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Analysis of MiRNA Expression and Biomarker Potential in Common Diseases: Breast Cancer and Obesity

A thesis submitted to the National University of Ireland Galway for the degree of Doctor of Philosophy in the School of Medicine

By

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This thesis is dedicated to my father Martin
and to the memory of my mother Mary.

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Helen Heneghan

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Abbreviations

A	adenine
bp	base pair(s)
C	cytosine
Ca^{++}	calcium ions
cDNA	complementary DNA
DCIS	ductal carcinoma *in situ*
ddH_2O	ultra-pure water
ddNTP	dideoxyribonucleotide(s)
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide(s)
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ER	oestrogen receptor
g	gram
G	guanine
HER2	Human Epidermal growth factor Receptor 2 (ErbB-2)
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
M	molar
mg	milligram
mM	millimolar
NaOH	sodium hydroxide
nm	nanometers
PCR	polymerase chain reaction
PR	progesterone receptor
RT PCR	reverse transcriptase PCR
RQ-PCR	relative quantification PCR
RNA	ribonucleic acid
SNP	single nucleotide polymorphism
T	thymine
TE	tris-EDTA buffer
TNM	Tumour, Node, Metastasis staging system
U	unit(s)
^{o}C	degrees centrigrade
μg	microgram
μL	microlitre
μM	micromolar

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Abstract

The two greatest health concerns worldwide at present are cancer and obesity; both of which continue to rise in incidence and are associated with high morbidity and mortality. Understanding the aetiology and mechanisms of these diseases is critical in order to develop clear and effective strategies for improving global health. Mi(cro)RNAs are a recently discovered class of short, non-coding, endogenous RNA molecules, only 18-25 nucleotides long. These small, ubiquitous molecules have been shown to play critical regulatory roles in a wide range of cellular processes. Aberrant miRNA expression has been observed in various pathological events including carcinogenesis, and in the aetiology of obesity and metabolic disorders. Furthermore, miRNAs have emerged as an exciting new class of disease biomarkers. The purpose of this study was to investigate the expression and dysregulation of miRNAs in two common diseases; breast cancer and obesity, with particular emphasis on exploring the potential of miRNAs as novel noninvasive biomarkers.

An early goal of this study was to define a protocol for optimal analysis of miRNA expression in human blood samples. This was achieved through RQ-PCR quantification of specific miRNA sequences, in total RNA isolated from whole blood using a modified copurification technique. Using this approach, it was established that cancer-specific miRNAs were dysregulated in the circulation of breast cancer patients compared to controls. Specifically, *miR-195* and *let-7a* were significantly increased in breast cancer patients (19.25 and 11.20 fold respectively), and levels correlated with tumour miRNA expression, tumour burden and other clinicopathological variables including hormone receptor and lymph node status. In addition, elevated systemic *miR-195* was observed to be specific to breast cancer patients. In combination with *let-7a* and *miR-155*, this panel of 3 circulating miRNAs could discriminate breast cancers from controls with a remarkably high sensitivity of 94%. A further novel finding from this work was the discovery that an inherited variation in the *let-7* binding site in the *KRAS* oncogene conferred increased susceptibility to breast cancer, particularly the 'triple negative' subtype, in premenopausal women (OR 4.78, CI 1.71 – 13.38, p=0.015). Finally, results from this work demonstrate that metabolic miRNAs are dysregulated in obese adipose tissue and also have potential to serve as novel non-invasive biomarkers for obesity and related metabolic conditions.

Although elucidating their mechanisms of action is still in its infancy, the discovery of miRNAs has uncovered a new and exciting repertoire of molecular factors upstream of gene expression, with great potential for new developments in current diagnostic and therapeutic strategies in the management of common diseases.

If the current momentum in miRNA translational research can be maintained, this has the potential to transform current practice to the ideal of individualized care for patients in the near future.

Chapter 1

Introduction

1. Introduction

1.1 Global burden of disease

Perhaps the two greatest health concerns worldwide at present are cancer and obesity; both of which are continuously rising in incidence and are associated with high morbidity and mortality. Understanding the aetiology and mechanisms of these diseases is critical for developing clear and effective strategies for improving global health [1].

As the second commonest cause of death in the developed world (after heart disease), cancer presents an epic health problem, with over 12 million new cases per annum worldwide and a predicted 27 million annual diagnoses expected by the year 2050 [2]. Amongst women, breast cancer is the commonest malignancy and the leading cause of death in the developed world.

Obesity and the metabolic syndrome are another major public health concern. The incidence of this disease spectrum is escalating rapidly, and contributes significantly to global morbidity, mortality and socioeconomic burden.

Although they are individual disease entities, associations between obesity and cancer are emerging. In particular the relationship between obesity and breast cancer in post-menopausal women is well established [3]. However the mechanism by which excess adiposity contributes to carcinogenesis remains poorly understood. Scientists and clinicians must focus on improving understanding of the molecular mechanisms underpinning major diseases such as cancer and obesity, in order to develop novel diagnostic and therapeutic strategies and thus contribute to lowering the global burden of disease significantly.

1.2 Breast Cancer

Breast cancer is now the commonest female malignancy in almost all of Europe and North America. Each year more than 1.3 million women are diagnosed with breast cancer worldwide and approximately 465,000 die from the disease [2] despite the fact that breast cancer is highly curable if diagnosed and treated appropriately at an early stage. In Ireland alone, the annual incidence is currently over 2300 and rising [4]. This increasing incidence of breast cancer will result in an unprecedented socioeconomic

burden. Consequently, there is a critical need to identify and develop more sophisticated and patient-specific means of diagnosing and treating the disease, in order to minimise its associated morbidity and mortality.

1.2.1 Breast cancer pathology

More than 95% of breast cancers arise from the breast epithelial elements. However the term "breast carcinoma" encompasses a diverse group of lesions which differ in microscopic appearance and biologic behaviour, although these disorders are often discussed and managed as a single disease. Breast carcinomas can be divided into two major groups:

- *In situ* carcinomas — the tumour cells remain confined to the ducts or lobules and show no evidence of microscopic invasion into the surrounding breast stroma. There are two types of *in-situ* carcinoma; ductal and lobular, named according to the predominant cell type from which the tumour arises.

- Invasive carcinomas — the tumour cells invade the breast stroma and have the potential to metastasise to distant sites. The invasive breast carcinomas consist of several histological subtypes; the commonest being infiltrating ductal adenocarcinoma (75-80%), followed in frequency by invasive lobular (10-15%), Mixed ductal-lobular (<5%), Inflammatory (2-3%), Colloid (2-3%), Tubular (<2%), Medullary (<2%), and Papillary (1%) [5]. Rarer subtypes, including metaplastic breast cancer and invasive micropapillary breast cancer, all account for less than 5 percent of cases overall.

Invasive breast cancer metastasises via local invasion to chest wall or skin, lymphatic infiltration to axillary nodes most commonly (Figure 1.1), or haematogenous spread to distant sites including bone, liver, lung and brain.
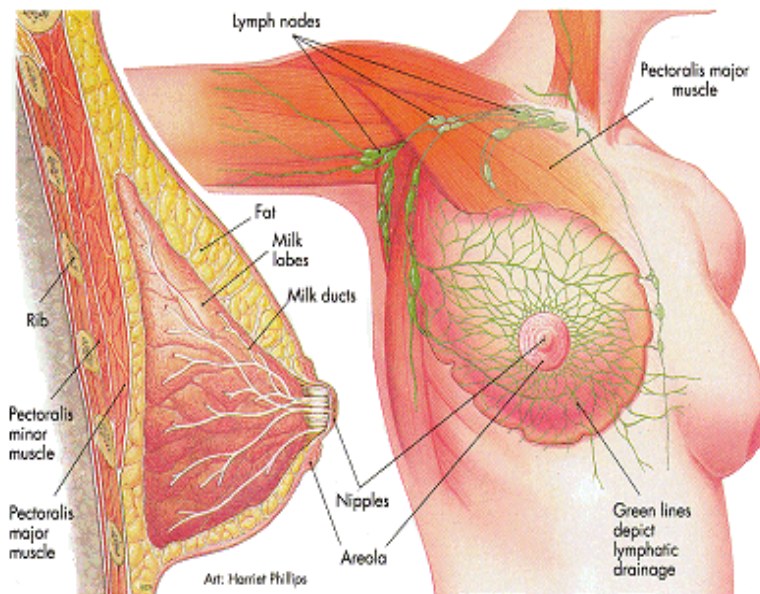
Figure 1.1 Lymphatic drainage of the female breast [1]

1.2.2   Breast tumour classification

The current methods of classifying breast cancer patients into prognostic groups, which influences therapeutic decisions, are based on clinicopathological parameters such as size and grade of the tumour, presence or absence of lymph node metastases, and distant metastases. The gold standard method in routine use is the TNM staging system [6] (Table 1.1). One of the most useful indices of prognosis in breast cancer is the Nottingham Prognostic Index (NPI) [7], which can be used to select patients for adjuvant treatment and is simply derived in the clinic from 3 proven prognostic parameters; tumour size, tumour grade and lymph node status, as follows:

NPI = [0.2 x tumour size (cm)] + tumour grade [1-3] + lymph node stage [1-3]

……...where:

0 nodes positive   =   1

1-3 nodes positive   =   2

>3 nodes positive   =   3

The interpretation of the NPI is illustrated in Table 1.2.

Table 1.1 TNM Classification for Breast Cancer		
Stage	**T, N, M staging**	**Prognosis (5 yr overall survival)**
Stage 0	Tis, N0, M0	93%
Stage I	T1, N0, M0	88%
Stage IIa	T0 or T1, N1, M0 or T2, N0, M0	74-81%
Stage IIb	T2, N1, M0 or T3, N0, M0	
Stage IIIa	T0-T2, N2, M0 or T3, N1 or N2, M0	
Stage IIIb	T4, N0-N2, M0	41-67%
Stage IIIc	T, N3, M0	
Stage IV	any T, any N, M1	15%

TNM Classification for Breast Cancer from the *AJCC Cancer Staging Manual*, 7th Edition.
Survival data from US National Cancer Database and American Cancer Society
www.cancer.org/Cancer/BreastCancer

T grading

TX: Primary tumour cannot be assessed

T0: No evidence of primary tumour

Tis: Carcinoma *in situ* eg. DCIS, LCIS, and Paget's disease

T1: Tumour ≤ 2 cm

T2: Tumour > 2 cm, ≤ 5 cm

T3: Tumour > 5 cm

T4: Tumour of any size with direct extension to chest wall or skin

N Grading

NX: Regional lymph nodes cannot be assessed (eg. removed previously)

N0: No regional lymph node metastases

N1: Metastases in 1-3 movable ipsilateral axillary lymph node(s)

N2: Metastases in 4-9 ipsilateral axillary nodes, fixed or matted axillary nodes, or metastases in ipsilateral internal mammary nodes

N3: Metastasis in 10 or more axillary lymph nodes, or in infraclavicular lymph nodes, or in clinically apparent ipsilateral internal mammary nodes in the presence of one or more positive axillary lymph nodes; or in more than three axillary nodes with clinically negative microscopic metastasis in internal mammary lymph nodes; or in ipsilateral supraclavicular lymph nodes

M grading

MX: Presence of distant spread (metastasis) cannot be assessed.

M0: No distant metastases

M1: Distant metastases present (sites commonly include bone, lung, brain, liver.)

Table 1.2 Interpretation of the Nottingham Prognostic index (NPI)			
NPI score	**Prognosis**	**5 year overall survival**	**Interpretation: Need for adjuvant chemotherapy**
≥2.0 to ≤2.4	Excellent	93%	Chemotherapy not indicated
>2.4 to ≤3.4	Good	85%	Need is doubtful
>3.4 to ≤5.4	Moderate	70%	May benefit from chemotherapy
>5.4	Poor	50%	Chemotherapy indicated

Although well established as prognostic and predictive markers, these histopathological indices have limitations. Their accuracy is imperfect, and their derivation is only possible following complete histopathological evaluation of the resected tumour and axillary specimens. Furthermore, the NPI specifically has unproven applicability in the era of screening-detected lesions, and in predicting behaviour of tumours less than 1.0 cm. No clinicopathological prognostic model has yet succeeded in fully capturing the diverse clinical course of breast cancer. Furthermore, these variables are not helpful in selecting adjuvant therapy regimes specifically tailored for an individual patient. The need for improved prognostic and predictive markers for breast cancer is obvious.

1.2.3   Breast tumour markers

To date, only three markers are established in the routine evaluation of breast tumours: oestrogen and progesterone receptors (ER/PR: for predicting response to endocrine therapies) and HER2/*neu* (for predicting response to Trastuzumab) [8]. Although these markers are assessed routinely, ER/PR and HER2/*neu* assessment is far from perfect [9].The most commonly employed technique at present, to evaluate the hormone receptor status of breast tumours, is immunohistochemistry (IHC) which relies on recognition of the receptor protein by specific antibodies. Although technically easy to perform and cost effective, this method is subjective and time consuming. More sophisticated methods are employed for HER2/*neu* assessment when its evaluation by IHC is equivocal. These include fluorescence *in-situ* hybridisation (FISH) which assesses for amplification of the HER2 gene, chromogenic *in-situ* hybridisation (CISH), and silver enhanced *in-situ* hybridisation

(SISH). Whilst enabling improved reliability and sensitivity compared to IHC, these methods are also more laborious and expensive to perform [10].

A number of circulating tumour markers (e.g., carcinoembryonic antigen [CEA] and carbohydrate antigen 15-3 [CA 15-3]) are widely used in the management of breast cancer, but the sensitivity of these markers is low [11-13], and so they are not useful as screening tools although they have long been in clinical use as prognostic markers and to monitor for disease progression or recurrence.

The ideal biomarker should be easily accessible such that it can be sampled relatively non-invasively, sensitive enough to detect early presence of tumours in almost all patients and absent or minimal in healthy tumour free individuals. The search for this 'holy grail' has been the focus of clinician scientists for many years. The advent of molecular profiling over the last two decades has opened up new opportunities in this field. Dedicated scientific research into the molecular mechanisms underlying breast cancer has identified novel molecular markers which may serve as potentially useful biomarkers, which could be useful for early diagnosis, prognostication and targeted therapy in breast cancer.

1.3 Molecular profiling of breast cancer

Over the last two decades there has been an increasing awareness that breast cancer is not a single disease, but rather a complex phenotypically diverse genetic disease, involving a variety of changes in gene expression and structure. The different clinical course and outcome of patients with clinically and pathologically similar tumours is now thought to be due to these genetic and molecular differences between cancers. Recent advances in molecular profiling technology have made great progress in unravelling the molecular taxonomy of breast cancer, which has shed new light on the aetiology of the disease and also heralded great potential for the development of novel biomarkers and therapeutic targets.

1.3.1 Gene expression profiling in breast cancer

Gene expression profiling is the measurement of the activity of thousands of genes at once, to create a global picture of cellular function. Using modern molecular profiling tools it is possible to measure this activity by counting the number of mRNA transcripts, which provides an estimate of the number of corresponding

proteins. High-throughput technologies, such as DNA microarray, serial analysis of gene expression (SAGE) and real-time reverse transcriptase polymerase chain reaction (RT-PCR) allow simultaneous counting of many gene transcriptions (up to tens of thousands) thus providing us with a 'snapshot' of a tissues global gene activity. This has facilitated a comprehensive analysis of transcriptional variation at the genomic level, resulting in vastly improved understanding of the molecular biology of diseases such as breast cancer. Indeed gene expression profiling of breast cancer has led to the development of new biological concepts, refined tumour classification, improve diagnostic and prognostic accuracy, and the identification of new therapeutic targets.

1.3.2 Epithelial subtypes of breast cancer

Pioneering work by Sorlie *et al* [14-16] identified molecular portraits of breast tumours based on microarray-generated gene expression signatures; tumours were classified according to their expression of a 496-gene 'intrinsic' gene subset. Five main subsets, or intrinsic subtypes, were identified which stratified breast cancers according to their ER, PR and HER2/*neu* receptor status. Subtypes were designated *Luminal A*, which strongly expressed ER and/or PR, but were HER2/*neu* negative; *Luminal B*, which were ER, PR and HER2/*neu* (triple) positive; *Basal* tumours which were negative for ER, PR and HER2/*neu* (i.e. 'triple negative'); and a *HER2* subset which was ER and PR negative but had high expression of several genes in the HER2/*neu* amplicon, including *HER2* and *GRB7*. Survival analyses showed significantly different outcome for patients depending on their tumour subtype, emphasising the clinical relevance of stratification by such molecular profiling (Figure 1.2). In this setting, gene expression profiling has been critical in demonstrating the extent of molecular differences between the various subtypes of breast tumours.
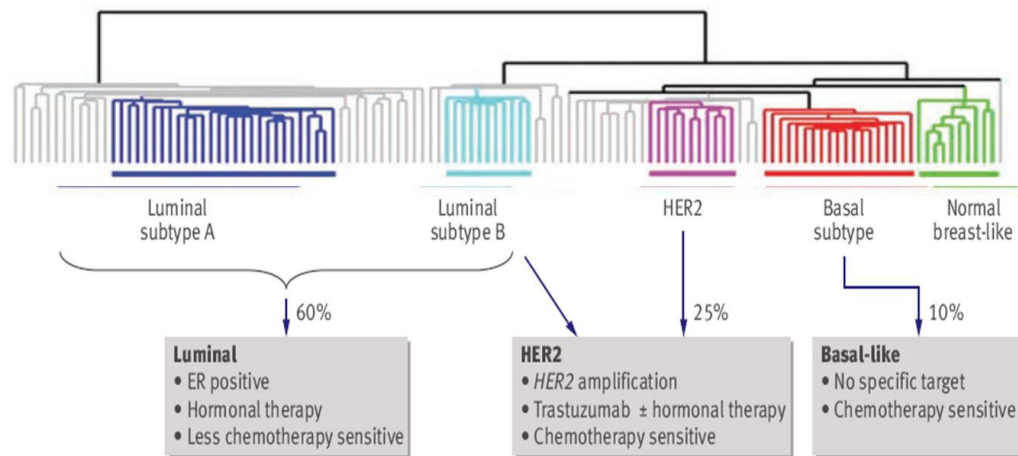
Proc Natl Acad Sci U.S.A. 2001;98(19): 10869-10874.

Figure 1.2 Intrinsic breast cancer subtypes as defined by Sorlie *et al.*

This method of classifying breast tumours into subtypes correlated well with a tumours hormone receptor status, and therefore has important prognostic and therapeutic implications.

1.3.3 Application of gene expression analysis for breast cancer prognostication

Translating laboratory research to clinical practice is a challenging process in any field of research. However molecular profiling technologies lend themselves very well to such applications. Clinical practice has readily adopted the concept of intrinsic subtypes and routinely, patients are stratified according to their ER, PR and HER2/*neu* status to improve diagnostic and prognostic accuracy, and better identify patients who will more likely respond to adjuvant therapies. Onco*type* DX ® [2] (Genomic Health Inc.) is a commercially available gene expression assay that represents the most successful introduction of molecular profiling technology into clinical application to date. This assay utilises a 21 gene profile (16 discriminator and 5 reference genes), detected by RQ-PCR in formalin-fixed paraffin embedded breast tumour sections from standard histopathology blocks. Developed by Paik *et al* [17], this assay is used to generate a recurrence score for each patient, by differentially weighting the constituent genes' expression. This score then accurately predicts 10-year breast cancer recurrence, as well as the clinical benefit of chemotherapy [18].

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[2] http://www.genomichealth.com/OncotypeDX/

Another multigene profile developed by van't Veer *et al*, which relies on a 70-gene prognostic signature, has also been commercially developed and markets as 'MammaPrint™ [3]'. In contrast to Onco*type* DX [®], this assay employs DNA microarray technology and requires fresh unfixed tumour tissue for assessment. Interestingly there is only one gene in common between Onco*type* DX [®] and MammaPrint™. Despite the immense popularity of these prognostic assays in clinical practice of late, further definitive proof of their clinical value is required. This will emerge as results from prospective studies evaluating their value appear.

The prospective evaluation of Onco*type* DX [®] has been ongoing in the TAILORx trial (**T**rial **A**ssigning **I**ndividua**L**ized **O**ptions for Treatment (**Rx**)) since May 2006. Almost 10,000 women with ER/PR positive, HER2/*neu* negative and node negative breast cancer have been enrolled at 900 sites in the United States, Canada and Europe. This trial is designed to determine whether adjuvant hormonal therapy alone is as effective as adjuvant hormonal therapy in combination with chemotherapy for certain women with early-stage breast cancer and it uses the recurrence score from Onco*type* DX [®] as a tool to randomise patients to the individual treatment options. The prospective evaluation of MammaPrint™ is underway in the MINDACT trial (**M**icroarray **I**n **N**ode-negative **D**isease may **A**void **C**hemotherapy **T**rial), under the guise of EORTC. This prospective, randomised study compares the 70-gene expression signature with common clinicopathological variables in selecting patients for adjuvant chemotherapy in node-negative breast cancer. Its primary objective is to assess whether 10-15% of 'low-risk' breast cancer patients can be spared form adjuvant chemotherapy without negatively affecting their distant metastases free survival, on the basis of a risk score obtained with the MammaPrint™ microarray [19].

These novel prognostic tests have several limitations which must be acknowledged [20]. Their assessment requires tumour sampling; the time lag from tumour biopsy until result is in excess of four weeks; and they are expensive, with a single test costing in excess of $3000 dollars on average. Furthermore the technologies employed in these tests, particularly the microarray approach used in MammaPrint™, are difficult to translate into routine clinical practice and the role or biological significance of many

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[3] http://www.agendia.com/pages/mammaprint/

of the genes included in these tests is not completely understood. Although conclusive evidence supporting the benefit of these molecular assays in guiding clinical decisions for breast cancer patients is pending, and they are not without limitations, their development has exposed an exciting new avenue for translational research with potentially important clinical implications.

1.4 Metabolic disease and obesity

Obesity and the metabolic syndrome are major public health concerns, and present a formidable management challenge. At present the World Health Organization estimates that 400 million adults are affected by obesity globally, as well as a significant number of children and adolescents[21]. The incidence of this disease spectrum continues to rise and contributes significantly to global morbidity, mortality and socioeconomic burden. Obesity is defined as a body mass index (BMI) of greater than 30 kg/m^2. The higher the BMI, the greater the severity of the disease, as indicated in Table 1.3.

Table 1.3 Body Mass Index and the categories of obesity	
BMI	**Classification**
< 18.5	Underweight
18.5–24.9	Normal weight
25.0–29.9	Overweight
30.0–34.9	Class I obesity
35.0–39.9	Class II obesity
≥ 40.0	class III obesity

The fundamental cause of obesity is an energy imbalance between calories consumed versus calories expended, and unquestionably the biggest contributing factors to its development are diet and lifestyle issues. However there is a growing body of evidence to suggest that aberrant genetic expression may play a significant predisposing and causative role in its pathogenesis [22-24]. The metabolic syndrome is defined as a state of metabolic dysregulation characterised by insulin resistance, inflammation, and a predisposition to type 2 diabetes mellitus, dyslipidemia, premature atherosclerosis, and other disorders [25]. Obesity, in particular central obesity, and physical inactivity are contributing factors, as are incompletely understood genetic determinants. At a molecular level, abnormalities in cellular lipid metabolism, as reflected by increases in intracellular triglycerides, are thought to be an early event [26-27]. The importance of the metabolic syndrome stems from the fact it precedes and probably largely contributes to the pathogenesis of the many disorders with which it is associated (i.e. diabetes mellitus type 2, atherosclerotic heart disease, cerebrovascular and peripheral vascular disease, amongst many others).

Current treatment modalities for obesity and the metabolic syndrome include lifestyle modification, diet and pharmacologic agents yet many patients remain recalcitrant to conventional medical therapy. Bariatric surgery has made laudable progress in the treatment of obesity and its related metabolic disorders, yet carries inherent risks. Appropriate patient selection for operative intervention is critical in order to minimize the surgical risks and achieve optimal outcomes. Weight loss, the metabolic response, and resolution of co-morbidities after intervention for morbid obesity have been reported to vary substantially between patients. There is no reliable clinical parameter or biomarker which currently predicts outcome after surgery and the absence of such a valuable tool is notable [28]. Investigation of the molecular mechanisms and genetic abnormalities underpinning metabolic disorders and obesity may identify new pathways involved in complex metabolic disease processes, improve our understanding of metabolic disorders and influence future approaches to the treatment of obesity.

1.5 Introduction to miRNAs

Mi(cro)RNAs are a recently discovered class of short, non-coding, endogenous RNA molecules, only 18-25 nucleotides long. Since their identification in 1993, these small and highly abundant molecules have been shown to play critical regulatory roles in a wide range of biological and pathological processes [29-30]. Elucidating their mechanisms of action is still in its infancy. Nonetheless, work in this area to date has demonstrated that miRNAs may regulate cellular gene expression at the transcriptional or post-transcriptional level; by suppressing translation of protein coding genes, or cleaving target mRNAs to induce their degradation, through imperfect pairing with target mRNAs of protein coding genes [31].

The interest in and understanding of miRNA-directed gene regulation has increased exponentially over the last five years in particular. This is reflected in the rapid expansion of literature describing novel miRNA genes, their regulatory targets and functions. At the time of writing, 10,581 mature miRNA sequences have been described in primates, rodents, birds, fish, worms, flies, plants, and viruses [324]; miRBase version 15, updated April 2010). This represents a growth of over 200 miRNAs in the last 2 years. In the human genome, over 940 mature miRNAs have been reported to date; however, computational prediction estimates that this could increase to >1,000 [33]. It is obvious that the miRNA story is in rapid evolution.

1.5.1 MiRNA biogenesis

MiRNA biogenesis in the human cell is a multistep complex process which begins in the nucleus where miRNA genes are transcribed by RNA polymerase II to form large capped and polyadenylated primary miRNA transcripts (pri-miRNAs). Pri-miRNA transcription occurs from distinct genomic locations; many are intergenic with independent promoters, others are clustered in polycistronic transcripts. Certain pri-miRNAs (approximately 50%) are located within introns of host genes suggesting that transcriptional regulation of miRNA biogenesis may be under host-gene promoter control in some instances [31,34]. These nascent pri-miRNAs are cleaved by the RNase III enzyme Drosha, coupled with its binding partner DGCR8, into 70-90 nucleotide precursors (pre-miRNA) which consist of an imperfect stem-loop

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[4] http://www.mirbase.org/

hairpin structure. Pre-miRNAs are then exported from the nucleus into the cytoplasm by Exportin 5. In the cytoplasm, the hairpin precursors are cleaved by another RNase III endonuclease, Dicer, and its binding partner the transactivator RNA-binding protein TRBP, into a small transient dsRNA duplex (miRNA:miRNA*) that contains both the mature miRNA strand (22 nucleotides in length) and its complementary sequence. This mature miRNA strand is preferentially incorporated into a miRNA-associated RNA-induced silencing complex (miRISC), containing Argonaute protein (Ago2). The other complementary strand is degraded. The mature miRNA strand guides its associated RISC to target mRNAs containing complementary sequences to the mature miRNA. A simplified representation is shown in Figure 1.3 [35].
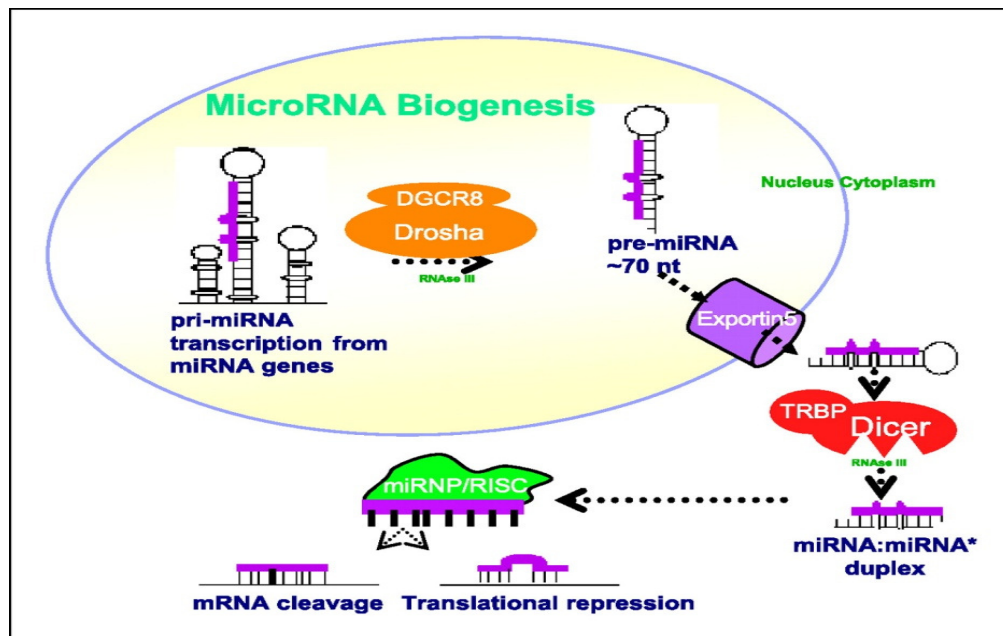
Figure 1.3 Schematic diagram illustrating miRNA biogenesis and processing in human cells

1.5.2 Function of miRNAs

MiRNAs have been demonstrated to exhibit high evolutionary conservation over a wide range of species although there is diversity in their expression profiles in different cell types and at different developmental stages, suggesting important regulatory functions [36]. MiRNAs exert their functionality via sequence-specific regulation of post-transcriptional gene expression, by targeting mRNAs for cleavage

or translational repression. The vast majority of animal miRNAs bind to the 3'UTR of target mRNA transcripts. Binding of miRNAs to the coding or 5'UTR of mRNA genes has also been demonstrated to be functional when it occurs [37-38]. The specific region of a miRNA sequence of critical importance for mRNA target recognition is located in the 5′-end of the mature miRNA strand, from bases 2 to 8, often referred to as the "seed-sequence" [31]. Governance of gene expression and protein translation by these non-coding RNA molecules occurs largely through one of two mechanisms, dependent upon the complementarity of the miRNA seed sequence with its target mRNA. Firstly, binding of miRNA to protein-coding mRNA sequences with perfect base-pairing homology induces the RNA-mediated interference (RNAi) pathway leading to cleavage of mRNA by Argonaute in the RISC. The alternative and more common mechanism by which miRNAs regulate their target genes, is through imperfect binding to partially complementary sequences in the 3' untranslated region (UTR) of downstream target coding mRNAs which leads to repression of protein translation, and subsequent reduction of steady state protein levels of targeted genes [30]. Thus miRNAs can reduce protein levels of their target genes without significantly altering the mRNA levels, consistent with translational control. However evidence suggests that miRNAs may also function before or after the initiation of translation [39-40] and so it appears that the precise mechanism of translational repression remains only partially understood. Associated reduction in mRNA abundance has also been reported. However it is unclear whether this is related to translational repression or is a consequence of a separate miRNA regulation mechanism; translationally silenced mRNAs may be sequestered in cytoplasmic P-bodies containing mRNA degradation enzymes [41]. Furthermore, de-adenylation and decapping of targeted mRNAs can occur independently of translational repression [42]. Similar to how small interfering RNAs (siRNAs) play a role in transcriptional gene silencing via epigenetic changes [43], miRNAs also have the potential to affect epigenetic mechanisms including methylation and histone deacetylation [44].

Elucidating miRNA targets and thus deepening our understanding of their functionality has proved challenging, due largely to the imperfect nature of the interaction between a miRNA and its target mRNA. Computational target prediction algorithms have been developed to identify putative miRNA targets. These

algorithms place considerable importance on the afore-mentioned miRNA 'seed-sequence', using it to search for complementary sequences in the 3′-UTRs of known genes that exhibit conservation across species. Such algorithms have predicted that each miRNA may potentially bind to as many as 200 targets, and it is estimated that miRNAs control the expression of over 30% of human protein coding genes [45]. However these computational methods of miRNA target prediction are limited; firstly as mentioned not all miRNAs target the 3′-UTR [37-38]; secondly some biological processes mediated by the miRNA-target interaction may be specific to humans and so the use of conservation across species as a limiting criterion may result in biologically significant targets being missed [46]. For these reasons, additional methods of target prediction and verification must be developed. The use of experimental techniques and functional analysis to manipulate miRNA expression in vitro, and observe the consequent downstream effects on gene expression, will be critical to validate the predictions of computer-generated targets.

1.5.3 Experimental techniques for miRNA analysis

The explosion of interest in miRNAs over the past few years necessitates effective tools for detecting their presence, quantification, and functional analysis. Isolation of miRNAs from specimens required modification of existing RNA extraction protocols, to take into account their tiny size and unique structure. Column based approaches were adapted to selectively capture and retain both the large and small RNA fractions e.g. using Qiagen RNeasy kits [5]. Copurification methods have also been developed to isolate total RNA, inclusive of the small RNA fraction. MiRNA expression profiling has been facilitated by the advent of high-throughput profiling techniques such as miRNA microarrays and bead-based miRNA profiling. These methods are far superior to existing low through-put techniques, such as Northern blotting and cloning.

1.5.3.1 Microarray

Microarray technology has also advanced to facilitate miRNA expression profiling. Labelling and probe design have improved to address the poor specificity initially observed when array technology could not distinguish between signals from pre-miRNA, pri-miR and mature miRNA sequences. Castoldi *et al* described a novel

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[5] http://www1.qiagen.com/

miRNA microarray platform using locked nucleic acid–modified capture probes [47-48]. Locked nucleic acid modification improved probe thermostability and increased specificity, thus enabling miRNAs with single nucleotide differences to be discriminated—an important consideration as sequence-related family members may be involved in different physiologic functions [49]. An alternative high-throughput miRNA profiling technique is the bead-based flow cytometric approach developed by Lu *et al* [50] ; a method which offers high specificity for closely related miRNAs because hybridisation occurs in solution. The high-throughput capability of array-based platforms make them an attractive option for miRNA studies compared with lower throughput techniques such as Northern blotting and cloning, which remain essential for the validation of microarray data.

1.5.3.2 Northern Blotting

Northern blotting, whereby RNA is fractionated on polyacramide gels, has traditionally been used for mRNA expression analysis. These techniques have also been applied to examine the expression of miRNAs, although they are severely limited in this application due to poor sensitivity, low-throughput and the need for large quantities of miRNA for assessment. Due to its reliance on hybridisation, northern blotting is severely limited in distinguishing between miRNAs that have only 1-2 nucleotide differences in their sequences. Efforts to improve this technique for the purpose of applying it more efficiently to miRNA analysis have been attempted. Valoczi *et al* reported improved sensitivity and reduced miRNA requirement when they modified the technique with partial substitution of DNA oligonucleotides by locked nucleic acid [51]. Despite some improvement, northern blotting is largely redundant now for miRNA expression profiling, as microarray profiling methods have advanced and address many of the limitations incurred by northern blots. The most common indication currently for northern blotting in miRNA analysis is to validate candidate miRNAs which have been identified by array-based expression profiles.

1.5.3.3 Next generation sequencing techniques

One of the limitations of microarray expression profiling is the requirement of prior sequence information to be used for probe design. Until recently, this sequence information has been limited mostly to that found in public databases (e.g.

miRBase). These data have been gathered mainly through a combination of bioinformatics, and extensive cloning experiments. In contrast, deep sequencing is not dependent on any prior sequence information. Instead it provides unbiased information about all RNA species in a given sample, thus allowing for discovery of novel miRNAs or other types of small RNAs that have eluded previous cloning and standard sequencing efforts. Next generation sequencing utilises massively parallel sequencing, generating millions of small RNA sequence reads from a sample [52]. This provides an excellent tool for those studying species where limited sequence information is currently available. Additionally, new sequence information generated using these techniques can be used to design improved microarray platforms for future large scale expression studies [53]. Currently available deep sequencing technologies include the Roche 454 and Illumina's Solexa[6] platforms. Roche 454's platform[7] utilises emulsion PCR for template amplification, and pyrosequencing technology on a high well-density picotiter plate. Illumina's Solexa platform uses bridge amplification on glass surface for template preparation and reverse terminator technology for sequencing. Both platforms provide high throughput and high quality sequencing production at low cost. In conjunction with the evolution of next generation sequencing technologies, which generate massive amounts of data, bioinformatic tools have had to evolve in concert. Several bioinformatics analysis programmes have been developed specifically to interpret and interrogate deep sequencing data. Examples include miRDeep, deepBase, miRExpress, and miRanalyzer [54-57]. As these highly sophisticated techniques continue to develop, the extent and significance of miRNA regulation of gene expression will become even more evident.

1.5.3.4 Real time Quantitative PCR

Quantitative real-time PCR (RQ-PCR) methodologies are considered the gold standard for quantification of nucleic acid levels [58] and have been widely applied to miRNA investigations. This technique is based on the quantitative relationship between the amount of starting target sample and the amount of PCR product at any given cycle number. The RQ-PCR process involves a reverse transcription (RT) reaction to convert isolated RNA into complementary DNA (cDNA), amplification

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[6] http://www.illumina.com/
[7] http://www.454.com/

of the cDNA using PCR, and quantification of the amplicons in real-time. Whilst the steps involved in RQ-PCR have largely remained constant since its conception [59-60], the technology has evolved to become increasingly sensitive, specific and versatile. The short length of mature miRNAs posed difficulties initially, but with the design of effective primers and probes with adequate specificity, this was overcome. Random primers and poly-T oligonucleotide primers are not suitable for RT of miRNA given their short length and the absence of a polyadenylated 3' tail. To overcome this, the extracted small RNA may be polyadenylated using a poly (A) polymerase followed by a RT reaction using a poly-T oligonucleotide primer. Alternatively, a gene-specific, stem-loop RT primer may be used [61]. When compared to linear RT primers, the stem-loop RT primers are far superior in discriminating between miRNA sequences that differ slightly and are at least 100 times better at discriminating between the mature miRNA and its longer precursor. The stem-loop creates steric hindrance that prevents priming of the precursor miRNA. To date, the most successful approach in terms of specificity and sensitivity is a two-step approach using looped miRNA-specific reverse transcription primers and TaqMan probes from Applied Biosystems[8, 62]. The stem-loop structure is specific to the 3'end of the mature miRNA. It extends the short mature miRNA and adds a universal 3' priming site for real-time PCR.

To correct for variables such as the amount of starting template and enzymatic efficiencies, RQ-PCR data is routinely normalised using endogenous control genes ('house-keeping genes') which are stably expressed across a sample set. The appropriate choice of endogenous control is critical to ensure validity and accuracy of the results generated. Evidence exists to support particular miRNAs as appropriate normalizers for given datasets, depending on the tissue of origin [63-64].

The many advantages of RQ-PCR in miRNA analysis include its efficiency, relatively low cost, low starting miRNA requirement, and the fact that both high and low abundance miRNAs can be detected. It is therefore particularly useful for validating the data obtained from miRNA microarray expression profiling.

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[8] http://www3.appliedbiosystems.com/AB_Home/index.htm

1.5.3.5 MiRNA *in situ* hybridisation

Whilst expression profiling techniques such as microarrays or Northern blots have generated insights into the tissue and/or developmental specificity of miRNAs [65] [66] [51], these methods do not to study miRNA expression with cellular resolution, thus making it impossible to localise the specific expression of miRNAs in non-homogenous tissues. Detection and localization of miRNAs by *in situ* hybridisation (ISH) is a technique which can overcome this issue, and allow direct visualization and localization of miRNAs in tissue specimens [67] [68]. The small size of miRNAs presents a particular challenge when attempting to detect and visualize them in clinical tissue sections. The recent development of LNA™-optimized detection probes for this purpose has resulted in the development of high-throughput automated ISH analysis, which enables large-scale screening of tissue sections including formalin-fixed and paraffin embedded (FFPE) specimens. This enables both the determination of miRNA expression levels in a semi-quantitative manner, as well as specific tissue localization (Figure 1.4[9]). *MiR-21* expression in cancerous and non-cancerous tissue (breast [8] and colorectal [69]) has been investigated using ISH which verifies greater *miR-21* expression in tumour compared to normal tissues. *MiR-21* appears to localize predominantly in cancer-associated fibroblasts, where staining intensities have been demonstrated to be even more intense than in adjacent malignant cells. It is hypothesised that this is a non–cell-autonomous phenomenon and that cytokines secreted from adjacent malignant tumours might contribute to *miR-21* induction [69].
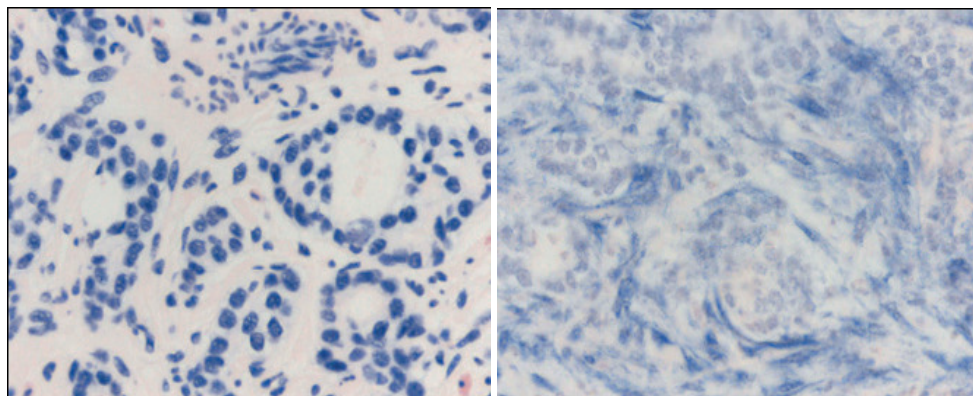
Figure 1.4 *In situ* hybridization analysis of FFPE breast cancer tissue using LNA™ Detection Probes. Left panel: stained with digoxigenin-labeled nuclear localization probe. Right panel: stained with digoxigenin-labeled *miR-21*-specifi c probe. *MiR-21* is expressed most strongly in fibroblast-like cells close to tumour cells (Image courtesy of Exiqon).

