MICRORNA INVOLVEMENT IN BREAST CANCER SUSCEPTIBILITY AND PROGRESSION

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Tese para obtenção do grau de Doutor em Ciências da Vida
na Especialidade em Genética, Oncologia e Toxicologia Humana
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e José Rueff, Professor Catedrático

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Silva S.N., Tomar M., Paulo C., **Gomes B.C.**, Azevedo A.P., Teixeira V., Pina J.E., Rueff, J., Gaspar J.F. (2010) Breast cancer risk and common single nucleotide polymorphisms in homologous recombination DNA repair pathway genes XRCC2, XRCC3, NBS1 and RAD51. Cancer Epidemiol.; 34(1):85-92

Anunciação, O. **Gomes, B.C**., Vinga, S., Gaspar, J., Oliveira, A.L. Rueff, J. (2010) A Data Mining Approach for the Detection of High-Risk Breast Cancer Groups. In Advances in Bioinformatics. (M.P. Rocha et als. Eds), Volume 74: pp 43-51. Springer-Verlag Berlin Heidelberg. ISBN 978-3-642-13213-1, DOI 10.1007/978-3-642-13214-8

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Gomes, B.C., Santos, B., Martins, M., Lopes, L., Morujão, I., Oliveira, M., Araújo, A., Rueff, J., and Rodrigues, A. S. miR-200c and miR-203 misexpression in a Portuguese breast cancer population and their association with clinicopathological characteristics. 18th meeting of the Sociedade Portuguesa de Genética Humana. November 2014, Lisboan, Portugal.

Santos, B., **Gomes, B.C.**, Martins, M., Lopes, L., Morujão, I., Oliveira, M., Araújo, A., Rueff, J., and Rodrigues, A. S. Immunohistochemistry detection of putative miR-200c and miR-203 Targets in Breast Cancer Patients. 18th meeting of the Sociedade Portuguesa de Genética Humana. November 2014, Lisboan, Portugal.

Rodrigues, A.S., Gromicho, M., **Gomes, B.C.**, Rueff, J. Methylation status of selected microRNAs in a Chronic Myeloid Leukaemia cell line (K562) and their relation with therapy response. 17th ECCO – 38th ESMO – 32nd ESTRO European Cancer Congress. 27th September – 1st October 2013, Amsterdam, Netherlands.

Gromicho, M., Rodrigues, A.S., **Gomes, B.C.**, Rueff, J. Epigenetic alterations of microRNAs 124-1 and 200c during acquired resistance to Imatinib in K562 CML cells. 15th International Conference on Chronic Myeloid Leukemia: Biology and Therapy. 26th – 29th September 2013, Estoril, Portugal.

Gomes, B.C., Rueff, J., Rodrigues, A.S. Epigenetic Regulation of microRNAs Expression in Breast Cancer Cell Lines. XXXVII Jornadas Portuguesas de Genética. 28th-30th May 2012, Lisbon, Portugal.

Gomes, B.C., Azevedo, A.P., Rueff, J., Rodrigues, A.S. Profiling of microRNA expression in breast cell lines of different tumourigenicity. 15th Meeting of the Sociedade Portuguesa de Genética Humana - SPGH. 10th-12th Novembro 2011, Lisbon, Portugal.

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Para os meus Pais

"– My life amounts to no more than one drop in a limitless ocean. Yet what is any ocean, but a multitude of drops?"

– David Mitchell in Cloud Atlas.

Agradecimentos

É minha opinião que um doutoramento é muito mais do que obter um grau. É também um amadurecimento psicológico e social. Durante o tempo decorrente de um doutoramento são muitas as pessoas que chegam, que partem, que ficam. Que nos fazem crescer que nos fazem perceber que a vida nem sempre é como idealizámos, pois precisamos que nos abram os horizontes para perceber o que realmente interessa, o que é a vida.

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List of abbreviations, genes, proteins and chemicals

All genes names are denoted in accordance with HUGO Gene Nomenclature Committee. All protein names are denoted in accordance with The Universal Protein Resource UniProt.

3'UTR	3'- untranslated region
4-OHT	4-hydroxytamoxifen
5-FU	5-fluorouracil
ABC	ATP-binding cassette
ABCB1	ATP binding cassette subfamily B member 1
ABCC1	ATP binding cassette subfamily C member 1
ABCC2	ATP binding cassette subfamily C member 2
ABCC5	ATP binding cassette subfamily C member 5
ABCF2	ATP-binding cassette sub-family F member 2
ABCG2	ATP binding cassette subfamily G member 2
ABHD10	Mycophenolic acid acyl-glucuronide esterase, mitochondrial
ABI	Applied Biosystems Instruments
ACBD3	Golgi resident protein GCP60
ACTB	Actin, beta
ACYP1	Acylphosphatase-1
ADCK3	Atypical kinase ADCK3, mitochondrial
ADME	Absorption, Distribution, Metabolism and Excretion
ADPGK	ADP-dependent glucokinase
AGO	Argonaute
AGO2	Argonaute 2, RISC catalytic component
AIB1/NCOA3	Nuclear receptor coactivator 3
AIP	AH receptor-interacting protein
AKAP12	A-kinase anchor protein 12
AKR1C2	Aldo-keto reductase family 1 member C2
AKR1D1	3-oxo-5-beta-steroid 4-dehydrogenase
AKT	V-akt murine thymoma viral oncogene homolog 1
ALKBH2	AlkB homolog 2, alpha-ketoglutarate-dependent dioxygenase
ALKBH3	AlkB homolog 3, alpha-ketoglutarate-dependent dioxygenase
ANKRD17	Ankyrin repeat domain-containing protein 17
ANOVA	Analysis of variance
ANXA1	Annexin A1

APEH	Acylamino-acid-releasing enzyme
APOBEC3C	DNA dC->dU-editing enzyme APOBEC-3C
ARAP1	Arf-GAP with Rho-GAP domain, ANK repeat and PH domain-containing protein 1
ARF6	ADP-ribosylation factor 6
ARFIP2	Arfaptin-2
ARFRP1	ADP-ribosylation factor-related protein 1
ARMT1	Protein-glutamate O-methyltransferase
ARPC5L	Actin-related protein 2/3 complex subunit 5-like protein
ASCO/CAP	American Society of Clinical Oncology/College of American Pathologists
ASL	Argininosuccinate lyase
ASPDH	Putative L-aspartate dehydrogenase
ATL2	Atlastin-2
ATM	Ataxia telangiectasia mutated, serine/threonine kinase
ATP	Adenosine triphosphate
ATP2B4	Plasma membrane calcium-transporting ATPase 4
ATP6V1B2	V-type proton ATPase subunit B, brain isoform
ATP6V1G1	V-type proton ATPase subunit G 1
ATP6V1H	V-type proton ATPase subunit H
ATXN10	Ataxin-10
B2M	Beta-2-microglobulin
BAG2	BAG family molecular chaperone regulator 2
BAK1	BCL2 antagonist/killer 1
BC	Breast cancer
BCAP29	B-cell receptor-associated protein 29
BCAS2	Pre-mRNA-splicing factor SPF27
BCL2	B-cell CLL/lymphoma 2
BCR-ABL	Breakpoint cluster region/ABL proto-oncogene 1, non-receptor tyrosine kinase
BER	Base excision repair
BIRC5	Baculoviral IAP repeat containing 5
BMI1	B cell-specific Moloney murine leukaemia virus integration site 1
bp	Base pairs
BRCA1	Breast cancer 1

BRCA2	Breast cancer 2
BSA	Bovine serum albumin
BUB3	Mitotic checkpoint protein BUB3
C11orf73	Protein Hikeshi
C12orf57	Protein C10
C14orf166	UPF0568 protein C14orf166
C2orf18	Chromosome 2 open reading frame 18, isoform CRA_c
C7orf50	Uncharacterized protein C7orf50
CA2	Carbonic anhydrase 2
CAB39	Calcium-binding protein 39
CALM1	Calmodulin
CALU	Calumenin
CAP1	Adenylyl cyclase-associated protein 1
CARHSP1	Calcium-regulated heat-stable protein 1
CASP3	Caspase-3
CAV2	Caveolin-2
CBR3	Carbonyl reductase [NADPH] 3
CCDC144CP	Putative coiled-coil domain-containing protein 144C
CCDC22	Coiled-coil domain-containing protein 22
CCR4-NOT	CCR4-NOT transcription complex subunit 1
CDC25C	Cell division cycle 25C
CDC42	Cell division cycle 42
CDH1	Cadherin 1
CDK2	Cyclin-dependent kinase 2
CDK6	Cyclin-dependent kinase 6
CDKN1A	Cyclin-dependent kinase inhibitor 1
cDNAs	Complementary DNAs
CENPE	Centromere-associated protein E
CEP170	Centrosomal protein of 170 kDa
CHAPS	3-((3-cholamidopropyl) dimethylammonium)-1-propanesulfonate
CHEK2	Checkpoint kinase 2
CHID1	Chitinase domain-containing protein 1
CHK2	Checkpoint kinase 2
CHMP2B	Charged multivesicular body protein 2b
CHMP3	Charged multivesicular body protein 3

CHMP4B	Charged multivesicular body protein 4b
CHP1	Calcineurin B homologous protein 1
CIRBP	Cold-inducible RNA-binding protein
CLL	Chronic lymphocytic leukaemia
CLPTM1	Cleft lip and palate transmembrane protein 1
CMAS	N-acylneuraminate cytidylyltransferase
CML	Chronic myeloid leukemia
CMPK1	Cytidine/uridine monophosphate kinase 1
c-MYC	V-Myc Avian Myelocytomatosis Viral Oncogene Homolog
CNOT7	CCR4-NOT transcription complex subunit 7
CNP	2',3'-cyclic-nucleotide 3'-phosphodiesterase
CO_2	Carbon dioxide
COA3	Cytochrome c oxidase assembly factor 3 homolog, mitochondrial
COMMD8	COMM domain-containing protein 8
COMT	Catechol O-methyltransferase
COX17	Cytochrome c oxidase copper chaperone
COX-2	Prostaglandin-Endoperoxide Synthase 2 (Prostaglandin G/H Synthase And Cyclooxygenase)
COX20	Cytochrome c oxidase protein 20 homolog
COX6C	Cytochrome c oxidase subunit 6C
CPD	Carboxypeptidase D
CpG	Cytosine-phosphate-Guanine
CPT1A	Carnitine O-palmitoyltransferase 1, liver isoform
c-RAF	Raf-1 proto-oncogene, serine/threonine kinase
CRK	V-crk avian sarcoma virus CT10 oncogene homolog
CRKL	Crk-like protein
CSC	Cancer stem cells
CSNK2A2	Casein kinase II subunit alpha'
CSRP2	Cysteine and glycine-rich protein 2
CST3	Cystatin-C
Ct	Cycle threshold
CTBP2	C-terminal-binding protein 2
CTNNB1	Catenin beta-1
CTNND1	Catenin delta-1
CUL3	Cullin-3

CUL4A	Cullin-4A
CXCR4	C-X-C motif chemokine receptor 4
CYP	Cytochrome P450
CYP1B1	Cytochrome P450 family 1 subfamily B member 1
CYP2E1	Cytochrome P450 family 1 subfamily E member 1
CYP3A4	Cytochrome P450 family 3 subfamily A member 1
DAB	3,3'-diaminobenzidine
DAC	5-Aza-2′-deoxycytidine
DCAKD	Dephospho-CoA kinase domain-containing protein
DCTD	Deoxycytidylate deaminase
DDR	DNA Damage Response
DERA	2-deoxyribose-5-phosphate aldolase homolog (C. elegans), isoform CRA_a
DERL1	Derlin
DFNA5	Non-syndromic hearing impairment protein 5
DGCR8	DiGeorge syndrome critical region 8
DGKA	Diacylglycerol kinase alpha
DICER1	Dicer 1, ribonuclease III
DME	Drug-metabolizing enzymes
DMEM	Dulbeccos's Modified Eagle's Medium Nutrient
DMEM/F-12	Dulbeccos's Modified Eagle's Medium Nutrient Mixture F-12 Ham
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNAJB11	DnaJ homolog subfamily B member 11
DNAJC3	DnaJ homolog subfamily C member 3
DNAJC5	DnaJ homolog subfamily C member 5
dNTPs	Nucleoside triphosphates
DOCK5	Dedicator of cytokinesis protein 5
DOCK9	Dedicator of cytokinesis protein 9
DOHH	Deoxyhypusine hydroxylase
DOX	Doxorubicin
DRAP1	Dr1-associated corepressor
DRG2	Developmentally-regulated GTP-binding protein 2
DROSHA	Drosha ribonuclease III
DSBs	Double strand breaks

DTT	Dithiothreitol
DUT	Deoxyuridine 5'-triphosphate nucleotidohydrolase, mitochondrial
E_2	17-β-estradiol
EBP	3-beta-hydroxysteroid-Delta(8),Delta(7)-isomerase
EDF1	Endothelial differentiation-related factor 1
EDTA	Ethylenediamine tetraacetic acid
EFHD2	EF-hand domain-containing protein D2
EFL1	Elongation factor-like GTPase 1
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGFR	Epidermal growth factor receptor
EIF4A2	Eukaryotic initiation factor 4A-II
ELOVL5	Elongation of very long chain fatty acids protein 5
EMC4	ER membrane protein complex subunit 4
EMC6	ER membrane protein complex subunit 6
EMC9	ER membrane protein complex subunit 9
EMT	Epithelial-to-mesenchymal transition
ENAH	Protein enabled homolog
endo-siRNAs	Endogenous small interfering RNAs
ENO1	Enolase 1
ENO2	Gamma-enolase
EPS8L2	Epidermal growth factor receptor kinase substrate 8-like protein 2
EPT1	Ethanolaminephosphotransferase 1
ER	Oestrogen receptor
ERCC1	Excision repair cross-complementation group 1
ERGIC3	Endoplasmic reticulum-Golgi intermediate compartment protein 3
ERLEC1	Endoplasmic reticulum lectin 1
ERLIN1	Erlin-1
EVI1	MDS1 and EVI1 complex locus
FAM114A1	Protein NOXP20
FAM120A	Constitutive coactivator of PPAR-gamma-like protein 1
FAM162A	Protein FAM162A
FAM210A	Protein FAM210A
FAM213A	Redox-regulatory protein FAM213A
FAM21C	WASH complex subunit FAM21C

FAM96A	MIP18 family protein FAM96A
FAM96B	Mitotic spindle-associated MMXD complex subunit MIP18
FBS	Foetal bovine serum
FBW7	F-box and WD repeat domain containing 7
FFPE	Formalin-fixed paraffin-embedded
FHL2	Four and a half LIM domains protein 2
FHL2	Four and a half LIM domains protein 2
FKBP10	Peptidyl-prolyl cis-trans isomerase FKBP10
FKBP2	cDNA FLJ52062, highly similar to Erythrocyte band 7 integral membrane protein
FKBP3	Peptidyl-prolyl cis-trans isomerase FKBP3
FKBP5	Peptidyl-prolyl cis-trans isomerase FKBP5
FMNL2	Formin-like protein 2
FMR1	Fragile X mental retardation 1
FOG2	Zinc finger protein, FOG family member 2
FOXO1	Forkhead box O1
FTL	Ferritin light chain
FXN	Frataxin, mitochondrial
FZD3	Frizzled class receptor 3
GAP	Growth Associated Protein
GATA3	GATA binding protein 3
GBAS	Protein NipSnap homolog 2
GC	Guanine Cytosine
GCSH	Glycine cleavage system H protein, mitochondrial
GE	General Electric
GLB1	Beta-galactosidase
GLS	Glutaminase kidney isoform, mitochondrial
GMIP	GEM-interacting protein
GMPPB	Mannose-1-phosphate guanyltransferase beta
GNB2l1	Guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1
GNL3	Guanine nucleotide-binding protein-like 3
GOLGA3	Golgin subfamily A member 3
GPS1	COP9 signalosome complex subunit 1
GRWD1	Glutamate-rich WD repeat-containing protein 1

GSK3B	Glycogen synthase kinase-3 beta
GSTP1	Glutathione S-transferase pi 1
GSTT2	Glutathione S-transferase theta 2, isoform CRA_a
GTF2F2	General transcription factor IIF subunit 2
GUK1	Guanylate kinase
GW182	Trinucleotide repeat-containing gene 6A protein
GYS1	Glycogen [starch] synthase, muscle
H2AFV	Histone H2A.V
H2AX	H2A histone family member X
HACD3	Very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase 3
HAT1	Histone acetyltransferase type B catalytic subunit
HCFC1	Host cell factor 1
HDAC1	Histone deacetylase 1
HEBP2	Heme-binding protein 2
HER2/ERBB2	Human epidermal growth factor receptor 2/erb-b2 receptor tyrosine kinase 2
HER3/ERBB3	Human epidermal growth factor receptor 3/erb-b2 receptor tyrosine kinase 3
HERPUD1	Homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member 1 protein
HIF1α	Hypoxia Inducible Factor 1, Alpha Subunit
HINT2	Histidine triad nucleotide-binding protein 2, mitochondrial
HLA-C	HLA class I histocompatibility antigen, Cw-12 alpha chain
HM13	Minor histocompatibility antigen H13
HMEpC	Human Mammary Epithelial progenitor Cell line
HMGA	High Mobility Group
HMGA1	High mobility group protein HMG-I/HMG-Y
HMGA2	High mobility group AT-hook 2
HMGCL	3-hydroxymethyl-3-methylglutaryl-Coenzyme A lyase (Hydroxymethylglutaricaciduria), isoform CRA_b
HMGN3	High mobility group nucleosome-binding domain-containing protein 3
HN1	Hematological and neurological-expressed 1 protein
HNRNPDL	Heterogeneous nuclear ribonucleoprotein D-like
HNRNPL	Heterogeneous nuclear ribonucleoprotein L

HOXD10	Homeobox D10
HR	Homologous recombination
HSAEpC	Human Small Airway Epithelial progenitor Cell line
HSD17B2	Estradiol 17-beta-dehydrogenase 2
HTRA2	Serine protease HTRA2, mitochondrial
HTT	Huntingtin
HuR	ELAV like RNA binding protein 1
IAH1	Isoamyl acetate-hydrolyzing esterase 1 homolog
ICLs	Interstrand DNA crosslinks
IEF	Isoelectric focusing
IFI16	Gamma-interferon-inducible protein 16
IFI35	Interferon-induced 35 kDa protein
IFT27	Intraflagellar transport protein 27 homolog
IHC	Immunohistochemistry
IL1A	Interleukin-1 alpha
ILK	Integrin-linked protein kinase
ILVBL	Acetolactate synthase-like protein
INF2	Inverted formin-2
IPG	Immobilized pH gradient
IPO11	Importin-11
IQGAP2	Ras GTPase-activating-like protein IQGAP2
IRGQ	Immunity-related GTPase family Q protein
IRS1	Insulin receptor substrate 1
ISCA1	Iron-sulfur cluster assembly 1 homolog, mitochondrial
IST	Instituto Superior Técnico
ITGA5	Integrin subunit alpha 5
ITGAV	Integrin alpha-V
ITGB3	Integrin subunit beta 3
KCl	Potassium chloride
KCTD12	BTB/POZ domain-containing protein KCTD12
KH_2PO_4	Potassium phosphate monobasic
KIAA0196	WASH complex subunit strumpellin
KIAA0391	Mitochondrial ribonuclease P protein 3
KIAA1033	WASH complex subunit 7
KPNA4	Importin subunit alpha-3

KRAS	GTPase KRas
KRT18	Keratin, type I cytoskeletal 18
LACTB2	Beta-lactamase-like protein 2
LAD1	Ladinin-1
LAMC1	Laminin subunit gamma-1
LASP1	LIM and SH3 protein 1
LC/MS	Liquid chromatography/Mass spectrometry
LIFR	Leukaemia inhibitory factor receptor alpha
LNA	Locked Nucleic Acid
lncRNAs	Long non-coding RNAs
LPXN	Leupaxin
LSM4	U6 snRNA-associated Sm-like protein LSm4
LSM5	U6 snRNA-associated Sm-like protein LSm5
LTV1	Protein LTV1 homolog
LUC7L2	Putative RNA-binding protein Luc7-like 2
LXN	Latexin
MAGI2	Membrane associated guanylate kinase, WW and PDZ domain containing 2
MALDI-TOF/TOF	Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometer
MAP1B	Microtubule-associated protein 1B
MAPK14	Mitogen-activated protein kinase 14
MAPK1IP1L	MAPK-interacting and spindle-stabilizing protein-like
MCM3	DNA replication licensing factor MCM3
MCM6	DNA replication licensing factor MCM6
MCMBP	Mini-chromosome maintenance complex-binding protein
MCT1	Monocarboxylate transporter 1
MDC1	Mediator of DNA damage checkpoint 1
MDR	Multidrug resistance
ME1	NADP-dependent malic enzyme
MED20	Mediator of RNA polymerase II transcription subunit 20
MERTK	MER proto-oncogene, tyrosine kinase
MET	Mesenchymal-to-epithelial transition
MF	Methylated forward
MGMT	O^6-methyl-guanine-DNA methyltransferase

MIG6	ERBB receptor feedback inhibitor 1
miRISC	miRNA induced silencing complex
miRNAs	Micro RNAs
MMGT1	Membrane magnesium transporter 1
MMR	Mismatch repair
$MnCl_2$	Manganese(II) chloride
MNT	MAX network transcriptional repressor
MOB4	MOB-like protein phocein
MR	Methylated reverse
mRNA	Messenger RNA
MRPL14	39S ribosomal protein L14, mitochondrial
MRPL18	39S ribosomal protein L18, mitochondrial
MRPL22	39S ribosomal protein L22, mitochondrial
MRPL30	39S ribosomal protein L30, mitochondrial
MRPL37	39S ribosomal protein L37, mitochondrial
MRPL38	39S ribosomal protein L38, mitochondrial
MRPL45	39S ribosomal protein L45, mitochondrial
MRPL47	39S ribosomal protein L47, mitochondrial
MRPL48	39S ribosomal protein L48, mitochondrial
MRPL50	39S ribosomal protein L50, mitochondrial
MRPL53	39S ribosomal protein L53, mitochondrial
MRPL9	39S ribosomal protein L9, mitochondrial
MRPS11	28S ribosomal protein S11, mitochondrial
MRPS12	28S ribosomal protein S12, mitochondrial
MRPS5	28S ribosomal protein S5, mitochondrial
MRPS6	28S ribosomal protein S6, mitochondrial
MRRF	Ribosome-recycling factor, mitochondrial
MSH2	MutS homolog 2
MSH6	MutS homolog 6
MSP	Methylation specific PCR
MSRB3	Methionine-R-sulfoxide reductase B3
MT1H	Metallothionein-1H
MT1L	Metallothionein-1L
MTA1	Metastasis associated 1
MTA2	Metastasis-associated protein MTA2

MTCH2	Mitochondrial carrier homolog 2
MTOR	Serine/threonine-protein kinase mTOR
MTT	Thiazolyl blue tetrazolium bromide
MUC1	Mucin 1, cell surface associated
MX	Mitoxantrone
MYC	V-Myc Avian Myelocytomatosis Viral Oncogene Homolog
MYL1	Myosin light chain 1/3, skeletal muscle isoform
MYO1E	Unconventional myosin-Ie
MYT1	Myelin transcription factor 1
Na_2HPO_4	Disodium phosphate
NAA20	N-alpha-acetyltransferase 20
NaCl	Sodium Chloride
NAE1	NEDD8-activating enzyme E1 regulatory subunit
NANOG/OCT4	Nanog homeobox/POU class 5 homeobox 1
NAV1	Neuron navigator 1
NCAPD2	Condensin complex subunit 1
NCEH1	Neutral cholesterol ester hydrolase 1
NCKAP1	Nck-associated protein 1
ncRNAs	Non-coding RNAs
NCSTN	Nicastrin
NDUFA12	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 12
NDUFA13	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 13
NDUFAF2	Mimitin, mitochondrial
NDUFB4	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 4
NECTIN1	Nectin cell adhesion molecule 1
NEDD4	E3 ubiquitin-protein ligase NEDD4
NER	Nucleotide excision repair
NFkB	Nuclear Factor of Kappa Light Polypeptide Gene Enhancer In B-Cells
NFS1	Cysteine desulfurase, mitochondrial
NHEJ	Non-homologous end joining
NIPSNAP3A	Protein NipSnap homolog 3A
NOP16	Nucleolar protein 16
NOS	Not otherwise specified
NPC1	Niemann-Pick C1 protein
NR2C2AP	Nuclear receptor 2C2-associated protein

NRDC	Nardilysin
NT5C2	Cytosolic purine 5'-nucleotidase
NT5C3A	Cytosolic 5'-nucleotidase 3A
NTMT1	N-terminal Xaa-Pro-Lys N-methyltransferase 1
NUBP2	Cytosolic Fe-S cluster assembly factor NUBP2
NUDT4	Diphosphoinositol polyphosphate phosphohydrolase 2
NUMA1	Nuclear mitotic apparatus protein 1
NUTF2	Nuclear transport factor 2
OARD1	O-acetyl-ADP-ribose deacetylase 1
OGFR	Opioid growth factor receptor
OGT	UDP-N-acetylglucosamine--peptide N-acetylglucosaminyltransferase 110 kDa subunit
oncomiRs	Oncogenic miRNAs
ORMDL3	ORM1-like protein 3
OSBPL8	Oxysterol-binding protein
OTUD6B	OTU domain-containing protein 6B
p27Kip1	Cyclin-dependent kinase inhibitor 1B
PABPC	Polyadenylate-binding protein complex
PABPN1	Polyadenylate-binding protein 2
PAGE	Polyacrylamide gel electrophoresis
PaK1	P21 Protein (Cdc42/Rac)-Activated Kinase 1
PALB2	Partner and localizer of BRCA2
PARK7	Parkinson disease (autosomal recessive, early onset) 7
PARP	Poly(ADP-ribose) polymerase
PASRs	Promoter-associated small RNAs
PAX	Paclitaxel
PBK	Lymphokine-activated killer T-cell-originated protein kinase
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCBD1	Pterin-4-alpha-carbinolamine dehydratase
PcG	Polycomb group
PCR	Polymerase chain reaction
PDCD4	Programmed cell death 4
PDCD5	Programmed cell death protein 5
PDDC1	Parkinson disease 7 domain-containing protein 1

PEPT1	Peptide transporter 1
PEX1	Peroxisome biogenesis factor 1
PEX11B	Peroxisomal membrane protein 11B
PFAS	Phosphoribosylformylglycinamidine synthase
PFDN1	Prefoldin subunit 1
PHPT1	14 kDa phosphohistidine phosphatase
PIKK	Phosphoinositide 3-kinase (PI3-kinase)-like family
PIR	Pirin
piRNAs	Piwi-interacting RNAs
PITPNA	Phosphatidylinositol transfer protein alpha isoform
PKM2	Pyruvate Kinase, Muscle
PLAA	Phospholipase A-2-activating protein
PLCG1	Phospholipase C gamma 1
PLEK2	Pleckstrin-2
PM20D2	Peptidase M20 domain-containing protein 2
PMSF	Phenylmethylsulfonyl fluoride
PNPLA4	Patatin-like phospholipase domain-containing protein 4
Pol II	RNA polymerase II
POLR2E	DNA-directed RNA polymerases I, II, and III subunit RPABC1
POTEE	POTE ankyrin domain family member E
PPCS	Phosphopantothenate--cysteine ligase
PPCS	Phosphopantothenate--cysteine ligase
PPCS	Phosphopantothenate--cysteine ligas
PPFIBP1	Liprin-beta-1
PPP1CC	Serine/threonine-protein phosphatase
PPP1R14B	Protein phosphatase 1 regulatory subunit 14B
PPP1R7	Protein phosphatase 1 regulatory subunit 7
PPP2R5D	Serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit delta isoform
PR	Progesterone receptor
pRb	Retinoblastoma 1
PRC1	Polycomb repressor complex 1
PRDX2	Peroxiredoxin 2
PRDX6	Peroxiredoxin 6
pre-miRNA	Precursor miRNA

pri-miRNAs	Primary miRNAs
PRKCI	Protein kinase C iota type
PRMT3	Protein arginine N-methyltransferase 3
PROMPTs	Promoter upstream transcripts
PSAP	Prosaposin
PSMA6	Proteasome subunit alpha 6
PSPH	Phosphoserine phosphatase
PTEN	Phosphatase and tensin homolog
PTMA	Prothymosin alpha
PTMS	Parathymosin
PTPRN2	Protein tyrosine phosphatase, receptor type N2
PTRH2	Peptidyl-tRNA hydrolase 2, mitochondrial
PUS1	tRNA pseudouridine synthase
PVDF	Polyvinylidene difluoride
PYCRL	Pyrroline-5-carboxylate reductase 3
qPCR	Quantitative PCR
R3HDM1	R3H domain-containing protein 1
RAB22A	Ras-related protein Rab-22A
RAB4A	Ras-related protein Rab-4A
RABAC1	Prenylated Rab acceptor protein 1
RABIF	Guanine nucleotide exchange factor MSS4
RAD23B	UV excision repair protein RAD23 homolog B
RAD51	RAD51 recombinase
RAD52	RAD52 homolog, DNA repair protein
RAN	RAN, member RAS oncogene family
RAS	Rat Sarcoma Viral Oncogene Homolog gene family
RBM8A	RNA-binding protein 8A
RBMXL2	RNA-binding motif protein, X-linked-like-2
RCC1	Regulator of chromosome condensation
RCC2	Protein RCC2
RCN1	Reticulocalbin-1
RDX	Radixin
REV1	REV1, DNA directed polymerase
RFC5	Replication factor C subunit 5
RHEB	GTP-binding protein Rheb

RHOA	Ras homolog family member A
RHOC	Ras homolog family member C
RNA	Ribonucleic acid
RPA2	Replication protein A 32 kDa subunit
RPL22L1	60S ribosomal protein L22-like 1
RPL26L1	60S ribosomal protein L26-like 1
RPS27L	40S ribosomal protein S27-like
RRM1	Ribonucleoside-diphosphate reductase large subunit
RTKN	Rhotekin
RT-qPCR	Reverse transcription qPCR
S100A13	Protein S100-A13
SACM1L	Phosphatidylinositide phosphatase SAC1
SATB1	Special AT-rich sequence binding protein 1
SCAMP1	Secretory carrier-associated membrane protein 1
SCC	Squamous Cell Carcinoma
SCO2	Protein SCO2 homolog, mitochondrial
SCRIB	Protein scribble homolog
SDC1	Syndecan-1
SDCBP	Syntenin-1
SDF2L1	Stromal cell-derived factor 2-like protein 1
SDS	Sodium dodecyl sulfate
SEC11A	Signal peptidase complex catalytic subunit SEC11
SEC16A	Protein transport protein Sec16A
SEC23IP	SEC23-interacting protein
SEC24D	Protein transport protein Sec24D
SEC63	Translocation protein SEC63 homolog
SEER	Surveillance, Epidemiology, and End Results
SRM	Selected Reaction Monitoring
SEP15	15 kDa selenoprotein
SEPHS1	Selenide, water dikinase 1
SEPT8	Septin-8
SERPINB5	Serpin family B member 5
SERPINB5	Serpin family B member 5
SERPINE1	Plasminogen activator inhibitor 1
SET	Protein SET

SETD1A	Histone-lysine N-methyltransferase SETD1A
SF3A3	Splicing factor 3A subunit 3
SF3B5	Splicing factor 3B subunit 5
SFN	Stratifin
SH2D4A	SH2 domain-containing protein 4A
SH3BGRL	SH3 domain-binding glutamic acid-rich-like protein
SIK1	Salt inducible kinase 1
SIRT1	Sirtuin 1
SIX1	Sine Oculis Homeobox Homolog 1
SKIV2L2	Superkiller viralicidic activity 2-like 2
SLC15A1	Solute carrier family 15 member 1
SLC16A1	Solute carrier family 16 member 1
SLC22A1	Solute carrier family 22 member 1
SLC22A5	Solute carrier family 22 member 5
SLC25A1	Tricarboxylate transport protein, mitochondrial
SLC25A22	Mitochondrial glutamate carrier 1
SLC25A6	ADP/ATP translocase 3
SLCO2B1	Solute carrier organic anion transporter family member 2B1
SMAD2	Mothers against decapentaplegic homolog 2
SMAD3	SMAD family member 3
SMARCA4	Transcription activator BRG1
SMCHD1	Structural maintenance of chromosomes flexible hinge domain-containing protein 1
SMN1	Survival motor neuron protein
SNAI2	Snail family zinc finger 2
SNAP29	Synaptosomal-associated protein 29
SNAPIN	SNARE-associated protein Snapin
SNPs	Single nucleotide polymorphisms
snRNA	Small nuclear RNAs
SNU13	NHP2-like protein 1
SNX1	Sorting nexin-1
SOCS3	Suppressor of cytokine signalling 3
SOX1	SRY-box 1
SOX2	SRY-box 2
SOX4	SRY-box 4

SP1	Sp1 transcription factor
SPC25	Kinetochore protein Spc25
SPIN1	Spindlin-1
SPSS	Statistical Package for the Social Sciences
SRC	SRC Proto-Oncogene, Non-Receptor Tyrosine Kinase
SRPRA	Signal recognition particle receptor subunit alpha
SRXN1	Sulfiredoxin-1
SSR3	Translocon-associated protein subunit gamma
SSU72	RNA polymerase II subunit A C-terminal domain phosphatase SSU72
ST14	Suppression of tumorigenicity 14
StarD10	StAR related lipid transfer domain containing 10
STAT1	Signal transducer and activator of transcription 1-alpha/beta
STAT3	Signal transducer and activator of transcription 3
STAT3	Signal transducer and activator of transcription 3
STK10	Serine/threonine-protein kinase 10
STK11	Serine/threonine kinase 11
STK4	Serine/threonine-protein kinase 4
STMN1	Stathmin
STX12	Syntaxin-12
SULT1A1	Sulfotransferase family 1A member 1
SUMO1	Small ubiquitin-related modifier 1
SUZ12	SUZ12 polycomb repressive complex 2 subunit
SYAP1	Synapse-associated protein 1
SYMPK	Symplekin
SYNGR2	Synaptogyrin-2
TBC1D9B	TBC1 domain family member 9B
TBCD	Tubulin-specific chaperone D
TBRG4	Protein TBRG4
TEMED	Tetramethylethylenediamine
TERT	Telomerase reverse transcriptase
TGF-β	Transforming Growth Factor, Beta
THEM6	Protein THEM6
THYN1	Thymocyte nuclear protein 1
TIAL1	Nucleolysin TIAR
TIGD2	Tigger transposable element-derived protein 2

TIMM8B	Mitochondrial import inner membrane translocase subunit Tim8 B
TIMP3	TIMP metallopeptidase inhibitor 3
tiRNAs	Transcription initiation RNAs
TLR7	Toll-like receptor 7
TMED4	Transmembrane emp24 domain-containing protein 4
TMED5	Transmembrane emp24 domain-containing protein 5
TMEM205	Transmembrane protein 205
TMEM41B	Transmembrane protein 41B
TMEM65	Transmembrane protein 65
TMEM70	Transmembrane protein 70, mitochondrial
TMSB10	Thymosin beta-10
TMX3	Protein disulfide-isomerase TMX3
TNBC	Triple-negative breast cancers
TNC	Tenascin C
TNM	Tumour Node Metastasis
TOP1	DNA topoisomerase 1
TP53	Tumour protein p53
TP53INP1	Tumour protein p53 inducible nuclear protein 1
TPBG	Trophoblast glycoprotein
TPI1	Triosephosphate isomerase 1
TPM	Tropomyosin
TPM1	Tropomyosin 1
TPM2	Tropomyosin beta chain
TPM3	Tropomyosin 3
TPP1	Tripeptidyl-peptidase 1
TPRKB	EKC/KEOPS complex subunit TPRKB
TRAPPC5	Trafficking protein particle complex subunit 5
TRBP	Transactivation-responsive RNA-binding protein
TRIM25	E3 ubiquitin/ISG15 ligase TRIM25
TRIM32	E3 ubiquitin-protein ligase TRIM32
Tris.base	Tris(hydroxymethyl)aminomethane
Tris.HCl	Tris(hydroxymethyl)aminomethane hydrochloride
TRKB	Neurotrophic receptor tyrosine kinase 2
TRMT1	tRNA (guanine(26)-N(2))-dimethyltransferase
TSFM	Elongation factor Ts

TSR3	Ribosome biogenesis protein TSR3 homolog
TSSa-RNAs	Transcription start site associated RNAs
TTC1	Tetratricopeptide repeat protein 1
TTP	Tristetraprolin
TUBA1b	Tubulin alpha 1b
TYMS	Thymidylate synthase
TYMS	Thymidylate synthase
UBA2	SUMO-activating enzyme subunit 2
UBA3	NEDD8-activating enzyme E1 catalytic subunit
UBC9	Ubiquitin conjugating enzyme E2 I
UBE2C	Ubiquitin-conjugating enzyme E2 C
UBE2D1	Ubiquitin-conjugating enzyme E2 D1
UBE2D2	Ubiquitin-conjugating enzyme E2 D2
UBE2D3	Ubiquitin-conjugating enzyme E2 D3
UBE2O	(E3-independent) E2 ubiquitin-conjugating enzyme
UBQLN2	Ubiquilin-2
UCSC	University of California Santa Cruz
UF	Unmethylated forward
UHRF2	Ubiquitin-like with PHD and ring finger domains 2
UQCRC1	Cytochrome b-c1 complex subunit 1, mitochondrial
UR	Unmethylated reverse
USP24	Ubiquitin carboxyl-terminal hydrolase 24
UV	Ultraviolet
VEGF-A	Vascular endothelial growth factor A
VMA21	Vacuolar ATPase assembly integral membrane protein VMA21
VPS26A	Vacuolar protein sorting-associated protein 26A
VPS28	Vacuolar protein sorting-associated protein 28 homolog
VPS37B	Vacuolar protein sorting-associated protein 37B
WDR77	Methylosome protein 50
XDH	Xanthine dehydrogenase/oxidase
xenomiRs	Exogenous origin miRNAs
XPO5	Exportin-5
YARS2	Tyrosine--tRNA ligase, mitochondrial
YBX3	Y-box-binding protein 3
YTHDF2	YTH domain-containing family protein 2

ZBTB1	Zinc finger and BTB domain containing 1
ZBTB10	Zinc finger and BTB domain containing 10
ZC3H4	Zinc finger CCCH domain-containing protein 4
ZC3HAV1	Zinc finger CCCH-type antiviral protein 1
ZCCHC6	Terminal uridylyltransferase 7
ZEB1	Zinc finger E-box-binding homeobox 1
ZEB2	Zinc finger E-box-binding homeobox 2
ZFP36L1	Zinc finger protein 36, C3H1 type-like 1
ZNF706	Zinc finger protein 706

Resumo

Os microRNAs (miRNAs) são pequenos RNAs não codificantes com função reguladora que regulam a expressão génica ao ligar-se a sequências específicas na região 3' UTR dos mRNAs. Diversos estudos mostraram que os miRNAs regulam mecanismos fundamentais para o normal funcionamento celular, como crescimento celular, proliferação, diferenciação e apoptose. A expressão de alguns miRNAs é alterada em diversos tipos de cancro, nomeadamente em cancro da mama. Estudos de análise funcional em linhas celulares mostraram que os miRNAs podem funcionar como supressores de tumor ou ter actividade oncogénica. Assim, o valor clínico dos miRNAs como potenciais marcadores para cancro da mama está a ser amplamente estudado de momento. No entanto, apenas se conhecem efeitos de alguns miRNAs. A maior dificuldade, neste âmbito, depreende-se com a identificação de possíveis alvos com relevância biológica para cancro da mama. Visto que os programas bioinformáticos predizem um elevado número de falsos positivos e falsos negativos, é de extrema importância identificar experimentalmente alvos relevantes.

Nesta tese procuramos explorar diferentes abordagens da influência de miRNAs em cancro da mama. Começamos por estudar os mecanismos que estão por trás da regulação dos próprios miRNAs. Colocámos a hipótese de serem mecanismos epigenéticos, tais como a metilação de citosinas no DNA, que estão a influenciar os níveis de expressão dos miRNAs. Para tal, tratámos linhas celulares de mama com um agente capaz de desmetilar o DNA e verificámos que os níveis de miRNAs são alterados. Contudo, não conseguimos encontrar uma associação entre a metilação de ilhas CpG nas regiões promotoras dos genes que codificam para os miRNAs. No entanto, não podemos excluir a possibilidade de os níveis de expressão de miRNAs estarem a ser regulados por metilação das suas zonas promotoras, dado que não estudámos todas as regiões promotoras existentes.

De seguida, abordámos a influência de dois miRNAs, miR-200c e miR-203, na resistência para fármacos dirigidos a cancro da mama, nomeadamente, Paclitaxel e 5-fluoruacil. Para

tal fizemos expressar ambos os miRNAs na linha celular MDA-MB-231 e inibir os mesmos na linha celular MCF-7. Infelizmente não fomos capazes de encontrar significado estatístico nos resultados obtidos. Contudo pudemos concluir que o miR-200c parece ter um efeito contrário nas linhas MCF-7 e MDA-MB-231 no que diz respeito ao tratamento com Paclitaxel e o miR-203 parece aumentar a resistência para o mesmo comporto na linha celular MDA-MB-231. O tratamento com 5-fluoruacil não mostrou qualquer diferença em ambas as linhas.

Dado que os estudos *in vitro*, nesta área, devem ser transpostos para humanos e/ou tecidos humanos, seguidamente procurámos estudar os níveis de expressão do miR-200c e do miR-203 em tecido tumoral mamário, bem como a expressão de dois alvos hipotéticos encontrados utilizando ferramentas bioinformáticas, SIX1 e SOX2. Relativamente ao miR-200c, não encontrámos quaisquer diferenças entre tecido normal e tecido tumoral de mama, nem conseguimos relacionar este miRNA com características clinicopatológicas. Comparativamente detectaram-se diferenças para o miR-203 e conseguimos relacionar este com os estadios iniciais de desenvolvimento tumoral. Conseguimos também demonstrar que o miR-203 pode ser um potencial marcador para discriminar os tumores lobulares invasivos. No que diz respeito à expressão do SOX1 e SOX2, observámos que ambos possuem uma incidência baixa na nossa população e que não se associam com a expressão dos miRNAs em estudo.

Por último, procurámos validar alguns alvos do miR-200c e do miR-203. Para tal, efectuámos um estudo comparativo de proteómica, onde fizemos expressar o miR-200c e o miR-203 na linha celular MDA-MB-231 e inibimos os mesmos miRNAs na linha celular MCF-10A. Este estudo exploratório, ainda por terminar, revelou aproximadamente 3000 proteínas diferentemente expressas nas linhas celulares. No entanto, até ao momento conseguimos reduzir esta vasta lista para uma menor com aproximadamente 10 proteínas para cada linha celular. De futuro, serão seguidas outras abordagens para validar estes alvos hipotéticos.

Devido ao facto da população recolhida ser recente, o seguimento clínico nos próximos anos permitirá tirar algumas conclusões relativas à resistência à terapia utilizada e a sua relação com a expressão dos miRNAs em estudo.

Palavras-chave: miRNAs, miR-200c, miR-203, cancro da mama, metilação, regulação, resistência a fármacos, IHC, SIX1, SOX2, RT-qPCR, proteómica, LC/MS, MALDI/TOF.

Abstract

MicroRNAs (miRNAs) are small non-coding regulatory RNAs that modulate gene expression by binding to their target mRNAs. By these means, miRNAs control normal rates of all major cellular pathways. A subset of miRNAs, which are differentially detected between normal and tumour tissue samples, has been identified in breast cancer, and functional analysis in cell line systems has revealed tumour suppressive and oncogenic functions of some of these miRNAs. Hence, the clinical value of these as novel biomarkers for cancer is being actively investigated. However, the function of only a few of these miRNAs in breast cancer has been investigated. One major difficulty is the identification of target mRNAs and proteins with biological significance in breast cancer and consequently the identifications of the pathways they influence. Given the relatively high rates of both false-positives and false-negatives from current miRNAs targets prediction programs, it is critically important to experimentally identify relevant miRNAs targets and the pathways involved in carcinogenesis.

In this thesis our main goal was to study the role of miRNAs in breast cancer. Thus, we started by addressing the mechanisms behind regulation of miRNAs expression levels. We hypothesized if that epigenetic mechanisms, such as DNA methylation, could influence miRNAs expression levels. Therefore, we treated breast cell lines with demethylating agents and observed that miRNAs expression levels were altered. However, we failed to prove a direct correlation between the methylation of CpG islands in promoter regions of the miRNAs studied and their expression. Nevertheless, we cannot exclude the possible regulation of miRNAs levels by methylation since we did not study all possible promoter regions of miRNAs genes as their promoter regions have not been fully identified.

Next, we addressed the possible effect of miRNAs in breast cancer therapy resistance. For that, we treated breast tumour cell lines with Paclitaxel and 5-fluoruacil, two known chemotherapeutics used in breast cancer, with the ectopic expression or inhibition of miR-

200c and miR-203. Unfortunately, we did not find any statistical difference between untreated and treated cells. However, miR-200c seems to have contrary effects in MCF-7 and MDA-MB-231 cells regarding treatment with Paclitaxel and miR-203 seem to augment resistance to Paclitaxel in MDA-MB-231 cells. Both miRNAs did not show any effect in cells treated with 5-Fluoruacil.

Since *in vitro* studies, always lack studies using human tissues. We subsequently studied the expression levels of miR-200c and miR-203 in breast tumour tissues and two putative targets found by bioinformatics tools, SIX1 and SOX2. Concerning miR-200c, we did not detect significant differences between normal breast and tumour tissues in our population. Additionally, we failed to correlate miR-200c with clinicopathological features. Regarding miR-203, we detect statistically differences between normal and tumour tissue and it seems that miR-203 is involved in breast cancer development, mainly in early stages of development. We also show that miR-203 might be a potential marker to discriminate stages in invasive lobular carcinoma. About the expression levels of SIX1 and SOX2, we observe relatively low levels of both proteins through immunohistochemistry and do not found any statistically difference between their expression and their regulators, miR-200c and miR-203.

Finally, we address the validation of putative targets of the miR-200c and miR-203. Thus, we conducted a comparative proteomic study to find differently expressed proteins when miR-200c and miR-203 were ectopically expressed or inhibited in breast cell lines. This exploratory study, until now, revealed a small list out of approximately 3000 proteins that are putative targets of both miRNAs and are differently expressed. Further studies will be conducted in order to validate these putative targets.

With this thesis, we believe we provide new insight into the involvement of miRNAs in breast cancer and also important knowledge of how miRNAs levels are being regulated and also in the discovery of new targets. We also gathered a considerable study population

during this thesis, which allow future correlations on therapy outcomes and survival with the biomarkers studied.

Keywords: miRNAs, miR-200c, miR-203, breast cancer, methylation, regulation, drug resistance, IHC, SIX1, SOX2, RT-qPCR, proteomics, LC/MS, MALDI/TOF.

1. Introduction

One of the most important landmarks of recent scientific history was the completion of the first draft of the human genome (Lander et al., 2001; Venter et al., 2001). The myriad opportunities that derive from the total sequencing of the human genome are priceless and the knowledge of human genetic diseases resulting therefrom is limitless. However, precaution is needed when interpreting the substantial data from human genome sequencing and subsequent studies. Indeed, no better words than those uttered by Venter and colleagues (Venter et al., 2001):

> *"(…) There are two fallacies to be avoided: determinism, the idea that all characteristics of the person are "hard-wired" by the genome; and reductionism, the view that with complete knowledge of the human genome sequence, it is only a matter of time before our understanding of gene functions and interactions will provide a complete causal description of human variability. (…)"*

The sequence of the human genome confirmed the idea that only approximately 2% of the genome codes for proteins, corresponding to 25.000 to 30.000 genes. The remaining of our genome was referred to as "junk DNA", a term first used by Susumu Ohno (Ohno, 1972). This term could not be less accurate. Indeed, the past few years revealed an immensity of data contradicting that notion and rewriting the central dogma of molecular biology postulated by Francis Crick (Crick, 1970). It is now clear that coding DNA isn't more important than non-coding DNA in cell homeostasis and that RNA is not only a molecule of information transmission, through messenger RNA (mRNA), but a group of molecules capable of other biological functions, namely in structural, catalytic and regulatory functions that are transcribed from all the genome [reviewed in (Alexander et al., 2010; Eddy, 2001; Esteller, 2011)]. Thus, every day, studies regarding the transcripts from non-coding DNA are increasingly becoming relevant, giving rise to a new meaning to the term "RNA World" (Cech, 2012).

New high-throughput sequencing technologies have enabled the identification of countless non-coding transcripts (ncRNAs), many of which are categorized into distinct RNA classes. Although classification of ncRNAs is not consensual, it can be classified in two main classes: 1) short or small ncRNAs; 2) and long ncRNAs (lncRNAs). Small ncRNAs are characterized as having less than 200 nucleotides in length and almost all seem to have regulatory function. This type of ncRNAs will be further discussed. LncRNAs have more than 200 nucleotides in length and only recently have emerged as key molecules in chromatin structure, transcriptional activity and mRNA stability, processing and translation (Esteller, 2011; Ponting et al., 2009; Quinn and Chang, 2015; Silva et al., 2015). Thus, aberrant expression of lncRNAs has been linked to several diseases (Kim et al., 2016; Pastori and Wahlestedt, 2012; Schonrock et al., 2012; Tye et al., 2015) including cancer (Shen et al., 2015; Silva et al., 2015).

1.1. Small non-coding RNAs

By far, the most studied small ncRNAs are microRNAs (miRNAs). Studies motivated by the discovery of miRNAs revealed other small ncRNAs, such as endogenous small interfering RNAs (endo-siRNAs), piwi-interacting RNAs (piRNAs), transcription start site associated RNAs (TSSa-RNAs), transcription initiation RNAs (tiRNAs), promoter-associated small RNAs (PASRs) and promoter upstream transcripts (PROMPTs) [revised in (Esteller, 2011)]. With the exception of PASRs, whose mechanism of action is not known, all small ncRNAs are involved in gene expression regulation. The focus of this thesis will be miRNAs and their regulatory action in cancer.

miRNAs are short (approximately 22 nucleotides) non-coding RNAs that were first described in 1993 when two independent groups (Lee et al., 1993; Wightman et al., 1993) published, in the same number of *Cell*, experiments on the *C. elegans* lin-4 gene which code for a pair of small RNAs with antisense complementarity to multiple sites on the 3'-untranslated region (UTR) of the lin-14 gene. This small RNA substantially reduced the

amount of lin-14 protein without noticeably changing the level of lin-14 mRNA. Soon the presence of another regulatory RNA, let-7, was observed in *C. elegans* (Reinhart et al., 2000) and in other species (Pasquinelli et al., 2000). This group of regulatory small RNAs were later designated as miRNAs (Lee and Ambros, 2001), an evolutionary conserved class of small RNAs that was found to control many developmental and cellular processes in eukaryotic organisms. Mammalian miRNAs are highly conserved and their genes also have multiple isoforms (paralogs), which are probably generated by duplication, one example is the human let-7 gene that accounts for 8 different isoforms distributed across 11 genomic loci (Lee et al., 2016). Most miRNA genes are located in regions distant from coding genes, suggesting that they represent single transcriptional units and several miRNA genes are clustered and normally transcribed as multicistronic RNA transcripts. Some miRNAs are instead intragenic, being located inside other genes and use the regular transcriptional machinery. miRNA paralogs that are clustered usually have identical seed regions and act redundantly. The biggest online database of miRNAs is the miRbase with the latest version of June 2014 (miRbase 21) listing 24521 miRNA loci from 206 species, processed to produce 30424 mature miRNA products. Of these, 1881 sequences belonged to the human genome (Kozomara and Griffiths-Jones, 2014).

1.1.1. miRNAs biogenesis and target regulation

The biogenesis of miRNAs is a complex process that takes place in the nucleus and in the cytoplasm (Figure 1.1). The main aspects and the proteins involved in this process have been already reviewed by several authors, some in a more broad perspective, some with a more specific viewpoint, but all with elucidating schemes (Breving and Esquela-Kerscher, 2010; Lin and Gregory, 2015; Ohtsuka et al., 2015; Pasquinelli, 2012). In the canonical pathway, miRNA genes are transcribed by RNA polymerase II (Pol II) into stem-loop structures called primary miRNAs (pri-miRNAs) and processed (capped, spliced and polyadenylated) in the nucleus. An individual pri-miRNA can either produce a single miRNA or contain clusters of two or more miRNAs that are processed from a common

primary transcript [e.g. miR-17-92 (He et al., 2005)]. Pri-miRNAs are then cleaved by the double-stranded RNase III enzyme DROSHA and its cofactor, DiGeorge syndrome critical region 8 (DGCR8). DROSHA contains two RNase III domains, each of which cleaves one strand of the double-stranded RNA towards the base of stem–loop secondary structures contained within pri-miRNAs to liberate a 60 to 70 nucleotide hairpin-shaped precursor miRNA (pre-miRNA). In the non-canonical pathway, miRNAs can be processed from introns of protein-coding genes and bypass DROSHA/DGCR8 process. In this case the pri-miRNA is designated by mirtron and is processed by the regular mRNA splicing mechanisms (Westholm and Lai, 2011). The pre-miRNAs are then exported to the cytoplasm by exportin 5 (XPO5) and processed by DICER1, an RNase III enzyme. DICER1 binds to the end of the pre-miRNA with its two catalytic RNase III domains cleaving the double-stranded RNA stem, close to the terminal loop sequence and producing the mature miRNA duplex with 2-nucleotide 3′ overhangs. DICER1 associates with transactivation-responsive RNA-binding protein (TRBP) which binds to double-stranded RNA. Although, TRBP is not essential to process pre-miRNA, it enhances the fidelity of DICER1-mediated cleavage and bridges DICER1 with Argonaute proteins that participate in the assembly of the miRNA induced silencing complex (miRISC), the final step of miRNAs biogenesis.

Target recognition and regulation by miRNAs is by itself a complex process. The small size of miRNAs and the pairing between a miRNA and a target site that does not need to be perfect, offers a wide selection of genes that can be subject to regulation. Indeed, one miRNA can regulate multiple mRNAs. However, the property that makes miRNAs versatile also hampers the prediction of putative targets. Although there are some exceptions, in animals, miRISC form partial duplexes with their targets and usually in the 3′ UTR. The ordinary pairing between miRISC and mRNA is perfect between nucleotides 2 and 8 at the 5′ end of the miRNA, known as the ‘seed’ region, and the target site (Figure 1.2). Due to the small size of the ‘seed’ region one miRNA can pair with mRNA in more than one location

and with different conformations of the bulge. These different conformations can alter miRNA repression efficiency.

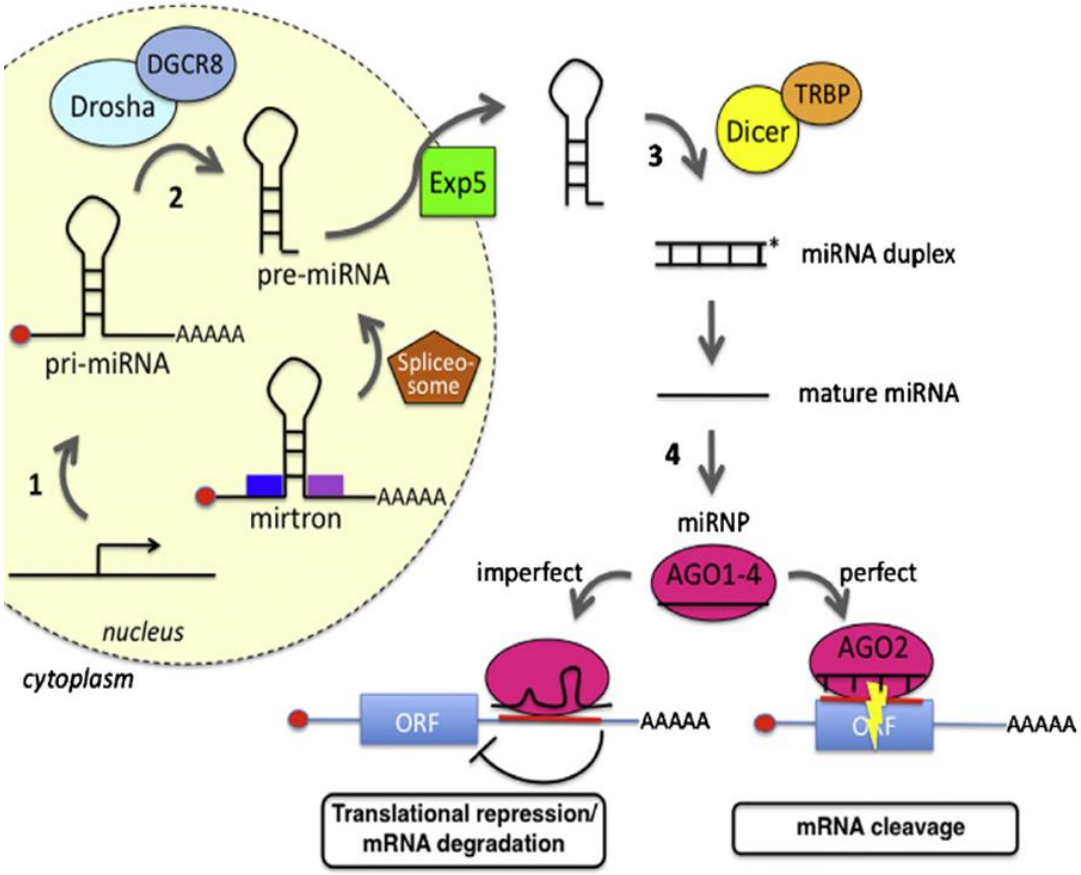

Figure 1.1 - miRNAs biogenesis. miRNAs genes are transcribed into pri-miRNA transcripts (1) that undergo processing by Drosha complexes (2). The resulting hairpin precursor, pre-miRNAs, are transported to the cytoplasm by XPO5. miRNAs can also be encoded in the introns of genes. These miRNAs can circumvent Drosha complexes and produce pre-miRNA precursors directly from byproducts of intron splicing, these miRNAs are denominated mirtrons. At the cytoplasm, the Dicer complex removes the loop region from pre-miRNAs (3), and one strand of the resulting duplex is bound by Argonaute to form miRISC (4), which targets mRNAs for regulation. Depending on the target recognition, gene repression can be by mRNA cleavage or translational repression followed by mRNA degradation. Scheme from (Breving and Esquela-Kerscher, 2010).

The mechanism of miRNAs action has been under scrutiny, since it can occur by mRNA destabilization, translational repression and contrary to what is assumed, activates gene expression. Perfect pairing of the miRNA–mRNA duplex leads to endonucleolytic cleavage of the mRNA by Argonaute. This mechanism is more common in plants, but nevertheless can occur also in animals. Destabilization of the target mRNA through other mechanisms is

a common outcome of miRNA regulation in animals (Figure 1.3). The miRISC complex usually associates with GW182 proteins. Depending on what proteins GW182 recruits next, the process of destabilization is different. Thus, if GW182 recruits CCR4-NOT complex, the mRNA becomes susceptible to exonucleolytic degradation of its poly(A) tail. If GW182 recruits CCR4-NOT complex and PABPC protein, mRNA repression will be prior to translation initiation, if it does not recruit PABPC it will be after translation initiation. Finally, miRISC can associates with fragile X mental retardation protein 1 (FMR1), stimulating gene expression (Cheever and Ceman, 2009). This is a much rarer destabilization mechanism.

