastasis. The same expression direction was found in colorectal cancer by Liu *et al.* (2016a), who correlated miR–1260b with lymph node metastasis and invasion, and consequently with the poor prognosis of the disease (Sand *et al.* 2013; Yan *et al.* 2013; Xu *et al.* 2015; Liu *et al.* 2016a).

On the other hand, Hirata *et al.* (2013) associated the downregulation of miR–1260b with the inhibition of a signal transduction pathway responsible for cell fate determination and cell migration in renal cancer (Hirata *et al.* 2013).

Taken together, diverse results have been reported for miR–1260a/b. For instance, Camps *et al.* (2014) found a downregulation of miR–1260a in MCF–7 cell line, while Park *et al.* (2014) registered an upregulation of the same miRNA in blood

samples. Specifically in TNBC, the downregulation of miR–1260a could be a reason for TNBC aggressiveness, since the miRNA leads to an upregulation of collagen 1A1, promoting metastasis. About miR–1260b, metastasis and cellular invasion was seen as a consequence of miR–1260b overexpression in lung and colorectal cancer (Xu *et al.* 2015; Liu *et al.* 2016a), whereas an opposite expression direction was reported to lead to cell migration in renal cancer (Hirata *et al.* 2013), and in hypoxic BC cells (Camps *et al.* 2014).

Concerning miR–203a–3p, it has been associated with tumor formation, cellular proliferation and metastasis in BC (Ding *et al.* 2013; Gomes *et al.* 2016). In TNBC, Ding *et al.* (2013), studying metastasis mechanisms, reported that in the cell lines MDA–MB–231 and MDA–MB–468, this miRNA was downregulated. They found that a transforming growth factor activated a TF, which in turn repressed miR–203a–3p. This suppression would then lead to the activation of EMT pathways, promoting tumor metastasis. Equally, Zhang *et al.* (2011) also reported a downregulation of this miRNA in TNBC cell line MDA–MB–231, associating it with TNBC aggressiveness.

Le *et al.* (2016) investigated cellular shape and matrix adhesion in the cell line MDA–MB–231. As extracellular matrix stiffness is associated with tumor formation, the group studied its effects in TNBC. They found that the augmentation of extracellular matrix stiffness lead to a downregulation of miR–203a–3p, which in turn upregulated the expression level of a protein coding gene responsible for mediating responses to cell migration signals (Le *et al.* 2016).

Gomes *et al.* (2016) investigated a Portuguese cohort, and described the miRNA as overexpressed in tumor tissues. They hypothesized that its upregulation may perform a defensive role in cell proliferation and invasiveness, since when downregulated, miR–203a–3p enhances a proto–metastatic gene expression, consequently increasing cell proliferation and metastasis (Gomes *et al.* 2016).

In meta–analysis studies, Liang *et al.* (2016) and Shao *et al.* (2017) found that an overexpression of this miRNA was associated with poor overall survival in BC patients. In addition, Liang *et al.* (2016) correlated miR–203a–3p with patients ethnicity. They described that this poor overall survival was characteristic of Caucasian patients, but that in Asian patients, the outcome was improved with miR–203a–3p upregulation (Liang *et al.* 2016; Shao *et al.* 2017).

An upregulation was also defined by Feng *et al.* (2014) when comparing TNBC cell lines MDA–MB–231 and BT–549 with two luminal BC cell lines, MCF–7 and BT474. Interestingly, they perceived that miR–203a–3p targeted a gene encoding a protein activator, *RASAL2*, which is oncogenic in TNBC (Feng *et al.* 2014).

Similar results were reported by Fite and Gomez-Cambronero (2015) while studying the invasiveness properties of the enzyme phospholipase D in the cell line MDA–MB–231. They found that an overexpression of this enzyme increased invasiveness of the cells, but that an overexpression of the miRNA suppressed the enzyme activity, thus decreasing the invasively aggressive properties (Fite & Gomez-Cambronero 2015).