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[9] http://www.exiqon.com/ls/Documents/booklet/Exiqon_Collection_Booklet_V5.pdf

1.5.3.6 Functional Analysis of MiRNAs

To complement miRNA profiling methods, and to address functional questions, necessitated the development of methods to manipulate miRNA expression. 2-*O*-Methyl antisense single-strand oligonucleotides and locked nucleic acid–modified oligonucleotides have been developed as miRNA inhibitors, making the suppression of endogenous miRNA activity and its downstream effect on mRNA expression achievable both *in vitro* and *in vivo* [70-72]. The effects of target miRNA knockdown on cell morphology and function can be determined using standard assays for cellular processes such as proliferation, migration, invasion, and angiogenesis. MiRNA inhibition can be studied in animal models via transfection with tumour cells treated with miRNA inhibitors [73] or by the intravenous injection of "antagomirs" (2-*O*-methyl–modified nucleotides with a cholesterol moiety at the 3'-end [74]. The most recent development in the field of miRNA inhibition, led by Naldini and colleagues, describes techniques to manipulate miRNA expression *in vivo* by expressing decoy miRNA targets via lentiviral vectors [75]. This new approach to examine loss-of-function *in vivo* complements the results obtained by classic knockout technology as described above. It allows inhibition of specific miRNAs by building in multiple different decoys in the same miRNA inhibitor. This exciting new development should provide answers to some interesting functional questions with clinical or therapeutic relevance. For example, one could now potentially knock down the oncogenic proprieties of the *miR-17-92-1* cluster which is well documented to be involved in human cancer [76]. This technique could also help to examine the *let-7* miRNA family, a well-known tumour suppressor miRNA family [77], thereby providing insights into the functional consequence of knocking down all *let-7* miRNAs [78].

MiRNA mimicry, a complementary technique to the aforementioned miRNA inhibition, has recently been used *in vitro* to identify the cellular processes and phenotypic changes associated with specific miRNAs transfected into cell lines [79]. Functional assays (e.g., proliferation, migration, invasion, and angiogenesis) then allow determination of the effects of miRNA up-regulation on tumourigenic or non-tumourigenic cell populations. These revolutionary technologies will undoubtedly

help to shed light on the functional roles of miRNAs and hold immense potential for application to the clinical arena as novel therapeutic strategies.

1.6   MiRNAs and disease

Profiling and functional investigation of miRNAs and their targets has identified miRNAs as critical regulators of a variety of cellular processes including differentiation, proliferation, apoptosis, adipogenesis and metabolic integration. Aberrant miRNA expression has been documented in many pathological processes, leading to both benign and malignant diseases [80-81]. Perhaps the greatest emphasis thus far has been on elucidating the precise role of miRNAs in carcinogenesis and great progress has been achieved in that field. Efforts to explore the role of miRNAs in benign diseases [e.g. diabetes mellitus, obesity, heart failure, infectious, inflammatory and auto-immune conditions including viral hepatitis, inflammatory bowel disease, and rheumatoid arthritis] have identified significant involvement of miRNAs in the pathogenesis of these conditions also [80, 82-88].

1.6.1   MiRNAs and cancer

Intensifying research in this field, using the range of techniques previously outlined, has resulted in the identification and confirmation of abnormal miRNA expression in most human malignancies (Table 1.4 [81]). In fact more than half of these cancer-associated miRNAs are located in cancer 'hotspot' chromosomal regions, including fragile sites, regions of loss of heterozygosity, amplification or common breakpoint regions [89]. The first evidence of involvement of miRNAs in malignancy came from the identification of a translocation-induced deletion at chromosome 13q14.3 in B-cell chronic lymphocytic leukaemia [90]. Loss of *miR-15a* and *miR-16-1* from this locus results in increased expression of the anti-apoptotic gene *BCL2*. A further example is the polycistron cluster *miR-17-92*, located at the chromosome 13 open reading frame locus on chromosome 13q31; a region known to undergo loss of heterozygosity in a number of different cancer types [91-93]. Several other miRNAs (*miR-196* and *miR-10a*) are located in homeobox clusters, known to be involved in carcinogenesis and associated with the malignant capacity of cancer cells [94-95]. MiRNA expression in tumours has been observed to be up- or down-regulated

compared with normal tissue, supporting their complex dual role as either 'oncomirs' or tumour suppressors respectively [50].

The ability to obtain miRNA expression profiles from human tumours has led to remarkable insight and knowledge regarding the developmental lineage and differentiation states of tumours. Even within a single developmental lineage it has been shown that distinct patterns of miRNA expression are observed, that reflect mechanisms of transformation, and further support the idea that miRNA expression patterns encode the developmental history of human cancers. In contrast to mRNA profiles it is also possible to successfully classify poorly differentiated tumours using miRNA expression patterns [96]. Furthermore, miRNAs remain largely intact in routinely collected, formalin-fixed, paraffin-embedded clinical tissues [50]. This has exciting implications clinically, in that miRNA expression may accurately diagnose poorly differentiated tumours which proved to be of uncertain histological origin thus facilitating treatment planning.

Table 1.4 MiRNAs with altered expression in malignancy		
Tissue/tumour type	**Increased expression**	**Decreased expression**
Breast [66,97]	miR-21, miR-29b-2	miR-125b, miR-145 miR-10b, miR-155, miR-17-5p, miR-27b
Ovarian [98-99]	miR-141, miR-200(a-c), miR-221	let-7f, miR-140, miR-145, miR199a, miR-424
Endometrial [100-102]	miR-103, miR-107, miR-185, miR-205, miR-210, miR-449	miR-99b, miR-152, miR-193, miR-204, miR-221, let-7i
Glioblastoma [35,96]	miR-221, miR-21	miR-181a, miR-181b, miR-181c
Chronic lymphocytic leukaemia [90]		miR-15, miR-16
Lymphoma [35,50]	miR-155, miR-17-92 cluster	miR-15a
Colorectal [35,50,96]	miR-10a, miR-17-92 cluster, miR-20a, miR-24-1, miR-29b-2, miR-31	miR-143, miR-145, let-7
Thyroid [35,96]	miR-221, miR-222, miR-146, miR-181b, miR-197, miR-346	
Hepatocellular [35,96]	miR-18, miR-224	miR-199a, miR-195, miR-200a, miR-125a
Testicular [50]	miR-372, miR-373	
Pancreatic [35,50,96]	miR-221, miR-376a, miR301, miR-21, miR-24-2, miR-100, miR-103-1,2, miR-107, miR-125b-1	miR-375
Cholangiocarcinoma [96]	miR-21, miR-141, miR-200b	
Prostate [50]	let-7d, miR-195, miR-203	miR-128a
Gastric [35,50,96]	miR-223, miR-21, miR-103-2	miR-218-2
Lung [35,50,96]	mir-17-92 cluster, miR-17-5p	let-7 family

1.6.2   Oncogenic miRNAs

The amplification or overexpression of miRNAs which target genes with tumour suppressor activities can lead to significant down-regulation of these tumour suppressors, or indeed other genes involved in cell differentiation. This may incite uncontrolled proliferation, loss of apoptotic activity, promote angiogenesis and/or invasion, and thereby contribute to tumour formation. In this way, miRNAs can act as oncogenes [103]. Specific examples of miRNAs with oncogenic activity follow:

Early profiling experiments identified *miR-21* as one of the most commonly dysregulated miRNAs in cancer. Its over-expression has been clearly demonstrated and extensively validated in a wide range of solid and haematogenous malignancies; hence it was one of the first miRNAs to acquire the 'oncomiR' label [104]. Functional analysis of *miR-21* has identified some of the downstream targets and pathways controlled by *miR-21*. Not surprisingly, a number of tumour-suppressor genes have been found to be targeted by this oncomiR. These include *TPM1* (tropomyosin-1), *PDCD4*, *maspin, PTEN*, and the p53 tumour suppressor[104].

The polycistronic *miR-17-92* cluster represents another example of miRNAs with oncogenic function. Over-expression of the seven miRNAs in this cluster (*miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-20a, miR-19b,* and *miR-92–1*) have been associated with a variety of malignancies (colon, lymphoma, breast, lung, pancreas, prostate and stomach [96]. The mechanism by which this cluster acts is likely to be due to suppression of *PTEN*, a tumour suppressor gene and negative regulator of the highly oncogenic prosurvival *PI3K/AKT* signalling pathway [105].

1.6.3   Tumour suppressor miRNAs

Conversely, several other miRNAs have been found to be under-expressed in cancerous compared to normal tissue. These same miRNAs are predicted to target oncogenic mRNA sequences, further implicating their role as tumour-suppressors. The seminal work in this field, by Johnson and colleagues at Slack's lab in Yale University, indicates that the *let-7* miRNA negatively regulates the oncogenic family of RAS proteins in both *C.elegans* and human tumour cell lines. Supporting evidence lies in the finding that the 3'UTRs of the human *RAS* genes contain multiple *let-7* complementary sites (LCSs), allowing *let-7* to bind and thereby regulate *RAS* expression. *Let-7* expression is also lower in lung tumours than in normal lung

tissue, while RAS protein is significantly higher in lung tumours, providing further evidence to support a mechanism for *let-7* as a tumour suppressor in certain cancer.

MiR-34a has been demonstrated to exert tumour suppressor properties in various cancers by conferring translational inhibition and inducing degradation of mRNA genes involved in cell cycle control and apoptosis [106]. Its loss is therefore coupled with uncontrolled cell growth and proliferation. These effects are mediated through loss of repression of the deacetylase sirtuin (*SIRT1)* and cyclin dependent kinase 6 (*CDK6*) [106].

In human bladder and colon cancer, expression of *miR-143* has been shown to be significantly lower in tumour compared to normal tissue [107, 108] . Furthermore, *miR-143* expression in cancer cells significantly inhibits cell proliferation, giving further evidence to support its role as a tumour suppressor. Several other miRNAs with tumour suppressor properties include *miR-101* (breast and prostate cancer), *miR-145* (colon and breast cancer), *miR-15a* (leukaemia), *miR-125a* and *miR-125b* (breast cancer).

1.6.4   MiRNAs and breast cancer

Elucidation of the molecular mechanisms involved in breast cancer pathogenesis has been the subject of extensive research, yet several dilemmas and major challenges still prevail in the management of breast cancer. These include unpredictable response and development of resistance to adjuvant therapies. The emergence of miRNAs as regulators of gene expression identifies them as obvious novel candidate diagnostic and prognostic indicators, and potential therapeutic targets. MiRNA expression studies in breast cancer indicate their importance and potential use as disease classifiers and prognostic tools in this field. In their analysis of 76 breast tumour and 34 normal specimens Iorio *et al* [66] identified 29 miRNAs that were differentially expressed in breast cancer tissue compared to normal, and a further set of 15 miRNAs that could correctly discriminate between tumour and normal breast tissue. In addition, miRNA expression correlated with biopathological features such as ER and PR expression (*miR-30*) and tumour stage (*miR-213* and *miR-203*). The differential expression of several *let-7* isoforms was associated with biopathologic features including PR status (*let-7c*), lymph node metastasis (*let-7f-1, let-7a-3, let-*

7a-2) or high proliferation index (*let-7c, let-7d*) in tumour samples. Mattie *et al* identified unique sets of miRNAs associated with breast cancers currently defined by their HER2/*neu* or ER/PR status [97]. Significantly, there was overlap between the miRNAs identified in both Iorio's and Mattie's studies.

The capacity of miRNA expression profiles to classify breast tumours according to clinicopathological variables currently used to predict disease progression highlights their potential as reliable prognostic indicators which may contribute to improved selection of patients for adjuvant therapy [109]. This approach has already shown clinical relevance for gene expression signatures, as outlined above in the description of Sorlie *et al's* derivation of intrinsic subtypes of breast cancer based on tumours' mRNA expression profiles. Furthermore, miRNA profiles may have superior accuracy to mRNA signatures in this regard [50]. Accordingly, a comprehensive interrogation of the breast cancer subtypes by miRNA expression profiling could further characterise the molecular determinants underlying these subtypes, perhaps define more precise subsets of breast cancer, and provide opportunities for identification of novel targets which could be exploited for targeted therapy [110]. To this end, Lowery *et al* have already identified a number of miRNAs of importance in breast cancer, which correlate with breast tumours' hormone receptor status. From a miRNA microarray profiling experiment performed on 29 early stage breast cancers, the authors identified miRNAs which were differentially expressed in breast tumours compared to adjacent normal breast tissue. The differential expression of these candidate miRNAs (including *miR-21, miR-10b, miR-145, mIR-155, miR-181b, miR-181c,* and *miR-195*) were validated in a larger cohort of almost 100 fresh frozen tumour specimens. Applying a novel artificial neural network approach to analyze their dataset, allowed the identification of 3 unique miRNA signatures which were predictive of a tumour's ER, PR, and HER2/*neu* status respectively [111] (Table 1.5).

Table 1.5 MiRNA signatures predictive of ER, PR & HER2/*neu* receptor status		
ER status	**PR status**	**HER2/*neu* status**
miR-342		
miR-299-3p	miR-520g	miR-520d
miR-217	miR-377	miR-181c
miR-190	miR-527 – 518a	miR-302c
miR-135b	miR-520f – 520c	miR-376b
miR-218		miR-30e-3p

Lowery AJ et al. Breast Cancer Res 2009;11(3):R27

1.6.5   SNPs in miRNA binding sites influence cancer susceptibility

The concept that single nucleotide polymorphisms (SNPs) in protein-coding genes can affect the functions of protein, and in turn influence the individual susceptibility to cancers, has been well documented. MiRNAs, as described above, exert their regulatory effect on gene expression by binding to the 3' untranslated region (UTR) of their target genes – regions which are evolutionarily highly conserved suggesting an important role for these regions in natural selection. Because a single miRNA can regulate hundreds of mRNAs simultaneously, the potential of cellular transformation resulting from dysfunction of a single miRNA is high. Very recently it has been recognised that SNPs in miRNA sequences, or indeed in their complementary binding site on mRNA, can result in diverse functional consequences, and therefore may represent ideal candidate biomarkers for disease diagnosis, prognosis and outcome [112] [113].

Firstly, SNPs that disrupt miRNA gene sequences have been associated with cancer risk. Inherited mutations or rare SNPs in the primary transcripts of *mir-15a* and *mir-16-1* have been linked to familial chronic lymphocytic leukaemia and familial breast cancer [114] . Several miRNA-associated SNPs have been shown to increase breast cancer susceptibility. For example, Hu *et al* demonstrated that SNPs in both *mir-196a2* (rs11614913:T>C) and *mir-499* (rs3746444:A>G) were associated with significantly increased risks of breast cancer (OR, 1.23; 95% CI, 1.02-1.48 for rs11614913:T>C; and OR, 1.25; 95% CI, 1.02-1.51 for rs3746444:A>G) in a case–control study of 1009 breast cancer cases and 1093 cancer-free controls in a population of Chinese women (p = 0.010 and 0.037, respectively) [115]. This study's

control population was carefully selected from a cohort of more than 30,000 participants in a Chinese community-based screening program for noninfectious diseases conducted in the same geographical region. All these women were cancer free, and a randomly selected group were frequency-matched to the cases for age and residential area (urban or rural) status. Shen *et al* identified a G to C polymorphism (*rs2910164*) within the sequence of the *mir-146a* precursor and demonstrated that a variant C allele led to increased levels of mature *miR-146* in patients with breast and ovarian cancer and predisposed them to an earlier age of onset of familial breast and ovarian cancer [116]. These findings suggested, for the first time, that SNPs in miRNAs may contribute to breast cancer susceptibility and may serve as novel biomarkers for breast cancer diagnosis. Secondly, there is also recent evidence that miRNA-binding site SNPs can influence cancer risk. Three recent papers report SNPs in miRNA target sites in human cancer genes, and show that allele frequencies vary between normal people and patients with cancer [117-119]. These papers have focused on colorectal, thyroid and non-small cell lung cancer respectively;

- Two miRNA-binding site polymorphisms (*rs17281995* and *rs1051690)* in the 3'UTRs of *CD86* and *INSR* genes, respectively, were significantly associated with increased CRC risk in a Czech Republic population [119].
- A SNP identified in a miRNA-binding site in the *kit* oncogene was associated with increased gene expression in papillary thyroid carcinoma [118].
- A SNP identified in the *let-7*-binding site in the *KRAS* oncogene disrupts *let-7* regulation of *KRAS,* and is also associated with altered cellular miRNA levels. This SNP *let-7* complementary site (LCS6SNP) has been shown to be a biomarker of an increased risk of developing NSCLC in two independent case–control studies [117]

These findings clearly demonstrate the utility of miRNA-associated SNP evaluation in cancer predisposition. Slack and Weidhaas' group in Yale hypothesise that the aforementioned SNP in the *let-7*-binding site in the *KRAS* oncogene (LCS6SNP) also imparts an increased risk of breast cancer. In a pilot study to test this hypothesis, carried out on a population of breast cancer patients in Connecticut, the prevalence of the LCS6SNP was 15-30%, compared with a background prevalence of 5.8% in

the general population. Interestingly, the SNP was most prevalent (30%) in patients with triple negative breast cancer (basal subtype). This finding remains to be investigated in a much larger population, to identify its true potential for determining breast cancer risk in young women. Nonetheless evidence thus far regarding the analysis of SNPs in miRNA binding sites indicates that they represent a new paradigm for cancer susceptibility.

1.7 MiRNAs and metabolic disease

MiRNAs play important regulatory roles in many other processes in addition to cell growth and proliferation. These include adipocyte differentiation, metabolic integration, insulin resistance, appetite regulation and control of the immune response [120] (Figure 1.5). Dysregulation of miRNAs involved in these processes is implicated in the pathogenesis of common benign conditions including diabetes mellitus, obesity, and hepatitis.
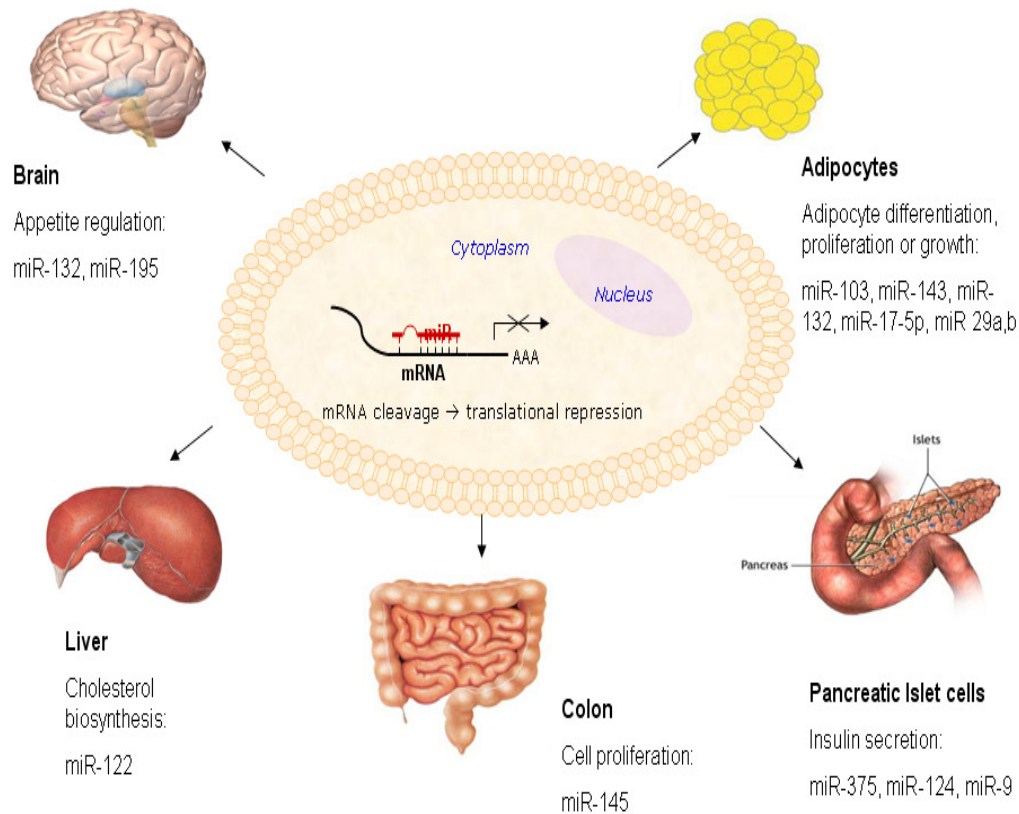
Figure 1.5 Target tissues of metabolic miRNAs

1.7.1   Dysregulated miRNA expression in diabetes mellitus

Poy *et al* originally identified several miRNAs which were differentially expressed in pancreatic endocrine cell lines, implying their role in glucose homeostasis. *MiR-375* overexpression was found to reduce beta cell number and viability and thereby suppress glucose-stimulated insulin secretion, events which induce a diabetic state. Conversely functional experiments showed that *miR-375* inhibition enhanced insulin secretion. These effects were shown to be mediated through *miR-375*'s gene targets which include myotrophin and PDK1 [121-122], and the results indicate that *miR-375* is a potentially important modulator of beta cell function.

1.7.2   Dysregulated miRNA expression in obesity

Xie *et al* provided the first experimental evidence for *miR-103* function in adipose biology [123]. Using 3T3-L1 cells (Mouse embryonic fibroblast - adipose like cell line), they demonstrated that expression of *miR-103* was induced approximately nine-fold during adipogenesis and consequently down regulated in adipose tissue harvested from obese mice. The accelerated *miR-103* differentiation during adipogenesis was accompanied by:

-        Increased expression of key transcription factors (*Pparγ2*)

-        Increased expression of key cell cycle regulators (G0/G1 switch 2 – *G0s2*)

-        Increased levels of molecules associated with lipid metabolism (*Fabp4*)

-        Increased levels of molecules associated with glucose homeostasis (*Glut4*)

-        Increased levels of molecules associated with endocrine function of adipocytes (adiponectin).

Computational studies predict that *miR-103* affects multiple mRNA targets in pathways that involve cellular acetyl-CoA and lipid metabolism. The inverse pattern of miRNA expression observed in differentiating adipocytes and obese tissue indicates that obesity leads to a loss of miRNAs that characterise fully differentiated and metabolically active adipocytes. Xie *et al* postulate that these changes are likely due to the chronic inflammatory environment in obese adipose tissue, which has been well described previously [124]. The authors also show that when differentiated 3T3-L1 adipocytes were treated with TNF-α (a macrophage produced cytokine involved in chronic inflammation, largely responsible for inducing insulin resistance

in obese adipose tissue) for 24 hours, levels of *miR-103* and *miR-143* reduced in the adipocytes, whilst levels of *miR-221* and *miR-222* were increased. They also observed similar miRNA expression patterns in adipose tissue from obese mice, as well as simultaneously increased levels of TNF-α. *MiR-132* has been shown to be highly expressed in brain tissue and neuronal cell types, and evidence exists to show that *miR-132* is involved in the regulation of cAMP response element-binding protein (CREB) which is also known to function in glucose homeostasis and appetite regulation [125].

1.7.3 Dysregulated miRNA expression in liver disease

MiR-122 is a liver specific miRNA implicated in cholesterol and lipid metabolism, and in hepatitis C virus replication [74,126]. Krutzfeldt *et al* provide evidence to support *miR-122* as a key regulator of the cholesterol biosynthetic pathway; in particular they observed that the expression of at least 11 genes involved in cholesterol biosynthesis was decreased between 1.4-fold and 2.3-fold in antagomir-122-treated mice, including hydroxy-3-methylglutaryl-CoA-reductase (*Hmgcr*), a rate-limiting enzyme of endogenous cholesterol biosynthesis. Observational and functional studies of *miR-122* have highlighted this miRNA as a potential therapeutic target for the treatment of hypercholesterolemia and hepatitis C.

1.8 MiRNAs as biomarkers

The emergence of miRNAs as modulators of gene expression identifies them as obvious novel candidate diagnostic and prognostic indicators, and potential therapeutic targets. In addition to their tissue specificity, miRNAs hold other unique characteristics that herald them as ideal tumour markers including their stability, ease of detection and association with established clinicopathological prognostic parameters. Acknowledgment of the exceptional stability of miRNAs in visceral tissue, instigated efforts to establish if these tiny molecules were also preserved, detectable, and quantifiable in the circulation and in other bodily fluids (e.g. urine, saliva, sputum). The last two years has seen an accumulating body of evidence to support circulating miRNAs as non-invasive, sensitive biomarkers of disease states, particularly cancers (including breast, lung, pancreas, ovarian, and prostate). This novel approach has immense potential to advance cancer diagnosis and stratification

beyond currently available methods. For those malignancies which still routinely present in advanced stages, and for which there is no reliable tumour marker available at present (lung, ovarian, pancreatic cancers in particular), this approach has the potential to transform presentation, management, and outcomes of these devastating diseases.

1.8.1   Unique biomarker characteristics of miRNAs

The ideal biomarker should be easily accessible such that it can be sampled relatively non-invasively, sensitive enough to detect early presence of tumours in almost all patients, and absent or minimal in healthy tumour free individuals [127]. MiRNAs have enormous potential to serve as an ideal class of cancer biomarkers for the following reasons:

a.   MiRNA expression is known to be aberrant in cancer tissues [50,90]

b.   MiRNA expression profiles are pathognomonic, or tissue-specific [50].

c.   MiRNAs are remarkably stable molecules that have been shown to be well preserved in formalin fixed, paraffin embedded tissues as well as fresh snap frozen specimens [128-129].

d.   MiRNAs can be quantified in the circulation and levels reflect miRNA expression in tumour.

1.8.2   Circulating miRNAs as novel minimally invasive biomarkers

This area of miRNA research is only currently emerging, and is generating much excitement in clinical and scientific communities, such is its potential. MiRNA presence in serum was described for the first time in March 2008, in patients with diffuse large B-cell lymphoma [130]. Subsequent to this, a small number of studies have reported similarly on the presence of miRNA in circulation, and illustrated the potential of circulating miRNAs as novel biomarkers of diseases and physiological states including malignancy, diabetes mellitus and pregnancy [131-135]. However these studies have been limited by small numbers and inconsistencies in methodologies [136]. Regarding the techniques employed to isolate and accurately quantify miRNAs in blood or its derivatives, the existing protocols are ill-defined. Of the few published reports in this domain, the techniques described therein are variable and difficult to reproduce. Several questions permeate this field – which circulating medium is preferable for systemic miRNA investigations, which purification technique retrieves

superior concentration of quality miRNA from blood, which concentration of miRNA is optimal for RQ-PCR analysis and which endogenous controls are appropriate for circulating miRNA studies.

1.9 Study rationale

The foremost involvement of miRNAs in the aetiology and progression of many common diseases heralds these molecules as significant novel markers with potential use as diagnostic, prognostic and therapeutic tools. The recent discovery that miRNAs are detectable and quantifiable in the circulation adds further scope to their potential, particularly as evidence accumulates to support their use as biomarkers of disease, both benign and malignant. It is anticipated that tissue and systemic miRNA expression could be harnessed as a new and improved means to detect and classify disease, determine prognosis, and predict response to existing therapies. The investigation of existing miRNA molecules coupled with the identification of novel miRNAs, and elucidation of their downstream targets, will provide a better understanding of their functional effects and thus provide greater insight into the complex and poorly understood mechanisms underlying diseases such as cancer and obesity. Furthermore this will provide clinicians and scientists with a new repertoire of molecular targets which could be exploited for potential therapeutic intervention.

The purpose of this study was to investigate the role of miRNAs in common and challenging diseases, both benign and malignant, using obesity and breast cancer respectively as representative disease states. In particular, the utility of circulating miRNAs as non-invasive disease biomarkers was a primary objective throughout the study. Specific study aims are outlined as follows:

1.   **Development of experimental techniques for circulating miRNA analysis**

   a.   The initial objective was to develop a reliable and reproducible protocol for optimal extraction, quantification and analysis of miRNA expression in human blood samples. This was essential, as at the time this study was initiated, very little methodological information pertaining to miRNA isolation from blood or its derivatives existed.

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b.   Identification of the most suitable circulating medium for systemic miRNA investigations was of paramount importance, as it was ill-defined which medium was more reliable for blood-based miRNA studies.

c.   Quantification of miRNA from blood samples by RQ-PCR required modification of the methods described previously for tissue miRNA analysis, given their lower abundance in peripheral circulating samples. This required investigating the optimal starting concentration of miRNA for RQ-PCR analysis, and which endogenous controls are appropriate for circulating miRNA studies

2.   Analysis of candidate miRNA expression in breast cancer

a.   A panel of 7 candidate miRNAs (*miR-10b, miR-21, miR-145, miR-155, miR-195, let 7a,* and *miR-16*) was chosen for investigation in the tumour tissues and blood samples of patients with primary breast cancer. These miRNAs were selected for two reasons. Firstly, a miRNA microarray experiment on these tumour tissues, conducted in the Department of Surgery NUI Galway, identified several candidate miRNAs which were differentially expressed in breast tumour compared to normal breast tissue. Secondly published reports also linked the miRNAs in this panel to breast cancer previously.

b.   We aimed to determine if these cancer-associated miRNAs were quantifiable in blood from breast cancer patients, and then establish if there was a correlation between the breast tumour and circulating miRNA expression profiles.

c.   The association of circulating miRNA expression with clinicopathological parameters such as tumour type, subtype, size, grade and stage of disease, was also explored to see if systemic

miRNA analysis had potential to classify patients into clinically relevant subgroups.

3.   **Analysis of circulating miRNAs as tumour specific biomarkers**

a.   Recent reports on circulating miRNAs as tumour markers indicate that specific miRNAs are dysregulated in blood from cancer patients. No study previously addressed whether elevated levels of these markers are tumour-specific or a general cancer phenomenon. We sought to investigate whether a panel of circulating miRNAs previously associated with breast cancer (*miR-10b, miR-21, miR-145, miR-155, miR-195*, and *let 7a*), was indeed breast cancer specific or generalised to several malignancies. Therefore we expanded our analysis of circulating miRNAs to include patients with prostate, renal, colorectal, bladder and melanoma cancers.

b.   The value of circulating miRNAs for early diagnosis of cancer, and thus their potential as cancer screening tools, requires investigating whether or not they are significantly dysregulated even in patients with pre-invasive and early stage disease. We sought to explore this concept by expanding our analysis of circulating miRNAs in cancer patients to include patients with *in-situ* carcinoma of the breast, as well as significant numbers of patients with early stage (TNM Stage I and II) cancer of the colon, kidney and prostate.

4.   **Analysis of a SNP in the *let-7* complementary site in the *KRAS* oncogene, as a biomarker of risk for developing breast cancer**

a.   Slack and Weidhaas' group at Yale hypothesise that a SNP in the *let-7*-binding site in the *KRAS* oncogene (LCS6SNP) imparts an increased risk of breast cancer. In a pilot study to test this hypothesis, carried out on a population of breast cancer patients in Connecticut, the prevalence of the LCS6SNP was 15-30%, compared with a background prevalence of 5.8% in the general population. In order to further test this hypothesis in a larger homogeneous population, the

Department of Surgery at NUI Galway collaborated with the Yale Molecular Biology Group, and conducted a population analysis of the association of LCS6SNP and breast cancer on a large cohort of breast cancer patients and age-matched non-cancer controls recruited in the West of Ireland, and New Haven, Connecticut.

5. Identification of miRNA expression profiles in adipose tissue and circulation of obese humans

a. The role of miRNAs in the deposition, accumulation and function of adipose tissue has only been conducted to date *in-vitro* or in model organisms (murine studies). A global miRNA profile was performed on a cohort of matched human omental and subcutaneous adipose tissues, form obese and non-obese individuals. This experiment was performed using a miRNA microarray platform devised at Systems Bioscience, California.

b. MiRNA array data were analysed using TIGR MeV bioinformatic algorithms, and validated using RQ-PCR and Taqman primers and probes. At the time of commencement of this study there was no published human adipose tissue miRNA microarray dataset, so this analysis of miRNA expression in obese humans will contribute significantly to the current knowledge of obesity and metabolic syndrome molecular biology.

c. The expression of candidate metabolic miRNAs was also investigated in blood samples form obese and non-obese individuals to explore the potential of circulating miRNAs as novel biomarkers of obesity and the components of the metabolic syndrome. In particular, the possibility of using systemic miRNA profiling in obese individuals as a predictor of good response to bariatric surgery, was explored by obtaining blood samples for miRNA analysis from bariatric patients preoperatively and again after at least 6 weeks postoperatively.

Chapter 2

Materials & Methodology

6.1 Department of Surgery Biobank

The Department of Surgery Biobank at Galway University Hospital has been in existence since 1992. Approval was granted by the Galway University Hospitals Clinical Research Ethics Committee to store breast cancer-related tissue specimens and clinically relevant data, pending gaining informed written consent form each patient. At the time of diagnosis, patients are informed of their opportunity to partake in the GUH research programme by the clinician and specialist breast care nurse. Following a detailed discussion of the programme, patients are given a written information leaflet (Appendix 1) further describing the Biobank process and its implications, and then asked for informed consent if they wish to participate. Consenting patients agree to blood and tissue specimens being taken prior to and during their treatment. Blood samples (whole blood, serum and plasma) are typically collected preoperatively, two weeks postoperatively, and at intervals over the course of their long term follow up. Tissue specimens are retrieved from patients at the time of surgical resection of their tumour. The type of breast tissue specimens routinely collected include primary breast tumour tissues retrieved from patients undergoing breast cancer-related surgical procedures, normal breast tissue retrieved during breast reduction surgery and benign breast tissues. Tissue samples are routinely snap-frozen in liquid nitrogen immediately following surgical excision and subsequently stored at -80ºC. Once data is input to the laboratory information management system (Shire) unique anonomised patient identifiers are generated, samples are coded alphanumerically and coded labels are printed for each specimen which is stored according to the storage location assigned by Shire.

The range of clinicopathological data entered into the database includes patient demographics, breast cancer risk factors, menopausal status at diagnosis, tumour size and grade, oestrogen and progesterone receptor status, HER2/*neu* status, nodal status, presence or absence of distant metastases, and finally stage of disease according to the TNM classification system. Breast carcinomas are generally graded according to the Nottingham modification of the Bloom Richardson system (Elston and Ellis, 1991, Elston, 1998) which examines the specimen's tubularity, nuclear size and shape, and hyperchromatic figures. In general, a grade of 1 indicates low

grade and well-differentiated cells, grade 2 indicates moderately-differentiated cells and grade 3 indicates high grade and poorly differentiated cells.

Since 2007 the Biobank has expanded, with approval from the Galway University Hospitals Clinical Research Ethics Committee, to include specimens from patients with cancers other than breast, as well as from patients with benign diseases. Blood, tumour and tumour-associated normal tissue specimens are now routinely collected from patients with colorectal cancer, prostate cancer, renal cell carcinoma, malignant melanoma and other non-melanoma skin cancers. With increasing numbers of obese patients presenting to GUH for bariatric procedures recently, the Biobank also stores blood and adipose tissue specimens from this cohort of patients. All bariatric surgical candidates are invited to participate in this programme; consenting patients agree to donate adipose tissue specimens at the time of their bariatric procedure, in addition to pre- and postoperative blood samples. Intra-operatively, the operating surgeon retrieves a 10-15gm specimen of omental fat and a similar specimen of subcutaneous fat from the infra-umbilical port insertion site. Fat samples are routinely snap-frozen in liquid nitrogen immediately after retrieval and subsequently stored at -80ºC until further processing.

2.2 Study Groups

2.2.1   Breast Cancer Study Groups

Clinical and pathological details in the breast cancer database were examined to determine suitable candidates for three study groups:

2.2.1.1   Breast tissue cohort

A cohort of breast tumour tissues (n=65) and matched tumour-associated normal tissues, representing all types, subtypes, and stages of breast cancer was identified. This group served as the cohort for the investigation of candidate breast cancer associated miRNA expression at tissue level. Breast tumour specimens were obtained from patients during primary curative resection. Matched tumour-associated normal breast tissue was also obtained from a subset of these patients where possible. Following excision, tissue samples were immediately snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction. The cohort of fresh-frozen

breast tumour (n = 65) and tumour-associated normal breast tissue (n = 17) specimens was representative of a typical breast cancer cohort with a mean age of 55.58 years, a predominance of invasive ductal carcinomas (81%) of Luminal A subtype (52%) and almost two thirds (65%) had early stage (TNM Stages I and II) disease. Clinical and pathological data relating to the clinical samples are presented in Table 2.1.

Table 2.1 Clinical details of breast cancer patients used for tissue miRNA analysis

	Breast cancer tissue cohort (n=65) n (%)
Mean age, years	55.58
[Range]	[33 -92]
Stage	
In-situ	0 (0)
I	13 (20)
II	29 (45)
III	17 (26)
IV	6 (9)
Invasive Tumour type	
Ductal	53 (81)
Lobular	9 (14)
Inflammatory	0 (0)
Other / n/a	3 (5)
Epithelial Subtype	
Luminal A	34 (52)
Luminal B	13 (20)
Basal	9 (14)
Her 2/neu	9 (14)
Nodal status	
Positive	31 (48)
Negative	34 (52)
Estrogen receptor status	
Positive	42 (65)
Negative	23 (35)
Her2/*neu* status	
Positive	22 (34)
Negative	43 (66)

2.2.1.2   Blood specimens

A cohort of patients was identified from whom preoperative blood samples were available, which was representative of a typical symptomatic group of breast cancer patients, and matched in age and pathological details to the cohort of breast tumour tissues. This group served as the cohort for testing the hypothesis that circulating miRNAs had potential as breast cancer biomarkers. Blood samples (whole blood, serum and plasma) were collected prospectively from 127 females, including 83 consecutive breast cancer patients at the time of diagnosis, and 44 healthy age-matched female volunteers who served as controls for this study. All patients had histologically confirmed breast cancer and their relevant demographic and clinicopathological details were obtained from the GUH Department of Surgery's prospectively maintained breast cancer database. The histological tumour profile of patients in this study reflected that of a typical breast cancer cohort, inclusive of a 10-15% proportion with *in situ* disease, with the majority of invasive tumours being of ductal type, and Luminal A epithelial subtype (Table 2.2). In addition, repeat blood samples were collected from a subset of this cohort, at their initial clinical review two weeks postoperatively (n=29) in order to assess serial changes in miRNA expression after surgical resection of the patients' breast tumours. The control blood samples were collected from healthy women residing in the same catchment area from which cases originated, and were collected on a contemporaneous basis with cases so as to minimize potential bias because of differential seasonal or environmental exposures. These women were carefully matched to the cancer cases for age (±5 years) and had no current or previous malignancy or inflammatory condition. All control individuals were interviewed by a clinician prior to being enrolled in this study to ensure they were eligible for inclusion in these studies. The mean age of the all-female control group for breast cancer circulating miRNA studies (n=44) was 56.1±14.1 years.

Table 2.2 Clinical details of breast cancer patients and controls in circulating miRNA studies		
	Breast Cancer Blood cohort (n=83) **n (%)**	**Control cohort (n=44)** **n (%)**
Mean age, years	55.1	56.1
[Range]	[30 -88]	[25 -80]
Stage		
In-situ	10 (12)	
I	14 (17)	
II	35(42)	n/a
III	18 (22)	
IV	6 (7)	
Invasive Tumour type		
Ductal	59 (71)	
Lobular	7 (8)	
Inflammatory	3 (4)	n/a
Other	4 (5)	
n/a (in-situ disease)	10 (12)	
Epithelial Subtype		
Luminal A	53 (63)	
Luminal B	8 (10)	
Basal	9 (11)	n/a
Her 2/neu	3 (4)	
n/a (in-situ disease)	10 (12)	
Nodal status		
Positive	38 (46)	n/a
Negative	45(54)	
Estrogen receptor status		
Positive	68 (82)	n/a
Negative	15 (18)	
Her2/neu status		
Positive	11 (13)	
Negative	62 (75)	n/a
n/a	10 (12)	

2.2.1.3   Population genetics breast cancer cohort

For the investigation of inherited variation in miRNA binding sites, a large cohort of patients with a history of breast cancer (all types, N=1132) was identified, from whom whole blood was available for genomic DNA analysis of specific germline mutations. Additionally, a large control group of disease-free healthy females (N=930), from whom genomic DNA was also available, were identified for comparative purposes. These case and control cohorts were recruited across two centres; GUH and Yale University. The entire study population consisted of subjects previously enrolled in a breast cancer case-control study in Connecticut[137], and in a separate breast cancer case control study in the west of Ireland[138-139]. Briefly, cases were incident, histologically confirmed breast cancer patients between 30-80 years, of various ethnicities (Caucasian, African-American, Hispanic) with no prior history of cancer (other than non-melanoma skin cancer). ER, PR and HER2/*neu* status were determined on all cases (Table 2.3). Controls were recruited from Yale New Haven Hospital (YNHH), Tolland County (New Haven), and the West of Ireland. An informed consent, family history of cancer, reproductive history, demographic factors and blood sample were obtained from all subjects (Table 2.4). A total of 1132 cases and 930 controls had DNA samples available for this study. All stages and histologic types of breast cancer, excluding pre-invasive carcinomas, were included. The controls were all of similar ethnic backgrounds to the cancer patients. Mixing Irish and New haven controls was deemed appropriate given that these populations have been found to share similar ethnic ancestry and have been subject to few demographic movements. Consequently, these populations are relatively homogenous, which reduces allelic and genotypic heterogeneity in case-control studies. The Irish controls (n=360) comprised women over the age of 60 years, with no self-reported personal history of any cancer and no family history of breast or ovarian cancer. This age range was chosen to reduce the possibility that these women may have undiagnosed or undeveloped familial breast cancers. The New Haven controls were slightly younger and age-matched to their breast cancer cases. These females also had no personal history of any cancer and no family history of breast or ovarian cancer.

Prior written, informed consent was obtained from each participant in these three breast cancer cohorts (breast tumor tissue cohort, breast cancer blood specimen

cohort, and population genetics breast cancer cohort). All studies were approved by the ethics review board of Galway University Hospital. The three breast cancer-related study groups were designed to represent a variety of tumour types, intrinsic subtypes and disease stage, and to reflect a typical cohort of patients presenting to a symptomatic breast cancer centre.