This mode of action makes miRNAs extremely versatile and relevant to many cell mechanisms. Thus, their expression deregulation easily becomes involved in many diseases. Indeed, miRNAs have already been described as being relevant in viral diseases (Jopling et al., 2005; Linnstaedt et al., 2010), bacterial infections (Xiao et al., 2009), multiple sclerosis (Keller et al., 2009), type 2 diabetes (Karolina et al., 2011), Parkinson's disease (Martins et al., 2011), Alzheimer's disease (Nunez-Iglesias et al., 2010) and cancer (Balatti et al., 2015; Masood et al., 2015; van Schooneveld et al., 2015), among others. Here, I will focus on how miRNAs influences cancer and how cancer influences miRNAs expression.

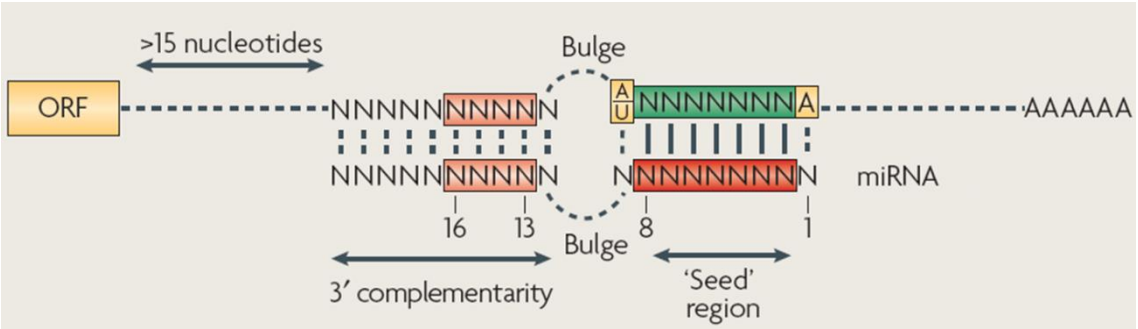

Figure 1.2 – Target recognition by miRNAs. Target base paring is perfect and contiguous from nucleotides 2 to 8. This region is called the 'seed' region. Although the pairing of nucleotide 1 and 9 isn't necessary, an A residue in position 1 of the miRNA, and an A or U in position 9 improve the site efficiency. Usually, bulges or mismatches are in the central region of the miRNA–mRNA duplex. Scheme from (Filipowicz et al., 2008).

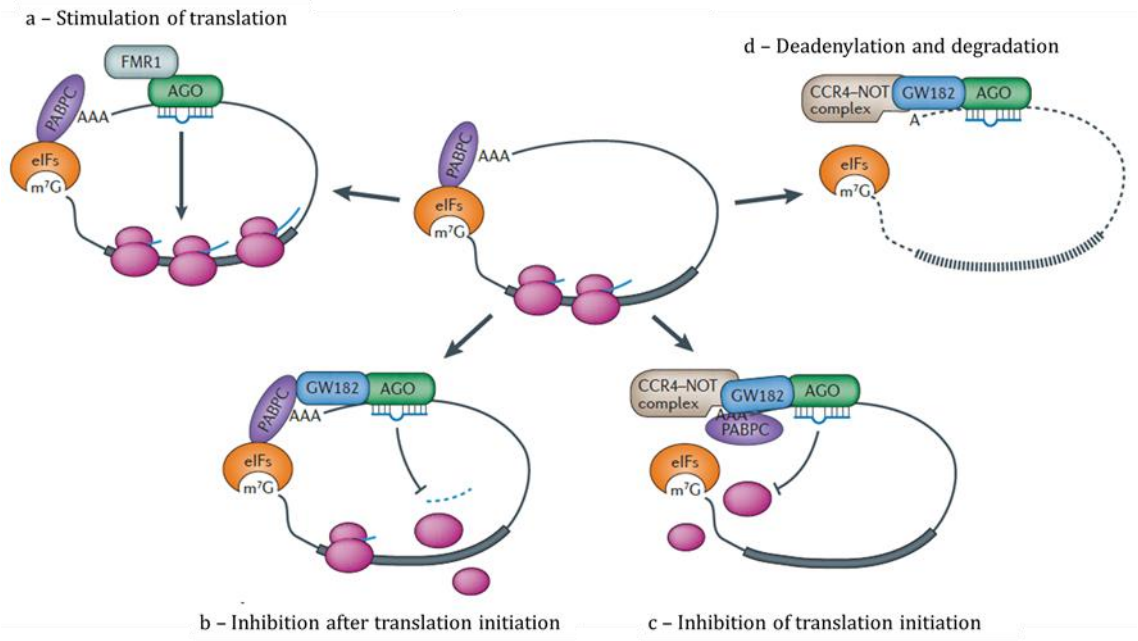

Figure 1.3 – Most frequent mechanisms of gene expression regulation by miRNAs in animals. Depending on the Argonaute (AGO) association with other accessory proteins, the mechanism of mRNA regulation can be different. (a) When AGO associates with fragile X mental retardation protein 1 (FMR1), it can stimulate gene expression. (b and c) When miRISC is in association with GW182 proteins, it represses translation. It can be after translation initiation or through inhibition of the translation process (when GW182 recruits CCR4-NOT complex and PABPC). (d) When AGO associates with GW182 and this one recruits only CCR4-NOT complex the repression is done by deanylation and degradation of mRNA. Scheme adapted from (Pasquinelli, 2012).

1.1.2. miRNAs and cancer

Due to their characteristics and their broad influence in cell homeostasis, soon after their discovery, miRNAs were associated with cancer (Calin et al., 2002; He et al., 2005; Johnson et al., 2005; O'Donnell et al., 2005). In the past years it became evident that miRNAs expression levels differ between normal and tumour cells, have tissue-specific expression signatures and promote or suppress tumour development and progression, thereby influencing all the hallmarks of cancer (Figure 1.4) postulated by Hanahan and Weinberg (Hanahan and Weinberg, 2011).

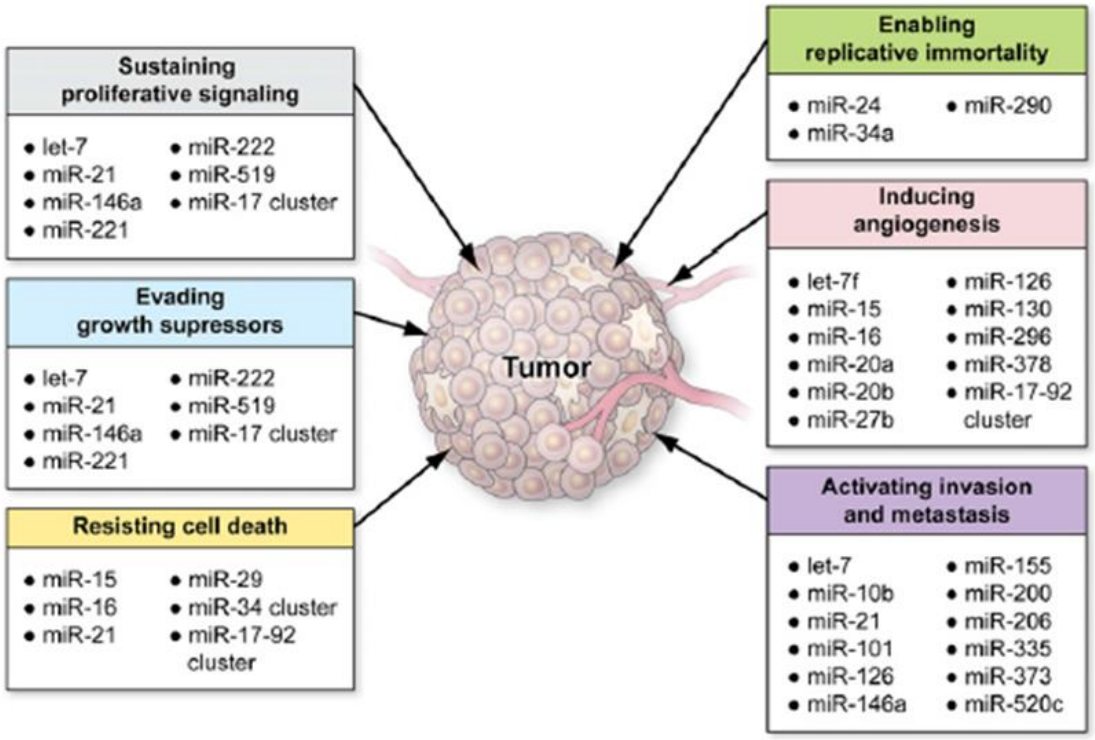

Figure 1.4 – miRNAs involvement in hallmarks of cancer. Several miRNAs can be involved in more than one hallmark. Scheme from (Ross and Davis, 2011).

Recently, miRNAs have also been found in body fluids [e.g. urine and plasma (Armstrong et al., 2015), saliva (Xie et al., 2015), sperm (Metzler-Guillemain et al., 2015) and milk (Melnik et al., 2014)] and seem to exert a role as intercellular messengers by exosome-mediated transfer between different cells in a 'hormone-like' manner (Nishida-Aoki and Ochiya, 2015). Also recently, the idea that miRNAs can have an exogenous origin (xenomiRs), like in milk or plants, and then exert their role in several cell types have come to discussion (Fabris and Calin, 2016). As a matter a fact, miR-200c present in cow milk has been shown to be absorbed in the human intestine and its presence then detected in peripheral blood mononuclear cells (PBMCs) (Baier et al., 2014). The implications of these studies are still not clear, yet they give a new perspective to exposure studies and how diet could influence cancer.

Cancer development involves multiple-step alterations in oncogenes and tumour suppressor genes over a period of time. Abundant data have already been published in

respect to how important the role of miRNAs is among many pathways involved in the pathogenesis of cancer. MiRNAs can function as oncogenes or tumour suppressors in the majority of cancers. Tumour suppressor miRNAs act by repressing oncogenes. These are usually down-regulated in cancer and the majority of the miRNAs are considered tumour suppressors. However, only few miRNAs have been already described as truly tumour suppressors, with functional data published. Curiously, some miRNAs can act as both suppressors and oncogenes, depending on the microenvironment. Oncogenic miRNAs, also known as oncomiRs, are much less frequent and tend to up-regulate oncogenes or supress tumour suppressor genes.

As stated before, the last revision of miRBase lists 1881 miRNAs in humans (Kozomara and Griffiths-Jones, 2014), many of these being connected to several types of cancer. One of the most known tumour suppressor miRNAs, because it was the first to be associated with cancer, is the miR-15/16 cluster. This cluster was first described by Calin and colleagues (Calin et al., 2002) in chronic lymphocytic leukaemia (CLL) and later confirmed that it has a pro-apoptotic action by binding to BCL2 (Cimmino et al., 2005). Other authors also confirmed a tumour suppressor effect of these miRNAs in other cancers like multiple myeloma (Roccaro et al., 2009) and prostate cancer (Bonci et al., 2016). Another known tumour suppressor miRNA is the let-7 family. It was first reported as influencing the oncogene RAS (Johnson et al., 2005), which is overexpressed in many tumour types. Although not directly, recently, it was showed that let-7a regulates PKM2 in gastric cancer (Tang et al., 2016) and exerts influence on cell proliferation, migration, and invasion of tumour cells. This protein is a key enzyme in aerobic glycolysis, being also associated with tumour size and stage. Even though the authors do not show a molecular interaction between let-7a and PKM2, they showed an inverse correlation of their expression. Due to the fact that PKM2 can be regulated by c-MYC and this in turn can be regulated by let-7a (He et al., 2010; Liu et al., 2012), the regulation must occur through c-MYC down-regulation in a let-7/c-MYC/PKM2 axis. The oncogenes high mobility group (HMGA), the

signal transducer and activator of transcription 3 (STAT3) gene and ubiquitin-like with PHD and ring finger domains 2 (UHRF2) gene are also targets of let-7 (Wang et al., 2012b), thus influencing cell cycle and cell proliferation. One further recognised group of tumour suppressor miRNAs is the miR-34 family (miR34a, miR-34b and miR-34c). BCL2 and c-MYC are described as miR-34a targets (Bommer et al., 2007; Christoffersen et al., 2010). Consequently, miR-34 family influences apoptosis and cell cycle regulation. Indeed, up-regulation of miR-34a was associated with oncogene-induced cellular senescence (Christoffersen et al., 2010). The most know feature of this family is that their promoter region is located in a CpG island, thus being methylated in many cancers (Vogt et al., 2011). Perhaps the most studied tumour suppressor miRNAs are from the miR-200 family (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) due to their critical role in the suppression of the epithelial-to-mesenchymal transition (EMT) and tumour cell adhesion, migration, invasion, and metastasis by targeting zinc finger E-box-binding homeobox 1 (ZEB1) and zinc finger E-box-binding homeobox 2 (ZEB2). Also, loss of miR-200 family members correlates with a lack of E-cadherin expression (Korpal et al., 2008). The mesenchymal-to-epithelial transition (MET) is considered to be critical for the late stages of metastasis, enabling the tumour cells to colonize and grow at distant sites. Of thirteen miRNAs with higher expression in metastases compared to primary tumours, four were members of the miR-200 family: hsa-miR-200a, hsa-miR-200b, hsa-miR-200c, and hsa-miR-141 (Gravgaard et al., 2012) once again suggesting their involvement in metastasis and contradicting the idea that the miR-200 family is tumour suppressive. Surprisingly, another study revealed the dual effect of miR-200 family. In this study the authors showed that tumour cells use the tumour suppressor ability of miR-200 family and overexpress it in metastasis. This will enable MET, thus enabling anchorage in distant organs (Banyard et al., 2013). A new published study suggested the same mechanism (Perdigao-Henriques et al., 2016). So, the miR-200 family is down-regulated in the primary tumour to enable cells to enter in EMT and then in distant organs the same cells up-regulate miR-200 family to

facilitate anchorage by MET. DNA methylation was also shown to play an important role in regulating the expression of the miR-200c/141 cluster (Vrba et al., 2010). The miR-200 family was associated with almost all cancer types and other targets were described, however the main action of this miRNA family is its influence in EMT/MET (Gao et al., 2016; Shi and Zhang, 2016). miR-203 is also designated as a tumour suppressor and is known to be regulated by epigenetic mechanisms in oral cancer (Kozaki et al., 2008), hepatocellular carcinoma (Furuta et al., 2010), breast cancer (Zhang et al., 2011), haematological malignancies (Chim et al., 2011) and rhabdomyosarcoma (Diao et al., 2014). miR-203 also influences metastasis by direct targeting of SNAI2 (Shi et al., 2015; Zhao et al., 2015). As for almost all miRNAs, contradictory data has been published regarding miR-203. Very recently, two articles were published showing that miR-203 induces proliferation, migration and invasion in pancreatic ductal adenocarcinoma (Ren et al., 2016) and inhibits proliferation and self-renewal of leukaemia stem cells (Zhang et al., 2016). Although these results seem to be contradictory, they are from different types of cancer and the targets are completely different. In the first study, the authors show that SIK1 is an inhibitor of proliferation, migration and invasion in pancreatic ductal adenocarcinoma and is a direct target of miR-203. Thus, high levels of miR-203 down-regulates SIK1 and consequently increases proliferation, migration and invasion. On the contrary, the second study shows that miR-203 is in an axis with BIRC5 and BMI1 in leukaemia stem cells and that its low levels induce a more oncogenic phenotype. This shows how versatile miRNAs can be, depending on the microenvironment and tissue.

1.1.2.1. Regulation of miRNAs expression levels in cancer

It is known that miRNAs are deregulated in cancer. Nevertheless, how this deregulation occurs is still not fully understood. Most probably, the deregulation occurs during miRNAs biogenesis (Figure 1.5) and tumour cells acquire mechanisms of 'defence' in order to down-regulate miRNAs with tumour suppresser capability and up-regulate miRNAs with oncogenic ability (Breving and Esquela-Kerscher, 2010; Kim et al., 2009; Lin and Gregory,

2015). The control of miRNAs expression can begin in the DNA by inhibiting the miRNAs genes from being transcribed. Tumour cells can acquire genetic alterations, such as mutations or deletions, in the miRNAs genes or Pol II, undergo epigenetic regulation, such as methylation of promoter sequences and histone modifications, and be repressed by oncogenes through targeting of transcription factors. Next in line, DROSHA/DGCR8 can be target of transcriptional regulation themselves by oncogenic proteins or each gene can be mutated in a tumour cell. This can deregulate both proteins levels, thus deregulating the initial processing into pre-miRNAs. Indeed, a feedback loop can occur in DROSHA/DGCR8 complex, where high levels of DROSHA and DGCR8 in the cell leads to a cleavage of *DGCR8* mRNA, which results in the reduction of DGCR8 and in turn reduction of DROSHA (Han et al., 2009). XPO5 and DICER1 can also be subject to genetic alterations and transcriptional regulation. Loss of TRBP, an accessory protein of DICER1, destabilizes DICER1/TRBP complex following impairment of miRNA processing and enhancement of cellular transformation. Finally, mutations in target recognition sites can inhibit the miRISC action. Furthermore single nucleotide polymorphisms (SNPs) in target sites have been studied as well as in miRNAs sequences. As an example, rs72552316 SNP in the miRNA binding sites of toll-like receptor 7 (TLR7) has been associated with bladder cancer (Cheng et al., 2014) and T8473C SNP of cyclooxygenase-2 (COX-2) interferes with miR-542-3p action (Moore et al., 2012). A recent meta-analysis was published showing an association of rs3746444 SNP in miR-499 with cancer (Xu et al., 2015), and that rs11614913 SNP of mir-196a-2, rs4919510 SNP of mir-608, rs6505162 of mir-423, rs11671784 SNP of mir-27a, rs2292832 of mir-149 and rs12355840 SNP of mir-202 are associated with many cancer types in several populations (Pipan et al., 2015). However, these studies need further confirmation in bigger populations, since the discrepancy of data is still large.

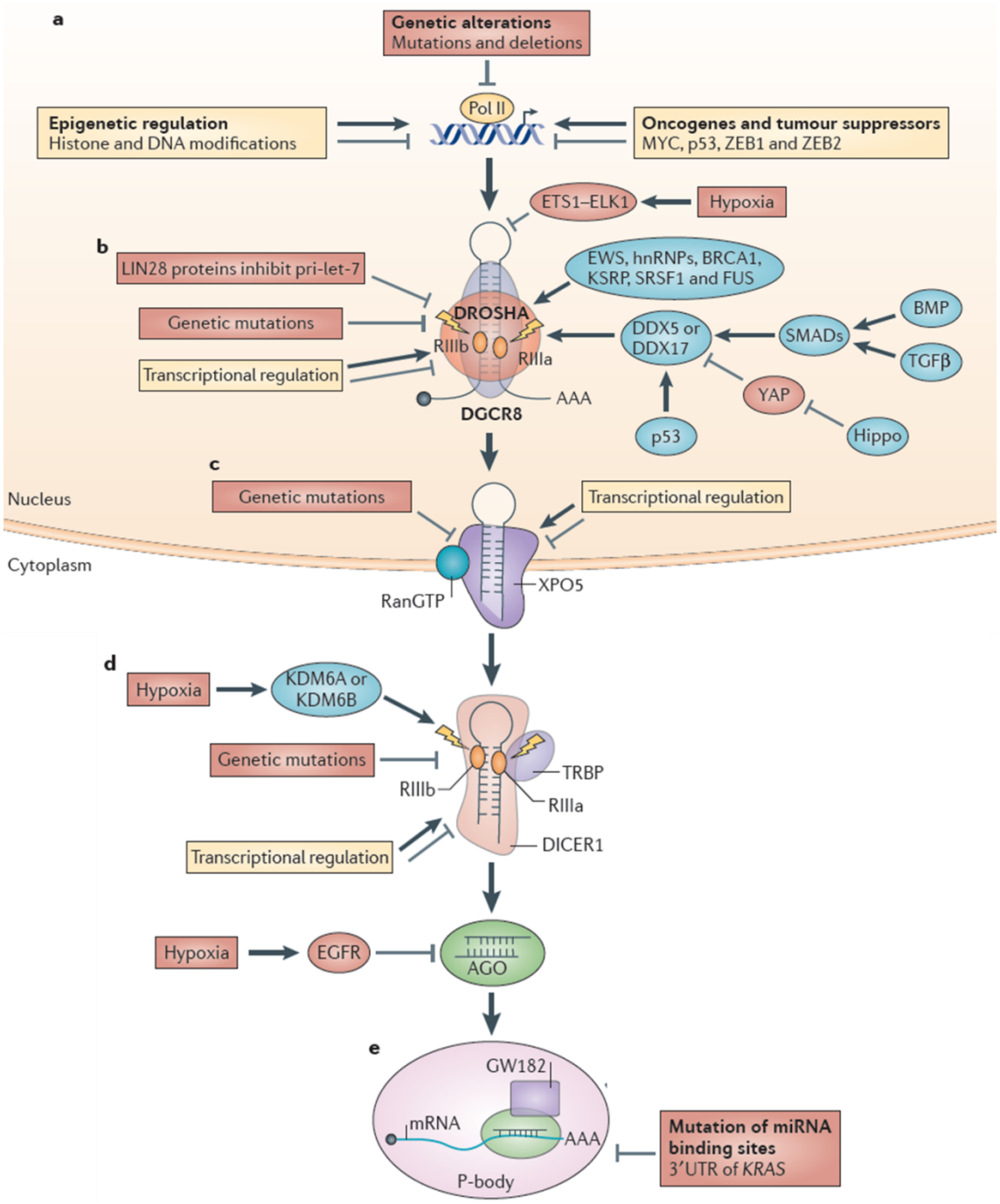

Figure 1.5 – Causes of deregulation of miRNAs expression levels in human cancer. miRNAs expression deregulation most probably happen during their biogenesis. It can happen at: a) DNA level, with genetic alterations, epigenetic modifications and negative or positive regulation by oncogenes and tumour suppressors, respectively; b) pri-miRNA processing, by genetic mutations and transcriptional regulation control of DROSHA and DGCR8 expression and by RNA-binding proteins and other cell signalling pathways; c) Genetic mutations and transcriptional regulation of XPO5; d) pre-miRNA processing, by genetic mutations and transcriptional regulation control of DICER1 expression and function to cleave pre-miRNA and phosphorylation of Argonaute inhibiting miRISC assembly; and e) mutations of miRNA-binding sites in target genes. Scheme adapted from (Lin and Gregory, 2015).

1.1.2.2. miRNAs and drug resistance

Perhaps one of the most studied topics in cancer is resistance to drugs. Soon after their discovery, miRNAs were associated with drug resistance. However, contradictory data have been published and what seemed to be a gold mine to personalized therapy revealed poor results and a lack of applicability. MiRNAs has been linked with all drug resistance pathways (drug metabolism, drug transporters, DNA repair, EMT and cancer stem cells). In Table 1.1 several miRNAs involved in drug resistance mechanisms are described, as well as the type of tumour associated and the drug.

1.1.2.2.1. Drug metabolism

Drug metabolism is a complex pathway of xenobiotic biotransformation that involves several proteins, and is a part of the general steps of drug fate in the organism: Absorption, Distribution, Metabolism and Excretion (ADME). Biotransformation involves two main phases before excretion: Phase I and Phase II. Xenobiotics are foreign compounds (such as drugs) that are not normally produced or expected to be present in an organism. Concerted actions of drug-metabolizing enzymes (DME) lead primarily to an increase in the polarity of xenobiotics, in Phase I reactions, followed by conjugation reactions (Phase II reactions) that increase their polarity but block the reactivity of polar groups introduced in Phase I reactions. Thereafter the transmembrane transport of the resulting metabolite is performed by membrane transporter proteins, essentially ATP-binding cassette (ABC) transporters (Phase III reactions). Besides an increase in transport mediated by ABC transporters, cancer cells tend to overexpress DMEs and evade cancer treatment thus becoming resistant to many drugs.

Although extensive studies have been performed on transcriptional regulation of the DMEs, there is a lack of understanding of their post-transcriptional regulation (Urquhart et al., 2007). One of the key players of the Phase I are cytochrome P450 (CYP) enzymes that catalyses oxidation reactions of the xenobiotics and occasionally reduction reactions

Table 1.1 - Pathways of drug resistance regulated by miRs. (NS - not specified). Table published in (Gomes et al., 2016)

Target gene	microRNA	Type of cancer/established cell line	Drug	Reference
Drug Metabolism				
CYP1B1	miR-27b	Human uterine cervix adenocarcinoma cell line HeLa; Human breast adenocarcinoma cell line MCF-7; Human embryonic kidney cell line HEK293; Human leukemic T-cell line Jurkat; Breast cancerous and adjacent noncancerous tissue	NS	(Tsuchiya et al., 2006)
CYP2E1	miR-378	Human embryonic kidney cell line HEK293	NS	(Mohri et al., 2010)
CYP3A4	miR-27b	Human pancreas cancer PANC1; Human colon carcinoma LS-180; Human embryonic kidney cell line HEK293	cyclophosphamide	(Pan et al., 2009a)
SULT1A1	miR-631	Human breast cancer cell lines ZR75-1 and MCF7; Human mammary epithelial cell line MCF10A;	actinomycin D	(Yu et al., 2010)
GSTP1	miR-133a	Human head and neck Squamous Cell Carcinoma (SCC); Human oesophageal SCC and bladder cell lines	cisplatin and carboplatin	(Moriya et al., 2012)
Drug transport				
ABCB1	miR-451	Human breast adenocarcinoma cell line MCF-7	doxorubicin	(Kovalchuk et al., 2008); (Zhu et al., 2008)
ABCB1	miR-200c	Breast cancerous tissue; Human breast adenocarcinoma cell line MCF-7	doxorubicin	(Chen et al., 2012)
ABCB1	miR-298	Human breast adenocarcinoma cell lines MCF-7 and MDA-MB-231	doxorubicin	(Bao et al., 2012)
ABCB1	miR-27a	Human breast adenocarcinoma cell line MCF-7	doxorubicin	(Zhu et al., 2008)

Table 1.1 (continued) - **Pathways of drug resistance regulated by miRs**. (NS - not specified). Table published in (Gomes et al., 2016)

ABCB1	miR-145	Human colon carcinoma cell line Caco-2; Human embryonic kidney cell line HEK293	NS	(Ikemura et al., 2013)
ABCB1	miR-381	Human chronic myelogenous leukemia cell line K562	adriamycin	(Xu et al., 2013)
ABCB1	miR-495	Human chronic myelogenous leukemia cell line K562	adriamycin	(Xu et al., 2013)
ABCG2	miR-181a	Human breast adenocarcinoma cell line MCF-7	mitoxantrone	(Jiao et al., 2013)
ABCG2	miR-328	Human breast adenocarcinoma cell line MCF-7	mitoxantrone	(Pan et al., 2009b)
ABCG2	miR-487a	Human breast adenocarcinoma cell line MCF-7	mitoxantrone	(Ma et al., 2013)
ABCG2	miR-519c	Human embryonic kidney HEK293; Human breast adenocarcinoma cell line MCF-7	mitoxantrone	(Li et al., 2011b)
ABCG2	miR-328	Human embryonic kidney HEK293; Human breast adenocarcinoma cell line MCF-7	mitoxantrone	(Li et al., 2011b)
ABCC1	miR-326	Normal and tumour breast tissues; Human breast adenocarcinoma cell line MCF-7	VP-16 and doxorubicin	(Liang et al., 2010)
ABCC1	miR-345	Human breast adenocarcinoma cell line MCF-7	cisplatin	(Pogribny et al., 2010)
ABCC1	miR-7	Human breast adenocarcinoma cell line MCF-7	cisplatin	(Pogribny et al., 2010)
ABCC1	miR-1291	Human pancreatic carcinoma cell line PANC-1; Human small lung cancer cell line H69; Human embryonic kidney cell line HEK293	doxorubicin	(Pan et al., 2013)
ABCC2	miR-297	Human ileocaecal colorectal adenocarcinoma cell line HCT-8 and HCT-116; Colorectal cancerous and adjacent noncancerous tissue	oxaliplatin and vincristine	(Xu et al., 2012b)
SLC15A1	miR-92b	Human colon carcinoma cell line Caco-2-BBE	NS	(Dalmasso et al., 2011)

SLC16A1	miR-29a	Hepatoma cell line mhAT3F; Pancreatic beta cell line MIN6; Human embryonic kidney cell line HEK293	NS	(Pullen et al., 2011)
SLC16A1	miR-29b	Hepatoma cell line mhAT3F; Pancreatic beta cell line MIN6; Human embryonic kidney cell line HEK293	NS	(Pullen et al., 2011)
SLC16A1	miR-124	Hepatoma cell line mhAT3F; Pancreatic beta cell line MIN6; Human embryonic kidney cell line HEK293	NS	(Pullen et al., 2011)
DNA Repair				
RAS	let-7 family	Human non-small cell lung cancer cells cell line A549		(Weidhaas et al., 2007)
ERCC1	miR-138	Human non-small cell lung cancer cells cell line A549	cisplatin	(Wang et al., 2011)
MSH2	miR-21	Human Dukes' type C, colorectal adenocarcinoma cell lines Colo-320 DM and SW620; Human colorectal adenocarcinoma cell line HCT-116; Human Dukes' type B, colorectal adenocarcinoma cell line SW480; Human colon carcinoma cell line RKO	5-fluorouracil	(Valeri et al., 2010a)
MSH6	miR-21	Human Dukes' type C, colorectal adenocarcinoma cell lines Colo-320 DM and SW620; Human colorectal adenocarcinoma cell line HCT-116; Human Dukes' type B, colorectal adenocarcinoma cell line SW480; Human colon carcinoma cell line RKO	5-fluorouracil	(Valeri et al., 2010a)
REV1	**miR-96**	**Human Bone Osteosarcoma Epithelial Cell line U2OS; Human uterine cervix adenocarcinoma cell line HeLa; Human breast cancer cell line HCC1937; Human breast adenocarcinoma cell line MDA-MB-231**	**cisplatin; PARP inhibitor AZD2281**	**(Wang et al., 2012c)**

Table 1.1 (continued) - **Pathways of drug resistance regulated by miRs**. (NS - not specified). Table published in (Gomes et al., 2016)

RAD51	**miR-96**	Human Bone Osteosarcoma Epithelial Cell line U2OS; Human uterine cervix adenocarcinoma cell line HeLa; Human breast cancer cell line HCC1937; Human breast adenocarcinoma cell line MDA-MB-231	cisplatin; PARP inhibitor AZD2281	(Wang et al., 2012c)
RAD51	miR-155	Human breast adenocarcinoma cell line MCF-7; triple-negative breast cancer tissue	NS	(Gasparini et al., 2014)
BRCA1	miR-182	Human acute promyelocytic leukemia cell line HL60; Human chronic myelogenous leukemia cell line K562; Human breast adenocarcinoma cell line MCF-7	PARP inhibitor	(Moskwa et al., 2011a)
BRCA1	miR-146a	Breast cancer tissue	NS	Garcia et al. 2011 [102]
BRCA1	miR-193a-5p	Human Mammary Epithelial progenitor Cell line HMEpC; Human Small Airway Epithelial progenitor Cell line HSAEpC; Human breast cancer cell lines MDA-MB-231, MDA-MB-157 and SK-BR-3	cisplatin	(van Jaarsveld et al., 2014)
BRCA1	miR-296-5p	Human Mammary Epithelial progenitor Cell line HMEpC; Human Small Airway Epithelial progenitor Cell line HSAEpC; Human breast cancer cell lines MDA-MB-231, MDA-MB-157 and SK-BR-3	cisplatin; doxorubicin and paclitaxel	(van Jaarsveld et al., 2014)
BRCA1	miR-183	Human Mammary Epithelial progenitor Cell line HMEpC; Human Small Airway Epithelial progenitor Cell line HSAEpC; Human breast cancer cell lines MDA-MB-231, MDA-MB-157 and SK-BR-3		(van Jaarsveld et al., 2014)
BRCA1	miR-16	HSAEpCs	cisplatin and doxorubicin	(van Jaarsveld et al., 2014)
EMT				
	miR-200c	Human breast adenocarcinoma cell line MCF-7	doxorubicin	(Chen et al., 2013b)
	miR-200b	Human breast adenocarcinoma cell line MCF-7 and resistant derivate	4-hydroxytamoxifen; fulvestrant	(Manavalan et al., 2013)

	miR-200c	Human breast adenocarcinoma cell line MCF-7 and resistant derivate	4-hydroxytamoxifen; fulvestrant	(Manavalan et al., 2013)
	miR-200c	Human breast cancer cell line SKBr-3	trastuzumab	(Bai et al., 2014)
MIG6	miR-200c	Several human cancer cell lines		(Izumchenko et al., 2014b)
MAGI2	miR-134/487b/655 cluster	Human lung adenocarcinoma cell lines A549, LC2/ad, PC3, PC9, RERF-LCKJ, RERF-LCMS, PC14, and ABC-1	gefitinib	(Kitamura et al., 2014)
	miR-147	Human colon cancer cell line HCT116 and SW480; Human lung cancer cell line A549	gefitinib	(Lee et al., 2014)
SMAD3	miR-489	Human breast adenocarcinoma cell line MCF-7	doxorubicin	(Jiang et al., 2014)
FBW7	miR-223	The human pancreatic cancer cells AsPC-1 and PANC-1	gemcitabine	(Ma et al., 2015)
Stem cells				
NANOG/OCT4	let-7a	Human head and neck cancer tissues	cisplatin	(Yu et al., 2011)
OCT4 and SOX2	miR-145	Glioblastoma	temozolomide	(Yang et al., 2012)
TP53INP1	miR-130b	Human liver tumour and adjacent non-tumour tissue	doxorubicin	(Ma et al., 2010b)
TP53/NANOG	miR-214	Human ovarian cancer A2780, OV2008, OV8 and SKOV3	cisplatin and doxorubicin	(Xu et al., 2012a)
ABCB1	miR-451	Human colon carcinoma cell lines DLD1, HT29, LS513, SW620, LoVo, and RKO; Colorectal cancer tissue	irinotecan	(Bitarte et al., 2011)
	miR-302	Human head and neck squamous cell carcinoma (HNSCC) cell line HSC-3	Cisplatin	(Bourguignon et al., 2012)

(Rodriguez-Antona and Ingelman-Sundberg, 2006). More than 90% of the reactions involved in the metabolism of all chemicals, whether environmental chemicals, natural ones, physiological compounds, and drugs, are catalysed by P450s (Rendic and Guengerich, 2014). Three-fourths of the human CYP reactions can be accounted for by a set of five CYPs: 1A2, 2C9, 2C19, 2D6, and 3A4, whit the largest fraction of the CYP reactions being catalysed by CYP 3A enzymes (Rendic and Guengerich, 2014). Therefore, the regulation of DMEs is crucial to drug efficacy and may be related to drug failure or drug resistance.

Recent studies have shown that miRNAs also control the expression of some DME (Ikemura et al., 2014; Koturbash et al., 2012; Tsuchiya et al., 2006). However few studies have shown a direct involvement of miRNAs and DME with drug resistance. Indeed, there are only some studies regarding the effect of miRNAs, such as on CYP1B1 (Tsuchiya et al., 2006), that is highly expressed in oestrogen target tissues, and catalyses the metabolic activation of various pro-carcinogens and the 4-hydroxylation of 17β-estradiol, and is also abundant in cancerous tissues (Crewe et al., 2002); on CYP2E1 (Mohri et al., 2010) that catalyses numerous low-molecular-weight xenobiotics and several pro-carcinogens, such as *N*-nitrosodimethylamine and *N*-nitrosomethylethylamine; on CYP3A4 (Pan et al., 2009a) that is highly important in metabolic reactions of several drugs; in the sulfotransferase isoform 1A1 (SULT1A1), a member of the sulfotransferase (SULT) family of phase II detoxification enzymes that catalyse a variety of xenobiotic and endogenous compounds, such as 4-hydroxytamoxifen (Duffel et al., 2001; Mercer et al., 2010; Yu et al., 2010); and on GSTP1 (Moriya et al., 2012), a member of the GST enzyme superfamily that catalyses the conjugation of electrophiles to glutathione in phase II detoxification reactions, including platinum drugs such as cisplatin and carboplatin (McLellan and Wolf, 1999). All these targets and respective miRNAs are summarized in Table 1.1 In spite of these results, miRNA-dependent regulation of expression in DMEs does not seem to be the most important mode of regulation as few miRNA-binding regions are found in the 3'-UTR

of DME genes. Furthermore, the miRNA binding sites described for most of the DMEs are poorly conserved, leading one to speculate that other forms of regulation are more important.

1.1.2.2.2. Drug transport

Drug transport through cell membranes is a critical step in allowing access of pharmacologic agents to intracellular targets and also extrusion of xenobiotics. The involvement of drug transport is probably the most studied mechanism in cancer drug resistance (Gottesman et al., 2002). Multidrug resistance (MDR) is frequently linked to over-expression of one or more of drug transport proteins present in the cytoplasmic membrane. The ABC transporters have an important cellular role in the efflux and influx of several substrates necessary to the cell and also in the efflux of toxic endogenous molecules and xenobiotics (Gromicho et al., 2013; Rodrigues et al., 2012). Up to now, 49 different ABC transporters were identified and classified in 7 families from ABCA through ABCG (Dean et al., 2001; Kathawala et al., 2014). The relevance of miRNAs in regulating the expression of ABC transporters has been recently reviewed (Haenisch et al., 2014; Ikemura et al., 2014).

One of the most well-known ABC transporters is ABCB1, also known as MDR1 or P-gp transporter. In chemotherapeutic-resistant cancer cell lines, ABCB1 is often observed to be up-regulated. Until now, some reports have been published regarding association of miRNAs, ABCB1 and resistance to doxorubicin. These miRNAs can be seen in Table 1.1 Most drug transport-associated miRNAs are down-regulated, which in turn increases the levels of ABCB1 and consequently enhances resistance to doxorubicin. As an example, diminished expression of miR-451 correlated with higher expression of ABCB1 in drug resistant cells (Kovalchuk et al., 2008; van Jaarsveld et al., 2010). On the contrary, in a human ovarian cancer cell line, and its multidrug resistant counterpart, as well as in a human cervix carcinoma cell line and its multidrug resistant variant, expressions of miR-

27a and miR-451 were up-regulated in multidrug resistant cells as compared with their parental lines, in effect down-regulating expression of the *ABCB1* gene (Zhu et al., 2008). These results show that at the moment caution should be taken in evaluating the involvement of specific miRNAs in drug resistance, since results could depend on various factors, including the cell lines under study.

One more ABC transporter is ABCG2 (also known as BCRP) that, in normal tissues, functions as a defence mechanism against xenobiotics. ABCG2 recognizes and transports a variety of chemotherapeutic drugs out of cancer cells, thereby resulting in reduced drug concentration, and subsequent drug resistance. Consequently ABCG2 plays a critical role in the development of MDR in breast cancer (Natarajan et al., 2012). Increased ABCG2 expression has been found in breast cancer cells that exhibit resistance to mitoxantrone (MX), topotecan and 7-ethyl-10-hydroxycamptothecin (SN-38) (Shiozawa et al., 2004). Up-regulation of ABCG2 also confers resistance to tamoxifen in breast cancer cells (Selever et al., 2011). In addition, ABCG2 expression correlates with chemotherapeutic response to anthracycline in patients with breast cancer (Burger et al., 2003). Differential expression patterns of miRNAs that target ABCG2 have been published (Table 1.1). Interestingly, most studies regarding ABCG2 and miRNAs only associates with MX.

Another well-known ABC transporter is ABCC1, also known as MRP1. The main subtracts of ABCC1 are vincristine and etoposide and ABCC1 also confers resistance to anthracyclines (doxorubicin, daunorubicin, epirubicin), MX, flutamide, and methotrexate. Curiously, many drugs are only transported in the presence of glutathione (Cole, 2014). Regarding ABCC1, only three reports were published to date showing a regulation by miRNAs (Table 1.1) (Liang et al., 2010; Pan et al., 2013; Pogribny et al., 2010).

ABCC2, also known as MRP2, and ABCC1 share a 49% amino acid identity. As ABCC1, this efflux pump need the presence of glutathione and can transport MX, cisplatin, irinotecan, paclitaxel and vincristine. ABCC2 is expressed in some solid tumours from the kidney,

colon, breast, lung, ovary, and as well as in cells from patients with acute myelogenous leukaemia (Chen and Tiwari, 2011). Regarding ABCC2, to our knowledge, only one article has been published associating miRNAs and ABCC2, showing that miR-297 targets the 3'UTR region of ABCC2 transcripts and consequently down-regulates its expression (Xu et al., 2012b).

Intestinal epithelial cells are responsible for the absorption of most cancer drugs, and they express a variety of influx transporters specific for drugs, amino acids, peptides, organic anions, organic cations, and other nutrients. Peptide transporter 1 (PEPT1/SLC15A1), organic cation/carnitine transporter 2 (SLC22A5), organic anion transporting polypeptide 2B1 (SLCO2B1), and monocarboxylate transporter 1(MCT1/SLC16A1) are expressed at the brush-border membrane, whereas organic cation transporter 1 (SLC22A1) is mainly expressed at the basolateral membrane in the small intestine (Ikemura et al., 2014). Recent studies have indicated that the regional differences in the expression of these transporters are dependent on the differentiation of intestinal epithelial cells (McKenna et al., 2010). Hence, misexpression of miRNAs could have a marked impact on absorption of cancer drugs. There are a limited number of reports on the SLC transporters regulated by miRNAs (Table 1.1). One example is mR-92b that was showed to regulate SLC15A1, causing decreased influx activity (Dalmasso et al., 2011). MiR-29a, miR-29b, and miR-124 can target SLC16A1 also, resulting in decreased expression at the protein level. The authors refer that this regulation mechanism is not the main regulator but complement other transcriptional mechanisms and mutations that alter SLC16A1 expression (Pullen et al., 2011).

1.1.2.2.3. DNA repair

DNA damage by endogenous or exogenous agents elicits a powerful cellular response called the DNA Damage Response (DDR), which evokes concerted molecular pathways to detect, repair, induce cell cycle arrest to allow repair, or in cases of high numbers of DNA

lesions or irreparable damage, apoptosis or cellular senescence (d'Adda di Fagagna, 2008; Harper and Elledge, 2007; Jackson and Bartek, 2009; Pearl et al., 2015). In the past years evidence has accumulated that drug resistance is also linked to alterations in these pathways (Helleday et al., 2008; Kelley, 2011; Kelley, 2012; Kelley and Fishel, 2008). The DDR pathways include DNA tolerance mechanisms by error-prone polymerases, the direct reversal of lesions, essentially de-alkylation of alkylated bases by O^6-methyl-guanine-DNA methyltransferase (*MGMT*), alkylation repair homolog 2 (*ALKBH2*) and alkylation repair homolog 3 (*ALKBH3*); nucleotide excision repair (NER); base excision repair (BER); mismatch repair (MMR); and the double strand break (DSBs) repair by homologous recombination (HR) and non-homologous end joining (NHEJ) (Hoeijmakers, 2001; Hoeijmakers, 2009). Besides these signalling cascades, the DDR also elicits the induction of several ncRNAs, including miRNAs. A large number of miRNAs are transcriptionally induced upon DNA damage and the level of induction is variable depending on cell type and the nature and the intensity of DNA damage and time after DNA damage (Chowdhury et al., 2013; d'Adda di Fagagna, 2014; Pothof et al., 2009; Sharma and Misteli, 2013; Templin et al., 2011; van Jaarsveld et al., 2014). Conversely, many miRNAs target DDR genes, thus controlling feed-back and feed-forward loops to fine-tune the response (for a review see (Bottai et al., 2014; Sharma and Misteli, 2013; Wouters et al., 2011)). Seventy-four (52% of the total) mammalian DNA repair and DNA damage checkpoint genes contain conserved miRNA target sites predicted in their 3'-UTR by the algorithms Targetscan, Miranda or both (Wouters et al., 2011).

The knockdown of the miRNA biogenesis pathway proteins DICER and AGO2 results in increased sensitivity to ultraviolet light (UV) and altered cell cycle after UV damage (Pothof et al., 2009). This was one of the first indications that miRNAs are implicated in the regulation of the DDR. Following this study many reports have shown that different DNA damaging agents induce different patterns of miRNAs expression (Wouters et al.,

2011). Thus, it is conceivable that alterations in miRNAs are involved in tumour response to anti-cancer agents.

A few examples indicate indeed that aberrant expression of miRNAs is associated with DNA-damage by radiation or chemicals (Blower et al., 2008; Weidhaas et al., 2007). Members of the let-7 family of miRNAs are rapidly down regulated upon ionizing radiation in A549 lung cancer cells. Conversely, over-expression of the let-7 family leads to radiosensitization *in vitro* of lung cancer cells and *in vivo* in a *C. elegans* model of radiation-induced cell death. In *C. elegans*, this was shown to occur partly through control of the proto-oncogene homologue let-60/RAS and genes in the DNA damage response pathway (Weidhaas et al., 2007). In another example, miR-138 was shown to target the ERCC1 gene, involved in NER, increasing the sensitivity of A549/DDP cells to cisplatin *in vitro* and increase apoptosis (Wang et al., 2011). Similarly, MMR proteins MSH2 and MSH6 are inhibited by miR-21 over-expression causing a reduction in 5-fluorouracil (5-FU) induced G2/M damage arrest and apoptosis, in vitro (Valeri et al., 2010b). REV1, an error-prone Y-family DNA polymerase required for translesion synthesis across interstrand crosslinks, was validated as target of miR-96. Equally, overexpression of miR-96 promoted cellular hypersensitivity to cisplatin *in vitro* and *in vivo* and enhanced sensitivity to the PARP inhibitor AZD2281. This miRNA also targets RAD51, a recombinase that promotes HR repair of DSBs and interstrand DNA crosslinks (ICLs) (Wang et al., 2012c). Over-expression of miR-155 was related with low levels of RAD51 and with better overall survival of patients with triple-negative breast cancers (TNBC) (Gasparini et al., 2014). This raises the possibility of personalized therapy in TNBC patients, knowing the miR-155 levels.

BRCA1 is an important component of the DDR pathway implicated in HR but also involved in other DNA repairs pathways. BRCA1 encodes a nuclear phosphoprotein and primarily functions to maintain genomic stability via critical roles in DNA repair, cell

cycle checkpoint control, transcriptional regulation, apoptosis and mRNA splicing (Savage and Harkin, 2015). Mutations in BRCA1 are associated with an increased risk of developing breast and ovarian cancer. BRCA1 is also a target of miR-182 (Moskwa et al., 2011b). Indeed, the authors showed that high expression of this miRNA in various breast tumour lines impacts BRCA1 levels and sensitivity to PARP1 inhibition. MiR-146a and miR-146-5p also bind to the same site in the 3'-UTR of BRCA1 and down-regulate its expression. In breast tumours, levels of these miRNAs are inversely correlated with BRCA1 protein. Likewise, these miRNAs are over-expressed in TNBC, the most common type of breast cancer in women with BRCA1 mutations (Garcia et al., 2011).

1.1.2.2.4. Epithelial to mesenchymal transition (EMT)

Metastasis is a dreadful event as it may lead ultimately to death in most cancers. The growth of cancer cells at distant organs from a different tissue requires complex processes of detaching from the original tissue; invasion through the basement membrane; movement in the bloodstream or lymphatic system; and anchorage in other organs. The initial process is called EMT and is characterized by a phenotypic change of the tumour cells from cell–cell adhesion and polarity to motility, invasiveness and some of the features of stem cells. This process not only enables the spread of the tumour cells but also their anchorage in distant organs, since tumour cells that undergo EMT can reverse this characteristic and acquires the epithelial phenotype again, in a process called mesenchymal-to-epithelial transition (MET). In EMT, cells lose the expression of E-cadherin and gain the expression of vimentin, N-cadherin, and fibronectin, markers of mesenchymal phenotype. Presumably, EMT is sustained by transient molecular changes and not by permanent genetic alterations. Indeed, the reversible nature of EMT must be associated with reversible epigenetic mechanisms, which allows stable but reversible modifications that do not directly affect the DNA primary sequence (Li and Li, 2015; Lindsey and Langhans, 2014; Zielinska et al., 2015).

MiRNAs, as post-transcriptionally regulators, are good candidates as EMT regulators and, as epigenetic mechanisms, do not affect the primary DNA sequence and can press tumour cells to acquire an EMT phenotype in the tumour microenvironment. The most studied case is the miR-200 family that targets at least two transcriptional repressors of E-cadherin, ZEB1 and ZEB2. This subject will be later detailed in chapter 4 of this thesis.

In lung adenocarcinoma it was shown that the miR-134/miR-487b/miR-655 cluster promotes the EMT through TGF-β signalling and induces resistance to gefitinib by directly targeting MAGI2, whose suppression encompassed loss of PTEN stability (Kitamura et al., 2014). Another example is the over-expression of miR-147, which alone induced reversal of EMT and consequently reversal of the native drug resistance of the colon cancer cell line HCT116 to gefitinib. Although the specific mechanism of action of miR-147 is still unknown, the authors found that miR-147 significantly up-regulates CDH1 and represses ZEB1, known EMT markers, and inhibited TGF-β1 expression and also repressed AKT phosphorylation, leading to gefitinib sensitivity (Lee et al., 2014). MiR-489 under-expression in a MCF7/DOX was also connected with EMT. On the contrary, SMAD3, involved in TGF-β-induced EMT, is over-expressed in the same cell line. Ectopic expression of mir-489 not only reversed mesenchymal features, but also sensitized the breast cell line to doxorubicin, through inhibition of SMAD3 (Jiang et al., 2014). These studies underline the importance of TGF-β signalling in EMT and the regulation of EMT influenced drug resistance by miRNAs. MiR-223 was also associated with drug resistance and EMT in pancreatic cancer. In fact, it is up-regulated in gemcitabine-resistant pancreatic cancer cells, thus acting as an oncogene, most probably, through inhibition of FBW7 which consequently overexpresses NOTCH1. The authors also showed that by inhibiting miR-223, pancreatic cancer cells were sensitized to gemcitabine (Ma et al., 2015).

1.1.2.2.5. Cancer stem cells and drug resistance

Although it is accepted that most tumours arise from a single mutated cell, a monoclonal event, the tumour itself is a sum of numerous types of cells, due to the heterogeneity derived from a continuous evolution of the primitive cancer cell. Although cancer stem cells (CSC) have been well characterized in haematological malignancies, their existence in other tissues has been much debated (for a review see (Pattabiraman and Weinberg, 2014)). Over the past years CSC have been identified using stem cell specific markers in several solid tumours including breast, brain, colon, prostate and pancreatic cancer (Al-Hajj et al., 2003; Li et al., 2007; O'Brien et al., 2007; Singh et al., 2003). Moreover, the possible existence of CSCs within tumours is intimately linked to tumour heterogeneity and tumour dedifferentiation.

Several miRNAs have been shown to regulate stemness, or what we consider as properties of tumour-initiating and maintaining cancer cells, of different cancer types. Recent studies showed differential expression of certain miRNAs between CSC and their differentiated counterparts (Liu and Tang, 2011; Shimono et al., 2009a; Tay et al., 2008), suggesting that miRNAs could also be involved in the regulation of CSC. Therefore, several miRNAs have been reported to regulate stem cell properties and drug resistance concomitantly (Table 1.1) (Raza et al., 2014).

As an example, was showed that let-7a expression was significantly decreased while NANOG/OCT4 expression was increased in head and neck cancer tissues as compared to adjacent normal cells (Yu et al., 2011). Likewise, the authors showed that these differently expressed proteins and miRNA is associated with resistance to cisplatin. Similarly, it was observed that human head and neck squamous cell carcinoma derived HSC-3 cells contain a subpopulation of CSCs characterized by the expression of stem cell markers (OCT4, SOX2, and NANOG) and shows an up-regulation of miR-302 which, in turn, up-regulates several survival proteins responsible for clonal formation, self-renewal and cisplatin

resistance (Bourguignon et al., 2012). However, the authors do not show a direct target of miR-302. Similarly, miR-214 regulates ovarian cancer cell stemness and chemoresistance towards cisplatin and doxorubicin treatment by targeting TP53/NNANOG, and expression of p53 abrogated miR-214-induced ovarian CSC properties (Xu et al., 2012a). In another study, expression of miR-145 was showed to be inversely correlated with the levels of OCT4 and SOX2 in glioblastoma-CD133+ (GBM-CD133+) cells and malignant glioma specimens (Yang et al., 2012). Additionally, miR-145-treated GBM-CD133$^+$ cells suppressed the expression of stemness (NANOG, c-MYC, and BMI1), drug-resistance (ABCG2, ABCC5, ABCB1) and anti-apoptotic genes (BCL2, BCL-xL) and increased the sensitivity of the cells to radiation and temozolomide (Yang et al., 2012). In hepatocellular carcinoma, miR-130b was shown to be associated with CSC growth that leads to worse overall survival and more frequent recurrence of cancer in patients. Also, cells transfected with miR-130b presented a higher resistance to doxorubicin (Ma et al., 2010b).

Several of these studies have used cell lines *in vitro* that express stem cell markers, however, one must keep in mind that these cell lines have vastly altered karyotypes (e.g. several translocations, insertions and deletions) that will obviously alter their biological behaviour. Therefore, caution must be exercised in interpreting the results described.

1.1.3. miRNAs and breast cancer

In spite of the huge scientific progression in breast cancer investigation, much is still needed to better understand this heterogeneous disease. After all these years of research, physicians still have a paucity of tools to a better diagnose breast cancer and its genetics can still be divided in high (*BRCA1*, *BRCA2*, *PTEN*, *TP53*, *CDH1*, and *STK11*), moderate (*CHEK2*, *BRIP1*, *ATM*, *PALB2*) and low (e.g. SNPs in several genes) penetrance genes (Shiovitz and Korde, 2015). Furthermore, physicians, more often than not, use gene panels currently available for breast cancer risk assessment in cases where a hereditary predisposition already exists (Foekens et al., 2006; Paik et al., 2004; van 't Veer et al.,

2002). Since hereditary breast cancer only accounts for approximately 10% of the cases, the remaining 90% still do not have accurate panels for risk assessment or to better diagnosis. In this matter, many studies attempted to correlate individual SNPs with individual susceptibility to sporadic breast cancer (Conde et al., 2009; Silva et al., 2009; Silva et al., 2006a; Silva et al., 2006b; Silva et al., 2007; Silva et al., 2010). In fact, since genetic variations may show very little effect individually but a strong effect conjointly, we explored the applicability of decision trees, in association with Professor Arlindo Oliveira in the Department of Computer Science and Engineering of Instituto Superior Técnico (IST), in order to ascertain significant differences when several variants of the same gene were analysed together (Anunciação et al., 2010). In this study we obtained a significant association between two SNPs of BRCA2 and alcohol consumption. Although these results were promising, as currently sequencing costs dropping to affordable prices, breast cancer genome sequencing can provide much more information at several levels, including point mutations, insertions, deletions, copy number variations and translocations (Campbell et al., 2008; Dieci et al., 2016; Dunning et al., 2016; Shah et al., 2009; Verigos and Magklara, 2015). Thus, further studies in bigger populations were abandoned. New technologies also allow a further classification of breast cancer into Luminal A, Luminal B, HER2 and Basal-like after the initial characterization in 2000 by Perou and colleagues (Perou et al., 2000). This molecular classification of breast cancer is routinely accomplished by pathologists through immunostaining of the tumour tissues for oestrogen receptor (ER), progesterone receptor (PR), human epidermal growth receptors (HER2), the proliferation marker Ki-67 and various cytokeratins (Figure 1.6) (Dowsett et al., 2011; Hammond et al., 2010; Wolff et al., 2013).

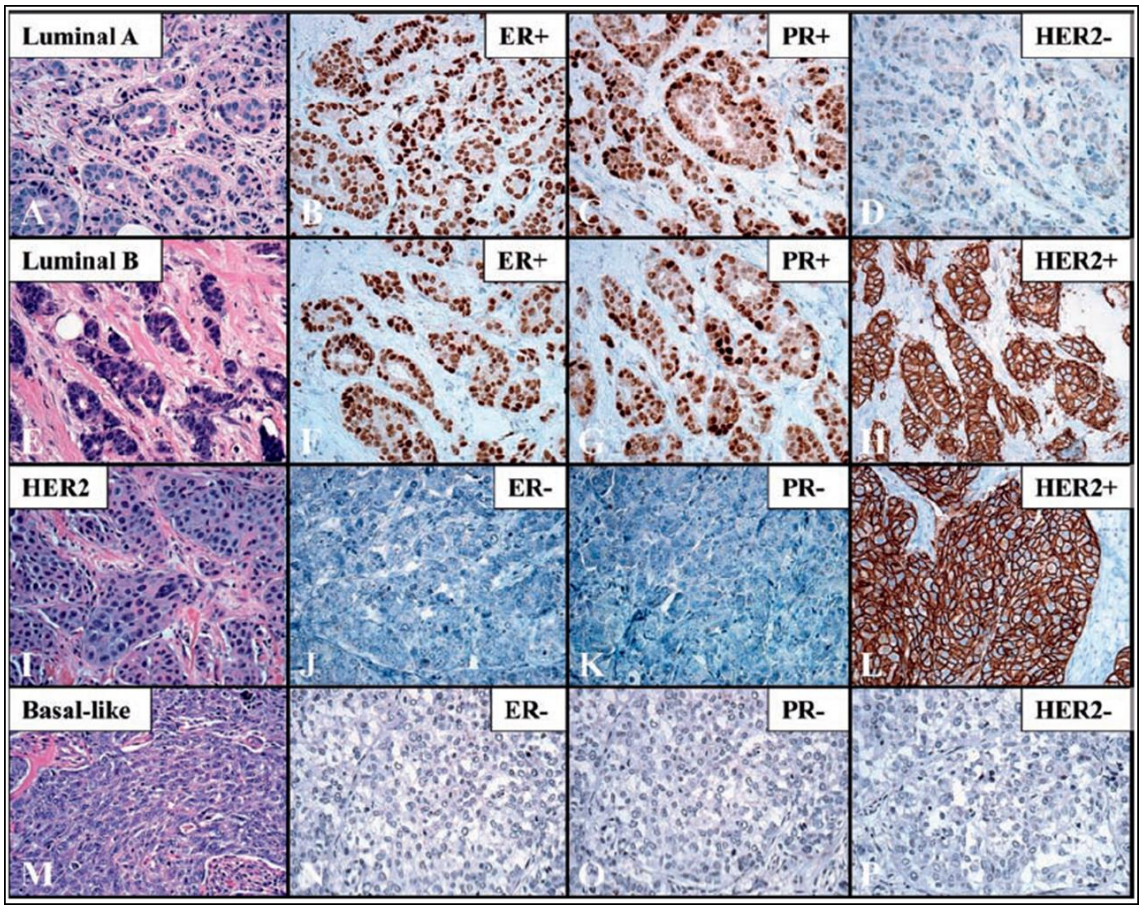

Figure 1.6 - Major molecular subtypes of breast cancer determined by gene profiling. Luminal A (Panels A-D): ER+ and/or PR+, HER2−, and low Ki67 (<14%); Luminal B (Panels E-H): ER+ and/or PR+ and HER2+ (luminal-HER2 group), or ER+ and/or PR+, HER2−, and high Ki67 (>13%); HER2 (Panels I-L): ER−, PR−, and HER2+; Basal-like (Panels M-P): ER−, PR−, HER2−, and CK5/6 and/or EGFR+**.** Figure from (Sandhu et al., 2010).