In the cell line MCF–7, Zhao *et al.* (2015) found that an overexpression of miR–203a–3p blocked cell growth and invasion by suppressing cell cycle activator cyclin D2 when compared with normal breast tissue. They also pointed out the role of this miRNA in metastasis cascades, since they observed that low levels of cyclin D2 enhanced cell–cycle suppressor p21 and p27, consequently increasing protein Bcl–2 expression level, which is associated with apoptosis (Zhao *et al.* 2015).

An association with cell proliferation and cell migration was not only found by Zhao *et al.* (2015) but also stated by Wang *et al.* (2012). When comparing miRNA expression profiles between TNBC cell lines MDA–MB–231 and MDA–MB–468 with a normal breast cell line, MCF–10A, they observed that miR–203a–3p overexpression lead to a decrease in BIRC5 and LASP1 genes, which are involved in cell proliferation and migration pathways (Wang *et al.* 2012).

Opposing results were found when He *et al.* (2016) evaluated cell proliferation patterns. They found an upregulation of miR–203a–3p in breast cancer tissues and, by knocking down miR–203a–3p, the expression of a growth factor decreased, thus inhibiting cell growth. Additionally, Ru *et al.* (2011) reported similar results to those of He *et al.* (2016). They stated that downregulation of miR–203a–3p in MCF–7 cell line combined with cisplatin treatment would enhance apoptotic cell death (Ru *et al.* 2011; He *et al.* 2016).

The conflict of results found in the literature may be a consequence of the heterogeneity of BC. According to the literature, two functions of miR–203a–3p are discussed: 1. miR–203a–3p is a tumor–suppressor miRNA that can be down– or upregulated depending on the BC type and cancer stage; 2. miR–203a–3p is an oncogenic miRNA. In our experimental results, miR–203a–3p was upregulated in the cell line MDA–MB–436, both in NGS and qPCR analysis. Consequently, the observed upregulation found in MDA–MB–436 in our study could either be a defense mechanism as stated by Gomes *et al.* (2016) or an oncogenic factor adding up to the aggressiveness of some BC subtypes as stated by Ru *et al.* (2011) and He *et al.* (2016).

6. Conclusion

TNBC, a heterogeneous and very aggressive BC subtype, characterized by its negative profile of ER, PR and HER2 receptors, exhibits high research and social importance due to the lack of an effective treatment.

As high GR expression was linked to poorer survival rates in TNBC patients, and it has been reported that about 25 % of TNBC cases are GR–positive, we aimed at identifying cellular miRNAs as GR–regulated factors in TNBC.

According to our NGS data, seven miRNA were significantly regulated by GR in TNBC, three of them upregulated (miR–221–5p, miR–576–3p, and miR–203a–3p), and four downregulated (let–7b–3p, miR–4746–5p, miR–1260a, and miR–1260b). Two miRNAs, miR–203a–3p and miR–1260a, could be further validated by RT–qPCR.

Consequently, our results show that there are indeed miRNAs regulated by GR in TNBC, of which some corroborate previous findings of associations with activation of oncogenic pathways, while others do not. Interestingly, from all seven GR–regulated miRNAs, none was found to be regulated in all of the three studied cell lines.

We speculate that the differences of the miRNA's regulation might be due to the fact that each cell line may belong to a specific TNBC subclass, as stated in chapter 1.1.4., and each class may show a unique miRNA pattern under GR–overexpression. Furthermore, our findings strengthen the assumption that miRNA expression in TNBC is subject to a complex regulation.

In light of our findings, further research is needed to unveil the exact functions of the miRNAs identified in this study on gene expression and cellular pathways to eventually develop effective therapies for patients affected by this aggressive BC subtype.