Table 2.3 Clinical details of breast cancer patients for analysis of inherited variation in the oncogenic let-7 complementary site *(LCS)* in *KRAS* (N=1132)

Mean age   54.1 years (27-90)

Tumour type	***n* *(%)***	**Nodal status**	***n* *(%)***
Ductal	883 (78)	Positive	509 (45)
Lobular	170 (15)	Negative	623 (55)
Inflammatory	11 (1)	**ER status**	
Other	68 (6)	Positive	917 (81)
		Negative	215 (19)
Epithelial Subtype		**HER2*/neu* status**	
Luminal A (ER/PR +, HER2 -)	770 (68)	Positive	226 (20)
Luminal B *(ER/PR +, HER2 +)*	147 (13)	Negative	906 (80)
Basal *(ER/PR -, HER2 -)*	136 (12)	**Stage of disease**	
HER2/neu *(ER/PR -, HER2 +)*	79 (7)	Stage I	329 (29)
		Stage II	509 (45)
		Stage III	249 (22)
		Stage IV	45 (4)

Table 2.4 Details of control cases for analysis of inherited variation in the oncogenic let-7 complementary site (LCS) in *KRAS* (N=930)

	Irish controls n=360	**Yale controls n=570**	**All controls n=930**	**p value***
Age	70.78 ± 6.8 yrs	55.14 ± 11.0 yrs	61.2 years	<0.001
Ethnicity				
Caucasian	360	521	881	<0.001
African-American	0	44	44	
Hispanic	0	5	5	

* Difference between Irish and Yale controls

2.2.2   Metabolic disease study groups

A consecutive cohort of patients with elevated BMI and/or components of the metabolic syndrome presenting to the GUH Obesity Clinic between June 2009 and December 2009 were recruited to this study (n=32). All consenting patients donated blood samples, for the purpose of miRNA analysis, at the time of routine biochemical, haematological and endocrinological investigations. A proportion of these patients, who were selected by a team of clinicians to undergo bariatric surgery as a therapeutic strategy for their obesity and its associated co-morbidities, also consented to having adipose tissue samples taken at the time of their bariatric procedure. An omental sample and an abdominal wall subcutaneous fat sample were obtained from each bariatric patient (n=19).

The controls for this study consisted of:

i.   Healthy volunteers from the community with BMI of less than 25, who donated blood samples

ii.   Patients undergoing elective laparoscopic abdominal procedures, who had a BMI of less than 25, donated adipose tissue specimens (omental and subcutaneous fat) for comparison with adipose tissue from the obese cohort. Examples of the surgical procedures patients in this control cohort underwent were laparoscopic hernia repairs, laparoscopic Nissen's fundoplication and laparoscopic cholecystectomy.

2.2.2.1   Obese and metabolically unhealthy

The obese cohort (n=32) were subdivided into those who did or did not have any component of the metabolic syndrome. These components included

- Hyperlipidaemia (fasting total cholesterol >6.2 mmol/L, and/or fasting triglycerides >2.25 mmol/L)
- Hypertension (systolic BP > 130 or diastolic BP >85 mm Hg, or treatment of previously diagnosed hypertension)
- Previously diagnosed type 2 diabetes, or impaired glucose tolerance (fasting plasma glucose > 5.6 mmol/L, or abnormal Oral Glucose Tolerance Test result).

The proportion of obese patients with any one or more of these components of the metabolic syndrome was 60%. This cohort was termed 'metabolically unhealthy' for the purpose of data analysis and interpretation. One of the aims of this study investigating miRNA expression in obesity was to determine if miRNA expression differed between those patients who were metabolically unhealthy compared to their obese counterparts who were free of metabolic complications.

2.2.2.2 Obese and metabolically healthy

Of the obese cohort, 40% had no metabolic co-morbidity, nor were taking any medication for the treatment of hyperlipidaemia, hypertension or Diabetes Mellitus. This subgroup was termed 'metabolically healthy'.

2.3   RNA extraction

Extraction of RNA from tumour tissue, normal breast tissue, adipose tissue and blood, was specific for the different tissue types. A separate purification of large (mRNA) and small RNA (miRNA) using column based technology was employed for breast tumour and normal breast tissue RNA isolation. For adipose tissue, total RNA was isolated from fat specimens using a co-purification Trizol-based technique.

Upon commencing this study, isolating quality miRNA from blood and its derivatives presented a significant challenge. No standardised or reproducible protocol existed, and the methods described in the early publications in this emerging field of miRNA research were vague and difficult to reproduce. We compared various methods including separate purification of large and small RNA from blood using column based methodology, and co-purification of total RNA using Trizol based techniques. We also compared each technique for serum, plasma and whole blood samples in order to identify which circulating medium was optimal for blood based miRNA studies.

2.3.1   Breast Tissue RNA extraction

A separate purification technique was applied to breast tissue (tumour and normal) to isolate miRNA and mRNA separately. Approximately 100 mg of tissue was homogenized in 1-2 mL QIAzol (Qiagen) using a bench-top homogenizer (Kinematica AG). Large (>200 nt) and small RNA (<200 nt) fractions were isolated separately using the RNeasy Plus mini kit and RNeasy MinElute cleanup kit (Qiagen) according to the Supplementary Protocol: Purification of miRNA from animal cells (Figure 2.1). An Eppendorf 5417C micro centrifuge was used throughout the RNA extraction process. An initial centrifugation step of 12,000 g for 10 minutes at 4°C was used, before addition of chloroform, to bring excess fat to the surface and allow for its removal by pipetting.
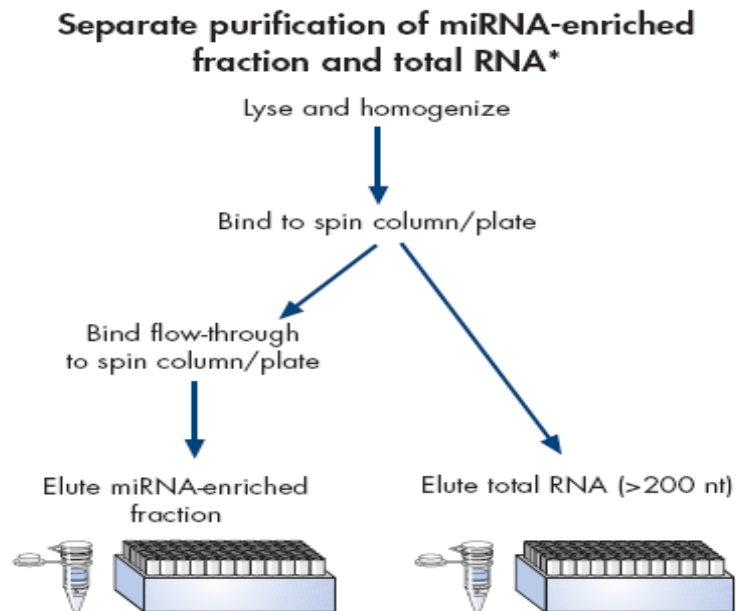
Figure 2.1 Separate purification protocol for isolation of large and small RNA from human samples, using RNeasy® Plus Mini Kit and RNeasy MinElute® Cleanup Kit (Qiagen)

One volume of 80% ethanol was added to the upper aqueous phase before being added to the RNeasy column. A centrifugation of 12,000g for 21 sec at 4°C was then used. 1.4 volumes of 100% ethanol were added to the flow-through from the RNeasy column and this was mixed thoroughly by vortexing. The RNeasy column was stored at 4°C for subsequent isolation of large RNA. The sample was passed through a MinElute column by centrifuging at 12,000g for 21 seconds at 4°C. The same

centrifugation conditions were used for two subsequent wash steps using 500μL Buffer RPE, from the Qiagen RNeasy mini kit, and 500μL 80% ethanol respectively. The small RNA was eluted from the MinElute column in 20μL RNase-free water by centrifuging at 12,000g for 1 minute at 4°C. The RNeasy column was removed from the 4°C and the column was washed with 350μL buffer RW1 by centrifuging at 12,000g for 21 seconds at 4°C.An optional DNase I treatment was then performed as follows. 80μL of DNase I mix, made using reagents from an RNase-free DNase set (Qiagen), were applied onto the membrane of the column and left at room temperature for 15 minutes. Following DNase treatment the buffer RW1 wash step was repeated. Two further wash steps, using 500μL of buffer RPE, were carried out. The second of these steps had an increased centrifugation time of 2 minutes to dry the membrane. The large RNA was eluted from the RNeasy column by applying 50μL RNase-free water to the membrane and centrifuging at 12,000g for 1 minute at 4°C. A portion of the purified large and small RNA was aliquoted for quantitative and qualitative analysis using NanoDrop1000 spectrophotometry (NanoDrop Technologies) and the Agilent 2100 Bioanalyzer (Agilent Technologies) respectively. The remaining RNA was stored at -80°C until further use.

2.3.2 Adipose Tissue RNA extraction

'Hot Trizol copurification technique':

A copurification technique, modified from the Tri Reagent BD (Molecular Research Centre) copurification protocol, was applied to adipose tissue to isolate total RNA. Approximately 100 mg of adipose tissue was homogenized in 1 mL of Trizol (Invitrogen) using a bench-top homogenizer as described in 2.3.1. In order to aid lysis of the adipose tissue, the homogenate was placed in a water-bath heated to 60°C, for 5 minutes[10]. To the heated lysate, 67μL of 1-bromo-4-methoxybenzene was added to augment the RNA phase separation process. The homogenous lysate was transferred to a 2 mL collection tube and then centrifuged at 12000g for 15 minutes, at 4°C. The clear aqueous phase (approximately 500μL) from each sample was carefully removed and transferred to fresh collection tubes respectively. RNA was precipitated by the addition of an equal volume of isopropanol followed by centrifugation of the solution at 12000g for 8 minutes at 18°C. Following removal of

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[10] http://www.ambion.com/techlib/basics/rnaisol/index.html

the supernatant, the RNA pellet was then washed with equal volume (approximately 500μL) of 75% ethanol. An additional ethanol wash was performed to improve the purity of RNA isolated, which was reflected in an improved 260/280 nm spectrophotometry ratio. Each RNA pellet was briefly air dried, then solubilised using 30μL of nuclease free water and transferred to storage tubes. A small volume (2 μL) of the total RNA solution was aliquoted for quantitative and qualitative analysis using NanoDrop1000 spectrophotometry and the Agilent 2100 Bioanalyzer (Agilent Technologies) respectively. The remaining RNA was stored at -80°C until further use.

2.3.3   Blood miRNA extraction

i.   Blood Collection

Whole blood was collected in Vacuette EDTA K3E blood bottles (Grenier Bio-one); one processed for plasma, another unprocessed, and a third sample collected in Vacutainer Serum Separator Tubes II (Becton Dickinson) for serum. Samples for serum collection were left to clot at room temperature for 30 minutes and then all samples destined for serum and plasma collection were centrifuged at 2000 rpm @ 4°C for 10 minutes. Plasma/serum was removed, aliquoted and stored at -20°C until required. The unprocessed whole blood sample was stored at 4°C until required.

ii.   RNA isolation

Protocol 1. Separate purification using column based methodology

Small RNA (miRNA) and large RNA (mRNA) was extracted from whole blood, serum and plasma samples as follows: In brief 250μL of thawed serum/plasma was mixed with 1 mL QIAzol® lysis reagent and 250μL of chloroform, and then centrifuged at 12000g for 15 minutes at 4°C. Large RNA fractions (> 200 nucleotides) and small RNA fractions (< 200 nucleotides) were isolated separately using Qiagen RNeasy kits (Qiagen) according to the manufacturer's instructions.

Protocol 2. Copurification of total RNA using Trizol

Total RNA was extracted from whole blood, serum and plasma samples using a modification of the Tri Reagent® BD (Molecular Research Centre) copurification protocol, as follows: Using 1 mL of whole blood, or its derivatives, phase separation was performed by the addition of 3 mL of Trizol and 200μL of 1-bromo-4-methoxybenzene to augment the RNA phase separation process. The homogenous lysate was divided between two 2 mL collection tubes and then centrifuged at 12000g for 15 minutes, at 4°C. The clear aqueous phase (approximately 1 mL) from each tube was then removed, transferred to fresh collection tubes respectively and RNA precipitated by the addition of 1 mL of isopropanol and centrifugation of the solution at 12000g for 8 minutes at 18°C. Following removal of the supernatant, the RNA pellet was then washed with 1 mL of 75% ethanol. An additional ethanol wash was performed to improve the purity of RNA isolated, which was reflected in an improved 260/280 nm spectrophotometry ratio. Each RNA pellet was briefly air dried and then solubilised using 30μL of nuclease free water. Hence each 1 mL of whole blood yielded 60μL of total RNA when the two matched RNA pellets were solubilised, combined, and finally transferred to storage tubes prior to storage at -80°C

2.4   Analysis of RNA concentration and integrity

2.4.1   MiRNA analysis

MiRNA concentration and purity was assessed using the NanoDrop1000® spectrophotometer (NanoDrop Technologies). A 1μL aliquot of RNA was pipetted onto the apparatus pedestal. The sample arm was used to compress the sample resulting in the formation of a sample column, held in place by surface tension. Spectral measurements were made with a tightly controlled pathlength of 0.1cm. RNA concentration was automatically calculated using the formula: RNA concentration (ng/μL) = (A260 x e)/b, where:

A260=Absorbance at 260nm, e=extinction coefficient (ng-cm/ml), b=pathlength(cm)

When analysing small RNA samples, 'Other' was selected as the sample type and an extinction coefficient of 33 was manually entered (Fig 2.2a). RNA with an absorbance ratio at 260 and 280 nm (A260/A280) between 1.8 and 2.2 was deemed indicative of pure RNA. The presence of protein or phenol results in high absorption at 280nm, producing a lower A260/A280 ratio. A ratio at 260 and 230 nm (A260/A230) between 1.8 and 2.2 was also considered acceptable. Lower ratios indicated the carry-over of guanidinium salts.
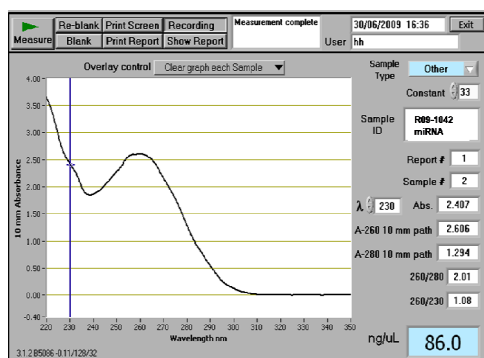
Figure 2.2a The concentration and purity of **miRNA** as assessed using the Nanodrop Spectrophotometer with a constant value of 33
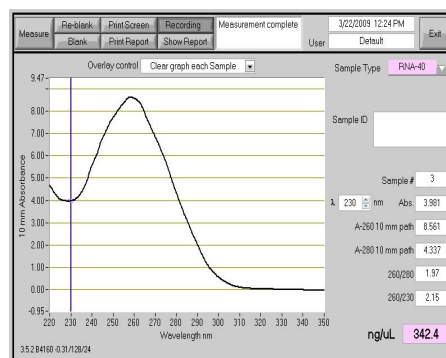
Figure 2.2b The concentration and purity of **large RNA** as assessed using the Nanodrop Spectrophotometer with a constant value of 40

The small-RNA enriched fractions were also analysed using the Small RNA Assay (Agilent Technologies), and the Agilent 2100 Bioanalyzer (Agilent Technologies). RNA samples loaded onto the Agilent chip were separated by capillary electrophoresis according to their molecular weight. The intensity of fluorescence on each sample's electropherogram represented the amount of RNA of a given size. The Small RNA Assay was chosen for its high resolution in the 6-150 nucleotide range, allowing verification of small RNA retrieval and comparison of the small RNA component between tissue samples. The Small RNA Assay was carried out according to the Agilent Small RNA kit guide. The electrodes were cleaned with RNase-free water for 5 minutes prior to use. To prepare the gel, the Small RNA gel matrix and small RNA dye concentrate were allowed to equilibrate to room temperature for 30 minutes; the latter reagent was protected from light throughout by covering the tube in tin-foil. The complete volume of gel was spun at 10,000g for 15

minutes. The dye concentrate was vortexed for 10 seconds and briefly centrifuged. In a new 0.5 mL RNase-free tube, 2μL of dye concentrate and 40μL of the filtered gel were mixed thoroughly by careful pipetting. The gel/dye mix was then spun at 13,000g for 10 minutes. Samples were diluted to 1 ng/μL, within the quantitative and qualitative range of the assay. The RNA samples and RNA ladder were denatured at 70ºC for 2 minutes and then placed on ice prior to use.

2.4.2   Total and large RNA Analysis

The concentration and purity of the large (>200 nt) and total RNA were assessed using the NanoDrop 1000 spectrophotometer (NanoDrop Technologies). The sample type 'RNA-40' was selected (Fig 2.2b).   Spectrophotometry was performed and interpreted as per section 2.4.1. Integrity of the total and large RNA fraction was assessed using the RNA 6000 Nano LabChip Series II Assay (Agilent Technologies) and the Agilent 2100 Expert software (Version B.02.03) This assay generates an RNA integrity number (RIN) for each large or total RNA sample based on the ratio of ribosomal bands and also the presence or absence of degradation products on the electrophoretic image. A threshold of RIN 7 was applied, ensuring that only RNA of good integrity was used in these experiments.

2.5   Extraction of high molecular weight genomic DNA

DNA was purified from 10 mL samples of whole blood using the Chemagic Magnetic Separation Module (Chemagen) using the manufacturer's reagents. The DNA extraction was performed on samples in batches of 12. Firstly, magnetic beads and DNA binding buffer were mixed by adding 1 mL of magnetic beads to each tube, followed by prefilled 28 mL DNA binding buffer 2. The blood lysate was prepared separately; 10 mL blood was added to a tube prefilled with lysate buffer, followed by 50 µL of protease, and the tubes vortexed for 10 seconds prior to a 10 minutes incubation period in a water bath at 65ºC. Immediately following this incubation period, the magnetic bead/DNA binding buffer solution was added to the blood lysate and vortexed for 10 seconds, which stops the protease reaction. The

DNA was then purified using the Chemagic Magnetic Separation Module[11]. Following transit through a horizontal tracking system incorporating 4 separate washing buffers, the purified DNA was eluted in TE and diluted to 25 ng/µL for genotype analysis (Figure 2.3). The DNA concentration and quality was analysed on a NanoDrop spectrophotometer at the 'DNA 50' extinction coefficients.

Figure 2.3 The Chemagic Magnetic Separation Module
The magnetic separation is achieved through the use of an electromagnet, and magnetisable and rotatable rods which are immersed into a magnetic bead suspension. Thereby the normally difficult resuspension step in manual DNA isolation techniques is very fast and efficient when using the Chemagic MSM, resulting in high consistency, yield and purity.

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[11] http://www.chemagen.com/fileadmin/downloads/EFI2006_chemagen_MHH.pdf

2.6   Reverse transcription: complementary DNA (cDNA) synthesis

2.6.1   Breast tissue: miRNA to cDNA

Small RNA (5 ng) was reverse transcribed using MultiScribe Reverse Transcriptase (Applied Biosystems). Each reaction was primed using a miRNA-specific stem-loop primer. Where sequences were available, primers were obtained from MWG Biotech. Otherwise, assays containing the RT stem-loop primer and the PCR primers and probes were used (Applied Biosystems). MiRNA was reverse-transcribed as follows:

Small RNA (1ng / μL)	5.0 μL
dNTPs (100 mM)	0.17 μL
10X RT Buffer	1.65 μL
Nuclease-free water	4.57 μL
RNase inhibitor (20U / μL)	0.21 μL
Stem-loop primer (50 nM)	3.1 μL
MultiScribe RT (50U / μL)	1.1 μL

Samples were incubated at 16ºC for 30 minutes, 42ºC for 30 minutes and finally 85ºC for 5 minutes to denature double-stranded duplexes. The reaction was performed using an AB9700 GeneAmp thermal cycler (Applied Biosystems). An RT-negative control was included in each batch of reactions.

2.6.2   Blood samples: miRNA to cDNA

RNA isolated from blood, serum and plasma samples was reverse transcribed as described above in section 2.6.1., but with starting concentrations of 100 ng of small RNA (as determined by measuring the concentration of the miRNA component of total RNA using the '33' setting on the spectrophotometer).

2.6.3   Adipose tissue cDNA synthesis

cDNA was synthesised from total RNA isolated from adipose tissue specimens as described in section 2.6.1., but with starting concentrations of 100 ng of small RNA per reaction.

2.7 Adipose Tissue miRNA Quantitative PCR Array

MiRNA profiling of adipose tissue was performed using a customised MicroRNA Array with QuantiMir system (System Biosciences), which is a real-time PCR-based array containing a panel of 95 metabolic-related miRNA assays and the U6 transcript as a proposed normalization signal.

2.7.1 Total RNA reverse transcription to cDNA

Total RNA from adipose tissue was reverse transcribed using QuantiMir™ RT System (System Bioscience) as follows:

RNA (100ng/µL)	5.0 µL	
PolyA Buffer (5X)	2.0 µL	
$MnCl_2$ (25mM)	1.0 µL	37°C 30 minutes
ATP (5mM)	1.5 µL	
PolyA Polymerase	0.5 µL	

After this initial RNA denaturation step, primers were annealed:

Oligo dT Adaptor (2.5pmol/ µL)   0.5 µL   60°C 5 mins / 21°C 2 mins

The following were then added to synthesise cDNA:

RT Buffer (5X)	4.0 µL	
dNTPs (10mM)	2.0 µL	
DTT (0.1M)	1.5 µL	42°C 60 mins / 95°C 10 mins
RNase-free H_2O	1.5 µL	
Reverse Transcriptase	1 µL	

This mixture (20.5 µL in total) was incubated for at 42°C for 60 minutes and at 95°C for 10 minutes on an AB9700 GeneAmp thermal cycler (Applied Biosystems).

2.7.2 Real-time qPCR reaction

Real-time quantitative PCR array profiling was performed for each sample in 96-well qPCR optical plates which contained sequence specific primers suspended in each well (Figure 2.4). For one entire 96-well qPCR plate the following mastermix was synthesised;

2X SYBR Green qPCR Mastermix	1750 μL
Universal Reverse Primer (10 μM)	60 μL
RNase-free water	1670 μL
QuantiMir™ cDNA	20 μL

RQ-PCR cycling was carried out using an AB7900HT instrument (Applied Biosystems). PCR reactions were initiated with a 2 minute incubation time at 95°C followed by 40 cycles of 95°C for 15 seconds and 60°C 60 seconds. After each PCR run, an additional melt analysis was performed to assess the Tm of the PCR amplicon; this verified the specificity of the amplification reaction.

Plate Array Arrangement

	1	2	3	4	5	6	7	8	9	10	11	12
A	let-7-family	miR-7	miR-92	miR-93	miR-9-1	miR-101-1	miR-103	miR-106a	miR-106b	miR-107	miR-10b	miR-1-1
B	miR-122a	miR-125a	miR-125b	miR-126	miR-128b	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-17-3p	miR-17-5p	miR-181a	miR-181b	miR-181c	miR-181d	miR-183	miR-185	miR-186	miR-188
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-3p	miR-30a-5p	miR-296	U6 snRNA

Figure 2.4. 96 human miRNA probes on the array have published implications with regard to potential roles in cell development, proliferation and apoptosis. The array plate also includes the U6 transcript as a normalization signal (well H12).

2.7.3 Array data processing

The qPCR arrays were performed on total RNA isolated from ten human adipose tissue samples; 6 of which were from morbidly obese patients (BMI > 40) and 4 from healthy individuals with BMI < 25 who served as controls. Analysis of the array data was performed as follows: The fold change of miRNA gene expression was calculated by the equation $2^{-\Delta\Delta Ct}$, where Ct is the cycle threshold. The cycle threshold (Ct) is defined as the number of cycles required for the fluorescent signal to cross the threshold in qPCR. ΔCt was calculated by subtracting the Ct values of the endogenous control (proposed EC for this array is U6 snRNA) from the Ct values of the miRNA of interest. $\Delta\Delta$Ct was then calculated by subtracting ΔCt of the control from ΔCt of disease (obese cases).

2.7.4 Bioinformatic analysis of array data; significance analysis of microarray

Significance analysis of microarrays (SAM) is a statistical technique establishes in 2001 by Tusher *et al* [140] for determining whether changes in gene expression are statistically significant. Normalised gene expression measurements from the microarray experiments were input to the TM4 MultiExperiment Viewer (MeV) freely available from TIGR (the Institute for Genomic Research)[12]. In addition, response variables from each experiment were input. The response variable may be a grouping [for example obese/not obese, omental fat/subcutaneous fat, or diabetic/not diabetic]. These can be paired or unpaired. SAM was used to identify genes with statistically significant changes in gene expression using a set of gene specific *t-tests*. The programme was designed to compute a statistic for each gene, measuring the strength of the relationship between gene expression and the response variable. This analysis uses non-parametric statistics, since the data may not follow a normal distribution. It uses repeated permutations of the data to determine if the expression level of any gene is significant in relation to the response. The cut-off for significance is determined by a tuning parameter *delta*, chosen by the user based on the false positive rate. One can also choose a fold change parameter to ensure that genes called 'significant' change at least a pre-specified amount. False Discovery Rate (FDR) is the percentage of such genes that are identified by chance. The *delta* threshold can be adjusted to obtain smaller/larger sets of genes and a new FDR is

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[12] http://www.tm4.org/documentation/TM4_Biotechniques_2003.pdf

calculated with each set. SAM was used in this miRNA array experiment to identify miRNAs that were differentially expressed in human adipose tissue according to the following response variables: obese status, omental vs. subcutaneous fat, presence of metabolic syndrome. This analysis was performed using TiGR Tools TMeV 4.0 Java version 1.5.0_04-b05. Differentially expressed miRNAs identified from these analyses were selected for validation by RQ-PCR in a larger cohort of fresh frozen adipose tissues from obese and non-obese individuals.

2.8   Real Time Quantitative Polymerase Chain Reaction (RQ-PCR)

RQ-PCR allows accumulating amplified DNA/cDNA to be detected and measured as the reaction progresses, i.e. in real time. It is possible to detect the amount of amplified product by incorporating a DNA-binding dye or fluorescently-labelled gene-specific probe in the reaction. The RQ-PCR reaction consists of an exponential phase, in which the amount of amplified product approximately doubles during each cycle of denaturation, primer annealing and template extension, and a non-exponential or plateau phase in which reduced reagents limit the reaction. The point at which enough amplified product has accumulated to produce a detectable fluorescent signal is known as the threshold cycle or Ct and the greater the amount of starting template, the lower the Ct value.

2.8.1 RQ-PCR of miRNA

RQ-PCR reactions were carried out in final volumes of 10 μL using an AB7900HT. Reactions consisted of

First strand miRNA-specific cDNA	0.7 μL
TaqMan Fast Master Mix (2X)	5.0 μL
TaqMan Probe (0.2μM)	0.5 μL
Forward primer (1.5μM)	1.5 μL
Reverse primer (0.7 μM)	0.7 μL
Nuclease-free water	1.68 μL

Standardised 'Fast' thermal cycling conditions were applied. This consisted of 40 cycles at 95ºC for 15 seconds and 60ºC for 60 seconds. CDNA, synthesised from

pooled normal breast tissue, was included on each 96-well plate as an interassay control and calibrator. All reactions were performed in triplicate to permit determination of intra-assay variation. The threshold standard deviation for intra- and inter-assay replicates was 0.3.

2.8.2 PCR amplification efficiencies

In a PCR reaction with optimised primer conditions, reagent concentrations *etc*, the amplification efficiency should approach 100% in the exponential phase, i.e. a doubling of amplification product for each cycle. To determine the amplification efficiency of the RQ-PCR miRNA assays, serial dilutions (neat to 10^{-6}) of cDNA template were prepared and amplified using the same conditions used for subsequent gene expression analyses. A dilution curve was constructed by plotting Ct versus the dilution factor of cDNA. Amplification efficiencies (E) were calculated for each RQ-PCR assay using the formula:

$$E = (10^{-1/slope} - 1) \times 100$$

(slope is that of the dilution curve)

The R^2 value of the dilution curve represents the linearity of the data. R^2 values should be ≥0.98 for each dilution curve. Amplification efficiencies between 90-110% indicate a relatively robust and reproducible RQ-PCR assay.

2.8.3 Endogenous controls

To produce reliable RQ-PCR data, corrections must be made for variation between reactions, which may have been introduced during any of the steps from sample preparation through to target amplification. The optimal means of correcting for technical and biological sources of variation (e.g. differences in sample procurement, RNA extraction, the amount of starting template, RNA quality, enzymatic efficiencies, or even sample-to-sample inconsistencies in cellular subpopulations), is to normalise RQ-PCR data to an endogenous control gene (EC). In fact the accuracy of RQ gene expression analysis is critically dependent on proper normalisation of the data in so far as inappropriate normalisation of RQ-PCR data can lead to incorrect conclusions [141]. A reliable EC gene is ideally stably expressed across a whole sample set. Ideally, a normaliser or EC is a single nucleic acid that exhibits invariant expression across all samples, is expressed along with the target in the samples of interest, and demonstrates equivalent storage stability, extraction, and quantification

efficiency as the target of interest. In reality, such a normaliser does not exist [142]. In the case of mRNA, multiple published reports have argued for combinations of transcripts or ribosomal RNA (rRNA) normalisers as part of an empirical strategy to minimise unwanted variation [142-143]. *MRPL19* and *PPIA* have previously been recommended EC genes for RQ-PCR expression of mRNA in breast tissue [144]. While ECs have been validated for quantification of mRNA expression in various experimental settings, similar reports of validated ECs for miRNA expression are limited. The Department of Surgery at NUI Galway published the first report documenting reliable EC genes for RQ-PCR expression of miRNAs in breast tissue; *miR-16* and *let-7a* [64]. As EC genes are tissue and organ specific, it would not be appropriate to infer that a normalising gene for one tissue type would be an equally reliable normaliser for another tissue type. At the time of writing, there was no consensus as to the appropriate ECs for miRNA expression in blood. Small nuclear RNA U6 (45 nucleotides) and rRNA 5S (121 nucleotides) had previously been claimed to have reasonable expression stability in a variety of tissue types and thus were used in several published miRNA RQ-PCR studies [145-146], however their stability in blood remained questionable [63,147]. *MiR-16* is abundantly and stably expressed in various tissue types from normal healthy individuals and patients with a variety of diseases [63]. Its expression in the circulation has also been documented to be unaltered in the presence of malignancy or other disease processes [130,132,135]. Hence circulating *miR-16* appears to be the most reliable miRNA reference gene in this context to date and was the EC used to normalise all circulating and adipose tissue miRNA RQ-PCR expression data in these studies. It is clear however that further work is needed to ascertain other reliable miRNA reference genes, in addition to *miR-16*, for the identification of an accurate normalisation protocol.

2.8.4 Relative quantitation of miRNA expression

To accurately and reliably determine gene expression values, raw fluorescence data (Ct values) generated by the real-time PCR instrument (Applied Biosystems) were exported to qBase plus software[13], the purpose of which was to scale raw data to an internally defined calibrator (Cal) and an endogenous control gene(s) so that the following formula could be applied to generate relative quantities:

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[13] http://www.biogazelle.com/products/qbaseplus

$$\Delta Ct = \text{Average Ct of test sample - Average Ct of calibrator}$$

The ΔCt values were converted to a linear form using the formula: $E^{-\Delta Ct}$, where E= amplification efficiency determined in section 2.9.2. In order to correct for non-biological variation in gene expression potentially introduced during the RQ-PCR process, *miR-16* was used as EC to normalise the RQ expression data (as described in detail in section 2.8.3) To calculate the expression of a target gene (TG) relative to the EC, the comparative Ct ($\Delta\Delta Ct$) method [148] was used as per the following equation:

$$\Delta\Delta Ct = \underbrace{\text{(Ct Target gene)-(Ct EC)}}_{\text{Test Sample}} - \underbrace{\text{(Ct Target gene)-(Ct EC)}}_{\text{Calibrator}}$$

The $\Delta\Delta Ct$ values were converted to a linear form using the formula: $E^{-\Delta\Delta Ct}$.

2.8.5 Statistical analysis of RQ-PCR data

All RQ-PCR data were log-transformed and exported to a statistical software package (SPSS Version 17.1 for Windows) for analysis. The distributions of data were tested for normality using the Kolmogorov-Smirnov test, and normal distribution of logged data was illustrated using histograms, prior to applying parametric analyses to the logged data. One way ANOVA and independent *t-tests* were used to compare the mean RQ expression values of independent samples. Correlation analysis using Pearson's correlation coefficient was performed where appropriate, and paired t-tests were used to assess related samples. All tests were two tailed and results with a $p<0.05$ were considered statistically significant. To determine sensitivity and specificity of miRNA expression levels in distinguishing cancer cases form controls, Receiver Operating Characteristic (ROC) curves were constructed and the area under the curve (AUC) was calculated. The AUC is a surrogate marker of the ability of each miRNA to differentiate between cancer cases and controls; by computing sensitivity and specificity for each possible cut-off point of the individual miRNAs. This was performed univariately for each individual miRNA, and multivariately for combinations of target miRNAs in our panel via logistic regression analysis.

2.9 *In situ* **hybridisation of miRNA**

In order to localise miRNA expression *in vivo,* and confirm miRNA expression levels in breast tumours, *in situ* hybridisation (ISH) analysis for *miR-195* was performed on formalin-fixed, paraffin-embedded (FFPE) sections of breast tumours using locked nucleic acid (LNA)/DNA probes (Exiqon) as follows.

6µm thin sections of FFPE tissues were adhered to glass slides as illustrated in Figure 2.5. Each section was then deparaffinized in three consecutive xylene baths for 1 min each, followed by 1 min each in serial dilutions of ethanol (100%, 100%, 95%, 95%), and three changes of diethyl pyrocarbonate–treated water. Each slide was then subjected to the following protocol:

- Immersed in 0.3% H_2O_2 for 30 min at room temperature
- Washed three times with diethyl pyrocarbonate–treated water
- Digested with proteinase K (2g/ml) (Roche) at 37°C for 15 min
- Washed three times with diethyl pyrocarbonate–treated water
- Submerged in 95% ethanol for 1 min, then air-dried completely

Slides were then hybridized to the LNA-modified miRNA probes (for *miR-195*, *miR-126* and *miR-205*) for 2hr at 55°C, using double digoxygenin (DIG)-labelled LNA oligonucleotide (60 nM) in a hybridization buffer (Exiqon). *MiR-195* was the primary miRNA of interest given its documented association with breast cancer; *miR-126* was selected because it recognizes endothelial cells therefore could distinguish vasculature from epithelium; *miR-205* is expressed in myoepithelial cells and could therefore be useful in differentiating invasive from non-invasive tumours by observing its expression in the myoepithelial layer next to the basement membrane.

The slides were then washed with 0.2x SSC at room temperature. DIG was detected using sheep anti-DIG-alkaline phosphates (APs) (Roche Diagnostics) according to the manufacturer's protocol. The slides were incubated with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate in AP buffer containing 0.2 mM levamisole for 2hr, counterstained with Nuclear Fast Red (Vector Laboratories) and mounted with Eukitt (VWR). Adjacent tissue sections were hybridized with *miR-205* and *miR-126*

probes to verify RNA preservation in the tissue, or a scrambled probe (negative control).
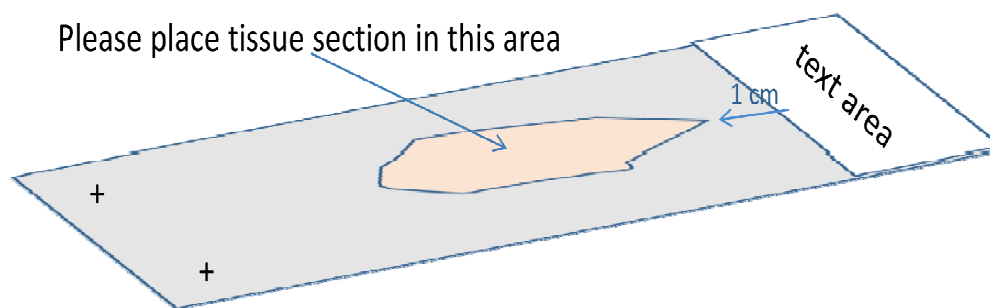
Figure 2.5. Preparation of FFPE tissue specimens for miRNA ISH analysis

2.10   SNP genotyping

2.10.1   SNP genotyping reactions

The DNA isolated from all blood samples was amplified using a customised TaqMan genotyping assay which was designed specifically to distinguish between the inheritance of either T or G alleles of the LSC6 single nucleotide polymorphism in the *KRAS* oncogene on chromosome 12 (Applied Biosciences)[14]. The assay mix contained fluorescently labelled primers and probes to detect and amplify variant alleles in the purified genomic DNA samples using the 5' exonuclease activity of Taq polymerase. In brief, the genotyping reactions were carried out as follows:

Purified gDNA (25 ng)	5 µL
TaqMan SNP assay (20X)	0.625 µL
Master Mix (2X)	6.25µL
Nuclease-free water	4.375 µL

The PCR reactions were run in on the AB7900HT instrument using the following thermal cycling conditions: PCR reactions were initiated with 10 minute incubation at 95ºC followed by 40 cycles of 92ºC for 15 sec and 60ºC for 60 sec.

Two no-template controls were included on each 96-well plate in addition to a known DNA control. The protocol is summarised in Figure 2.6.

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[14] www.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_042998.pdf

2.10.2   Determination of genotype

A standard curve was generated to quantify the amount of DNA in each sample. After PCR amplification, an endpoint plate read was performed using the SDS software on the AB7900HT instrument. The Sequence Detection System (SDS) Software uses the fluorescence measurements made during the plate read to plot fluorescence (Rn) values based on the signals from each well. The plotted fluorescence signals indicate which alleles are in each sample (Figure 2.7). Samples heterozygous or homozygous for the variant G allele were considered positive for the *KRAS*-variant, based on prior studies published by the group at the Department of Molecular and Cellular Biology at Yale University, USA [149].
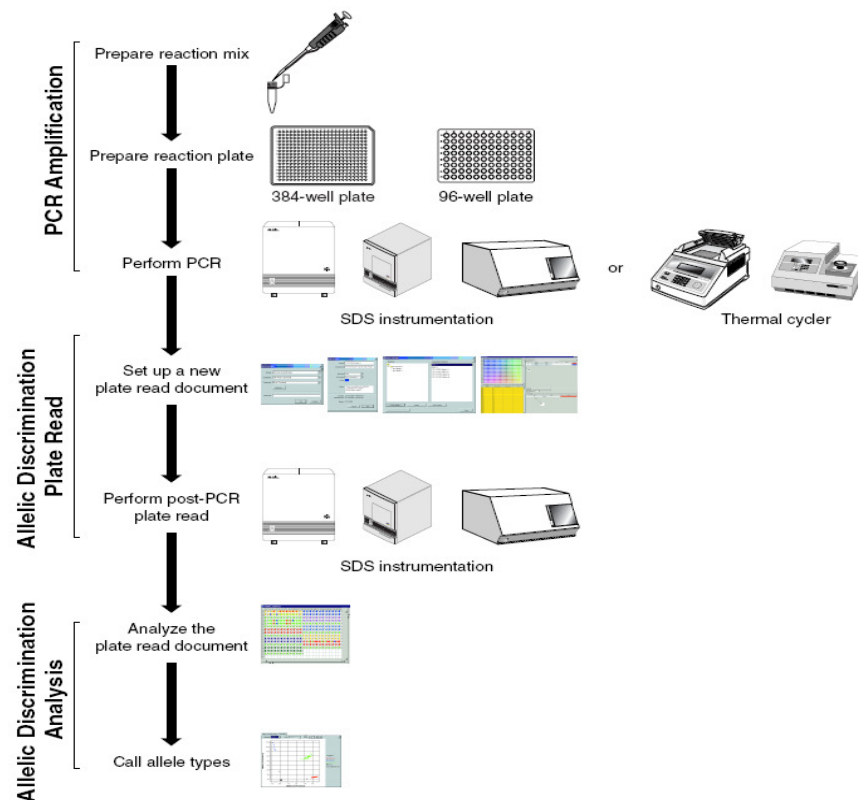
Figure 2.6 SNP genotyping procedure
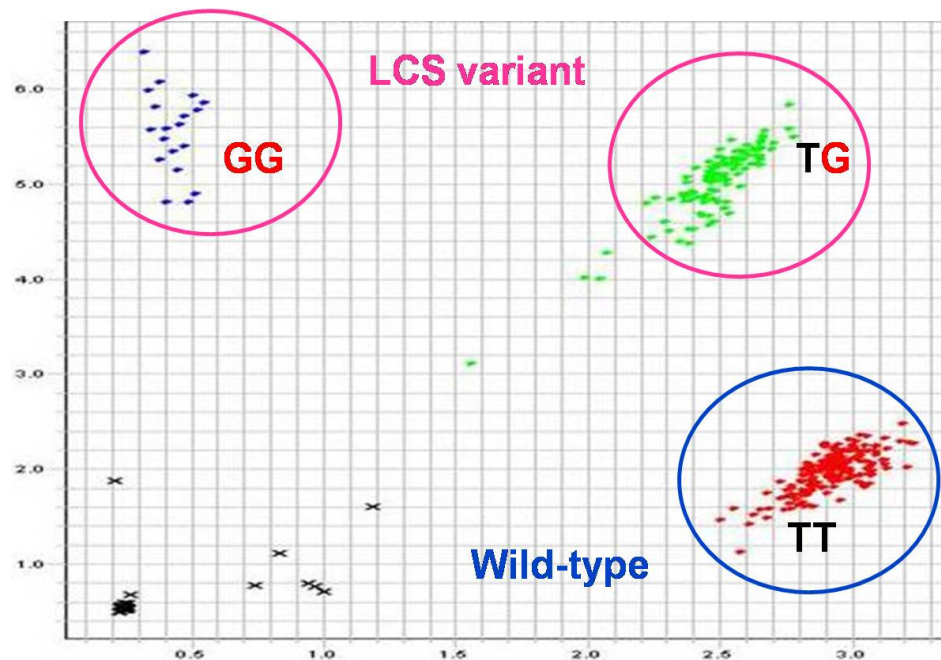
Figure 2.7 Determination of SNP genotype by PCR
Samples heterozygous or homozygous for the variant G allele were considered positive for the *KRAS*-variant

2.10.3   Statistical analysis

The genotype distributions of the controls were tested for Hardy-Weinberg equilibrium. Unconditional logistic regression was performed to estimate the relative risk associated with each genotype. Controls were adjusted for age and ethnicity (Caucasian, African-American, Hispanic, or other). The population was stratified by menopausal status, and separate risk estimates were obtained by ER and PR status using multinomial logistic regression with a three-level outcome variable (coded as 0, 1, or 2 for controls, ER/PR positive cases, and ER/PR negative tumours, respectively). Tests for interaction were conducted using a Wald chi-square comparing the parameter estimates obtained for each genotype in ER and/or PR positive cases versus ER/PR negative cases.

In order to determine the association of the *KRAS*-variant with a particular breast cancer subtype, the patients were stratified according to the subtype of breast cancer and a χ^2 test was performed using SPSS software to calculate two-sided p-values, OR, and 95% CI. The dominant model was used for all genetic association analysis due to the low frequency of the rare allele.