Approximately 70% of invasive breast cancers are ER positive. These are classified as luminal cancers which are sub-classified into luminal A and luminal B subtypes based on their proliferation rate (Ki-67 expression) and HER2. The majority of ER positive tumours also express PR. The ER negative breast cancers are sub-classified as HER2 positive (approximately 15% of all the breast cancer) and as triple-negative, based on HER2 over-expression/gene amplification. Basal-like breast cancers (approximately 15% of all breast carcinomas) are distinguished from other kind of triple-negative breast cancers by expression of citokeratins 5, 6, 14, 17, 34 or P63. More recently, additional sub-types, such as the claudin-low, have been identified, while the existence of the normal-like subtype is still debatable as it could be an artefact of gene profiling due to a disproportionally high content of normal cells. Usually, the clinical outcome of the patients with normal-like

subtype is better than basal-like and HER2 but worse than that of luminal group (Lavasani and Moinfar, 2012; Perou, 2010; Prat and Perou, 2011; Sabatier et al., 2014).

The ER positive breast cancer subtypes (luminal A and luminal B) usually show a good prognosis and excellent long-term survival (approximately 80%–85% 5-year survival). This must be due to the availability of effective therapy, mainly, tamoxifen and aromatase inhibitors. The low-grade luminal A tumours are regularly treated only with anti-oestrogens, whereas the high proliferative luminal B tumours often have lower expression levels of ER, lower or no PR expression, and are considered to have lower sensitivity to endocrine treatment and higher sensitivity to chemotherapy. In contrast, HER2 positive and basal-like tumours are difficult to treat and are associated with poor prognosis (approximately 50%–60% 5-year survival). Although HER2 positive tumours have an aggressive progression, the survival rate has improved in the last decade due to the use of targeted therapies like trastuzumab, an antibody against HER2, which have been shown to be effective in 20% of patients. Basal-like tumours, although more aggressive than other tumour types, can be especially sensitive to chemotherapy but promising strategies are being developed to treat these type of cancer, such as poly-ADP ribose polymerase-1 (PARP) inhibitors. Regarding claudin-low breast cancer, this subtype has a poor long-term prognosis (Ignatiadis and Sotiriou, 2013).

As stated before, these protein biomarkers are used as surrogates for gene expression analysis to determine molecular subtype. If from one side it has been argued that the definition of breast cancer subtypes using immunohistochemistry (IHC) is not able to recapitulate the information provided by the gene expression, from the other side it has been suggested that the additional clinical value of the molecular classification is limited by its close correspondence to ER and HER2 status and proliferation markers defined by IHC. Nevertheless, these biomarkers have shown limited capacity to predict individual patient outcomes since patients with the same clinicopathological features can have different clinical outcomes.

In recent years miRNAs have been extensively associated with breast cancer. The use of genome-wide approaches has enabled the production of miRNAs fingerprints in tumours and in its normal counterpart. As a result, miRNA expression signatures (miRNome) allowed different types of cancer to be discriminated with high accuracy and the tissue of origin of poorly differentiated tumours to be identified (Volinia et al., 2006). With this, improved prognostics and better decisions regarding therapy can be made. Indeed, many miRNAs have been associated with different subtypes of breast cancer. A comprehensive review by Serpico and colleagues (Serpico et al., 2014) show a vast list of miRNAs differently expressed in luminal, HER2, basal-like and normal-like subtypes of breast cancer (Table 1.2). In this case the targets regulated by these miRNAs are not the most important, but the signature in these subtypes. In a near future these signatures could enhance treatment due to a better classification.

Table 1.2 – Differently expressed miRNAs in breast cancer subtypes. Table adapted from (Serpico et al., 2014)

Subtype	miRNAs signature	
Luminal A	Up-regulated	miR-191, -26, -126, -136, -100, -99a, -145, -146b, -10a, -199a/b, -130a, -30a, -224, -214, let-7a/b/c/f, -342
	Down-regulated	miR-206, -15b, -107, -103
Luminal B	Up-regulated	miR-191, -26, -106a/b, -93, -25, -10a, -30a, -224, let-7b/c/f and -342, -15b, -107, -103
	Down-regulated	miR-206, -100, -99a, -130, -126, -136, -146b
HER2 positive	Up-regulated	miR-150, -142, -148a, -106b, -93, -155, -25, -187, -375
	Down-regulated	miR-125a/b
Basal-like	Up-regulated	miR-150, -142, 148a, -106a/b, -18a, -93, -155, -25, -187, -135b
Normal-like	Up-regulated	miR-142-5p, -135b, -126, -136, -100, -99a, -145, -10a, -199a/b, -130a, -30a-3p, -214, -7a/c

Many of these miRNAs have been described as influencing cancer and particularly, breast cancer (Hemmatzadeh et al., 2016; Serpico et al., 2014). In spite of several studies performed through the years, few miRNAs have been associated with breast cancer and with validated targets. In Table 1.3, some miRNAs known to have oncogenic or tumour suppressor effects in breast cancer and their respective validated targets are described. As

shown in Table 1.3, many miRNAs has redundant target regulation and can influence many cell pathways. Others have been associated, however the mechanisms of action are not known.

As mentioned before, miRNAs have an important role in regulating multi-drug resistance. Table 1.1 shows several miRNAs affecting DME in breast cancer, mostly in human breast cell lines. Few studies though have shown an association between miRNAs and drug resistance in patients. As an example, a recent study revealed that miR-200c was up-regulated in BC tissues from chemoresistant patients compared to responders (Lv et al., 2014). Conversely, Chen and colleagues (Chen et al., 2012) showed a correlation of the low expression of miR-200c with poor response to neoadjuvant chemotherapeutics using breast cancer tissues. In these cases the actual mechanism behind the drug resistance is not known, however through studies performed in cell lines, some clues can be identified. Other pathways can influence drug resistance and consequently be target of miRNAs. As an example, miR-15a/16 down-regulation leads to an increase of the anti-apoptotic protein BCL2 and consequently resistance to tamoxifen. In fact, this happens through an alternative regulation of miR-15a/16 cluster by oncogenic HER2Δ16 that is detected in more than 30% of the ER positive tumours (Cittelly et al., 2010). Consequently, this could be a justification for the poor therapy results in some patients. On the contrary, miR-221/222 cluster functions as an oncogenic miRNA by targeting the cell cycle inhibitor $p27^{Kip1}$. Low levels of $p27^{Kip1}$ were associated with tamoxifen resistance (Miller et al., 2008). Interestingly, increased level of miR-221/222 cluster, probably by targeting the same protein mentioned before, influences fulvestrant resistance. In this case, miR-221/222 can function as a tumour suppressor and an oncogenic miRNA. With fulvestrant treatment, miR-221/222 levels increase and by targeting ERα acts like a tumour suppressor. However, long periods of estradiol deprivation can lead to a constitutive increased expression of miR-221/222 that becomes oncogenic by targeting cell cycle inhibitors like $p27^{Kip1}$. The dual action of a miRNA or in this case a miRNA cluster was

demonstrated (Rao et al., 2011). Another example is miR-128a, a hormone-responsive miRNA, which is over-expressed in letrozole-resistant cell lines and by targeting TGFβ can enhance cell growth and resistance to letrozole. Indeed, inhibiting miR-128a, the inhibitory effect of TGFβ is restored and cells became sensitized to letrozole. Here, the potential effect of miR-128a in drug resistance and also the mechanism of miR-128a expression regulation was demonstrated, since it is highly expressed in cells with high levels of hormones (Masri et al., 2010). Indeed, miR-125b is up-regulated in a drug resistant cell line. Precisely, miR-125b is over-expressed in taxol-resistant cell lines inhibiting the apoptotic effect of taxol. This resistance must due to the fact that miR-125b targets BAK1, a pro-apoptotic protein (Zhou et al., 2010). Interestingly, the concomitant administration of a miR-21 inhibitor and taxol, improved the toxicity of this chemotherapeutic agent and consequently apoptosis in the MCF-7 cell line. This study shows the potential of using miRNAs inhibitors in some tumours. However, precaution and much more studies must be performed regarding possible side effects of using miRNAs/anti-miRNAs as a therapy (Mei et al., 2010). Regarding targeted therapy to HER2/3, trastuzumab seems to be influenced by miR-21(Gong et al., 2011) and lapatinib and gefitinib by miR-205 (Iorio et al., 2009).

Table 1.3 – miRNAs with known oncogenic and tumour suppressor effect in breast cancer.
Table adapted from (Hemmatzadeh et al., 2016) and (Serpico et al., 2014)

miRNA effect	miRNA	Target in breast cancer
Oncogenic	miR-9	LIFR, E-cadherin
	miR-10b	RHOC, HOXD10
	Cluster 17/20	AIB1, cyclin D1
	miR-21	BCL2, PDCD4, PTEN, TPM, SERPINB5, TIMP3
	miR-27a/96/182	ST14, ZBTB10/MYT1, FOXO1
	miR-29	TTP
	miR-92	ERβ
	miR-103/107	DICER
	miR-106a	AIB1
	miR-106b/93/25	pRb, p21
	miR-127a	ZBTB1
	miR-132	p120, RAS, GAP
	miR-181 family	ATM
	miR-191	SATB1, CDK6
	miR-196a	ANXA1
	miR-155	RHOA, SOX1
	miR-221/222	ERα, p27(kip1), p57
	miR-210	MNT, RAD52
	miR-335	SOX4, TNC, PTPRN2, MERTK
Tumour suppressor	miR-205	HER3, VEGF-A
	miR-206	CyclinD2, Erα, SRC1/2, GATA3
	miR-224	CDC42, CXCR4
	miR-34a	BCL2, SIRT1, NOTCH1
	miR-7	PaK1, MRP1, EGFR
	miR-200 family	BMI1, ZEB1, ZEB2, FOG2, PLCG1, SUZ12, Moesin, Fibronectin, TRKB
	miR-30a	Vimentin
	miR-30e	ITGB3, UBC9
	miR-17-5p	AIB1
	let-7	RAS, HMGA2
	miR-20b	HIF1α
	miR-22	CDK6, HER3, ERα, CDC25C, SP1, EVI1
	miR-27b	CYP1B1
	miR-31	RHOA, RDX, ITGA5, FZD3
	miR-125a/b	HER2/HER3, HuR, c-RAF, MUC1, BAK, RTKN, CYP24, ERα
	miR-126	VEGF, IRS1, CRK
	miR-145	RTKN, ERα, MUC1
	miR-335	SOX4, TNC
	miR-146	NFkB
	miR-448	SATB1
	miR-661	MTA1, Nectin1, StarD10

2. Aim of this thesis

Fourteen years have passed since George Calin first published the association of miRNAs and cancer (Calin et al., 2002). At that time, there was a suspicion that a deletion on chromosome 13 was implicated in leukemogenesis. Indeed, this deletion is the most frequent abnormality in patients with CLL and back then, several studies failed to identify a causal gene in this region. Calin and colleagues demonstrated that two miRNAs genes (miR-15 and miR-16) were located within that region and that patients with CLL have a downregulation or absence of these two miRNAs when compared to normal tissue or lymphocytes. Shortly after, three other studies came to associate cancer and miRNAs. Two reporting the relationship of miR-17-92 cluster and the MYC oncogenic pathway (He et al., 2005; O'Donnell et al., 2005) and a third demonstrating that let-7 directly regulates Ras (Johnson et al., 2005). Since then, hundreds of studies are published every year showing an association of miRNAs and several oncogenic pathways.

There are three interesting questions about miRNAs in neoplastic diseases:

1. *How do tumour cells deregulate miRNAs expression in order to benefit themselves?*

2. *How do miRNAs influence tumour cells?*

3. *What is the actual expression of miRNAs in patient tumour cells?*

It is established that miRNAs are post-transcription regulators of gene expression and that in most tumour cells they are differently expressed when compared with normal cells. However, the mechanisms involved in this expression deregulation are still poorly understood. Thus, in Chapter 3 of this thesis we tried to address this topic by studying methylation statuses in three human breast cell lines representing three different stages of

mammary cells (MCF-10A – non-tumour cell line; MCF-7 – tumour cell line; MDA-MB-231 – tumour with metastatic capability) and how these statuses influence miRNAs expression.

Once the deregulation of miRNAs is established, cells start to behave differently and acquire new phenotypes, including invasiveness, metastasis and drug resistance, for example. Thus, in Chapters 4 and 6 of this thesis our approach was to study the effect of two miRNAs (miR-200c and miR-203) in human breast cell lines (MCF-10A, MCF-7 and MDA-MB-231).

Last but not least, although functional studies in cell lines are extremely important and give us plenty of knowledge, they need to be confirmed in tumour cells of patients with cancer, and in this case breast cancer. Thus, in Chapter 5, our approach was to study the expression patterns of two miRNAs (miR-200c and miR-203) in breast tumour cells of several patients and some putative targets of these miRNAs.

Finally, I summarize the main results and some conclusions about the work showed in this thesis as well as future perspectives about this work in Chapter 7.

3. Regulation of miRNAs expression in human breast cell lines

3.1. State of the art

Expression of miRNAs is deregulated in cancer and their impact in disease progression is now well documented [for further reading about miRNAs expression deregulation in specific types of tumours see: (Balatti et al., 2015; Chruscik and Lam, 2015; Lyra-Gonzalez et al., 2015; Masood et al., 2015; Rusek et al., 2015; van Schooneveld et al., 2015)]. However, how miRNAs expression became deregulated is still poorly understood, mainly because their behaviour in cells depends on the tissue and microenvironment. Thus, the influence of a miRNA in breast tissue may not be the same for lung cancer.

Some articles have been published reporting evidences that miRNAs might be regulated post-transcriptionally by proteins involved in their biogenesis, such as XPO5 (Iwasaki et al., 2013), DICER (Ueda et al., 2009) or other biogenesis partners (Shen and Hung, 2015). Oestrogens are also regulators of miRNAs expression. Indeed, Carolyn M. Klinge recently published an excellent review with all miRNAs regulated by estradiol, tamoxifen and other endocrine disrupting chemicals (Klinge, 2015). There is also evidence that telomerase reverse transcriptase (TERT) regulates miRNAs expression in early biogenesis, however the real mechanism is not known but certainly is not related with the canonical function of the TERT (Lassmann et al., 2015).

One of the most studied but less conclusive regulators of miRNAs expression is DNA methylation (Lopez-Serra and Esteller, 2012), nevertheless much is still needed to understand DNA methylation and its role in miRNAs expression.

Regarding breast cancer, DNA methylation-silencing associated repression of some miRNAs has been already described. Specifically, miR-9-1 (Lehmann et al., 2008; Lujambio

et al., 2008), miR-34a (Lodygin et al., 2008), miR-148a (Lujambio et al., 2008), cluster miR-200c/141 (Neves et al., 2010), miR-335 (Png et al., 2011), and let-7a-3 (Vrba et al., 2013). Indeed, a study published by Vrba and colleagues described a set of miRNAs promoters aberrantly methylated in breast cancer cell lines and also aberrantly methylated in breast cancer tissues, being thus clinically relevant.

Therefore, we decided to study basal levels of several miRNAs in MCF-10A, MCF-7 and MDA-MB-231 breast cell lines and then verify if the expression levels of these miRNAs are altered by challenging the cell lines with 5-Aza-2′-deoxycytidine (DAC), a demethylating agent. Additionally we performed a profile of the differentially expressed proteins after treatment with DAC.

3.2. Materials and methods

3.2.1. Cell lines

Human breast cell lines MCF-10A (non-tumour), MCF-7 (tumour) and MDA-MB-231 (tumour with metastatic capability) were kindly provided by Professor Nuno Oliveira from Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa. MCF-10A cells were cultured in Dulbeccos's Modified Eagle's Medium Nutrient Mixture F-12 Ham (DMEM/F-12; Sigma-Aldrich D8437), 5% horse serum (Sigma-Aldrich H1270), 1% penicillin-streptomycin (with 10,000 units penicillin and 10 mg streptomycin per mL) (Sigma-Aldrich P0781), 10 μg/mL insulin (Sigma-Aldrich I9278), 20 ng/mL epidermal growth factor (EGF; Sigma-Aldrich E9644), 0.5 μg/mL hydrocortisone (Sigma-Aldrich H0888) and 100 ng/mL cholera toxin (Sigma-Aldrich C8052). MCF-7 cells were cultured in DMEM (Sigma-Aldrich D6046), 10% foetal bovine serum (FBS; Sigma-Aldrich F7524), 10 μg/mL insulin (Sigma-Aldrich I9278) and 1% penicillin-streptomycin (with 10,000 units penicillin and 10 mg streptomycin per mL) (Sigma-Aldrich P0781). MDA-MB-231 cells were cultured in DMEM (Sigma-Aldrich D6046), 10% FBS (Sigma-Aldrich F7524)

and 1% penicillin-streptomycin (with 10,000 units penicillin and 10 mg streptomycin per mL) (Sigma-Aldrich P0781).

All cell lines were incubated at 37 °C with 5% CO_2 in a humidified chamber.

In order to test the demethylating effect of 5-Aza-2′-deoxycytidine (DAC; Sigma-Aldrich A3656), the breast tumour cell lines were cultured for five days in the presence of 2.5 μM of DAC. As a control we used dimethyl sulfoxide (DMSO; Merck Millipore #1029522500) at a percentage of 0.1% (v/v).

3.2.2. Nucleic acid purification

RNA, miRNAs and DNA were purified with AllPrep DNA/RNA Mini kit (Qiagen # 80204) and RNeasy MinElute Cleanup Kit (Qiagen # 74204). Cells were harvested and lysed in RLT buffer in a proportion of 350 μL per 3×10^6 cells. Up to 700 μl lysed cells were loaded into an AllPrep DNA spin column and centrifuged for 30 seconds at ≥8000 × g. The flow-through was used for RNA purification and the DNA spin column stored at room temperature until further use. One volume of 70% ethanol was added to the flow-through and mixed by pipetting. Up to 700 μl of the sample, including any precipitate that may have formed, was loaded into an RNeasy spin column and centrifuged for 30 seconds at ≥8000 × g. The flow-through was stored at room temperature to later miRNAs purification. A wash was done by adding 700 μl of buffer RW1 to the RNeasy spin column and centrifuged for 30 seconds at ≥8000 × g. The flow-through was discarded. A second wash with 500 μl of RPE buffer was done, followed by a centrifugation for 30 seconds at ≥8000 × g. The flow-through was also discarded. A third wash with 500 μl of RPE buffer was done, followed by a centrifugation for 2 minutes at ≥8000 × g. The flow-through was again discarded. Next, 30 μl of nuclease-free water was added directly to the spin column membrane and centrifuged for 1 min at ≥8000 × g to elute the RNA. This RNA was then stored at -80 °C until further use. One volume of 100% ethanol was added to the flow-through previously stored for miRNAs purification. This mixture was then loaded into an

RNeasy MinElute Cleanup column and centrifuged for 30 seconds at ≥8000 × g. The flow-through was discarded. A wash was done by adding 500 µl of RPE buffer and centrifuged for 30 seconds at ≥8000 × g and discarding the flow-through. A second wash with 500 µl of 80% ethanol was done and centrifuged for 2 minutes at ≥8000 × g. The flow-through was discarded and a centrifugation for 5 minutes at full speed was done in order to dry the filter. Next, 14 µl of nuclease-free water was added to the column and centrifuged for 1 minute at full speed. The miRNAs were then stored at -80 °C until further use. At last, the genomic DNA was purified by adding 500 µl of AW1 buffer to the AllPrep DNA spin column that was stored before and centrifuged for 30 seconds at ≥8000 × g. The flow-through was discarded and 500 µl of AW2 buffer were added to the column and centrifuged for 2 minutes at full speed. After discarding the flow-through, 100 µl of elution buffer was added to the column and incubated for 1 minute at room temperature. Then, the column was centrifuged for 1 minute at ≥8000 × g. The genomic DNA was then stored at -80 °C until further use.

All samples were quantified using a NanoDrop™ spectrophotometer.

3.2.3. Reverse transcription qPCR

A profiling of 95 miRNAs in MCF-10A, MCF-7 and MDA-MB-231 was done by using QuantiMir Cancer Array from System Biosciences (#RA610A-1) in a real time PCR 7300 system (ABI). The protocol was divided into 4 steps: *a) polyA tail formation* - 100 ng of purified miRNAs were added to a mix of 2 µl of 5× polyA buffer, 1 µl of 25 mM $MnCl_2$, 1.5 µl of 5 mM ATP and 0.5 µl of polyA polymerase to a total volume of 10 µl. This mixture was then incubated at 37 °C during 30 minutes in a thermal cycler (9700 ABI); *b) Anneal Anchor dT Adaptor* - 0.5 µl Oligo dT Adaptor was added to the mixture in step a), followed by an incubation for 5 minutes at 60 °C and a cooling to room temperature for 2 minutes; *c) Syntheses of cDNAs* – to the mixture of step b) were added 4 µl of 5× RT buffer, 2 µl of dNTPs mix, 1.5 µl of 0.1 M dithiothreitol (DTT), 1.5 µl of RNase-free water and 1 µl of

reverse transcriptase. Then, an incubation for 60 minutes at 42 °C and an enzyme inactivation for 10 minutes at 95 °C was done. The cDNAs were stored at - 20 °C until further use; and *d) qPCR Reaction* – A mixture of 1,750 µl of 2× 2X SYBR® Green qPCR Mastermix buffer, 60 µl of 10 µM Universal Reverse Primer, 20 µl of cDNA and 1,670 µl of RNase-free water was done. From this mixture, 29 µl were loaded per well in a 96-well plate and then 1 µl of specific primers were loaded per well. U6 snRNA was used as endogenous control. The miRNAs studied and the plate arrangement can be seen in Table 3.1. This methodology was used with all cell lines in normal conditions and MCF-7 cells treated with 2.5 µM of DAC for 5 days.

Table 3.1 - microRNAs studied with QuantiMir Cancer Array and plate arrangement

	1	2	3	4	5	6	7	8	9	10	11	12
A	let7-family	miR-7	miR-92a	miR-93	miR-9	miR-101	miR-103	miR-106a	miR-106b	miR-107	miR-10b	miR-1
B	miR-122	miR-125a-5p	miR-125b	miR-126	miR-128	miR-132	miR-133a	miR-134	miR-135b	miR-136	miR-137	miR-140
C	miR-141	miR-142-3p	miR-143	miR-145	miR-146a	miR-149	miR-150	miR-151	miR-153	miR-154	miR-155	miR-15a
D	miR-15b	miR-16	miR-17*	miR-17	miR-181a	miR-181b	miR-181c	miR-181d	miR-183	miR-185	miR-186	miR-188-5p
E	miR-18a	miR-190	miR-191	miR-192	miR-194	miR-195	miR-196a	miR-197	miR-198	miR-199a+b	miR-30b	miR-19a+b
F	miR-95	miR-20a	miR-200a	miR-200b	miR-200c	miR-202	miR-203	miR-204	miR-205	miR-206	miR-21	miR-210
G	miR-214	miR-215	miR-372	miR-373	miR-218	miR-219	miR-22	miR-488	miR-221	miR-222	miR-223	miR-224
H	miR-23a	miR-24	miR-25	miR-26a	miR-26b	miR-27a+b	miR-30c	miR-29a+b+c	miR-30a*	miR-30a	miR-296	U6 snRNA

Individual reverse transcription qPCR was done by using Universal cDNA synthesis kit (Exiqon # 203300) and SYBR® Green master mix, Universal RT (Exiqon # 203450). This methodology was performed in order to confirm the expression of miR-199b, miR-203, miR-200c, miR-24, miR-154 and let-7a in breast cell lines cultured in normal conditions.

Universal cDNA synthesis started with a dilution of purified miRNAs to a concentration of 1 ng/µl in nuclease-free water. A reverse transcription reaction mix was then prepared by mixing 4 µl of 5× Reaction buffer, 10 µl of nuclease-free water, 2 µl of enzyme mix and 4 µl

of template RNA at 1 ng/µl. This mixture was incubated at 42 °C for 60 minutes, followed by an enzyme inactivation for 5 minutes at 95 °C and immediately cooled to 4 °C. The cDNA was then stored at -20 °C until further use. In order to proceed to real-time PCR, cDNA previously synthesized was diluted 80×. Then, a PCR master mix was done by mixing 5 µl of SYBR® Green master mix, 1 µl of PCR primer mix and 4 µl of diluted cDNA template to a total volume of 10 µl. The mixture was loaded into a 96-well PCR plate and then the real-time PCR was performed in a Roche 480 LightCycler instrument with the following conditions: polymerase activation at 95 °C for 10 minutes; 45 cycles of amplification at 95 °C for 10 seconds and 60 °C for 1 minute (with a ramp-rate of 1.6 °C /s). The primers used were: hsa-miR-199b-5p (Exiqon, LNA™ PCR primer set # 204152); hsa-miR-203 (Exiqon, LNA™ PCR primer set # 204285); hsa-miR-24 (Exiqon, LNA™ PCR primer set # 204260); hsa-miR-154 (Exiqon, LNA™ PCR primer set # 204518); hsa-let-7a (Exiqon, LNA™ PCR primer set # 204775); hsa-miR-200c-3p (Exiqon, LNA™ PCR primer set # 204482) and as endogenous control U6 snRNA (Exiqon, PCR primer set # 203907).

Relative expression of miRNAs was determined by $2^{-\Delta Ct}$, where ΔCt is Ct (miRNA) - Ct (U6 snRNA).

QuantiMir Cancer Array was analysed in duplicate and individual reverse transcription qPCR was analysed in triplicate independent experiments.

3.2.4. Primer selection and design

All promoter sequences of miRNAs genes and CpG islands were selected in The UCSC Genome Browser (http://genome.ucsc.edu/index.html) and miRStart (http://mirstart.mbc.nctu.edu.tw/). In all, 20 promoter regions were selected for 12 miRNAs, Table 3.2. CpG islands were predicted with 50% or greater GC content, length greater than 200 base pairs (bp) and a ratio greater than 0.6 of observed number of CG dinucleotides to the expected number on the basis of the number of G's and C's in the segment. All this regions were loaded into methyl primer express® software v1.0 (ABI).

This software retrieves specific primers to methylated and non-methylated regions by *in silico* transforming DNA sequences. In the case of the promoter regions of XPO5 and RAN was UCSC Genome Browser was used. The selection of CpG islands was done as described before and the primer design made in methyl primer express® software v1.0. All primers can be seen in Table 3.2.

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Table 3.2 - Selected gene promoters; primer sequences for unmethylated and methylated regions of the promoters; and annealing temperatures for each pair of primers.

Gene		primer sequence	Annealing temperature ºC
miR-26b	**UF**	*5' TTTTTATAAATTTTGTGTTATGT 3'*	50
	UR	*5' TACATACCCACTAAACACACAA 3'*	
	MF	*5' TTATAAATTTCGCGTTACGC 3'*	45
	MR	*5' TACGTACCCACTAAACGCA 3'*	
miR-34a	**UF**	*5' GGAGTTTTTTTTTTATGGTGGT 3'*	50
	UR	*5' CCCTTTCCACACTACCCTACA 3'*	
	MF	*5' AGTTTTTTTTTTATGGCGGC 3'*	
	MR	*5' CTTTCCACGCTACCCTACG 3'*	
let-7a-3	**UF**	*5' GAGGAGATGGTATGTTTGTGAAGTTG 3'*	55
	UR	*5' AAACATACAAATACCCACCCTACTCA 3'*	
	MF	*5' GACGGTACGTTCGTGAAGTCG 3'*	
	MR	*5' CATACGAATACCCACCCTACTCG 3'*	
miR-145	**UF**	*5' AGTTGAGTGTTAATTTGTTAGTGTT 3'*	51
	UR	*5' ATTTTACCCAACAAAACATCTCC 3'*	
	MF	*5' CGAGCGTTAATTCGTTAGC 3'*	
	MR	*5' ATTTTACCCGACGAAACATC 3'*	
miR-150	**UF**	*5' TTTGAAGGTTAAGGTGGATTTT 3'*	51
	UR	*5' ATAAAAAAACCCAAACACTACTA 3'*	
	MF	*5' GAAGGTTAAGGCGGATTTC 3'*	
	MR	*5' AAAAAACCCGAACGCTACTA 3'*	
miR-200c	**UF**	*5' GTTTTTTGTTAGATGGATGTGG 3'*	51
	UR	*5' ATCAACCATCATCTCAATACTTT 3'*	
	MF	*5' CGTTTTTCGTTAGACGGAC 3'*	
	MR	*5' GTCAACCGTCATCTCGATAC 3'*	
miR-203	**UF**	*5' GTTAGGTGTGTTTAGGTTAGGT 3'*	53
	UR	*5' CCCATAAATCCCACAACTAATCC 3'*	
	MF	*5' AGGTGCGTTTAGGTTAGGC 3'*	
	MR	*5' CATAAATCCCGCGACTAATC 3'*	
miR-199b	**UF**	*5' GGGATTTAGTGTTTTTTTGGGGG 3'*	53
	UR	*5' CCAAAACCAACCATTTCTATCCC 3'*	
	MF	*5' CGGGATTTAGCGTTTTTTCG 3'*	
	MR	*5' CAAAACCGACCGTTTCTATC 3'*	
miR-124-1 (*)	**UF**	5' ATAAGGAGAGTAGTGGGGATTT 3'	58
	UR	5' ACAACAAACAAATTCCAAAAAAC 3'	
	MF	5' AGGAGAGTAGCGGGGATTC 3'	
	MR	5' CAACAAACGAATTCCGAAAA 3'	

UF – unmethylated forward; UR – unmethylated reverse; MF – methylated forward; MR – methylated reverse. (*) CpG island located at the transcription binding site of TP53 gene.

Table 3.2 - (continued) – Selected gene promoters, primer sequences for unmethylated and methylated regions of the promoters and annealing temperatures for each pair of primers.

Gene		primer sequence	Annealing temperature ºC
miR-124-1 (**)	**UF**	*5′ GTTTAGGTTTTTGGTTTGGTTT 3′*	56
	UR	*5′ ACTCAACAATCAACATTAAAAAT 3′*	
	MF	*5′ TAGGTTTTCGGTTTGGTTC 3′*	
	MR	*5′ CGACGATCAACGTTAAAAAT 3′*	
miR-124-1 (***)	**UF**	*5′ TTTGGTTGGGTTGGTTGAATT 3′*	62
	UR	*5′ CACAACAACCACACATATTCTAAA 3′*	
	MF	*5′ GGTTGGGTCGGTTGAATC 3′*	
	MR	*5′ AACGACCACGCGTATTCTAAA 3′*	
miR-124-2	**UF**	*5′ GGTGTATTTTGGGGTTTTTGT 3′*	58
	UR	*5′ TACAAACAAAACCCTCTACACA 3′*	
	MF	*5′ CGTATTTTGGGGTTTTTGC 3′*	
	MR	*5′ TACGAACGAAACCCTCTACG 3′*	
miR-124-3	**UF**	*5′ GTTGGGATTGGTAATTATGTTT 3′*	56
	UR	*5′ CAAAAAAACACTCAAACTATTC 3′*	
	MF	*5′ CGGGATTGGTAATTACGTTC 3′*	
	MR	*5′ CGAAAAAACGCTCGAACTAT 3′*	
miR-17-92	**UF**	*5′ GGTTTTTTAAATTTTGTATGTGT 3′*	50
	UR	*5′ ACTACCCACACAAACTAACAAA 3′*	
	MF	*5′ TTTTTAAATTTTGTACGCGC 3′*	
	MR	*5′ ACTACCCACGCGAACTAAC 3′*	
miR-219-1	**UF**	*5′ AATTGAGGTTAAGGTTGTTGGTT 3′*	51
	UR	*5′ ATAAAACAAAACATAAACACCACA 3′*	
	MF	*5′ CGAGGTTAAGGTTGTTGGTC 3′*	
	MR	*5′ ATAAAACGAAACGTAAACGCC 3′*	
miR-219-2	**UF**	*5′ TTTGTTTTTTTGTGGTTGAGTT 3′*	50
	UR	*5′ CACAAACACTACAAATAACCCA 3′*	
	MF	*5′ CGTTTTTTTGTGGTTGAGTC 3′*	
	MR	*5′ CACGAACGCTACAAATAACC 3′*	
miR-126 (‡)	**UF**	*5′ ATTTTGGAAGATGTTATGTTTTT 3′*	47
	UR	*5′ TACCATAAACAACACATTATTAC 3′*	
	MF	*5′ TTGGAAGACGTTACGTTTTC 3′*	51
	MR	*5′ TACCGTAAACGACGCATTAT 3′*	
miR-126 (‡‡)	**UF**	*5′ GAATTTTGGAGTTAGTAGTGT 3′*	50
	UR	*5′ AACACTAACAAACCCCTCA 3′*	
	MF	*5′ AATTTCGGAGTTAGTAGCGC 3′*	53
	MR	*5′ ACACTAACGAACCCCTCG 3′*	

UF – unmethylated forward; UR – unmethylated reverse; MF – methylated forward; MR – methylated reverse. (**) CpG island 70. (***) CpG island 170. (‡) CpG island 29. (‡‡) CpG island 97.

Table 3.2 - (continued) – Selected gene promoters, primer sequences for unmethylated and methylated regions of the promoters and annealing temperatures for each pair of primers.

Gene		primer sequence	Annealing temperature ºC
miR-24-1	**UF**	*5' TTTATGGAGTTTTTAGTTGAGGT 3'*	52
	UR	*5' CCTCAAACACTTACAAACACAAA 3'*	
	MF	*5' ACGGAGTTTTTAGTTGAGGC 3'*	
	MR	*5' TCGAACACTTACAAACACGA 3'*	
miR-24-2	**UF**	*5' GAAGGGAATAGAGGTTGGGTT 3'*	52
	UR	*5' TCCCCAAAAACATTACAAAAAAA 3'*	
	MF	*5' GGGAATAGAGGTCGGGTC 3'*	
	MR	*5' CCCAAAAACGTTACGAAAAA 3'*	
RAN (†)	**UF**	*5' AGTTGGTGTTGTTATGGTAATT 3'*	51
	UR	*5' AACTCCCACTAAAAAACACTACC 3'*	
	MF	*5' CGGCGTTGTTACGGTAATC 3'*	54
	MR	*5' CTCCCGCTAAAAAACGCTAC 3'*	
RAN (††)	**UF**	*5' TTATGTTTGTTTGAGTGAGTATT 3'*	49
	UR	*5' ACACACATACACTAAAAACAAAA 3'*	
	MF	*5' CGTTTGTTCGAGCGAGTATC 3'*	52
	MR	*5' CGCATACGCTAAAAACGAAA 3'*	
XPO5 (§)	**UF**	*5' TTTATTTTTTAGATGGGGTGGT 3'*	53
	UR	*5' AACTATCTCAATCTTTACCACCAC 3'*	
	MF	*5' ATTTTTTAGACGGGGTGGC 3'*	
	MR	*5' CTATCTCAATCTTTACCGCCG 3'*	
XPO5 (§§)	**UF**	*5' TTATTTTAGTTATAGGTGGTGTT 3'*	47
	UR	*5' TCAAATAAACACACTATACAAAC 3'*	
	MF	*5' TTTTAGTTATAGGCGGCGTC 3'*	51
	MR	*5' TCAAATAAACGCGCTATACG 3'*	

UF – unmethylated forward; UR – unmethylated reverse; MF – methylated forward; MR – methylated reverse. (†) CpG island 75. (††)CpG island 126. (§) CpG island 46. (§§) CpG island 94.

3.2.5. Methylation specific PCR

Methylation specific PCR (MSP) was done by using EZ DNA Methylation-Gold™ Kit (Zymo Research D5005) to perform a bisulfite conversion of GC-rich DNA and ZymoTaq™ DNA Polymerase (Zymo Research E2002) to perform PCR.

Bisulfite conversion was done by adding 130 µl of CT conversion reagent to 20 µl of DNA sample (500 ng). The CT conversion reagent was previously prepared by adding 900 µl of

nuclease-free water, 300 µl of M-Dilution buffer, and 50 µl M-Dissolving buffer to a tube of CT conversion reagent that was provided with the kit. Next, the mixture was incubated for 10 minutes at 98 °C, followed by 150 minutes at 64 °C and a cooling to 4 °C. After cooling the sample, the converted DNA was cleaned up by loading it to a Zymo-Spin™ IC Column and 600 µl of M-Binding buffer. The mixture was then mixed by inverting the column several times and centrifuged during 30 seconds at full speed (>10,000 × g). Then, 100 µl of M-Wash buffer was added to the column and centrifuged at full speed for 30 seconds. Next, 200 µl of M-Desulphonation buffer was added to the column and let to stand at room temperature (20-30°C) for 15 minutes, followed by a centrifugation at full speed for 30 seconds. Two successive washes were done by adding 200 µl of M-Wash buffer to the column and centrifuged for 30 seconds at full speed. Finally, 10 µl of M-Elution buffer was directly added to the column matrix and centrifuged for 30 seconds at full speed to elute the DNA. The cleaned up converted DNA was then stored at -20 °C until further use.

In order to do the PCR, two separately mixes were prepared using primers for unmethylated sequence and primers for methylated sequence (all primers used can be seen in Table 3.2). The mixes only differed in the primer sequences used. Thus, 50 µl reaction mixes containing 25 µl of 2× buffer, 0.5 µl dNTPs (0.25 mM each dNTP), 3 µl of reverse and forward primer at 10 µM each, 0.4 µl of ZymoTaq™ DNA polymerase (5 U/µl), 17.1 µl of nuclease-free water and 1 µl of converted DNA (50 ng) was prepared and placed into a thermal cycler. The PCR conditions were: initial denaturation at 95 °C for 10 minutes; 40 cycles of denaturation at 94 °C for 30 seconds, annealing at the primers specific temperatures referred in Table 3.2 for 30 seconds and extension at 72 °C for 30 seconds; followed by a final extension at 72 °C for 7 minutes and a cooling to 4 °C. The PCR products were then subject to electrophoresis in 2% (w/v) agarose gel stained with ethidium bromide (1µg/ml) and visualized under ultra-violet light. All PCR reactions were carried out twice in independent experiments.

3.2.6. Protein purification and quantification

In order to proceed to protein purification, human breast cell lines in ordinary medium culture and treated with 2.5 μM of DAC and 0.1% (v/v) of DMSO were harvested and washed twice with cold phosphate-buffered saline (PBS) solution. Next, a lysis buffer (50 mM Tris.base pH=8; 150 mM NaCl; 5 mM Ethylenediamine tetraacetic acid (EDTA); 1% (v/v) Nonidet P-40; 1 mM phenylmethylsulfonyl fluoride (PMSF); and protease inhibitor cocktail (Roche # 11 697 498 001)) was added to pelleted cells (in a proportion of 25 μl per 1×10^6) and roughly mixed by vortex and left on ice for 30 minutes. The lysis buffer containing the cells was then centrifuged for 10 minutes at 4 °C and 14,000 × g. The supernatant containing total protein extract was recovered and stored at -80 °C until further use.

Protein quantification was done by Bradford assay using protein assay dye reagent concentrate (Bio-Rad # 500-0006) and bovine serum albumin (BSA) standard (Bio-Rad #500-0206). Standard concentrations of protein (0; 1; 5; 10; 20; 25; 30; 35; 40 μg) were prepared in deionized water to a final volume of 800 μl. Then, 200 μl of protein assay dye reagent concentrate were added. The samples were then loaded in a 96-well plate and read in a plate-reader spectrophotometer at 595 nm. The same procedure was done using the protein extracts and then by linear regression the exact concentration determined.

3.2.7. Protein analysis by 2-D SDS-PAGE Gels and MALDI-TOF/TOF

After protein quantification, total protein was cleaned-up by using 2D clean-up kit from GE Healthcare Life Sciences (#80-6484-51). To 100 μg of total protein were added 300 μl of precipitant reagent, mixed by vortex and left on ice for 15 minutes. Then, 300 μl of co-precipitant were added and mixed by pipetting. The mixture was centrifuged at 12,000 × g for 5 minutes and the supernatant discarded. 40 μl of co-precipitantwere added to the pellet layer and left on ice for 5 minutes. The mixture was centrifuged at 12,000 × g for 5 minutes. Next, the supernatant was removed and 25 μl of deionized water added to the

pellet and dispersed by vortex. Then, 1 ml of chilled wash buffer and 5 µl of wash additive were added to the dispersed pellet and mixed by vortex. This mixture was left on ice for 30 minutes, assuring that every 10 minutes a mixture by vortex was done. After 30 minutes the mixture was centrifuged at 12,000 × g for 5 minutes and the supernatant removed. The pellet was air dried and finally resuspended in 150 µl of rehydration buffer (2% (v/v) CHAPS detergent; 7 M urea; 2 M tiurea; 0.5% (v/v) immobilized pH gradient (IPG) buffer; 0.5% (v/v) DeStreak reagent; 1 mM PMSF; 15 mM DTT; protease inhibitor cocktail). After 2 hours at room temperature the samples were ready to load in IPG gel (Immobiline DryStrip Gels pH 3-10 non-linear 7 cm, #17-6001-12 from GE Healthcare Life Sciences). Isoelectric focusing (IEF) was then made using the following conditions: 30 V for 12 hours, 100 V for 30 minutes, 500 V for 30 minutes, 1000 V for 30 minutes and finally 5000 V for 1 hour. After protein separation by its isoelectric point, strips were submerged in equilibration buffer (Tris.HCl 1.5 M pH 8.8; 6 M Urea; 2% (w/v) sodium dodecyl sulfate (SDS); glycerol 30%; and traces of bromophenol blue) with 1% (w/v) DTT for 15 minutes and then 15 minutes in equilibration buffer with 2.5% (w/v) iodoacetamide. After equilibration, the strips were positioned in the polyacrylamide gel (2.5 ml Tris.HCl 0.4 M pH 8.8; 75 µl APS 10%; 10 µl TEMED; 4 ml bisacrylamide 30%; and 3.5 ml deionized water) and subject to electrophoresis at 150 V. After electrophoresis, the gels were stained with Coomassie blue and scanned in order to acquire a picture of the gel. Pictures were then analysed in Progenesis Samespots software. This software aligned different pictures from different experimental conditions and analysed differences between them. After triplicates from independent experiments the differentially expressed spots from different cell conditions were excised from gels and sent to analysis to identify proteins with MALDI-TOF/TOF under the responsibility of Professor Deborah Penque from Laboratory of Proteomics, Human Genetics Department, Instituto Nacional de Saúde Dr Ricardo Jorge.

3.2.8. Protein analysis by western blot

Total protein was denatured in Laemmli buffer 2× (4% (w/v) SDS 10%; 20% (v/v) glycerol 50%; 0.02% (w/v) bromophenol blue; 125 mM Tris.HCl pH 6.8; and 10% (v/v) 2-Mercaptoethanol) in a proportion of 1:1 and heated at 95 °C for 10 minutes. Samples were then loaded into precast gels (4–20% Mini-PROTEAN® TGX™ Precast Protein Gels, Bio-Rad #4561093S) and subject to electrophoresis at 100 V for 90 minutes in running buffer 1× (25 mM Trizma-base; 192 mM Glycine; 0.1% (w/v) SDS; pH 8.3). The gels were equilibrated in transfer buffer 1× (25 mM Trizma.base; 192 mM Glycine; 0.1% (w/v) SDS; and 10% methanol) for 20 minutes as well as the PVDF membranes. Next, proteins were transferred from gel to PVDF membrane in transfer buffer 1× for 60 minutes at 100 V. Membranes were then blocked using blocking buffer from WesternDot™ 625 Goat Anti-Mouse Western Blot Kit (# W10132) for 1 hour and then washed with wash-buffer also provided with the kit. The membranes were incubated at room temperature for 1 hour with primary antibodies for RAN (GeneTex #GTX13049) and β-actin (Santa Cruz # sc-47778) and after three successive washes incubated at room temperature for one hour with secondary antibody provided with the kit. At last, and after three more washes, the membranes were incubated at room temperature with streptavidin conjugate for one hour. The membranes were then visualized under ultra-violet light and photographed.

3.2.9. Statistical analysis

Data were analysed with GraphPad Prism 5 software, using one-way ANOVA with Dunn's multiple comparison post hoc test and two-way ANOVA with Bonferroni multiple comparison post hoc test with confidence intervals of 95%. All graphs were made with GraphPad Prism 5 software and values represented are mean expression + range or mean expression ± standard deviation.

3.3. Results

MCF-10A, MCF-7 and MDA-MB-231 cell lines have different patterns of miRNAs expression

In order to ascertain how miRNAs expression is regulated, we used three human breast cell lines with different phenotypes and measured the expression of 95 miRNAs known to have importance in tumourigenesis (in general and not only in breast cancer). Considering the 95 miRNAs as a whole we can observe that MDA-MB-231 levels of miRNAs are much lower when comparing with MCF-10A and MCF-7 ($p < 0.05$; Figure 3.1). In contrast, the MCF-7 cell line has a higher expression of miRNAs, but without statistically significant differences when compared with MCF-10A. Figure 3.2 shows the expression levels of the miRNAs individually.

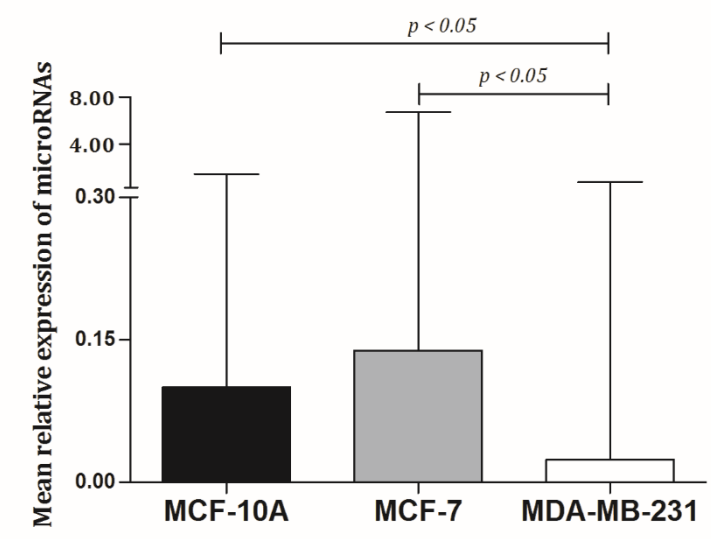

Figure 3.1- Relative expression of all miRNAs from QuantiMir Cancer Array in MCF-10A, MCF-7 and MDA-MB-231. The values represented are mean values + range of relative expression of 95 miRNAs to U6 snRNA. *p* values were determined by one-way ANOVA and Dunn's multiple comparison post hoc test with 95% confidence interval.

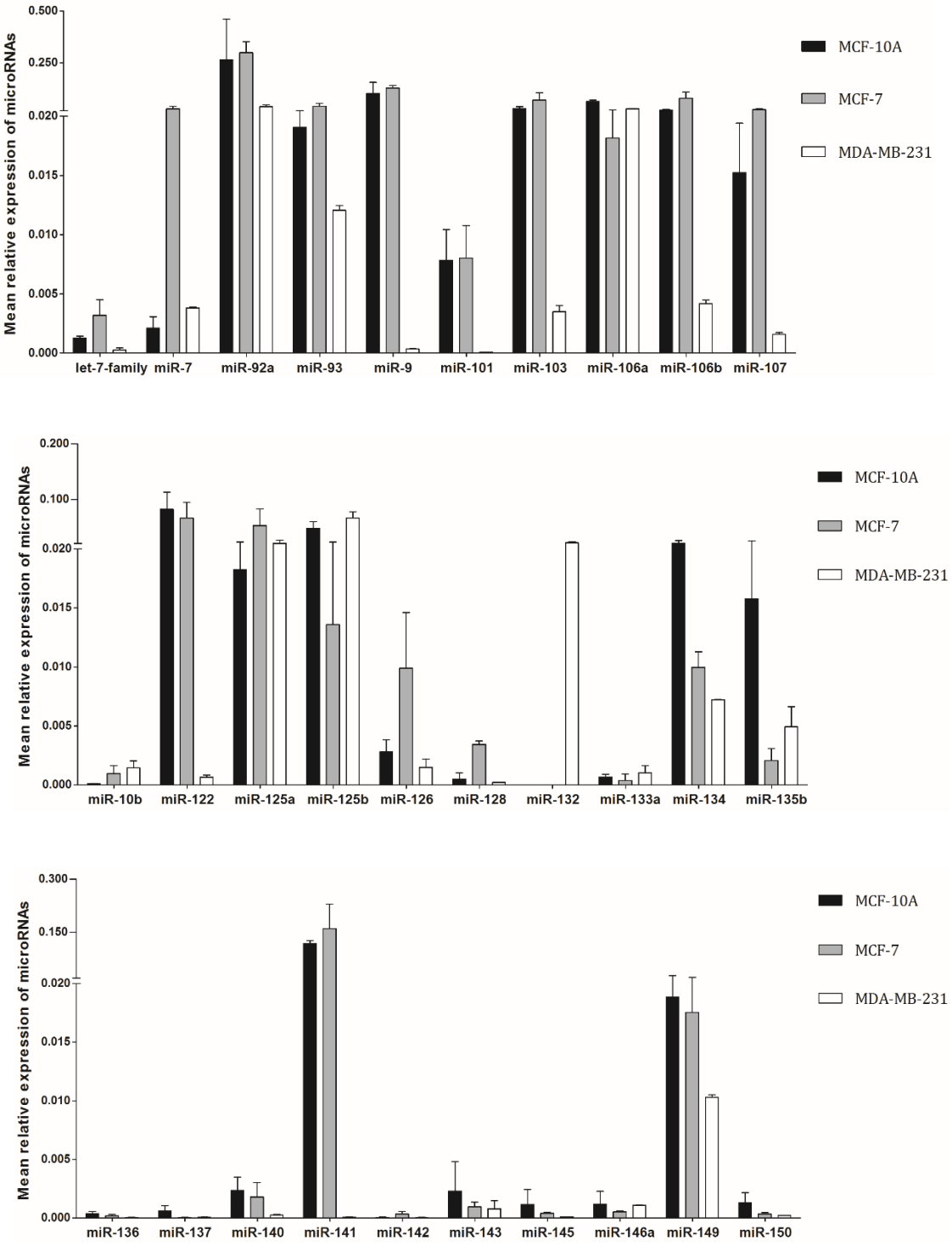

Figure 3.2 - Expression patterns of the miRNAs from the QuantiMir Cancer Array in MCF-10A, MCF-7 and MDA-MB-231 cell lines. Values are represented as mean relative expression to U6 snRNA ± standard deviation.

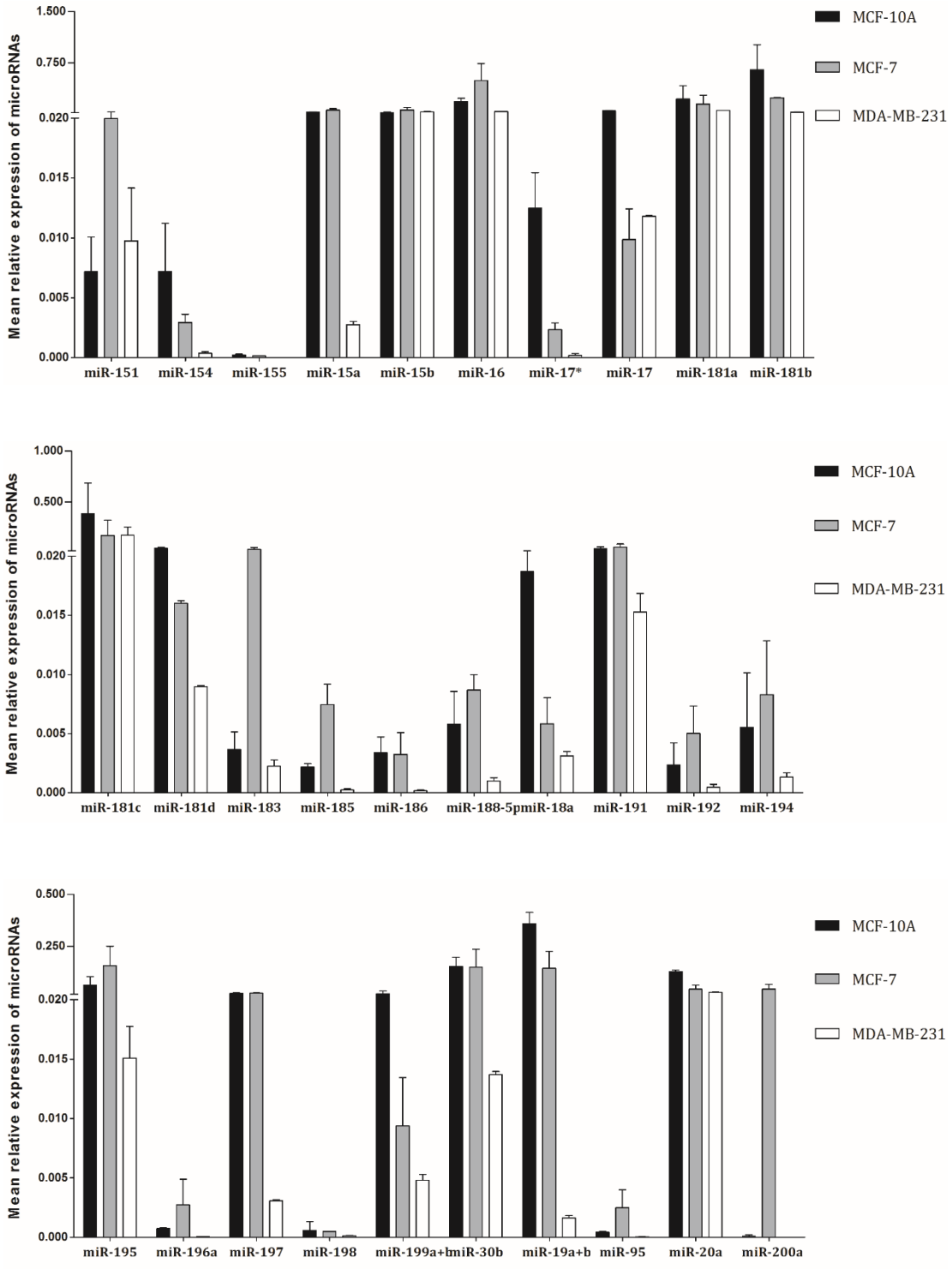

Figura 3.2 (continued) - Expression patterns of the miRNAs from the QuantiMir Cancer Array in MCF-10A, MCF-7 and MDA-MB-231 cell lines. Values are represented as mean relative expression to U6 snRNA ± standard deviation.

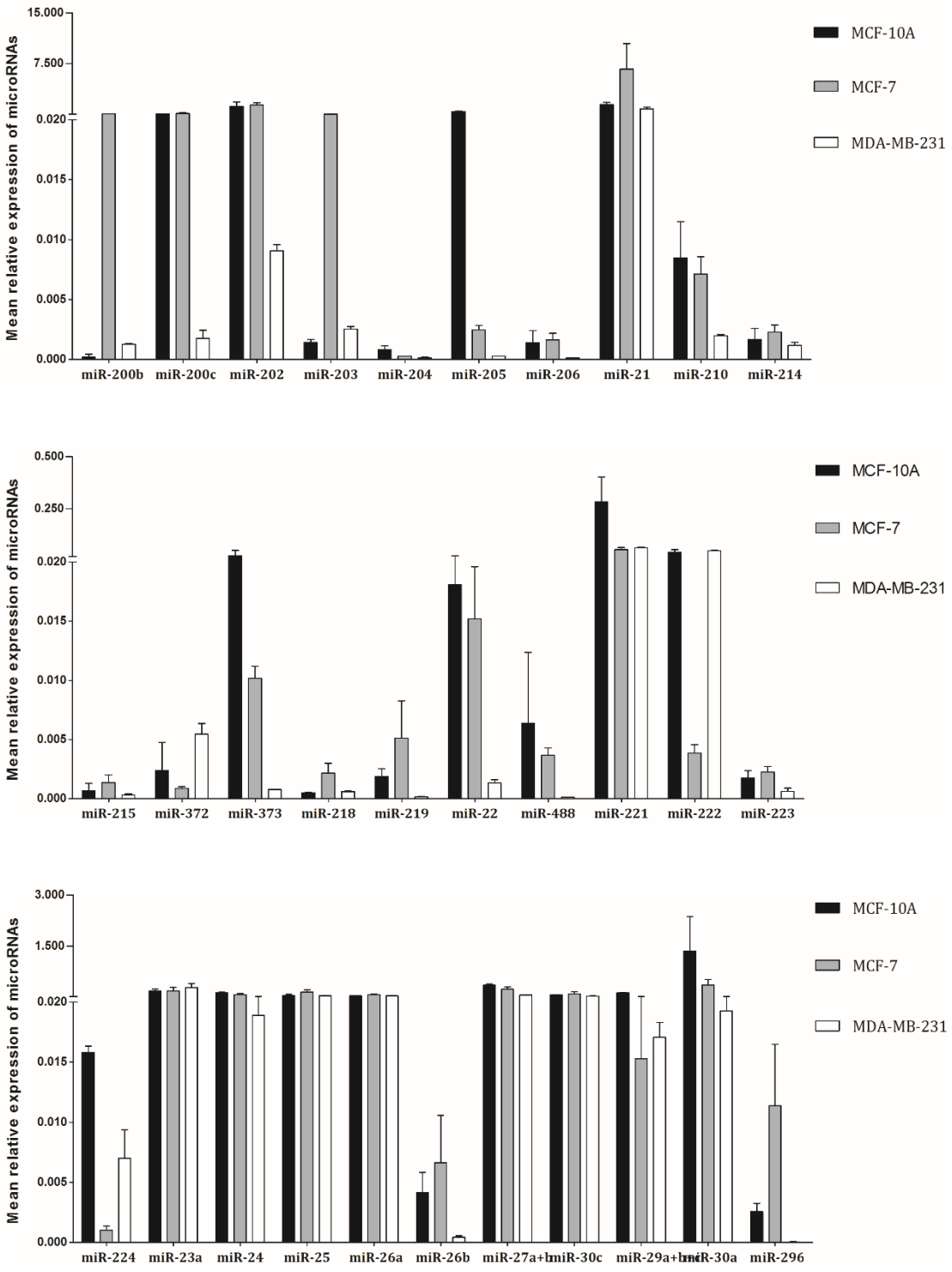

Figura 3.2 (continued) - Expression patterns of the miRNAs from the QuantiMir Cancer Array in MCF-10A, MCF-7 and MDA-MB-231 cell lines. Values are represented as mean relative expression to U6 snRNA ± standard deviation.