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8. Annexes

8.1. Table A1: miRNAs Average Size and Concentration

Sample	% of total cDNA	Average Size [bp]	miRNA concentration [ng/ul]
MDA–MB–231 R1 parental	8	142	2,35
MDA–MB–231 R2 parental	8	141	4,12
MDA–MB–231 R3 parental	6	143	2,15
MDA–MB–231 R1 NR3C1	9	143	4,41
MDA–MB–231 R2 NR3C1	10	144	7,17
MDA–MB–231 R3 NR3C1	9	143	3,03
MDA–MB–231 R1 siRNA	8	142	3,97
MDA–MB–231 R2 siRNA	9	144	4,7
MDA–MB–231 R3 siRNA	11	144	3,9
MDA–MB–468 R1 parental	7	141	4,64
MDA–MB–468 R2 parental	5	141	2,16
MDA–MB–468 R3 parental	5	143	3,09
MDA–MB–468 R1 NR3C1	9	143	4,48
MDA–MB–468 R2 NR3C1	8	143	6,24
MDA–MB–468 R3 NR3C1	7	143	4,02
MDA–MB–468 R1 siRNA	11	141	7,25
MDA–MB–468 R2 siRNA	7	144	6,37
MDA–MB–468 R3 siRNA	5	142	2,76
MDA–MB–436 R1 parental	8	142	4,42
MDA–MB–436 R2 parental	7	143	2,63
MDA–MB–436 R3 parental	6	142	1,45
MDA–MB–436 R1 NR3C1	7	142	3,7
MDA–MB–436 R2 NR3C1	8	143	4,46
MDA–MB–436 R3 NR3C1	7	142	7,14
MDA–MB–436 R1 siRNA	8	142	4,27
MDA–MB–436 R2 siRNA	8	143	3,67
MDA–MB–436 R3 siRNA	6	142	2,49

8.2. Table A2: Reference miRNA Output of GenEx Professional Software

Cell line	GeNorm – BaseMean ≥ 50		NormFinder – BaseMean ≥ 50	
	miRNA	***M-value***	***miRNA***	***SD***
MDA–MB–231	let–7a–5p	0.0693	miR–148b–3p	0.0551
	let–7c–5p	0.0693	miR–25–3p	0.0559
	let–7b–5p	0.1011	let–7a–5p	0.1189
	let–7i–5p	0.1116	let–7f–5p	0.1272
	miR–148b–3p	0.1251	let–7c–5p	0.1334
	miR–25–3p	0.1324	miR–218–5p	0.1362
	miR–9–5p	0.1420	let–7b–5p	0.1438
	miR–625–3p	0.1469	miR–378a–3p	0.1454
	miR–30c–2–3p	0.1514	miR–9–5p	0.1513
	miR–378a–3p	0.1571	miR–26a–5p	0.1524
MDA–MB–436	miR–24–3p	0.0700	miR–25–3p	0.1079
	miR–28–5p	0.0800	miR–589–5p	0.1085
	miR–503–5p	0.1064	let–7b–5p	0.1299
	miR–25–3p	0.1119	miR–196b–5p	0.1315
	miR–196b–5p	0.1145	miR–24–3p	0.1319
	let–7g–5p	0.1187	miR–126–3p	0.1381
	miR–193a–5p	0.1254	miR–28–5p	0.1412
	miR–340–5p	0.1351	miR–193a–5p	0.1418
	miR–185–3p	0.1435	miR–98–5p	0.1433
	miR–22–3p	0.1556	miR–96–5p	0.1475
MDA–MB–468	let–7a–5p	0.0979	miR–25–3p	0.0529
	let–7c–5p	0.0979	let–7e–5p	0.0974
	miR–192–5p	0.1112	miR–149–5p	0.1150
	miR–25–3p	0.1188	let–7a–5p	0.1275
	miR–374a–3p	0.1292	miR–26a–5p	0.1287
	miR–149–5p	0.1372	miR–192–5p	0.1435
	let–7e–5p	0.1412	let–7d–5p	0.1451
	miR–126–3p	0.1477	let–7c–5p	0.1461
	miR–320a	0.1537	miR–126–3p	0.1463
	miR–7–5p	0.1604	miR–374a–3p	0.1501

Legend: blue, candidate reference miRNAs.