2.11 Candidate miRNA selection

2.11.1 Candidate miRNAs for breast cancer studies

The selection of candidate miRNAs for expression analysis in these breast cancer studies was based on the following rationale:

i. The miRNAs chosen were previously documented to be dysregulated in breast tumour tissues, in literature reporting early miRNA microarray studies and subsequent validation reports.

ii. A breast tumour miRNA microarray performed in the Department of Surgery at NUI Galway yielded a number of aberrantly expressed miRNAs in breast tumour tissue [111]. These included *miR-10b, miR-21, miR-145, miR-155,* and *miR-195,* to name but a few. These 5 miRNAs, as well as two validated endogenous control miRNAs for breast tissue (*let-7a* and *miR-16*), were selected for validation by RQ-PCR in a larger, more heterogeneous group of breast tumour tissues. Investigating for the expression of these same miRNAs in the circulation of breast cancer patients was another important objective.

Table 2.5 illustrates the panel of miRNAs selected for investigation in breast cancer patients in these studies, with justification for their inclusion.

2.11.2 Candidate miRNAs for obesity and metabolic syndrome studies

The investigation of miRNAs in obesity and the metabolic syndrome is still in the embryonic phase and few miRNAs are known to be associated with these disease processes. A miRNA microarray was therefore performed to identify candidate miRNAs which are dysregulated in the adipose tissue and circulation of obese patients.

Table 2.5 Candidate miRNAs for investigation in breast cancer patients (tumour tissue and the circulation)	
miRNA of interest	**Previous association with breast cancer**
miR-10b	Decreased expression in breast tumour tissue compared to normal breast tissue
miR-21	Increased expression in breast tumour tissue compared to normal breast tissue. Also increased in other solid cancers: colorectal, pancreas, gastric, lymphomas
miR-145	Decreased expression in breast tumour tissue compared to normal breast tissue
miR-155	Increased expression in breast tumour tissue compared to normal breast tissue
miR-195	Reported by *Mattie et al* to be associated with hormone receptor status, as part of a 'miRNA signature'
let 7a	Reliable endogenous controls for analysis of miRNA by RQ-PCR in human breast tissue, with *miR-16*
miR-16	Reliable endogenous controls for analysis of miRNA by RQ-PCR in human breast tissue, with *let-7a* and used as a single endogenous control for investigating serum miRNA levels in recent studies.

2.12 Target prediction of miRNAs

2.12.1 Computational target prediction

Identification of genes targeted by miRNAs is a critical step toward understanding the role of miRNAs in gene regulatory networks. Identifying miRNA targets *in vivo* has been very challenging largely because of the limited complementarity between miRNAs and their mRNA targets, which can lead to the finding of hundreds of potential targets per miRNA [150]. As part of the effort to understand interactions between miRNAs and their targets, computational algorithms have been developed based on observed rules for features such as the degree of hybridisation between the two RNA molecules [151].

There are 4 principle steps involved in the *in silico* approach to miRNA target prediction [151]:

   i.   Extraction of rules related to formation of miRNA–mRNA duplexes (See Figure 2.8).

   ii. Incorporation of those rules in computational algorithms

iii.   Prediction of novel miRNA target sites using those algorithms

iv.   Validation of the results, and thus the algorithm itself, using computational and experimental approaches.
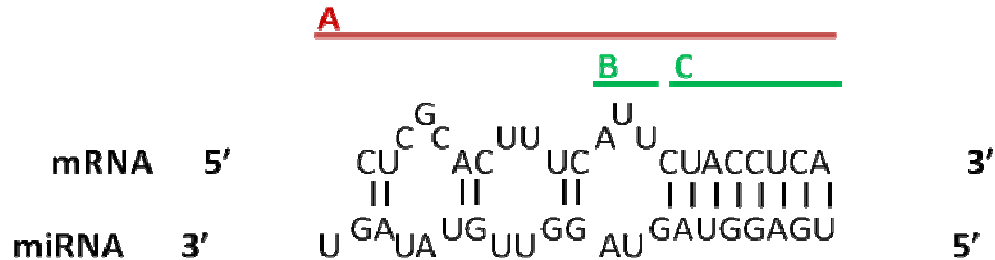
Figure 2.8 A typical pattern of base pairing between miRNA and its target mRNA
Typically, the miRNA binds to a specific site or sites within the 3′UTR region of the mRNA sequence. According to thermodynamic analysis, some degree of complex formation occurs along the entire miRNA–mRNA duplexed region (A). Base pairing is particularly weak in the central region (B) and particularly strong at the 5′ end (seed region) of the miRNA (C). These aspects are commonly used to identify putative novel binding sites. Base pairing between *let-7* miRNA and *hbl-1* mRNA in *C. elegans* is shown as an example (Lin *et al,* 2003).

Computational and experimental approaches have revealed that not only is the interaction between an individual miRNA and its mRNA target important, but also the relationship between several miRNAs targeting a single gene sequence [152-153]. Numerous target prediction resources and software programmes for miRNA target prediction are available, all of which place emphasis on the seed sequence of miRNA and the 3'untranslated (UTR) of the mRNA sequence (Table 2.6). In most of these bioinformatic algorithms the thermodynamic properties of the miRNA–mRNA duplex formation are determined by calculation of free energy (ΔG), which is considered an important aspect for evaluation and target determination. The Vienna package, which can be used to estimate free energy and secondary structure, is the most commonly used software tool for thermodynamic analysis employed by the target prediction resources [154]. Whilst most tools are designed to reduce the false-positive rate of target identification and maximize the accuracy at the same time, there are wide variations between tools because their individual sets of predicted target genes do not overlap well.

2.12.2   Target prediction tools

Three of the most commonly used programmes for miRNA target prediction in vertebrates are:

- miRanda (miRBase) [32,155-156]
- TargetScan [45,157-158]
- PicTar [159]

These three are frequently used for performance comparisons or as pre-processors for other tools to obtain initial putative target sites.

2.12.3   miRBase

The miRNA registry, miRBase, is a well-known and widely used database of miRNA sequences [155-156] initially developed to assign uniform names to miRNAs and it remains open to log newly identified miRNAs[15]. MiRBase has been expanded to include not only miRNA sequence data, but also information about the potential genomic targets of miRNAs. The miRBase Target database provides predicted miRNA target genes for various species, relying on the miRBase sequence database as a data source for miRNA sequences and on the miRanda software as a miRNA target prediction algorithm [161,175]. To determine putative targets the miRNA sequence of interest is scanned against the 3'UTR of all available species in Ensembl [176-177]. It scores complementary sites between 0 and 100, where a score of 0 represents no complementarity and 100 represents perfect complementarity. The scoring system is weighted for complementarity at the 5' end of the miRNA. An algorithm score (S score) is then calculated based on base pairing complementarity. The next step is to determine a free energy (ΔG) score of the resulting duplex; this is performed using the RNA lib package [154]. Cut-offs for S and ΔG score must be met before conservation of the 3' UTR target sites is examined across species. For a site to be conserved it must be present at the same position in a cross-species orthologous UTR alignment by a miRNA of the same family. The positions of the target site are permitted a shift of ±10 residues, and the sequence identity need not be perfect (e.g. 90% identity may be a sufficient requirement). Each target must also be conserved in at least two species for inclusion in the miRBase database.

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[15] http://microrna.sanger.ac.uk/

2.12.4   TargetScan

TargetScan is an algorithm developed by Lewis *et al* in 2003, for prediction of miRNA targets in vertebrates [158]. It requires conservation in the human, mouse, rat, dog and chicken genomes, in determining putative mRNA targets for a miRNA. It requires a 6 nucleotide match strictly comprising nucleotides 2-7. A mRNA is declared a target of a miRNA if there is a conserved seed match and a conserved anchoring adenosine nucleotide on the 3'UTR downstream of the seed region, or a conserved m8-t8 match (i.e. An A:U or G:C match between the eight nucleotide of the miRNA, and the corresponding position in the 3'UTR, or both). This algorithm can predict over 5300 human genes as potential targets of miRNAs which suggests that over one third of human genes are controlled by miRNAs.

2.12.5   PicTar

PicTar software fully relies on comparative data from several species to identify common targets for miRNAs [159]. PicTar also computes the maximum likelihood that a given sequence is bound by one or more miRNAs. The PicTar algorithm identifies 'seed matches' which are 7 nucleotide segments in the 3'UTR region of a mRNA and which have perfect Watson-Crick complementarity to the miRNA of interest. The seed match region must commence at nucleotide 1 or 2 of the miRNA. Conservation of this seed match sequence is compared across species (usually human, mouse, chimp, rat, dog and chicken) the free energy of the miRNA:mRNA duplex is calculated and compared to an established cut-off. A score is computed for each alignment and the average of these scores across all species is reported in the PicTar predictions. Target genes are first predicted using common criteria, such as optimal binding free energy, and are then tested statistically using genome-wide alignment of eight vertebrate genomes to filter out false positives. The false-positive rate for PicTar has been estimated to be about 30%, and known miRNA target sequences were identified correctly using this software. This algorithm suggests that on average approximately 200 transcripts are regulated by a single miRNA. TargetScan appears to be the superior programme currently, as it consistently demonstrates the best performance in comparisons; however because it only considers stringent seed sequences, there is a possibility that it ignores many potential targets. In practice it is routine to perform miRNA target searches in all

three software programmes, and the common mRNA targets that emerge from all three target lists are considered the most likely putative gene targets.

Table 2.6 Computational software programmes for microRNA target prediction			
Software programme	**URL**	**Organism(s)**	**Reference(s)**
TargetScan	http://genes.mit.edu/targetscan/	Vertebrates	[45]
miRanda	http://www.microrna.org/	Flies, vertebrates	[160-162]
DIANA-microT	http://diana.pcbi.upenn.edu/DIANA-microT/	Vertebrates	[163]
RNAhybrid	http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/	Flies	[164]
GUUGle	http://bibiserv.techfak.uni-bielefeld.de/guugle/	Flies	[165]
PicTar	http://pictar.bio.nyu.edu/	Nematodes, flies, vertebrates	[159,166-167]
MicroInspector	http://mirna.imbb.forth.gr/microinspector/	Any	[168]
MovingTargets	Available by request on DVD	Flies	[169]
FastCompare	http://tavazoielab.princeton.edu/mirnas/	Nematodes, flies	[170]
miRU	http://bioinfo3.noble.org/miRNA/miRU.htm	Plants	[171]
TargetBoost	https://demo1.interagon.com/demo/	Nematodes, flies	[172]
rna22	http://cbcsrv.watson.ibm.com/rna22.html	Nematodes, flies, vertebrates	[173]
miTarget	http://cbit.snu.ac.kr/~miTarget/	Any	[174]

2.12.6   Putative mRNA targets for breast cancer and metabolic diseases

Predicted targets of specific miRNAs associated with breast cancer (including *miR-195, let-7a, miR-342* and *miR-181c*) and obesity (*miR-17-5p, miR-34a, miR-99a, miR-122, miR-132, miR-143,* and *miR-145*) were determined by searching the aforementioned validated target programmes (miRBase, TargetScan and PicTar). This process yielded putative mRNAs with a known role in breast cancer or other cancer-associated signalling cascades, and in metabolic diseases (Table 2.7).

Table 2.7 Putative mRNA targets of miRNAs involved in obesity and the metabolic syndrome

MiRNA	Target Tissue	Function	Target Gene
miR-103	Adipose	Adipocyte differentiation	PANK1
miR-143	Adipose	(pre)Adipocyte differentiation	MAPK7
miR-132	Adipose	Adipocyte proliferation and growth, insulin resistance	CREB
miR-17-5p	Adipose	Adipocyte clonal expansion, insulin resistance	RBL2
miR-99a	Adipose, Liver	Fatty acid metabolism, Cholesterol biogenesis	IGF1R, CYP26B1
miR-29a, b	Adipose, Liver, Kidney, Muscle	Glucose transport, Amino acid metabolism, insulin resistance	INSIG1, CAV2, BCKHA
miR-122	Liver	Cholesterol biosynthesis, cellular stress response, Hepatitis C virus replication	PMVK, TRPV6, BCL2L2, CCNG1, HMGCR
miR-145	Colon	Cell proliferation	IRS1
miR-375	Pancreas	Insulin secretion, Pancreatic islet development	MTPN, USP1, JAK2, ADIPOR2
miR-124a	Pancreas	Pancreatic islet development	FOXA2, RAB27A
miR-9	Pancreas	Insulin secretion	ONECUT2
miR-133	Heart	Long QT syndrome, cardiac hypertrophy,	HERG, RHOA, CDC42, WHSC2
miR-192	Kidney	Kidney and diabetic nephropathy development	SIP1

Chapter 3

Evaluation of Circulating MiRNAs in Breast Cancer

3.1 Introduction

Current challenges in the management of breast cancer include a continuing search for sensitive and specific minimally invasive biomarkers that can be exploited to detect early neoplastic changes, thus facilitating the detection of breast cancer at an early stage, as well as for monitoring the progress of patients with breast cancer and their response to treatments. Existing diagnostic tools and biomarkers for breast cancer have many inherent deficiencies. Mammography is currently the gold standard diagnostic tool however it is not without limitations, including its use of ionizing radiation and a false positive rate of 8-10% [178]. To date, only three markers are established in the routine evaluation of breast tumours: ER and PR (for predicting response to endocrine therapies) and HER2/*neu* (for predicting response to Trastuzumab)[9]. Although these markers assessed routinely, ER, PR and HER2/*neu* assessment is far from perfect [8]. A number of circulating tumour markers (e.g., carcinoembryonic antigen [CEA] and carbohydrate antigen 15-3 [CA 15-3]) are widely used in the management of breast cancer, but the sensitivity of these markers is low [11-13], and so they are not useful as screening tools although they have long been in clinical use as prognostic markers and to monitor for disease progression or recurrence. The ideal biomarker should be easily accessible such that it can be sampled relatively non-invasively, sensitive enough to detect early presence of tumours in almost all patients and absent or minimal in healthy tumour free individuals.

The recent discovery that miRNA expression is frequently dysregulated in cancer [50] has uncovered a new repertoire of molecular factors, upstream of gene expression, which warrants investigation to further elucidate their precise role in malignancy. MiRNA expression studies in breast cancer clearly indicate their importance and potential use as disease classifiers and prognostic tools in this field [66,97]. A relevant and important feature of miRNAs is their remarkable stability. They are known to be well preserved in tissue samples even after years of formalin-fixation and paraffin-embedding, and can be efficiently extracted from and quantified in such specimens [128]. Investigation of cancer-specific miRNAs in the circulation is an emerging and exciting field of study. It is hypothesized that if miRNAs are present in the circulation of cancer patients,

their unique stability and resilience should allow their detection and quantification to be practicable. The first report of circulating miRNAs, by Lawrie et al [130], described elevated serum levels of *miR-21* in patients with diffuse large B-cell lymphoma. Subsequently, circulating miRNAs have been postulated as novel biomarkers for cancer, and other disease processes including diabetes, liver disease, heart failure, myocardial infarction and sepsis [130-132,135,147,179-184]. However this concept needs investigation to validate the theory. To date there has been no report on the role of circulating miRNAs in breast cancer. We hypothesized that levels of specific cancer associated miRNAs in circulation would differ between breast cancer patients and healthy individuals. If this hypothesis held truth, it would signify a major breakthrough in breast cancer management, bringing us ever closer to finding a novel, sensitive and non-invasive biomarker for this common disease.

3.2 Aims

The initial aims of this study were to develop and optimize a protocol for miRNA extraction and quantification in blood and its derivatives; serum and plasma. This was essential as at the time this study was initiated, no standardized or reproducible protocol existed for this purpose. Of the few published reports in this domain, the techniques of extracting miRNAs from the circulation, and subsequent quantification of systemic miRNA levels, are ill-defined, variable and difficult to reproduce. Several questions permeate this field – which circulating medium is preferable for systemic miRNA investigations, which purification technique retrieves superior concentration of quality miRNA from blood, which concentration of miRNA is optimal for RQ-PCR analysis and which endogenous controls are appropriate for circulating miRNA studies.

After developing a method for circulating miRNA analysis, the primary aim of this study was to investigate whether cancer specific miRNAs were detectable in the circulation of breast cancer patients. If present, then we aimed to determine if systemic miRNA levels in breast cancer patients were altered compared to age matched healthy controls.

Levels of miRNAs in the circulation of breast cancer patients were compared to matched breast tumour miRNA expression levels, to ascertain whether the systemic miRNA profile reflected the tumour miRNA expression profile.

Circulating miRNAs were further investigated as potential biomarkers for the management of breast cancer patients by investigating if levels in the blood changed postoperatively, following curative tumour resection. The decrease to basal level of a biomarker, after successful treatment of a tumour, would be an important property of the ideal tumour marker.

Finally a potential relationship between circulating miRNA levels and existing clinicopathological features of breast cancer such as tumour subtype, stage of disease, nodal status or hormone receptor status, was investigated.

3.3 Materials and methods

3.3.1 Study groups

Following ethical approval and written informed consent, blood samples (whole blood, serum and plasma) were collected prospectively from 127 females, including 83 consecutive breast cancer patients and 44 healthy age-matched female volunteers who served as controls for this study. All patients had histologically confirmed breast cancer and their relevant demographic and clinicopathological details were obtained from our prospectively maintained breast cancer database. The histological tumour profile of patients in this study reflects that of a typical breast cancer cohort, inclusive of a 10-15% proportion with *in situ* disease, with the majority of invasive tumours being of ductal type, and Luminal A epithelial subtype (Table 2.2). In addition, repeat blood samples were collected from a subset of this cohort, at their initial clinical review two weeks postoperatively (n=29). The control blood samples (n=44) were collected from healthy women with no current or previous malignancy, or inflammatory condition. Further detail on this control group is given in section 2.2.1.2 and in Table 2.2. A similar cohort of age and stage-matched breast cancer patients (n=65) were identified from whom tumour and tumour associated normal (TAN)

tissues were prospectively collected (Table 2.1). These tumour and TAN specimens were obtained from the breast cancer patients at the time of primary curative resection at Galway University Hospital, Galway, Ireland; here they were snap frozen and then stored in the Department of Surgery Biobank at NUI Galway as previously described (Section 2.1.1).

3.3.2 Blood collection

Whole blood was collected in Vacuette EDTA K3E blood bottles (Grenier Bio-one); one processed for plasma, another unprocessed, and a third sample collected in Vacutainer Serum Separator Tubes II (Becton Dickinson) for serum. Unprocessed whole blood samples were stored at 4°C until required. All samples were prepared and processed as described in 2.3.3.

3.3.3 Candidate miRNA targets

A panel of 7 cancer associated miRNAs was chosen for investigation in this study, on the basis of their reported relevance to breast cancer as described in section 2.1.1.1 and in Table 2.5 [35,64,66,97,132].

3.3.4 RNA isolation

Extraction of RNA from tumour tissue, normal breast tissue, and blood, was modified for the different tissue types. A separate purification of large and small RNA, using column based technology, was employed for breast tissue RNA isolation. For blood miRNA isolation, a variety of techniques were investigated to determine the optimal method for this purpose given that no standardized method existed at the initiation of this study. These are described below.

3.3.4.1 MiRNA extraction from breast tissue

Column based technology (RNeasy® Plus Mini Kit and RNeasy MinElute® Cleanup Kits, Qiagen) was used to isolate the micro and large RNA fractions separately from breast tissue specimens, as described in Section 2.3.1.

3.3.4.2 MiRNA extraction from blood, serum and plasma

Two protocols were used to extract RNA from blood specimens; a separate purification technique similar to that which is routinely employed for breast

tissue and a copurification method based on a modification of the Tri Reagent®
BD (Molecular Research Centre) protocol. Both are described in detail in
Section 2.3.3 and are summarised briefly as follows:

Protocol 1. Separate purification using column based methodology

250µl of thawed serum/plasma/whole blood was mixed with 1ml QIAzol® lysis
reagent and 250µl of chloroform, and then centrifuged at 12000g for 15 minutes
at 4°C. Large RNA and small RNA fractions were isolated separately using
Qiagen RNeasy kits according to the manufacturer's instructions.

Protocol 2. Copurification of total RNA using Trizol:

Using 1ml of whole blood, or its derivatives serum and plasma, phase separation
was performed by the addition of 3 ml of Trizol, and 200µl of 1-bromo-4-
methoxybenzene to augment the RNA phase separation process. Total RNA was
precipitated using isopropanol and washed with 75% ethanol prior to
solubilisation with 60 µl of nuclease free water.

3.3.5 Analysis of RNA concentration and integrity

RNA concentration was determined using a Nanodrop® spectrophotometer
(NanoDrop Technologies). The wavelength-dependent extinction coefficient '33'
was taken to represent the micro- component of all RNA in solution. In general
concentrations ranging between 30-300 nanograms per microlitre of miRNA
were obtained per sample. Integrity was assessed using RNA 6000 Nano
LabChip Series II Assays (for small RNA) on a 2100 Bioanalyzer (Agilent
Technologies) as described in Section 2.4.

3.3.6 Analysis of miRNA gene expression by RQ-PCR

RNA samples were reverse transcribed using primers specific to each miRNA
target, and real-time quantitative PCR (RQ-PCR) was carried out using TaqMan®
miRNA primers and probes as described by the manufacturer (Applied
Biosystems, USA), as described in Section 2.7. For breast tissue cDNA
synthesis, 5ng of small RNA was reverse transcribed using MultiScribe™ and
miRNA specific RT primers. For blood and its derivatives, various
concentrations of total RNA (5ng, 50ng, 100ng) were reverse transcribed, again

using MultiScribe™ and target specific RT primers, in order to determine which was the optimal starting concentration for reliable amplification. Reverse transcription was followed by RQ-PCR on a 7900 HT Fast Real-Time PCR System (Applied Biosystems). Triplicate samples, validated endogenous controls and inter-assay controls were used throughout. MiRNA expression levels were calculated using QbasePlus software.

3.3.7 Analysis of miRNA expression in breast tumours by *in situ* hybridisation
MiR-195 expression in breast tumours was further analysed using *in situ* hybridisation (ISH) with LNA-modified oligonucleotide probes, to verify expression levels as quantified by RQ-PCR and also to localise its expression in-vivo. Six breast tumours were selected from the breast tissue cohort (described in Table 2.1); in each *miR-195* levels had been quantified by RQ-PCR as described in Section 3.3.6. The tumours with highest (n=3) and lowest (n=3) *miR-195* expression levels respectively were included for ISH analysis. FFPE blocks from these 6 breast tumours were obtained and prepared for sectioning. From the tumour blocks, 6µm-thick sections were collected, observing RNA handling techniques. ISH with LNA-probes for *miR-195, miR-205* and *miR-126* was performed using the protocol described in detail in Section 2.9, and hybridised sections were inspected by light microscopy.

3.3.8 Statistical analysis
Data were analysed using the software package SPSS 15.0 for Windows. Due to the magnitude and range of relative miRNA expression levels observed, results data were log transformed for analysis. Data are presented as Mean ± SD. There was no evidence against normality for the log transformed data as confirmed using the Kolmogorov-Smirnov test. The two-sample t-test was used for all two sample comparisons and ANOVA, followed by Tukey HSD Post Hoc test, to compare the mean response between the levels of the between subject factors of interest. All tests were two tailed and results with a $p < 0.05$ were considered statistically significant.

3.4 Results

3.4.1 Development of optimal method for blood miRNA quantification

3.4.1.1 Comparison of techniques and blood specimens for RNA isolation

Separate miRNA purification vs. total RNA copurification:

The separate purification technique, based on column based methodology, for isolation of miRNA from whole blood, serum and plasma yielded relatively low concentrations of miRNA per sample (Range 1.9 – 86.1 ng / µL, for 75 specimens including 25 matched sets of serum, plasma and whole blood samples). Applying a co-purification protocol to the same patients' matched blood, serum and plasma samples, yielded consistently higher concentrations of miRNA with this method, in particular for whole blood (Range 20.3 – 221.6 ng / µL, Table 3.1). The concentration and integrity of the miRNA component of these samples was confirmed using an Agilent Bioanalyzer. Additionally, RQ-PCR quantitation of miRNA extracted from blood (whole, serum and plasma) samples using the latter technique, indicated that earlier amplification (implicating higher yields) of miRNAs were observed in whole blood compared to matched serum and plasma (Table 3.2). Under standard conditions, *miR-16* amplified at an average CT of 17.00 in whole blood compared to CTs of 31.80 and 32.37 for plasma and serum respectively.

Table 3.1 Comparison of miRNA concentrations obtained from two different RNA isolation techniques in a cohort of 10 blood samples		
	Whole blood miRNA conc. (ng/uL)	**Serum miRNA conc. (ng/ uL)**	**Plasma miRNA conc. (ng/ uL)**
Qiagen separate purification N=10	13.73	12.91	36.49
Trizol copurification N=10	80.92	36.71	25.03

Table 3.2 Mean CT values for *miR-16* expression in 10 matched whole blood, serum and plasma samples (100ng miRNA per reaction)

	Whole blood	Serum	Plasma
CT value	17.00	32.37	31.8

When comparing whole blood differential cell counts across all participants in this study, cancer cases and controls, there was no significant difference between these two groups with regard to their mean white cell count (SD): 6.9(2.11) *vs.* 7.61(2.14) x 10^3/µL respectively, p = 0.122, haemoglobin levels 13.02(0.95) *vs.* 13.24(1.03) g/dL respectively, p = 0.272 or haematocrit levels 39(2.4)% *vs.* 40(2.5)% respectively, p = 0.617. Based on these findings, whole blood was identified as the preferred medium for investigation of miRNAs in circulation.

3.4.1.2 Whole blood miRNA yield from fresh and stored blood samples

A comparative analysis of whole blood samples which had been stored for varying lengths of time indicated that fresher whole blood samples yielded higher concentrations of miRNA compared to blood specimens stored at 4°C for several weeks or months (Figure 3.1). When RNA was extracted from blood specimens within 3 days of collection, the miRNA concentration was as high as 170.9 ng/µL. For samples stored for up to 12 months the miRNA yield was, on average, 23.8 ng/µL.

3.4.1.3 Starting concentration of miRNA per reaction

The concentration of miRNA per cDNA synthesis reaction which returned superior RQ-PCR amplification was 100ng per reaction. At this concentration the average CT at which *miR-16* amplified in whole blood was 16.93. Starting miRNA concentrations of 5ng, 10ng or 50ng resulted in later amplification of miRNAs at RQ-PCR (Table 3.3).

Table 3.3 Mean CT cycle at which *miR-16* amplified, using differing concentrations of miRNA per reaction

	100ng	50ng	10ng	5ng
CT value	16.9	18.4	23.3	24.4
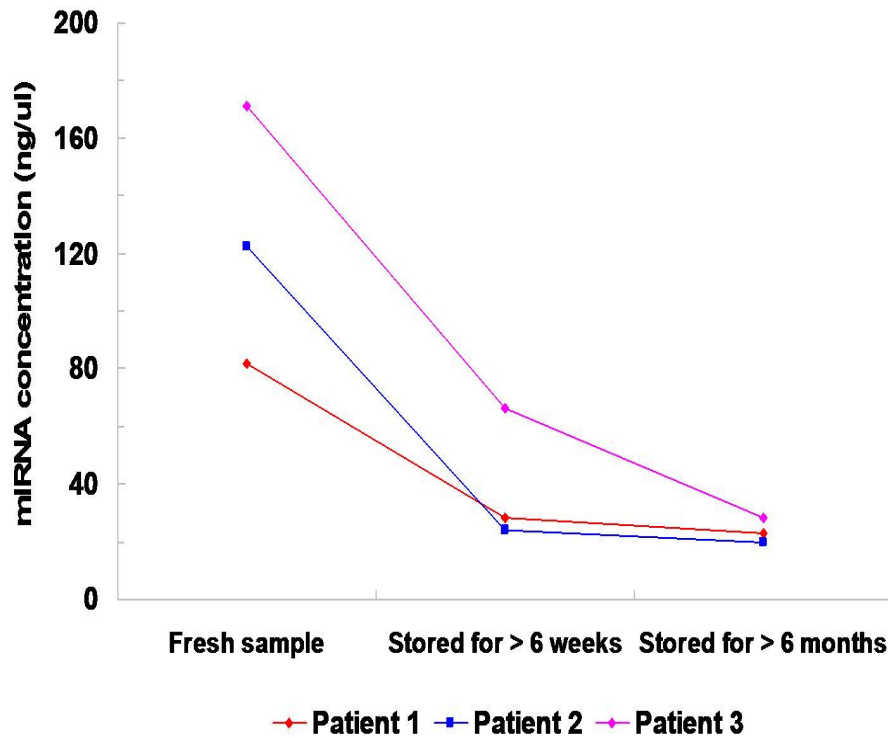
Figure 3.1 Concentrations of miRNA in nanograms per microlitre, yielded from whole blood which had been freshly taken* or stored for more than 6 weeks and 6 months respectively. This illustrates that higher yields of miRNA were attainable when the RNA was isolated soon after blood sampling. However reasonable yields are still attainable even in blood samples stored at 4°C for over 6 months.
(*RNA isolated from sample within 3 days of blood collection*)

3.4.1.4 Endogenous control for circulating miRNA investigations

MiR-16 was found to be abundantly expressed in all samples analyzed in this study; in cancer patients and healthy controls alike, with very little variability between samples. Thus *miR-16* was identified as a suitable endogenous control in whole blood, for normalisation of RQ-PCR data in a blood based miRNA study (Figure 3.2).
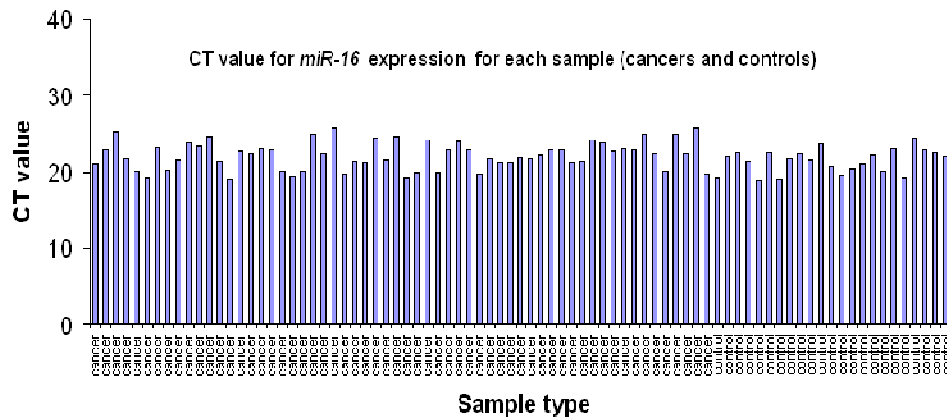
Figure 3.2 *MiR-16* expression in all 127 participants (83 breast cancer patients and 44 disease free, age-matched female controls) is stably expressed in all samples and thus is an ideal endogenous control for blood.

3.4.2 Detection of miRNAs in the circulation of breast cancer patients

Expression of seven miRNAs, chosen for their established relevance to breast cancer, (*miR-10b, miR-21, miR-145, miR-155, miR-195, miR-16* and *let-7a*) were detectable in whole blood samples from all breast cancer patients (n=83) as well as healthy controls (n=44). *MiR-16* expression was used to normalise RQ-PCR data and expression levels of the other six miRNAs relative to *miR-16* and to the lowest expressing sample ($\Delta\Delta$CT) are shown in Table 3.4. The levels of two tumour-associated miRNAs (*miR-195* and *let-7a*) were significantly higher, on average, in the breast cancer cohort than in healthy controls ($p < 0.001$ and $p < 0.001$), corresponding to an average fold-change of 19.25 and 11.20 respectively (Figure 3.3). Furthermore, blood levels of *miR-195* could detect individuals with breast cancer with 85.5% sensitivity and 100% specificity; whilst blood *let-7a* levels could detect breast cancer with 77.6% sensitivity and 100% specificity (Figure 3.4). Within this breast cancer cohort, a subset of patients had postoperative blood samples collected (n=29) to assess the effect of curative tumour resection on circulating miRNA levels. Thus it was found that *miR-195* and *let-7a* expression in the blood had decreased significantly to levels comparable with control subjects ($p<0.001$, Figure 3.5). Expression of preoperative circulating *miR-10b, miR-21, miR-145* and *miR-155* did not differ significantly between the breast cancer cohort and controls ($p = 0.449$, 0.606, 0.062, 0.280 respectively, Figure 3.3).

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Table 3.4 Mean RQ expression levels (SD) of target miRNAs in blood from breast cancer patients compared with blood from healthy controls.				
Target miRNA	**Breast Cancer blood (n=83)**	**Control blood (n=44)**	**Mean change** fold	*p-value*
miR-10b	1.05 (3.03)	0.83 (0.83)	1.27	*0.449*
miR-21	3.52 (10.30)	2.69 (7.47)	1.31	*0.606*
miR-145	3.58 (7.29)	1.65 (4.14)	2.17	*0.062*
miR-155	2.92 (6.23)	1.77 (4.48)	1.65	*0.280*
miR-195	6.91 (12.17)	0.36 (0.43)	19.25	***<0.001***
let-7a	5.05 (24.33)	0.45 (0.9)	11.20	***<0.001***

Figure 3.3 Expression levels of 6 cancer-associated miRNAs in preoperative blood samples from breast cancer patients (n=83) and disease-free age matched controls (n=44). Levels of the target miRNAs are expressed relative to *miR-16* in each sample. Levels of two miRNAs, *miR-195* and *let-7a,* were significantly elevated in blood form the cancer patients compared to controls – 19.25 fold and 11.20 fold changes respectively (p<0.001).
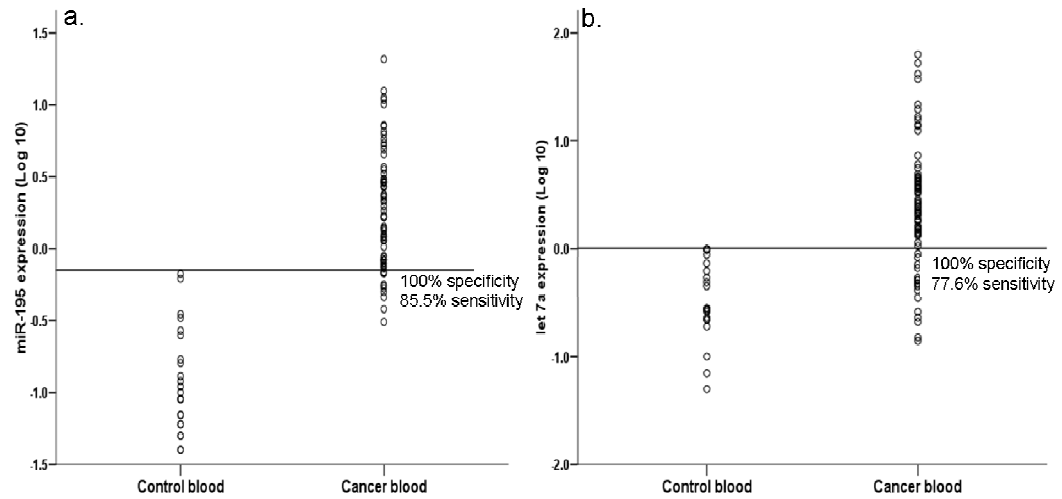
Figure 3.4 Blood *miR-195* (a) and *let-7a* (b) levels discriminate patients with breast cancer from healthy age matched controls. The horizontal lines indicate a 100% specificity threshold. Sensitivities of 85.5% and 77.6% were observed for blood *miR-195* and *let-7a* respectively
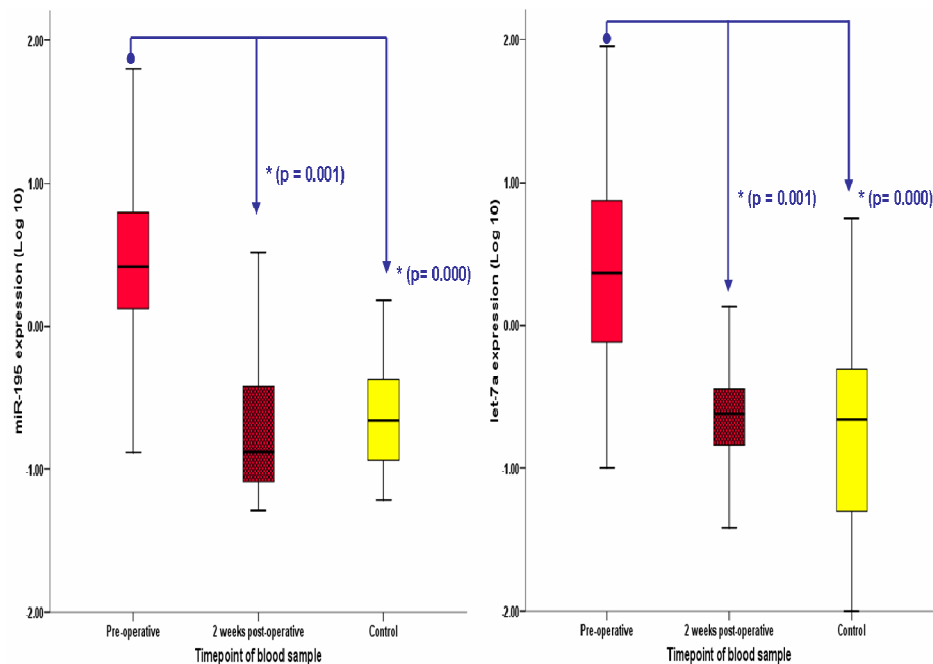
Figure 3.5 Expression levels of *miR-195* (a) and *let-7a* (b) in preoperative (n=83) and postoperative (n=29) blood samples from breast cancer patients and controls (n=44). At two weeks postoperatively a significant decrease in mean circulating *miR-195* and *let-7a* levels was observed, reaching levels comparable with control subjects.

3.4.3 Relationship of systemic and tumour miRNA expression profiles

Given that circulating *miR-195* was so significantly elevated in breast cancer patients (19.25 fold), we proceeded to investigate *miR-195* expression in a similar cohort of stage and age-matched invasive breast tumours (n=65), and in a cohort of tumour associated normal (TAN) controls (n=18). Tumour expression of *miR-195* was significantly higher compared to that in TAN: 1.23(0.43) *vs.* 0.49(0.37), p<0.001 (Figure 3.6). Tumour *miR-195* expression was also significantly higher in Stage IV compared to Stages I & II tumours (p= 0.006 and 0.039 respectively ANOVA and Tukey post hoc analysis). A similar trend of increasing systemic *miR-195* expression with advancing stage of breast cancer was also observed, although statistically significant differences were only observed between Stage I and Stage IV cancer patients (Figure 3.7). Conversely, systemic *let-7a* levels were significantly higher among patients with in-situ and early stage disease (Stages I and II) compared to patients with advanced or metastatic disease (Stage IV) (Figure 3.8). Controlling for age and stage of disease, *miR-195* expression in tumour tissue showed a significant positive correlation with circulating *miR-195* levels (Pearson's correlation coefficient 0.326, p=0.021) (Figure 3.9).
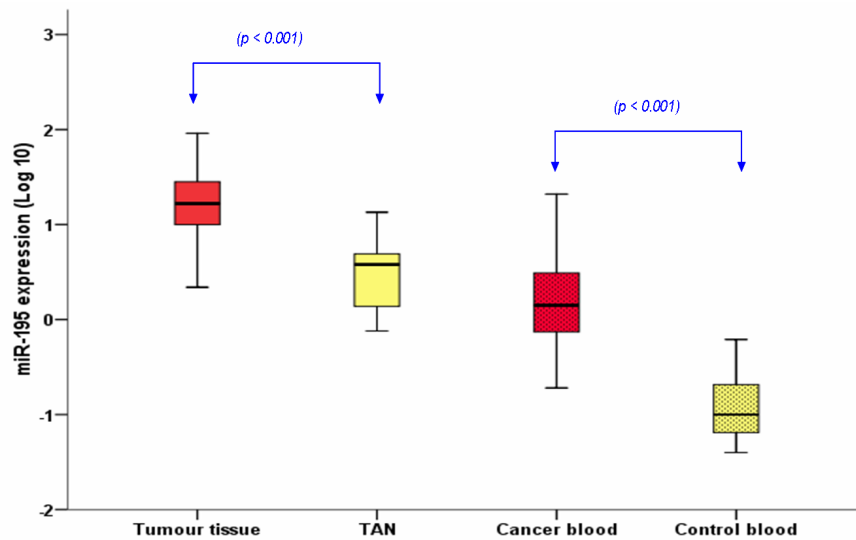
Figure 3.6 *MiR-195* expression in breast cancer tissues (n=65), tumour associated normal (n=18), preoperative invasive breast cancer blood samples (n=73) and healthy control bloods (n=44). *Mir-195* expression in tumour tissue differed significantly to TAN (p<0.001) and similarly its expression in blood from breast cancer patients differed significantly to healthy control blood (p < 0.001).