MCF-7 cell line overexpress miRNAs levels after DAC treatment

In order to ascertain if miRNAs levels increase with DAC treatment, MCF-7 cell line was treated with 2.5 μM of DAC for five days and then the miRNAs levels were measured with the QuantiMir Cancer Array. Compared with its counterpart without treatment (only with 0.1% of DMSO as negative control) we observed that almost all levels of miRNAs increased ($p < 0.001$ given by two-way ANOVA; Figure 3.3). Definitely, DAC influences miRNAs expression levels and Figure 3.3 shows which miRNAs are significantly increased. Thus, we also treated MCF-10A and MDA-MB-231 cell lines with 2.5 μM of DAC for 5 days and analysed 20 putative promoter regions of 12 miRNAs (miR-26b, miR34a, let-7a-3, miR-145, miR-200c, miR-203, miR-199b, miR-124, miR-17-92, miR-219, miR-126 and miR-24) in the three cell lines. Not all miRNAs overexpressed with DAC treatment were analysed because their promoter regions weren't described or didn't have predicted CpG islands. Thus, the miRNAs and promoter regions selected were those which were previously described as methylated in several types of cancer or those for which we obtained overexpressed with DAC treatment and had promoter regions described. In order to analyse if the selected promoter regions were methylated we performed a bisulfite conversion of the DNA, where unmethylated cytosines are replaced by uracil and allows us to distinguish between unmethylated and methylated sequences. Through PCR with specific primers for both sequences we obtained the methylation status of each promoter region. Figure 3.4 shows the PCR products we obtained. Among the 20 promoter regions we shall focus on let-7a-3, miR-199b, miR-124 and miR-24.

The putative promoter region studied of Let-7a-3 seems to be totally methylated in MDA-MB-231 and partially methylated in the other two cell lines. Indeed, looking at Figure 3.4 we can see that the unmethylated sequence disappears with the aggressiveness of the cell line when not treated with DAC. When treated with DAC, the unmethylated sequence became more intense in MCF-10A and MCF-7 and in the case of MDA-MB-231 becomes

visible. In order to confirm if the methylation status is related with let-7a expression, we performed RT-qPCR and found contradictory results. The MDA-MB-231 is the cell line that expresses higher levels of let-7a (Figure 3.5). Regarding the putative gene promoter of miR-199b, we found that in MCF-10A it was totally unmethylated while being partially methylated in MCF-7 and MDA-MB-231 cells (Figure 3.4). With DAC treatment we were unable to detect changes in band intensity. Once again, we confirmed the methylation status through RT-qPCR of miR199b. However we did not detect expression of this miRNA in any of the cell lines. With respect to miR-124, we studied 3 promoter regions of miR-124-1, one of miR-124-2 and another of miR-124-3. All these regions express the same miR-124, the only difference between miR-124-1, miR-124-2 and miR-124-3 is the chromosome region. Thus, the human genome has three copies of this gene but the one transcribed is not known. For that reason we studied the 3 regions and found CpG islands in all of them. From all regions, the most revealing were 124-1 (CpG island 70), miR124-1 (CpG island 170) and miR-124-3 (Figure 3.4). However, these results are contradictory with the miR-124 expression levels (Figure 3.5). MiR-24 also displays differences in methylation status of the promoter sequence, but once again the expression of miR-24 does not confirm the methylation status. Levels of miR-200c and miR-203 were also assessed in spite of being totally unmethylated. Interestingly, we obtained visible differences between the cell lines (Figure 3.5).

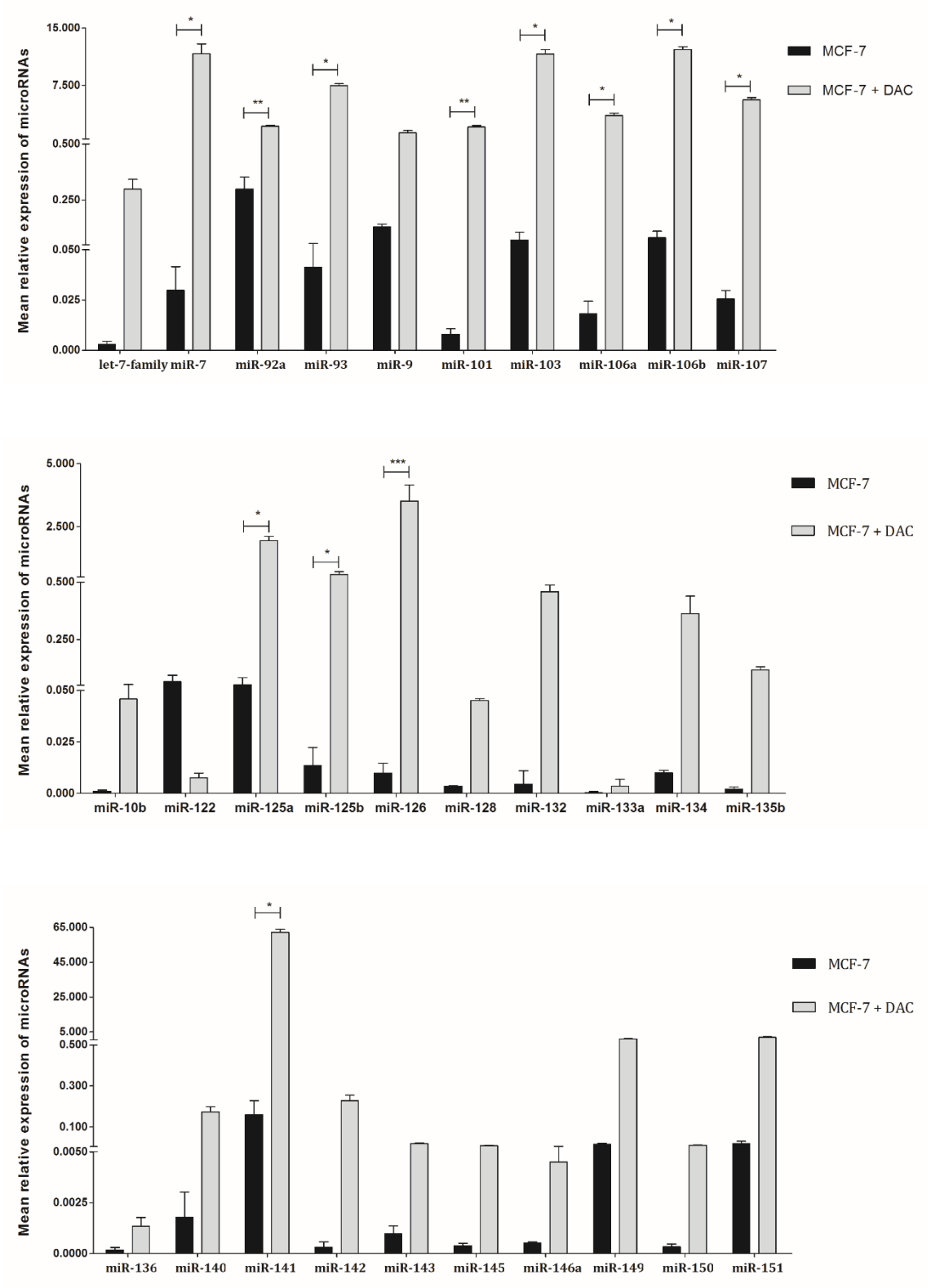

Figure 3.3 - Expression patterns of the miRNAs from the QuantiMir Cancer Array in MCF-7 and MCF-7 treated with 2.5 µM DAC for 5 days. Values are represented as mean relative expression ± standard deviation. *p* values were determined by two-way ANOVA and corrected with Bonferroni multiple comparison post hoc test with 95% confidence interval. * $p < 0.001$; ** $p < 0.01$; *** $p < 0.05$

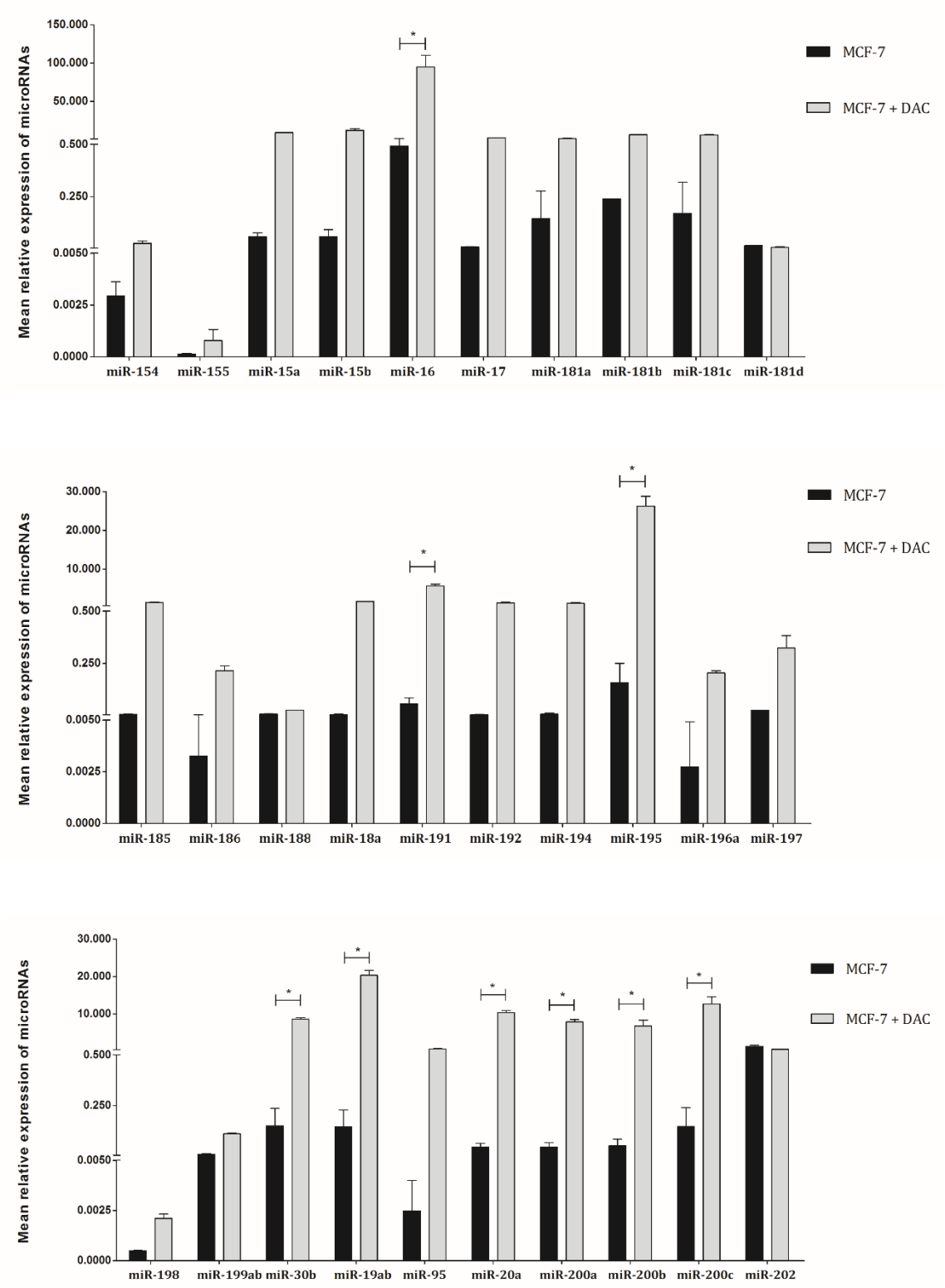

Figura 3.3 (continued) - Expression patterns of the miRNAs from the QuantiMir Cancer Array in MCF-7 and MCF-7 treated with 2.5 µM DAC for 5 days. Values are represented as mean relative expression ± standard deviation. *p* values were determined by two-way ANOVA and corrected with Bonferroni multiple comparison post hoc test with 95% confidence interval. * $p < 0.001$; ** $p < 0.01$; *** $p < 0.05$

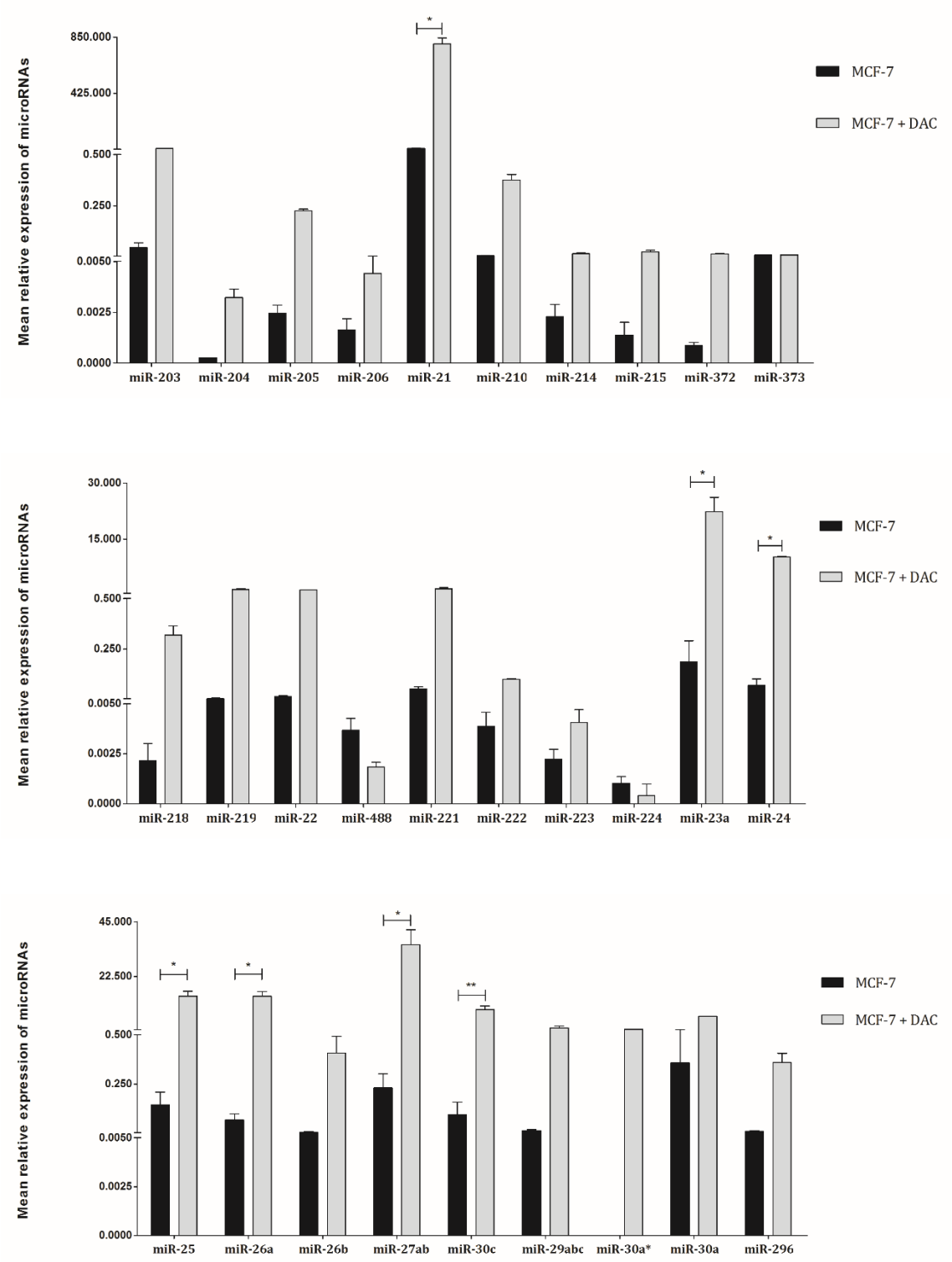

Figura 3.3 (continued) - Expression patterns of the miRNAs from the QuantiMir Cancer Array in MCF-7 and MCF-7 treated with 2.5 µM DAC for 5 days. Values are represented as mean relative expression ± standard deviation. *p* values were determined by two-way ANOVA and corrected with Bonferroni multiple comparison post hoc test with 95% confidence interval. * $p < 0.001$; ** $p < 0.01$; *** $p < 0.05$

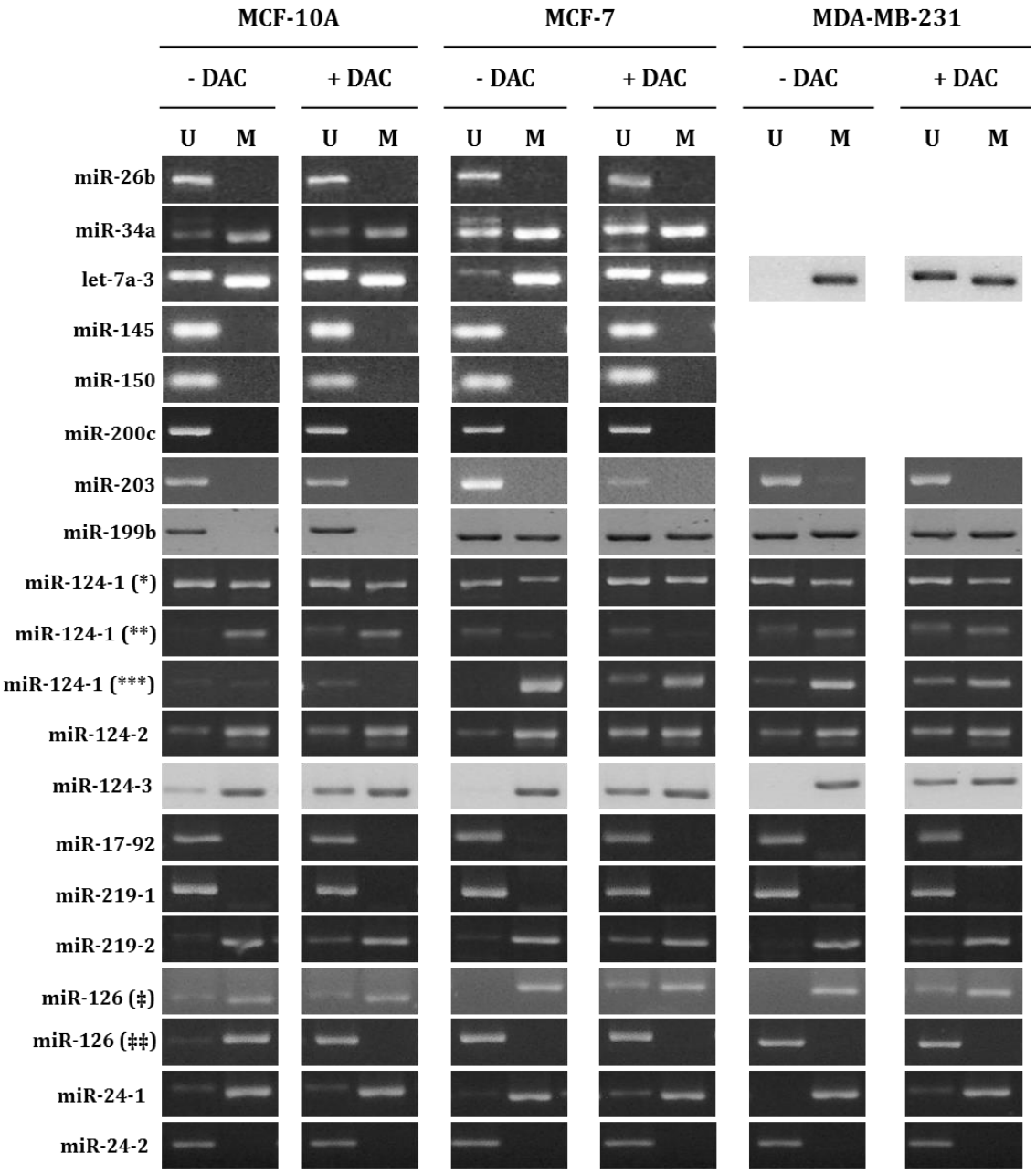

Figure 3.4 - Methylation status of the miRNAs gene promoters studied. UF – unmethylated forward; UR – unmethylated reverse; MF – methylated forward; MR – methylated reverse. (*) CpG island located at the transcription binding site of P53 gene; (**) CpG island 70; (***) CpG island 170; (‡) CpG island 29; (‡‡) CpG island 97.

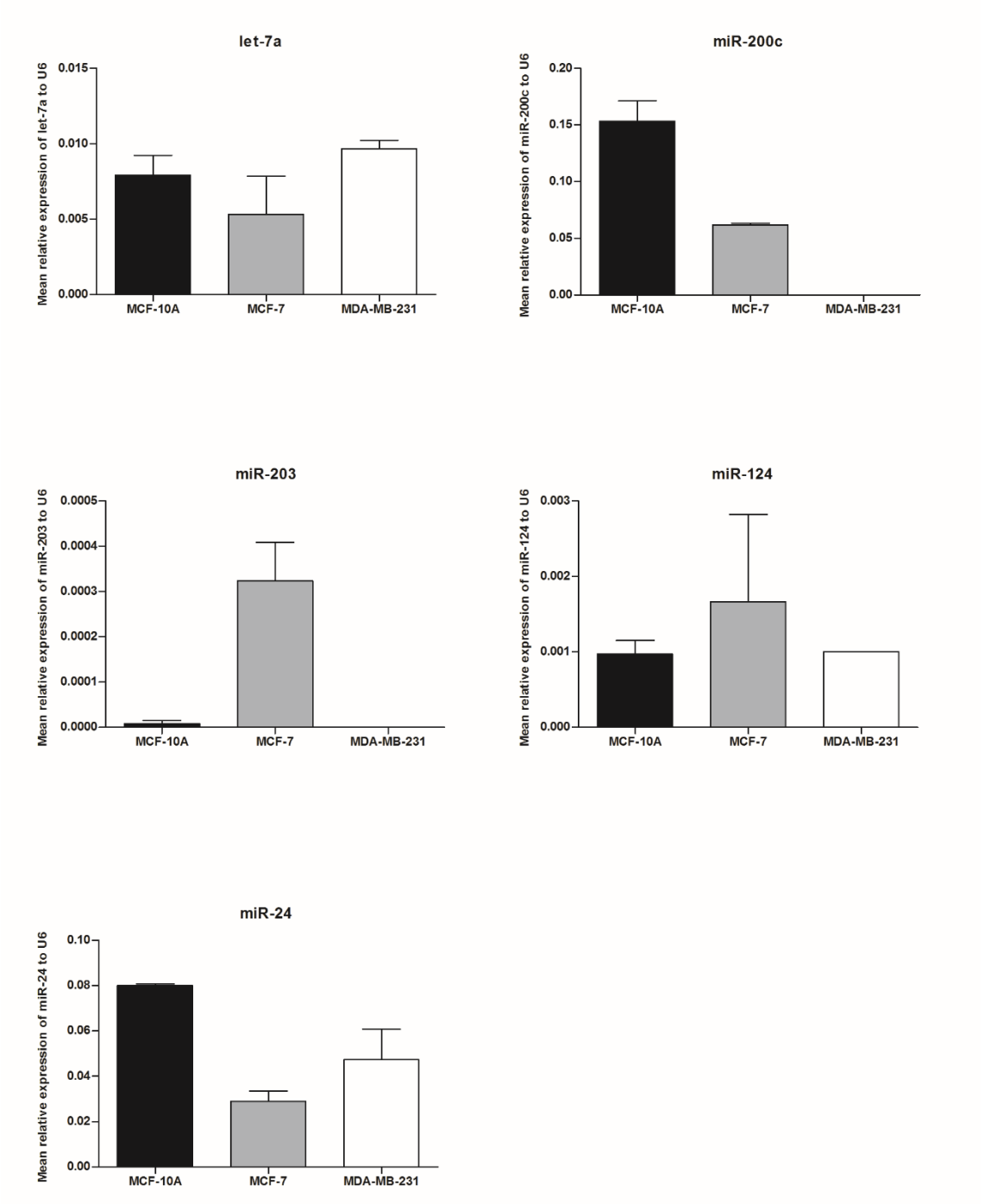

Figure 3.5 – Expression levels of let-7a, miR-203, miR-200c, miR-124 and miR-24 in MCF-10A, MCF-7 and MDA-MB-231 cell lines assessed by RT-qPCR. Values are mean relative expression to U6 snRNA ± standard deviation.

DAC influences protein expression in MCF-10A, MCF-7 and MDA-MB-231

After treatment with 2.5 μM of DAC, proteins were extracted in order to see if there are differences between cell lines in respect to protein profile. Figure 3.6 and Figure 3.7 represents the differentially expressed proteins in MCF-10A, MCF-7 and MDA-MB-231 cells. Interestingly, we obtained more differences in MCF-10A. After separation with 2-D SDS PAGE and protein analysis with MALDI-TOF/TOF, we observed that TPI1, PRDX2, CMPK1, ENO1, PRDX6, PSMA6, SERPINB5, ACTB, SFN, TPM1 and TPM3 were overexpressed in cells treated with DAC. While in MCF-7 + DAC, TUBA1b and RAN were overexpressed and in MDA-MB-231, GNB2l1, PARK7 and RAN were overexpressed.

As we observed that RAN seems to be methylated in MCF-7 and MDA-MB-231 and it is one of the genes involved in miRNAs biogenesis we hypothesized if this could be a mechanism of regulation of miRNAs. Thus we performed MSP of two promoter regions of two biogenesis genes - RAN and XPO5 (Figure 3.8). Only the XPO5 CpG island 46 was methylated. However, treatment with DAC seems to be ineffective. This could be a methodological problem rather than a positive result. The experiment was repeated with a different batch of cells with treatment and the results remain the same. In order to confirm the sequencing results with MALDI-TOF/TOF we performed western blot of RAN (Figure 3.9). We did not find differences in MCF-7 and MDA-MB-231 after treatment with DAC, in contrast with the sequencing results.

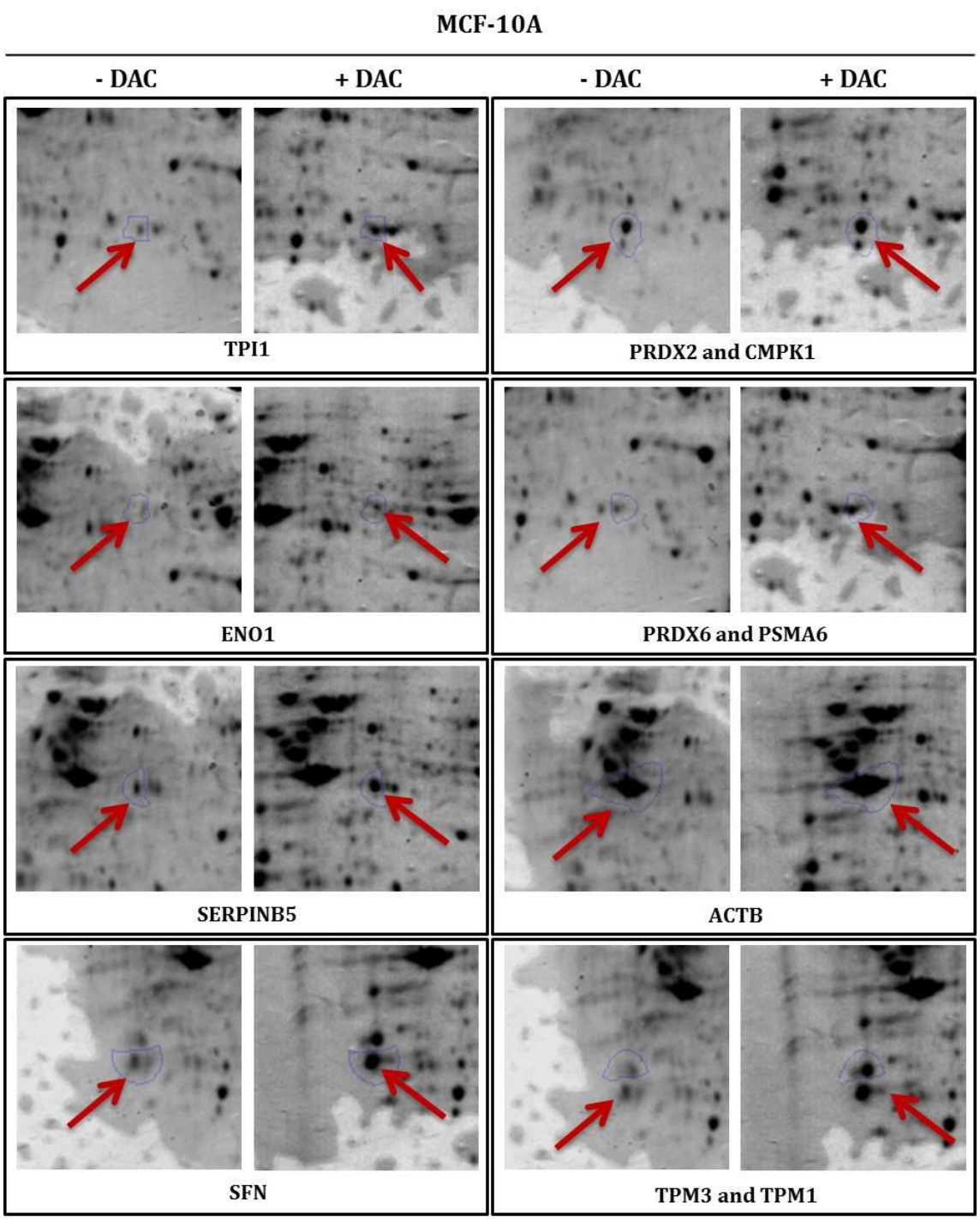

Figure 3.6 - Differentially expressed proteins in the MCF-10A cell line with and without treatment with 2.5 µM DAC for 5 days.

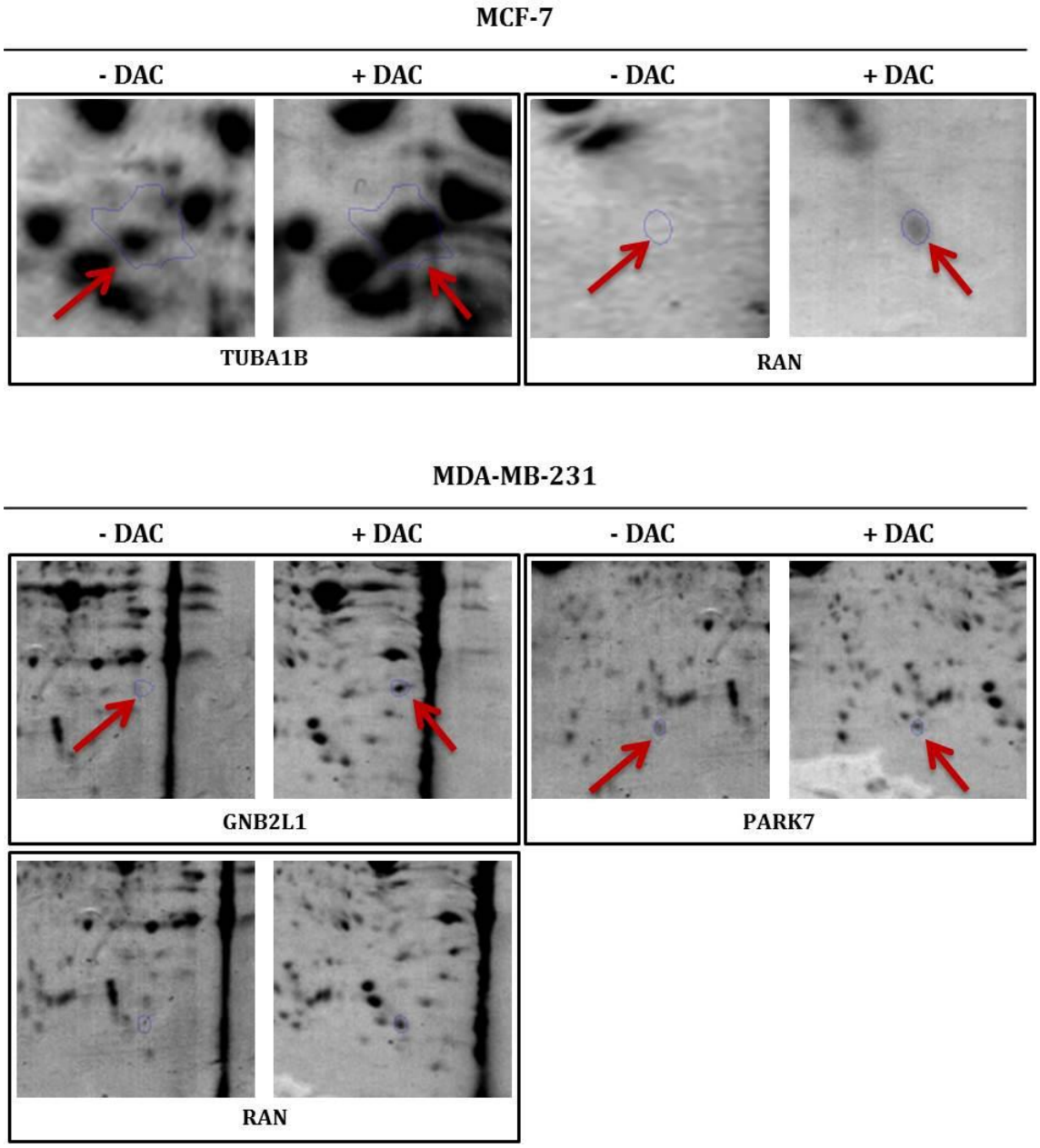

Figure 3.7 - Differentially expressed proteins in the MCF-7 and MDA-MB-231 cell lines with and without treatment with 2.5 µM DAC for 5 days.

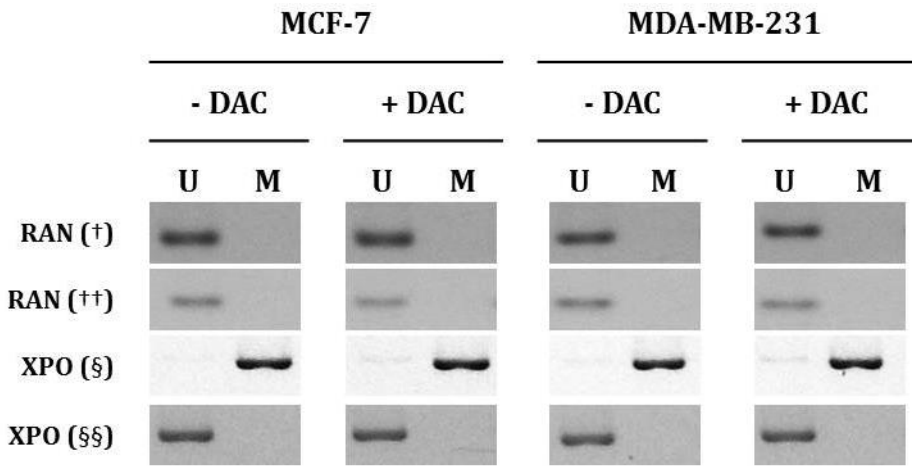

Figure 3.8 - Methylation status of the gene promoters of RAN and XPO5. UF – unmethylated forward; UR – unmethylated reverse; MF – methylated forward; MR – methylated reverse. (†) CpG island 75. (††)CpG island 126. (§) CpG island 46. (§§) CpG island 94.

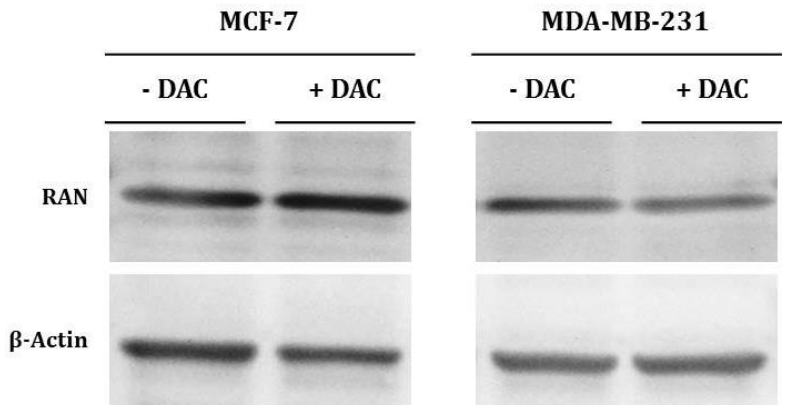

Figure 3.9 - Confirmation of the RAN protein expression by western blot.

3.4 Discussion

Gene expression can be regulated at various levels. Indeed, from gene to protein synthesis, there are many steps suitable for being regulated. Post-transcriptional regulation is one of those levels and since the discovery of miRNAs it has been markedly studied. In normal conditions, steady miRNAs levels can preserve cell homeostasis by regulating genes that if not regulated can lead to several diseases. However, any system can be disturbed and

miRNAs are not an exception. In fact, miRNAs can be regulated through the same mechanisms of coding genes, like epigenetic and post-transcriptionally modifications that concomitantly deregulate proteins involved in miRNAs biogenesis.

Based on this assumption and taking into account our exploratory results regarding this matter, we decided to study epigenetic changes in putative miRNAs gene promoters and one protein involved in their biogenesis as possible reasons for misexpression.

In an exploratory study we analysed 95 miRNAs in MCF-10A, MCF-7 and MDA-MB-231. These three cell lines represent, respectively, non-tumour mammary epithelium, tumour mammary epithelium and tumour with metastatic capability mammary mesenchymal-like. These cell lines have distinct morphologic aspects and as we showed their miRNAs expression pattern is also distinct (Figure 3.1 and Figure 3.2). Overall, MDA-MB-231 has low levels of miRNAs compared with the other two cell lines. These results are in accordance with a previous report (Lee et al., 2008) showing that that a large number of miRNAs are transcribed as precursors but are not processed to mature miRNA in cancer cell lines. The authors also state that is unlikely that a reduction in DROSHA or DICER levels, *per se*, explain the under-expression of miRNAs since other miRNAs are efficiently processed to mature in the same cell lines or tissues. Thus, some other aspect must be interfering with the process. One explanation is the transport of pre-miRNAs from the nucleus to the cytoplasm. Supporting this data was our exploratory results with respect to protein expression. Indeed, after treatment with DAC, tumour cell lines seem to over-express RAN (Figure 3.7), an accessory protein of XPO5 that enables the miRNAs export to the cytoplasm. With this data, we also hypothesized that RAN promoter region must be hypermethylated. However, after studying the promoter methylation status of RAN we found that it was not hypermethylated (Figure 3.8). Thus, our explanation to this fact is that we observed an over-expression of RAN not because its methylation status but because other macromolecules need to be transported to the cytoplasm after treatment with DAC (Matchett et al., 2014). Thus, we observed an adaptation of the cell to the new

conditions and not a demethylation of this gene. This explanation is probably the same for the increased expression of RAN in MCF-7 cell line (Figure 3.7). Further studies of this protein through western blot did not show increasing levels of RAN after treatment with DAC (Figure 3.9). These discrepancies may be related with the specificity of the techniques and most probably with antibody affinities, impeding us to confirm the results obtained with mass spectrometry. Although, we were unable to infer if RAN is actually regulating miRNAs expression in breast cancer cell lines, the augmented expression of RAN is associated with cancer. In fact, several reports have been published showing that RAN is associated with poor prognosis in several types of cancer such as prostate (Harada et al., 2008), breast (Kurisetty et al., 2009), colon (Hung et al., 2009), renal (Abe et al., 2008) and lymphoma (Hartmann et al., 2008). Interestingly, cancer cells seem to be RAN dependent, due to its role in mitotic spindle and mitosis (Xia et al., 2008).

We then decided to study methylation status of miRNAs genes promoters. After the exploratory results that showed usndicated that miRNAs are differentially expressed in the cell lines, we studied the effect of DAC in the expression of miRNAs. The generalized increase of miRNAs expression levels after treatment (Figure 3.3) that we observed was expected and prompted us to look for promoter regions of miRNAs genes. After an exhaustive search in the data bases and publications we found 20 putative promoter regions in 16 miRNAs genes and studied their methylation status. Unfortunately, it was not possible to study all overexpressed miRNAs existing in the quantification array since the promoter regions were not described. In spite of our efforts, we cannot associate methylation status with miRNAs expression due to the fact that both results are contradictory (Figure 3.4 and Figure 3.5). After we initiated this approach a study was published regarding let-7a-3 (Vrba et al., 2013). The authors showed that the promoter region of let-7a-3 in MCF-7 and MDA-MB-231 is hypermethylated, which is in agreement with our results. However, the authors do not show let-7a-3 expression level. It would be interesting if the authors had confirmed the methylation status with expression levels.

Another article confirms our results of miR-124 promoter region (Lv et al., 2011) and assume that miR-124 is regulated by methylation. In fact, the authors used the same regions as we and found that the promoter regions of miR-124-1, miR-124-2 and miR-124-3 were highly methylated in MDA-MB-231. However, the authors used bisulfite sequencing instead of MSP. In spite of the technique, the methylation status showed by the authors is quite similar to ours and they detected an increased expression of miR-124 after treatment with DAC. Without treatment the authors showed that miR-124 expression is attenuated in MDA-MB-231, as we do. If the promoter regions are methylated and yet miR-124 is expressed we can only conclude that these regions are not influencing miR-124 expression levels. Otherwise, the miR-124 must not be expressed or is expressed in much lower levels. Thus, the assumption made by these authors is not fully correct and further studies regarding miR-124 and methylation status are needed.

Interestingly, miR-200c (Neves et al., 2010) and miR-203 (Sandhu et al., 2012; Sandhu et al., 2014) were described as being hypermethylated in breast cancer and in MDA-MB-231 cell lines, disagreeing from our results. However, like these authors we did not find any expression level of miR-200c and miR-203, as well.

In summary, we can conclude that neither RAN nor methylation of the studied regions seems to influence miRNAs expression levels. However none of the hypothesis should be discarded but studied through other approaches, like high resolution melting or bisulfite sequencing in order to more effectively detect methylated regions. After the approach described in this Chapter, many other promoter regions of miRNAs were described. Thus, a new analysis of these regions concomitantly with the ones already performed by us but with new technological approaches should be of utmost value.

4. Functional analysis of miR-200c and miR-203 in breast cancer cell lines MCF-7 and MDA-MB-231

The work showed in this chapter allowed us to publish a review chapter about the involvement of miRNAs in several tumours and drugs resistance:

- Gomes, B. C., Rueff, J., and Rodrigues, A. S. (2016). MicroRNAs and Cancer Drug Resistance. Methods Mol Biol 1395, 137-162.

4.1. State of the art

Breast cancer therapy is usually performed using radiotherapy, hormonotherapy (e.g. tamoxifen, anastrazole, letrozole and exemestane), chemotherapy (e.g. doxorubicin, cyclophosphamide, docetaxel, fluorouracil, epirubicin and methotrexate) and targeted therapy (e.g. trastuzumab). Frequently the three types of therapy are used together, or in a combination of several chemotherapeutics, in order to achieve better outcomes. Nevertheless, in spite of the advances in drug discovery, many patients relapse due to drug resistance.

As stated in Chapter 1, miRNAs expression deregulation can lead to altered phenotypes and consequently different cell behaviour. Being characterized as oncomiRs or tumour suppressors, miRNAs can influence all hallmarks of cancer (Hanahan and Weinberg, 2011; Ross and Davis, 2011). Drug resistance is an acquired phenotype of the tumour cells that is most undesired. The confirmation that it is influenced by miRNAs led quickly to studies that assessed the influence of miRNAs in drug resistance. Indeed, miRNAs can regulate drug resistance-related genes, alter drug targets, change drug concentrations, influence therapeutic-induced cell death, regulate angiogenesis and be involved in the development of tumour stem cells. Thereafter many groups have focused on the role of these small regulatory RNAs in the development of cancer drug resistance (Table 1.1).

Tumour suppressor miR-200c and miR-203 have been associated with several targets, including in cancer drug resistance. It is known that the sensitivity to some cancer drugs like etoposide, taxol and epidermal growth factor receptor inhibitors is increased with E-cadherin expression restoration. Chen and colleagues (Chen et al., 2013b) showed that miR-200c increases drug sensitivity of breast cancer cells to doxorubicin through the E-cadherin-mediated up regulation of PTEN. Similarly, an increased expression of miR-200b and miR-200c enhances the sensitivity to growth inhibition by 4-hydroxytamoxifen (4-OHT) and fulvestrant in breast cancer cells (Manavalan et al., 2013). Although it is known that miR-200 family regulates EMT through ZEB1 and E-cadherin, the real mechanism through which the miR-200 family regulates drug resistance is not known, thus being necessary further studies to understand these phenomena. In response to this question, interesting data about miR-200c and feedback circuits of miR-200c/ZEB1 and miR-200c/ZNF217/TGF-β/ZEB1 were recently published (Bai et al., 2014). The authors showed that these circuits contribute to trastuzumab resistance and metastasis of breast cancers. Interestingly, this feedback circuits might be related with reverse EMT in metastases formation, since ZEB1 can inhibit miR-200c expression. The authors also showed that low levels of miR-200c activate the TGF-β signalling pathway and consequently trastuzumab resistance in breast cancer cells. Indeed, restoring miR-200c was sufficient to re-sensitize cells to trastuzumab and reverse the mesenchymal phenotype by inhibiting TGF-β signalling and ZEB1 expression. Similarly, another published report showed that a high MIG6 expression and a suppression of miR-200c expression is a consequence of TGF-β-induced EMT and a signature for resistance to erlotinib (Izumchenko et al., 2014a).

Drug transport through cell membranes is a critical step in allowing access of pharmacologic agents to intracellular targets. The involvement of drug transport is probably the most studied mechanism in cancer drug resistance and one of the most well-known transporters is ABCB1, also known as MDR1 or P-gp transporter. In

chemotherapeutic-resistant cancer cell lines, ABCB1 is often observed to be up-regulated. The increased expression of ABCB1 leads to an increased resistance to several chemotherapeutics, such as taxanes (e.g. paclitaxel and docetaxel), epipodophyllotoxins derivatives (e.g. etoposide and teniposide), anthracyclines (e.g. doxorubicin), antibiotics (e.g. actinomycin D), vinca alkaloids (e.g. vinblastine and vincristine) and tyrosine kinase inhibitors (e.g. imatinib and erlotinib) (Gromicho et al., 2011; Gromicho et al., 2012; Kathawala et al., 2014). To date, some authors have published data about misexpression of miRNAs or ABCB1in breast cancer (Chen et al., 2012; Kovalchuk et al., 2008). Kovalchuck and colleagues (Kovalchuk et al., 2008) showed that the *ABCB1* gene is highly expressed in the MCF-7/DOX breast tumour cell lines resistant to doxorubicin when compared with wild type MCF-7, while Chen and colleagues (Chen et al., 2012) showed a correlation of miR-200c with poor response to neo-adjuvant chemotherapeutics using breast cancer tissues. Thus, this poor prognosis could be due to misregulation of *ABCB1* by miR-200c. However, the confirmation of an inverse correlation between ABCB1 and miR-200c in breast cancer need further validation.

Relatively to miR-203, there is no direct evidence of its effect on drug resistance in breast cancer. However, association with other tumours like colon and CML has been published. Indeed, it was demonstrated that miR-203 can sensitize cells to paclitaxel (PAX) in colon cancer (Li et al., 2011a) and imatinib mesylate in CML (Li et al., 2013b) by targeting BCL2 and BCR-ABL, respectively. Ectopic expression of miR-203 in colorectal cancer cell lines lead to an increased resistance to oxaliplatin by targeting ATM (Zhou et al., 2014) and sensitises cells to 5-fluorouracil (5-FU) by targeting thymidylate synthase (TYMS) (Li et al., 2015).

Paclitaxel and 5-FU are two chemotherapeutic agents used in breast cancer. Due to the fact that there is no data regarding miR-200c and miR-203 and its influence in breast tumour cells and taking into account the expression of these two miRNAs in MCF-7 and MDA-MB-

231 cell lines (Figure 3.5), we aimed to assess the effect of the ectopic up-regulation and down-regulation of miR-200c and miR-203 in breast tumour cell lines in resistance to PAX and 5-FU.

4.2. Material and Methods

4.2.1. Cell lines and nucleic acid purification

Cell lines culture and nucleic acid purification was done according to *Cell lines* and *Nucleic acid purification* from chapter 3, section Material and Methods.

4.2.2. Ectopic expression and inhibition of miR-200c and miR-203

Mimetic miRNAs (Pre-miR™ miRNA Precursor hsa-miR-200c-3p # PM11714 and hsa-miR-203a #PM10152; Life Technologies) and inhibiting miRNAs (Anti-miR™ miRNA Inhibitor hsa-miR-200c-3p # AM11714 and hsa-miR-203a-3p # AM10152; Life Technologies) were transfected in MDA-MB-231 and MCF-7, respectively. As negative controls we used Pre-miR™ miRNA Precursor Negative Control #1 (Life Technologies # AM17110) and Anti-miR™ miRNA Inhibitor Negative Control #1 (Life Technologies # AM17010). These negatives controls are oligonucleotides similar to miRNA precursors and miRNA inhibitors but without biological effect. The transfection agent used was FuGENE HD transfection reagent (Promega # E2311). Pre-miR™ miRNA Precursor with respective negative control was used in a concentration of 30 nM and Anti-miR™ miRNA Inhibitor with respective negative control was used in a concentration of 50 nM. The transfection agent was used in a concentration of 0.3% (v/v). The transfection complex (oligo + transfection agent) was prepared in DMEM without any supplementation and incubated at room temperature for 15 minutes. After this time, the transfection complex was added to the cells that already had complete culture medium in a proportion of 1:1 and incubated at 37 °C for 24 h.

4.2.3. Cell viability assay (MTT)

Approximately 3000 cells were cultured in complete medium in 96-well plates. The cells were allowed to grow for 24 h and then transfected as stated in *4.2.2*. After 24 h of transfection, cells were exposed to different concentrations of paclitaxel (PAX; Sigma-Aldrich # T7402) (dissolved in DMSO, not exceeding 0.1%, in concentrations of 0, 10, 100, 250, 500, 750, 1000 and 1250 nM for 72 h) and 5-Fluorouracil (5-FU; Sigma-Aldrich # F6627) (dissolved in DMSO, not exceeding 0.1%, in concentrations of 0, 5, 10, 20, 40, 80, 160, 200 and 250 μM for 72 h). DMSO at 0.1% (v/v) was added to the wells without chemical (control cultures). After 72 h, the medium was removed and thiazolyl blue tetrazolium bromide (MTT; Sigma-Aldrich # M5655) dissolved in culture medium was added to each well at a concentration of 0.5 mg/ml. Cells were then incubated for 3 h and the MTT discarded. Next, 200 μl of DMSO was added to each well to dissolve the formazan crystals. Absorbance was read at 595 nm in a Zenyth 3100 microplate reader. Absorbance values presented by control cultures correspond to 100% cell viability. At least three independent experiments were performed.

4.2.4. Reverse transcription qPCR

Reverse transcription qPCR was done by using Universal cDNA synthesis kit II (Exiqon # 203301) and ExiLENT SYBR® Green master mix (Exiqon # 203403). This methodology was performed to detect miR-200c and miR-203 expression levels in MCF-7 and MDA-MB-231 cell lines after ectopic inhibition or over-expression of the miR-200c and miR-203. The methodology was done as stated in *3.2.3*. The real-time PCR was performed in ABI 7300 real time PCR instrument with the following conditions: polymerase activation at 95 °C for 10 minutes; 40 cycles of amplification at 95 °C for 10 seconds and 60 °C for 1 minute.

4.2.5. Statistical analysis

Data were analysed with GraphPad Prism 5 software, using one-way ANOVA with Dunn's multiple comparison post hoc test and two-way ANOVA with Bonferroni multiple comparison post hoc test with confidence intervals of 95%. All graphs were made with GraphPad Prism 5 software and values represented are mean expression ± standard deviation.

4.3. Results

Ectopic expression or inhibition of miR-200c and miR-203 in human breast cell lines does not affect drug resistance

In order to confirm if miR-200c and miR-203 influence PAX and 5-FU resistance in MCF-7 and MDA-MB-231 cell lines, we performed a transfection on both cell lines with anti-miRNAs (200c and 203) and pre-miRNAs (200c and 203), respectively. By itself, the transfection conditions do not affect cell viability when compared with the negative control that was transfected with an oligonucleotide with similar properties of anti-miRNAs and pre-miRNAs but does not have a biological effect (Figure 4.1). In fact, cell viability remains practically the same after 24 h of transfection and 72 h of normal growing conditions in both cell lines. Subsequently, we tested the effect of PAX and 5-FU. Thus, after 24 h of transfection, cell lines were incubated with PAX and 5-FU for 72 h and then cell viability tested using MTT assay. As showed in Figure 4.2 and Figure 4.3, we do not detect any significant difference with the transfection of anti-miRNAs and pre-miRNAs. However some aspects need to be considered in what regards PAX (Figure 4.2). Although not statistically different, we can observe that MCF-7 cell line transfected with anti-miR-200c has 50% cell viability at a lower dose of PAX then the other two conditions. While MCF-7 plus anti-miR-200c has 50% viability at approximately 500 nM of PAX, MCF-7 plus anti-miR-203 and negative control have 50% viability at approximately 1000 nM. In MDA-MB-231 cell line we observe the same pattern, although, cells were transfected with pre-miR-200c and pre-miR-203. With the ectopic expression of miR-200c cells have 50%

viability at approximately 500 nM of PAX while transfection with negative control has 50% viability at 750 nM. When MDA-MB-231 was transfected with pre-miR-203 it does not reach the 50% viability, even at the higher dose of 1250 nM. In Figure 4.4 we show the expression of miR-200c and miR-203 after transfection with inhibitory (anti-miR-200c and anti-miR-203) and precursor (pre-miR-200c and pre-miR-203) miRNAs. These graphs prove an effective transfection of MCF-7 and MDA-MB-231 cell lines.

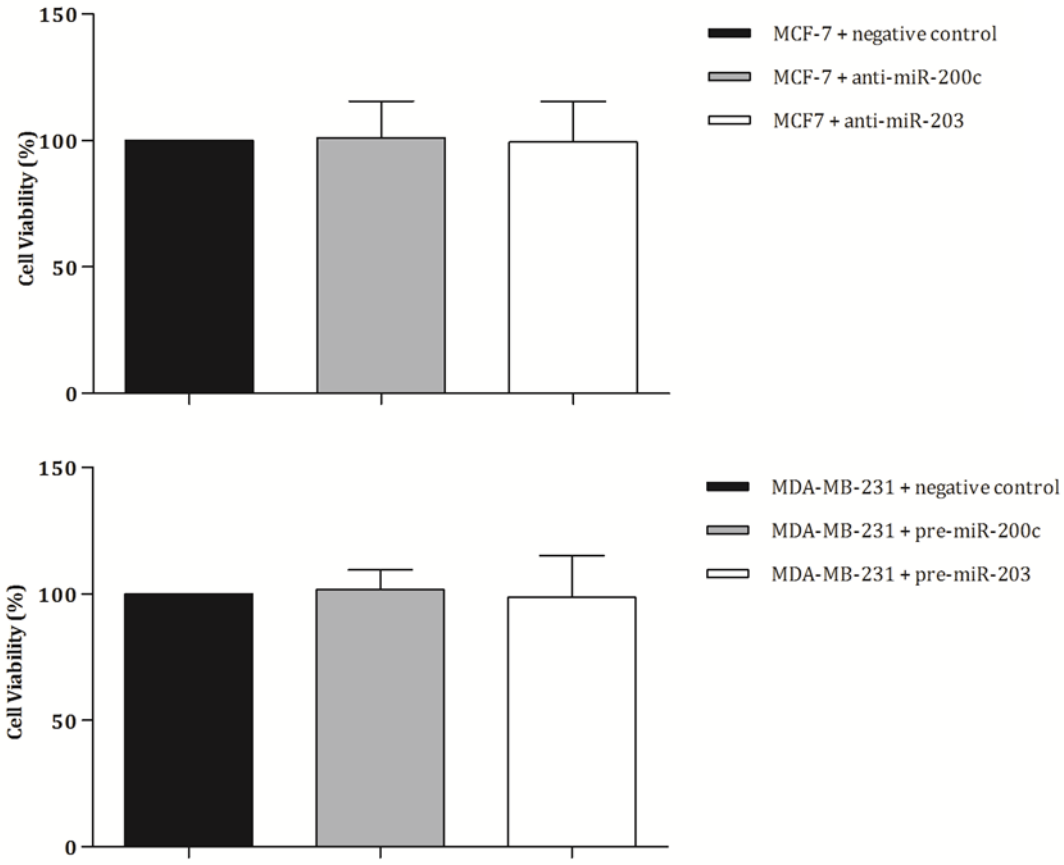

Figure 4.1 – Cell viability after ectopic inhibition of miR-200c and miR-203 in MCF-7 cell line and ectopic over-expression of miR-200c and miR-203 in MDA-MB-231. Values represent mean values of three independent experiments ± standard deviation. There were no significant differences between negative control and inhibition or over-expression of miR-200c and miR-203 in both cell lines.

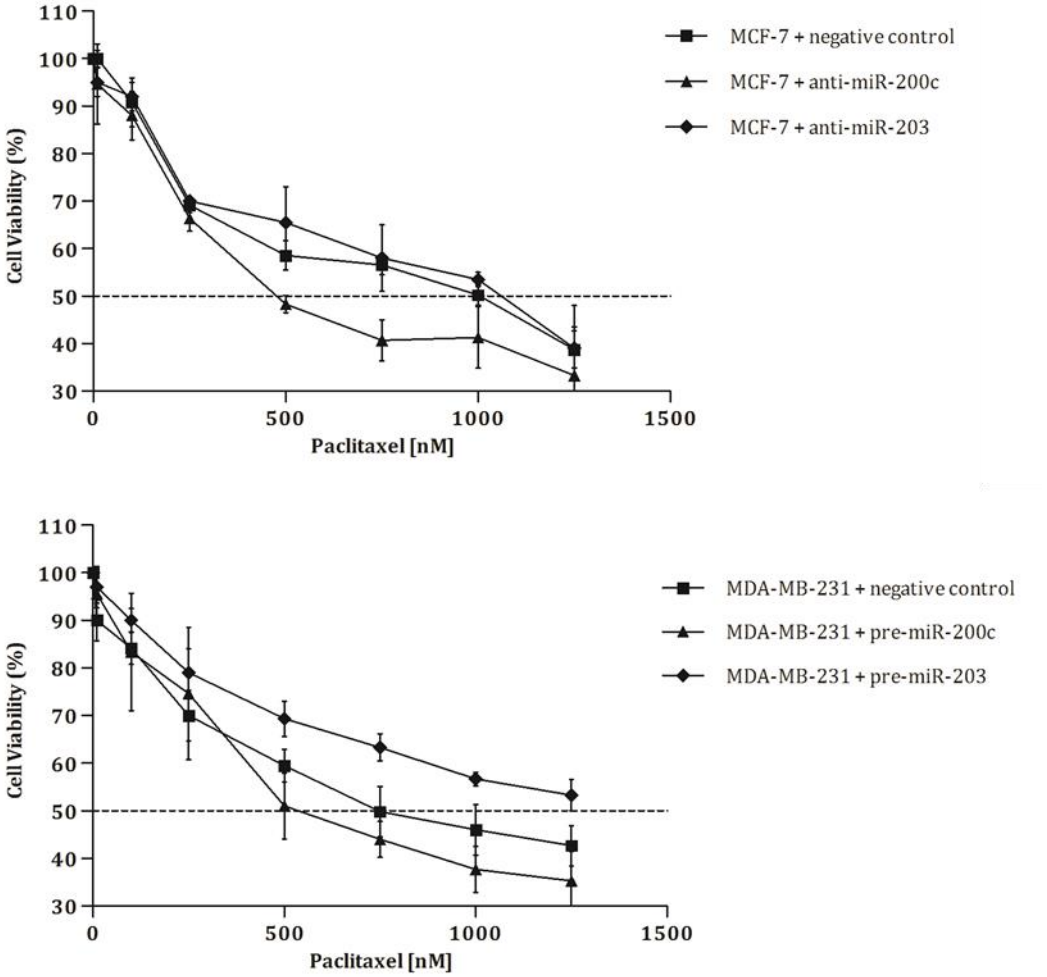

Figure 4.2 - Cell viability after ectopic inhibition of miR-200c and miR-203 in MCF-7 cell line and ectopic over-expression of miR-200c and miR-203 in MDA-MB-231 with Paclitaxel treatment. Cells were treated with Paclitaxel at concentrations of 0, 10, 100, 250, 500, 750, 1000 and 1250 nM for 72 h. Values represent mean values of five independent experiments ± standard deviation. There were no significant differences between negative control and inhibition or over-expression of miR-200c and miR-203 in both cell lines.