8.3. Table A3: qPCR Validation Results for the Seven Dysregulated miRNAs

miRNA	Fold change	*p*–value	Cell line
miR–221–5p	0.7829	0,00467	MDA–MB–231
miR–576–3p	1.1925	0,31974	MDA–MB–231
let–7b–3p	1.1995	0,01698	MDA–MB–231
miR–203a–3p	1.5060	0.00003	MDA–MB–436
miR–4746–5p	1.3524	0,00304	MDA–MB–436
miR–1260a	0.7673	0,00044	MDA–MB–468
miR–1260b	1.1329	0,28884	MDA–MB–468

8.4. Abstract for the XLI Jornadas Portuguesas de Genética Poster Presentation

Glucocorticoid receptor regulates specific microRNAs in triple-negative breast cancer

Ricardo González[1,2*], Dominik Buschmann[2,3], Benedikt Kirchner[3,4], Michael W Pfaffl[3], Gustav Schelling[5], Ortrud Steinlein[2], Marlene Reithmair[2]

[1]Departmento de Biologia, Faculdade de Ciências da Universidade do Porto, Universidade do Porto, Rua Campo Alegre, 823, 4150-170 Porto, Portugal.
[2]Institute of Human Genetics, University Hospital, Ludwig-Maximilians-University Munich, Germany
[3]Division of Animal Physiology and Immunology, TUM School of Life Sciences Weihenstephan, Technical University Munich, Munich, Germany
[4]Dr. von Hauner Childrens's Hospital, Ludwig Maximillian University, Munich, Germany
[5]Department of Anesthesiology, University Hospital, Ludwig-Maximilians-University, Munich, Germany

*up201502177@fc.up.pt

Breast cancer (BC) is the most prevalent type of cancer in women and leads to high mortality rates [1].
In particular, triple-negative breast cancer (TNBC) is known as a heterogeneous and very aggressive BC subtype, characterized by its negative profile of progesterone receptor (PR), estrogen receptor (ER), and human epidermal growth factor receptor 2 (HER2). These features are the main reason why there is still no effective treatment available.
Glucocorticoids (GCs) are a group of corticosteroid hormones that act by binding to glucocorticoid receptors (GRs). GRs are crucial transcriptional factors involved in gene regulation. High GR expression in TNBC was recently linked to poorer survival rates in TNBC patients [2]. It is known that GRs are not only capable of influencing the expression of protein coding genes but also modulate microRNA (miRNAs) expression. MiRNA are small noncoding elements that likewise regulate gene expression. The initiation and progression of BC are associated with miRNA dysregulation, which can either act as oncogenic or tumor suppressor factors [3].
To broaden the knowledge in this research field, the project aimed to identify cellular miRNAs regulated by GR in TNBC.
Experimental procedures included: cell culture of three TNBC cell lines in three different conditions (endogenous GR expression; transfected with a *NR3C1* plasmid, encoding the GR; transfected with silencing RNA (siRNA), silencing endogenous *NR3C1* gene expression); isolation of RNA, including quality control, and quantification; preparation of a library for Next-Generation Sequencing (NGS); bioinformatics analysis of NGS data.
Seven miRNAs were found to be significantly regulated by GR in TNBC. In MDA-MB-231: upregulation of miR-576-3p and miR-221-5p, downregulation of let-7b-3p. In

MDA-MB-436: upregulation of miR-203-3p, downregulation of miR-4746-5p. In MDA-MB-468: downregulation of miR-1260a and b.
We conclude that there are indeed miRNAs regulated by GR in TNBC, of which some corroborate previous findings of associations with activation of oncogenic pathways. Our results further indicate that GR-regulated miRNA expression may be TNBC subtype specific.

In light of our findings, further research is needed to unveil the exact functions of these miRNAs on gene expression and cellular pathways in order to eventually develop effective therapeutics for patients affected by this aggressive BC subtype.

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