Figure 3.7(a) Tissue miR-195 vs. Stage

Figure 3.7(b) Systemic miR-195 vs. Stage
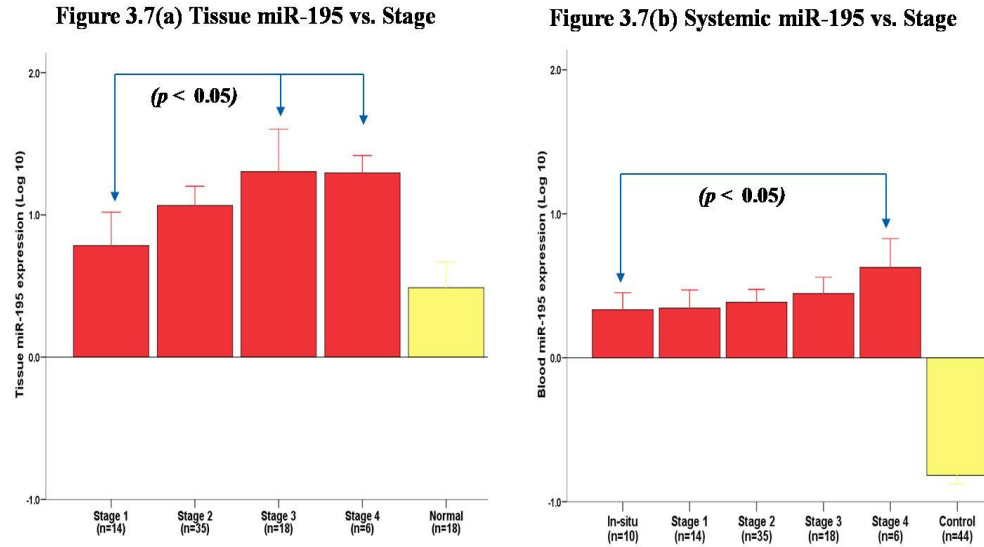
Figure 3.7 *MiR-195* expression according to stage of disease, in invasive breast cancer tissue specimens (n=65) (a) and breast cancer preoperative blood samples (n=83) (b). Tumour *miR-195* expression was significantly higher in Stages III and IV compared to early stage cancers (p< 0.05 respectively - ANOVA and Tukey post hoc analysis). There was a similar trend for increasing systemic *miR-195* levels as the stage of disease advanced, which was statistically significant between *in situ* and Stage IV cancers (p<0.05).
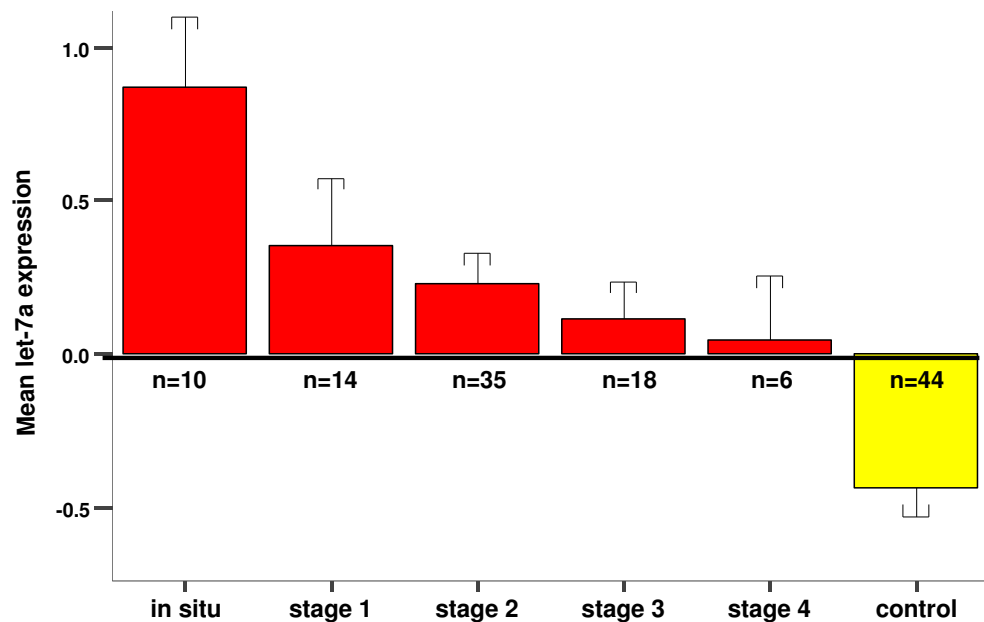
Figure 3.8 Systemic *let-7a* expression according to stage of disease in breast cancer preoperative blood samples (n=83). Systemic *let-7a* expression was significantly higher in *in-situ* and early stage cancers, compared to that among patients with Stage 4 breast cancer (p< 0.05 respectively - ANOVA and Tukey post hoc analysis).
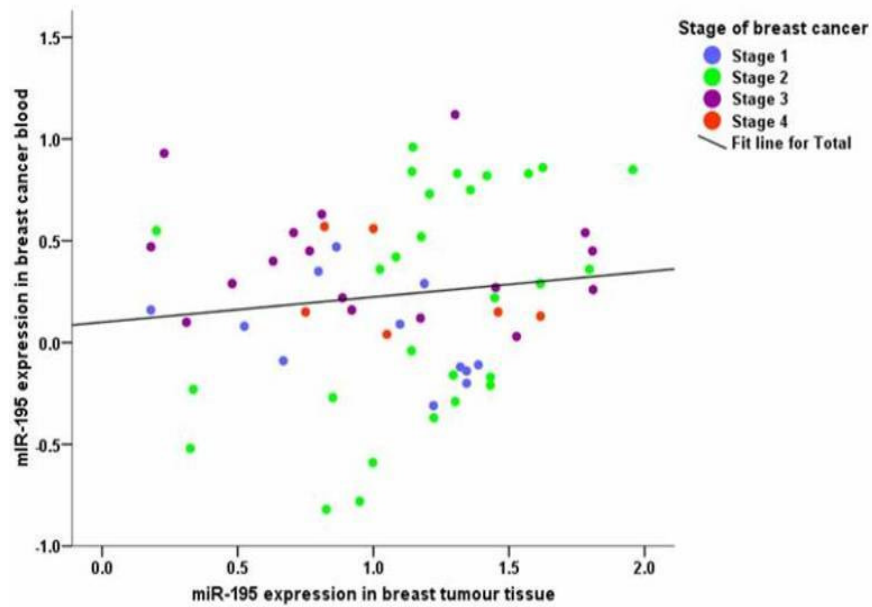
Figure 3.9 Correlation of *miR-195* levels in blood (n=65) and tumour tissue (n=65) from stage-matched invasive breast cancer patients. [Stage 1 (n=13), Stage 2 (n=29), Stage 3 (n=17), Stage 4 (n= 6)]. There was a significant correlation between blood and tumour *miR-195* levels, for stages of disease. (Pearson's correlation coefficient r = 0.326, p=0.021).

3.4.4 Localization of miRNAs in breast tumour tissue by *in-situ* hybridisation

To confirm the RQ-PCR data and to localise miRNA expression in breast tumour tissue, ISH analysis was performed on sections from 6 breast tumours using LNA-modified oligonucleotide probes for *miR-195*. Using *miR-126* and *miR-205* as positive controls, and scrambled miRNA probe from Exiqon as a negative control, *miR-195* expression was determined in the breast tumour sections. The three tumours with higher *miR-195* expression by RQ-PCR also demonstrated greater staining intensity for *miR-195* by ISH (Figure 3.10). Additionally, *miR-195* was predominantly localised to epithelial cells within the tumour section. *MiR-195* expression was also evident within the vasculature of breast tumour sections.
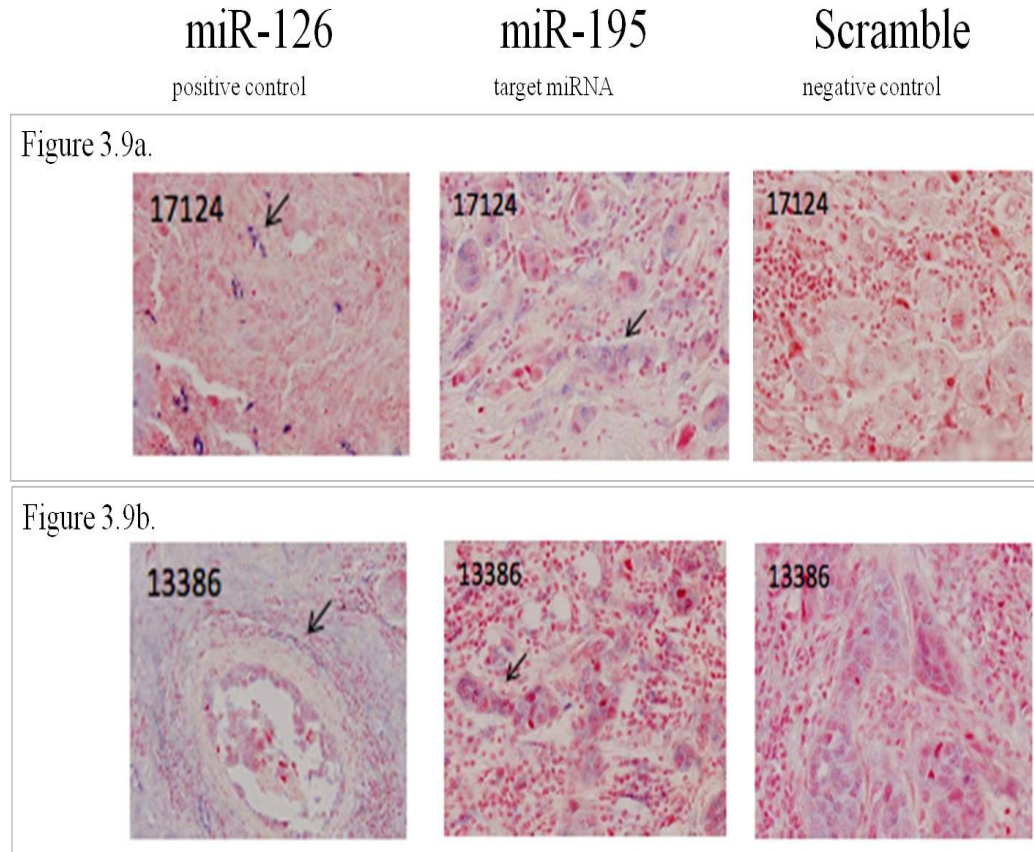
Figure 3.10. *MiR-195* **localization in breast tumour tissue by ISH.**
17124 (Figure 3.9a) and 13386 (Figure 3.9b) are sections from Her2/*neu* positive breast tumours respectively, both of which also expressed high levels of *miR-195* by RQ-PCR. As illustrated in the images on the far left *miR-126* staining (blue), which is specific for endothelial cells, demonstrates the vasculature within the breast tumour and stroma. On the far right the negative control (miR scramble) demonstrated no detectable labeling in either tumour. *MiR-195* expression is illustrated in the middle column and is evidently abundant in epithelial cells, and within vessels, of both tumours.

3.4.5 Relationship of circulating miRNAs to clinicopathological parameters

In addition to assessing the relationship of breast tumour and systemic miRNA profiles to the stage of disease, other relevant biopathologic associations of circulating miRNAs were investigated. Lymph node positive patients were found to have significantly lower levels, on average, of circulating *let-7a* compared to those with node negative disease (n = 38 and n = 45 respectively, p=0.002).

Higher circulating levels of *miR-10b* and *miR-21* were observed in patients with ER negative disease (n=15), compared to those with ER positive breast cancer (n=68), (p=0.028 and p=0.004 respectively). A potential relationship between circulating miRNA levels, type of disease (ductal vs. lobular), intrinsic subtype, or invasiveness (*in situ* vs. invasive cancers) was also investigated but no statistically significant differences were identified for any of these parameters. The associations between systemic *miR-195* and *let-7a* and many of the clinicopathologic variables commonly used to classify breast cancers are illustrated in Table 3.5.

Table 3.5 Associations between systemic *miR-195* and *let-7a* and various clinicopathological variables pertinent to breast cancer.		
Clinicopathological variable	**p-value for association with systemic *miR-195***	**p-value for association with systemic *let-7a***
Stage of disease^	0.005	<0.001
Nodal status*	0.999	0.002
ER positivity*	0.425	0.196
PR positivity*	0.645	0.206
Her2/neu positivity*	0.354	0.102
Tumour grade^	0.363	0.581
Tumour size^	0.226	0.525
Tumour type^	0.823	0.920
Intrinsic subtype^	0.794	0.463

^ ANOVA, * t-test

At tissue level, HER2/*neu* over-expressing tumours expressed higher *miR-195* compared to HER2/*neu* negative tumours, p=0.002. A similar significant relationship was not observed in the circulation of HER2/*neu* positive patients compared to HER2/*neu* negative patients, p=0.354 (Figure 3.11).

(a) Tissue *miR-195* vs. HER2/*neu* status   **(b) Systemic *miR-195* vs. HER2/*neu* status**
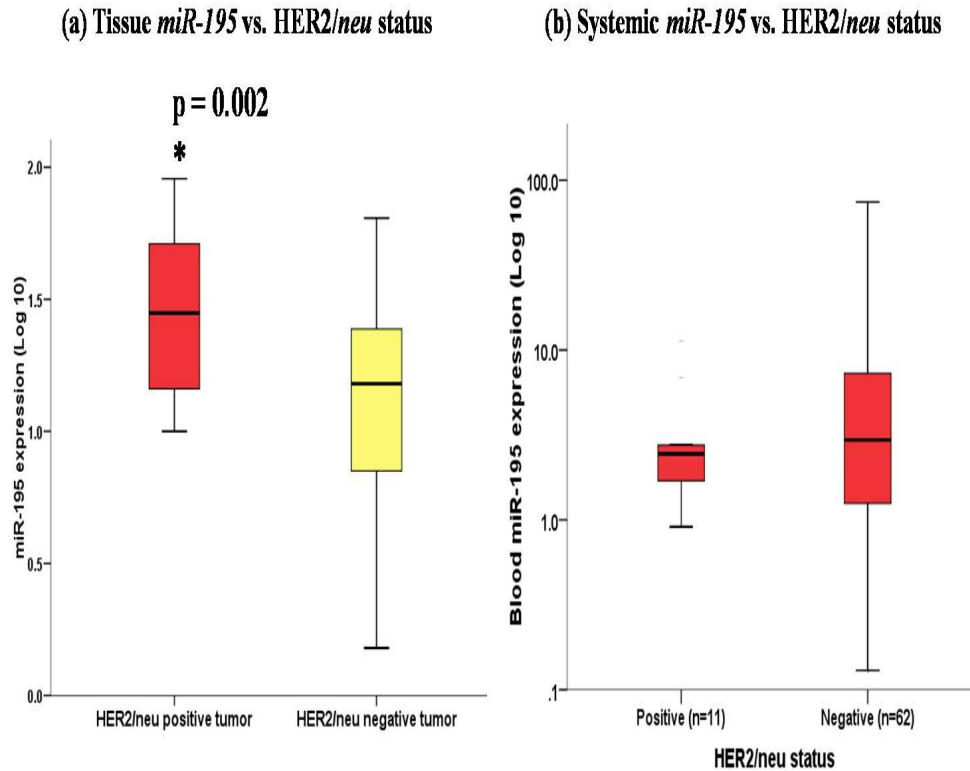
Figure 3.11 (a) *miR-195* expression in invasive breast cancer tissue (n=65), according to HER2/*neu* status. HER2/*neu* positive tumours (n=20) expressed higher *miR-195* than HER2/*neu* negative tumours (n=45), p=0.002.
 (b) *miR-195* expression in circulation of patients with invasive breast cancer (n=73), according to HER2/*neu* status. There was no difference in circulating *miR-195* expression in patients who over-expressed HER2/*neu* compared to those who were HER2/*neu* negative (p=0.354)

3.5 Discussion

This observational case-control study is the first report of circulating miRNAs in breast cancer patients and our results demonstrate that cancer-associated miRNAs in blood can potentially serve as novel non-invasive biomarkers for breast cancer. We demonstrate that *miR-195* and *let-7a* are significantly increased in the blood of breast cancer patients in comparison to disease-free control subjects (19.25 fold and 11.20 fold changes respectively), and can discriminate breast cancer patients from healthy controls with high specificity and sensitivity. *MiR-195* expression in a similar cohort (age- and stage-matched) of breast tumours and TAN specimens shows a similar significant increase in

tumour tissue over TAN. When profiling tumour and systemic *miR-195* levels according to the stages of breast cancer, we demonstrate similar profiles in both tissue types (Figure 3.6). Interestingly, circulating miRNAs were detectable in patients with *in-situ* disease, at levels comparable with early stage breast cancer patients (Figure 3.7). This finding has important clinical implications; for the first time a biomarker is shown to be present in the circulation of patients with non-invasive breast tumours, at levels significantly higher than healthy controls and similar to early stage cancers. Detection of breast cancer in this early pre-invasive stage has important consequences in the context of breast screening.

In addition, this is the first report of an optimal protocol for quantification of miRNAs in the circulation. We present evidence that extracting RNA from whole blood using a novel copurification technique, and amplifying for specific miRNA targets using specific Taqman primers and probes, at a starting concentration of 100ng of miRNA, is a reliable and reproducible method for blood based miRNA analysis. Whole blood samples from patient and control subjects in this study were comparable for white cell counts, haemoglobin and haematocrit levels, thereby eliminating potential bias in the results due to cellular and protein components. With regard to the optimal circulating medium for miRNA analysis, this is the first report that whole blood is superior to serum and plasma for this purpose. Whole blood holds several advantages over serum or plasma, as a medium for RNA isolation; most notably that no additional processing of the sample is required prior to RNA extraction and therefore is less labour intensive to work with. It is also possible to scale down the volumes of reagents and whole blood used in our copurification method for miRNA isolation, which implies that this technique has potential applicability in the clinical setting as a point-of-care blood test.

It is not known why the separate purification of miRNAs from blood and its derivatives results in lesser yields of miRNA. Whilst this method works effectively for tissue miRNA isolation, results are less successful when it is applied to fluid media such as blood, serum or plasma. One potential explanation may be that the silica-membrane of the RNeasy spin columns, with its maximal binding capacity of 100 µg RNA may not have the capacity to bind the maximal

miRNA component in a small volume of a dilute circulating medium such as blood [185]. Perhaps modification of the column membrane may alter its affinity for miRNA molecules in fluid specimens and improve the ability of this technique to yield higher quantities of quality miRNA. Another potential explanation of why separate purification using column-based kits yields low miRNA concentration is that the recommended volume of blood/serum/plasma (250 μL) to be filtered through the columns contains only dilute miRNA amount. If miRNAs in the circulation represent what is shed from the tumour or released from lysed tumour cells, then one may hypothesize that the miRNA complement in the entire blood volume is at much lower concentration than at tissue level. By sampling only a tiny volume of the circulation such as 250μl, obviously the miRNA concentration within such a small volume could be expected to be very low. Perhaps if the columns and their silica membranes were modified to hold larger blood volumes such as 1ml, or enrich for miRNA, then the resulting yield could be increased.

In recent years, tissue miRNA quantitation by RQ-PCR has developed substantially. The technique is now sensitive enough to reliably quantify miRNAs from a minute starting RNA volume, as is the case with tumour biopsy specimen where RNA quantities as low as 5ng are used for tissue miRNA quantitation. The same specimen size limitation does not apply to blood based miRNA investigations, as the sample is easier to obtain in larger quantities and can be resampled at various time-points without great difficulty or inappropriate distress to patients. Authors of existing circulating miRNA articles describe using varied starting miRNA concentrations in their expression experiments; ranging from 5-50 ng per reaction[130-132,134-135,179,181]. The authors have not justified their individual choices of initial concentrations, nor has any study previously attempted to determine which concentration is optimal. This study is the first to address this specific and important methodological concern. Based on the data presented here, where starting miRNA concentrations of 100ng resulted in significantly earlier amplification at RQ-PCR compared to starting concentrations of 5ng, 10ng, or 50ng (Table 3.3), the higher starting concentration is recommended for blood based miRNA studies.

There is no consensus from published circulating miRNA studies regarding the ideal internal normalisation control for these investigations. Small nuclear RNA U6 (45nt) and rRNA 5S (121nt) have previously been claimed to have reasonably stable expression in a variety of tissue types and thus used in several published miRNA RQ-PCR studies [145-146,186], however their stability in blood is questionable [63,181]. *MiR-16* has been shown to be abundantly expressed in normal healthy individuals as well as in patients with a variety of diseases, and levels in the circulation have been documented several times to be unaltered in the presence of malignancy [130,132,135]. Our data supports this finding (Figure 3.2). Hence circulating *miR-16* is the most commonly used miRNA reference gene in this context to date. It is clear that further work is needed to ascertain other reliable miRNA reference genes, in addition to *miR-16*, for the identification of an accurate normalization protocol for blood-based miRNA studies.

The two miRNAs found to be significantly increased in the blood of breast cancer patients in this study, *miR-195* and *let-7a,* have previously been described in breast cancer miRNA studies. *MiR-195* was reported by *Mattie et al* to be significantly higher in HER2/*neu* positive compared to HER2/*neu* negative breast cancers [97], a finding which was also true for the cohort of 65 invasive breast tumours analysed for *miR-195* expression in this study (HER2/*neu* positive n=22, HER2/*neu* negative n=43, p=0.002, Figure 3.11). Furthermore we observed a significant increase in tumour *miR-195* levels in metastatic breast cancers, compared to early stage tumours. This pattern was reflected in the circulation, although to a lesser (non-significant) extent. Interestingly circulating levels of *miR-195* also decreased significantly two weeks following curative tumour resection (Figure 3.5). Such observations support the concept of utilising systemic miRNA profiling as a novel and non-invasive biomarker for breast cancer. Further evidence to support *miR-195* as an important player in breast tumourigenesis, and additionally as a circulating biomarker, comes from our ISH analysis of this miRNA in breast tumours. This novel technique enables localisation of miRNA expression *in-vivo*, as well as verifying expression levels obtained by RQ-PCR. We demonstrate that *miR-195* is expressed predominantly in epithelial cells of breast tumours and it is also evident in vasculature. It is plausible that the cells expressing *miR-195* in blood vessels (as illustrated in

Figure 3.10) are circulating tumour cells, or that this represents cell-free *miR-195* in the circulation. Further studies are merited to scrutinise these findings, and to explore the exciting potential of ISH analysis as a novel technique to investigate miRNA expression *in vivo*.

The finding that *let-7a* was increased over 11-fold in breast cancer patients was unexpected. *Let-7a* is well described as having a functional role as a tumour suppressor [187] and has been shown to be down-regulated in many solid organ cancers, including lung, colorectal and gastric cancer [188-189]. In relation to breast cancer, *let-7a* in conjunction with *miR-16* has been described as a reliable endogenous control for analysis of miRNAs by RQ-PCR in human breast tissue [64]. As endogenous control genes are tissue and organ specific, it is acceptable that a house keeping gene for one tissue type can be investigated as a target gene in another. The finding that *let-7a* was greatly increased in the blood of breast cancer patients raises an interesting question concerning the origin of circulating miRNAs. Whilst recent blood-based miRNA reports, including the present study, clearly show that malignancy alters miRNA levels in the circulation, it is still unknown how tumour associated miRNAs make their way into the bloodstream. Slack *et al* raised two hypotheses in a recent report [136], firstly that tumour miRNAs may be present in circulation as a result of tumour cell death and lyses, or alternatively that tumour cells release miRNAs into the tumour microenvironment, where they enter newly formed blood vessels, and thereby make their way into the circulation. Our findings fit generally with the first hypothesis; however it is clear that further studies are needed to gain greater insight into the origin of circulating miRNAs. Finally, regarding *let-7a*, our finding that systemic levels of this miRNA were highest in patients with noninvasive and early stage breast cancer is noteworthy. One possible explanation for this finding is that *let-7a* may be involved in breast tumour initiation and so is highest in cancers which are in the earliest stages of development. This observation could hold great clinical importance, as to date there is no sensitive tumour marker for non-invasive breast cancer, so a blood test which detects *in-situ* disease could be a useful cancer screening tool.

We identified higher circulating levels of *miR-21* and *miR-10b* in patients with ER-negative disease. *MiR-21* has been described as an oncomir, and is up-regulated in many solid and haematological cancers. In relation to breast cancer, higher levels of *miR-21* have previously been associated with advanced disease, poorer prognosis, and lymph node metastasis [190-193]. However the relationship of tumour *miR-21* level to ER status has been inconsistently described; Mattie *et al* found higher *miR-21* levels to be associated with ER positive breast cancer in their study of 20 breast tumour biopsies, 11 of which were ER positive [97]. More recently Qian *et al* showed high *miR-21* levels to be associated with estrogen receptor negative disease in a much larger study of 344 breast tumours, 218 of which were known to be ER positive and 120 ER negative [194]. Our findings in blood correspond to those of Qian *et al* in relation to breast tissue. Functional studies have shown that *in vitro* manipulation of *miR-21* expression can alter the responsiveness of ER negative cell lines to hormonal therapies. This further highlights the importance of *miR-21* expression in human breast cancer. Although this study did not find circulating *miR-21* to differ significantly between breast cancer patients and controls, its association with clinicopathological parameters such as ER status indicates that circulating *miR-21* may serve as a prognostic molecular marker for breast cancer and disease progression.

The role of *miR-10b* in breast cancer has also been addressed with varying conclusions on its precise function. Early studies collectively found *miR-10b* to be down-regulated in breast tumour compared to normal breast tissue [66,97]. More recently, *Ma et al* contested these findings, and reported that *miR-10b* played a part specifically in the metastatic process but not in primary tumour formation, having found this miRNA to be highly expressed in metastatic breast cancer cells [192]. To our knowledge, this is the first report of a significant association between *miR-10b* and the hormonal status of breast cancers. Given that hormone receptor negative status is considered a poor prognostic factor for breast cancer [195], our observation that circulating *miR-10b* is higher in ER negative disease is in keeping with the findings of *Ma et al*.

This study has several limitations. In addition to the aforementioned deficiencies common to all blood-based miRNA studies (no standardised methodology for miRNA quantification from blood, no validated endogenous controls), we acknowledge that this is a pilot study with a relatively small cohort size. In particular, the number of control patients used in this study was limited. Future prospective studies in this field will need to include much larger numbers of control patients in order to determine what the normal range for each miRNA is in the circulation. Only then will it be possible to truly establish the level of a systemic miRNA which would be concerning for a pathological state. This study also needs to be repeated in larger independent breast cancer cohorts in order to determine the reproducibility of the data.

3.6 Conclusion

Inherent characteristics of miRNAs such as their lower complexity, tissue specific expression profiles, stability, and ease with which they are amplified and quantified, make these molecules ideal candidates as biomarkers to reflect various physiological and pathological states. The results presented here showing significantly altered circulating miRNA levels in breast cancer patients compared to healthy individuals, with similar profile for *miR-195* in breast tumour tissues compared to TAN, and the associations of particular circulating miRNAs with commonly used prognostic indicators, highlights the potential of these molecules as novel non-invasive biomarkers for breast cancer. Circulating tumour-associated miRNAs have the potential to detect breast cancer even in its earliest stages, and can differentiate tumours according to histological features such as hormone receptor and lymph node status. Further prospective evaluation of blood-based miRNAs, in breast and other cancers is needed, to validate these findings and to further explore the exciting potential of circulating miRNAs to emerge as clinically useful novel biomarkers for cancer.

Chapter 4

Tumour Specificity of Circulating MiRNAs

4.1 Introduction

Early diagnosis of cancer remains a compelling challenge for clinicians; it is the ultimate goal in order to minimize treatment-associated morbidity and mortality, and achieve maximal long-term survival. Recently the concept of individualized therapeutic regimens for cancer patients is in vogue, as clinicians and translational researchers attempt to tailor treatment regimens in order that each patient receives maximal benefit in the neoadjuvant and adjuvant settings. The discovery of novel classes of molecular markers in cancer has provided exciting, potentially viable biomarkers which may have utility in early cancer detection. These biomarkers may facilitate accurate tumour stratification, predict response to treatments, risk of disease recurrence or progression, or even represent novel therapeutic targets. One notable example in recent years was the exciting discovery by Slamon *et al* in 1985, that the HER2/*neu* oncogene was overexpressed in 20-30% of human breast cancers, and that the prognosis of patients whose tumours overexpress HER2/*neu* is poor [196-197]. This finding led to the development of a specific recombinant monoclonal antibody against HER2/*neu*, Trastuzumab, to treat patients whose tumours overexpressed this oncogene. Subsequent trials evaluating this antibody, involving more than 10,000 women, established that adjuvant Trastuzumab therapy halves the recurrence rate and reduces mortality by 30% [8,198-199]. Currently, HER2/*neu* expression in breast tumour tissue is routinely evaluated, and when overexpressed, Trastuzumab is considered for inclusion in individual adjuvant therapy regimens.

Despite stellar efforts in probing the molecular biology of common cancers, similar progress to that observed in breast cancer has not been mirrored; there remains an enormous dearth of knowledge regarding the molecular taxonomy and complex pathways of other malignancies Currently, there are few known or validated biomarkers for early detection, treatment planning, follow-up, or targeted therapy of cancer. The utility of currently available tumour markers is limited by disappointing sensitivities and specificities, even in the case of prostate specific antigen (PSA) which is widely used in routine clinical practice for the screening and management of prostate cancer (Table 4.1).

Table 4.1 Existing non-invasive tumour markers which are in routine clinical use for common cancers

Cancer	Tumour marker	Sensitivity	Specificity	Reference
Breast	CEA Ca15-3	29-53%* 54-90%*	70-99% 86-99%	Harris et al [11]
Prostate	PSA (>4ng/ml)	20-72%	90-94%	Thompson et al [200] Mettlin et al [201]
Lung	None	-	-	Arenberg [202]
Colon	CEA (>5ng/ml) Ca19-9	26% 18%	72% 89%	Moertel et al [203] Herszényi et al [204]
Uterine	Ca125	34.6%	90%	Moore et al [205]
Melanoma	S-100 protein	15-65%*	97%	Wollina et al [206]
Non-Hodgkin's	None	-	-	
Ovarian	Ca125	71%*	98%	Skates et al [207]
Bladder	Urine cytology	71%	97%	Stonehill et al [208]
Renal	None	-	-	De la Taille et al [209]
Pancreas	Ca19-9	69-93%	78-98%	Tian et al [210]

The potential of miRNAs as novel tumour markers has been the focus of much recent attention due to their tissue specificity and unique ability to predict clinicopathological parameters with superior accuracy to mRNA expression profiling [50]. This recognition led to the exploration of these tiny molecules in the circulation, in the hope that if present, systemic miRNA analysis could herald a breakthrough in clinical practice, where the quest for sensitive and specific non-invasive cancer biomarkers persists. Recent reports have documented altered serum or plasma miRNAs in a variety of cancers including prostate, colon, lung and gastric [130-132,179,183]. In our previous study (Chapter 3. Evaluation of Circulating MiRNAs in Breast Cancer) we demonstrated that circulating *miR-195* and *let-7a* are significantly elevated in breast cancer patients. However it is unknown whether the specific miRNAs reported to be altered in these studies are disease specific or a global cancer phenomenon.

4.2 Aims

The overall objective of this study was to further investigate the utility of a panel of circulating miRNAs (*miR-10b, miR-21, miR-145, miR-155, miR-195*, and *let-7a*), known to be dysregulated in cancer, as potential tumour markers.

The initial aim of the study was to evaluate the expression of these miRNAs in the circulation of a diverse cohort of cancer patients. Therefore we expanded our analysis of circulating miRNAs to include patients with breast, prostate, renal, colon and melanoma cancers.

Following determination of systemic miRNA expression in these various cancer patients, the primary aim of this study was to determine the specificity of *miR-195* and *let-7a* (both of which were demonstrated to be elevated in blood from breast cancer patients compared to healthy controls) for breast cancer.

The value of circulating miRNAs for early diagnosis of cancer, and thus their potential as cancer screening tools, requires investigating whether or not they are significantly dysregulated even in patients with pre-invasive and early stage disease. We sought to explore this concept by including in this analysis patients with *in-situ* carcinoma of the breast, as well as significant numbers of patients with early stage (TNM Stage I and II) cancer of the breast, colon, kidney and prostate.

4.3 Materials and methods

4.3.1 Study groups

Following ethical approval and written informed consent, whole blood samples were collected prospectively from 226 participants; including 83 consecutive breast cancer patients, 30 colon cancers, 20 prostate cancers, 20 renal cell carcinomas and 10 individuals with malignant melanoma. The control group comprised 63 healthy age-matched individuals from the community (44 female, 19 male). Of note, the breast cancer patients and the female control patients were the same cohort of patients used in the previous study (Chapter 3, and described

in Table 2.2). All 163 cancer patients presented to the tertiary referral cancer centre in the West of Ireland for management of their malignancy. Each case had a histologically confirmed diagnosis and the histological tumour profiles reflect those of typical cohorts for the respective malignancies (Table 4.2).

4.3.1.1 Breast cancer cohort

As shown in Table 2.2, the breast cancer cases were predominantly of ductal type (71%), and the epithelial subtype composition was typical for a symptomatic cohort of patients:

Luminal A	63%
Luminal B	10%
HER2/*neu* overexpressing	4%
Basal	11%
n/a (*in situ* disease)	12%

Almost three-quarters of the cohort had early stage disease (71%) inclusive of a 12% proportion with pre-invasive carcinoma. Lymph node positivity was observed in 46% of the cohort and 82% of breast tumours were oestrogen receptor positive. A smaller proportion (14%) over-expressed the HER2/*neu* receptor.

Table 4.2 Study participants demographic and clinicopathological details						
Total number study participants: N = 226						
	Breast Cancer (n=83)	**Colon cancer (n=30)**	**Prostate Cancer (n=20)**	**Renal Cancer (n=20)**	**Melanoma (n=10)**	**Controls (n=63)**
	n (%)	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**n (%)**
Mean age, years	55.1	68.8	60.6	64.5	52.9	52.1
[Range]	[30 -88]	[45 -88]	[50-68]	[31-78]	[17-78]	[24–80]
Sex						
Male	0 (0)	19 (63)	20 (100)	11 (55)	5 (50)	19 (30)
Female	83 (100)	11 (37)	0 (0)	9 (45)	5(50)	44 (70)
Stage						
In situ	10 (12)	0 (0)	0 (0)	0 (0)	0 (0)	
I	14 (17)	7 (24)	1 (5)	6 (30)	3 (30)	
II	35(42)	13 (43)	10 (50)	6 (30)	5 (50)	*n/a*
III	18 (22)	9 (30)	9 (45)	6 (30)	1 (10)	
IV	6 (7)	1 (3)	0 (0)	2 (10)	1 (10)	

4.3.1.2 Prostate cancer cohort

The prostate cancer cohort comprised 20 males with adenocarcinoma of the prostate, 55% of whom had early stage I and II disease. The average Gleason score was 7 (n=13) with a range as follows:

Gleason score	N (%)
6	1 (5)
7	13 (65)
8	4 (20)
9	2 (10)

The mean serum PSA level at diagnosis was 7.67 (Range 3.1 - 17.5).

4.3.1.3 Colon cancer cohort

Colon cancer cases (n=30; 19 male, 11 female) were all infiltrating adenocarcinomas, predominantly left sided tumours (73%) and 67% had early stage I and II disease.

4.3.1.4 Renal carcinoma cohort

The renal cell carcinoma (RCC) cohort (n=20, 11 male, 9 female) had an average tumour size of 5.9cm (Range 2-12cm) at presentation. 23% had extra-capsular invasion. Lymph node positive disease was recorded in 29%. The Fuhrman Nuclear Grade is the most widely used and most predictive grading system for renal cell cancer [211] which classifies RCC into four categories (I-IV) according to nuclear size and morphology, the presence of nucleoli, and extent of chromatin clumping. Grade I represents low cellular mitotic activity and has been widely established to carry the best prognosis; the converse is true for grade IV RCC. In this cohort of RCCs the Fuhrman Nuclear Grade was as follows:

Fuhrman Nuclear Grade	N (%)
I	0 (0)
II	13 (65)
III	4 (20)
IV	3 (15)

4.3.1.5 Malignant melanoma cohort

Of the 10 malignant melanoma cases (5 male, 5 female) 7 patients presented with Clarkes level IV or V lesions, mean Breslow's thickness was 2.3mm (Range 0.9 – 5.0). Two patients (20%) had a positive sentinel node at presentation; one of which was located in the axilla in conjunction with a primary melanotic lesion on the upper arm, and the second was located in the groin in the presence of a primary lesion on the leg.

4.3.1.6 Control cohort

The control blood samples were collected from age-matched healthy men and women residing in the same catchment area from which cases originated, and were collected on a contemporaneous basis with cases so as to minimize potential bias because of differential seasonal or environmental exposures. Control individuals were interviewed by a clinician prior to being enrolled in this study to ensure they had no current or previous malignancy, or concurrent inflammatory condition.

All patients' demographic and clinicopathological details were entered in a prospectively maintained cancer database.

4.3.2 Blood collection

In the preceding study it was observed that systemic miRNA analysis is optimally performed on unclotted whole blood samples, compared to serum or plasma (Chapter 3. Evaluation of circulating miRNAs in breast cancer). Venous whole blood samples (non-fasting) were collected from each participant in a Vacuette EDTA K3E blood bottle (Grenier Bio-One International AG). Unprocessed whole blood sample were stored at 4°C until required.

4.3.3 Candidate miRNA targets

A panel of seven miRNAs was chosen for investigation in this study *(miR-10b, miR-21, miR-145, miR-155, miR-195, let 7a,* and *miR-16).* As explained in Section 2.11, these miRNAs were chosen based on their previously documented associations with malignancies [35,81] or for their potential as endogenous controls

in the circulation [132,179,212] (Table 2.5). This is also the same panel of miRNA targets investigated in the preceding breast cancer case-control study.

4.3.4 RNA isolation from whole blood

Copurification of total RNA using Trizol:

Total RNA was extracted from 1ml of whole blood respectively using TRI Reagent® BD (Molecular Research Centre, Inc., USA), as previously described (Section 2.3.4).

4.3.5 Analysis of RNA concentration and integrity

RNA concentration was determined using a Nanodrop® spectrophotometer (NanoDrop Technologies Inc). The wavelength-dependent extinction coefficient '33' was taken to represent the micro-component of all RNA in solution. Each 1 mL of whole blood yielded 60 µL of total RNA, with yields ranging between 30-300 nanograms per µL of small RNA, which was then transferred to storage tubes prior to storage at -80°C. Integrity was assessed using RNA 6000 Nano LabChip Series II Assays (for small RNA) on a 2100 Bioanalyzer (Agilent Technologies) as described in Section 2.4.

4.3.6 Analysis of miRNA gene expression by RQ-PCR

RNA samples were reverse transcribed using primers specific to each miRNA target, and real-time quantitative PCR (RQ-PCR) was carried out using TaqMan® miRNA primers and probes as described by the manufacturer (Applied Biosystems, USA), as described in Section 2.8. 100ng of total RNA per sample was reverse-transcribed using the MultiScribe™-based High-Capacity cDNA Archive kit (Applied Biosystems). RT-negative controls were included in each batch of reactions. PCR reactions were carried out in final volumes of 10 µL using an ABI 7900 HT Fast Real-Time PCR System (Applied Biosystems). Briefly, reactions consisted of 0.7 µL cDNA, 5 µL TaqMan® Universal PCR Fast Master Mix, 0.2 µM TaqMan® primer–probe mix (Applied Biosystems). Reactions were initiated with 10-minute incubation at 95°C followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. An interassay control derived from a breast cancer cell line (ZR-75-1) was included on each plate and all reactions were performed in triplicate. *MiR-16* was again used as the

endogenous control to standardize miRNA expression. The threshold standard deviation for intra-assay and inter-assay replicates was 0.3. The percentage PCR amplification efficiencies (E) for each assay were calculated, using the slope of the semi-log regression plot of cycle threshold versus log input of cDNA (10-fold dilution series of five points), with the following equation and a threshold of 10% above or below 100% efficiency was applied: E = (10 - 1 / slope - 1) x 100. The relative quantity of miRNA expression was calculated using the comparative cycle threshold ($\Delta\Delta$Ct) method [64], normalised to miR-16 levels and the lowest expressed sample was used as a calibrator.

4.3.7 Statistical Analysis

Data were analysed using the software package SPSS 17.0 for Windows. Due to the magnitude and range of relative miRNA expression levels observed, results data were log transformed for analysis. There was no evidence against normality for the log transformed data as confirmed using the Kolmogorov-Smirnov test and so data are presented as Mean ± SD. ANOVA, followed by Tukey HSD Post Hoc test, was used to compare the mean response between the levels of the between subject factors of interest whilst the two-sample t-test was used for any two sample comparisons. All tests were two tailed and results with a $p < 0.05$ were considered statistically significant. Receiver operating characteristic (ROC) curves were constructed and the area under the curve (AUC) was calculated to assess the ability of each miRNA to differentiate between cancer cases and controls, by computing sensitivity and specificity for each possible cut-off point of the individual miRNAs. This was performed univariately for each individual miRNA, and multivariately for combinations of the six target miRNAs in our panel via logistic regression analysis.

4.4 Results

4.4.1 Dysregulated expression of miRNAs in the circulation of cancer patients

Expression of seven miRNAs, selected for their established relevance to cancer (*miR-10b, miR-21, miR-145, miR-155, miR-195*, *miR-16,* and *let-7a*), were detectable at variable levels in the circulation of all 226 study participants (163 cancer patients and 63 healthy age-matched controls, Table 4.3). *MiR-16* expression was stable and reproducible across all 226 participants' peripheral blood samples and was therefore used to normalise RQ-PCR data.