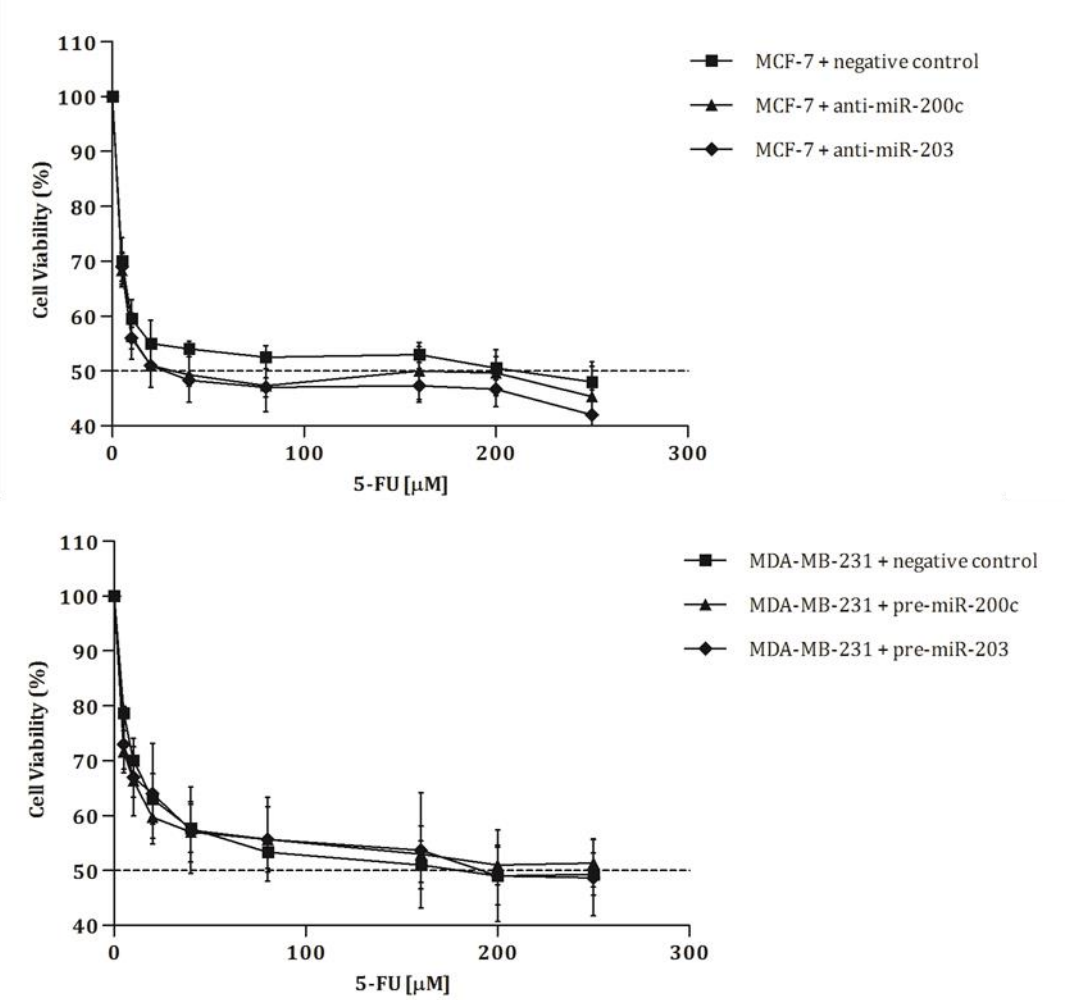

Figure 4.3 - Cell viability after ectopic inhibition of miR-200c and miR-203 in MCF-7 cell line and ectopic over-expression of miR-200c and miR-203 in MDA-MB-231 with 5-FU treatment. Cells were treated with 5-fluorouracil at concentrations of 0, 5, 10, 20, 40, 80, 160, 200 and 250 μM for 72 h. Values represent mean values of five independent experiments ± standard deviation. There were no significant differences between negative control and inhibition or over-expression of miR-200c and miR-203 in both cell lines.

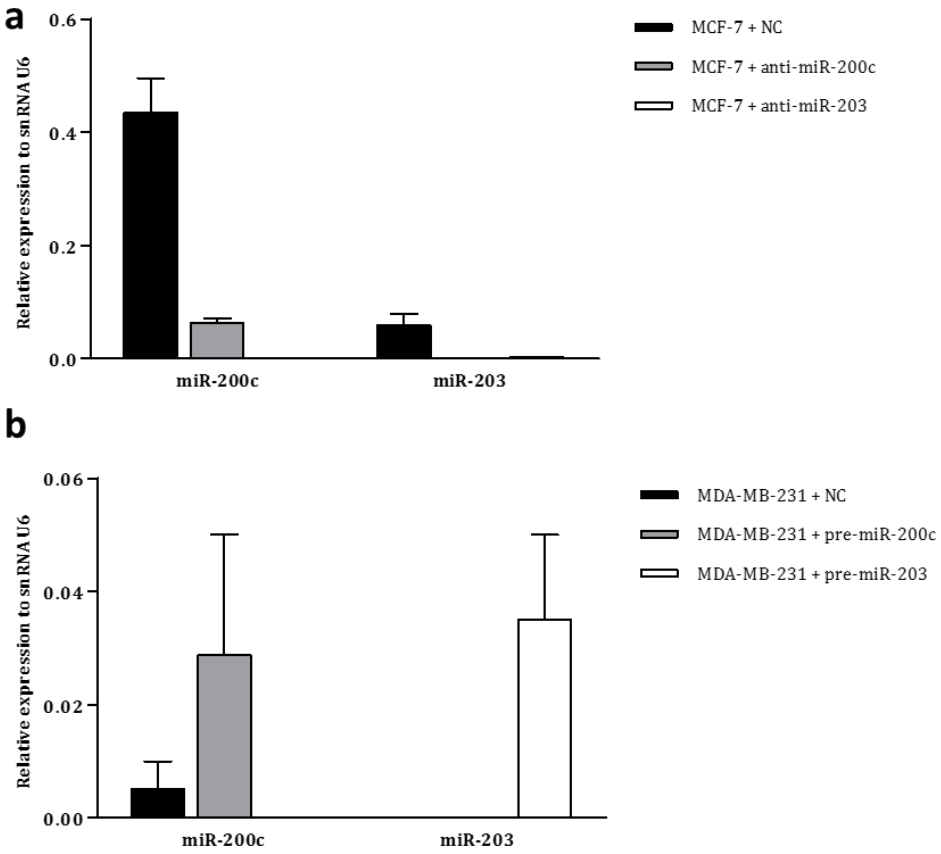

Figure 4.4 – Expression of miR-200c and miR-203. Inhibition was done through anti-miRNAs transfection (a) and insertion was done through pre-miRNAs transfection (b) using FUGENE HD. Values represent mean values of two independent experiments ± standard deviation.

4.4. Discussion

Cancer drug resistance still a burden worldwide and a reason for cancer recurrence and ultimately, death. In spite of the numerous progresses made in the last decades in what regards cancer drug resistance knowledge, many other aspects still await discover. In fact, a comprehensive knowledge of cancer drug resistance can lead to a more effective personalized therapy and consequently better outcomes for cancer patients. MiRNAs have been pointed out as regulators of drug resistance, thus many studies have been published in the last few years (Table 1.1). MiR-200c and miR-203 are two tumour suppressor miRNAs widely studied as modulators of drug resistance. However, there are few reports published associating these miRNAs and resistance to PAX or 5-FU, two cancer drugs

broadly used in breast cancer, manly in more advanced and aggressive types of breast cancer. Thus, we decided to study the effect of these miRNAs in breast cancer cell lines MCF-7 and MDA-MB-231. Through exploratory data, we observed that miR-200c and miR-203 are expressed in MCF-7 cells but not in MDA-MB-231, a more aggressive type of breast cancer cell line. Thus, we decided to inhibit these miRNAs in MCF-7 cells and ectopically express both miRNAs in MDA-MB-231 cells. Transfection with anti-miRNAs and pre-miRNAs, respectively, *per se* does affect viability in cell lines. Consequently, any differences found after treatment with a drug would be indicative that these miRNAs could be the reason for such differences. Under this assumption, we treated both cell lines with several concentrations of PAX and 5-FU. Interesting results were obtained regarding treatment with PAX. According to our data miR-200c has different roles in the resistance of PAX in MCF-7 and MDA-MB-231. While in MCF-7 cells the inhibition of miR-200c confers sensitivity to PAX, in MDA-MB-231 the insertion of miR-200c also confers sensitivity to PAX. In fact, comparing MCF-7 negative control treated cells with MCF-7 miR-200c inhibited cells, the 50% survival concentration in inhibited cells is half (500 nM) of that showed by the negative control (1000 nM). Although there were no published results regarding miR-200c, PAX and breast cancer, the results obtained with MCF-7 cells are contrary to those published in other types of cancer. In particular, in ovarian cancer was demonstrated that restoration of miR-200c not only decreased tumour growth but also significantly enhanced the response to PAX (Brozovic et al., 2015; Cittelly et al., 2012). Furthermore, the study also suggested that restoration of miR-200c immediately before treatment with PAX could enhance the therapy and that miR-200c could be given concomitantly with paclitaxel as second-line therapy upon relapse (Cittelly et al., 2012). Similarly, a study published interesting data showing that patients with ovarian cancer that do not achieve a complete clinical response to PAX had much lower levels of miR-200c than those that had complete clinical response (Leskela et al., 2011). The results published in these studies are in accordance with ours in MDA-MB-231 cell line, where the

insertion of miR-200c also reduced the concentration (500 nM) of PAX needed to achieve 50% of cell viability.

Regarding our miR-203 results there are also differences between both cell lines treated with PAX. While in MCF-7 cells, where miR-203 expression was supressed, we did not obtain any difference when compared to the negative control; in MDA-MB-231 cells, where miR-203 expression was ectopically increased, we observed an increased resistance to PAX. Regarding miR-203, the only study to our knowledge reports that miR-203 ectopic expression in colon cancer cell lines chemosensitize cells to PAX (Li et al., 2011a).

We highlight the fact that to date there is no data regarding these miRNAs effect in resistance to PAX in breast cancer. These contradictory results obtained from two different breast cancer cell lines lead us to conclude that miR-200c and miR-203 are regulating different targets and consequently affecting different pathways depending on the aggressiveness of the cell line. Since PAX promotes the assembly of microtubules and reduces the concentration of tubulin subunits necessary for polymerization into microtubules, we hypothesize that these miRNAs target genes that exacerbate (MDA-MB-231) or inhibit (MCF-7) microtubules polymerization in the presence of PAX, protecting it from the disassembly. Thus, cells entering mitosis produce abnormal spindles that lead to a long-term mitotic arrest and division of their chromosomes in multiple directions. This leads to aneuploidy and consequently cell death, presumably due to loss of essential chromosomes (Weaver, 2014). Therefore, we intend to study possible targets of these miRNAs involved in mitotic spindle formation and associate that with PAX resistance.

Regarding 5-FU, we did not obtain any differences after inhibition or ectopic expression of miR-200c and miR-203. Although not expected, this might be due to the mechanism of action of 5-FU. 5-FU is an analogue of uracil with a fluorine atom in place of hydrogen that is converted in the cell to several active metabolites (e.g. fluorodeoxyuridine

monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP)). These active metabolites disrupt RNA and DNA synthesis and the action of Thymidylate synthase (TS) (Longley et al., 2003). This mode of action might explain the sharp drop of cell viability at low 5-FU doses. Perhaps assessment of longer incubation periods could allow us to observe different outcomes of cell viability. In fact, due to the fact that these cell lines have their doubling time between 24 and 35 hours, the time of drug treatment, 72 hours, could be insufficient. Thus, in the future longer periods of treatment must be used. This approach has not been done yet because of technical problems in respect to miRNAs transfection. In our transfection technique optimization, 72 hours was the longest time period that can assure reproducible results with transfected cell lines (data not showed).

In conclusion, miR-200c seems to have contrary effects in MCF-7 cell line and MDA-MB-231 and miR-203 seem to augment resistance to PAX in MDA-MB-231 cell line. Both miRNAs did not showed any effect in cells treated with 5-FU.

5. Analysis of miR-200c and miR-203 expression levels, and their putative targets in human breast cancer tissues

Part of the results and techniques presented in this chapter were published in:

- Gomes, B.C., Santos, B., Rueff, J., and Rodrigues, A. S. (2016). Methods for Studying MicroRNA Expression and Their Targets in Formalin-Fixed, Paraffin-Embedded (FFPE) Breast Cancer Tissues. Methods Mol Biol 1395, 189-205.
- Gomes, B.C., Martins, M., Lopes, L., Morujão, I., Oliveira, M., Araújo, A., Rueff, J., and Rodrigues, A. S. (2016) Prognostic importance of microRNA-203 expression in breast cancer. Oncology Reports. *In Press*

Or are submitted and under revision:

- Gomes, B.C., Santos, B., Martins, M., Lopes, L., Morujão, I., Oliveira, M., Araújo, A., Rueff, J., and Rodrigues, A. S. (2016) Immunohistochemistry detection of Six1 and Sox2 in a Portuguese breast cancer patient cohort. Submitted to BMC Cancer.

5.1. State of the art

Breast cancer (BC) is still a worldwide burden with an estimated incidence of more than 1.5 million new cases and approximately half a million deaths per year (Torre et al., 2015). Due to early detection, improvement in treatment options and changes in life style paradigms, mortality rates have been decreasing in developed countries. Conversely, developing countries are witnessing an increase in BC incidence and mortality rates, most probably due to weak awareness campaigns and changes in daily habits such as sedentary lifestyle, high consumption of sugars and fat that lead to overweight and obesity, known risk factors of BC (Torre et al., 2015). Moreover the proportion of cases diagnosed in less developed countries is meagre when compared to developed regions thus leading to higher mortality rates (De Abreu et al., 2014).

Although the molecular mechanisms that underlie the development of breast cancer are being boldly investigated, our current knowledge is far from complete. BC is a heterogeneous malignancy and clinical diagnosis and prescribed therapy still reliy primarily on the TNM staging system based on tumour (T), node status (N) and metastasis (M). Oestrogen and progesterone receptor status, human epidermal growth factor receptor 2 (HER2/neu) status and the Ki-67 proliferative index, besides tumour-infiltrating lymphocytes, as well as the age of the patient, are used to classify BC into various subtypes (Badve et al., 2011; De Abreu et al., 2014; Gyorffy et al., 2015). Nonetheless, these conventional breast cancer prognostic factors have intrinsic limitations, and their use does not allow an accurate prediction of treatment resistance or relapse. Defining new molecular prognostic factors to refine BC classification could be useful in improving the therapeutic schemes.

Recently, various miRNAs have been characterized and identified as regulators and/or biomarkers in breast cancer development, including initiation, metastasis, and therapy resistance (Chen et al., 2008; Cheng et al., 2015; He et al., 2005; Ma et al., 2010a; Takamizawa et al., 2004; Vasudevan et al., 2007). Due to their size, miRNAs are stable in human samples and can easily be used as molecular signatures in cancer (Andreasen et al., 2010; Azam et al., 2015; Graveel et al., 2015; Hui et al., 2011; Sadeghian et al., 2015; Wang et al., 2014). Their targets although not so easily detected are nonetheless good tools to develop molecular signatures in cancer.

MiR-203 was originally described as a keratinocyte-specific miRNA (Sonkoly et al., 2007) but was soon shown to play an important role in bladder cancer (Gottardo et al., 2007). It was also shown that is epigenetically silenced in hematopoietic cancers (Bueno et al., 2008). Several studies have shown an association of miR-203 and chemotherapeutic resistance to cisplatin (Ru et al., 2011), invasiveness (Chen et al., 2015; Zhang et al., 2011), proliferation (Chen et al., 2015; Hailer et al., 2014; Wang et al., 2012a; Yu et al., 2012), metastases (Ding et al., 2013; Taipaleenmaki et al., 2015) and as a biomarker (Imaoka et

al., 2015; Madhavan et al., 2012). Some miR-203 targets have been identified, such as *SNAI2, SOCS3, BIRC5 and LASP1* (Chang et al., 2015; Chen et al., 2015; Ru et al., 2011; Wang et al., 2012a; Zhang et al., 2011), but a complete picture of the expression of miR-203 in different cohorts of BC, its mechanisms of action and the circuitry of its effects still remains by and large to be fully clarified. Recently, it was also shown that miR-203 directly binds to ATM (Zhou et al., 2014). ATM, encodes a serine/threonine kinase, a member of the phosphoinositide 3-kinase (PI3-kinase)-like family (PIKK), that is activated in response to double strand DNA breaks damage and is responsible for maintaining the stability of the genome by phosphorylation and activation of several downstream targets (e.g. H2AX, CHK2, BRCA1, p53 and MDC1) known to be implicated in cancer [for further reading see a very recent and complete review (Guleria and Chandna, 2016)].

MiR-200c belongs to miR-200 family, a known family of miRNAs with tumour suppressor activity by inhibiting EMT by binding to ZEB1 and ZEB2 (Gregory et al., 2008; Wellner et al., 2009). However, many other putative targets can be found in on-line databases specialized in *in silico* detection of miRNAs targets (e.g. TargetScan v7.0 (Agarwal et al., 2015)). Sine Oculis Homeobox Homolog 1 (SIX1) is one of the putative targets described. It has been described as involved in the development of many tissues and organs, and its levels vary, throughout developmental stages from embryo to adulthood (Wu et al., 2015b). In adult life and health conditions, SIX1 is not expressed in somatic cells. However, some authors showed that gene amplification of SIX1 can increase BC risk (Reichenberger et al., 2005) and induce tumour cells to undergo EMT (Micalizzi et al., 2009), indeed a feedback loop between miR-204-5p and SIX1 promotes EMT in BC (Zeng et al., 2015). Recently, it was also described that SIX1 expression can be regulated by miR-185 (Imam et al., 2010), thus suppressing tumour growth and progression. Another putative target of miR-200c is SRY (Sex Determining Region Y)-Box 2 (SOX2). This is a transcription regulator that controls pluripotency and self-renewal in embryonic stem cells by physically interacting with Octamer-binding transcription factor 4 (OCT4) and Homeobox

Transcription Factor Nanog (NANOG), forming a protein complex that binds the promoters of numerous stem cell differentiation factors, suppressing their expression (Zhang and Cui, 2014). A meta analyses done by Li and colleagues showed that SOX2 is a good prognostic factor for head and neck cancer (Li et al., 2014) and non-small cell lung cancer (Chen et al., 2013a). Some studies showed that SOX2 has some relevance in promoting metastatic potential in breast cancer (Huang et al., 2014; Lengerke et al., 2011). Functional studies also support a pivotal role of miR-200c in regulating self-renewal of mammary stem cells through a direct targeting of B cell-specific Moloney murine leukemia virus integration site 1 (BMI1) (Shimono et al., 2009b). BMI1 was identified as a proto-oncogene cooperating with c-Myc during the initiation of lymphomas. It has subsequently been identified as a transcriptional repressor belonging to the polycomb group (PcG) proteins, and is also a key factor in the polycomb repressor complex 1 (PRC1), which serves as an important epigenetic regulatory complex for modulation of chromatin remodelling. Over-expression of BMI1 has been found in a large number of human cancers, including breast cancer, which indicates that BMI1 might play important roles in cancer initiation and progression (Benetatos et al., 2014). BMI1 is also a putative target of miR-203, however there are no functional studies showing their association.

With the aim of contributing to a better understanding of the role of miR-203a and miR-200c in breast cancer, we assessed here miR-203a and miR-200c expression and clinicopathological features in a Portuguese population with breast carcinoma. Since these two miRNAs have 4 targets (putative or/and proven with few studies done so far) we also aimed to study the expression of SIX1, SOX2, ATM and BMI1 in breast cancer samples by immunohistochemistry and correlate their expression with expression levels of their regulators and clinicopathological features.

5.2. Material and methods

5.2.1. Human FFPE samples collection

Patients with breast carcinoma were recruited for the study at the Hospital de São José, from Centro Hospitalar de Lisboa Central, during 2013 and 2014. Each patient signed a written informed consent form and this study was reviewed and approved by the Ethical Committees of the NOVA Medical School and of the Centro Hospitalar de Lisboa Central. All clinical information was gathered by trained and specialized clinicians. All samples were originated from surgical sections (mastectomy or tumorectomy). A total of 109 formalin-fixed paraffin-embedded (FFPE) paired normal and tumour tissue samples were collected. Normal tissue was adjacent to the tumour and in all cases was confirmed by the pathologist team as being only normal mammary tissue. Diagnosis and common immunohistochemical markers for breast cancer classification such as oestrogen and progesterone receptor (ER and PR), HER2 amplification status and Ki-67 proliferative index were evaluated by 2 highly trained and independent pathologists. Staging was done by tumour (T), node (N) and metastasis (M) classification (Singletary et al., 2002). Procedure for immunohistochemical detection was done according to the recommendations of the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines (Hammond et al., 2010; Wolff et al., 2013) and the International Ki67 in Breast Cancer Working Group (Dowsett et al., 2011) at the time of sample collection. With the existing canons at the time, the molecular classification of breast tumours was as follows: Luminal A - ER or PR positive and Ki67 <13%; Luminal B - ER or PR positive and Ki67 ≥13%; HER2 positive - ER and PR negative, HER2 positive; Triple negative – ER, PR and HER2 negative.

5.2.2. Total RNA purification from FFPE breast tissues

Total RNA was purified from FFPE tissues using RecoverAll™ Total Nucleic Acid Isolation Kit (Ambion® # AM1975) and according to the manufacturer's protocol with slight alterations. One ml of 100% xylene was added to approximately 80 μm of tissue slices

(eight slices of 10 μm) and heated for 3 minutes at 50 °C to melt the paraffin. The samples where then centrifuged for 2 minutes at room temperature at 14,000 rpm to pellet the tissue. The xylene was discarded without disturbing the pellet and two successive washes with 1 ml of 100% ethanol were done. The samples where centrifuged for 3 minutes at 14,000 rpm. Any remaining drops of ethanol were removed and let to air dry for 30 minutes at room temperature. Next, 200 μl of digestion buffer and 4 μl of protease, both provided with the kit, were added in each sample. The samples were then homogenized and macerated with a pestle and incubate for 15 minutes at 50 °C and next an extra 15 minutes at 80 °C. An additive (from the kit)/ethanol mixture (240 μl/550 μl) was added to the mixtures and mixed by pipetting. Then, up to 700 μL of the mixture was added to a filter cartridge and centrifuged at 10,000 × g for 30 seconds. The flow-through was discarded and the process repeated until all the mixture had passed through the filter. Successive washes with 700 μl of wash 1 buffer (from the kit) and 500 μl of wash 2/3 buffer (from the kit) were done and centrifuged at 10,000 × g for 30 seconds. After discarding the flow-through, 60 μl of DNase mix was added to the center of each filter cartridge and left to incubate for 30 minutes at room temperature. Again, successive washes with wash 1 buffer and wash 2/3 buffer were done and centrifuged at 10,000 × g for 30 seconds. Finally, 60 μl of nuclease-free water was added to each filter cartridge, incubated for 60 seconds at room temperature and centrifuged at full speed for 60 seconds. Total RNA containing miRNAs was then stored at -80 °C until further use.

5.2.3. Reverse transcription qPCR

Reverse transcription qPCR was done by using Universal cDNA synthesis kit II (Exiqon # 203301) and ExiLENT SYBR® Green master mix (Exiqon # 203403). This methodology was performed to detect miR-200c and miR-203 expression levels in human cancer breast tissues and their counterpart adjacent normal tissues.

First of all, a dilution of purified total RNAs to a concentration of 5 ng/μl in nuclease-free water was done. In order to make a universal cDNA synthesis, a reverse transcription

reaction mix was then prepared by mixing 4 µl of 5× Reaction buffer, 10 µl of nuclease-free water, 2 µl of enzyme mix and 4 µl of template RNA at 1 ng/µl. This mixture was incubated at 42 °C for 60 minutes, followed by an enzyme inactivation for 5 minutes at 95 °C and immediately cooled to 4 °C. The cDNA was then stored at -20 °C until further use. In order to proceed to real-time PCR, cDNA previously synthesized was diluted 80×. Then, a PCR master mix was done by mixing 5 µl of SYBR® Green master mix, 1 µl of PCR primer mix, 4 µl of diluted cDNA template and 0.2 µl of ROX passive reference dye (Bio-Rad # 1725858) to a total volume of 10.2 µl. The mixture was loaded into a 96-well PCR plate and then the real-time PCR was performed in ABI 7300 real time PCR instrument with the following conditions: polymerase activation at 95 °C for 10 minutes; 40 cycles of amplification at 95 °C for 10 seconds and 60 °C for 1 minute. The primers used were: hsa-miR-203a (Exiqon, LNA™ PCR primer set # 204285); hsa-miR-200c-3p (Exiqon, LNA™ PCR primer set # 204482) and as endogenous control U6 snRNA (Exiqon, PCR primer set # 203907).

Relative expression of miRNAs in each FFPE sample was determined by $2^{-\Delta Ct}$, where ΔCt is Ct (miR-203a) - median Ct (U6 snRNA). Fold change was determined by $2^{-\Delta\Delta Ct}$, where ΔΔCt is ΔCt (tumor) – ΔCt (normal). All samples were analysed in duplicate.

5.2.4. Immunohistochemistry

Serial 2-3µm paraffin sections of 44 FFPE breast cancer samples were cut onto Superfrost® Plus slides (Thermo Scientific, # J1800AMNZ). For proper adhesion, sections were left overnight at 37 °C and then put in microwave for 2 minutes at full potency. Then, they were deparaffinised in Xylene, and rehydrated in decreasing concentrations of ethanol (100%, 96% and 70%). Antigen retrieval was achieved in microwave at 80% for 20 minutes with Tris-EDTA Buffer (10 mM Trizma.base, 1 mM EDTA solution with 0.05% Tween 20, pH 9.0), and endogenous peroxidase activity blocked with 3% hydrogen peroxide. Protein block was performed with ready-to-use (2.5 %) normal horse blocking solution for 20 minutes (Vector Laboratories # MP-7402), and then sections were

incubated at room temperature with the following antibodies: Anti-ATM, rabbit, monoclonal, clone Y170 (Millipore # 04-200); Anti-BMI1, mouse, monoclonal, clone 229F6 (Nordic-MUbio # MUB2004S); Anti-BMI1, mouse, monoclonal, clone 10H8 (Cell Applications # CB16351); Anti-SIX1, rabbit, polyclonal (Sigma-Aldrich # HPA001893); Anti-SIX1, mouse, monoclonal, clone CL0185 (Sigma-Aldrich # AMAb90544); Anti-SOX2, rabbit, polyclonal (Sigma-Aldrich # S9072); and Anti-SOX2, mouse, monoclonal, clone 10F10 (Sigma-Aldrich # SAB5300177). Antibodies were diluted in phosphate buffer saline (PBS) (137mM NaCl, 2,7mM KCl, 10mM Na_2HPO_4, 1,8mM KH_2PO_4, pH 7.4) with 0.05% Tween 20. Bound antibody was visualized using ready-to-use ImmPRESS™ anti-mouse Ig detection kit (Vector Laboratories # MP-7402) for 30 minutes and next to two successive washes with PBS with 0.05% Tween 20 the slides were incubated with 3,3'-diaminobenzidine (DAB, Invitrogen # 88-2014) for 5 minutes and then washed in running water for 2 minutes. Subsequently, slides were counterstained with Mayer's Hematoxylin (Sigma-Aldrich # MHS80) for 2 minutes, followed by wash in running water for 5 minutes. Dehydrate through increasing concentrations of ethanol (70%, 96%, 100%) clarified in xylene and mounted with Entellan® (Merck # 1.07961.0500). As positive controls we used normal breast tissue for ATM and BMI1, normal cervix for SIX1, and normal tonsil for SOX2.

All slides were further evaluated by 3 observers that used the same criteria, previously validated by a pathologist. Sections were evaluated in its totality and positivity was considered when 10% or more of tumour cells stained specifically with a moderate to strong intensity. After an initial independent observation, all results were reviewed in group and a final result assigned to each case by consensus. Slide evaluation was blind to tumour characteristics and other clinical data. Subsquently images were captured at the Pathology Department of Egas Moniz Hospital with a Leica DFC320 digital camera coupled to a Leica DM1000 microscope using Leica IM50 software.

5.2.5. Statistical analysis

All statistical analyses were performed using the SPSS statistical software package version 21.0 (SPSS Inc., Chicago, IL, USA). Non-parametric Wilcoxon signed-rank test was used to analyse the differences between matched samples (normal vs. tumour tissues). The Mann–Whitney U test and Kruskal-Wallis test were used to analyse the differences of miR-203a and miR-200c expression levels in the tumour tissue according to clinicopathological characteristics. For nominal variables, the relationships between clinicopathological characteristics and miR-203a and miR-200c status were studied using the chi-square test and Fisher's exact test. The Mann–Whitney U test was used to analyse the differences of miR-200c expression levels in the tumour tissue according to SIX1 and SOX2 status. The relationships between clinicopathological features and SIX1 and SOX2 status were studied using the chi-square test and Fisher's exact test. All graphs were made with GraphPad Prism 5 software.

5.3. Results

Study population

Breast tumour tissue and adjacent normal mammary tissue was collected from 109 patients. The study population comprised only Caucasian woman from the area of Lisbon and on the day of diagnosis the median age was 62 years (range: 30 – 85). The median age of menarche and menopause was 13 years (range: 8 – 17) and 50 years (range: 36 – 59), respectively, and approximately 66% of the population was diagnosed with breast carcinoma in post menopause status. Seventy seven percent of the women had one or more pregnancies and around 73% had one or more children. Forty five percent claim to have taken birth control pills. Around 50% were overweight or obese. Regarding general tumour characteristics, the median size was 18.5 mm (range: 6 – 130), about 51% showed no invasion of the lymph nodes, approximately 80% were ER positive, 72.5% were PR positive, and 13.8% showed high amplification of HER2 and 44% showed high Ki-67

proliferation index. The most common histological type was Invasive Carcinoma NOS (83.5% of the cases), followed by invasive lobular carcinoma (9.2%), ductal carcinoma in situ (6.4%) and invasive lobular and ductal carcinoma (0.9%). The most common molecular subtype was Luminal A (47.4% of all cases). Luminal B represented about 40% of the remaining cases. Triple negative subtype represented approximately 12% of the cases. The most frequent stage was II (46.8%) followed by I (37.6%) and III (8.3%). Of all patients, 69.2% had radiotherapy, 43% had chemotherapy (of which 38.7% took doxorubicin + cyclophosphamide, 39.6% docetaxel, 2.8% fluorouracil + epirubicin + cyclophosphamide and 0.9% cyclophosphamide + methotrexate + fluorouracil and 12.3% took trastuzumab), 87.9% had hormonotherapy (of which 42.1% took tamoxifen, 29.9% took anastrazole, 13.1% took letrozole and 3.7% took exemestane). Only 5.6% had disease recurrence (0.9% local, 0.9% local and metastasis, 3.7% metastasis) and 3.8% died.

All this data, together and with the stratification of some variables, is displayed in Table 5.1, Table 5.2 and Table 5.3.

MiR-203a is overexpressed in tumour tissue compared to normal tissue

Analysing the levels of miR-200c and miR-203a, we detected a significant overexpression of miR-203a in tumour tissue (1.7 fold higher) compared to normal adjacent tissue from the 109 patients (p = 0.003; Wilcoxon signed-rank test to matched samples) (Table 5.1; Figure 5.1). Regarding miR-200c, we did not detect significant differences between normal tissue and tumour tissue (Table 5.1; Figure 5.1).

Table 5.1 - Association of miR-203a and miR-200c relative expression with clinical characteristics. *p* value < 0.05 was considered significant according to Non-parametric Wilcoxon signed-rank test.

			Median relative expression of miR-200c				Median relative expression of miR-203			
			Normal Tissue	Tumour Tissue	Tumour /Normal	*p* value	Normal Tissue	Tumour Tissue	Tumour /Normal	*p* value
No. of cases	109		8.76	10.54	1.2	0.202	0.07	0.12	1.7	**0.003**
Age at diagnosis, median (range)	62	(30 - 85)								
30 - 39, N (%)	3	(2.8)	5.04	4.42	0.88	0.285	0.04	0.07	1.75	0.593
40 - 49, N (%)	17	(15.6)	14.84	10.68	0.72	0.266	0.14	0.13	0.93	0.906
50 - 59, N (%)	27	(24.8)	10.17	11.15	1.10	0.946	0.14	0.15	1.07	0.657
> 60, N (%)	54	(49.5)	5.94	9,65	1.62	**0.038**	0.04	0.11	2.75	**0.001**
missing	8	(7.3)								
Age of menarche, median (range)	13	(8 - 17)								
≤ 13, N (%)	65	(59.6)	7.82	10.88	1.39	0.320	0.06	0.12	2.00	0.066
> 13, N (%)	35	(32.1)	9.11	10.33	1.13	0.177	0.06	0.14	2.33	**0.003**
missing	9	(8.3)								
Age of menopause, median (range)	50	(36 - 59)								
≤ 50, N (%)	44	(52.4)	6.95	11.14	1.60	**0.003**	0.04	0.14	3.5	**< 0.001**
> 50, N (%)	29	(34.5)	5.63	9.35	1.66	0.471	0.05	0.10	2.0	0.539
missing	11	(13.1)								
Menopause status, N (%)										
pre	25	(22.9)	10.89	9.72	0.89	0.130	0.14	0.12	0.86	0.607
post	72	(66.1)	6.44	10.88	1.69	**0.018**	0.05	0.11	2.20	**0.003**
peri	1	(0.9)								
missing	11	(10.1)								
Breastfeeding, N (%)										
No	32	(29.4)	9.24	10.44	1.13	0.184	0.04	0.11	2.75	0.170
Yes	67	(61.5)	7.95	10.75	1.35	0.253	0.08	0.13	1.62	**0.002**

Table 5.1 (continued) - Association of miR-203a and miR-200c relative expression with clinical characteristics. p value < 0.05 was considered significant according to Non-parametric Wilcoxon signed-rank test.

missing	10	(9.2)								
No. of pregnancies, N (%)										
0	15	(13.8)	9.23	9.73	1.05	0.807	0.07	0.15	2.14	0.972
1 - 2	42	(38.5)	9.70	11.28	1.16	0.368	0.06	0.11	1.83	**0.046**
3 - 4	27	(24.8)	7.44	7.86	1.06	0.211	0.08	0.11	1.38	0.416
> 4	15	(13.8)	5.41	18.59	3.44	**0.004**	0.04	0.16	4.00	**0.001**
missing	10	(9.2)								
No. of children, N (%)										
0	21	(19.3)	9.24	10.33	1.12	0.184	0.04	0.12	3.00	0.295
1 - 2	64	(58.7)	9.11	10.88	1.19	0.549	0.08	0.11	1.38	**0.012**
3 - 4	12	(11.0)	9.74	12.13	1.25	0.575	0.08	0.12	1.50	0.657
> 4	4	(3.7)	5.33	8.68	1.63	0.068	0.03	0.21	7.00	0.068
missing	8	(7.3)								
Oral contraceptive, N (%)										
No	48	(44.0)	5.81	8.90	1.53	**0.047**	0.04	0.11	2.75	0.130
Yes	49	(45.0)	9.46	11.1	1.17	0.584	0.08	0.12	1.50	**0.004**
missing	12	(11.0)								

Table 5.2 - Association of miR-203a and miR-200c relative expression with life style habits. *p* value < 0.05 was considered significant according to Non-parametric Wilcoxon signed-rank test.

			Median relative expression of miR-200c				Median relative expression of miR-203			
			Normal Tissue	Tumour Tissue	Tumour/ Normal	*p* value	Normal Tissue	Tumour Tissue	Tumour/ Normal	*p* value
Body Mass Index, N (%)										
Underweight	2	(1.8)	48.02	11.59	0.24	0.180	0.22	0.31	1.41	0.655
Normal	42	(38.5)	9.58	12.91	1.35	0.060	0.08	0.13	1.63	0.252
Overweight	30	(27.5)	5.81	8.61	1.48	0.441	0.04	0.11	2.75	**0.006**
Obese	23	(21.1)	9.11	11.39	1.25	0.550	0.06	0.11	1.83	0.073
missing	12	(11.1)								
Smoking habit, N (%)										
No	73	(67.0)	6.43	10.43	1.62	0.221	0.05	0.13	2.6	**0.001**
Yes	22	(20.2)	9.59	10.75	1.12	0.088	0.09	0.11	1.22	0.465
missing	14	(12.8)								
Alcohol habits, N (%)										
No	54	(49.5)	9.27	9.74	1.05	0.532	0.07	0.13	1.86	0.059
Sporadically	24	(22.0)	6.80	10.14	1.49	0.568	0.04	0.12	3.00	**0.037**
Daily	17	(15.6)	10.89	9.72	0.89	0.130	0.14	0.12	0.86	0.607
missing	14	(12.8)								

Table 5.3 - Association of miR-203a and miR-200c relative expression with pathological characteristics. *p* value < 0.05 was considered significant according to Non-parametric Wilcoxon signed-rank test.

			Median relative expression of miR-200c				Median relative expression of miR-203			
			Normal Tissue	Tumour Tissue	Tumour/ Normal	*p* value	Normal Tissue	Tumour Tissue	Tumour/ Normal	*p* value
Size of the tumour, median mm (range)	18.5	(6 - 130)								
≤ 18.5 mm, N (%)	54	(49.5)	9.11	9,29	1.02	0.843	0.08	0.12	1.50	**0.019**
> 18.5 mm, N (%)	54	(49.5)	8.77	10.94	1.25	0.173	0.06	0.12	2.00	0.076
missing	1	(1.0)								
Lymph node invasion, N (%)										
No	56	(51.4)	7.95	11.02	1.39	0.459	0.05	0.12	2.40	**0.004**
Yes	52	(47.7)	9.10	9.39	1.03	0.315	0.09	0.11	1.22	0.199
missing	1	(0.9)								
Oestrogen receptor status, N (%)										
Negative	16	(14.7)	5.78	10.28	1.78	0.221	0.07	0.15	2.14	0.074
Positive	87	(79.8)	8.78	10.54	1.20	0.476	0.07	0.12	1.71	**0.042**
missing	6	(5.5)								
Progesterone receptor status, N (%)										
Negative	22	(20.2)	7.05	10.28	1.46	0.117	0.04	0.12	3.00	0.091
Positive	79	(72.5)	8.62	10.54	1.22	0.666	0.08	0.12	1.50	**0.046**
missing	8	(7.3)								
HER2 status, N (%)										
Negative	87	(79.8)	7.05	10.03	1.42	0.450	0.08	0.12	1.50	**0.016**
Positive	15	(13.8)	9.74	10.68	1.09	0.433	0.72	0.73	1.01	0.609
missing	7	(6.4)								
Ki67 index status, N (%)										
Negative	53	(48.6)	7.05	9.73	1.38	0.845	0.05	0.13	2.60	**0.024**

Positive	48	(44.0)	9.10	10.94	1.20	0.218	0.08	0.10	1.25	0.253
missing	8	(7.3)								
Histological type, N (%)										
Ductal Carcinoma *In Situ*	7	(6.4)	9.24	17.86	1.93	0.128	**0.05**	**0.11**	**2.20**	**0.028**
Invasive Carcinoma NOS	91	(83.5)	7.82	10.61	1.36	0.230	**0.07**	**0.12**	**1.71**	**0.009**
Invasive Lobular Carcinoma	10	(9.2)	14.48	9.29	0.64	0.173	0.13	0.11	0.84	0.575
Invasive Lobular and Ductal Carcinoma	1	(0.9)								
Molecular Type, N (%)										
Luminal A	48	(47.5)	6.80	9.29	1.37	0.717	0.07	0.13	1.86	0.054
Luminal B (HER2-)	27	(26.7)	8.78	10.94	1.25	0.339	0.08	0.11	1.38	0.527
Luminal B (HER2+)	13	(12.9)	9.43	10.68	1.13	0.239	0.07	0.08	1.14	0.221
Triple Negative	12	(11.9)	5.46	12.90	2.36	0.203	0.06	0.15	2.50	0.139
HER2+	1	(1.0)								
Stage, N (%)										
0	7	(6.4)	9.24	17.86	1.93	0.128	**0.05**	**0.11**	**2.20**	**0.028**
I	41	(37.6)	11.03	9.14	0,83	0.483	0.08	0.12	1.50	0.126
II	49	(44.9)	6.44	12.13	1.88	0.010	**0.06**	**0.13**	**2.17**	**0.009**
III	9	(8.3)	10.52	8.18	0.78	0.260	0.10	0.11	1.10	0.678
missing	3	(2.8)								

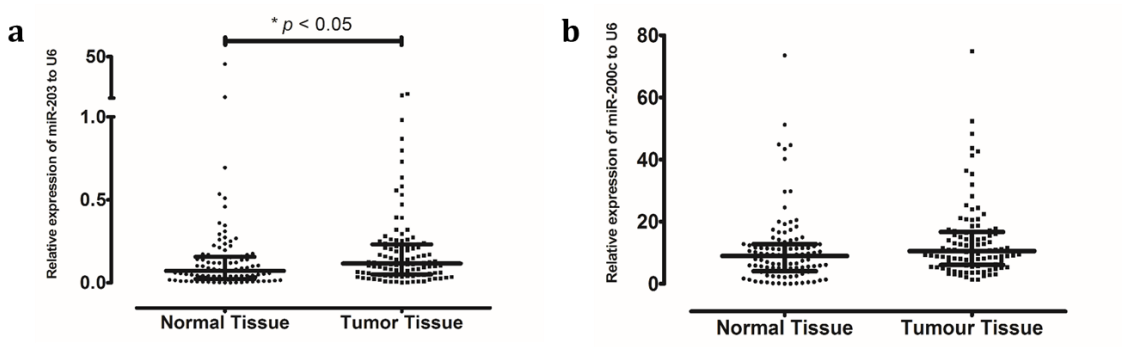

Figure 5.1 - Differences in miR-203a (a) and miR-200c (b) relative expression in tumour tissue and adjacent normal tissue. The expression levels are shown in arbitrary units determined by $2^{-\Delta Ct}$ method [ΔCt = Ct (miRNA) - median Ct (U6 snRNA)]. Lines represent median with interquartile range. *p* value < 0.05 was considered significant according to non-parametric Mann-Whitney test.

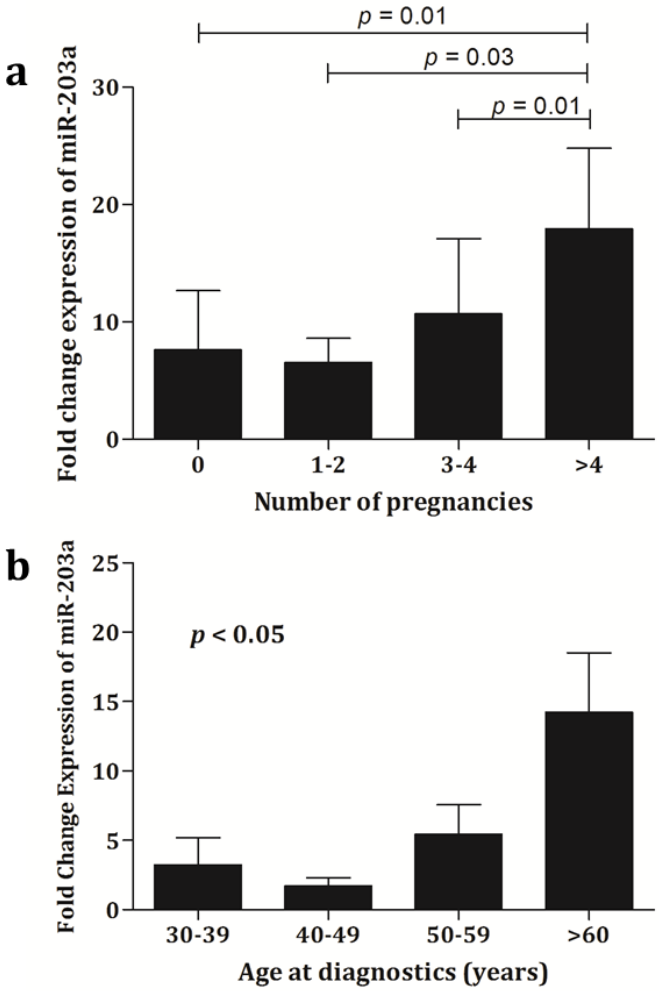

Figure 5.2 – Fold change expression of miR-203 regarding number of pregnancies (a) and age at diagnosis (b). *p* value < 0.05 was considered significant according to non-parametric Kruskal-Wallis.

Association between miR-203a expression and reproductive characteristics

The evaluation of clinical variables (Table 5.1) revealed a significantly different distribution of the fold change expression of miR-203a when considering the number of pregnancies (Kruskal-Wallis p = 0.006; Figure 5.2a). Specifically, there was a higher fold change expression in woman with four or more pregnancies comparing to the other classes (no pregnancies vs. >4 – p = 0.01; 1-2 vs. >4 – p = 0.03; 3-4 vs. >4 – p = 0.01; Figure 5.2a). Significant differences in age classes were also found (Figure 5.2b).

Using Wilcoxon signed-rank test to matched samples with the same variables, patients with more than 60 years on the day of diagnosis (fold change = 2.75; p = 0.001), menarche age over 13 years (fold change = 2.33; p = 0.003) and menopause under 50 years (fold change = 3.50; p < 0.001), showed a significant overexpression of miR-203a when comparing tumour tissue with normal adjacent tissue. Patients diagnosed in post menopause status (fold change = 2.20; p = 0.003) and who had less than 40 years of fertile status (< 30 years: fold change = 3.75; p = 0.041; 30-40 years: fold change = 2.40; p = 0.005) also presented an increased expression of miR-203a in tumour tissues compared to adjacent normal tissue. Regarding the number of pregnancies, patients with more than 4 pregnancies showed a significantly increased expression of miR-203a in tumour tissue (fold change = 4.00; p = 0.001). These results are in accordance with the ones described above where the Kruskal-Wallis test was applied. In accordance, although not significantly, women with more than four children showed an increased expression of miR-203a. Patients with first childbirth before 20 years of age also showed an increased expression of miR-203a (fold change = 3.40; p = 0.033). Breastfeeding status and oral contraceptive consumption also showed statistically significant results (Table 5.1).

Association between miR-200c expression and reproductive characteristics

Regarding miR-200c, we did not obtain a significantly different distribution of the fold change expression with respect to variables in Table 5.1. However, applying Wilcoxon signed-rank test to matched samples with the same variables we obtained significant differences in patients with more than 60 years on the day of diagnosis (fold change = 1.62; p = 0.038), menopause under 50 years (fold change = 1.60; p = 0.003), patients diagnosed in post menopause status (fold change = 1.69; p = 0.018), with more than 4 (fold change = 3.44; p = 0.004) and oral contraceptive consumption (fold change = 1.53; p = 0.047).

Association between miR-203a expression and lifestyle characteristics

It is known that some lifestyle habits can be a risk factor for cancer. In our series we included body mass index and smoking and alcohol habits. Over-weighted patients showed an increase of miR-203a expression in tumour tissues (fold change = 2.75; p = 0.006) and those who do not smoke (fold change = 2.60; p = 0.001) or sporadically drink (fold change = 3.00; p = 0.037) also showed an increased expression of miR-203a (Table 5.2). We did not obtain significant results regarding miR-200c and lifestyle characteristics.

Association between miR-203a expression and clinicopathological characteristics

Several clinicopathological characteristics showed an association with miR-203a expression (Table 5.3). Tumours with diameter smaller or equal to 18.5 mm, showed significant difference, albeit with a slight fold change of 1.5 compared with adjacent normal tissue (p = 0.019), together with tumours positive for ER (fold change = 1.71; p = 0.042), PR (fold change = 1.50; p = 0.046), negative for HER2 (fold change = 1.50; p = 0.016) and Ki-67 index (fold change = 2.60; p = 0.024). Tumours that do not invade lymph nodes also presented higher expression of miR-203a (fold change = 2.40; p = 0.004). With regard to histological classification, ductal carcinomas in situ (fold change = 2.20; p = 0.028) and invasive carcinoma NOS (fold change = 1.71; p = 0.009) showed a significantly

higher expression of miR-203a. Stage 0 and II also showed significant increased expression (fold change = 2.20; p = 0.028; fold change = 2.17; p = .009, respectively). When considering only invasive lobular tumours significant differences were found in staging, mainly when comparing stage III with stage I (Figure 5.3).

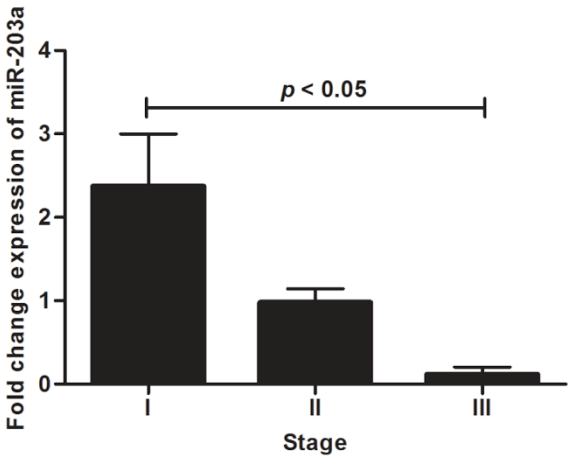

Figure 5.3 - Fold change expression of miR-203 regarding number of pregnancies **(a)** and age at diagnostics **(b)**. p value < 0.05 was considered significant according to non-parametric Kruskal-Wallis.

Immunohistochemistry optimization

Due to a nonexistence of accurate protocols regarding ATM, BMI1, SIX1 and SOX2 immunohistochemistry and in order to proceed with breast tumour samples characterization of these proteins it would be necessary to optimize all antibodies. Of the antibodies mentioned in the Material and Methods section, only Anti-SIX1, mouse, monoclonal, clone CL0185 (Sigma-Aldrich # AMAb90544) and Anti-SOX2, mouse, monoclonal, clone 10F10 (Sigma-Aldrich # SAB5300177) performed properly and with reproducible results. Regarding ATM antibodies, both revealed wrong stained structures. Mouse BMI1, monoclonal, clone 229F6 (Nordic-MUbio # MUB2004S) showed lack of staining; BMI1 mouse, monoclonal (Cell Applications # CB16351) showed excessive staining; SIX1 rabbit, polyclonal (Sigma-Aldrich # HPA001893) showed unspecific staining; and finally, SOX2 rabbit, polyclonal (Sigma-Aldrich # S9072) showed excessive staining. Slide captures of optimization can be seen in Figure 5.4.

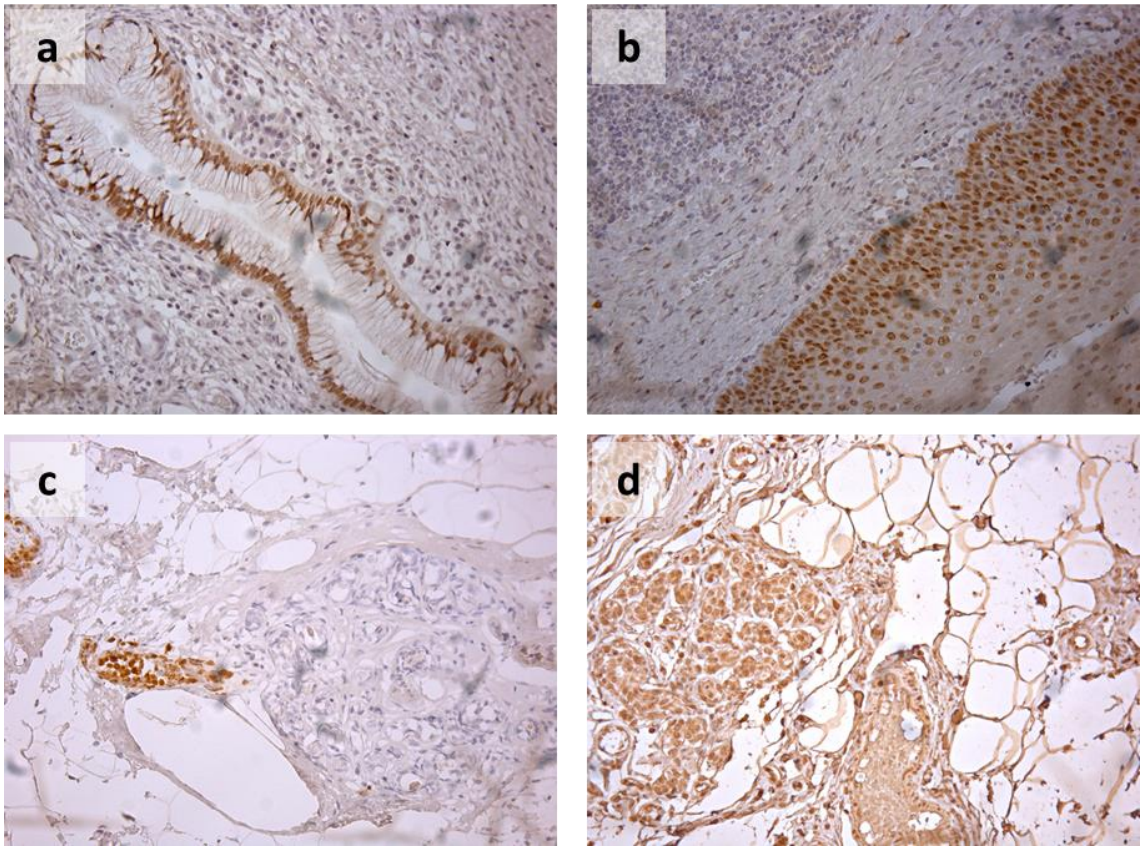

Figure 5.4 – Slide captures of SIX1 (a), SOX2 (b), ATM (c), and BMI1 (d). (a) – optimal staining of SIX1, only the nuclei of glandular epithelium of normal cervix is stained (20×). **(b)** – optimal staining of SOX2, only nuclei of stratified epithelium of normal tonsil is stained (20×). **(c)** – wrong structures stained by ATM antibodies, nuclei of normal breast glandular epithelium should be marked, however appears in blue. Only neutrophils appear to be stained in cytoplasm (20×). **(d)** – Excessive staining of BMI1 in slides with normal breast tissue. All structures appear to be stained, even at an antibody dilution of 1/1500, clearly indicating its unspecificity (20×).

Six1 and Sox2 do not correlate with miR-200c and clinicopathological features

Due to the fact that not all antibodies seemed to achieve a good staining, it was not possible to correlate ATM with miR-203 and BMI1 with miR-203 and miR-200c. Thus, we only report results regarding SIX1, SOX2 and their correlation with miR-200c and clinicopathological features.

A total of 44 samples from 43 patients were analysed concerning SIX1 and SOX2 through IHC. Of that, 13.6% showed positive staining for SIX1 and 9.1% for SOX2 (Figure 5.5 and Table 5.4). In table 5.4 we show the distribution of frequencies of SIX1 and SOX2

expression in the clinicopathological features. No statistical significance was reached with $\chi 2$ test. Association between SIX1 and SOX2 expression and miR-200c expression was not also verified.

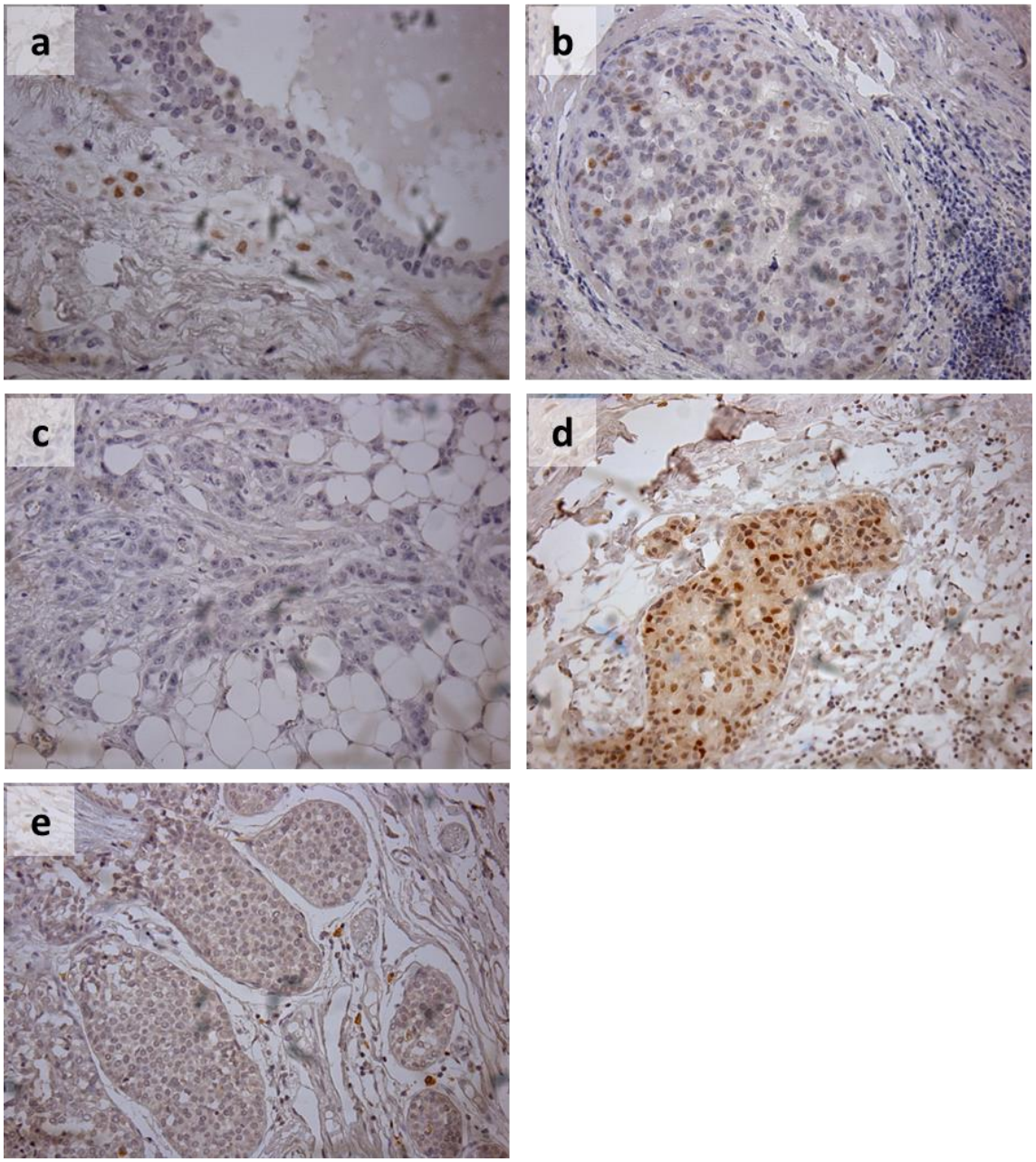

Figure 5.5 - Slide captures of SIX1 (a, b, c) and SOX2 (d, e). (a) - SIX1 positive invasive lobular carcinoma (40×). **(b)** – SIX1 positive invasive carcinoma NOS (20×). **(c)** - SIX1 negative invasive carcinoma NOS (20×). **(d)** - SOX2 positive invasive carcinoma NOS (20×). **(e)** - SOX2 negative invasive carcinoma NOS (20×).

Table 5.4 – Distribution of SIX1 and SOX2 expression in a Portuguese breast cancer population and their clinicopathological features. No statistical differences within each feature, thus, *p* values are not shown.

		Six1		Sox2	
	Total*	**Negative**	**Positive**	**Negative**	**Positive**
Total Population, N (%)	44	38 (86.4)	6 (13.6)	40 (90.9)	4 (9.1)
Tumor Size, N (%)					
≤ 18.5 mm	25 (56.8)	24 (54.5)	1 (2.3)	23 (52.3)	2 (4.5)
> 18.5 mm	19 (43.2)	14 (31.8)	5 (11.4)	17 (38.6)	2 (4.5)
Histological Type, N (%)					
Ductal Carcinoma In Situ	2 (4.5)	2 (4.5)	0	2 (4.5)	0
Invasive Carcinoma NOS	38 (86.4)	33 (75.0)	5 (11.4)	34 (77.3)	4 (9.1)
Invasive Lobular Carcinoma	4 (9.1)	3 (6.8)	1 (2.3)	4 (9.1)	0
Molecular Type, N (%)					
Luminal A	19 (43.2)	16 (38.1)	3 (7.1)	18 (42.9)	1 (2.4)
Luminal B (HER2-)	14 (31.8)	11 (26.2)	3 (7.1)	11 (26.2)	3 (7.1)
Luminal B (HER2+)	5 (11.4)	5 (11.9)	0	5 (11.9)	0
Triple-Negative	4 (9.1)	4 (9.5)	0	4 (9.5)	0
Stage, N (%)					
0	2 (4.5)	2 (4.7)	0	2 (4.7)	0
I	19 (43.2)	16 (37.2)	3 (7.0)	17 (39.5)	2 (4.7)
II	21 (47.7)	18 (41.9)	3 (7.0)	19 (44.2)	2 (4.7)
III	1 (2.3)	1 (2.3)	0	1 (2.3)	0
Lymph Node Invasion, N (%)					
No	19 (43.2)	15 (36.6)	4 (9.8)	17 (41.5)	2 (4.9)
Yes	22 (50.0)	20 (48.8)	2 (4.9)	21 (51.2)	1 (2.4)
ER status, N (%)					
Negative	6 (13.6)	6 (14.0)	0	6 (14.0)	0
Positive	37 (84.1)	31 (72.0)	6 (14.0)	33 (76.7)	4 (9.3)
PR status, N (%)					
Negative	9 (20.5)	6 (14.4)	3 (7.1)	8 (19.1)	1 (2.4)
Positive	33 (75.0)	30 (71.4)	3 (7.1)	30 (71.4)	3 (7.1)
HER2 status, N (%)					
Negative	38 (86.4)	32 (72.0)	6 (14.0)	33 (76.7)	4 (9.3)
Positive	6 (13.6)	6 (14.0)	0	6 (14.0)	0
Ki67 status, N (%)					
Negative	24 (54.5)	20 (46.5)	4 (9.3)	22 (51.1)	2 (4.7)
Positive	19 (43.2)	17 (39.5)	2 (4.7)	17 (39.5)	2 (4.7)
Age of Menarche, N (%)					
≤ 13 years	25 (56.8)	21 (52.5)	4 (10.0)	22 (55.0)	3 (7.5)
> 13 years	15 (34.1)	14 (35.0)	1 (2.5)	14 (35.0)	1 (2.5)
Age of Menopause, N (%)					
≤ 50 years	21 (47.7)	19 (61.2)	2 (6.5)	18 (58.1)	3 (9.7)
> 50 years	10 (22.7)	8 (25.8	2 (6.5)	9 (29.0)	1 (3.2)

Table 5.4 (continued) – Distribution of SIX1 and SOX2 expression in a Portuguese breast cancer population and their clinicopathological features. No statistical differences within each feature, thus, *p* values are not shown.

		Six1		Sox2	
	Total*	**Negative**	**Positive**	**Negative**	**Positive**
Age at diagnosis, N (%)					
40-49 years	6 (13.6)	5 (11.6)	1 (2.3)	6 (14.0)	0
50-59 years	13 (29.5)	12 (27.9)	1 (2.3)	13 (30.2)	0
> 60 years	24 (54.5)	20 (46.5)	4 (9.4)	20 (46.5)	4 (9.3)
Menopausal status, N (%)					
Pre	8 (18.2)	7 (17.5)	1 (2.5)	8 (20.0)	0
Post	32 (72.7)	28 (70.0)	4 (10.0)	28 (70.0)	4 (10.0)
Body Mass Index, N (%)					
Normal	17 (38.6)	15 (38.5)	2 (5.1)	17 (43.6)	0
Overweight	14 (31.8)	12 (30.8)	2 (5.1)	11 (28.2)	3 (7.7)
Obese	8 (18.2)	7 (17.9)	1 (2.6)	7 (17.9)	1 (2.6)

* The sum of the number of individuals in the clinicopathological features may not be 44 due to the fact that some individuals does not answered to all questions of the form.

5.4. Discussion

Several studies have established that specific miRNA expression patterns can be correlated with biological and clinical features. Studies of miRNA expression patterns in different cell populations are of utmost importance in order to unveil the significance of these molecules in the diagnosis and prognosis of breast cancer. In the present study we show that miR-203a is overexpressed in tumour tissues when compared to adjacent normal tissue in a Portuguese cohort. To our knowledge this is the first study reporting miR-203a expression in a Portuguese breast cancer population. Our results are in accordance with another study published by Ru and colleagues (Ru et al., 2011). However, they did not compare adjacent normal tissue with tumour tissue but an independent disease-free population. The same pattern of overexpression was also observed in ovarian cancer (Iorio et al., 2007), cervical cancer (Gocze et al., 2013), kidney and bladder cancers (Gottardo et al., 2007), colon adenocarcinoma (Schetter et al., 2008) and head and neck squamous cell carcinoma (de Carvalho et al., 2015). Conversely, miR-203 expression levels seem to be decreased in hepatocellular carcinoma (Liu et al., 2015b). Altogether these data

support the notion that miR-203a plays an important role in the development of cancer in a tissue specific manner.

With this study, we compared miR-203a expression levels of ductal carcinoma *in situ* (N = 7), invasive carcinoma NOS (N = 91) and invasive lobular carcinoma (N = 10). Our sample population also comprised one mixed tumour (invasive ductal and lobular carcinoma) but this was not considered in the statistical analysis of histological subtypes. Comparing matched samples, we observed significant differences between miR-203a levels in tumour tissue and adjacent normal tissue in ductal carcinoma *in situ* and invasive carcinoma NOS. However, there were no significant differences between the two groups. Due to the fact that we are in the presence of two different types of breast tumours, ductal and lobular, it makes more sense to analyse ductal carcinoma *in situ* and invasive carcinoma NOS separately. However, we did not find statiscally significant differences either. Nevertheless, we highlight the fact that there is a decrease of miR-203a expression levels in invasive carcinoma NOS when compared with ductal carcinoma *in situ*. These results suggest that during tumour development, miR-203a might be down regulated, thus suggesting that miR-203a might be implicated in early tumour development stages. Indeed, this involvement of miR-203a in invasiveness through the inhibition of the polycomb group gene *BMI1* was already reported in melanoma (Chang et al., 2015) and non-small cell lung cancer (Chen et al., 2015), in which miR-203a expression levels are inversely correlated with *BMI1* expression levels according to the cell type. Zhang et al. (Zhang et al., 2011) also reported an increased expression of miR-203a in breast tumours compared to matched adjacent normal tissue, even though their cohort was smaller. Additionally, the authors determined miR-203a expression levels in several non-tumorigenic, non-metastatic and metastatic breast cell lines and showed an increased expression of miR-203a in non-metastatic compared to non-tumorigenic and metastatic lines. These results led the authors to speculate that miR-203a is overexpressed in a protective mechanism to deal with cell proliferation and invasiveness, and thereafter,

most probably through epigenetic mechanisms, the tumour cells repress miR-203a expression to enable proliferation, invasion and metastasis through increased expression of the pro-metastatic gene *SNAI2*. In fact, our data are in accordance with this report, since when we stratify the tumours according to lymph node invasion, the tumours that metastasize have a decreased expression of miR-203a (fold change expression = 1.22; N = 53; Table 5.3) compared to those that did not metastasize to lymph node (fold change expression = 2.40; N = 52; Table 5.3), although they do not statistically differ. Additionally, we also found that miR-203a has a decreased expression in tumours positive for HER2 and high levels for the proliferation index Ki67 (Table 5.3). Altogether these data are in accordance with the fact that miR-203a may act as a tumour suppressor and in early stages of cancer development may play a protective role but throughout tumour development it might be repressed in order to enable tumour cells to proliferate, invade and metastasize.

Interestingly, miR-203a expression decreases from stage 0 to stage I, then increases in stage II, and again decreases in stage III. This up and down regulation across stages was unexpected, since as a putative tumour suppressor, miR-203a expression should decrease within stages. Petrovic et al. (Petrovic et al., 2014) observed a similar pattern in invasive breast carcinomas but with miR-21. MiRNA levels are dependent on cell differentiation, thus displaying differential expression levels according to stage. Analysing only invasive lobular carcinoma tumours, there are significant differences between stage I (N = 2), II (N = 6) and III (N= 2). Although the number of samples is small, we can observe a pronounced decrease in miR-203a expression within the stages (Figure 5.3). We can thus speculate that miR-203a expression levels have influence in invasive lobular carcinoma and it could be used as a marker to distinguish different stages.

Breast cancer risk increases with age. However, individual risk depends on other factors, including reproductive history, lifestyle habits and family history, among others. Our data show significant differences with age stratification. Indeed, women over 60 years at

diagnosis presented an increased expression of miR-203a when tumour tissue was compared with adjacent normal tissue. As estimated by The Surveillance, Epidemiology, and End Results (SEER) Program of the National Cancer Institute (Howlader et al., 2012), there is a risk increment for developing breast cancer with age. Our stratification was done using the same criteria, and we observed a higher expression of miR-203a in patients above 60 years. Regarding age of menarche and age of menopause, known players for breast cancer, the expression levels were higher for matched samples for age of menarche above 13 years and for age or menopause less than 50 years. Although we observed significant differences in matched samples for the classes referred, there are no differences within age of menarche and menopause classes indicating that miR-203a expression levels are not influenced by it.

Furthermore, it is known that female hormones, such as 17-β-estradiol (E_2), regulate gene expression by binding to oestrogen receptors (Marino et al., 2006). Indeed, Yu and colleagues (Yu et al., 2012), showed that E_2 can regulate miRNAs expression and thus control cell proliferation. The authors showed that miR-16, miR-143 and miR-203a expression is suppressed after E_2 stimulation hence up-regulating BCL2, cyclin D1 and BIRC5. Thus, the authors propose a mechanism whereby cells that undergo stimulation by E_2 increase proliferation by inhibiting tumour suppressor miRNAs involved in cell proliferation and survival. Additionally, the authors evaluated the expression levels of these miRNAs in triple positive and triple negative breast tumours and showed that they have increased levels of expression in triple positive tumours, indicating that these miRNAs may function as tumour suppressors in triple positive breast tumours. In contrast, our data shows that triple positive samples have lower expression of miR-203a than triple negative ones. Indeed, when we stratified our data according to hormone receptor status, individually, we always obtained an increased expression level of miR-203a in negative receptor status. When sample matching is analysed we find significant differences between tumour tissue and adjacent normal tissue of positive status. To confirm these

data, when we analysed the samples by stratifying them by molecular subtype, we observed that basal-like tumours have higher expression of miR-203a. Although the terms basal-like tumour and triple negative tumours are not used interchangeably (Badve et al., 2011), in this case we can consider that all basal-like are triple negative tumours. Interestingly, women who have used oral contraceptives have less expression of miR-203a. Thus, miR-203a expression might be influenced by oestrogen and progesterone (Yu et al., 2012).

Summarizing miR-203a data, it seems that miR-203a is involved in breast cancer development, mainly in early stages of development. Early tumour cells might up-regulate miR-203a in a self-protective manner in order to manage the augmented cell proliferation and then, most probably, through epigenetic mechanisms or E_2 mediated suppression, miR-203a might be down-regulated and its targets up-regulated. Accordingly, it could represent a potential marker for invasiveness. Here, we also show that miR-203a might be a potential marker to discriminate stages in invasive lobular carcinoma. Further studies with larger populations of invasive lobular carcinoma cases must be performed in order to validate these results.

In what concerns miR-200c, we did not detect significant differences between normal breast and tumour tissue in our population. Moreover, we failed to correlate miR-200c with clinicopathological features. On the contrary, other authors had previously published results associating miR-200c and some clinicopathological features in cancer. Actually, (Xu et al., 2016) and (Wang et al., 2013b) reported contrary reports, the first showing an association between miR-200c under expression and an increased number of lymph node metastasis in breast cancer and the second the opposite. Another study associates poor outcome in patients without progesterone receptors expression and high levels of miR-200c (Tuomarila et al., 2014). One possible explanation for our results is that our population only had 5.6% disease recurrence. In fact, 3.7% had long distance recurrence. Since miR-200c is associated with EMT and poor prognosis and our population have a

good survival rate, we failed to detect significant alterations of miR-200c. However, these results are in accordance with a recent meta-analysis published by Wu and colleagues (Wu et al., 2015a). The authors stated that in the 17 studies included in the meta-analysis miR-200c expression was not significantly associated with cancer prognosis. However, miR-200c expression in blood serum it was significantly associated with prognosis, TNM stage, lymph node metastasis and distant metastasis. Indeed, another study showed the same pattern (Antolin et al., 2015) and stated that the miR-200c detection in blood holds promise as a diagnostic marker. Due to the fact that until now our population have a low frequency of recurrences and that we only studied tumour tissue, we cannot exclude miR-200c as marker of disease and we need to perform further studies regarding circulating miRNAs and not only in tumour tissue.

The gold standard to characterize breast cancer, according to molecular status, is ER, PR, HER2 and Ki-67 detection by IHC. This characterization helps in therapy decisions, however, as all methods of diagnosis, is not error free and some patients do not respond well to therapy. In order to better characterize tumours and consequently to improve therapy decisions, new molecular markers are needed. MiRNAs have been described as good markers as also their putative regulated genes. Functional studies showing interaction between miRNAs and putative targets have been published. Although some studies showing association between miRNAs and regulated targets in cancer tissues have been done, most of the studies are designed and performed in cell lines. Because miRNAs regulate their targets at a post-transcriptional level and regulatory complex does not undergo to an immediate degradation, it is more logical to analyse protein expression than mRNA levels of putative targets. Here, we correlate the expression of miR-200c and two putative targets, SIX1 and SOX2, by RT-qPCR and IHC, respectively.

Given the fact that SIX1 and SOX2 have the ability to regulate cell proliferation, we expected a higher number of cases overexpressing both proteins. However, only 13.6%

and 9.1%, respectively, of the cases showed expression of these proteins. On the contrary, we expected a decreased expression of miR-200c in tumour tissues. In fact, we observed the opposite.

SIX1 has been described as an important player in BC aetiology, being overexpressed by gene amplification (Reichenberger et al., 2005) and inducing EMT (Micalizzi et al., 2009). Recently, Jin and colleagues (Jin et al., 2014) showed that 61.8% of their BC population has strong positive rates of SIX1. They also determined that SIX1 protein expression was significantly correlated with clinical stage, lymph node metastasis and HER2 expression status, suggesting that SIX1 may be a useful marker for prognostic evaluation of breast cancer. Conversely, our results showed no association with any of the clinicopathological characteristics analysed. Additionally, there seems to be a variation in the accurate location of SIX1 using different antibodies since the protein has been seen in both cytoplasm and nucleus. Hence, further studies are required to elucidate the mechanism that regulates SIX1 localization in order to properly understand its expression and influence in a cellular context. Moreover, according to TargetScan v7.0 (Agarwal et al., 2015), SIX1 mRNA is targeted by several miRNAs including miR-200c but, in our study it was not possibly to conclude that miR-200c levels could be related to SIX1 protein levels. Even more, none of the positive cases for SIX1 had miR-200c downregulated, as should be expected.

With regard to SOX2 expression, our results do not agree with those of other studies such as the one of Lengerke and colleagues (Lengerke et al., 2011) performed in Germany in 2011 with 95 patients, wherein 27.9% of analysed samples of invasive breast carcinoma were SOX2 positive. In another study of Huang and colleagues (Huang et al., 2014), carried out in 2014 in China with 609 samples, SOX2 was detected in 19.0% of invasive breast carcinomas. This study also showed that SOX2 expression was associated closely with high histological grade, large tumour size, molecular subtypes with adverse outcome

(preferably expressed in basal-like breast cancers), negative hormone receptors status and high proliferation index. Hence, SOX2 contributes to a less differentiated state, tumour progression and lymph node metastasis in breast cancer. However, in our study SOX2 expression could not be associated with any of those characteristics, neither with the other characteristics assessed. Interestingly, all the SOX2 positive cases are also ER positive, even though this association is not statically relevant. Nonetheless, the number of positive cases did not allow us to draw a conclusion. Furthermore, although SOX2 mRNA is, according to Targetscan, a target of miR-200c and it is described in vivo in colorectal carcinoma as involved in a feedback loop regulation (Lu et al., 2014), we did not find any association between the two of them in our study and again, no positive cases were found with miR-200c downregulation.

There are few studies regarding the expression of SIX1 and SOX2 in human patients with breast cancer and as far as we know, there are no studies that characterize the Portuguese population for expression of both proteins. A conclusion we can draw from our study, though is that, in our population of breast cancers we observe relatively low levels of SIX1 and SOX2. Furthermore, we could not find a statistically significant association between the expression of both proteins and various clinicopathological parameters. Regarding the relationship between levels of miR-200c and expression of their putative targets, SIX1 and SOX2, it was also not possible to find a statistically significant association. This may be due to the small sample size used in this study, which restricts us from drawing well established conclusions.

6. Identification of putative miR-200c and miR-203 targets

6.1. State of the art

Throughout this thesis we have been showing that miRNAs are differentially expressed in breast cancer and can influence carcinogenesis by deregulating several pathways of cellular homeostasis. We have also been showing some examples of experimentally validated targets of miRNAs. However, these targets do not explain all the cellular effects of miRNAs, leading us to conclude that other targets are being deregulated. In fact, as stated before, one miRNA can have more than one target and one target can be targeted by several miRNAs.

Most studies rely on the study of one miRNA and one or two targets [as example please see: (He et al., 2010; Ikemura et al., 2013; Iorio et al., 2009; Johnson et al., 2005)] that are previously identified by bioinformatics tools [as example TargetScan (Agarwal et al., 2015)]. Although this approach is effective and important, due to the mechanism of action of miRNAs, it does not show the real influence of a selected miRNA or a group of miRNAs in the cell. Thus, we believe that approaches that give us the notion of the cellular context in precise specific conditions, as ectopically expression or inhibition of miRNAs, can provide much more information about what pathways are being influenced by a miRNA. These approaches rely on the observation of several miRNAs targets through quantitative proteomics.

In Chapter 3 we have shown a presumably adequate approach for the identification of putative cellular targets of microRNAs in MCF-10A, MCF-7 and MDA-MB-231 cell lines by protein analysis by 2-D SDS-PAGE Gels and MALDI-TOF/TOF. Also in Chapter 5 we attempted to analyse miR-200c and miR-203 expression levels, and their putative targets in human breast cancer tissues by usage of breast cancer samples from FFPE. This showed

low levels of expression of SIX1 and SOX2, at least at the threshold's levels of detection of immunostaining methods. In order to attempt a deeper insight into the possible molecular targets of miR-200c and miR-203, our group has established a collaboration with Professor Peter James (Lund University, Sweden) and analysed differentially expressed proteins in MDA-MB-231 and MCF-10A cell lines when miR-200c and miR-203 were ectopically expressed or inhibited, respectively, by quantitative proteomics.

Quantitative proteomics is widely used to show deregulated proteins in a given condition or to determine molecular portraits of different cancer subtypes. As an example, the group of Professor Peter James has been publishing data showing that breast-cancer-derived cell lines reveal poor similarity with breast tumours (Cifani et al., 2015), showing that proteomic analysis is capable of confirm the mRNA molecular subtypes in breast cancer (Waldemarson et al., 2016) and showing protein differences in cellular transformation in ovarian cancer (Waldemarson et al., 2012). This approach together with by miRNAs expression can be a powerful tool to experimentally identify new targets and/or confirm putative targets retrieved by bioinformatics tools. In fact, there were already published data regarding this matter. As an example, Geng and colleagues (Geng et al., 2016) published some results showing that miR-125a, miR-143, miR-150, miR-181c, miR-182, miR-183, miR-199a, miR-429 are capable of influencing proteins involved in cell apoptosis, cell survival, cell cycle, inflammatory response and metabolism in rat liver regeneration. It was also shown that the integration of miRNA and protein expression data gives new insights into molecular processes that are related with differences in the genesis and clinical evolution of triple negative breast tumours (Gamez-Pozo et al., 2015). Lai and colleagues (Lai et al., 2012) do a more concise study and ectopically expressed miR-372 and then through comparative proteomics analysed differently expressed proteins in lung adenocarcinoma. Thus, the authors identified new potential diagnostic biomarkers for lung adenocarcinoma in several pathways. Taking into account these studies and the potentiality of comparative proteomics in cancer research, we describe in the present

Chapter the proteomic results of differentially expressed proteins in MDA-MB-231 and MCF-10A cell lines when miR-200c and miR-203 were ectopically expressed or inhibited, respectively.

6.2. Materials and methods

6.2.1. Cell lines

Cell lines culture was done according to Cell lines and Nucleic acid purification from Chapter 3, section Material and Methods.

6.2.2. Ectopic expression and inhibition of miR-200c and miR-203

Ectopic expression and inhibition of miR-200c and miR-203 was done according to the section Materials and Methods from Chapter 4.

6.2.3. Protein purification and quantification

Protein purification and quantification was done according to the section Protein purification and quantification from Chapter 3.

6.2.4. Protein identification through LC/MS

Total protein was denatured in Laemmli buffer 2× (4% (w/v) SDS 10%; 20% (v/v) glycerol 50%; 0.02% (w/v) bromophenol blue; 125 mM Tris.HCl pH 6.8; and 10% (v/v) 2-Mercaptoethanol) in a proportion of 1:1 and heated at 95 °C for 10 minutes. The samples were then sent to Professor Peter James and proteins identified through LC/MS.

6.2.5. Bioinformatics and Statistical analysis

After identification of the proteins through LC/MS, we received a list of 2956 proteins that were then analysed according to their ontology using PANTHER internet tools. After this, we statistically tested all eligible proteins using two-way ANOVA with Bonferroni post-test (p value $< 0,05$) with GraphPad Prism 5 software. All graphs were made using Microsoft Excel 2010 software.

6.3. Results

Differently expressed proteins ontology

Through LC-MS we identified 2956 misexpressed proteins after transfection of MDA-MB-231 cell line with pre-miR-200c and pre-miR-203; and MCF-10A cell line with anti-miR-200c, anti-miR-203 and pre-miR-203. All these conditions compared with a negative control that was in the exact same conditions but without the pre-or anti-miR. However, not all proteins were eligible to study. Thus, we filtered and selected proteins according to their q value (lower than 0.01), that had concordant replicates and p value (Two-way ANOVA with Bonferroni post-test) lower than 0.05. In tables 6.2 to 6.5, we show this selection of proteins as well as their expression values under different conditions of cell culture (Appendix).

Regarding the transfection of MDA-MB-231 cell line with pre-miR-200c, we obtained 47 differently expressed proteins, being 14 up-regulated and the remaining down-regulated (Table 6.2). In order to classify according to their ontology, we used PANTHER internet tools. Thus, we observed that many proteins have catalytic or binding function (Figure 6.1); and most proteins are involved in metabolic or cellular processes (Figure 6.2), being metabolic processes characterized as primary metabolic processes and cellular processes characterized as cell communication, cell cycle, cellular component movement and chromosome segregation. Most proteins are localized in cytoplasm or associated to sub-cellular structures (nucleus, cytoskeleton, endoplasmic reticulum and endosome) (Figure 6.3); most proteins have nucleic acid binding, transferase or hydrolase activity (Figure 6.4); and finally, the majority of proteins are members of the Huntington disease pathway (Figure 6.5). After transection with pre-miR-203, we obtained 43 differently expressed proteins, being 7 up-regulated and the remaining 36 down-regulated (Table 6.3). The majority of proteins are involved in catalytic or binding functions (Figure 6.6); metabolic or cellular processes (Figure 6.7), where metabolic processes are herein defines as

primary metabolic processes and cellular processes characterized as cell communication, cell cycle and cellular component movement; are localized in cytoplasm (cell part) or associated with sub-cellular structures (cytoskeleton, endoplasmic reticulum and mitochondrion)(Figure 6.8); and are classified as hydrolases (Figure 6.9); and belong to integrin signaling pathway (Figure 6.10).

Regarding MCF10A cell line, it was transfected with anti-miR-200c, anti-miR-203 and pre-miR-203. This last transfection was done due to the fact that miR-203 is poorly expressed in this cell line. Thus, we decided to lower its expression even more and augment it in order to see the differences. Considering the transfection with anti-miR-200c, we obtained 82 differently expressed proteins, of which 54 were down-regulated and the remaining 28 were up-regulated (Table 6.4). Of these 82 proteins, the most common belongs to, in what regards molecular function, to binding and catalytic activity proteins (Figure 6.11); to metabolic and cellular process (Figure 6.12), where metabolic processes is characterized as primary metabolic processes and cellular processes characterized as cell communication, cell cycle and cellular component movement; to cytoplasm and organelle-associated (cytoskeleton, endoplasmic reticulum, mitochondrion, nucleus and cytoplasmic membrane-bounded vesicle) proteins (Figure 6.13); to nucleic acid binding class of proteins (Figure 6.14); and to ubiquitin proteasome pathway proteins (Figure 6.15). After transfection with anti-miR-203, we obtained 130 proteins differently expressed, of which 93 were down-regulated and 37 up-regulated (Table 6.5). The majority of proteins are classified as having catalytic and binding activity (Figure 6.16); involved in metabolic and cellular processes (Figure 6.17), where metabolic processes is characterized as primary metabolic processes and cellular processes characterized as cell communication, cell cycle, cellular component movement and cytokinesis; are localized in cytoplasm or are organelle-associated (nucleus, cytoskeleton, endoplasmic reticulum and endosome) (Figure 6.18); and also belong to nucleic acid binding and hydrolase class of proteins (Figure 6.20); or finally, belong to the p53 pathway (Figure 6.20). The last cell culture

condition is with the transfection of pre-miR-203. After this, 200 proteins were found differentially expressed when transfection was performed. Of these, 104 were up-regulated and 96 were down-regulated (Table 6.6). The most common proteins have catalytic and binding activity (Figure 6.21); are involved in involved in metabolic and cellular processes (Figure 6.22) where metabolic processes is characterized as primary metabolic processes and cellular processes characterized as cell communication, cell cycle, cell proliferation, cellular component movement, chromosome segregation and cytokinesis; and with respect to their localization, are localized in cytoplasm or are organelle-associated (Figure 6.23) (chromosome, cytoplasmic membrane-bounded vesicles, cytoskeleton, endoplasmic reticulum, endosome, mitochondrion and nucleus); and last, belong to nucleic acid binding and transferase class of proteins (Figure 6.24).

Putative targets of miR-200c and miR-203

Of the differently expressed proteins in each condition we selected those with opposite expression of the respective miRNA. With this we select the putative direct targets of miR-200c and miR-203. After this selection we performed an analysis of these putative targets in TargetScan and determined which one has homology with miR-200c and miR-203. These proteins are shown in Table 6.1.

Table 6.1 – Putative direct targets of miR-200c and miR-203 identified using TargetScan (Release 7.1 – June 2016). * - Down-regulated; ‡ - Up-regulated

	Putative target	
	miR-200c	**miR-203**
MDA-MB-231	IAH1*	YARS2* IAH1* FKBP5* CAB39* GLS*
MCF10A	DGKA‡ CRKL‡ TIGD2‡	HAT1‡ CTBP2‡ HNRNPL‡ PLAA* PRKCI* HDAC1* RRM1*

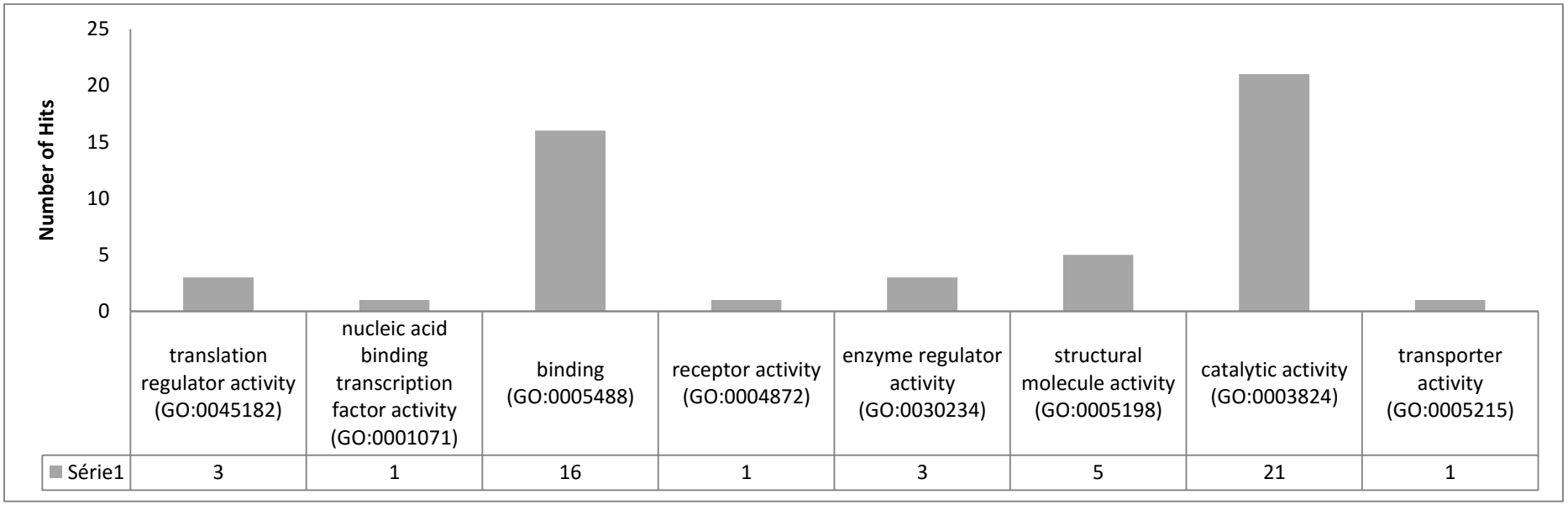

Figure 6.1 – Molecular function of the differently expressed proteins in MDA-MB-231 after transfection with pre-miR-200c. Of the 47 proteins identified, PANTHER and the Gene Ontology Consortium retrieved 51 hits in molecular functions. Of these, the most common are proteins with catalytic and binding activity. PANTHER™ GO slim (version 10.0, released 2015-05-15).

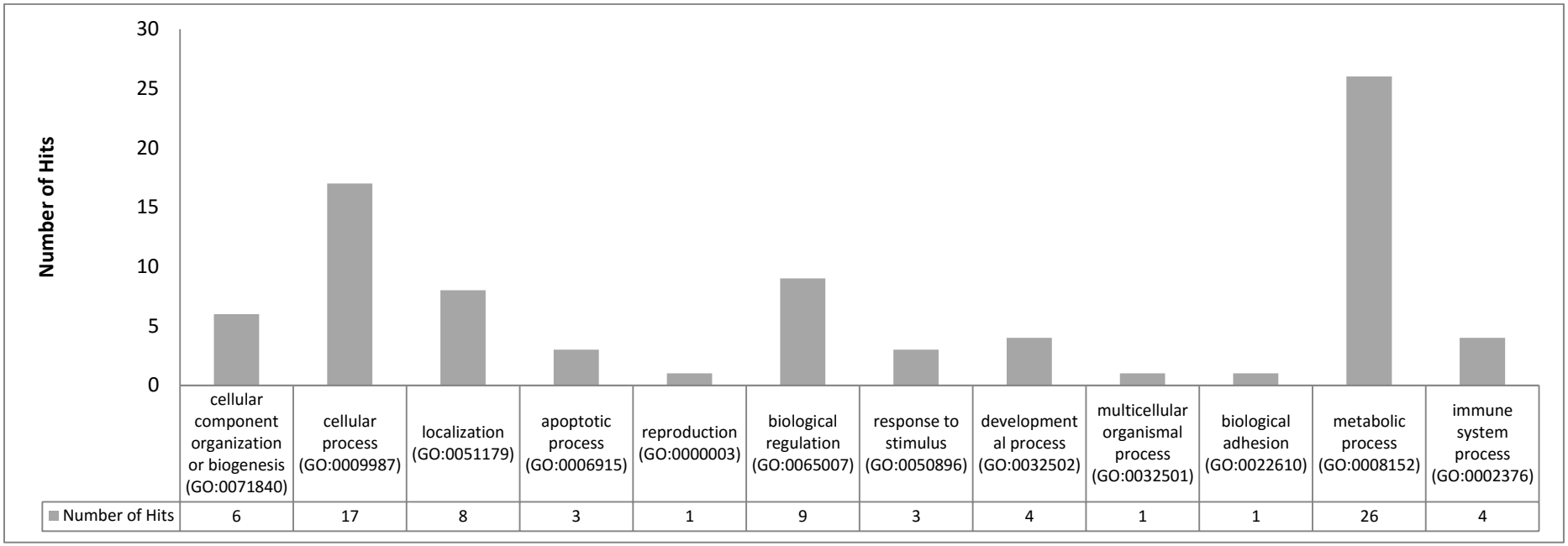

Figure 6.2 – Biological processes of the differently expressed proteins in MDA-MB-231 after transfection with pre-miR-200c. Of the 47 proteins identified, PANTHER and the Gene Ontology Consortium retrieved 83 hits in biological processes. Of these, metabolic and cellular processes are the most common. Metabolic processes are characterized in greater number as primary metabolic processes and cellular processes are characterized as cell communication, cell cycle, cellular component movement and chromosome segregation. PANTHER™ GO slim (version 10.0, released 2015-05-15).

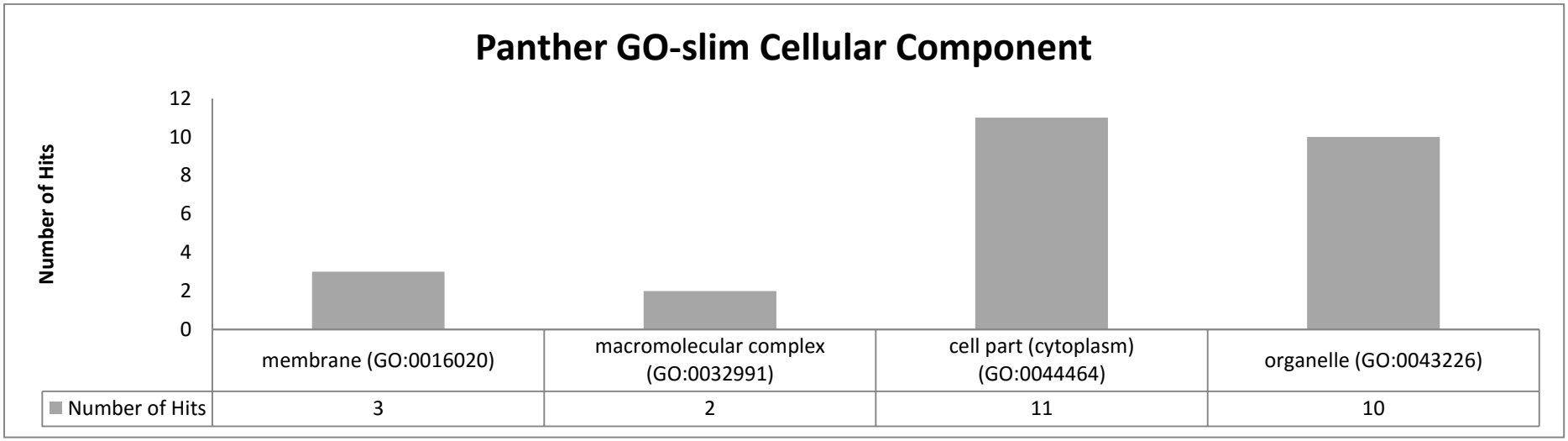

Figure 6.3 – Cellular components of the differently expressed proteins in MDA-MB-231 after transfection with pre-miR-200c. Of the 47 proteins identified, PANTHER and the Gene Ontology Consortium retrieved 26 hits in cellular components. Of these, cell part (cytoplasmic proteins) and organelle-associated (nucleus, cytoskeleton, endoplasmic reticulum and endosome) proteins are the most common. PANTHER™ GO slim (version 10.0, released 2015-05-15).

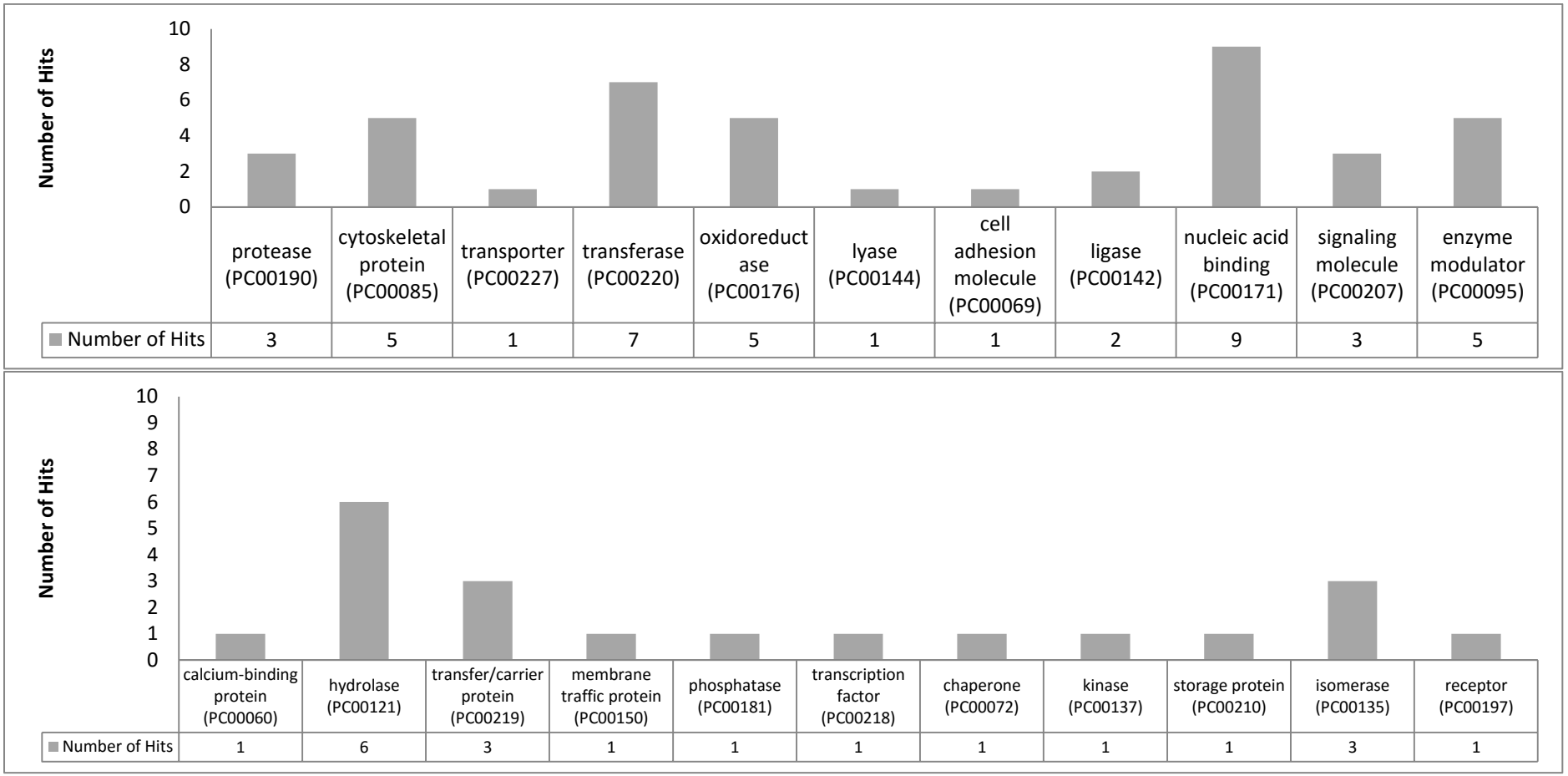

Figure 6.4 – Protein classes of the differently expressed proteins in MDA-MB-231 after transfection with pre-miR-200c. Of the 47 proteins identified, PANTHER and the Gene Ontology Consortium retrieved 62 hits in protein classes. Of these, nucleic acid binding, transferase and hydrolase proteins are the most common. PANTHER™ Protein Class (version 10.0, released 2015-05-15).

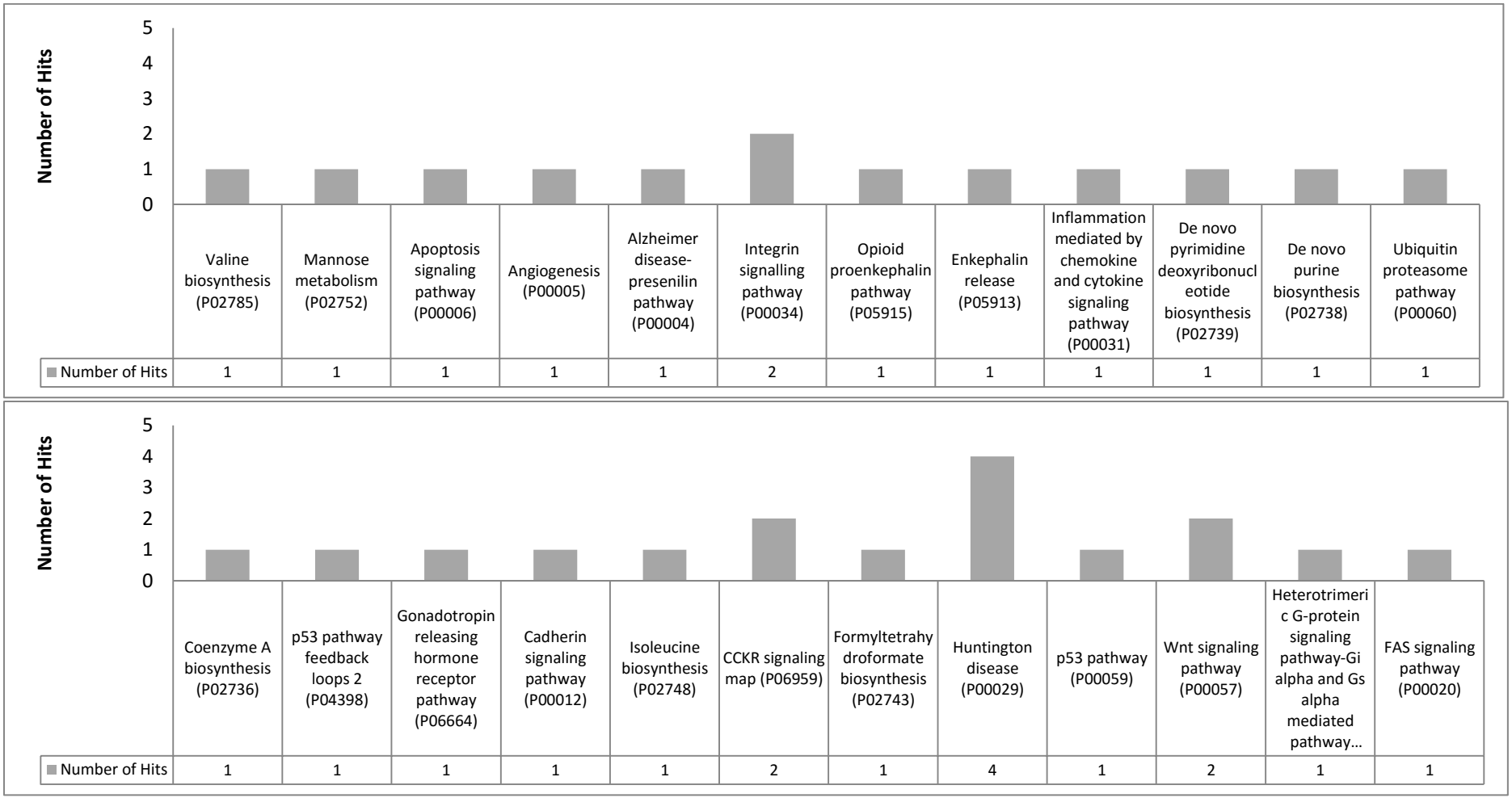

Figure 6.5 - Pathways of the differently expressed proteins in MDA-MB-231 after transfection with pre-miR-200c. Of the 47 proteins identified, PANTHER and the Gene Ontology Consortium retrieved 30 hits in pathways. Of these, Huntington disease related proteins were the most common. PANTHER™ Pathway 3.4, released 2015-05-15.

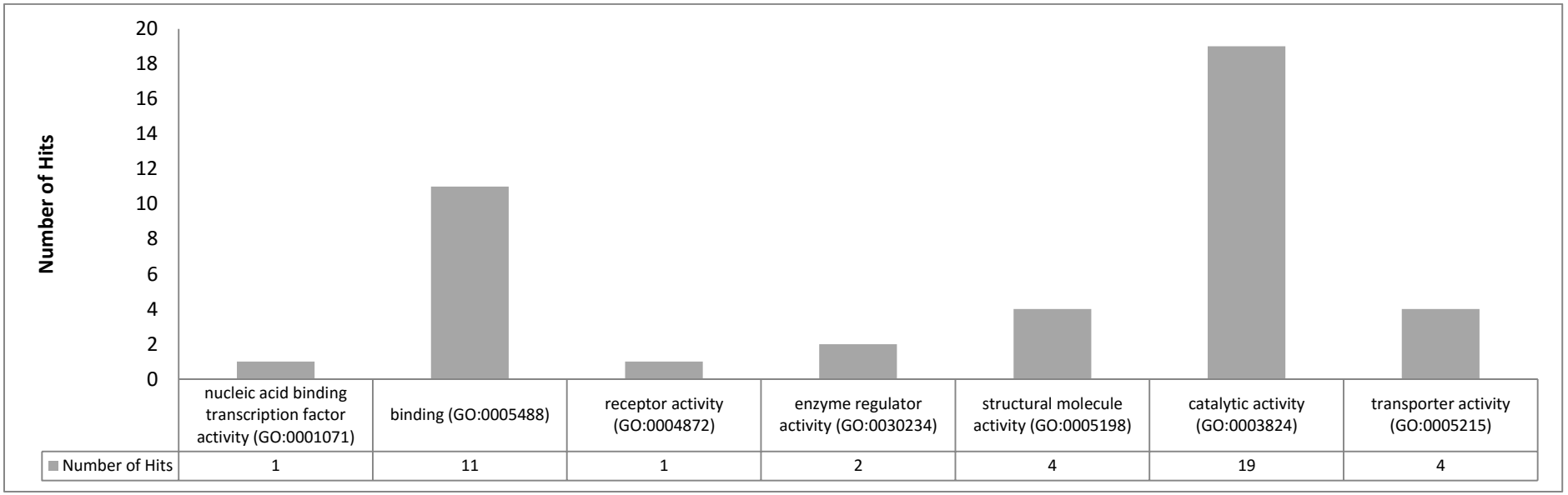

Figure 6.6 – Molecular function of the differently expressed proteins in MDA-MB-231 after transfection with pre-miR-203. Of the 43 proteins identified, PANTHER and the Gene Ontology Consortium retrieved 42 hits in molecular functions. Of these, the most common are proteins with catalytic and binding activity. PANTHER™ GO slim (version 10.0, released 2015-05-15).

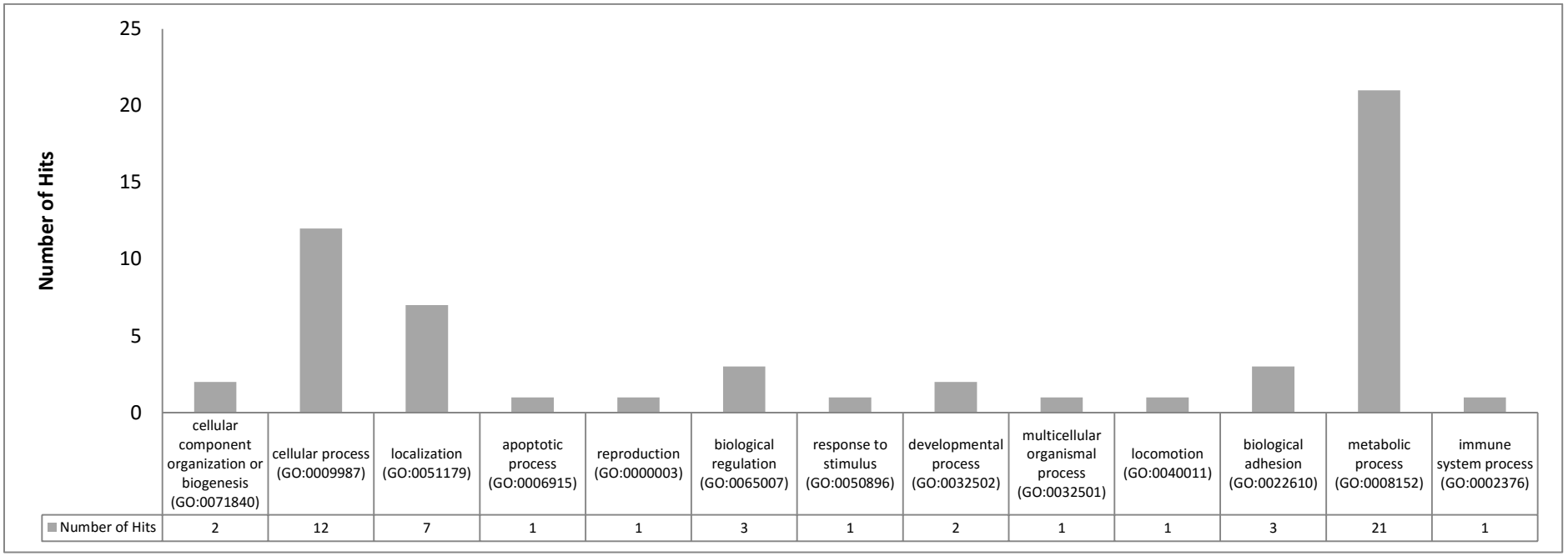

Figure 6.7 - Biological processes of the differently expressed proteins in MDA-MB-231 after transfection with pre-miR-203. Of the 43 proteins identified, PANTHER and the Gene Ontology Consortium retrieved 56 hits in biological processes. Of these, metabolic and cellular processes are the most common. Metabolic processes are characterized in greater number as primary metabolic processes and cellular processes are characterized as cell communication, cell cycle and cellular component movement. PANTHER™ GO slim (version 10.0, released 2015-05-15).

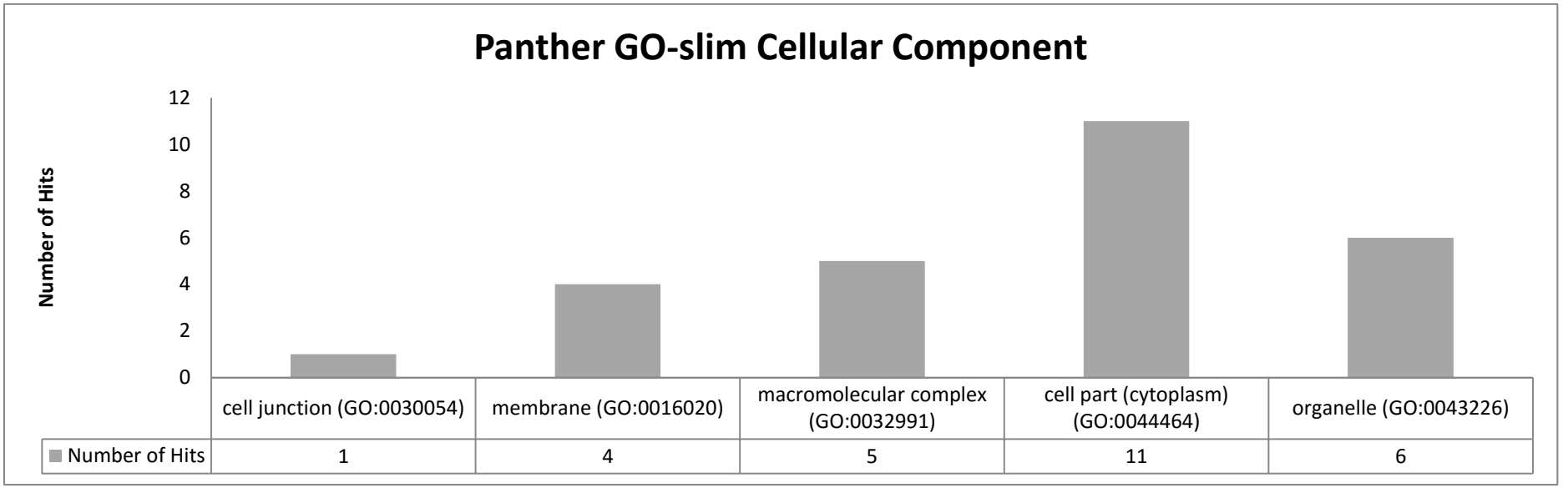

Figure 6.8 - Cellular components of the differently expressed proteins in MDA-MB-231 after transfection with pre-miR-203. Of the 43 proteins identified, PANTHER and the Gene Ontology Consortium retrieved 27 hits in cellular components. Of these, cell part (cytoplasmic proteins) and organelle-associated (cytoskeleton, endoplasmic reticulum and mitochondrion) proteins are the most common. PANTHER™ GO slim (version 10.0, released 2015-05-15).

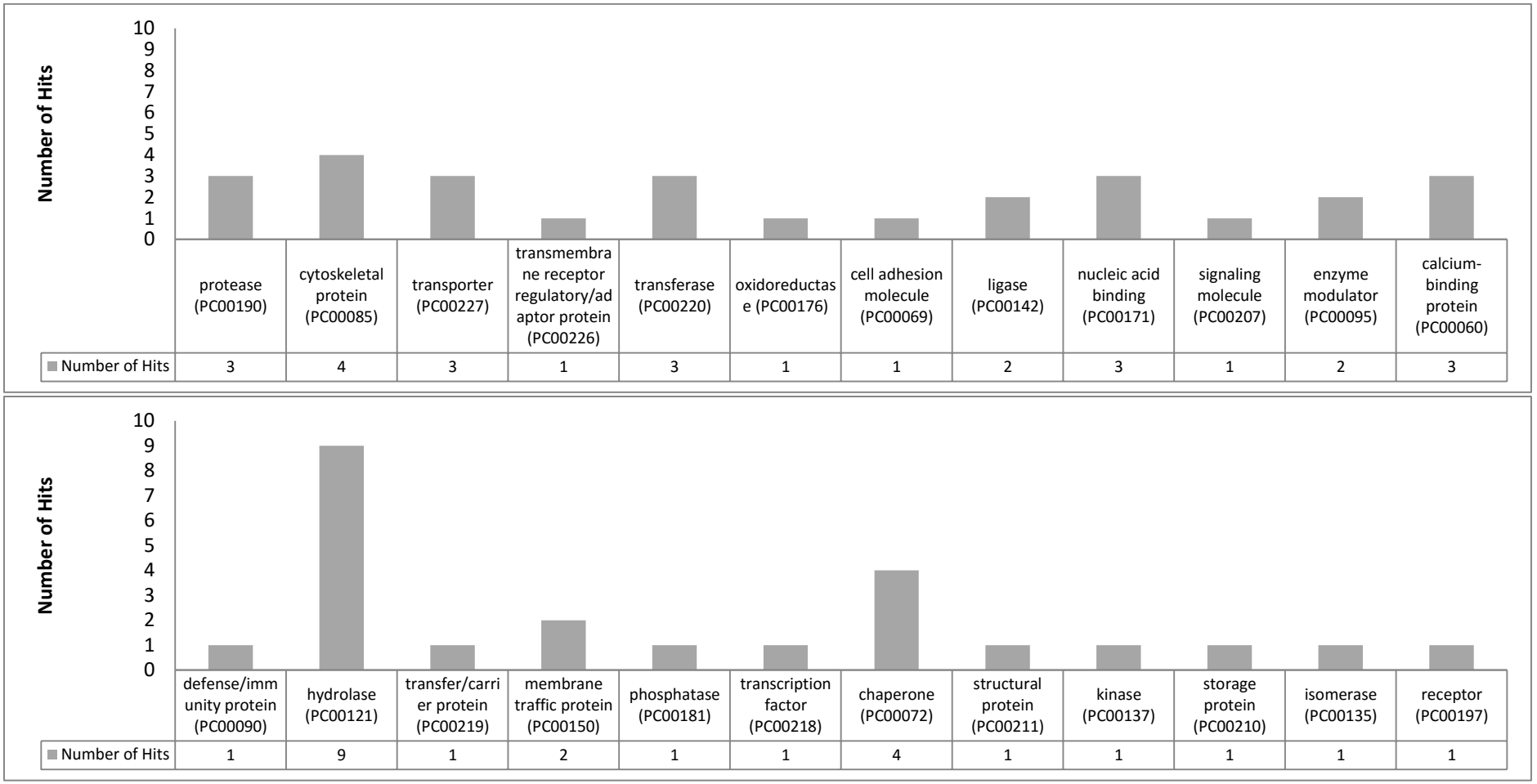

Figure 6.9 – Protein classes of the differently expressed proteins in MDA-MB-231 after transfection with pre-miR-203. Of the 43 proteins identified, PANTHER and the Gene Ontology Consortium retrieved 51 hits in protein classes. Of these, hydrolase, cytoskeletal and chaperone proteins are the most common. PANTHER™ Protein Class (version 10.0, released 2015-05-15).