Table 4.3 Mean logged RQ expression levels (SD) of target miRNAs in blood from all cancer patients compared with blood from healthy controls						
Target miRNA	Breast (n=83)	Colon (n=30)	Prostate (n=20)	Renal (n=20)	Melanoma (n=10)	Controls (n=63)
miR-10b	3.0 (0.8)	1.9 (0.6)*	2.6 (0.8)	2.0 (0.4)*	1.0 (0.4)*	3.1 (0.6)
miR-21	3.3 (0.5)	2.3 (1.2)	2.9 (0.8)	2.6 (0.6)	1.5 (0.3)*	2.9 (0.6)
miR-145	4.0 (0.4)	2.7 (0.8)*	3.0 (0.6)*	3.5 (0.7)	1.6 (0.9)*	3.7 (0.7)
miR-155	3.1 (0.6)	2.1 (0.5)*	1.3 (0.5)*	1.9 (0.5)*	1.4 (0.7)*	2.9 (0.7)
miR-195	4.2 (0.6)*	2.6 (0.6)	2.7 (0.9)	3.2 (0.4)	1.9 (0.3)	3.0 (0.4)
let 7a	3.4 (0.7)*	3.1 (0.9)*	3.3 (1.1)*	3.5 (0.7)*	2.3 (0.5)	2.2 (0.8)

*** Significantly altered (p<0.01) compared to control miRNA levels**

To explore the potential of using this panel of miRNAs as specific biomarkers for breast cancer, we compared levels of the six 6 target miRNAs in the circulation of 83 consecutive breast cancer patients with those of 80 other cancer patients and 63 healthy control subjects. Circulating levels of five cancer-associated miRNAs (*let-7a, miR-10b, miR-145, miR-155,* and *miR-21*) were generally dysregulated in the presence of several cancers, including breast. No single one of these five markers in isolation denoted a particular malignancy. By contrast elevated levels of systemic *miR-195* was a unique finding in the breast cancer cohort (p < 0.001), indicating that it may be a breast cancer specific marker (Figure 4.1). This expression pattern held true for cancer patients with early stage disease specifically (TNM Stages: *in situ*, I and II) (Figure 4.2).
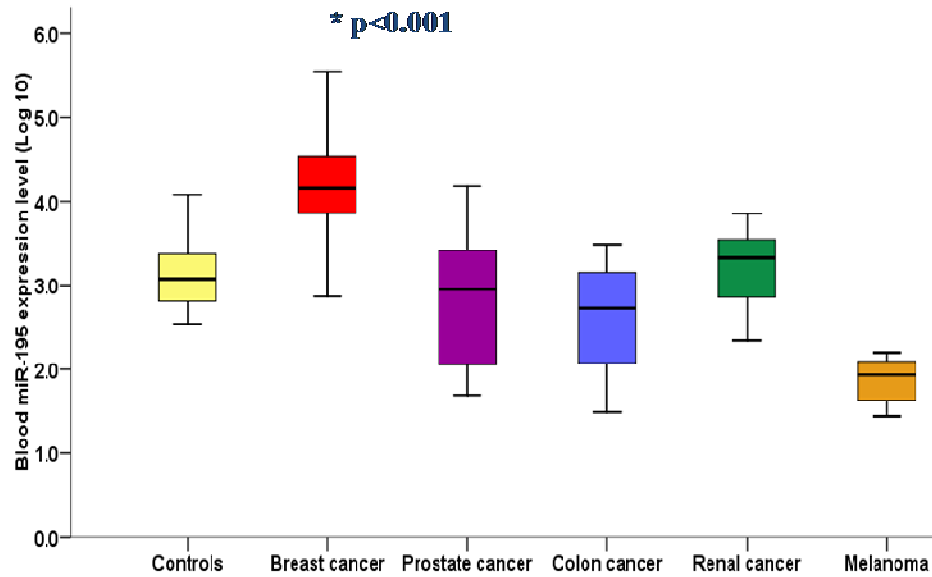
Figure 4.1 *MiR-195* expression in controls, breast cancers, prostate cancers, colon cancers, renal cancers and malignant melanoma cases. *MiR-195* is significantly elevated only in the breast cancer group (p<0.001).
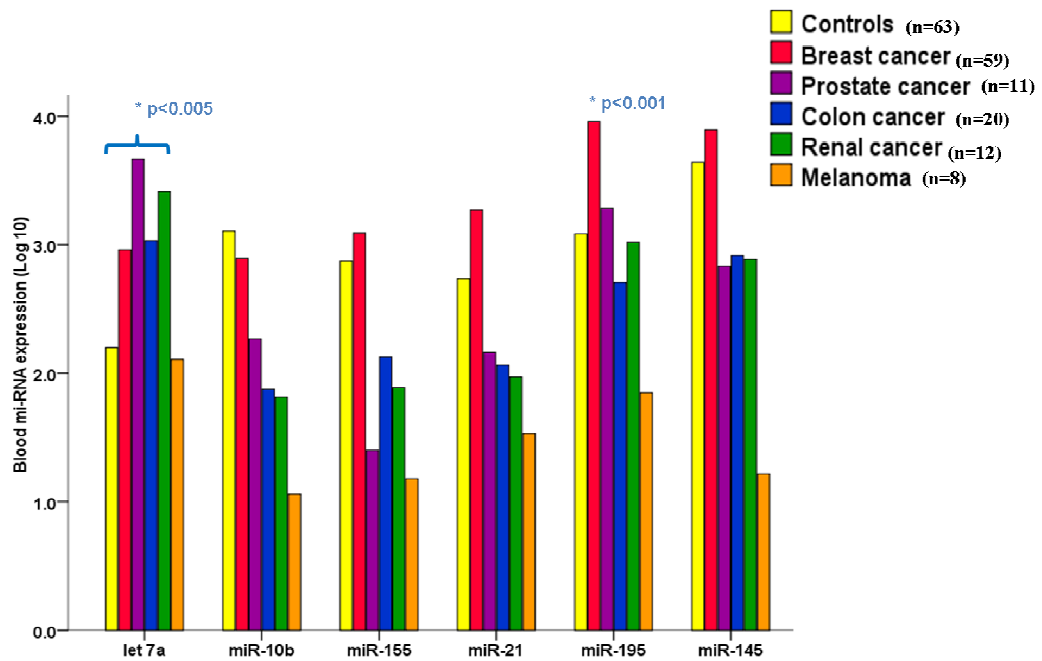
Figure 4.2 In **early stage cancers** (TNM Stages: *in situ*, I and II, n=110) versus controls (n=63) *miR-195* expression was significantly elevated only in the breast cancer patients (p<0.001). Circulating *let-7a* levels were significantly elevated in patients with several visceral malignancies of early stage, when compared to the control group.

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4.4.2 Generic 'oncomiRs'

4.4.2.1 Systemic *let-7a* expression in various cancers

Let-7a levels were observed to be significantly increased in the circulation of patients with several visceral malignancies (breast, prostate, colon and renal cancers), when each group was compared to controls (p<0.001, ANOVA). Systemic *let-7a* levels were similarly increased in all malignancies considered here, with no difference between the various cancer patients (Figure 4.3). Patients with malignant melanoma were not observed to have altered circulating *let-7a* levels (*p=1.00*).
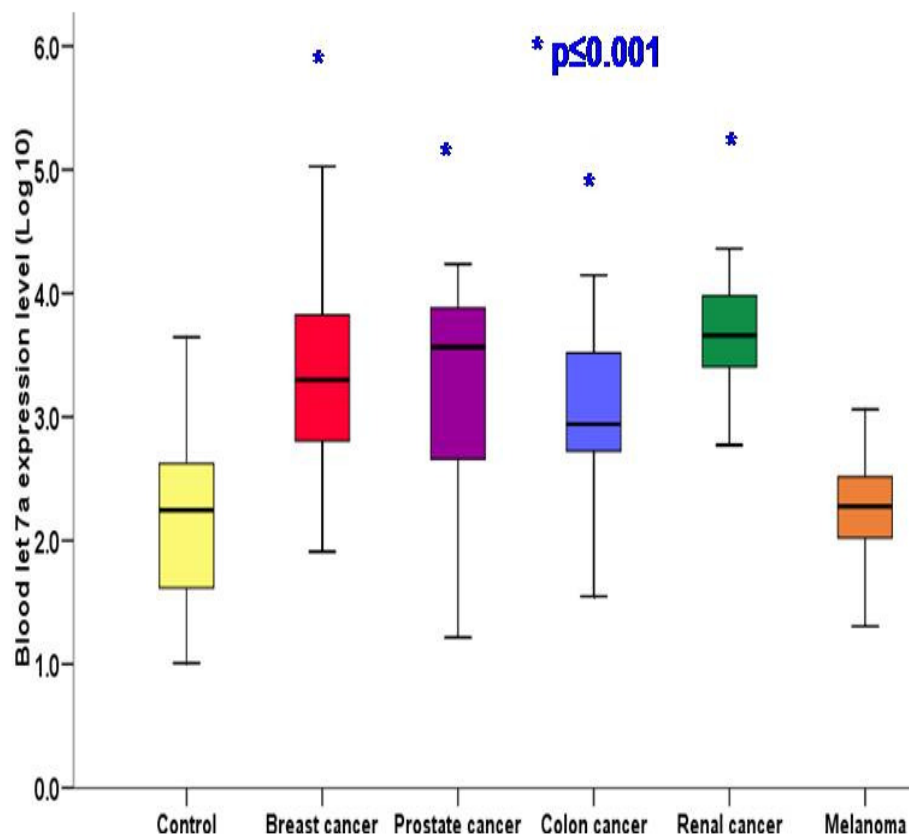
Figure 4.3 *Let-7a* levels are significantly elevated in patients with a variety of visceral malignancies including breast, colon, prostate and renal cancers, compared to controls (p<0.001).

4.4.2.2 Systemic *miR-10b* expression in various cancers

MiR-10b, a pro-metastatic miRNA[190], was found to be significantly decreased, on average, in blood from colon and renal cancers as well as melanoma patients, for all stages of disease. Levels of circulating *miR-10b* did not differ significantly according to the stage of disease in these 3 groups of cancer patients (p=0.525, 0.741 and p=0.54 respectively). There was no relationship between circulating *miR-10b* levels and size or grade of tumours in the colon cancer and RCC cohorts. In the malignant melanoma cases, there was no significant correlation between *miR-10b* levels and Clarke's level or Breslow's thickness (Pearson's correlation coefficient: r = 0.03 and r = 0.23 respectively, p=0.94 and p=0.552 respectively). Systemic *miR-10b* levels were within the normal range in patients with breast & prostate cancers (Figure 4.4).
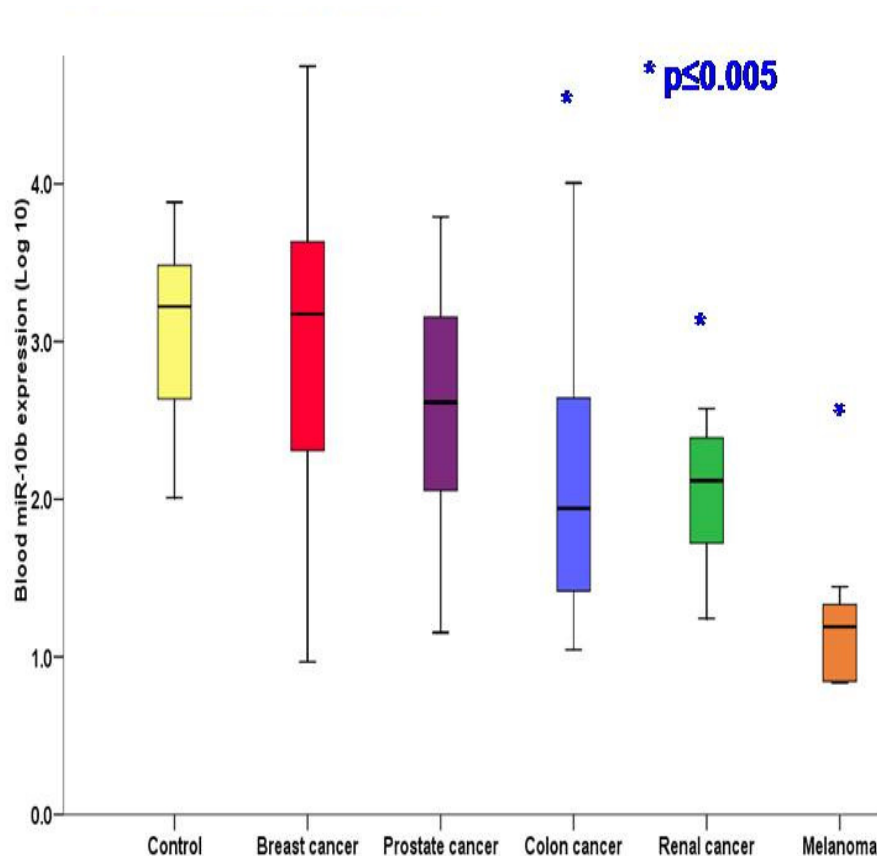
Figure 4.4 Circulating *miR-10b* expression in cancer patients compared to controls.

4.4.2.3 Systemic *miR-145* expression in various cancers

MiR-145, a tumour suppressor miRNA [213], was significantly decreased in blood from colon cancer, prostate cancer and melanoma patients compared with the control group (p=0.001). There was no significant relationship between *miR-145* expression levels in the circulation and the stages of disease for these 3 cancers (p=0.165, p=0.932 and p=0.323 respectively). Levels in breast cancer patients did not differ from the control group (p=0.162).

4.4.2.4 Systemic *miR-155* expression in various cancers

MiR-155, a miRNA associated with a variety of malignant tumours [214], was observed to be significantly decreased systemically in all malignancies (p<0.001) except breast cancer, where levels were similar to the control group (p=0.38, Figure 4.5). There was no relationship between systemic *miR-155* levels in cancer patients and any of the following clinico-pathological parameters: tumour size, grade, stage of disease, node positivity.
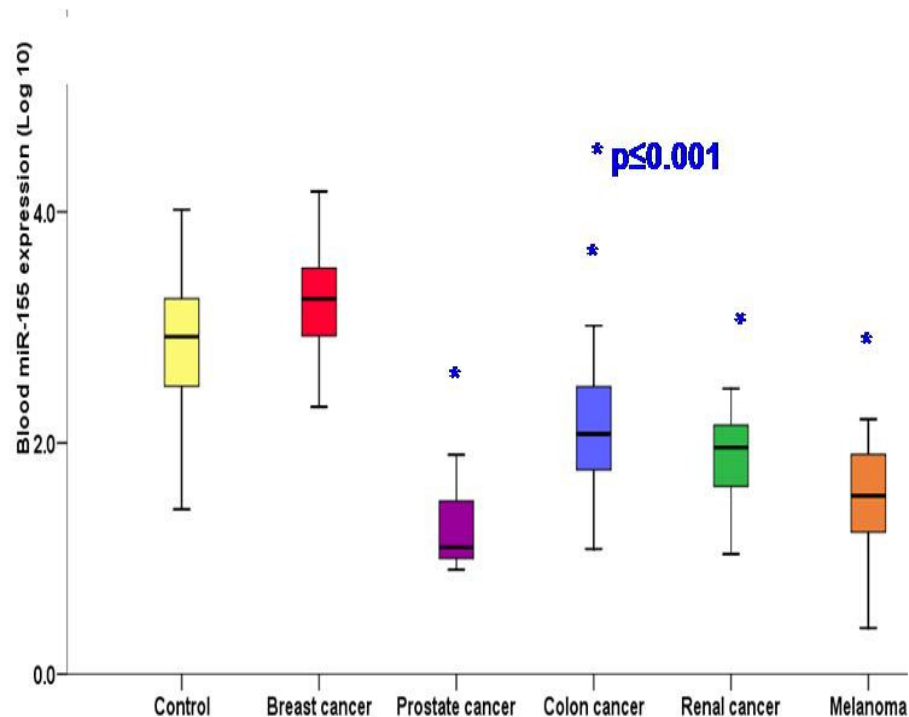
Figure 4.5 Circulating *miR-155* expression in cancer patients and controls. With the exception of breast cancers, there was a significant decrease in *miR-155* expression in all other cancer cases compared to the control group.

4.4.2.5 Systemic *miR-21* expression in various cancers

MiR-21, a well described oncogenic miRNA[215], was observed to be expressed at generally higher levels in the circulation of breast, colon, prostate and renal cancer patients compared to the healthy controls, however this increase did not reach statistical significance in this study cohort (Figure 4.6). In the breast cancer cohort the increase in *miR-21* levels above controls almost met statistical significance with a p-value of 0.056. Malignant melanoma patients were observed to express significantly lower levels of *miR-21* in the circulation when compared to controls p<0.001).
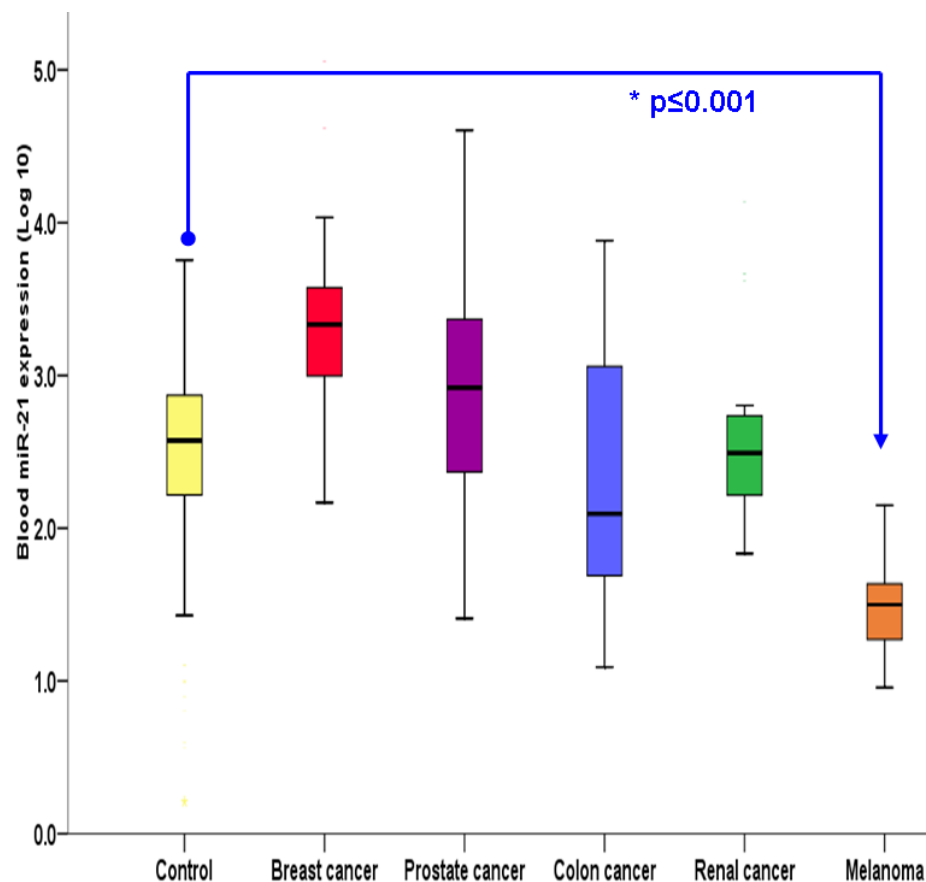
Figure 4.6 Circulating *miR-21* expression in cancer patients and controls. No significant difference in expression was observed between *miR-21* expression in breast, colon, renal, and prostate cancers compared to the controls. Although the elevated *miR-21* levels observed in the breast cancer group in comparison to the controls, almost met statistical significance (p=0.056). Melanoma patients has significantly lower levels of *miR-21* in their circulation compared to controls p<0.001)

4.4.3 Breast specific tumour marker

4.4.3.1 *MiR-195*; breast cancer biomarker properties

Only one of this panel of seven miRNAs, *miR-195*, was exclusively over-expressed in a specific cancer population. *MiR-195* was observed to be significantly over-expressed only in blood from the breast cancer patients ($p<0.001$), with levels in other cancer patients largely comparable to healthy controls (Figure 4.1). On average levels of *miR-195* in breast cancer patients were 25-fold (unlogged fold change) higher compared to levels in control subjects. Circulating *miR-195* levels correlated significantly with tumour size (Pearson's correlation coefficient 0.446, $p<0.001$) and all tumours, irrespective of size, expressed significantly higher levels of *miR-195* in the circulation compared to the control cohort (Figure 4.7). We observed a significant incremental increase in systemic *miR-195* levels between small tumours (T1 and T2) and both T3 and T4 tumour sizes ($p=0.002$ and $p<0.001$ respectively; ANOVA and Tukey post-hoc analysis, Figure 4.8). *MiR-195* was also detectable in patients with non-invasive disease; at levels significantly higher than the control group.
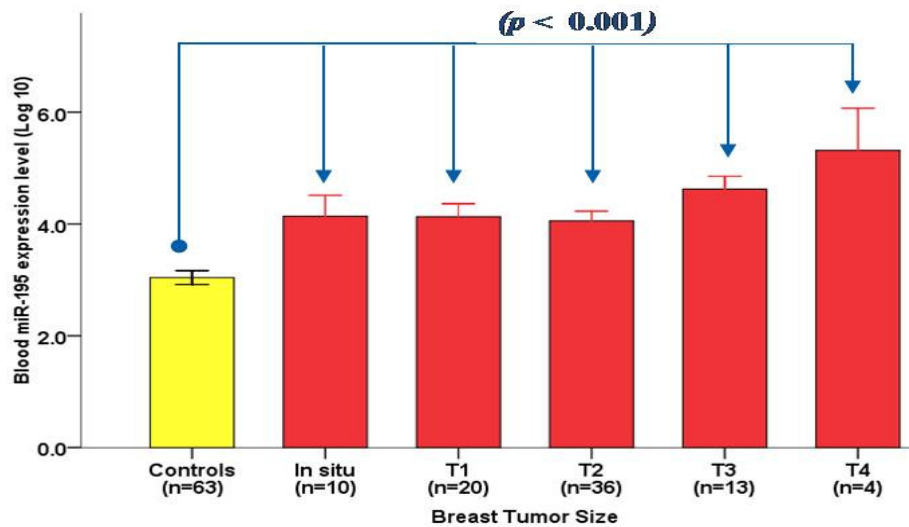
Figure 4.7 Circulating *miR-195* according to breast tumour size*. All tumours, irrespective of size, expressed significantly higher levels of *miR-195* in the circulation compared to the control cohort.
*Tumour size is documented as per the American Joint Committee on Cancer (AJCC) TNM system: **Tis:** Carcinoma in situ. **T1:** Tumour ≤ 2 cm. **T2:** Tumour 2.1 cm – 4.9 cm. **T3:** Tumour ≥ 5 cm. **T4:** Tumour of any size penetrating skin or chest wall.
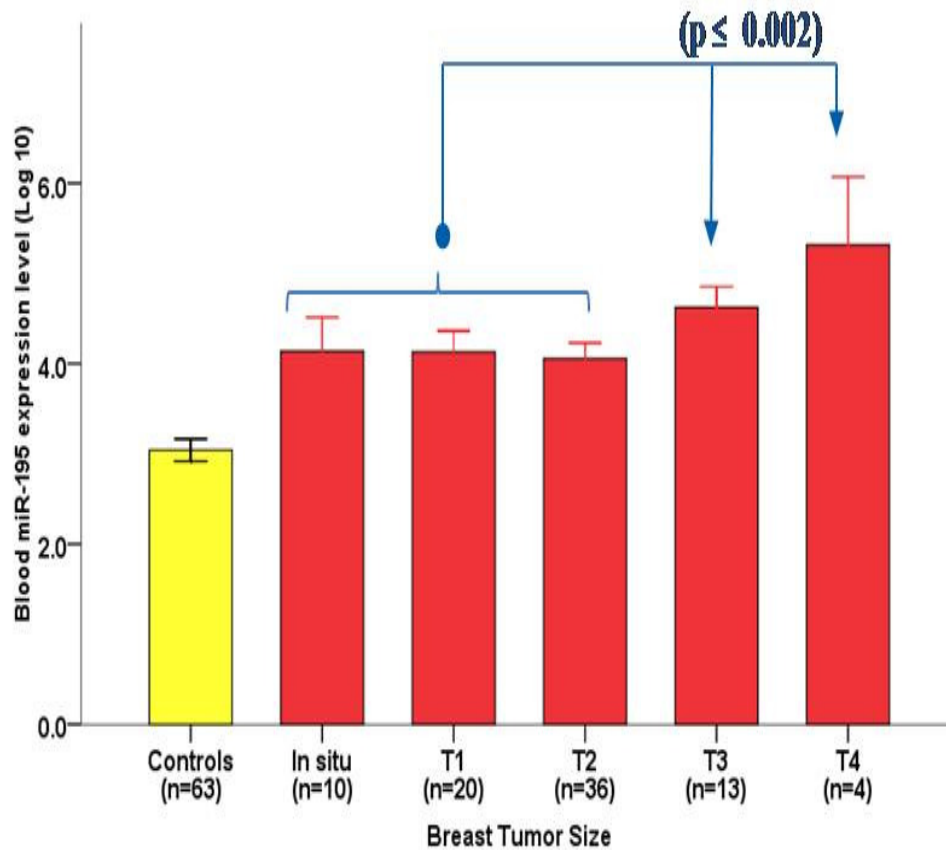
Figure 4.8 Circulating *miR-195* according to breast tumour size. A significant incremental increase in systemic *miR-195* levels was observed between small tumours (T1 and T2) and both T3 and T4 tumour sizes (p=0.002 and p<0.001 respectively; ANOVA and Tukey post-hoc analysis)

4.4.3.2 Sensitivity and specificity of systemic miRNAs for breast cancer

ROC analysis determined the optimal cut-off value for *miR-195* to differentiate breast cancer cases from controls, and from this analysis the sensitivity of circulating *miR-195* alone was determined to be 87.7%, at a specificity of 91%, with area under ROC of 0.937 (Figure 4.9). A panel of 3 circulating miRNAs further increased the sensitivity compared to individual markers in isolation. The combination of circulating levels of *miR-195, let-7a* and *miR-155* increased the sensitivity for differentiating breast cancer cases from controls to 94% (logistic regression analysis; p<0.001). Circulating *miR-195* levels did not correlate with the existing breast tumour marker cancer antigen 15.3 (CA 15.3) (Pearson's correlation coefficient 0.138, p=0.346).
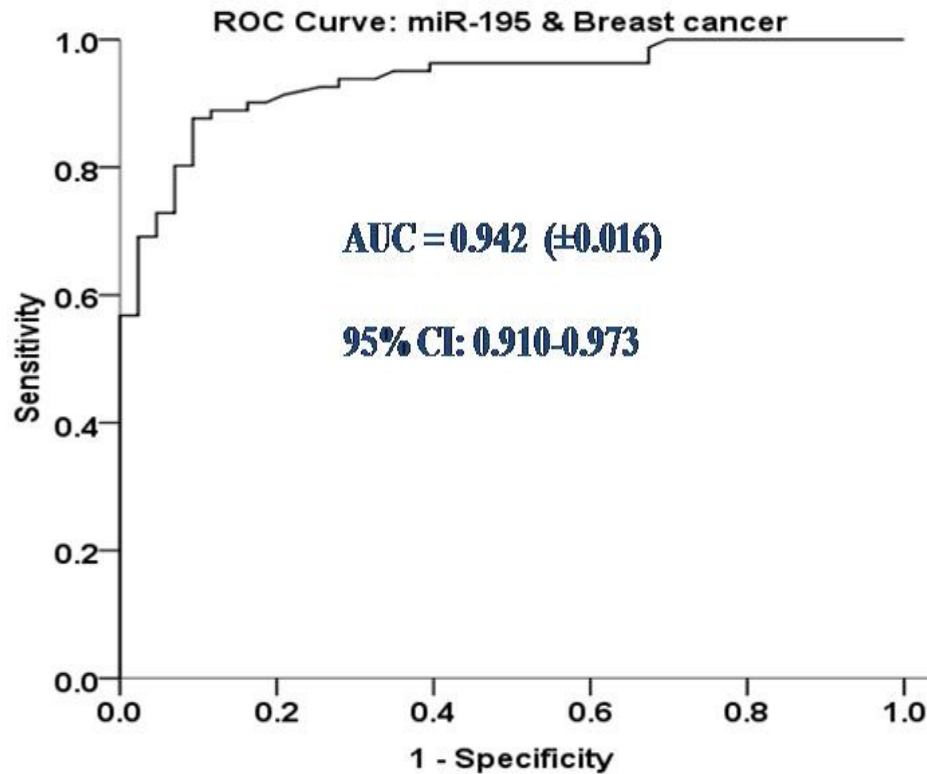
Figure 4.9 ROC curve of the breast cancer sample set analyzed for systemic *miR-195*.

4.5 Discussion

The results from this study demonstrate that cancer-associated miRNAs are generally dysregulated in the circulation of patients with visceral malignancy; this dysregulation appears to be relatively non-specific. This is somewhat predictable, given that the majority of miRNAs investigated in this study have been associated with a variety of common cancers, and are known to be involved in multiple critical stages of carcinogenesis. However a growing body of evidence, based on high-throughput miRNA microarray studies in many cancer types, has identified some miRNAs which appear to be specific for a given tissue type [50]. Furthermore it has been shown that miRNAs have the ability to identify the origin of poorly or undifferentiated tumours [216]. The data presented here for circulating *miR-195*, which was observed to be significantly elevated only in breast cancer patients, supports the hypothesis that certain miRNAs are site

specific. *MiR-195* had previously been demonstrated to be overexpressed in primary breast cancer tissue, which prompted its inclusion in the panel of miRNAs investigated in this study. Mattie *et al* identified *miR-195* levels to be significantly higher in HER2/*neu* positive compared to HER2/*neu* negative breast cancers [97]. Subsequently, Zhang *et al* identified *miR-195* to be significantly elevated in breast tumour compared to normal breast tissue [217], a finding which was reproducible in our breast cancer cohort thus validating these results (Chapter 3; Section 3.4.3) [212].

Several findings from this and the preceding study, provide convincing evidence to support circulating *miR-195* as a breast specific tumour marker. *MiR-195* expression is increased in breast tumours compared to normal breast tissue, a finding mirrored in the circulation where *miR-195* levels are considerably higher (19-fold) in breast cancer patients compared to healthy controls. Two weeks following curative resection, systemic levels decreased to a basal level, comparable with the control group. Furthermore both tumour and circulating levels correlated with disease burden (tumour size and stage of disease). Finally, *miR-195* was not elevated in blood from patients with other malignancies (prostate, colon, renal, melanoma).

Let-7a, one of the well established cancer-associated miRNAs, was observed to be significantly elevated in almost all of the cancer patients included in this study with the exception of those with malignant melanoma. These findings support previously published data which implicates *let-7a* as a protagonist in many cancers, particularly lung, breast, colon, gastric and ovarian. However we observed a paradoxical effect in the circulation (i.e. a significant increase in systemic *let-7a* levels in cancer patients compared to controls), to that described previously at tumour tissue level where *let-7a* is most commonly found to be under-expressed in tumour compared to normal tissue, for each specific cancer [218]. Although unexpected this finding may be explained by the interaction of *let-7a* with its target mRNA– the *KRAS* oncogene, at cellular level. Recent evidence proposes that dysfunctional interaction between *let-7* and *KRAS*, due to a single nucleotide polymorphism in the *let-7* complementary site in the *KRAS* 3' UTR, prevents *let-7* from binding and exerting its tumour suppressor effect resulting in

overexpression of the oncogene [117]. A plausible hypothesis is that this particular failure of micro- and messenger RNA to bind could lead to lower expression levels of *let-7a* in tumour tissues [77], and a reciprocal increase of free *let-7a* sequences entering the tumour microenvironment and subsequently, the circulation. This hypothesis warrants further elucidation in order to define the precise mechanism by which miRNAs enter the circulation.

MiR-155 levels were observed to be significantly decreased in the circulation of all cancer patients with the exception of breast cancers, in contrast to expression levels observed in primary breast tumour tissue [214]. Volinia *et al* identified *miR-155* as an oncogenic miRNA over-expressed in several solid tumours including colon, lung, lymphoma and breast [96]. Subsequent functional studies have further defined its critical role in carcinogenesis [219]; it is known to promote cell migration and invasion by targeting *RhoA*, a gene involved in cell junction formation and stabilization. *MiR-155* also mediates TGF-beta induced epithelial to mesenchymal transition – a remarkable process central to the development of tumour invasion and metastasis that involves the dissolution of epithelial tight junctions, intonation of adherens junctions, remodelling of the cytoskeleton, and loss of apical-basal polarity. The cohort of prostate, renal, colon and malignant melanoma cancers investigated in this study included a greater proportion of advanced cancers (TNM stages 2, 3 and 4) compared to our breast cancer subgroup, 29% of whom had *in situ* or stage I disease. This may have contributed to the disparity in circulating *miR-155* expression between breast and other cancers. However our data suggest that *miR-155* does have specific value as a biomarker for breast cancer. In the breast cancer subgroup, circulating levels of *miR-155* in combination with *miR-195* and *let-7a*, did increase the ability of these miRNAs to discriminate cancer cases from controls, above the sensitivity of either miRNA alone. The analysis of this panel of miRNAs in blood could achieve sensitivity as high as 94%.

Although these findings demonstrate the potential utility of circulating miRNAs as cancer-specific biomarkers, it is important to acknowledge that the sample size of cancers evaluated in this study is relatively small, particularly for the cancers other than breast cancer, and that the panel of miRNAs selected for

evaluation was biased towards our search for breast cancer specific markers. Additionally, the control group was small and perhaps not ideal for some of the cancers studied. For prostate cancer studies for example, an older group of males would need to be included in the control population in order to determine the normal reference range for circulating miRNAs, before one could define what levels were abnormal. Nonetheless, these data suggest sustained effort toward developing circulating miRNAs as cancer specific biomarkers is warranted.

4.6 Conclusion

Unique properties of miRNAs including their remarkable stability, tissue specific expression profiles, and ease with which they are quantified herald these molecules ideal cancer biomarkers. The findings presented here demonstrate the specificity of elevated circulating *miR-195* for breast cancer, and remarkably high sensitivity of *miR-195* in combination with the general oncomirs *let-7a* and *miR-155* for discriminating breast cancer cases from controls, thus prompting their potential utility as unique, non-invasive breast tumour markers. Further evaluation of blood-based miRNAs in larger cancer cohorts is necessary to validate these findings, and to further elucidate the feasibility of developing circulating miRNA assays specific for individual cancers, as clinically useful tools to detect even early stage malignancy.

Chapter 5

Inherited Variation in MiRNA Binding Sites

5.1 Introduction

A significant challenge in the management of common diseases such as breast cancer is the identification of 'at risk' individuals early, so that those at highest risk may benefit from targeted screening and therapeutic programmes. The advent of genetic testing presents one solution to this challenge. Breast cancer has long been known to have a significant genetic component; females with an affected first degree relative carry an approximately 1.8 times increased relative risk compared to the general population [220]. Singularly the most important step in our current understanding of breast cancer genetics to date has been the discovery of rare high penetrance genes, *BRCA1* and *BRCA2*, which confer up to a 30-fold increase in relative risk of developing the disease. However mutations in these predisposition genes account for only 5-8% of familial breast cancer overall [221]. Familial clustering has been observed in an additional 20% of breast cancer cases but is as yet unexplained. Subsequently, large genome wide association studies have identified several candidate moderate and low penetrance breast cancer susceptibility variants, such as single nucleotide polymorphisms (SNPs), which also confer smaller though important increases in risk of developing breast cancer [222]. Examples of genetic variations which have a documented association with breast cancer are illustrated in Figure 5.1. There remain a large proportion of breast cancer patients, with significant family histories, who are likely to have as yet unidentified inherited variations which contributed to their increased risk of developing breast cancer. The identification of these novel variants would enable improved risk estimation of breast cancer in those with a family history, and also at a population level.

Breast cancer is not a single disease, and stratifying breast tumours according to their hormone receptor status or cellular origin (i.e. basal or luminal) helps to reduce the underlying biologic complexity. Many of the currently known breast cancer genetic risk factors and SNPs associate preferentially, and often strongly, with oestrogen receptor (ER) positive or negative subtypes [223-224]. Unraveling any heterogeneity among patients in terms of genetic variations which may be unique to epithelial subtypes of breast cancer could be rewarding. Further large

genomewide association studies are warranted in an effort to detect novel associations simply by focusing on specific breast-cancer subtypes.
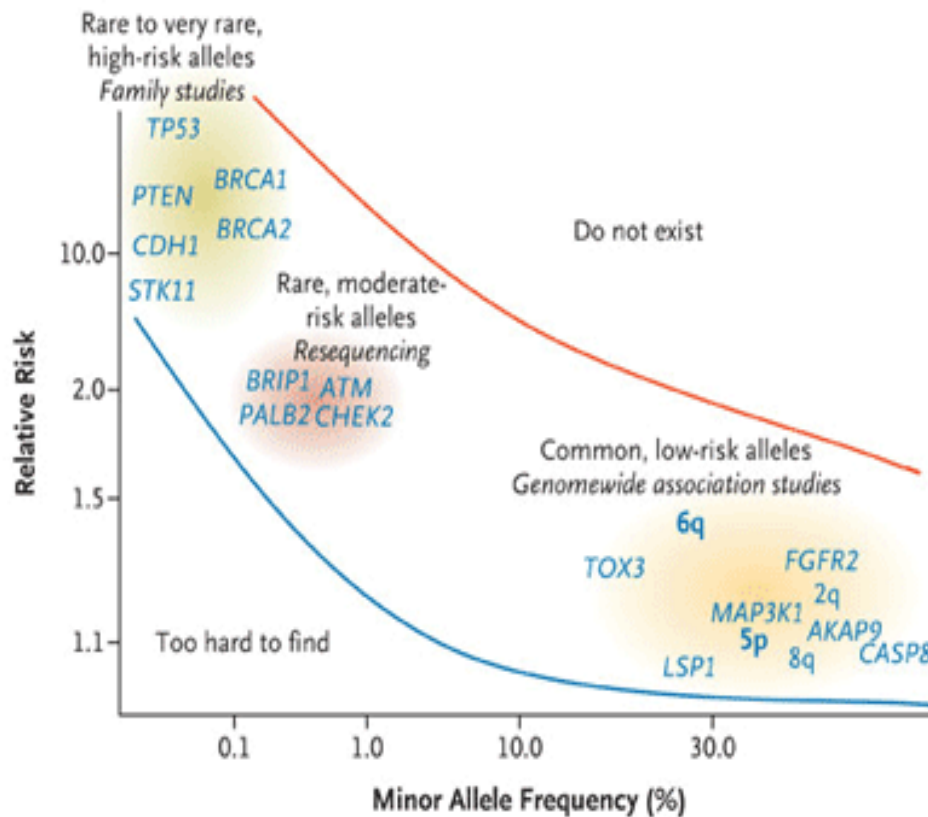
Figure 5.1 Breast-Cancer Susceptibility Loci and Genes[225].
All known breast-cancer susceptibility genes are shown between the red and blue lines. No genes are believed to exist above the red line, and no genes have been identified below the blue line. High-risk syndromic genes are highlighted in green. Mutations in serine threosine kinase 11 (*STK11*) cause the Peutz–Jeghers syndrome; in patients with this syndrome, the risk of breast cancer can be as high as 32% by the age of 60 years[226]. Similar or higher risks are seen in association with germ-line mutations in *PTEN* and the E-cadherin gene (*CDH1*). Mutations in *PTEN* are associated with florid bilateral fibrocystic breast disease and a substantially increased risk of breast cancer. Mutations in *CDH1* are associated with an approximate 40% risk of lobular breast cancer and diffuse gastric cancer[227-228]. The moderate-penetrance genes (highlighted in red) have an approximate relative risk of 2.0. There are probably many more genes in this class, but they can be identified only by resequencing candidate genes in affected persons in families with breast cancer. Mutations in *BRIP1, PALB2,* and *BRCA2* predispose to Fanconi's anemia. The common, low-risk genes are shown in orange. SNPs in *FGFR2* and *TOX3,* and those on chromosomes 5p and 2q specifically increase the risk of estrogen-receptor–positive breast cancer.

Although the Luminal A subtype of breast tumours (ER/PR positive, HER2/*neu* negative) are most prevalent (85%), it is the triple-negative (TN: ER, PR and HER2/*neu* negative) tumours which are the most challenging cohort to treat. Gene expression profiles have identified these tumours as a molecularly distinct subtype of breast cancer, characterized by low expression of hormone receptor and HER2-related genes. This subtype is termed 'basal-like' because of the resemblance of their gene expression patterns to that of the myoepithelial cell of the breast, and so triple-negative breast cancer has become a commonly used proxy for this subtype. There are currently no specific targeted therapies for the aggressive TN subclass of breast tumours despite laudable efforts to identify novel molecular therapeutic targets for this cohort [229]. Consequently the prognosis for these patients is dismal [230].

The significant association of the TN phenotype with younger age of onset and particularly higher prevalence among African American (AA) women suggests that there may be some genetic predisposition [195]. Unfortunately, few genetic markers of increased risk of developing TN breast cancer exist. None of the potentially modifiable risks or reproductive factors that have been associated with other breast cancer subtypes apply consistently to the TN tumours [231]. *BRCA1* gene mutations are often associated with TN tumours; at least three-quarters of *BRCA1*-related breast cancers are basal-like by microarray [15] or by immunohistochemistry [232]. However these mutations still only account for 10-15% of TN breast cancers [233-234]. These findings suggest that there may be additional genetic factors that predispose individuals to TNBC. Identifying those individuals at risk of developing this particularly aggressive phenotype of breast cancer will aid in targeted screening and therapeutic programmes.

MiRNAs regulate gene expression by base pairing with sequences within the 3'-untranslated regions (UTR) of target mRNAs; thereby causing mRNA cleavage and/or translational repression [35,235]. MiRNAs are dysregulated in almost every cancer, and it has recently emerged that a potential mechanism by which miRNAs effect carcinogenesis is the presence of polymorphisms in oncogenic miRNA coding sequences [137] or 3'UTR miRNA binding sites. The presence of SNPs in miRNA sequences or binding sites on their target mRNAs has been

associated with risk of developing a variety of malignancies, including breast cancer [117,236-237]. 3'UTR binding site SNPs in *BRCA1* have been identified as biomarkers of breast cancer risk in Thai familial breast and ovarian cancer families [236]. Another genetic variant associated with increased familial breast cancer risk, particularly in premenopausal and high risk women, is a SNP in a putative *miR-453* target site in the estrogen receptor (ESR-1) [237]. To date, none of these miRNA-altering biomarkers of disease risk have been associated with the TN subtype of breast cancer.

Researchers at the Slack and Weidhaas laboratories in Yale University (New Haven, CT) have previously identified a novel germline polymorphism (*rs61764370*) in a *let-7* miRNA binding site within the 3'UTR of the *KRAS* oncogene, referred to as the '*KRAS*-variant' or LCS6SNP [117]. This baseline frequency of this variant is approximately 6% - in a global population of non-cancerous controls. Their previous studies demonstrate that this *KRAS*-variant is functional; presence of this variant in the *let-7* complementary site within the 3' UTR of *KRAS* prevents *let-7* from binding to *KRAS* to exert its regulatory tumour suppressor effect, thereby disrupting *KRAS* regulation (Figure 5.2 a & b)[117]. Chin *et al* have demonstrated that this particular variant is a genetic marker of increased risk of ovarian cancer in the general female population, most strongly for patients from hereditary breast and ovarian cancer families (paper submitted). Because of this apparent association of the *KRAS*-variant with hereditary breast and ovarian cancer, we wished to further investigate the association of the *KRAS*-variant with breast cancer. Compared to elsewhere on mainland Europe, the west of Ireland has been undisturbed by secondary migrations throughout history therefore has a relatively homogenous population which makes it well suited for the study of complex genetic diseases. In collaboration with researchers at the Molecular Biology Laboratory at Yale University, we aimed to test the hypothesis that the recently identified SNP in the *let-7* complementary site within the 3' UTR of *KRAS* confers susceptibility to breast cancer.

Figure 5.2a
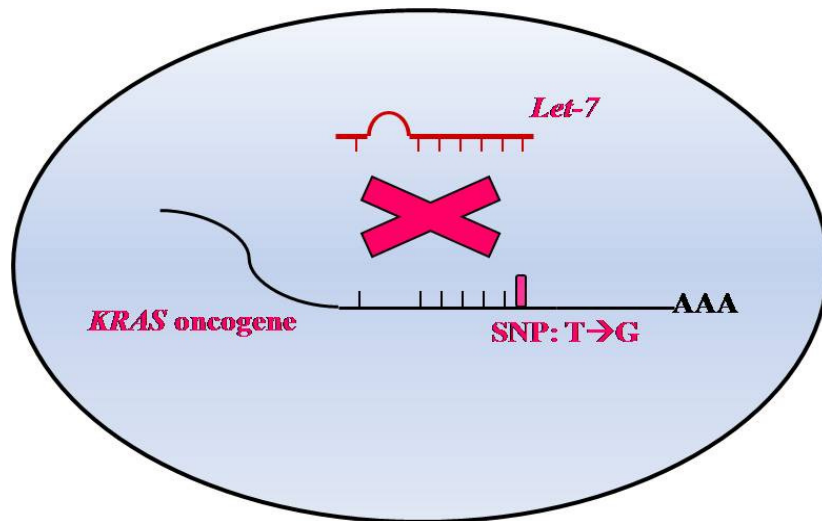
Figure 5.2b
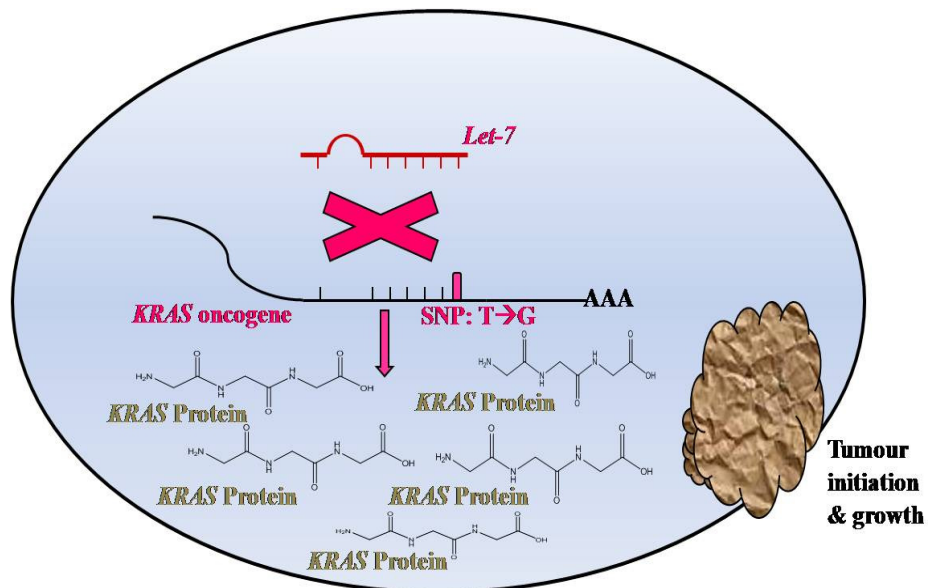
Figure 5.2(a) A polymorphism in a miRNA binding site at the 3' UTR of messenger RNA will prevent the miRNA target from binding here and thus exerting its important regulatory effects. This could lead to over activation of mRNA (eg. the *KRAS* oncogene) & uncontrolled protein translation (eg. the *KRAS* protein Fig 5.2b) which can induce tumour formation

5.2 Aims

The primary aim of this study was to assess for inherited variation in the oncogenic *let-7* binding site in *KRAS* in a large homogeneous breast cancer population, and thereby determine the risk this *KRAS*-variant imparts for developing breast cancer.

Secondary aims were to determine if the *KRAS*-variant was associated with the triple negative subtype of breast cancer or particular ethnic groups.

5.3 Materials and methods

A population analysis of the association of the *KRAS*-variant and breast cancer was conducted across two sites (Department of Surgery at Galway University Hospital and the Department of Molecular and Cellular Biology at Yale University) on a cohort of 1132 breast cancer patients and 930 age-matched non-cancer controls.

5.3.1   Study groups

5.3.1.1 Breast cancer cases

Two breast cancer populations were included in this analysis. The first consisted of subjects previously enrolled in a breast cancer case-control study in Connecticut [137]. This cohort comprised 415 consecutive patients with histologically confirmed breast cancer. Patients were aged between 30-80 years, of varied ethnicity, and had no prior history of cancer (other than non-melanoma skin cancer). Hormone receptor status was determined on all cases by immunohistochemistry (IHC) and Fluorescent *In Situ* Hybridisation (FISH) where IHC was equivocal. The second cohort consisted of 717 cases form the west of Ireland, each of whom also had histologically confirmed disease. All stages and histologic types of breast cancer, excluding pre-invasive carcinomas, were included. Full ER, PR and HER2/*neu* status was again determined on all samples.

Clinicopathological details of these combined cohorts (N=1132) are summarised in Table 2.3. Included in this breast cancer cohort were 136 patients (12%) with TN breast cancer. Informed consent, a detailed family history of breast and/or ovarian cancer and a blood sample for genomic DNA analysis were obtained from all cases in addition to all relevant demographic and clinical information.

5.3.1.2 Control cohort

Controls (n=570) were recruited from Yale New Haven Hospital (YNHH), Tolland County, or Fox Chase University Hospital for a previous study in Connecticut which formed a unique human genetic resource at Yale University (an allele frequency database for diverse populations and DNA polymorphisms) [238]. An additional cohort of healthy Caucasian controls were recruited in the West of Ireland (n=360). Demographic details of all 930 control females genotyped for the *KRAS*-variant in this study are outlined in Table 2.4. Although the New Haven and Irish controls differed in race and age, they were deemed suitable for inclusion in this study for the following reasons: these populations are known to share similar ethnic ancestry and have been subject to few demographic movements. Consequently, both populations are relatively homogenous, which reduces allelic and genotypic heterogeneity in case-control studies. The Irish controls (n=360) comprised women over the age of 60 years, with no self-reported personal history of any cancer and no family history of breast or ovarian cancer. This age range was chosen to reduce the possibility that these women may have undiagnosed or undeveloped familial breast cancers. The New Haven controls were slightly younger and age-matched to their breast cancer cases. These females also had no personal history of any cancer and no family history of breast or ovarian cancer.

In total, 930 control females had DNA samples available for genotyping and so were used for this study. An informed consent, family history of cancer, reproductive history, demographic factors and blood sample were obtained from all control subjects. Control individuals were interviewed by a clinician prior to being enrolled in this study to ensure they had no current or previous malignancy prior to inclusion in this study.

5.3.2 Blood collection

Venous whole blood samples (10ml; non-fasting) were collected from each participant in a Vacuette EDTA K3E blood bottle (Grenier). Unprocessed whole blood sample were stored at 4°C until required for genomic DNA isolation

5.3.3 Genomic DNA isolation from whole blood

DNA was extracted from 10ml samples of whole blood using the Chemagic Magnetic Separation Module (Chemagen) and manufacturer's reagents. Extractions were performed in batches of 12 samples. The extraction protocol is described in detail in Section 2.5.

5.3.4 Analysis of DNA concentration and integrity

DNA concentration was determined using a Nanodrop spectrophotometer and the wavelength-dependent extinction coefficient '50' was taken to represent the DNA- component.

5.3.5 SNP genotyping

All samples were genotyped for the *KRAS*-variant using a custom Taqman SNP genotyping assay (Applied Biosystems) which was specifically designed to identify the T or G allele of the *KRAS*-variant. Details of the SNP genotyping reactions are presented in Section 2.9.1.

5.3.6 Determination of the genotype

Samples heterozygous or homozygous for the variant G allele were considered positive for the *KRAS*-variant, based on prior studies published by the group at Yale University [117].

5.3.7 Data analysis

The genotype distributions of the controls were tested for Hardy-Weinberg equilibrium. Unconditional logistic regression was performed to estimate the relative risk associated with each genotype. Controls were adjusted for age and ethnicity. The population was stratified by menopausal status, and separate risk estimates were obtained by ER and PR status using multinomial logistic regression with a three-level outcome variable. Tests for interaction were

conducted using a Wald chi-square comparing the parameter estimates obtained for each genotype in ER/PR positive cases versus ER/PR negative cases. In order to determine the association of the *KRAS*-variant with a particular breast cancer subtype, the patients were stratified according to the subtype of breast cancer and a χ^2 test was performed to calculate two-sided p-values, OR, and 95% CI. The dominant model was used for all genetic association analysis due to the low frequency of the rare allele.

5.4 Results

5.4.1 The *KRAS*-variant predicts increased risk of breast cancer

The prevalence of the variant allele in the breast cancer cohort overall (all ethnic groups, all subtypes) was 13.7%, which is significantly higher than expected in any existing geographic population and higher than the 7.6% prevalence of the *KRAS*-variant among the control group in this study. This supports the hypothesis that that this variant allele is a marker of an increased risk of breast cancer [OR 1.92, CI 1.48 - 2.49, p<0.001] (Figure 5.3).
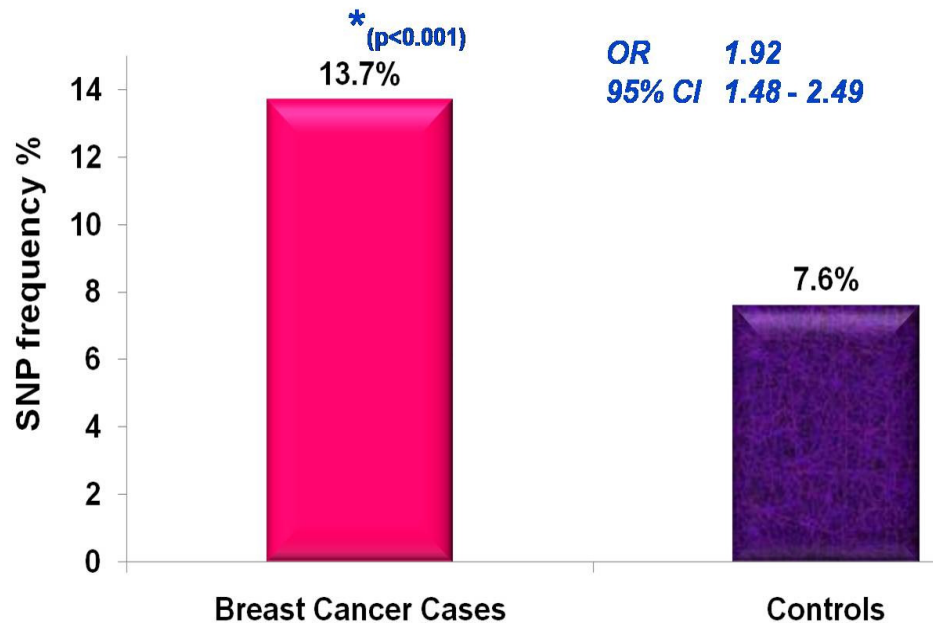
Figure 5.3 The *KRAS*-variant is significantly associated with all types of breast cancer compared to a global control population. The bar graph depicts the percentage of *KRAS*-variant positive breast cancer subjects (155 of 1132 females) compared to the proportion of all controls who are positive for the variant (71 of 930) [p<0.001, OR: 1.92, 95% CI: 1.48-2.49].

5.4.2 *KRAS*-variant is significantly associated with triple negative breast cancer

When analyzed by subtype, the *KRAS*-variant was found in 22% (*n* = 30/136) of women in the TN subgroup compared to less than 12-14% of women with the other breast cancer subtypes (*n* = 102/770 for Luminal A, 21/147 for Luminal B, and 4/79 for HER2/*neu* positive subgroups, p=0.02, Figure 5.4). These results indicate that *KRAS*-variant carriers with breast cancer are significantly more likely to have triple negative breast cancer compared to other subtypes (OR=1.94, 95% CI=1.12-3.36). Interestingly, the *KRAS*-variant was rarely associated with the HER2/*neu* positive subtype (ER-/PR-/HER2+), being significantly less common in this subtype compared to TNBC (5.26% vs. 22%, p = 0.0068). These findings indicate that the *KRAS*-variant is not only associated with ER and PR negativity, but is specifically associated with the triple negative subtype of breast cancer.
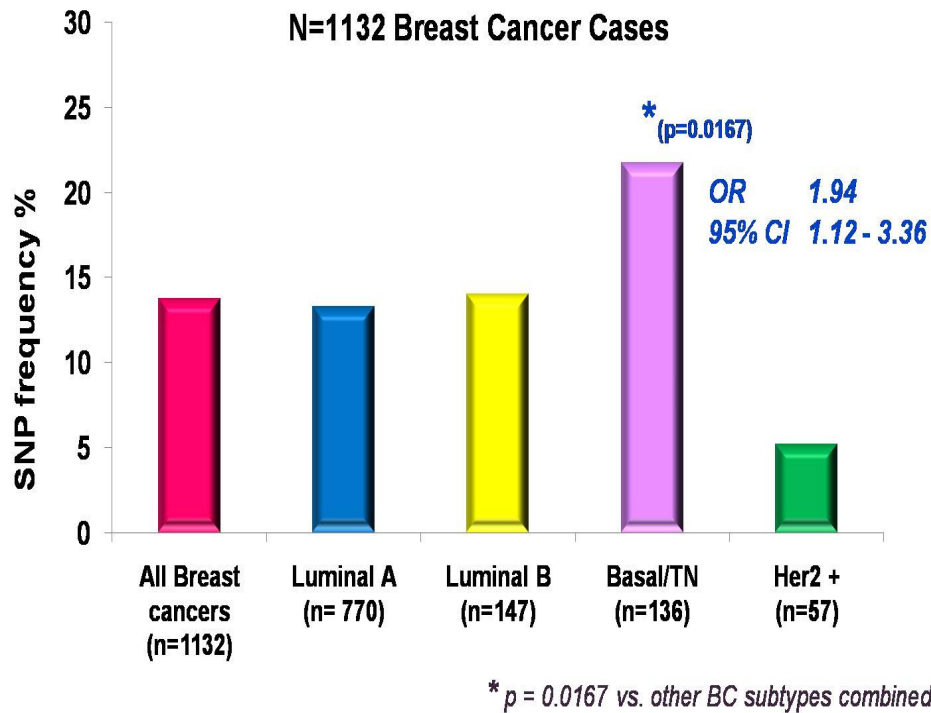
Figure 5.4 Distribution of the *KRAS*-variant in breast cancer subtypes. The triple negative subtype exhibited the *KRAS*-variant allele more frequently than all other subtypes (p=0.0167).

5.4.3 The *KRAS*-variant is associated with TNBC for women of all ethnicities

The subgroup of TN breast cancer cases (n=136) comprised not only Caucasian women, but also African American (AA) women (n=35%) and Hispanic women (7%). In order to determine the association of the *KRAS*-variant with the risk of TN breast cancer in different ethnic groups, the frequency of the *KRAS*-variant in the cohort of 136 TN patients with known ethnicities was compared to the frequency of the variant in ethnicity-matched controls. In this TN patient cohort 35% of women were AA (*n* = 48) and 7% Hispanic (*n* = 10) (Figure 5.5a). It was observed that 31% of Caucasian TN patients, 6.25% of the AA TN patients and 40% of Hispanic TN patients carried the *KRAS*-variant compared to 7.8%, 2.2% and 0% of ethnicity-matched controls respectively (Figure 5.5b). Because the numbers of patients in these groups were small, it was not possible to display significance between ethnic groups with regard to *KRAS*-variant TN breast cancers. However, these findings suggest that the *KRAS*-variant is a genetic marker of an increased risk for developing TNBC for women of all ethnicities.

Figure 5.5a
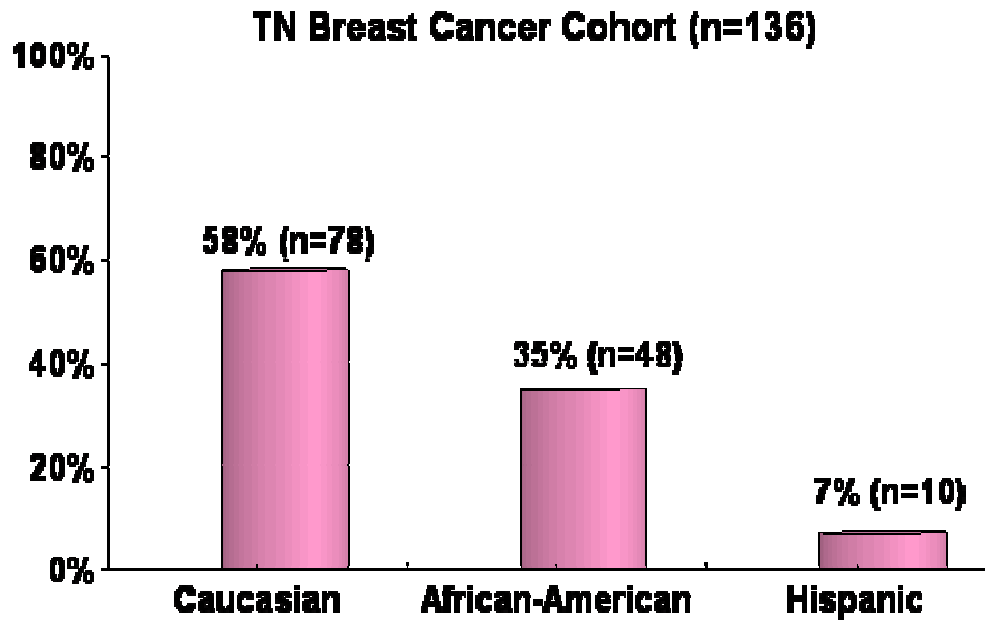
Figure 5.5b
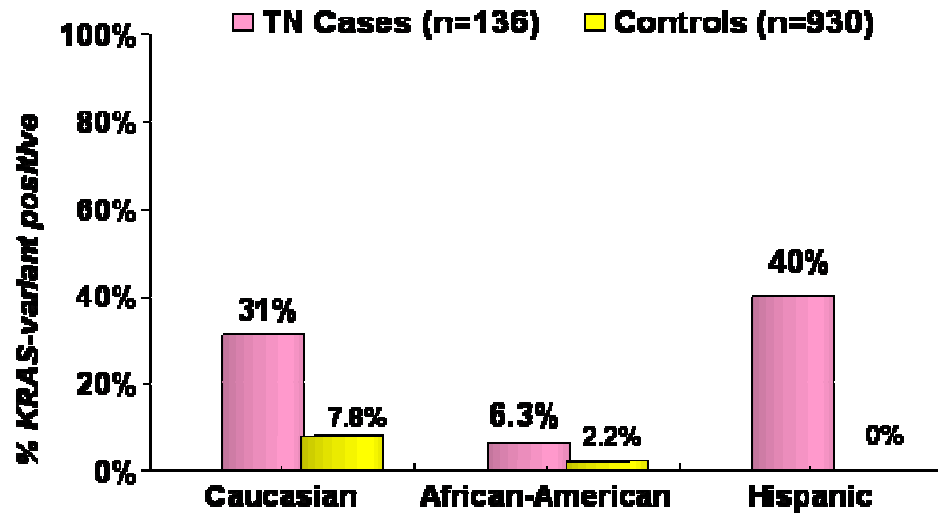
Figure 5.5 The *KRAS*-variant in a triple negative breast cancer cohort according to ethnicity of the subjects. **(a)** Ethnic composition of the TN breast cancer cohort. **(b)** Prevalence of the *KRAS*-variant in the Caucasian, African-American and Hispanic TN breast cancer patients, compared to respective ethnicity-matched controls.

5.4.4 The *KRAS*-variant predicts increased risk of TN breast cancer for pre-menopausal women of all ethnicities

A significant association of the *KRAS*-variant with breast cancer among pre-menopausal women with TN breast cancer was also observed (OR: 2.32; 95% CI: 1.11-4.90, p = 0.015, Figure 5.6). This association was not observed in women with ER and/or PR positive tumours, or for postmenopausal women. The frequency of the *KRAS*-variant was 27% in pre-menopausal ER/PR/Her2 negative breast cancer patients vs. 10.6% for controls and 9.1% for pre-menopausal ER and/or PR positive cases. These findings indicate that the *KRAS*-variant is a genetic marker of an increased risk of developing TN breast cancer for younger women.
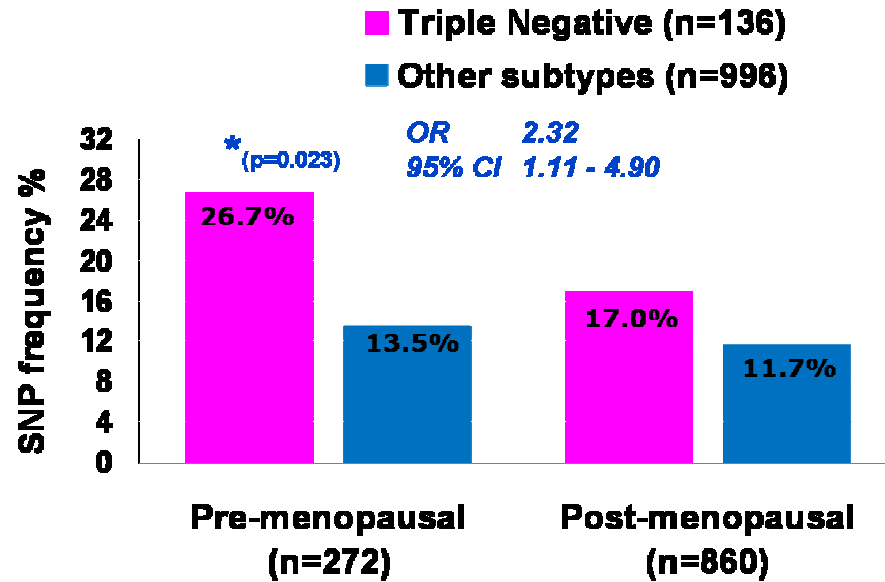
Figure 5.6 The *KRAS*-variant in a triple negative breast cancer cohort according to menopausal status. The *KRAS*-variant is far more prevalent in the pre-menopausal TN cohort compared to post-menopausal patients with the same subtype (p=0.023, OR: 2.32, 95% CI: 1.11-4.9).

5.4.5 Characteristics of *KRAS*-variant TN breast cancer patients

The mean age of TN cancer patients with the *KRAS*-variant appeared to be lower for Caucasian and Hispanic TN patients, but not for AA TNBC patients. Because of the trend for the *KRAS*-variant TN breast cancers to be younger, an association between these cancers and *BRCA1* mutations was investigated. 22 women with TN breast cancer had known *BRCA1* status; 2 of whom had *BRCA1* mutations and 5 of whom had the *KRAS*-variant. None of the TN patients in this small cohort were both *BRCA1* and *KRAS*-variant positive. While this does not rule out an association of *BRCA1* and the *KRAS*-variant in enhanced TN breast cancer risk, it does confirm that the *KRAS*-variant is not simply a surrogate marker for *BRCA1* mutations, suggests it identifies a separate group of women at risk, and by itself likely represents a true genetic marker of TN breast cancer risk.

5.5 Discussion

The results of this study indicate that a germline polymorphism in the *KRAS* 3'UTR, the '*KRAS*-variant', is a genetic marker of increased risk of developing breast cancer, in particular the triple negative subtype in younger women. These findings have been demonstrated in two independent breast cancer populations; one in the West of Ireland and the other in New Haven, Connecticut. Furthermore it is evident from this study that the *KRAS*-variant is more prevalent in particular ethnic groups such as the Hispanic triple negative breast cancer cohort where the KRAS variant was present in 40% of breast cancer cases compared to 0% of ethnicity- matched controls. The variant was also 3 times more prevalent in African American patients with triple negative breast cancer compared to their respective controls. Due to limited controls for these cohorts we were unable to prove that this association of the *KRAS*-variant with African American and Hispanic women was statistically significant, but our findings support the hypothesis that the *KRAS*-variant is a genetic marker of TNBC risk for women of all ethnicities. Although TN breast cancer is associated with *BRCA1* mutations; in that at least three-quarters of *BRCA1*-related breast cancers are basal-like by microarray and negative for hormone receptors by IHC [239], these mutations are rare and of all TN breast tumours, *BRCA1* mutations account for only ~10-15% [234,240]. This indicates that there are likely to be significant risk factors, other than high penetrance *BRCA* mutations, predisposing to this aggressive phenotype of breast cancer.

In contrast, the *KRAS*-variant is found in almost 30% of premenopausal ER/PR/ HER2 negative breast cancer patients, and in over 20% of unselected TN patients. The finding of no overlap between *BRCA1* mutations and the *KRAS*-variant in our small subset of TN patients indicates that the *KRAS*-variant is not just a surrogate marker for *BRCA1* mutations, but identifies a unique set of women at risk for developing TN breast cancer. This finding is consistent with other recent data in ovarian cancer from the research team at Yale University, where the *KRAS*-variant was highly enriched in uninformative (*BRCA1* and *BRCA2* negative) ovarian cancer patients (paper submitted).

It has been demonstrated that this *KRAS*-variant directly affects regulation of *KRAS*, resulting in higher *KRAS* levels in tumours with the variant allele [117]. Activating tumour-acquired *KRAS* coding sequence mutations resulting in *KRAS* over-activity are common in human tumours, yet are rare in breast cancer. However, high expression of *KRAS* is observed in 70% of TN/basal tumours but not in other breast cancer subtypes [241]. One could hypothesize that the *KRAS*-variant may contribute to the over-expression of *KRAS* that is frequently seen in TN tumours. Further investigations are warranted to test this possibility and to delineate precisely the role of the *KRAS*-variant in cancer predisposition.

This study has several strengths, including the large numbers of breast cancer cases and control subjects and the availability of clinicopathological data on the cancer cases. However, a number of weaknesses permeate the study. Firstly, the diverse origins of females in the control group may be deemed problematic. As described in Section 2.2.1.3, the large control group used in this study was comprised of two independent cohorts of healthy females; one from Connecticut in the United States and the other from the West of Ireland. While their nationalities may appear unrelated, these populations are well known to share similar ethnic ancestry and have been subject to few demographic movements so in fact they are quite a homogeneous population. This is an important consideration in case-control studies as it reduces allelic and genotypic heterogeneity. Secondly, there is limited clinical data available on the control individuals. It is known from the questionnaires they completed at entry into the study that they did not have and current or prior malignancy, but information on other clinical illnesses and demographic data is lacking. Thirdly, no follow-up data is included in this study. It has been shown in other studies that the *KRAS*-variant is a biomarker of poor outcome in several cancers including head and neck cancer. Given the unavailability of survival data for the cancer cohort in this study, it was not possible to assess if *KRAS*-variant positivity was independently associated with poorer outcome or survival in these breast cancer patients.

5.6 Conclusion

These results provide the first evidence that the *KRAS*-variant is a genetic marker of risk for TN breast cancer. The association of the *KRAS*-variant particularly with this aggressive subtype of breast cancer is of potential clinical significance, as few genetic risk factors are known for this subtype of breast cancer, and those that are known account for only a minority of cases. Because the TN subtype carries the poorest prognosis, predicting the risk of this particular subtype of breast cancer is of utmost importance, to allow early screening and intervention for these patients. Furthermore, identifying the genetic causes of TN breast cancer are the first steps towards fully elucidating the biology of this disease in order to develop targeted effective therapies for these patients. Further work is necessary to determine the importance of the presence of the *KRAS*-variant in TN and other breast cancers. In addition to obtaining survival data on breast cancer patients with the variant allele, it is critical that concerns about the prevalence of the *KRAS*-variant in the general healthy population are addressed. Larger studies involving genotyping of extensive numbers of healthy individuals, representing various ethnicities, countries and ages, will have to be conducted before the true significance of the *KRAS*-variant in cancer patients can be determined.

Chapter 6

MiRNA Expression in Obesity

6.1 Introduction

Obesity and the metabolic syndrome are major public health concerns, and present a formidable therapeutic challenge. At present the World Health Organization estimates that 400 million adults are affected by obesity globally, as well as a significant number of children and adolescents [21]. The incidence of this disease spectrum continues to rise and contributes significantly to global morbidity, mortality and socioeconomic burden. Undoubtedly the leading factors contributing to its development are dietary excesses and lifestyle issues. However there is a growing body of evidence to suggest that aberrant genetic expression may play a significant predisposing and causative role in its pathogenesis [22-24]. The metabolic syndrome - a state of metabolic dysregulation characterised by insulin resistance, inflammation, and a predisposition to type 2 diabetes mellitus, dyslipidemia, premature atherosclerosis, and other disorders [25] – is also postulated to have underlying genetic determinants which are incompletely understood. Furthermore the development of this syndrome is closely linked to depot-specific distribution of fat, with central adiposity conferring greatest risk, and genetic factors are thought to predispose to this particular phenotype [242]. Whilst numerous studies describe an association between excess intra-abdominal (omental) fat and metabolic comorbidities, particularly non-insulin dependent diabetes mellitus, few have examined the specific role of omental fat on metabolic syndrome development.

Current treatment modalities for obesity and the metabolic syndrome include lifestyle modification, diet and pharmacologic agents though many patients remain recalcitrant to conventional medical therapy. Bariatric surgery is perhaps the most effective treatment, resulting in sustainable and significant weight loss and resolution of metabolic comorbidities in up to 80% [243-244], yet it carries inherent risks. Appropriate patient selection for operative intervention is critical in order to minimize the surgical risks and achieve optimal outcomes. Weight loss, the metabolic response, and resolution of comorbidities after intervention for morbid obesity have been reported to vary substantially between patients. At present there is no reliable clinical parameter or biomarker which predicts outcome after bariatric surgery[28]. Investigation of the molecular mechanisms and

genetic abnormalities underpinning metabolic disorders may identify new pathways involved in complex metabolic disease processes, improve our understanding of metabolic disorders, and influence future approaches to the treatment of obesity.

Profiling and functional investigation of miRNAs and their targets has identified them as critical regulators of a variety of cellular processes including differentiation, proliferation, and apoptosis [45]. Thus far, significant progress has been made in elucidating the precise role of miRNAs primarily in carcinogenesis [80-81]. More recently similar efforts in the study of benign disease processes have also identified miRNAs as key players in a variety of non-malignant, though common and similarly challenging diseases [e.g. diabetes mellitus, obesity, heart failure, infectious, inflammatory and auto-immune conditions including viral hepatitis, inflammatory bowel disease and rheumatoid arthritis] [80,82-88]. The cellular processes implicated in these conditions which are proposed to be regulated by miRNAs include adipocyte differentiation, metabolic integration, insulin resistance, appetite regulation and control of the immune response [120].

In addition to controlling the expression of up to one-third of all protein-coding genes [45], and regulating complex cellular pathways, miRNAs are emerging as ideal biomarkers of disease consequent to their tissue specific expression and association with clinicopathologic parameters [81,131]. The ability to quantify miRNAs in circulation has uncovered a new repertoire of biomarkers with potential to aid in disease diagnosis, stratification, prognostication and follow-up. In the field of metabolic diseases such as obesity and its associated comorbidities, an ongoing challenge is this search for novel non-invasive markers that are reflective or predictive of one's metabolic state, and prognostic for individuals being considered for bariatric surgery. MiRNA analysis has the potential to fill that void in current clinical practice. Unlike in cancer, few studies have investigated miRNA expression in human omental or subcutaneous fat, or in the circulation, as potential biomarkers for obesity and the metabolic syndrome. Until now evidence supporting the critical role of miRNAs in metabolic pathways has emanated from investigating their effects in-vitro or in model organisms.

6.2 Aims

The primary aim of this study was to characterise the expression of miRNAs in human fat tissue; both in omental and matched subcutaneous fat samples from obese and non-obese individuals.

We wished to determine if miRNA expression was similar in the two main fat depots (omentum and subcutaneous fat), or whether these two fat types displayed unique miRNA profiles. We then aimed to identify whether miRNA expression in fat tissue (either omental or subcutaneous) differed between obese and non-obese subjects, and between obese patents who were 'metabolically unhealthy' compared to obese individuals who were free of metabolic comorbidities. Finally we wished to establish if circulating miRNAs have potential as biomarkers for selecting appropriate management strategies for the obese.

6.3 Materials and methods

6.3.1 Study design and patient cohorts

This study was divided into three phases: Phase I – adipose tissue marker discovery, Phase II - marker selection and validation, Phase III – circulating biomarker evaluation.

Phase I: Adipose tissue marker discovery

Following ethical approval and written informed consent, paired omental and subcutaneous adipose tissue samples were obtained from 5 patients at the time of elective abdominal surgery procedures; 3 of this cohort were morbidly obese (BMI > 40 kg/m^2) and underwent bariatric surgical procedures whilst 2 individuals underwent elective laparoscopic Nissen's fundoplication and had BMI of < 25 kg/m^2. MiRNA expression profiles were generated from all 10 adipose tissue samples. Analysis of the miRNA expression profiles obtained from obese and non-obese adipose tissue permitted the establishment of two differential miRNA expression patterns which were then compared. Dysregulated miRNAs in obese adipose tissue were identified for further analysis in Phase II.

Phase II: MiRNA marker selection and validation in adipose tissue

Paired omental and subcutaneous adipose tissue samples were obtained from a larger cohort of 19 bariatric surgery patients (all with BMI > 40 kg/m^2) and 10 control subjects all with BMI < 25 kg/m^2 who underwent elective laparoscopic abdominal procedures (Nissen's fundoplication, cholecystectomy, para-oesophageal hernia repairs). Demographic and baseline clinical details of these cohorts are illustrated in Table 6.1. 68% of the obese cohort was deemed 'metabolically unhealthy' based on the diagnosis of one or more metabolic comorbidities including:

- Hyperlipidaemia (fasting total cholesterol >6.2 mmol/L, and/or fasting triglycerides >2.25 mmol/L)
- Hypertension (systolic BP > 130 or diastolic BP >85 mm Hg, or treatment of previously diagnosed hypertension)
- Previously diagnosed type 2 diabetes, or impaired glucose tolerance (fasting plasma glucose > 5.6 mmol/L, or abnormal Oral Glucose Tolerance Test result).

Of the obese cohort, 32% had no metabolic comorbidity, nor were taking any medication for the treatment of hyperlipidaemia, hypertension or Diabetes Mellitus. This subgroup was termed 'metabolically healthy'. Candidate miRNA markers were identified from the profiling experiment in Phase I and from literature documenting important roles of specific miRNAs in adipogenesis and other metabolic pathways (Table 6.2). The expression of these putative markers was validated in the larger cohort of adipose tissue samples from 19 obese and 10 non-obese individuals.

Table 6.1 Demographic and metabolic characteristics of adipose tissue study groups [Means (SD)]

	Obese (n=19)	*Controls (n=10)*	*p-value*
Male/Female	32% / 68%	40% / 60%	*0.415*
Age (yrs)	43.9 (11.9)	44.5 (16.9)	*0.935*
BMI (kg/m^2)	48.7 (9.0)	23.9 (1.4)	*<0.001*
DM Type II	48%	20%	*<0.001*
Hypertension	63%	30%	*0.09*
Hyperlipidaemia	68%	30%	*0.05*
HbA1C	6.1 (1.3)	6.2 (1.9)	*0.90*
Lipid profile			
LDL	2.8 (0.8)	2.9 (0.6)	*0.69*
Tg	2.1 (1.0)	1.3 (1.0)	*0.03*
HDL	1.3 (0.6)	1.6 (0.4)	*0.04*

Table 6.2 Candidate miRNAs for investigation in obese patients and their putative mRNA targets

MiRNA	Target Tissue	Function	Target Gene
miR-143	Adipose	(pre)Adipocyte differentiation	MAPK7
miR-132	Adipose	Adipocyte proliferation and growth, insulin resistance	CREB
miR-17-5p	Adipose	Adipocyte clonal expansion, insulin resistance	RBL2
miR-99a	Adipose Liver	Fatty acid metabolism, Cholesterol biogenesis	IGF1R, CYP26B1
miR-34a	Pancreas	Beta cell function in human pancreas	IL-1beta, TNF-alpha
miR-122	Liver	Cholesterol biosynthesis, cellular stress response, Hepatitis C virus replication	PMVK, TRPV6, BCL2L2, CCNG1, HMGCR
miR-145	Colon	Cell proliferation	IRS1
miR-195	Brain	Appetite regulation, energy balance regulation	BDNF

Phase III: Candidate miRNA evaluation as circulating metabolic biomarkers

Putative miRNA markers identified in Phase II were analysed in the circulation of an expanded cohort of obese patients (n=30) and non-obese controls (n=20). The controls for this phase consisted of age-matched healthy volunteers from the community with BMI of less than $25kg/m^2$, and the non-obese surgical patients who donated a whole blood sample in addition to omental and subcutaneous fat samples at the time of elective abdominal surgery.

Written informed consent was obtained from all participants for the use of their tissue and blood specimens. Exclusion criteria included any current or previous history of malignancy, infectious or inflammatory process.

6.3.2 Adipose tissue and blood collection

Paired omental fat and subcutaneous fat (from the peri-umbilical area) samples were harvested intra-operatively from each surgical patient, and snap frozen in liquid nitrogen prior to storage at -80°C until required for analysis.

A fasting venous blood sample (10ml) was collected from each participant in a Vacuette EDTA K3E blood bottle (Grenier Bio-One International AG, Kremsmünster, Austria) for miRNA quantification[212]. Glycosylated haemoglobin (HbA1C) was also measured in unclotted whole blood. Fasting serum samples were also obtained from each participant in Vacutainer Serum Separator Tubes II (Becton Dickinson) for the purpose of blood lipids and glucose analysis.

6.3.3 RNA isolation from adipose tissue and blood

Total RNA was prepared from all fat specimens (omental and subcutaneous) using a copurification technique, modified from the Tri Reagent BD (Molecular Research Centre) copurification protocol. In brief approximately 100 mg of tissue was homogenized in 1 mL of Trizol (Invitrogen), then heated to 60°C for 5 minutes in a water-bath to augment lysis of the adipose tissue prior to centrifugation at 12000g for 15 minutes, at 4°C. The clear aqueous phase (approximately 500μL) from each sample was removed and RNA was precipitated by the addition of an equal volume of isopropanol followed by centrifugation of the solution at 12000g for 8 minutes at

18°C. Following removal of the supernatant, the RNA pellet was then washed with equal volume (approximately 500 μL) of 75% ethanol. An additional ethanol wash was performed to improve the purity of RNA isolated. Each RNA pellet was solubilised using 30 μL of nuclease free water and transferred to storage tubes prior to storage at -80°C. Total RNA was extracted from 1mL of whole blood using an adaptation of the TRI Reagent® BD technique, as previously described [212]. RNA concentration of all samples was determined using a Nanodrop® spectrophotometer. The wavelength-dependent extinction coefficient '33' was taken to represent the micro- component of all RNA in solution as detailed earlier (Chapter 2). In general concentrations ranging between 30-300 ng/μL of miRNA were obtained per sample, (adipose tissue and blood RNA concentrations were comparable). Integrity was assessed using RNA 6000 Nano LabChip Series II Assays (for small RNA) on a 2100 Bioanalyzer as described previously (Chapter 2).

6.3.4 MiRNA microarray profiling

Adipose tissue samples (n=10) from 3 obese patients and 2 age-matched non-obese controls (paired omental and subcutaneous fat samples from each case) were selected for miRNA profiling in the biomarker discovery phase of the study. MiRNA expression profiling was performed using a customized real-time PCR-based miRNA array containing a panel of 95 miRNA assays and the U6 snRNA transcript as a proposed endogenous control (Systems Biosciences). Briefly, 100 ng of total RNA isolated from adipose tissue specimens was polyadenylated then reverse transcribed to cDNA. Real time quantitative PCR was performed using Power SYBR MasterMix (Applied Biosystems) and miRNA specific primers, in a an ABI 7900 HT Fast Real-Time PCR System (Applied Biosystems). PCR reactions were initiated with a 2 minute incubation time at 95°C followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. After each PCR run, an additional melt analysis was performed to assess the Tm of the PCR amplicon; this verified the specificity of the amplification reaction.   The fold change of miRNA gene expression was calculated by the equation $2^{-\Delta\Delta Ct}$, where Ct is the cycle threshold. The cycle threshold (Ct) is defined as the number of cycles required for the

fluorescent signal to cross the threshold in qPCR. ΔCt was calculated by subtracting the Ct values of the endogenous control from the Ct values of the miRNA of interest. ΔΔCt was then calculated by subtracting ΔCt of the control from ΔCt of disease (obese cases).

6.3.5 Validation of miRNA expression in adipose tissue and blood by RQ-PCR

RQ-PCR quantification of miRNA expression was performed using TaqMan MicroRNA® Assays (Applied Biosystems) according to the manufacturer's protocol. 100ng of total RNA was reverse-transcribed using the MultiScribe™-based High-Capacity cDNA Archive kit (Applied Biosystems). RT-negative controls were included in each batch of reactions. PCR reactions were carried out in final volumes of 10 μL using an ABI 7900 HT Fast Real-Time PCR System (Applied Biosystems,). Briefly, reactions consisted of 0.7 μL cDNA, 5 μL TaqMan® Universal PCR Fast Master Mix, 0.2 μM TaqMan® primer–probe mix (Applied Biosystems). Reactions were initiated with 10-minute incubation at 95°C followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. An interassay control was included on each plate and all reactions were performed in triplicate. *MiR-16* was used as endogenous control for quantitative PCR analysis of miRNA expression in fat and blood specimens given the stability of its expression in all samples, and a consensus that it is an appropriate normalizing gene for miRNA biomarker investigations [64,132,245]. The threshold standard deviation for intra-assay and inter-assay replicates was 0.3. Relative quantities of miRNA expression were calculated using the comparative cycle threshold (ΔΔCt) method [64], normalised to *miR-16* levels, and the lowest expressed sample was used as a calibrator.

6.3.6 Statistical analysis

Significance Analysis of Microarrays (SAM) was employed to analyse data from the miRNA profiling experiment in order to identify miRNAs that were differentially expressed in human fat tissue according to the following response variables: obese status and omental vs. subcutaneous fat. This analysis was performed using TiGR Tools TMeV 4.0 Java version 1.5.0_04-b05 software as described in section 2.7.3.

Differentially expressed miRNAs identified from these analyses were selected for validation by RQ-PCR in the larger cohort of fresh frozen adipose tissues from obese (n=19) and non-obese (n=10) individuals, and for analysis in the circulation of obese and non-obese individuals.

Data were then analysed using the software package SPSS 17.0 for Windows. Due to the magnitude and range of relative miRNA expression levels observed, results data were log transformed for analysis. There was no evidence against normality for the log transformed data as confirmed using the Kolmogorov-Smirnov test and so data are presented as Mean ± SD. ANOVA, followed by Tukey HSD Post Hoc test, was used to compare the mean response between the levels of the between subject factors of interest whilst the two-sample t-test was used for any two sample comparisons. All tests were two tailed and results with a $p<0.05$ were considered statistically significant.