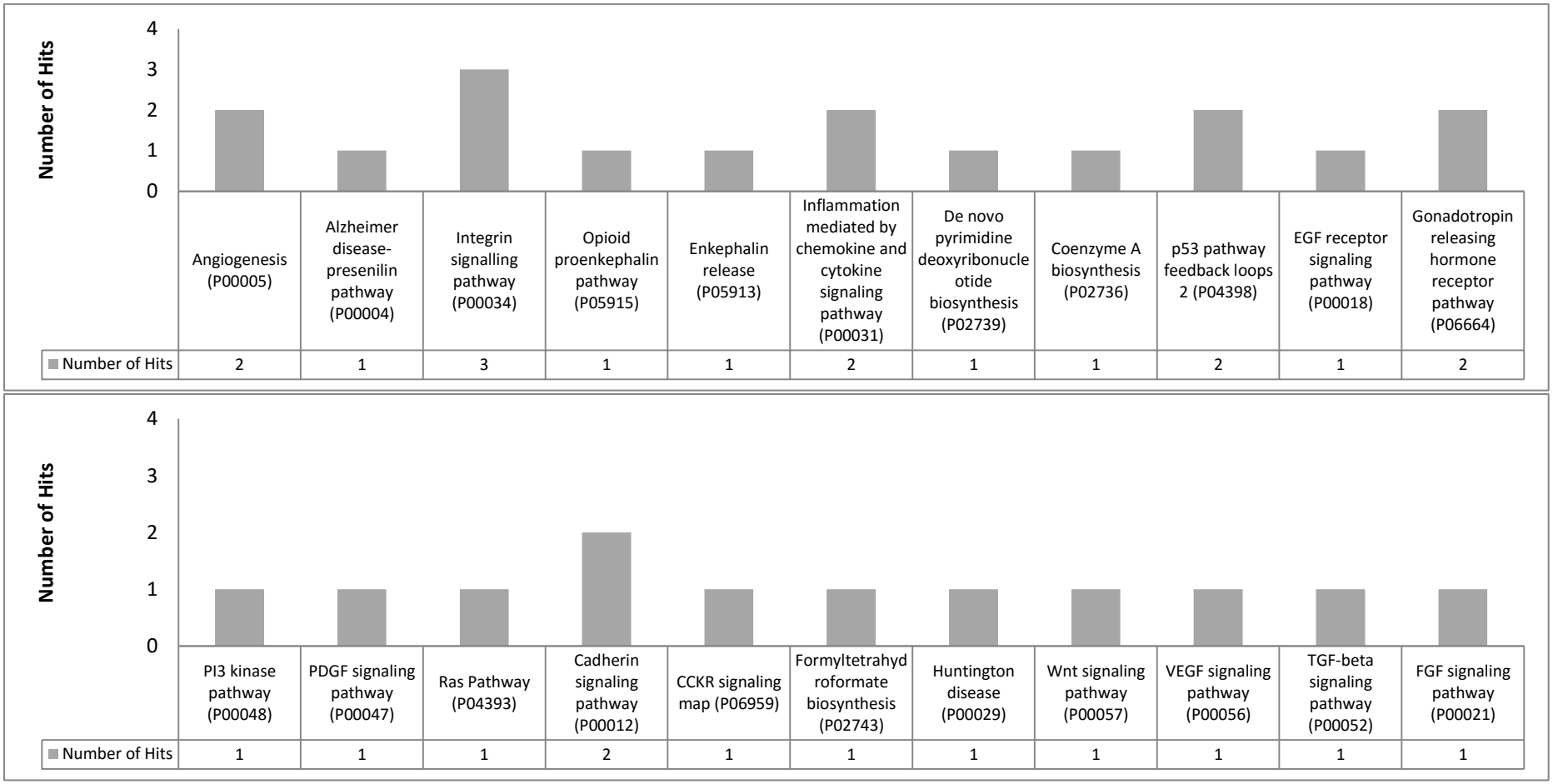

Figure 6.10 - Pathways of the differently expressed proteins in MDA-MB-231 after transfection with pre-miR-203. Of the 43 proteins identified, PANTHER and the Gene Ontology Consortium retrieved 29 hits in pathways. Of these, Integrin signaling pathway related proteins were the most common. PANTHER™ Pathway 3.4, released 2015-05-15.

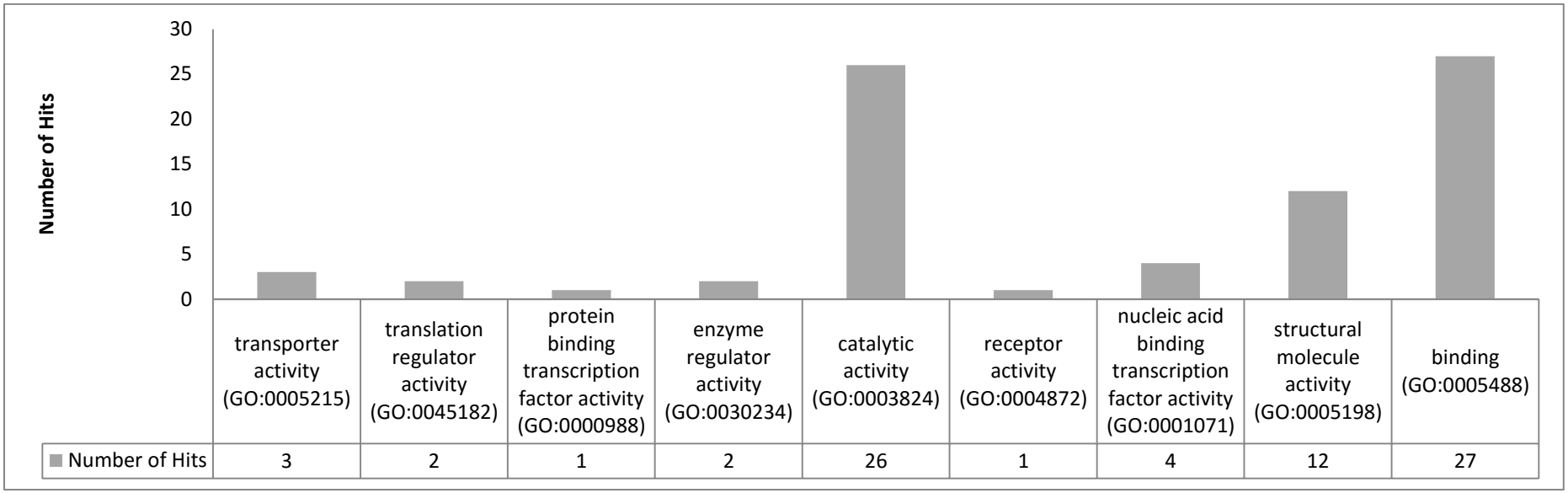

Figure 6.11 – Molecular function of the differently expressed proteins in MCF-10A after transfection with anti-miR-200c. Of the 82 proteins identified, PANTHER and the Gene Ontology Consortium retrieved 78 hits in molecular functions. Of these, the most common are proteins with binding and catalytic activity. PANTHER™ GO slim (version 10.0, released 2015-05-15).

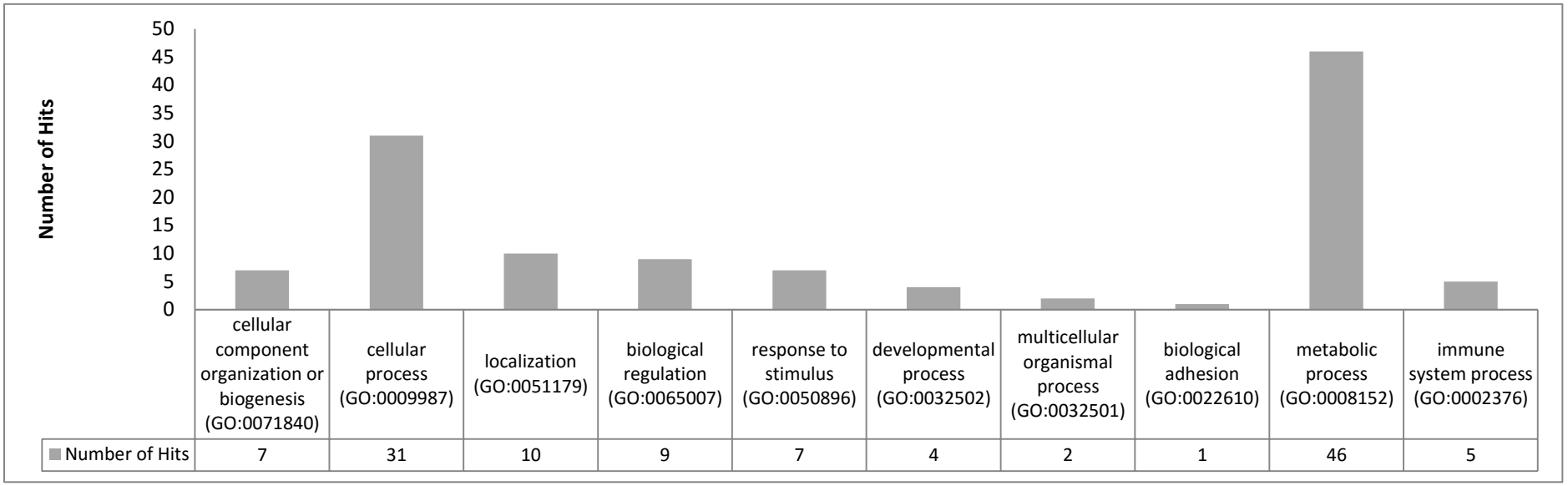

Figure 6.12 - Biological processes of the differently expressed proteins in MCF10A after transfection with anti-miR-200c. Of the 82 proteins identified, PANTHER and the Gene Ontology Consortium retrieved 122 hits in biological processes. Of these, metabolic and cellular processes are the most common. Metabolic processes are characterized in greater number as primary metabolic processes and cellular processes are characterized as cell communication, cell cycle and cellular component movement. PANTHER™ GO slim (version 10.0, released 2015-05-15).

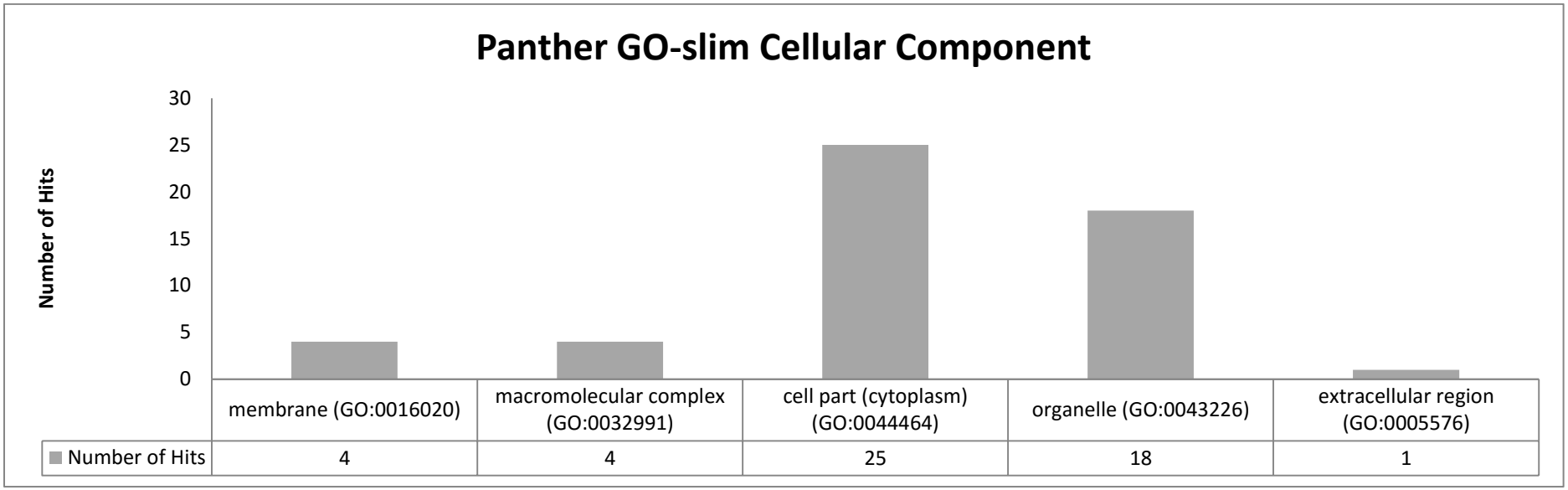

Figure 6.13 - Cellular components of the differently expressed proteins in MCF10A after transfection with anti-miR-200c. Of the 82 proteins identified, PANTHER and the Gene Ontology Consortium retrieved 52 hits in cellular components. Of these, cell part (cytoplasmic proteins) and organelle-associated (cytoskeleton, endoplasmic reticulum, mitochondrion, nucleus and cytoplasmic membrane-bounded vesicle) proteins are the most common. PANTHER™ GO slim (version 10.0, released 2015-05-15).

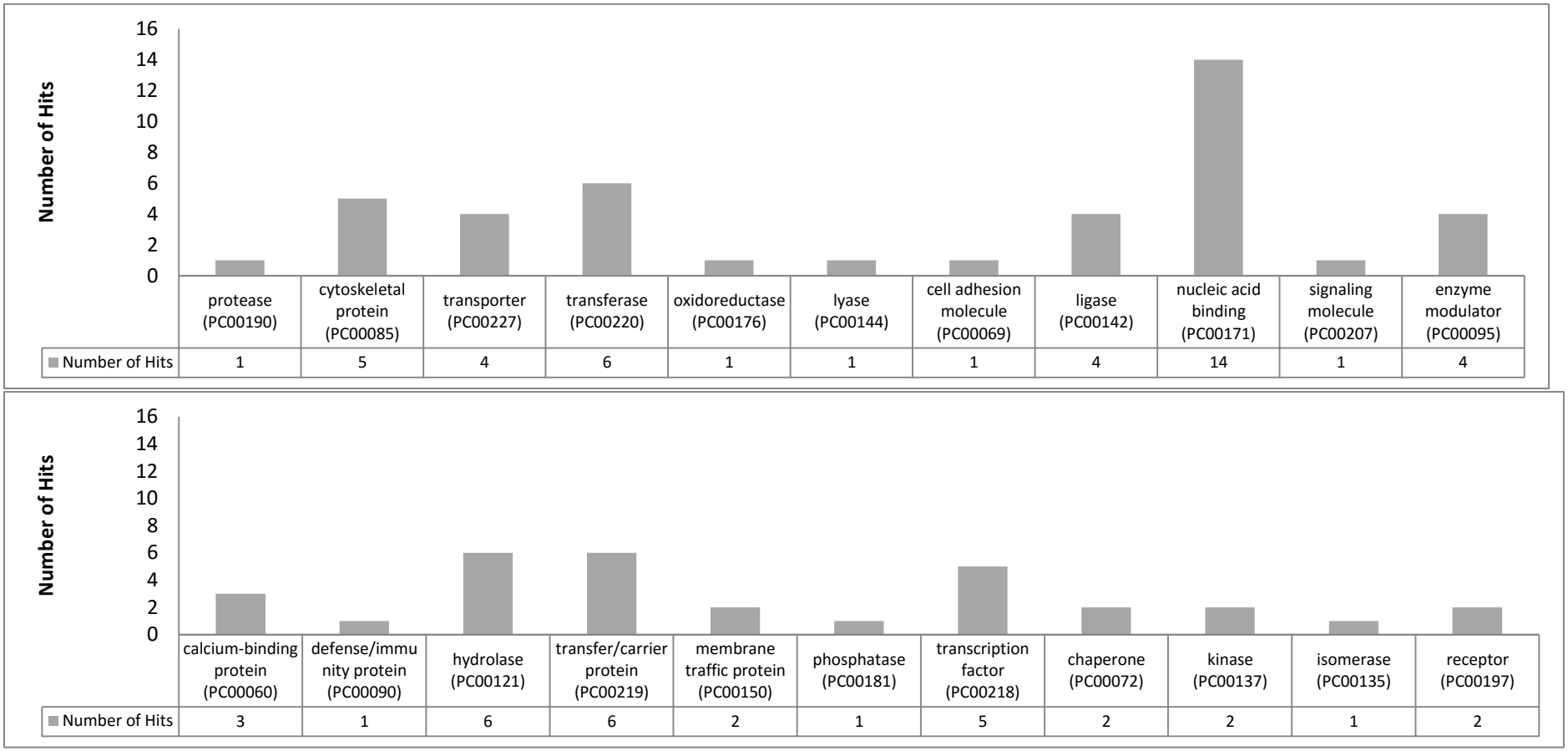

Figure 6.14 – Protein classes of the differently expressed proteins in MCF10A after transfection with anti-miR-200c. Of the 82 proteins identified, PANTHER and the Gene Ontology Consortium retrieved 73 hits in protein classes. Of these, nucleic acid binding proteins are the most common. PANTHER™ Protein Class (version 10.0, released 2015-05-15).

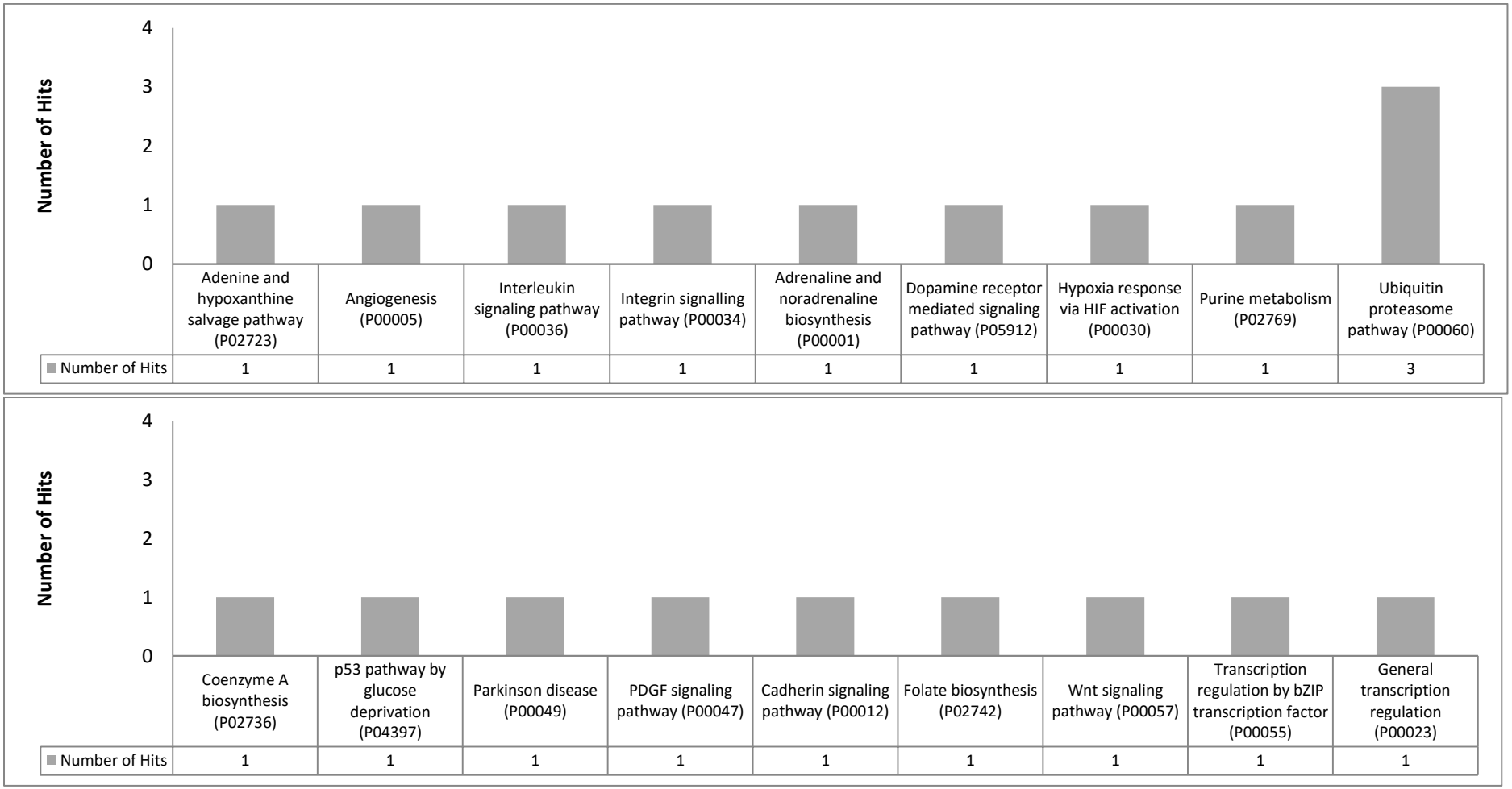

Figure 6.15 - Pathways of the differently expressed proteins in MCF10A after transfection with anti-miR-200c. Of the 82 proteins identified, PANTHER and the Gene Ontology Consortium retrieved 20 hits in pathways. Of these, ubiquitin proteasome pathway related proteins were the most common. PANTHER™ Pathway 3.4, released 2015-05-15.

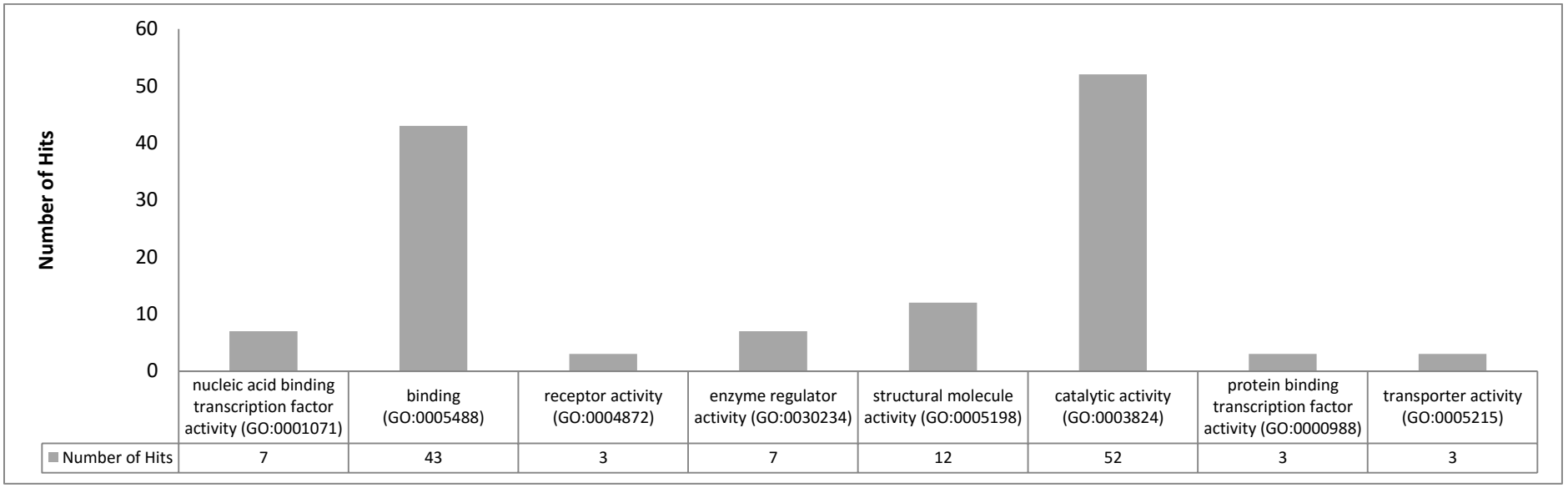

Figure 6.16 – Molecular function of the differently expressed proteins in MCF-10A after transfection with anti-miR-203. Of the 130 proteins identified, PANTHER and the Gene Ontology Consortium retrieved 126 hits in molecular functions. Of these, the most common are proteins with catalytic and binding activity. PANTHER™ GO slim (version 10.0, released 2015-05-15).

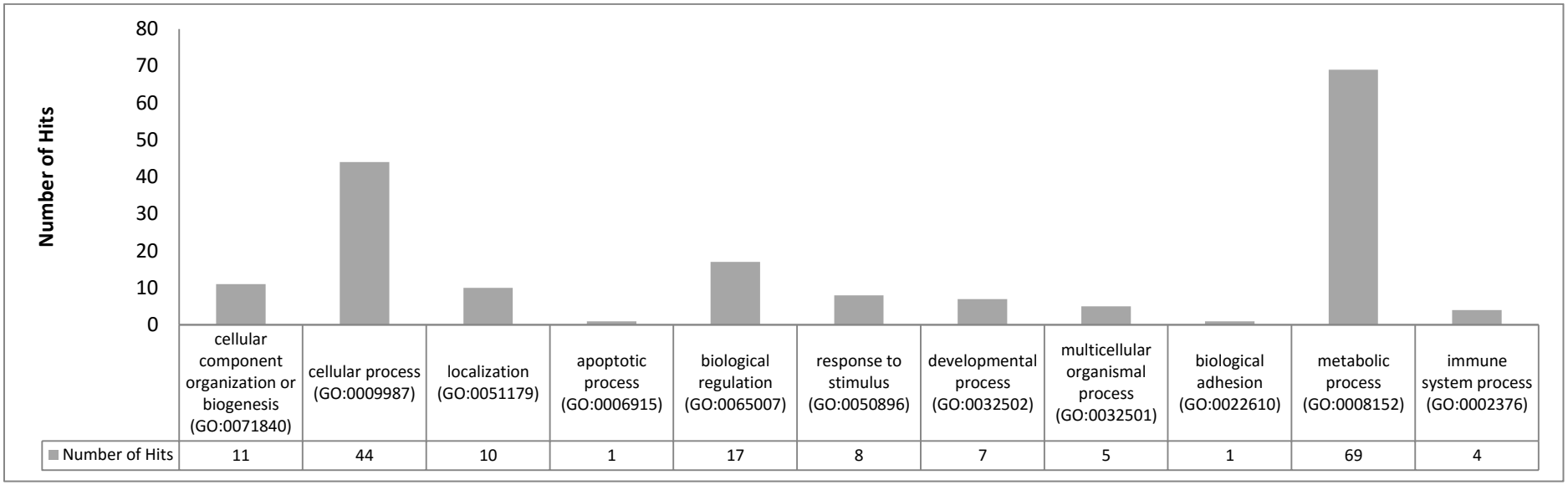

Figure 6.17 - Biological processes of the differently expressed proteins in MCF10A after transfection with anti-miR-203. Of the 130 proteins identified, PANTHER and the Gene Ontology Consortium retrieved 176 hits in biological processes. Of these, metabolic and cellular processes are the most common. Metabolic processes are characterized in greater number as primary metabolic processes and cellular processes are characterized as cell communication, cell cycle, cellular component movement and cytokinesis. PANTHER™ GO slim (version 10.0, released 2015-05-15).

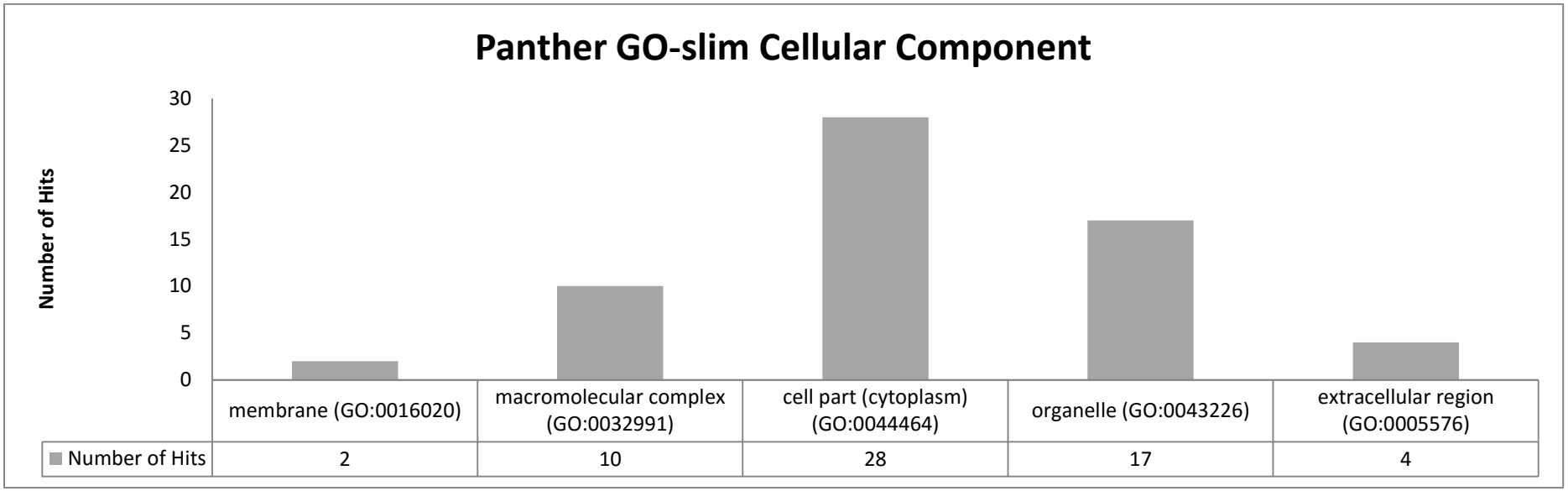

Figure 6.18 - Cellular components of the differently expressed proteins in MCF10A after transfection with anti-miR-203. Of the 130 proteins identified, PANTHER and the Gene Ontology Consortium retrieved 61 hits in cellular components. Of these, cell part (cytoplasmic proteins) and organelle-associated (chromosome, cytoskeleton, mitochondrion and nucleus) proteins are the most common. PANTHER™ GO slim (version 10.0, released 2015-05-15).

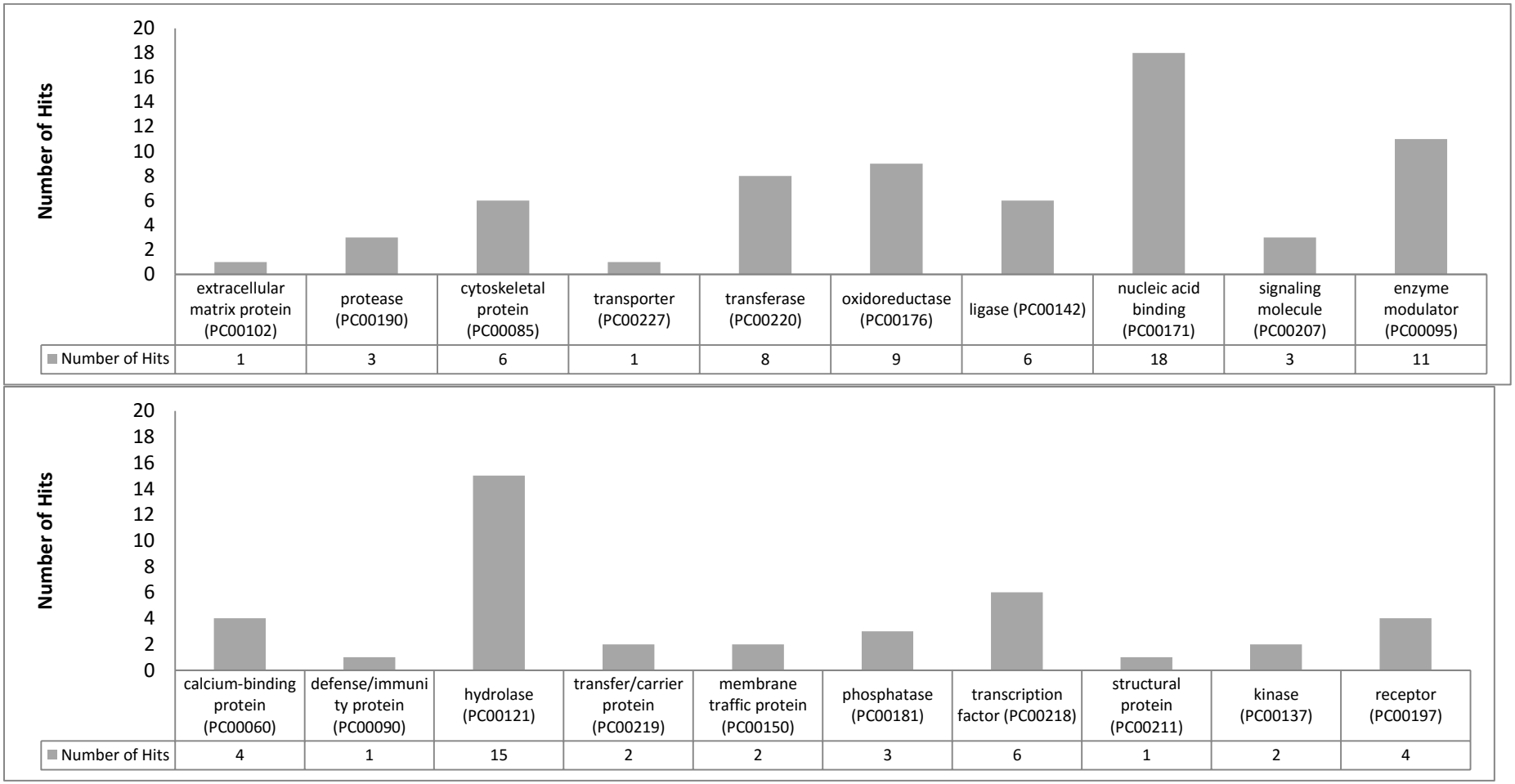

Figure 6.19 – Protein classes of the differently expressed proteins in MCF10A after transfection with anti-miR-203. Of the 130 proteins identified, PANTHER and the Gene Ontology Consortium retrieved 104 hits in protein classes. Of these, nucleic acid binding proteins and hydrolase are the most common. PANTHER™ Protein Class (version 10.0, released 2015-05-15).

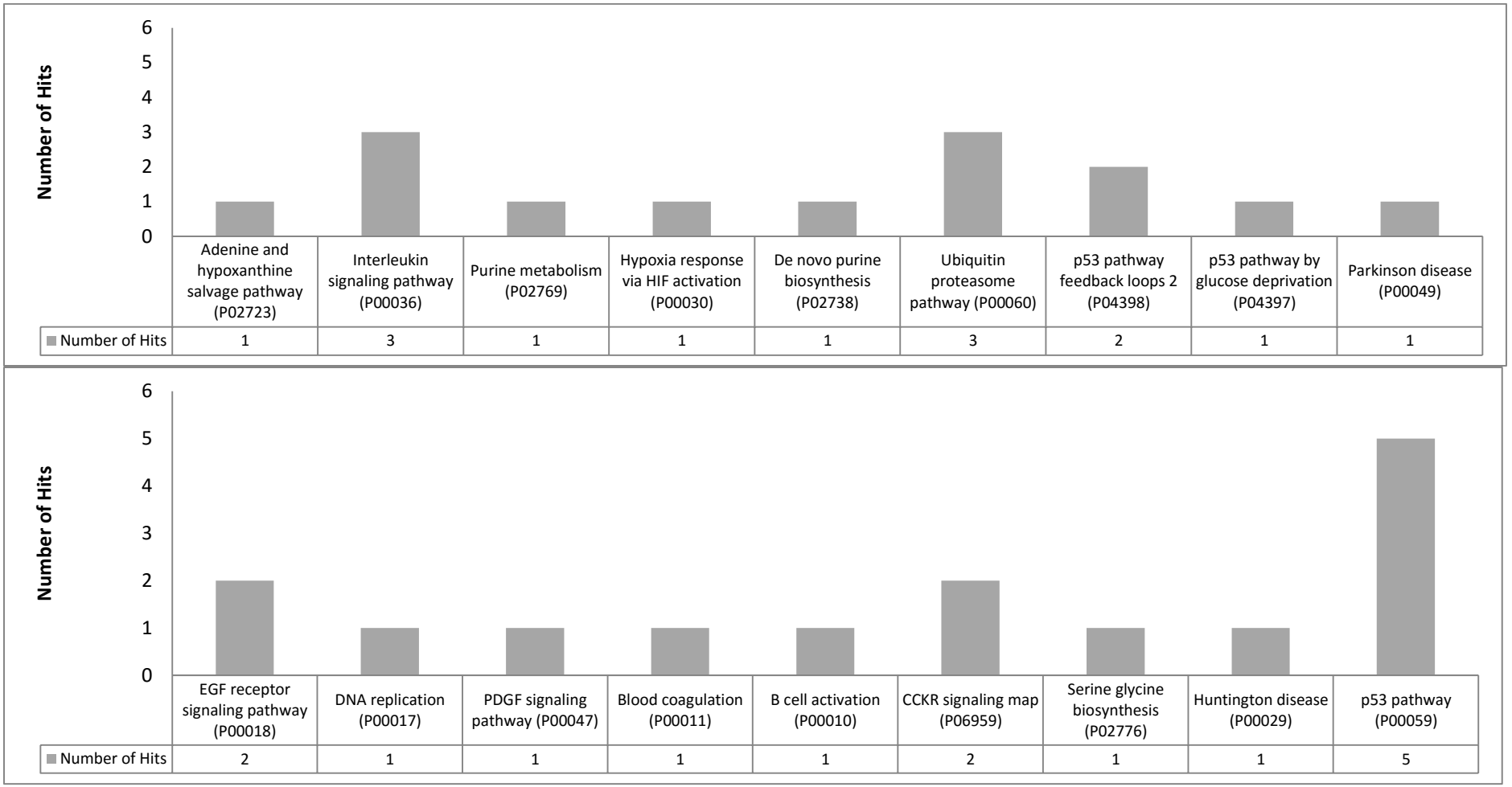

Figure 6.20 - Pathways of the differently expressed proteins in MCF10A after transfection with anti-miR-203. Of the 130 proteins identified, PANTHER and the Gene Ontology Consortium retrieved 42 hits in pathways. Of these, p53 pathway related proteins were the most common. PANTHER™ Pathway 3.4, released 2015-05-15.

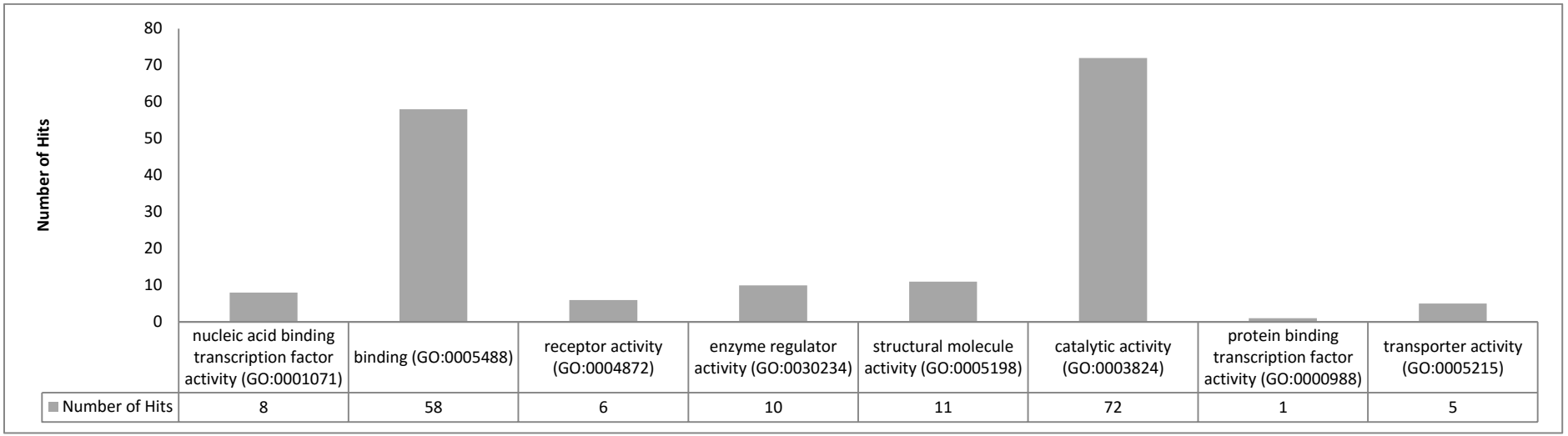

Figure 6.21 - Molecular function of the differently expressed proteins in MCF-10A after transfection with pre-miR-203. Of the 200 proteins identified, PANTHER and the Gene Ontology Consortium retrieved 171 hits in molecular functions. Of these, the most common are proteins with catalytic and binding activity. PANTHER™ GO slim (version 10.0, released 2015-05-15).

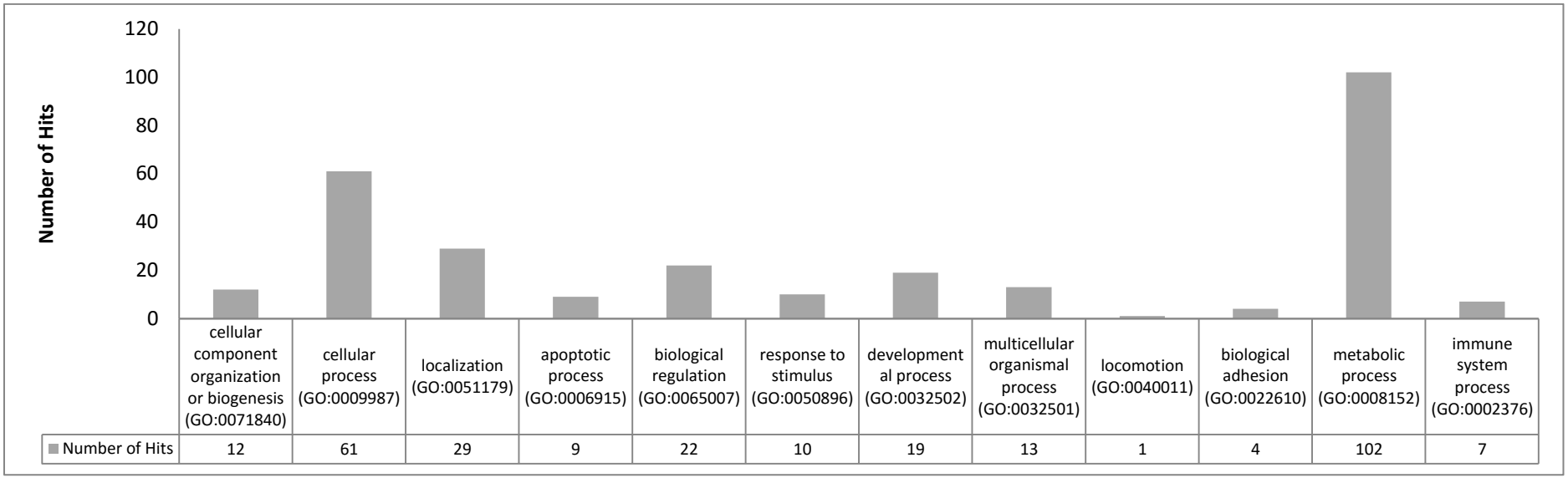

Figure 6.22 - Biological processes of the differently expressed proteins in MCF10A after transfection with pre-miR-203. Of the 200 proteins identified, PANTHER and the Gene Ontology Consortium retrieved 289 hits in biological processes. Of these, metabolic and cellular processes are the most common. Metabolic processes are characterized in greater number as primary metabolic processes and cellular processes are characterized as cell communication, cell cycle, cell proliferation, cellular component movement, chromosome segregation and cytokinesis. PANTHER™ GO slim (version 10.0, released 2015-05-15).

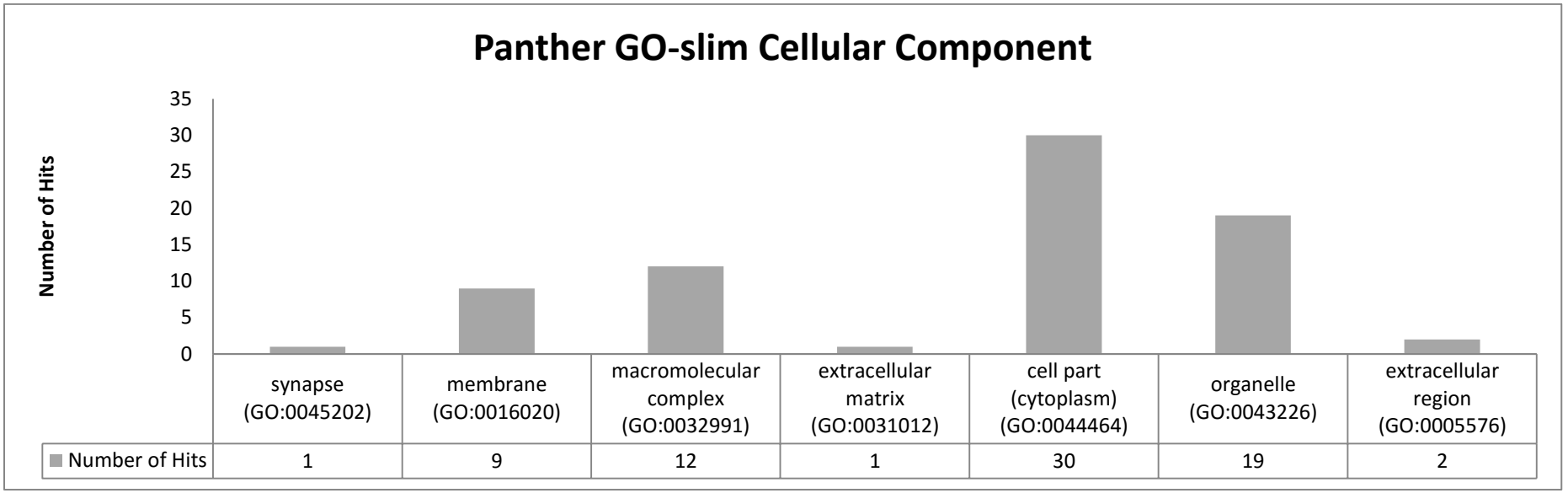

Figure 6.23 - Cellular components of the differently expressed proteins in MCF10A after transfection with pre-miR-203. Of the 200 proteins identified, PANTHER and the Gene Ontology Consortium retrieved 74 hits in cellular components. Of these, cell part (cytoplasmic proteins) and organelle-associated (chromosome, cytoplasmic membrane-bounded vesicles, cytoskeleton, endoplasmic reticulum, endosome, mitochondrion and nucleus) proteins are the most common. PANTHER™ GO slim (version 10.0, released 2015-05-15).

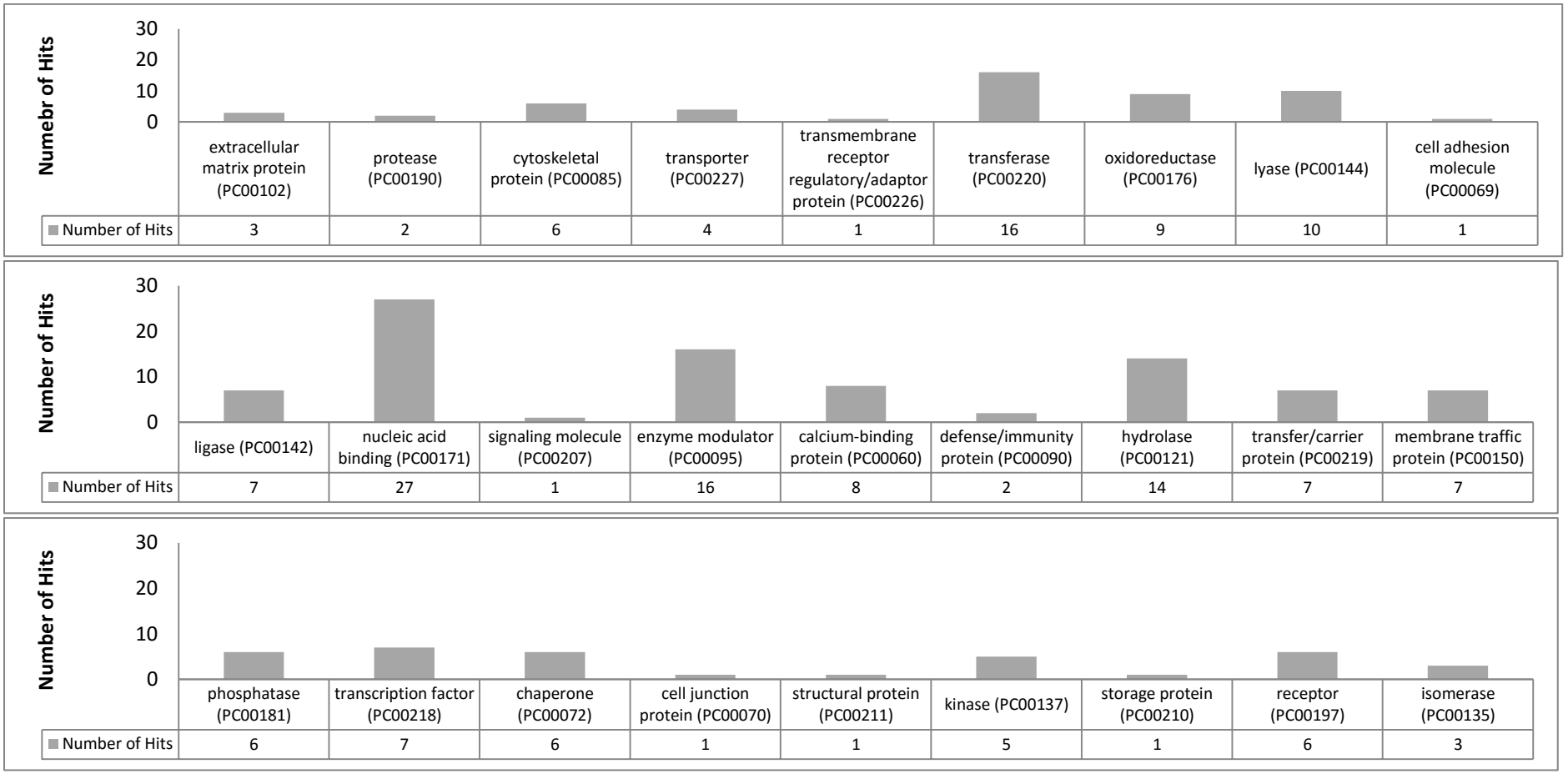

Figure 6.24 – Protein classes of the differently expressed proteins in MCF10A after transfection with pre-miR-203. Of the 200 proteins identified, PANTHER and the Gene Ontology Consortium retrieved 177 hits in protein classes. Of these, nucleic acid binding proteins and hydrolase are the most common. PANTHER™ Protein Class (version 10.0, released 2015-05-15).

6.4. Discussion

MicroRNAs are conspicuous members of the cell circuitry of regulation of protein expression. Epigenetic regulation, particularly by microRNAs, besides DNA methylation or histone acetylation, plays an important role in carcinogenesis and therapy. There are approximately 2000 different human microRNA species identified that form a intertwined network of concurrently regulated proteins that mediate cancer cell survival even upon a challenge by cancer drugs (Rueff and Rodrigues, 2016).

Validation of targets of miRNAs is a slow field of study since they can have virtually hundreds of targets. High-throughput analysis and the increasing resolution of some methodologies allowed investigators in the past few years to achieve better and trustworthy results. One of these techniques was mass spectrometry. This allows the identification of several proteins in a given condition, such as a condition where we know the variation of a single miRNA. Assuming that this is the only variable, we can then make some assumptions regarding the differentially expressed proteins found by mass spectrometry. With this assumption, in collaboration with Professor Peter James, we designed experiments where we varied only one miRNA in a previous known expression cell line. This allowed us to infer that the differentially expressed proteins are in principle, subject to regulation by that miRNA.

Analysing according to the ontology, we can conclude that most deregulated proteins after transfection with pre-miR-200c, pre-miR-203, anti-miR-200c and anti-miR-203 belong to metabolic processes and are located in the cytoplasm or associated with organelles or the nucleus. This is in accordance with their activity, that is catalytic and nucleic acid binding. Recent studies have been showing that metabolomics is a new field of study to better understand tumour biology (Huang et al., 2016; Kelly et al., 2016; Shao et al., 2016; Siminska and Koba, 2016). This field not only analyse metabolites (Hsu et al., 2016) but also proteins involved in the cellular metabolism (Wright et al., 2016). This way, new

biomarkers are being found and new approaches to study cancer are being made, in particular, new therapeutic targets are being studied (Wishart, 2016). Thus, our work here presented reveals that miRNAs also modulate metabolism in breast cancer cell lines. Surprisingly, there are few studies showing association of miRNAs with metabolomics. This must be due to technological complexity and the fact that this is a recent field of study. However, a recent study already sowed that miR-155 regulates thiamine levels in breast cancer (Kim et al., 2015) and with the levels of serum lipids, metabolites associated with glucose metabolism and insulin resistance in cardiovascular risk (Raitoharju et al., 2014). Therefore, we intend to prioritize our future work to this matter and study the association of these miRNAs with metabolic pathways.

Along with ontology study of the deregulated proteins, with the approach here presented we can study putative direct targets of miR-200c and miR-203. In Table 6.1 we show a list of possible miR-200c and miR-203 targets that were already identified with bioinformatics tools and we show here an experimentally possible association.

In MDA-MB-231 we show that IAH1 is a putative direct target of miR-200c. Until now there isn't any study showing this association, thus our results need further studies with other approaches to prove that IAH1 is a direct target of miR-200c. Regarding miR-203, we identified more putative direct targets and of these, FKBP5 and CAB39 were already experimentally associated with miRNAs. FKBP5 is a chaperone protein and is regulated by miR-511 which then regulates neuronal differentiation (Zheng et al., 2016). Interestingly, FKBP5 also interact with AGO2 to facilitate RISC assembly in the miRNAs action pathway (Martinez et al., 2013). CAB39 is a calcium binding protein that acts like a scaffold protein and interacts with AMPK/AKT/mTOR pathway. Until now, only one miRNA was associated with CAB39. Several studies were already published regarding miR-451 and CAB39 and its involvement in different types of cancer, such as colorectal (Chen et al., 2014; Li et al., 2013a) and glioma (Godlewski et al., 2010; Tian et al., 2012). Some liver diseases (Hur et

al., 2015) and cardiac hypertrophy (Kuwabara et al., 2015) are also influenced by miR-451 and CAB39 by targeting AMPK/AKT pathway.

In MCF-10A transfected with anti-miR-200c we detected three putative targets, being CRKL the only one that is associated with miR-200c *in silico*. CRKL is an oncogene by promoting cell proliferation, survival, migration and invasion and also by promoting growth factor independence (Kim et al., 2010). It was also proved that CRKL is a substrate of BCR-ABL in CML, thus prompting leukaemogenesis (Hemmeryckx et al., 2001). Previously, miR-200s family, in which miR-429 is included, was already experimentally associated with CRKL. In fact, miR-200s family were associated with cervical cancer (Wang et al., 2016b), nasopharyngeal carcinoma (Wang et al., 2016a) and breast cancer (Ye et al., 2015). This data is in accordance with our results. However, we need to perform more assays to prove that CRKL is an effective target of miR-200c in breast cancer. CRKL is also regulated by miR-126 in gastric cancer (Wang et al., 2013a) and hepatocellular carcinoma (Ghosh et al., 2016). Regarding the transfection of MCF-10A with anti-miR-203, we detected also 3 proteins over-expressed. Of these only CTBP2 has miR-203 as putative regulator. Although not by miR-203, there were already some results published regarding the regulation of CTBP2 by miRNAs. In particular, regulated by miR-342 (Wang et al., 2015), miR-132 (Salta et al., 2014) and miR-101(Cui et al., 2013). All of these seem to influence stem cells and development. When the MCF-10A cell line was transfected with pre-miR-203 we detected 4 putative targets of miR-203 and all of these have already been described as being regulated by miRNAs. Specifically, miR-203 seems to be regulator of keratinocyte growth, differentiation, and skin inflammation in a skin incision through PLAA (Sun et al., 2012). Here we demonstrate a possible association with breast cancer. For PRKCI the only experimentally demonstration of miRNAs regulation was under miR-219. This study was in tongue squamous cells carcinoma (Song et al., 2014). Regarding RRM1, it is regulated by miR-101 in pancreatic cancer and drug resistance. The authors also suggest that the co-delivery of microRNA-101 and gemcitabine could increase the

therapy outcome (Fan et al., 2016). Finally, HDAC1 is regulated by several miRNAs, including miR-874 (Nohata et al., 2013), miR-34a (Wu et al., 2014) and miR-449a (Liu et al., 2015a). Interestingly, and showing the scheme of an intertwined epigenomic programme in the cell, is the demonstrated capability of HDAC1 to regulate some miRNAs through epigenetic mechanisms. Indeed, HDAC1 is capable of regulate miR-133a in cardiac fibrosis (Renaud et al., 2015), miR-34a/CD44 pathway in gastric cancer (Lin et al., 2015), miR-146a in osteoarthritis, where the authors showed that using HDAC1 inhibitors the expression of miR-146a was increased (Wang et al., 2013c) and miR-449 and miR-224 in hepatocellular carcinoma (Buurman et al., 2012; Wang et al., 2012d).

Although not with miR-200c and miR-203, all these studies show that somehow these putative targets in breast cancer are good candidates to further studies. Thus, we intend to proceed with this approach and will perform a Selected Reaction Monitoring (SRM), once more in collaboration with Professor Peter James. This technique will allow us to perform a quantitative approach of selected proteins. Therefore we will select all inversely expressed proteins when compared with miRNAs expression and analyse through SRM. After this, we will proceed with more cell lines studies in order to confirm association of these miRNAs and the respective targets through luciferase assay. Not less important with this study we can conclude whether the miR-200c and miR-203 are important mediators in metabolic processes and homeostatic activity of the cell. Further studies concerning metabolomics are also being equated.

6.5. Appendix

Table 6.2 – Differently expressed proteins after transfection of MDA-MB-231 cell line with pre-miR-200c. Values are presented as the mean of log_2(Intensity) of two independent experiments. Protein and gene nomenclature is according to UNIPROT database.

	log_2(Intensity) MEAN			
ID_UNIPROT	**Negative control**	**pre-miR-200c**	**PROTEIN**	**GENE**
A1L0T0	0,00	23,78	Acetolactate synthase-like protein	ILVBL
O15126	0,00	24,60	Secretory carrier-associated membrane protein 1	SCAMP1
O43847	0,00	24,11	Nardilysin	NRDC
O60826	0,00	22,40	Coiled-coil domain-containing protein 22	CCDC22
Q8WYK3	0,00	25,42	Thymidylate synthase	TYMS
Q6ICJ4	0,00	21,15	Glutathione S-transferase theta 2B	GSTT2
P13807-2	0,00	24,59	Glycogen [starch] synthase, muscle	GYS1
P20962	0,00	21,85	Parathymosin	PTMS
P42574	0,00	25,41	Caspase-3	CASP3
P42858	0,00	27,19	Huntingtin	HTT
Q13418	0,00	26,03	Integrin-linked protein kinase	ILK
H0YK42	0,00	24,87	Sorting nexin-1	SNX1
F5H702	0,00	21,81	39S ribosomal protein L48, mitochondrial	MRPL48
Q5VVM0	0,00	25,20	Phosphopantothenate--cysteine ligase	PPCS
Q9UI26	21,07	0,00	Importin-11	IPO11
C9JQV0	21,08	0,00	Uncharacterized protein C7orf50	C7orf50
B4DGU4	21,13	0,00	Catenin beta-1	CTNNB1
Q9Y5V0	21,39	0,00	Zinc finger protein 706	ZNF706
Q9BZ29-3	21,51	0,00	Dedicator of cytokinesis protein 9	DOCK9
H0YNH6	21,52	0,00	ER membrane protein complex subunit 9	EMC9

Table 6.2 (continued) – Differently expressed proteins after transfection of MDA-MB-231 cell line with pre-miR-200c. Values are presented as the mean of log2(Intensity) of two independent experiments. Protein and gene nomenclature is according to UNIPROT database.

Q5JUV4	21,58	0,00	Cytosolic purine 5'-nucleotidase	NT5C2
Q9Y2Z4	21,76	0,00	Tyrosine--tRNA ligase, mitochondrial	YARS2
B4DMV3	21,79	0,00	Isoamyl acetate-hydrolyzing esterase 1 homolog	IAH1
Q16774	21,80	0,00	Guanylate kinase	GUK1
Q969Z0	21,83	0,00	Protein TBRG4	TBRG4
O75976	22,05	0,00	Carboxypeptidase D	CPD
Q9BPX5	22,34	0,00	Actin-related protein 2/3 complex subunit 5-like protein	ARPC5L
E9PPY7	22,86	0,00	Arfaptin-2	ARFIP2
O00762	22,90	0,00	Ubiquitin-conjugating enzyme E2 C	UBE2C
Q13451	23,32	0,00	Peptidyl-prolyl cis-trans isomerase FKBP5	FKBP5
Q9BQA1	23,66	0,00	Methylosome protein 50	WDR77
Q01518-2	23,87	0,00	Adenylyl cyclase-associated protein 1	CAP1
O00629	23,98	0,00	Importin subunit alpha-3	KPNA4
Q9BS40	23,99	0,00	Latexin	LXN
O94925-3	24,07	0,00	Glutaminase kidney isoform, mitochondrial	GLS
P07602	24,24	0,00	Prosaposin	PSAP
Q9UBB4-2	24,28	0,00	Ataxin-10	ATXN10
Q9NUJ1	24,41	0,00	Mycophenolic acid acyl-glucuronide esterase, mitochondrial	ABHD10
J3QT28	24,85	0,00	Mitotic checkpoint protein BUB3	BUB3
Q13547	24,90	0,00	Histone deacetylase 1	HDAC1
Q9H444	25,00	0,00	Charged multivesicular body protein 4b	CHMP4B
E9PNC7	25,01	0,00	Dr1-associated corepressor	DRAP1
Q12874	25,20	0,00	Splicing factor 3A subunit 3	SF3A3
Q9Y5P6	25,33	0,00	Mannose-1-phosphate guanyltransferase beta	GMPPB

Table 6.3 - Differently expressed proteins after transfection of MDA-MB-231 cell line with pre-miR-203. Values are presented as the mean of log_2(Intensity) of two independent experiments. Protein and gene nomenclature is according to UNIPROT database.

	log2(Intensity) MEAN			
ID_UNIPROT	**Negative control**	**pre-miR-203**	**PROTEIN**	**GENE**
O43464-2	0,00	22,87	Serine protease HTRA2, mitochondrial	HTRA2
Q8WYK3	0,00	25,05	Thymidylate synthase	TYMS
P13798	0,00	24,50	Acylamino-acid-releasing enzyme	APEH
P20962	0,00	21,99	Parathymosin	PTMS
Q13418	0,00	24,90	Integrin-linked protein kinase	ILK
Q9BZE1	0,00	24,18	39S ribosomal protein L37, mitochondrial	MRPL37
Q5VVM0	0,00	25,40	Phosphopantothenate--cysteine ligase	PPCS
Q9UPU5	19,86	0,00	Ubiquitin carboxyl-terminal hydrolase 24	USP24
F5H1F6	20,37	0,00	Vacuolar protein sorting-associated protein 37B	VPS37B
P16989	20,58	0,00	Y-box-binding protein 3	YBX3
H3BTA8	21,03	0,00	Homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member 1 protein	HERPUD1
B4DGU4	21,13	0,00	Catenin beta-1	CTNNB1
P51636-2	21,21	0,00	Caveolin-2	CAV2
J3KTL8	21,35	0,00	Structural maintenance of chromosomes flexible hinge domain-containing protein 1	SMCHD1
Q9Y5V0	21,39	0,00	Zinc finger protein 706	ZNF706
Q9BTW9	21,56	0,00	Tubulin-specific chaperone D	TBCD
Q5JUV4	21,58	0,00	Cytosolic purine 5'-nucleotidase	NT5C2
Q9Y2Z4	21,76	0,00	Tyrosine--tRNA ligase, mitochondrial	YARS2
P01116-2	21,77	0,00	GTPase KRas	KRAS
B4DMV3	21,79	0,00	Isoamyl acetate-hydrolyzing esterase 1 homolog	IAH1
Q9P0J0	21,89	0,00	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 13	NDUFA13

Table 6.3 (continued) - Differently expressed proteins after transfection of MDA-MB-231 cell line with pre-miR-203. Values are presented as the mean of log2(Intensity) of two independent experiments. Protein and gene nomenclature is according to UNIPROT database.

O75976	22,05	0,00	Carboxypeptidase D	CPD
Q9Y2R0	22,06	0,00	Cytochrome c oxidase assembly factor 3 homolog, mitochondrial	COA3
Q9HBM1	22,16	0,00	Kinetochore protein Spc25	SPC25
Q96ND0	22,22	0,00	Protein FAM210A	FAM210A
Q9BPX5	22,34	0,00	Actin-related protein 2/3 complex subunit 5-like protein	ARPC5L
P09543-2	22,78	0,00	2',3'-cyclic-nucleotide 3'-phosphodiesterase	CNP
O60716-8	22,95	0,00	Catenin delta-1	CTNND1
Q9UGP8	22,98	0,00	Translocation protein SEC63 homolog	SEC63
Q96A49	23,00	0,00	Synapse-associated protein 1	SYAP1
Q13451	23,32	0,00	Peptidyl-prolyl cis-trans isomerase FKBP5	FKBP5
Q53H82	23,45	0,00	Beta-lactamase-like protein 2	LACTB2
Q9NZB2	23,57	0,00	Constitutive coactivator of PPAR-gamma-like protein 1	FAM120A
Q9Y376	23,60	0,00	Calcium-binding protein 39	CAB39
P12236	23,91	0,00	ADP/ATP translocase 3	SLC25A6
O94925-3	24,07	0,00	Glutaminase kidney isoform, mitochondrial	GLS
Q9BV86-2	24,18	0,00	N-terminal Xaa-Pro-Lys N-methyltransferase 1	NTMT1
O75348	24,20	0,00	V-type proton ATPase subunit G 1	ATP6V1G1
O75436	24,23	0,00	Vacuolar protein sorting-associated protein 26A	VPS26A
P21281	24,66	0,00	V-type proton ATPase subunit B, brain isoform	ATP6V1B2
Q9Y5A9	24,69	0,00	YTH domain-containing family protein 2	YTHDF2
Q14192	24,72	0,00	Four and a half LIM domains protein 2	FHL2
Q8IYA2	26,97	0,00	Putative coiled-coil domain-containing protein 144C	CCDC144CP

Table 6.4 - Differently expressed proteins after transfection of MCF-10A cell line with anti-miR-200c. Values are presented as the mean of log_2(Intensity) of two independent experiments. Protein and gene nomenclature is according to UNIPROT database.

	log2(Intensity) MEAN			
ID_UNIPROT	**Negative control**	**anti-miR-200c**	**PROTEIN**	**GENE**
O14737	21,70	0,00	Programmed cell death protein 5	PDCD5
O14773-2	24,15	0,00	Tripeptidyl-peptidase 1	TPP1
O14979-3	22,42	0,00	Heterogeneous nuclear ribonucleoprotein D-like	HNRNPDL
P20338	21,83	0,00	Ras-related protein Rab-4A	RAB4A
P20962	18,51	0,00	Parathymosin	PTMS
P21964-2	22,19	0,00	Catechol O-methyltransferase	COMT
P30508	20,75	0,00	HLA class I histocompatibility antigen, Cw-12 alpha chain	HLA-C
P46934-4	20,07	0,00	E3 ubiquitin-protein ligase NEDD4	NEDD4
P53007	23,36	0,00	Tricarboxylate transport protein, mitochondrial	SLC25A1
P80217	20,99	0,00	Interferon-induced 35 kDa protein	IFI35
F5GWE5	23,95	0,00	Phosphatidylinositol transfer protein alpha isoform	PITPNA
Q00688	25,70	0,00	Peptidyl-prolyl cis-trans isomerase FKBP3	FKBP3
E7EWC2	25,48	0,00	Ras GTPase-activating-like protein IQGAP2	IQGAP2
Q13618-3	23,86	0,00	Cullin-3	CUL3
Q14258	20,82	0,00	E3 ubiquitin/ISG15 ligase TRIM25	TRIM25
Q16527	22,33	0,00	Cysteine and glycine-rich protein 2	CSRP2
Q27J81-2	23,08	0,00	Inverted formin-2	INF2
Q71UM5	22,68	0,00	40S ribosomal protein S27-like	RPS27L
Q7Z7H5-3	21,26	0,00	Transmembrane emp24 domain-containing protein 4	TMED4
Q8WUY1	21,07	0,00	Protein THEM6	THEM6
C9JQV0	21,46	0,00	Uncharacterized protein C7orf50	C7orf50
F5H702	21,60	0,00	39S ribosomal protein L48, mitochondrial	MRPL48
Q92797	21,43	0,00	Symplekin	SYMPK

Table 6.4 (continued) - Differently expressed proteins after transfection of MCF-10A cell line with anti-miR-200c. Values are presented as the mean of log2(Intensity) of two independent experiments. Protein and gene nomenclature is according to UNIPROT database.

Q96C90	21,61	0,00	Protein phosphatase 1 regulatory subunit 14B	PPP1R14B
Q96E11-8	20,31	0,00	Ribosome-recycling factor, mitochondrial	MRRF
Q96GA3	21,69	0,00	Protein LTV1 homolog	LTV1
Q96P48-3	22,30	0,00	Arf-GAP with Rho-GAP domain, ANK repeat and PH domain-containing protein 1	ARAP1
Q96PY5-3	22,78	0,00	Formin-like protein 2	FMNL2
Q99584	19,66	0,00	Protein S100-A13	S100A13
Q9C0C9	21,60	0,00	(E3-independent) E2 ubiquitin-conjugating enzyme	UBE2O
Q9H0U6	22,33	0,00	39S ribosomal protein L18, mitochondrial	MRPL18
Q9H5X1	21,21	0,00	MIP18 family protein FAM96A	FAM96A
Q9H993	22,20	0,00	Protein-glutamate O-methyltransferase	ARMT1
Q9HAV4	21,73	0,00	Exportin-5	XPO5
Q9NTJ5	22,32	0,00	Phosphatidylinositide phosphatase SAC1	SACM1L
Q9NX08	21,41	0,00	COMM domain-containing protein 8	COMMD8
Q9P107	20,05	0,00	GEM-interacting protein	GMIP
Q9Y3D0	20,98	0,00	Mitotic spindle-associated MMXD complex subunit MIP18	FAM96B
B4DLN1	21,74	0,00	Uncharacterized protein	
F5H1S9	22,04	0,00	tRNA pseudouridine synthase	PUS1
Q9Y6C9	21,94	0,00	Mitochondrial carrier homolog 2	MTCH2
Q9UG63	23,70	0,00	ATP-binding cassette sub-family F member 2	ABCF2
B4DJG8	23,90	0,00	Charged multivesicular body protein 2b	CHMP2B
Q9UBT2	24,89	0,00	SUMO-activating enzyme subunit 2	UBA2
Q93083	18,42	0,00	Metallothionein-1L	MT1L
H7C585	19,46	0,00	Frataxin, mitochondrial	FXN
G3V108	20,98	0,00	CCR4-NOT transcription complex subunit 7	CNOT7
Q9H0P0-3	22,52	0,00	Cytosolic 5'-nucleotidase 3A	NT5C3A

P47989	22,50	0,00	Xanthine dehydrogenase/oxidase	XDH
P42345	23,06	0,00	Serine/threonine-protein kinase mTOR	MTOR
C9JG32	23,83	0,00	Elongation factor Ts	TSFM
P42285	22,94	0,00	Superkiller viralicidic activity 2-like 2	SKIV2L2
H3BM18	22,42	0,00	Gamma-interferon-inducible protein 16	IFI16
H0YAT1	22,67	0,00	SH2 domain-containing protein 4A	SH2D4A
Q9BWS9	23,11	0,00	Chitinase domain-containing protein 1	CHID1
P41247-2	0,00	20,20	Patatin-like phospholipase domain-containing protein 4	PNPLA4
Q9Y3C1	0,00	21,33	Nucleolar protein 16	NOP16
Q8WVC6	0,00	21,38	Dephospho-CoA kinase domain-containing protein	DCAKD
Q13043-2	0,00	21,38	Serine/threonine-protein kinase 4	STK4
O00762	0,00	21,39	Ubiquitin-conjugating enzyme E2 C	UBE2C
Q9BYD2	0,00	21,79	39S ribosomal protein L9, mitochondrial	MRPL9
B5MCY6	0,00	22,30	Protein phosphatase 1 regulatory subunit 7	PPP1R7
O14929-2	0,00	22,62	Histone acetyltransferase type B catalytic subunit	HAT1
O94804	0,00	22,66	Serine/threonine-protein kinase 10	STK10
Q9Y2A7	0,00	22,77	Nck-associated protein 1	NCKAP1
Q5VYS8-4	0,00	22,84	Terminal uridylyltransferase 7	ZCCHC6
Q9NYT0	0,00	22,87	Pleckstrin-2	PLEK2
Q5JVY0	0,00	22,97	E3 ubiquitin-protein ligase TRIM32	TRIM32
Q14980-4	0,00	23,03	Nuclear mitotic apparatus protein 1	NUMA1
P19784	0,00	23,29	Casein kinase II subunit alpha'	CSNK2A2
Q9NWU5	0,00	23,32	39S ribosomal protein L22, mitochondrial	MRPL22
O00560-2	0,00	23,34	Syntenin-1	SDCBP
Q9BTE3-2	0,00	23,52	Mini-chromosome maintenance complex-binding protein	MCMBP
Q14240	0,00	23,72	Eukaryotic initiation factor 4A-II	EIF4A2
Q9UHD9	0,00	23,97	Ubiquilin-2	UBQLN2
Q08378-4	0,00	24,12	Golgin subfamily A member 3	GOLGA3

Table 6.4 (continued) - Differently expressed proteins after transfection of MCF-10A cell line with anti-miR-200c. Values are presented as the mean of log2(Intensity) of two independent experiments. Protein and gene nomenclature is according to UNIPROT database.

A8MU28	0,00	24,20	NEDD8-activating enzyme E1 regulatory subunit	NAE1
P23743	0,00	24,36	Diacylglycerol kinase alpha	DGKA
Q96JJ7	0,00	24,44	Protein disulfide-isomerase TMX3	TMX3
P46109	0,00	24,50	Crk-like protein	CRKL
Q99614	0,00	24,58	Tetratricopeptide repeat protein 1	TTC1
P13984	0,00	24,68	General transcription factor IIF subunit 2	GTF2F2
Q4W5G0	0,00	25,01	Tigger transposable element-derived protein 2	TIGD2

Table 6.5 - Differently expressed proteins after transfection of MCF-10A cell line with anti-miR-203. Values are presented as the mean of log_2(Intensity) of two independent experiments. Protein and gene nomenclature is according to UNIPROT database.

	log2(Intensity) MEAN			
ID_UNIPROT	**Negative control**	**anti-miR-203**	**PROTEIN**	**GENE**
O00625	22,02	0,00	Pirin	PIR
O14979-3	22,42	0,00	Heterogeneous nuclear ribonucleoprotein D-like	HNRNPDL
O15067	25,54	0,00	Phosphoribosylformylglycinamidine synthase	PFAS
O15118	22,31	0,00	Niemann-Pick C1 protein	NPC1
O60711	22,30	0,00	Leupaxin	LPXN
O75368	21,58	0,00	SH3 domain-binding glutamic acid-rich-like protein	SH3BGRL
O75934	22,51	0,00	Pre-mRNA-splicing factor SPF27	BCAS2
O96011-2	21,77	0,00	Peroxisomal membrane protein 11B	PEX11B
P01583	24,39	0,00	Interleukin-1 alpha	IL1A
P05121	24,46	0,00	Plasminogen activator inhibitor 1	SERPINE1
B8ZZQ6	20,84	0,00	Prothymosin alpha	PTMA
P11387	25,76	0,00	DNA topoisomerase 1	TOP1
C9J4G9	23,07	0,00	Beta-galactosidase	GLB1
B7Z708	22,41	0,00	cDNA FLJ58698, highly similar to cAMP-dependent protein kinase, alpha-catalytic subunit (EC 2.7.11.11)	
P20962	18,51	0,00	Parathymosin	PTMS
P23434	21,16	0,00	Glycine cleavage system H protein, mitochondrial	GCSH
P25205	23,45	0,00	DNA replication licensing factor MCM3	MCM3
P30508	20,75	0,00	HLA class I histocompatibility antigen, Cw-12 alpha chain	HLA-C
H3BS44	23,73	0,00	Estradiol 17-beta-dehydrogenase 2	HSD17B2
P38936	17,91	0,00	Cyclin-dependent kinase inhibitor 1	CDKN1A
P46821	24,84	0,00	Microtubule-associated protein 1B	MAP1B

Table 6.5 (continued) - Differently expressed proteins after transfection of MCF-10A cell line with anti-miR-203. Values are presented as the mean of log2(Intensity) of two independent experiments. Protein and gene nomenclature is according to UNIPROT database.

P46934-4	20,07	0,00	E3 ubiquitin-protein ligase NEDD4	NEDD4
P51610-2	22,28	0,00	Host cell factor 1	HCFC1
P52895	24,46	0,00	Aldo-keto reductase family 1 member C2	AKR1C2
E9PPY7	22,35	0,00	Arfaptin-2	ARFIP2
P61599	19,36	0,00	N-alpha-acetyltransferase 20	NAA20
P62158	22,89	0,00	Calmodulin	CALM1
B8ZZJ0	20,44	0,00	Small ubiquitin-related modifier 1	SUMO1
C9JBI3	23,28	0,00	Phosphoserine phosphatase	PSPH
Q13217	24,14	0,00	DnaJ homolog subfamily C member 3	DNAJC3
E7EWC2	25,48	0,00	Ras GTPase-activating-like protein IQGAP2	IQGAP2
Q13618-3	23,86	0,00	Cullin-3	CUL3
Q14011	20,63	0,00	Cold-inducible RNA-binding protein	CIRBP
Q14258	20,82	0,00	E3 ubiquitin/ISG15 ligase TRIM25	TRIM25
Q14738-3	23,19	0,00	Serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit delta isoform	PPP2R5D
Q15021	21,94	0,00	Condensin complex subunit 1	NCAPD2
Q15293	24,45	0,00	Reticulocalbin-1	RCN1
Q16527	22,33	0,00	Cysteine and glycine-rich protein 2	CSRP2
Q27J81-2	23,08	0,00	Inverted formin-2	INF2
B4DMV3	20,54	0,00	Isoamyl acetate-hydrolyzing esterase 1 homolog	IAH1
Q5J8M3-3	21,34	0,00	ER membrane protein complex subunit 4	EMC4
Q5SW79	23,97	0,00	Centrosomal protein of 170 kDa	CEP170
Q66K14-2	21,26	0,00	TBC1 domain family member 9B	TBC1D9B
Q6PIU2	24,94	0,00	Neutral cholesterol ester hydrolase 1	NCEH1
Q7Z2Z2-2	20,48	0,00	Elongation factor-like GTPase 1	EFL1

B4DLH2	23,97	0,00	Chromosome 2 open reading frame 18, isoform CRA_c	C2orf18
Q8IWE2	25,22	0,00	Protein NOXP20	FAM114A1
Q8N183	21,35	0,00	Mimitin, mitochondrial	NDUFAF2
Q8NDC0	23,45	0,00	MAPK-interacting and spindle-stabilizing protein-like	MAPK1IP1L
Q8NEY1-7	23,58	0,00	Neuron navigator 1	NAV1
Q8NFW8	24,57	0,00	N-acylneuraminate cytidylyltransferase	CMAS
Q8TCT9-5	24,55	0,00	Minor histocompatibility antigen H13	HM13
C9JQV0	21,46	0,00	Uncharacterized protein C7orf50	C7orf50
E5RGY0	23,54	0,00	Derlin	DERL1
F5H702	21,60	0,00	39S ribosomal protein L48, mitochondrial	MRPL48
H0YLY7	21,41	0,00	Calcineurin B homologous protein 1	CHP1
Q96C19	23,49	0,00	EF-hand domain-containing protein D2	EFHD2
Q96C90	21,61	0,00	Protein phosphatase 1 regulatory subunit 14B	PPP1R14B
Q96E11-8	20,31	0,00	Ribosome-recycling factor, mitochondrial	MRRF
Q96GA3	21,69	0,00	Protein LTV1 homolog	LTV1
Q96P48-3	22,30	0,00	Arf-GAP with Rho-GAP domain, ANK repeat and PH domain-containing protein 1	ARAP1
Q99622	18,50	0,00	Protein C10	C12orf57
Q9BVP2-2	24,36	0,00	Guanine nucleotide-binding protein-like 3	GNL3
H0Y6C7	20,42	0,00	Intraflagellar transport protein 27 homolog	IFT27
Q9C0C9	21,60	0,00	(E3-independent) E2 ubiquitin-conjugating enzyme	UBE2O
Q9H5X1	21,21	0,00	MIP18 family protein FAM96A	FAM96A
A6PVP4	20,67	0,00	Mediator of RNA polymerase II transcription subunit 20	MED20
Q9H993	22,20	0,00	Protein-glutamate O-methyltransferase	ARMT1
Q9HAV4	21,73	0,00	Exportin-5	XPO5
Q9HCN8	21,85	0,00	Stromal cell-derived factor 2-like protein 1	SDF2L1

Table 6.5 (continued) - Differently expressed proteins after transfection of MCF-10A cell line with anti-miR-203. Values are presented as the mean of log2(Intensity) of two independent experiments. Protein and gene nomenclature is according to UNIPROT database.

Q9HD33-2	22,29	0,00	39S ribosomal protein L47, mitochondrial	MRPL47
Q9NRW3	20,20	0,00	DNA dC->dU-editing enzyme APOBEC-3C	APOBEC3C
Q9NTJ5	22,32	0,00	Phosphatidylinositide phosphatase SAC1	SACM1L
Q9NX08	21,41	0,00	COMM domain-containing protein 8	COMMD8
Q9UNX3	20,90	0,00	60S ribosomal protein L26-like 1	RPL26L1
Q9Y3A6	19,30	0,00	Transmembrane emp24 domain-containing protein 5	TMED5
C9JA28	20,00	0,00	Translocon-associated protein subunit gamma	SSR3
Q9Y5S9-2	21,22	0,00	RNA-binding protein 8A	RBM8A
Q9Y5Y2	21,33	0,00	Cytosolic Fe-S cluster assembly factor NUBP2	NUBP2
Q9UPT8	22,28	0,00	Zinc finger CCCH domain-containing protein 4	ZC3H4
B4DLN1	21,74	0,00	Uncharacterized protein	
H0Y621	23,25	0,00	Endoplasmic reticulum-Golgi intermediate compartment protein 3	ERGIC3
Q9UBT2	24,89	0,00	SUMO-activating enzyme subunit 2	UBA2
H7C585	19,46	0,00	Frataxin, mitochondrial	FXN
H0YE25	18,95	0,00	Parkinson disease 7 domain-containing protein 1	PDDC1
Q14160	22,30	0,00	Protein scribble homolog	SCRIB
Q9H0P0-3	22,52	0,00	Cytosolic 5'-nucleotidase 3A	NT5C3A
P47989	22,50	0,00	Xanthine dehydrogenase/oxidase	XDH
P42345	23,06	0,00	Serine/threonine-protein kinase mTOR	MTOR
Q92599-2	24,73	0,00	Septin-8	SEPT8
P42285	22,94	0,00	Superkiller viralicidic activity 2-like 2	SKIV2L2
P51857-2	24,66	0,00	3-oxo-5-beta-steroid 4-dehydrogenase	AKR1D1
Q6S8J3	24,99	0,00	POTE ankyrin domain family member E	POTEE
H3BM18	22,42	0,00	Gamma-interferon-inducible protein 16	IFI16

Q9H3Z4-2	23,51	0,00	DnaJ homolog subfamily C member 5	DNAJC5
D6RAX2	22,84	11,49	C-terminal-binding protein 1	CTBP1
P51532-2	0,00	19,08	Transcription activator BRG1	SMARCA4
Q9UI09	0,00	19,56	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 12	NDUFA12
Q9UFN0	0,00	20,11	Protein NipSnap homolog 3A	NIPSNAP 3A
Q9BU89	0,00	21,07	Deoxyhypusine hydroxylase	DOHH
O15294-4	0,00	21,29	UDP-N-acetylglucosamine--peptide N-acetylglucosaminyltransferase 110 kDa subunit	OGT
Q9Y2R0	0,00	21,34	Cytochrome c oxidase assembly factor 3 homolog, mitochondrial	COA3
O95168-2	0,00	21,56	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 4	NDUFB4
P82675	0,00	21,63	28S ribosomal protein S5, mitochondrial	MRPS5
B7Z358	0,00	21,80	39S ribosomal protein L50, mitochondrial	MRPL50
E7ESI2	0,00	21,82	Cyclin-dependent kinase 2	CDK2
Q14980-4	0,00	21,87	Nuclear mitotic apparatus protein 1	NUMA1
Q13043-2	0,00	21,91	Serine/threonine-protein kinase 4	STK4
Q9P0J0	0,00	22,56	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 13	NDUFA13
O00762	0,00	22,59	Ubiquitin-conjugating enzyme E2 C	UBE2C
Q13619-2	0,00	22,67	Cullin-4A	CUL4A
Q9Y3E7-2	0,00	22,78	Charged multivesicular body protein 3	CHMP3
Q71UI9	0,00	22,90	Histone H2A.V	H2AFV
P09669	0,00	22,92	Cytochrome c oxidase subunit 6C	COX6C
Q9UI12-2	0,00	23,12	V-type proton ATPase subunit H	ATP6V1H
O14929-2	0,00	23,13	Histone acetyltransferase type B catalytic subunit	HAT1
B4DKZ7	0,00	23,19	cDNA FLJ55704, highly similar to Transcriptional repressor p66 alpha	
Q9NXH9-2	0,00	23,21	tRNA (guanine(26)-N(2))-dimethyltransferase	TRMT1
Q5RI15	0,00	23,24	Cytochrome c oxidase protein 20 homolog	COX20
E7EUV3	0,00	23,24	Ankyrin repeat domain-containing protein 17	ANKRD17

Table 6.5 (continued) - Differently expressed proteins after transfection of MCF-10A cell line with anti-miR-203. Values are presented as the mean of log2(Intensity) of two independent experiments. Protein and gene nomenclature is according to UNIPROT database.

O75828	0,00	23,46	Carbonyl reductase [NADPH] 3	CBR3
Q8WZA9	0,00	23,49	Immunity-related GTPase family Q protein	IRGQ
O00560-2	0,00	23,53	Syntenin-1	SDCBP
O00515	18,31	23,70	Ladinin-1	LAD1
O60678-2	0,00	23,75	Protein arginine N-methyltransferase 3	PRMT3
P56545	0,00	23,97	C-terminal-binding protein 2	CTBP2
Q8NHH9-3	0,00	24,22	Atlastin-2	ATL2
P14866-2	0,00	24,22	Heterogeneous nuclear ribonucleoprotein L	HNRNPL
Q9UHD9	0,00	24,26	Ubiquilin-2	UBQLN2
P13984	0,00	24,30	General transcription factor IIF subunit 2	GTF2F2
Q9BTE3-2	0,00	24,49	Mini-chromosome maintenance complex-binding protein	MCMBP
B7Z8U9	0,00	24,56	cDNA FLJ52831, highly similar to Tripartite motif-containing protein 29	
Q86U42-2	0,00	24,84	Polyadenylate-binding protein 2	PABPN1
O94776	0,00	25,03	Metastasis-associated protein MTA2	MTA2
Q96JJ7	0,00	25,13	Protein disulfide-isomerase TMX3	TMX3

Table 6.6 - Differently expressed proteins after transfection of MCF-10A cell line with pre-miR-203. Values are presented as the mean of log_2(Intensity) of two independent experiments. Protein and gene nomenclature is according to UNIPROT database.

	log2(Intensity) MEAN			
ID_UNIPROT	**Negative control**	**pre-miR-203**	**PROTEIN**	**GENE**
O43760	0,00	21,98	Synaptogyrin-2	SYNGR2
O43852	0,00	25,55	Calumenin	CALU
O60613	0,00	19,06	15 kDa selenoprotein	SEP15
O60869-2	0,00	19,23	Endothelial differentiation-related factor 1	EDF1
E5RGS4	0,00	19,02	Prefoldin subunit 1	PFDN1
O75323	0,00	23,73	Protein NipSnap homolog 2	GBAS
O75368	0,00	20,34	SH3 domain-binding glutamic acid-rich-like protein	SH3BGRL
O75477	0,00	23,64	Erlin-1	ERLIN1
O94855	0,00	23,34	Protein transport protein Sec24D	SEC24D
O95295	0,00	20,88	SNARE-associated protein Snapin	SNAPIN
O95721	0,00	21,88	Synaptosomal-associated protein 29	SNAP29
O95816	0,00	21,94	BAG family molecular chaperone regulator 2	BAG2
P00533	0,00	22,91	Epidermal growth factor receptor	EGFR
E7EWZ6	0,00	21,81	Integrin alpha-V	ITGAV
E9PJS4	0,00	21,41	Signal recognition particle receptor subunit alpha	SRPRA
F8VZY9	0,00	24,63	Keratin, type I cytoskeletal 18	KRT18
P07311	0,00	19,24	Acylphosphatase-1	ACYP1
P09104	0,00	24,75	Gamma-enolase	ENO2
Q5TCU3	0,00	23,70	Tropomyosin beta chain	TPM2
P11047	0,00	22,87	Laminin subunit gamma-1	LAMC1
P15927	0,00	22,83	Replication protein A 32 kDa subunit	RPA2
P16949	0,00	22,03	Stathmin	STMN1
P17096	0,00	21,13	High mobility group protein HMG-I/HMG-Y	HMGA1

Table 6.6 (continued) - Differently expressed proteins after transfection of MCF-10A cell line with pre-miR-203. Values are presented as the mean of log2(Intensity) of two independent experiments. Protein and gene nomenclature is according to UNIPROT database.

P19388	0,00	21,66	DNA-directed RNA polymerases I, II, and III subunit RPABC1	POLR2E
P20962	0,00	23,23	Parathymosin	PTMS
B4DIT7	0,00	24,74	cDNA FLJ58187, highly similar to Protein-glutamine gamma-glutamyltransferase 2(EC 2.3.2.13)	
P23634-5	0,00	24,79	Plasma membrane calcium-transporting ATPase 4	ATP2B4
P31930	0,00	23,94	Cytochrome b-c1 complex subunit 1, mitochondrial	UQCRC1
B1AK13	0,00	22,74	3-hydroxymethyl-3-methylglutaryl-Coenzyme A lyase (Hydroxymethylglutaricaciduria), isoform CRA_b	HMGCL
P40937-2	0,00	21,75	Replication factor C subunit 5	RFC5
P42858	0,00	21,72	Huntingtin	HTT
B4DF38	0,00	24,05	cDNA FLJ52123, highly similar to Platelet-activating factor acetylhydrolase IB alpha subunit	
P46934-4	0,00	20,26	E3 ubiquitin-protein ligase NEDD4	NEDD4
B4DWK0	0,00	21,58	Selenide, water dikinase 1	SEPHS1
P51610-2	0,00	22,23	Host cell factor 1	HCFC1
E9PPY7	0,00	21,51	Arfaptin-2	ARFIP2
P54727	0,00	22,43	UV excision repair protein RAD23 homolog B	RAD23B
J3QR71	0,00	21,90	Developmentally-regulated GTP-binding protein 2	DRG2
P62158	0,00	22,03	Calmodulin	CALM1
B8ZZJ0	0,00	20,64	Small ubiquitin-related modifier 1	SUMO1
F5GWE5	0,00	20,53	Phosphatidylinositol transfer protein alpha isoform	PITPNA
Q01085	0,00	23,52	Nucleolysin TIAR	TIAL1
Q01105	0,00	27,60	Protein SET	SET
Q07352	0,00	23,04	Zinc finger protein 36, C3H1 type-like 1	ZFP36L1
Q12768	0,00	21,17	WASH complex subunit strumpellin	KIAA0196

Q12874	0,00	22,47	Splicing factor 3A subunit 3	SF3A3
Q12965	0,00	21,78	Unconventional myosin-Ie	MYO1E
Q13217	0,00	23,11	DnaJ homolog subfamily C member 3	DNAJC3
Q13641	0,00	22,41	Trophoblast glycoprotein	TPBG
Q13795	0,00	20,57	ADP-ribosylation factor-related protein 1	ARFRP1
Q14061	0,00	20,75	Cytochrome c oxidase copper chaperone	COX17
Q14192	0,00	22,88	Four and a half LIM domains protein 2	FHL2
Q16539	0,00	23,59	Mitogen-activated protein kinase 14	MAPK14
H0Y6C3	0,00	21,88	Pyrroline-5-carboxylate reductase 3	PYCRL
B1AJQ6	0,00	22,97	Syntaxin-12	STX12
Q8N138-4	0,00	19,14	ORM1-like protein 3	ORMDL3
Q8TBC4-2	0,00	24,48	NEDD8-activating enzyme E1 catalytic subunit	UBA3
Q92542-2	0,00	24,09	Nicastrin	NCSTN
Q99622	0,00	19,92	Protein C10	C12orf57
Q9C0C9	0,00	20,32	(E3-independent) E2 ubiquitin-conjugating enzyme	UBE2O
C9JAG1	0,00	21,98	Ethanolaminephosphotransferase 1	EPT1
Q9H3P7	0,00	23,78	Golgi resident protein GCP60	ACBD3
Q9H444	0,00	23,98	Charged multivesicular body protein 4b	CHMP4B
A6PVP4	0,00	19,37	Mediator of RNA polymerase II transcription subunit 20	MED20
Q5VVM0	0,00	22,98	Phosphopantothenate--cysteine ligas	PPCS
Q9NP77	0,00	20,00	RNA polymerase II subunit A C-terminal domain phosphatase SSU72	SSU72
Q9NRX4	0,00	24,71	14 kDa phosphohistidine phosphatase	PHPT1
Q9UBS4	0,00	23,19	DnaJ homolog subfamily B member 11	DNAJB11
Q9UJK0	0,00	19,42	Ribosome biogenesis protein TSR3 homolog	TSR3
E9PQR7	0,00	21,94	Vacuolar protein sorting-associated protein 28 homolog	VPS28
J3KT51	0,00	21,98	Hematological and neurological-expressed 1 protein	HN1
Q9UL26	0,00	22,09	Ras-related protein Rab-22A	RAB22A
C9JA28	0,00	20,07	Translocon-associated protein subunit gamma	SSR3

Table 6.6 (continued) - Differently expressed proteins after transfection of MCF-10A cell line with pre-miR-203. Values are presented as the mean of log2(Intensity) of two independent experiments. Protein and gene nomenclature is according to UNIPROT database.

G3V158	0,00	21,94	2-deoxyribose-5-phosphate aldolase homolog (C. elegans), isoform CRA_a	DERA
Q9Y3A3-2	0,00	22,64	MOB-like protein phocein	MOB4
B8ZZF8	0,00	20,65	U6 snRNA-associated Sm-like protein LSm5	LSM5
C9JNE2	0,00	18,39	O-acetyl-ADP-ribose deacetylase 1	OARD1
Q9Y5J9	0,00	19,38	Mitochondrial import inner membrane translocase subunit Tim8 B	TIMM8B
Q9Y5Z4	0,00	20,60	Heme-binding protein 2	HEBP2
B4DLN1	0,00	21,79	Uncharacterized protein	
B5MC72	0,00	20,01	Endoplasmic reticulum lectin 1	ERLEC1
Q9BWJ5	0,00	19,25	Splicing factor 3B subunit 5	SF3B5
O15091-3	0,00	20,10	Mitochondrial ribonuclease P protein 3	KIAA0391
F5H871	0,00	20,51	WASH complex subunit FAM21C	FAM21C
H0YBZ9	0,00	19,61	Survival motor neuron protein	SMN1
F5GZP6	0,00	21,11	Liprin-beta-1	PPFIBP1
Q9Y657	0,00	20,49	Spindlin-1	SPIN1
Q8WVC6	0,00	21,16	Dephospho-CoA kinase domain-containing protein	DCAKD
Q9Y6Y8	0,00	21,99	SEC23-interacting protein	SEC23IP
Q96AY3	0,00	22,23	Peptidyl-prolyl cis-trans isomerase FKBP10	FKBP10
F8W943	0,00	22,83	Argininosuccinate lyase	ASL
Q9BRR6-2	0,00	22,92	ADP-dependent glucokinase	ADPGK
O43819	0,00	22,50	Protein SCO2 homolog, mitochondrial	SCO2
Q9Y697-2	0,00	22,54	Cysteine desulfurase, mitochondrial	NFS1
P51857-2	0,00	25,35	3-oxo-5-beta-steroid 4-dehydrogenase	AKR1D1
H7C207	0,00	20,46	R3H domain-containing protein 1	R3HDM1
Q8NI60	0,00	17,76	Atypical kinase ADCK3, mitochondrial	ADCK3

O15027-2	0,00	19,73	Protein transport protein Sec16A	SEC16A
P63313	0,00	19,79	Thymosin beta-10	TMSB10
Q6PI78	0,00	19,58	Transmembrane protein 65	TMEM65
Q15651-2	0,00	19,61	High mobility group nucleosome-binding domain-containing protein 3	HMGN3
F8VUA7	0,00	20,96	Oxysterol-binding protein	OSBPL8
H3BM18	0,00	21,84	Gamma-interferon-inducible protein 16	IFI16
P33316-2	0,00	20,80	Deoxyuridine 5'-triphosphate nucleotidohydrolase, mitochondrial	DUT
Q8IYS1	0,00	22,43	Peptidase M20 domain-containing protein 2	PM20D2
P49841	0,00	23,40	Glycogen synthase kinase-3 beta	GSK3B
O43933	0,00	25,73	Peroxisome biogenesis factor 1	PEX1
Q02224-3	0,00	26,40	Centromere-associated protein E	CENPE
A6ND91	0,00	28,94	Putative L-aspartate dehydrogenase	ASPDH
F5H7C4	18,51	0,00	Methionine-R-sulfoxide reductase B3	MSRB3
Q9P016-2	20,33	0,00	Thymocyte nuclear protein 1	THYN1
P80294	20,38	0,00	Metallothionein-1H	MT1H
Q8IYA2	20,64	0,00	Putative coiled-coil domain-containing protein 144C	CCDC144CP
Q9Y3C4	20,71	0,00	EKC/KEOPS complex subunit TPRKB	TPRKB
Q15382	21,20	0,00	GTP-binding protein Rheb	RHEB
P02792	21,42	0,00	Ferritin light chain	FTL
O60443	21,42	0,00	Non-syndromic hearing impairment protein 5	DFNA5
Q9Y2V2	21,74	0,00	Calcium-regulated heat-stable protein 1	CARHSP1
P82912-2	21,82	0,00	28S ribosomal protein S11, mitochondrial	MRPS11
C9JTE9	21,85	0,00	B-cell receptor-associated protein 29	BCAP29
Q9UI14	21,93	0,00	Prenylated Rab acceptor protein 1	RABAC1
Q9BRJ2	21,94	0,00	39S ribosomal protein L45, mitochondrial	MRPL45
Q5TBE9	21,94	0,00	Iron-sulfur cluster assembly 1 homolog, mitochondrial	ISCA1
Q9BRX8-2	22,02	0,00	Redox-regulatory protein FAM213A	FAM213A

Table 6.6 (continued) - Differently expressed proteins after transfection of MCF-10A cell line with pre-miR-203. Values are presented as the mean of log2(Intensity) of two independent experiments. Protein and gene nomenclature is according to UNIPROT database.

B4DQ47	22,11	0,00	cDNA FLJ58036, highly similar to Homo sapiens sterile alpha motif and leucine zipper containing kinase AZK (ZAK), transcript variant 2, mRNA	
P61769	22,16	0,00	Beta-2-microglobulin	B2M
Q9BV81	22,21	0,00	ER membrane protein complex subunit 6	EMC6
Q9BYN0	22,26	0,00	Sulfiredoxin-1	SRXN1
E9PP29	22,32	0,00	Transmembrane protein 41B	TMEM41B
Q2M389-2	22,35	0,00	WASH complex subunit 7	KIAA1033
B4E2V5	22,38	0,00	cDNA FLJ52062, highly similar to Erythrocyte band 7 integral membrane protein	FKBP2
B8ZZV5	22,38	0,00	39S ribosomal protein L30, mitochondrial	MRPL30
Q9BQ67	22,40	0,00	Glutamate-rich WD repeat-containing protein 1	GRWD1
P18827	22,42	0,00	Syndecan-1	SDC1
Q96DV4	22,44	0,00	39S ribosomal protein L38, mitochondrial	MRPL38
Q02952-3	22,47	0,00	A-kinase anchor protein 12	AKAP12
P55769	22,50	0,00	NHP2-like protein 1	SNU13
Q9Y224	22,52	0,00	UPF0568 protein C14orf166	C14orf166
A1L0T0	22,53	0,00	Acetolactate synthase-like protein	ILVBL
H0YBW4	22,54	0,00	Phospholipase A-2-activating protein	PLAA
Q8N4V1	22,59	0,00	Membrane magnesium transporter 1	MMGT1
O14737	22,62	0,00	Programmed cell death protein 5	PDCD5
Q9BX68	22,62	0,00	Histidine triad nucleotide-binding protein 2, mitochondrial	HINT2
P62330	22,65	0,00	ADP-ribosylation factor 6	ARF6
Q8IUR0	22,69	0,00	Trafficking protein particle complex subunit 5	TRAPPC5
P51668	22,74	0,00	Ubiquitin-conjugating enzyme E2 D1	UBE2D1

P61457	22,78	0,00	Pterin-4-alpha-carbinolamine dehydratase	PCBD1
P41743	22,78	0,00	Protein kinase C iota type	PRKCI
P09669	22,80	0,00	Cytochrome c oxidase subunit 6C	COX6C
Q9H7D0	22,83	0,00	Dedicator of cytokinesis protein 5	DOCK5
Q3ZAQ7	22,85	0,00	Vacuolar ATPase assembly integral membrane protein VMA21	VMA21
Q96EL3	22,87	0,00	39S ribosomal protein L53, mitochondrial	MRPL53
H0YNG3	22,89	0,00	Signal peptidase complex catalytic subunit SEC11	SEC11A
P00918	22,92	0,00	Carbonic anhydrase 2	CA2
C9JW69	22,93	0,00	Regulator of chromosome condensation	RCC1
Q15796-2	22,94	0,00	Mothers against decapentaplegic homolog 2	SMAD2
O15235	22,94	0,00	28S ribosomal protein S12, mitochondrial	MRPS12
Q86WQ0	22,95	0,00	Nuclear receptor 2C2-associated protein	NR2C2AP
Q9BUB7	22,96	0,00	Transmembrane protein 70, mitochondrial	TMEM70
Q53FT3	23,12	0,00	Protein Hikeshi	C11orf73
P47224	23,18	0,00	Guanine nucleotide exchange factor MSS4	RABIF
F8VYE8	23,22	0,00	Serine/threonine-protein phosphatase	PPP1CC
C9JFE4	23,24	0,00	COP9 signalosome complex subunit 1	GPS1
Q9H936	23,24	0,00	Mitochondrial glutamate carrier 1	SLC25A22
B4DRF4	23,28	0,00	Very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase 3	HACD3
P82932	23,30	0,00	28S ribosomal protein S6, mitochondrial	MRPS6
P32321	23,31	0,00	Deoxycytidylate deaminase	DCTD
P01034	23,32	0,00	Cystatin-C	CST3
B4DZ70	23,33	0,00	NADP-dependent malic enzyme	ME1
Q8N6M0	23,33	0,00	OTU domain-containing protein 6B	OTUD6B
P05976	23,35	0,00	Myosin light chain 1/3, skeletal muscle isoform	MYL1
Q9NZT2-2	23,39	0,00	Opioid growth factor receptor	OGFR
Q71UM5	23,43	0,00	40S ribosomal protein S27-like	RPS27L

Table 6.6 (continued) - Differently expressed proteins after transfection of MCF-10A cell line with pre-miR-203. Values are presented as the mean of log2(Intensity) of two independent experiments. Protein and gene nomenclature is according to UNIPROT database.

F8VRL4	23,44	0,00	Diphosphoinositol polyphosphate phosphohydrolase 2	NUDT4
P61970	23,66	0,00	Nuclear transport factor 2	NUTF2
Q96KB5	23,68	0,00	Lymphokine-activated killer T-cell-originated protein kinase	PBK
Q5TGH5	23,75	0,00	Elongation of very long chain fatty acids protein 5	ELOVL5
O14979-3	23,76	0,00	Heterogeneous nuclear ribonucleoprotein D-like	HNRNPDL
Q9Y3E5	23,76	0,00	Peptidyl-tRNA hydrolase 2, mitochondrial	PTRH2
P62837	23,77	0,00	Ubiquitin-conjugating enzyme E2 D2	UBE2D2
Q13547	23,89	0,00	Histone deacetylase 1	HDAC1
P40763-2	23,95	0,00	Signal transducer and activator of transcription 3	STAT3
Q7Z2W4	24,00	0,00	Zinc finger CCCH-type antiviral protein 1	ZC3HAV1
Q9Y4Z0	24,06	0,00	U6 snRNA-associated Sm-like protein LSm4	LSM4
Q6P1L8	24,20	0,00	39S ribosomal protein L14, mitochondrial	MRPL14
Q9P258	24,34	0,00	Protein RCC2	RCC2
Q9H6S3	24,43	0,00	Epidermal growth factor receptor kinase substrate 8-like protein 2	EPS8L2
Q96CX2	24,53	0,00	BTB/POZ domain-containing protein KCTD12	KCTD12
O00170	24,55	0,00	AH receptor-interacting protein	AIP
P50416-2	24,77	0,00	Carnitine O-palmitoyltransferase 1, liver isoform	CPT1A
P42224	24,95	0,00	Signal transducer and activator of transcription 1-alpha/beta	STAT1
O96005	25,01	0,00	Cleft lip and palate transmembrane protein 1	CLPTM1
F8W7Q4	25,04	0,00	Protein FAM162A	FAM162A
E9PL69	25,05	0,00	Ribonucleoside-diphosphate reductase large subunit	RRM1
E5RGY0	25,20	0,00	Derlin	DERL1
Q6UW68	25,34	0,00	Transmembrane protein 205	TMEM205
O75526	25,39	0,00	RNA-binding motif protein, X-linked-like-2	RBMXL2

Q14566	25,45	0,00	DNA replication licensing factor MCM6	MCM6
Q8N8S7-2	25,92	0,00	Protein enabled homolog	ENAH
Q15125	26,43	0,00	3-beta-hydroxysteroid-Delta(8),Delta(7)-isomerase	EBP
P61077	26,47	0,00	Ubiquitin-conjugating enzyme E2 D3	UBE2D3
C9JYQ9	26,53	0,00	60S ribosomal protein L22-like 1	RPL22L1
P13807-2	27,21	0,00	Glycogen [starch] synthase, muscle	GYS1
O15047	27,71	0,00	Histone-lysine N-methyltransferase SETD1A	SETD1A

7. Conclusions and future perspectives

Precision cancer medicine is more than a glimmer of hope on the horizon to become a desirable objective of cancer research. It can lead to a combination of diagnosis and treatment strategies that can be used to integrate patient and tumour variables with an outcome oriented approach. In fact, this approach can guide to personalized therapy, improve drug response and avoid the morbidity associated with treatments that could hamper the desired effect due to side effects.

Many approaches have been followed to attain precision medicine. Its lexicon embraces genomics, transcriptomics, proteomics and more recently metabolomics (Lumachi et al., 2015; Shajahan-Haq et al., 2015; Song et al., 2015; Xu et al., 2014). All these approaches aim to find a biomarker or a set of biomarkers that can help define which patients have a clinical indication for a selected therapy, whom can have a relapse that lead to a poor outcome and if a tumour has gained the capability to metastasize to a different organ.

MiRNAs have also been used in this pursuit for precision medicine, however we have much to learn about miRNAs biology. In fact, much is still unknown about miRNAs biogenesis, which includes expression regulation and maturation, mode of action and target regulation.

In order to better understand the biology of miRNAs this thesis aimed to answer the relevant questions previously stated in Chapter 2 of this thesis:

How do tumour cells deregulate miRNAs expression in order to gain a benefit?

The work performed in order to answer this question is presented in Chapter 3. We followed two different approaches, analysis of methylated CpG islands in miRNAs genes promoter regions and proteomics in non-tumoural and tumoural breast cell lines.

Many DNA methylation changes are observed between benign and tumour cells (Gyorffy et al., 2016; Pouliot et al., 2015). This mechanism can be reversible, thus conferring to the

tumour cell an adaptive phenotype depending on the microenvironment and the needs in a given condition. In fact, tumours can take advantage of this mechanism in embryo development where genes are switched on and off by epigenetic mechanisms like methylation (Bird, 2002). The discovery of miRNAs and the development of better techniques to analyse methylation statuses allowed studies comparing normal and tumour tissues and tumour cell lines to find possible biomarkers. Here we analysed a set of 20 promoter region of several miRNAs in breast cell lines. With this study we can conclude that these regions may not be the promoter regions of the miRNAs studied, since DNA methylation statuses does not correlate with the respective miRNAs expression levels. However, we cannot exclude the fact that methylation is not affecting miRNAs expression levels. Thus, a possible future approach will be to find new regions upstream in the miRNAs coding sequences but now using bisulfite sequencing a more sensitive technique of detecting methylated CpG islands.

Through proteomics analysis we also can conclude that RAN is not regulating miRNAs expression levels. Also, with this approach we cannot exclude the role of other miRNAs biogenesis machinery proteins in the regulation of it expression levels. In fact, as stated before, there are already evidences that regulatory-like proteins affect the levels of miRNAs in the cell. In order to gain deeper insights in the proteome controlled by miR-200c and miR-203 we searched through LC/MS analysis the proteome putatively controlled by those two miRNAs. If this approach could lead to a set of proteins whose expression is dependent upon the levels of miR-200c and miR-203, it still awaits further data to deeper interpret the role those proteins might have in breast cancer pathogenesis and, also what proteins may be candidates of the regulation circuitry involving miRNAs.

How do miRNAs influence tumour cells?

After analysing the possible mechanisms of miRNAs expression regulation we analysed the influence of the different expression of miRNAs in cell phenotype, mainly in the

resistance to some chemotherapeutic agents used in breast cancer. In Chapter 1 and 4 of this thesis we stated the importance of miRNAs in modulating drug resistance with several examples in many types of tumours. With our experiments we can conclude that miR-200c and miR-203 are differently expressed in breast tumour cell lines. Specifically, miR-200c and miR-203 are not expressed in MDA-MB-231 while they are in the MCF-7 cells. With these data we decided to ectopically express miRNA-200c and miR-203 in MDA-MB-231 cells and inhibit the expression of the same miRNAs in MCF-7 cells. This strategy allowed us to analyse the possible effect of these miRNAs in resistance to PAX and 5-FU. Thus, miR-200c seems to have an opposite effect in MCF-7 and MDA-MB-231 cells and miR-203 seem to increase resistance to PAX in MDA-MB-231 cell line. None of the miRNAs showed any effect in cell treated with 5-FU. To our knowledge this was the first study showing these results in breast cancer.

What is the real expression of miRNAs in patient tumour cells?

Last but not least, we analysed a set of 109 patients' tissues in order to profile the miR-200c, miR-203 and putative targets SIX1 and SOX2. With this approach we aimed to understand if these molecules could function as biomarkers to better classify cancer, which later could end up in a better therapy scheme. To our knowledge the work here presented was the first done in a Portuguese population on the role of the miR-200c, miR-203 as regulators of SIX1 and SOX2. According to our data, miR-203a could be involved in breast cancer development, mainly in early stages of development. We also observed that miR-203a might be a potential marker to discriminate stages in invasive lobular carcinoma. Regarding miR-200c we did not detect significant differences between normal breast and tumour tissues and also cannot correlate miR-200c with clinicopathological features. Further studies with larger populations are needed to validate our data regarding miRNAs expression.

Concerning the putative targets of our studied miRNAs, we could not substantiate a control of expression of the two studied proteins by the miRNAs under study. However, due to the small sized population we used in the IHC study, we cannot exclude a possible association.

Three years have passed since we started to collect our samples of FFPE from patients with breast cancer. Thus, until now we do not have sufficient data to infer if there are any patients with drug resistance. Our close collaboration with the department of Pathology and Breast Pathology Unit from Central Lisbon Hospital Centre will allow us in the future to correlate our miRNAs expression levels and putative targets with therapy and possibly associate them with a specific outcome, ending in a better prognostic.

What other proteins may be controlled or dependent on miR-200c and miR-203?

In order to gain insight into this question and more thoroughly clarify the role of miR-200c and miR-203 in the proteomic profile of cells, we analysed the role of ectopically expression of miRNA-200c and miR-203 in MDA-MB-231 cells and inhibition of the expression of the same miRNAs in MCF-10A cells and the respective effect in the proteomic profile of those cells under miRNAs control within a close collaboration with Professor Peter James of Lund University. The data allowed the identification of a set of proteins differentially expressed under control of any of the miRNAs. Although this is still a first approach, it suggests that a number of these proteins are involved in breast cancer and may potentially be used for earlier detection of cancer relapse and as putative biomarkers of prognosis.

Future Perspectives/Work

We believe that this work adds more knowledge in the pursuit of the personalized medicine. In fact, as future perspectives we aim to follow several “omics” and validate the current diagnostic methodology. Currently, breast cancer diagnosis relies, apart from clinical examination, mostly on imaging techniques such as mammography and local

biopsy in order to validate imaging results. Although breast cancer can be detected earlier, there is some concern about the dose of radiation necessary to perform a mammography. Moreover, some breast cancers are not found by mammograms, either because the test was not done or because even under ideal conditions mammograms do not detect every breast cancer. Although breast cancers found during screening exams are more likely to be smaller and still confined to the breast, the size of a breast cancer and how far it has spread are some of the most important factors in predicting the prognosis of a woman with this disease. Early detection of breast cancer improves the chances that breast cancer can be treated successfully. Thus, early detection tests for breast cancer help save thousands of lives each year, and many more lives could be saved if even more women and their health care providers took advantage of these tests, provided they are minimally invasive, low cost, usable in primary care settings, and sensitive to early, curable, clinically significant lesions. Within this framework, the new medical and scientific paradigm of precision medicine is claiming new minimally invasive methods which may reliably help in a personalized diagnosis of cancer and allow an early detection of recurrence of the disease guiding in the therapeutic schemes to be adopted. Recent technology development allows the identification of molecules in blood shed from tumours. These molecules include metabolites of sugars, lipids, amino acids, fatty acids, phenolic compounds, alkaloids, DNA and RNA. In spite of the great efforts worldwide and the elucidating studies in this matter, to our knowledge, there are no studies validating medical imaging and biopsy analysis with metabolomics and liquid biopsies. Thus, in the future we propose a comprehensive study with women undergoing medical imaging, with or without tumour detection, of metabolites shed from primary tumour cells, ctDNA, circulating tumour cells (CTC's) and exosomes (containing microRNAs, mRNAS and proteins shed from primary tumour cells) in blood samples, in order to validate the imaging and biopsy diagnosis (Figure 7.1). If successful we shall develop a minimally-invasive methodology to detect breast cancer early and with more precision. We also aim to follow longitudinally the

same patients after surgery, performing the same biomedical analysis in order to compare and validate our data with pathology-driven IHC classification and follow-up medical imaging, thus gaining insight on the prognosis of disease relapse and metastization of the primary tumour to other organs. Concomitantly we intend to continue the analyses of tumour and adjacent normal tissue of the same patients in order to find new biomarkers that aid in the classification of the tumour.

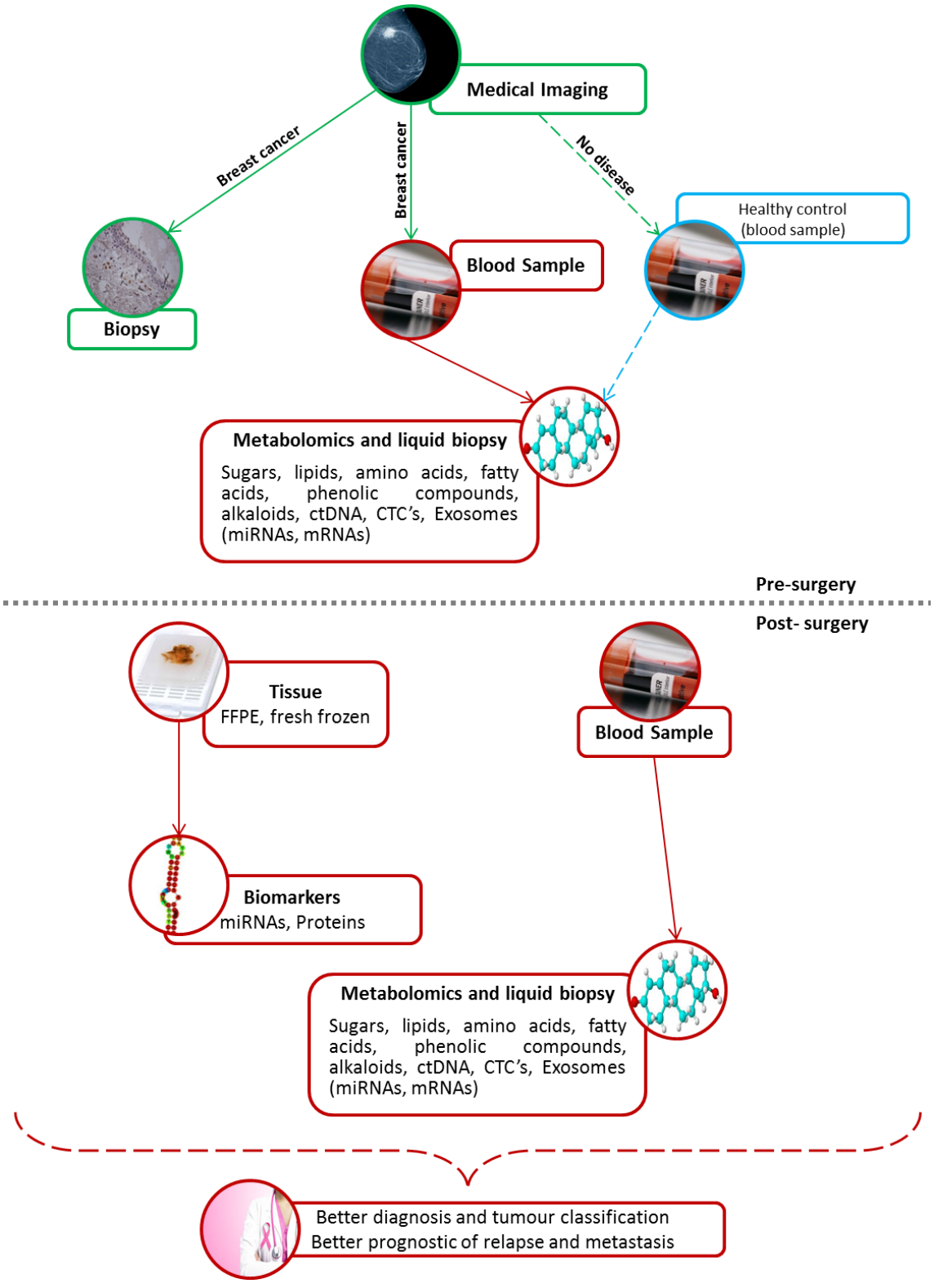

Figure 7.1 – Future approach in order to achieve a better diagnosis, tumour classification and prognostic of relapse and metastasis in patients with breast cancer.

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