6.4 Results

6.4.1 Identification of differentially expressed miRNAs in omental fat

In the marker discovery phase, real-time PCR-based miRNA expression profiling arrays were performed on 10 human adipose tissues to identify differentially expressed miRNAs between omental and subcutaneous fat depots, as well as between obese and non-obese individuals. The obese and non-obese patients included in this profiling experiment were age and sex matched, with mean BMI of 43.75 kg/m^2 and 23.5 kg/m^2 respectively. Comparing omental and subcutaneous expression profiles, no single miRNA in the array was exclusively expressed in either fat depot. Overall a poor correlation in miRNA expression was observed for paired omental and subcutaneous fat samples (Pearson's correlation coefficient 0.13, p=0.33) and miRNA expression was largely underexpressed in omental fat compared to paired subcutaneous fat samples (Figure 6.1). Only 6 of the 95 miRNA targets were similarly expressed in paired omental and subcutaneous fat samples

(*miR-107, miR-136, miR-153, miR-17-5p, miR-185,* and *miR-95*, p>0.90 for all 6 miRNAs).

6.4.2 Differential miRNA expression in obese vs. non-obese omental fat

Firstly, the profiling experiment identified no difference in miRNA expression in subcutaneous fat between obese and non-obese subjects (p=0.83). However a number of miRNAs were differentially expressed in omental fat from obese compared to non-obese controls. Using a 2-fold expression difference as a cut-off level, 6 upregulated and 10 down-regulated miRNAs were identified in obese omentum (Table 6.3). Of these 16 putative markers, 5 had supporting evidence from existing literature documenting a role for these candidate miRNAs in metabolic pathways (*miR-122, miR-17-5p, miR-132, miR-143, miR-145*) [80,246-247].
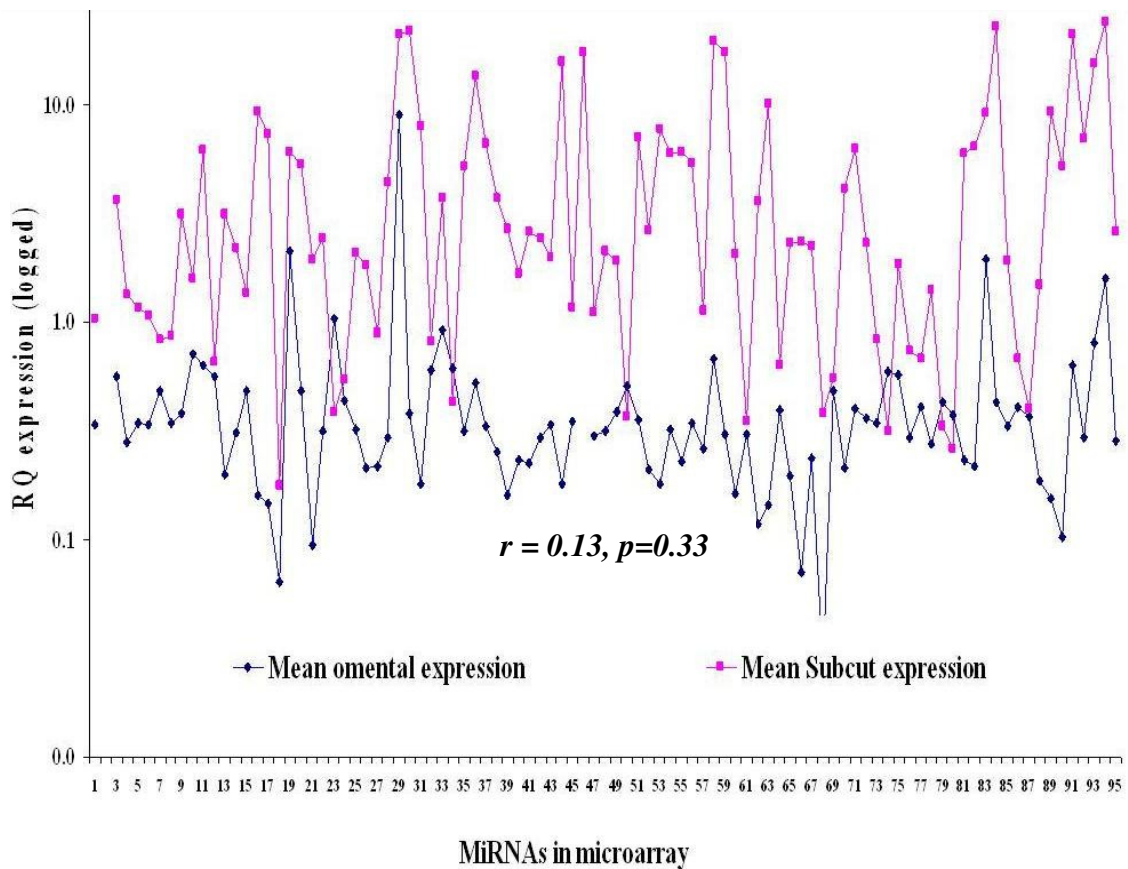
Figure 6.1 MiRNA expression profiling in paired omental and subcutaneous adipose tissue samples (N=10). There is a poor correlation in miRNA expression between these two fat depots (r=0.13, p=0.33).

Table 6.3 List of miRNAs upregulated (> 2 fold) or downregulated (> 2 fold) in obese adipose tissue (omentum) compared to non-obese adipose tissue.

miRNA	Fold change	
miR-7	2.06	
miR-204	2.09	
miR-219	2.22	up-regulated (> 2 fold)
miR-122a	*2.27*	
miR-15b	2.41	
miR-488	2.47	
miR-26b	0.06	
miR-372	0.29	
miR-205	0.31	
miR-132	*0.34*	
miR-19 a&b*	0.35	down-regulated (> 2 fold)
miR-22	0.37	
miR-143	*0.43*	
miR-27 a&b*	0.45	
miR-17-5p	*0.45*	
miR-145	0.50	

* Codetection of miRNA family members in this miRNA profiling array

The 5 markers in **bold italic** are also documented in the literature to be implicated in metabolic pathways [80].

6.4.3 Selection and validation of candidate miRNAs in human fat by RQ-PCR

The 5 markers identified from the array experiment, and which had previously been shown to be implicated in metabolic functions, were chosen for validation in a larger cohort of fat specimens. A further 3 miRNAs were selected for investigation in this larger cohort of adipose tissue specimens, based on compelling evidence from existing literature that they were implicated in adipogenesis (*miR-34a, miR-99a* and *miR-195*). Probes for *miR-34a* and *miR-99* were not included on the array platform used in this study. *MiR-195* expression in the profiling cohort was observed to be 1.33-fold higher in adipose tissue from obese compared to non-obese patients which did not meet the cut-off level of a 2-fold difference in expression to merit inclusion in the validation study from that perspective; however *miR-195* was selected as a putative marker for further investigation given its postulated role in appetite regulation and glucose homeostasis [248]. *MiR-16* was selected as the preferred endogenous control for the validation study, given its stable expression in all samples in the profiling experiment. Its expression was observed to be more stable than that of the mammalian U6 snRNA transcript which was proposed as a normalising gene by the manufacturers of the array (Figure 6.2). To validate the 8 candidate miRNAs in adipose tissue, RQ- PCR was applied using specific miRNA assays for each target. Expression levels of the 8 miRNAs were validated on 58 fat samples; paired omental and subcutaneous specimens from 19 morbidly obese patients and 10 healthy non-obese controls.

MiR-16 expression in adipose tissues

U6 expression in adipose tissues
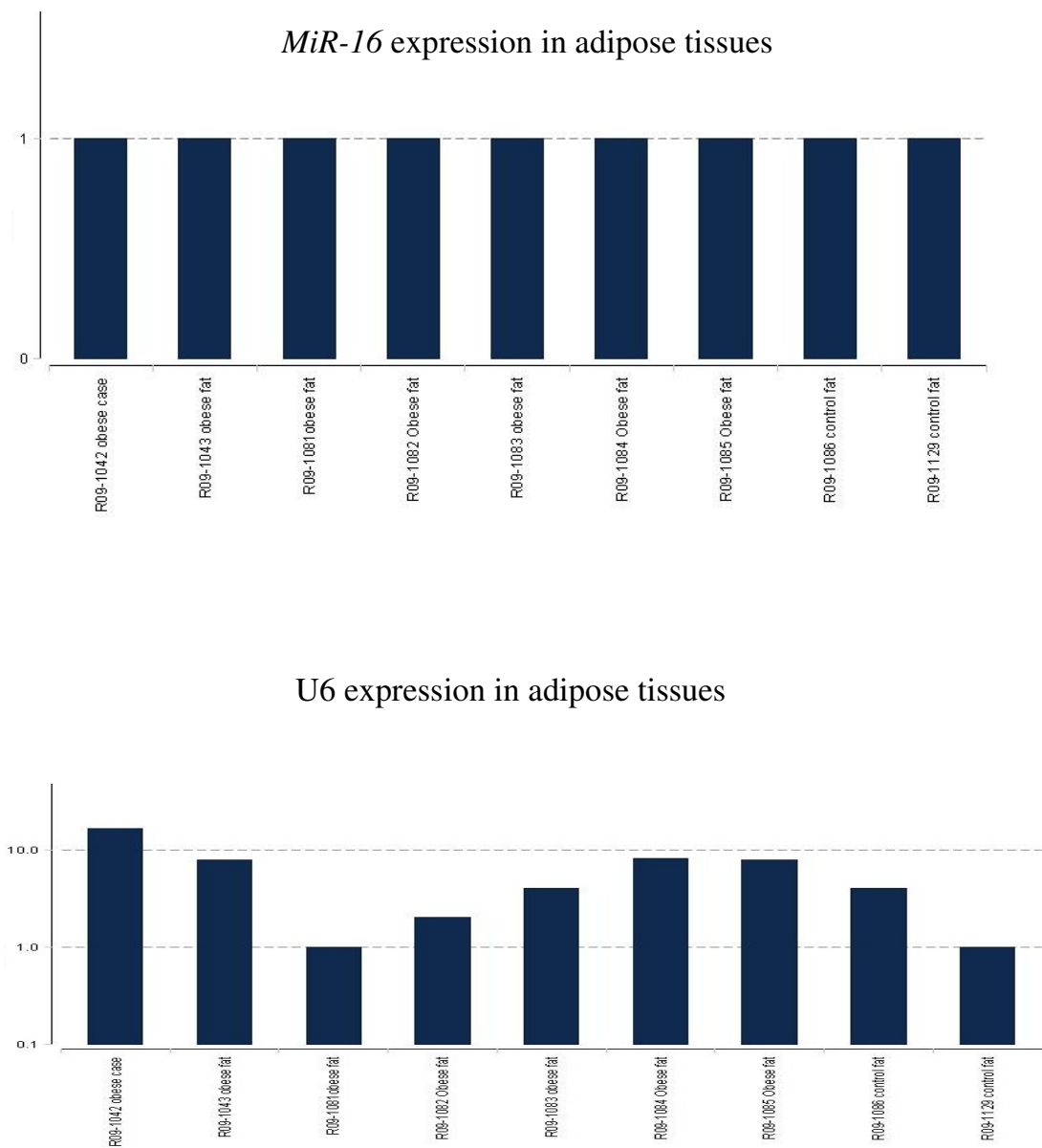
Figure 6.2 Expression of the U6 snRNA transcript and *miR-16* in adipose tissue samples. *MiR-16* is observed to be more stably expressed than U6, and is therefore more suitable as an endogenous control for miRNA investigations in fat tissue.

Similar to results from the profiling experiment, expression of the 8 candidate miRNAs in subcutaneous fat was similar between obese and non-obese subjects (Table 6.4). In omental fat however, two of the 8 miRNA targets were significantly under-expressed in obese individuals compared to controls (Table 6.5). *MiR-17-5p* expression was on average 19-fold lower, and *miR-132* expression 25-fold lower, in obese omentum compared to non-obese omentum (p=0.035 and p=0.009 respectively, Figure 6.3). Expression levels of *miR-34a, miR-99a, miR-143, miR-145,* and *miR-195* were all decreased in omental adipose tissue from obese patients, but not significantly so, Table 6.5). Only one miRNA, *miR-122*, was upregulated in obese compared to non-obese omental fat although the 11.8-fold increase did not reach statistical significance (p=0.57).

Table 6.4 Mean RQ expression levels (Log10) of target miRNAs in subcutaneous fat from obese vs. non-obese individuals				
Target miRNA	**Obese subcutaneous fat (n=19)**	**Non-obese subcutaneous fat (n=10)**	**Mean fold change***	*p-value*
miR-17-5p	3.06	3.24	↓ 1.1	*0.629*
miR-132	5.39	6.98	↓ 1.3	*0.105*
miR-34a	5.26	6.21	↓ 1.2	*0.282*
miR-99a	4.81	6.03	↓ 1.3	*0.161*
miR-122	7.80	8.62	↓1.1	*0.411*
miR-143	7.18	8.79	↓1.2	*0.214*
miR-145	8.63	9.49	↓ 1.1	*0.295*
miR-195	7.17	6.64	↑ 1.1	*0.607*

Target miRNA	Obese omentum (n=19)	Non-obese omentum (n=10)	Mean fold change*	p-value	Obese bloods (n=30)	Non-obese bloods (n=20)	Mean fold change*	p-value
Table 6.5 Mean RQ expression levels (Log10) of target miRNAs in omentum and blood from obese vs. non-obese individuals								
miR-17-5p	2.33	3.02	↓ 19.8	*0.035*	1.79	2.28	↓ 18.78	*0.050*
miR-132	4.48	6.33	↓ 25.9	*0.009*	3.33	5.92	↓ 59.95	*0.048*
miR-34a	5.26	6.21	↓ 7.0	*0.511*	4.55	4.79	↓ 11.29	*0.339*
miR-99a	4.89	5.34	↓ 12.4	*0.500*	1.89	2.10	↓ 12.92	*0.333*
miR-122	7.76	7.24	↑ 11.8	*0.505*	5.75	5.52	↑ 11.01	*0.625*
miR-143	6.77	7.32	↓ 12.1	*0.891*	2.59	2.99	↓ 14.27	*0.205*
miR-145	7.92	9.11	↓ 14.1	*0.297*	5.42	5.92	↓ 12.37	*0.037*
miR-195	6.20	7.58	↓ 16.7	*0.711*	5.70	6.04	↓ 11.47	*0.287*
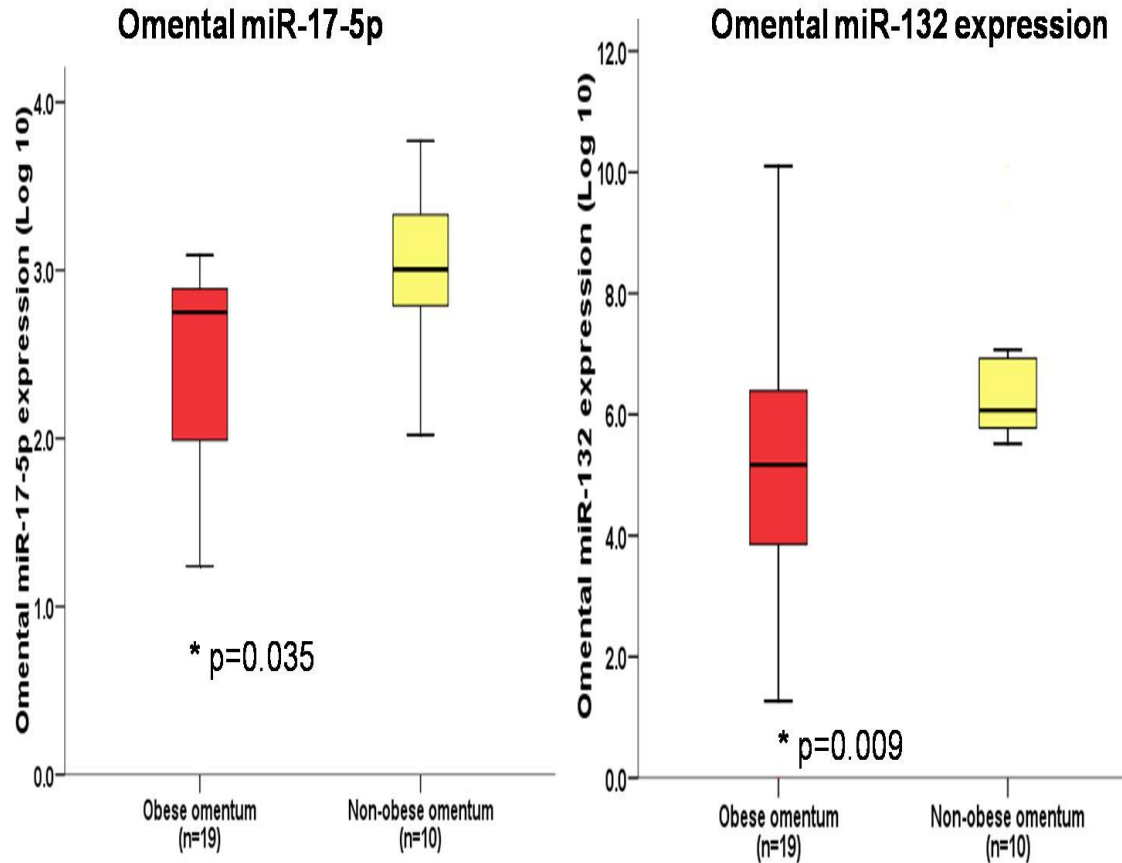
Figure 6.3 Expression of *miR-17-5p* and *miR-132* were both significantly under-expressed in omental adipose tissue from obese compared to non-obese individuals (p=0.035 and p=0.009 respectively).

6.4.4 Circulating miRNAs reflect expression in omental fat tissue

We proceeded to investigate expression of the 8 target miRNAs in the circulation of a larger cohort of obese (n=30) and non-obese individuals (n=20). Systemic levels of *miR-17-5p* and *miR-132* were significantly under-expressed in the obese cohort compared to controls (p=0.05 and p=0.048 respectively), reflecting the same expression pattern as was observed in omentum.

MiR-145 expression, which was decreased 14.1 fold in obese compared to non-obese omentum (p=0.297), was also underexpressed in the circulation of obese patients compared to controls, and this difference reached statistical significance (p=0.037).

Systemic levels of *miR-34a, miR-99, miR-143* and *miR-195* were under-expressed in obese vs. non-obese individuals (similar to omental expression of these 4 miRNAs) though again the difference in circulating levels between these two cohorts did not reach statistical significance (Table 6.5). *MiR-122* levels in blood were 11-fold higher in obese individuals, similar to levels in obese omentum (p=0.625).

6.4.5 Circulating miRNAs as predictors of bariatric surgery success

Pre-operative systemic miRNA analysis in the cohort of patients who underwent bariatric surgery (n=19) identified a number of markers which were associated with favourable response to surgery. At a mean postoperative follow-up of 6 months (Range 4 - 12 months); mean excess body weight lost was 41.3% (Range 16.7 – 55.8%), metabolic comorbidities had resolved in 61.5% (8 of 13 patients with metabolic comorbidities) with minor improvement in a further 15% and no improvement in 23%. In patients who had an improvement or complete remission of metabolic comorbidities following bariatric surgery, preoperatively blood *miR-132* and *miR-145* levels were observed to be significantly higher compared to those who had no metabolic response to surgical intervention (p=0.033 and p=0.009 respectively). There was no significant correlation with % of excess body weight lost and preoperative circulating miRNA levels.

6.4.6 Correlation of miRNA expression with metabolic clinical parameters

Expression of *miR-17-5p*, which was significantly decreased in omental fat and in the circulation of obese patients, displayed a significant inverse correlation with BMI in both adipose tissue and in blood samples (Pearson's correlation coefficients -0.419 and -0.346, p=0.037 and p=0.023 respectively, Figure 6.4). Omental and systemic *miR-143* levels were significantly elevated in patients with diabetes mellitus compared to non-diabetic patients (p<0.001 and p=0.037 respectively) (Figure 6.5a & b). Furthermore systemic *miR-143* levels correlated positively with glycosylated haemoglobin (r=0.595, p<0.001) and fasting blood glucose (r=0.632, p<0.001) (Figure 6.5c & d). No significant relationship was observed between blood cholesterol levels and omental or circulating miRNA levels.
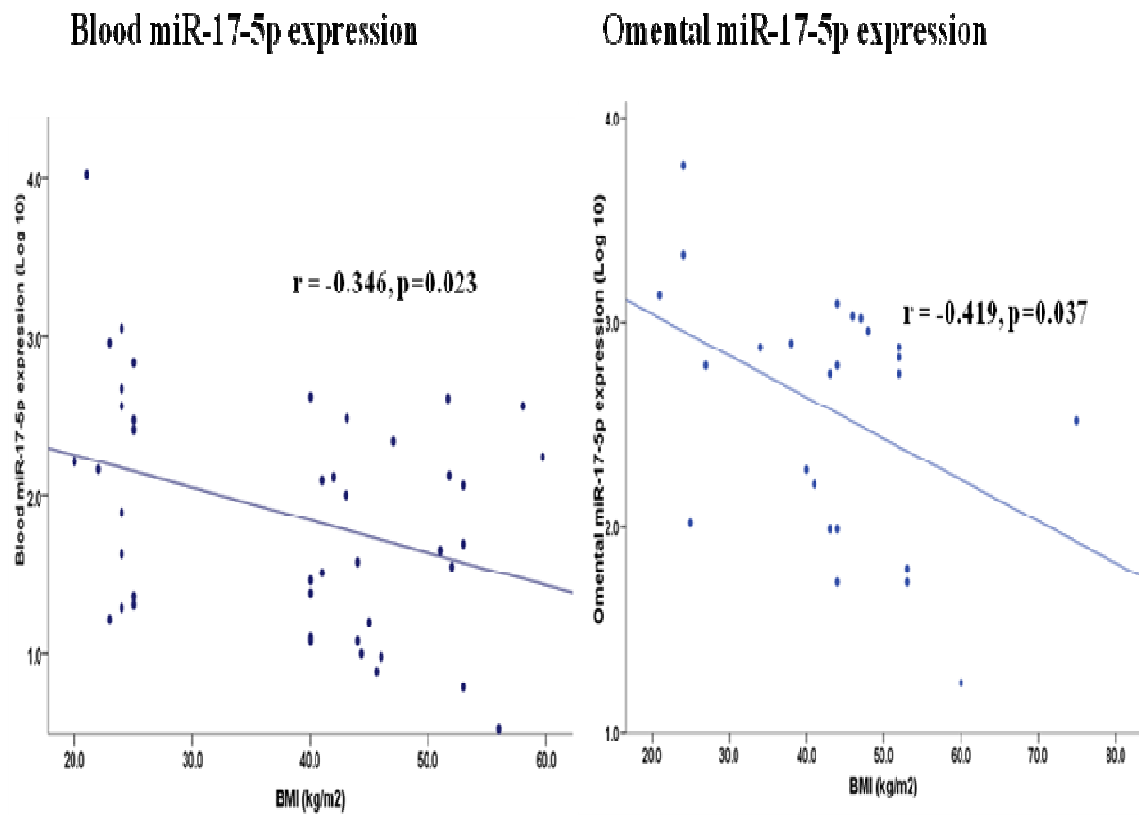
Figure 6.4 *MiR-17-5p* expression in omentum and in the circulation correlated inversely with patients' body mass index (p=0.037 and p=0.023 respectively).
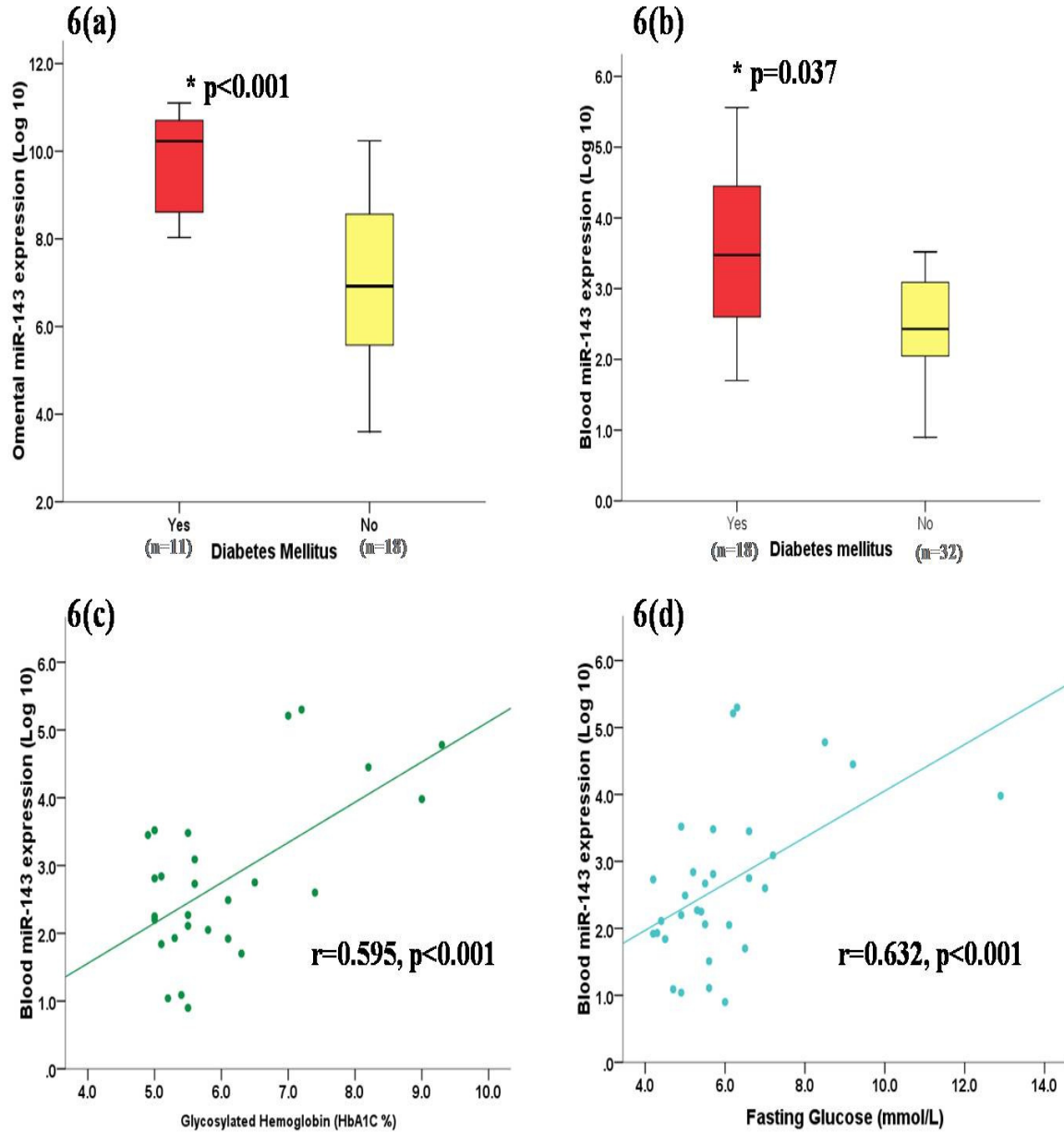
Figure 6.5 *MiR-143* expression in patients according to the presence or absence of diabetes mellitus (DM). **(a)** Omental *miR-143* expression in patients with DM (n=11) is significantly higher than in patients without DM (n=18) (p<0.001). **(b)** Circulating *miR-143* expression in patients with DM (n=18) is also significantly higher than in patients without DM (n=32) (p=0.037). Systemic *miR-143* levels correlate positively with **(c)** glycosylated haemoglobin levels (p<0.001) and **(d)** with fasting blood glucose (p<0.001).

6.5 Discussion

This is the first report of miRNA expression in adipose tissue and in the circulation of obese humans, and our results demonstrate that metabolic miRNAs can potentially serve as novel non-invasive biomarkers for obesity and related metabolic conditions, and represent novel therapeutic targets for these common diseases. Firstly we demonstrate that omental and subcutaneous fat depots display significantly different miRNA profiles, with largely no correlation in gene expression between these two fat types (Figure 6.1). We observed similar miRNA expression in subcutaneous fat from all subjects irrespective of their body habitus, but omental fat from obese patients displayed significantly different gene expression profiles to non-obese omentum. This study identified that *miR-17-5p* and *miR-132* expression in omental fat from obese patients was significantly lower than in non-obese omental tissue (19-fold and 25-fold respectively). Interestingly, circulating miRNA profiles reflected omental fat miRNA expression; systemic *miR-17-5p*, *miR-132* and *miR-145* were significantly decreased in venous blood from obese compared to non-obese subjects. Overall a down-regulation of miRNA expression was observed, in adipose tissue and systemically, in obese individuals (Table 6.5). Furthermore we identified significant correlations between miRNA expression and existing metabolic parameters such as BMI, glycosylated haemoglobin and blood glucose, and levels of two systemic miRNAs (*miR-132* and *miR-145*) were found to be predictive of a favourable metabolic response to bariatric surgery.

A global decrease in miRNA expression in omental fat from obese individuals, as observed in this study, has been previously documented. Estep *et al* identified significant downregulation of miRNAs in omentum from obese patients with non-alcoholic steatohepatitis (NASH), including two miRNAs which were underexpressed in obese omental fat in this study; *miR-132* and *miR-99a* [249]. Xie *et al* describe a significant down-regulation of *miR-143* and *miR-103* in adipose tissue from leptin deficient *ob/ob* and diet-induced obese mice [123]. This pattern of miRNA downregulation in obese omentum has been shown to occur in association with

significantly increased levels of inflammatory cytokines such as TNF-α and Interleukin-6 [123,249]. It is postulated that the chronic inflammatory environment of obese adipose tissue, which has been well described previously and thought to be responsible for insulin resistance in obese subjects [124], may be induced by dysregulated expression of miRNAs involved in regulation of the inflammatory response.

It has long been recognized that omental and subcutaneous fat are anatomically and physiologically distinct tissues, and presumed that intrinsic cellular and molecular features of omental fat explain why abdominal obesity is such a significant risk factor for metabolic and cardiovascular diseases. However few studies have investigated the molecular variations between these two fat depots. We identified significantly different miRNA expression profiles between paired omental and subcutaneous fat from obese individuals (n=19). Profiling of 95 miRNA targets in both tissues revealed that no miRNA was exclusively expressed in either fat depot, suggesting perhaps their common developmental origin; a finding also recently reported by Klöting *et al* in a smaller number (n=15) of paired adipose tissue samples [246]. Only 6 miRNAs in our profiling experiment were similarly expressed in the two fat depots (*miR-107, miR-136, miR-153, miR-17-5p, miR-185,* and *miR-95*). The observed poor correlation between miRNA expression in omental and subcutaneous fat in this study gives further impetus to the hypothesis that the omentum is a distinct tissue, under separate regulatory influences than subcutaneous fat, with important functional consequences particularly for obese individuals with extensive central adiposity.

The finding that omental and circulating levels of *miR-17-5p* and *miR-132* were significantly decreased in obese compared to non-obese individuals, and that *miR-17-5p* expression correlates inversely with BMI, is consistent with results reported by Klöting *et al* which document a negative correlation between omental expression of *miR-17-5p* and *miR-132* and visceral fat area [246]. *MiR-17-5p,* one of 6 miRNAs in the *miR-17-92* cluster, has been reported to be upregulated 2-fold in the early clonal

expansion stage of adipogenesis. *In vitro* data indicates that this miRNA cluster induces acceleration of adipocyte differentiation by negatively regulating the key cell cycle regulator Rb2/p130 [250]. Whilst the converse expression pattern (i.e. a down-regulation of *miR-17-5p* in obese omental fat and in the circulation) was observed in this study, the inverse relationship of miRNA expression in differentiating adipocytes and fully differentiated obese tissue is previously described [123]. Known gene targets for *miR-132* include cAMP response element-binding protein (CREB) which has a role in glucose homeostasis [125], and brain-derived neurotrophic factor (BDNF) which is implicated in appetite regulation and energy homeostasis [251], further supporting the important role of this miRNA in the pathogenesis of obesity.

Given the significant risks associated with bariatric surgery, careful patient selection is critical in order to minimize the associated morbidity and mortality [252]. Whilst the benefits of surgical intervention for morbidly obese patients with metabolic comorbidities are well documented, a proportion of patients will derive little clinical benefit despite technical success and behavioural modifications by the patient postoperatively [253]. Valid outcome predictors are lacking. We identified two miRNAs in circulation of obese patients which have potential as predictive factors for diabetes resolution following bariatric procedures. Preoperative systemic levels of *miR-132* and *miR-145* were significantly higher in patients who had no improvement in glycaemic control or blood pressure reduction at a mean follow-up of 6 months post-operatively (p=0.033 and p=0.009 respectively). The fact that *miR-132* is implicated in glucose homeostasis through its regulation of the cAMP-responsive transcription factor CREB, and that putative targets of *miR-145* include insulin receptor substrate-1 and beta-actin, further highlight the potential for these miRNAs as predictive markers of diabetes remission in bariatric patients.

MiR-122 was identified in our adipose tissue profiling experiment as one of only 6 miRNAs upregulated more than 2-fold in obese vs. non-obese omental fat. Whilst our tissue array validation and circulating miRNA studies demonstrated that this increase in *miR-122* expression in omentum and in the circulation of obese patients held true (11-fold increase in obesity), the differences between obese and non-obese individuals did not meet statistical significance in either tissue type (p=0.505 and p= 0.625 respectively). Nonetheless this is the first report to our knowledge of increased *miR-122* expression in omental fat and blood from obese subjects. This liver specific miRNA is well documented to be implicated in cholesterol and lipid metabolism. Krutzfeldt *et al* provide evidence to support *miR-122* as a key regulator of the cholesterol biosynthetic pathway; in particular they observed that the expression of at least 11 genes involved in cholesterol biosynthesis was decreased between 1.4-fold and 2.3-fold in antagomir-122-treated mice, including hydroxy-3-methylglutaryl-CoA-reductase (Hmgcr), a rate-limiting enzyme of endogenous cholesterol biosynthesis [74]. Furthermore silencing of *miR-122* by systemic administration of high affinity LNA anti-miRs resulted in dose dependent lowering of plasma cholesterol in mice and non-human primates (monkeys) without adverse sequelae or hepatic toxicity. These findings, in addition to our in-vivo *miR-122* expression results, indicate the potential for *miR-122* antagonism as a potential anti-obesity therapeutic strategy.

Converse to miRNA antagonism as a therapeutic approach, where miRNA expression is known to be under-expressed in obese adipose tissue (e.g. *miR-17-5p* and *miR-132),* induction of miRNA expression using viral or liposomal delivery of tissue-specific miRNAs could potentially result in restoration of catabolic activity to the tissue. This concept of 'miRNA replacement therapy' has yet to be extrapolated in this setting. Further studies are necessary to examine the efficacy and safety of these novel therapeutic approaches however evidence to date is encouraging.

Although our findings are promising, there are a number of limitations in this study. Firstly, our sample size is relatively small, and further validations of these markers in larger cohorts of patients are necessary, particularly with regard to the utilization

of circulating miRNAs as predictive markers of favourable outcomes from bariatric surgery. A post-analysis power and sample size calculation based on the results of this pilot study indicates that in order to detect a two fold change in circulating or omental fat miRNA levels (the response variable) with 80% power, and find significant changes between two subject factors (e.g. obese patients grouped according to presence or absence of diabetes mellitus) then a sample size of 5 per group would be required. This analysis suggests that our sample sizes were adequate to detect such a large difference in miRNA levels according to the clinicopathological variables discussed above. However for smaller (and likely clinically relevant) differences in miRNA expression to be detected, larger numbers would be required. Secondly, functional analysis of miRNAs in adipose tissue is required to further elucidate their role in the pathogenesis of obesity. Thirdly, the profiling array platform utilized in this study was limited in that it probed for only 95 human miRNA sequences. Given that there are over 750 human miRNAs currently listed in miRBase version 14[16], it is likely that there are several other metabolic miRNAs which remain to be identified. Perhaps the application of next generation deep sequencing technologies in this field will uncover the true extent and significance of miRNA regulation of gene expression in obesity and the metabolic syndrome.

6.6 Conclusion

The results presented here provide strong evidence in support of the role of miRNAs as key players in the regulation of complex metabolic pathways. Additionally, the dysregulation of miRNAs in omental fat and in the circulation of obese individuals highlights the potential for manipulating miRNAs as a novel therapeutic strategy for the management of obesity and the metabolic syndrome. Further dedicated, focused research in this field is imperative to ascertain the full potential of miRNAs as novel metabolic biomarkers and therapeutic agents against obesity.

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[16] http://www.mirbase.org/, updated Sept 2009

Chapter 7

Discussion

7.1 Introduction

With cancer and obesity representing the two greatest global health concerns at present, it is incumbent upon clinicians and scientists to investigate the molecular biology of these diseases, in order to better understand their aetiology and mechanisms and thereby develop novel diagnostic, prognostic and therapeutic strategies. Since their discovery in 1993, miRNAs have emerged as an exciting new class of disease biomarker with further potential as the next generation of therapeutics. These small, endogenous, non-coding RNAs have play important regulatory roles in governing gene expression and numerous cellular processes, whilst aberrant expression of miRNAs has been observed in a diversity of pathological events. Importantly, they have been critically implicated in the pathogenesis of all human cancers and more recently in the aetiology of obesity and metabolic disorders. Although elucidating their mechanisms of action is still in its infancy, the discovery of miRNAs has uncovered an entirely new and exciting repertoire of molecular factors upstream of gene expression, with great potential for new developments in current diagnostic and therapeutic strategies in the management of common diseases.

7.2 Summary and implications of results

It was the aim of this work to investigate the expression and dysregulation of miRNAs in two common diseases; breast cancer and obesity, with particular emphasis on exploring the potential of miRNAs as novel disease biomarkers.

7.2.1 MiRNAs as breast cancer biomarkers

Although laudable progress has been made in unravelling and understanding breast cancer biology over the last decade, similar advances have not occurred in terms of early diagnosis, improved screening tests, or more targeted and less toxic cancer therapies. Sensitive and specific biomarkers are critical tools for early detection and monitoring of breast cancer, in addition to representing potential therapeutic targets. Unique characteristics of miRNAs include their remarkable stability, tissue specific expression, and relative ease of detection and quantification. Such properties imply that miRNAs hold great potential as disease biomarkers. It is also well established that dysregulated expression of miRNAs is a critical event in breast tumour development and progression. Upon commencing this project, a number of miRNAs were known to be associated with breast cancer. This work aimed to further elucidate the role of these candidate miRNAs as novel breast tumour markers.

Development of a technique for blood miRNA analysis:

Upon commencing this study no standardized protocol existed for isolation and quantification of miRNAs in the circulation. Given the interest in miRNAs as disease biomarkers it quickly became a priority of the scientific community to develop effective and reproducible method for this purpose. An early goal of this study was to define a protocol for optimal extraction, quantification and analysis of miRNA expression in human blood samples. We demonstrate that the optimal miRNA extraction technique for blood specimens is a modified version of the Trizol co-purification protocol. We have identified whole blood as preferable to serum or plasma for circulating miRNA analysis, and that using higher concentrations (100 ng per reaction) of systemic miRNA in RQ-PCR studies yields superior results

compared to lower concentrations. Finally, we observed that *miR-16* is stably expressed across all analyzed blood samples, including those obtained from cancer cases, patients with benign disease, and healthy controls. Thus *miR-16* appears to be a suitable endogenous control for blood based miRNA investigations.

Circulating miRNAs as breast tumour markers:

From our analysis of circulating miRNAs in 83 cancer patients and 44 healthy age-matched females, we identified two miRNAs which were significantly altered in blood from breast cancer patients compared to controls. *MiR-195* and *let-7a* levels were significantly higher in blood from the breast cancer cohort than in healthy controls (p < 0.001 and p < 0.001), corresponding to an average fold-change of 19.25 and 11.20 respectively. Several findings from this work provide convincing evidence to support circulating *miR-195* as a breast specific tumour marker. *MiR-195* expression was increased in breast tumours compared to normal breast tissue, a finding mirrored in the circulation where *miR-195* levels were shown to be considerably higher (19-fold) in breast cancer patients compared to healthy controls. Furthermore we observed a significant increase in tumour *miR-195* levels with advancing stage of disease; a pattern also reflected in the circulation. Two weeks following curative resection, systemic levels of *miR-195* and *let-7a* had decreased to basal levels, comparable with the control group. These two miRNAs had previously been described in breast cancer miRNA studies. *MiR-195* was reported by *Mattie et al* to be significantly higher in HER2/*neu* positive compared to HER2/*neu* negative breast cancers[9], a finding which was also true for the cohort of 65 invasive breast tumours analysed for *miR-195* expression in this study (p=0.002). *Let-7a* is well described as a tumour suppressor and has been implicated in many human solid organ malignancies. Several findings from this study with regard to systemic *let-7a* expression in breast cancer patients were surprising. Firstly the expression of *let-7a* has been documented to be relatively stable in breast tumour tissue[64]therefore systemic levels do not appear to be reflective of tumour burden. Secondly, let-7a levels in tumour tissues are usually down-regulated – consistent with its reported function as a tumour-suppressor. Our finding that *let-7a* levels were significantly

higher in breast cancer patients is converse to what may have been expected and draws attention to the prevailing question – where miRNAs in the circulation originate from, and what (if any) is their function. It is clear that further studies are needed to gain greater insight into the origin of circulating miRNAs.

Tumour specificity of circulating miRNAs:

A further aim of this work was to investigate the site/tumour specificity of circulating miRNAs. The knowledge that *miR-195* and *let-7a* were elevated in breast cancer patients raised the question as to whether these miRNAs would also be elevated in patients with other malignancies. We demonstrate that cancer-associated miRNAs are generally dysregulated in the circulation of patients with visceral malignancy; this dysregulation appears to be relatively non-specific with the exception of systemic *miR-195* which was over-expressed only in breast cancer patients. The non-specific dysregulation of *let-7a, miR-10b, miR-145, miR-155* and *miR-21* in unselected cancer patients was not surprising; a substantial body of evidence documents an association and even a functional role for these 5 miRNAs in various common cancers. In contrast the finding that *miR-195* was significantly elevated only in breast cancer patients, supports the hypothesis that certain miRNAs are indeed site specific and therefore have potential as tumour markers.

Inherited variation in miRNA binding sites and risk of breast cancer:

A potential mechanism by which miRNAs instigate carcinogenesis is impaired binding to their mRNA target, consequent to the presence of polymorphisms in either the oncogenic miRNA coding sequences[137] or 3'UTR miRNA binding sites. The presence of genetic variants in miRNA sequences or binding sites has already been associated with increased risk of developing a variety of malignancies, including breast cancer [117,236-237]. A recently identified genetic variation in the *let-7* complementary site within the 3' UTR of *KRAS* has been shown to confer susceptibility to breast cancer (*Ratner et al. In press*). An aim of this study was to determine the prevalence of this *KRAS*-variant in a large breast cancer population, and to determine whether the *KRAS*-variant predisposes to any specific subtype of

breast cancer. The results indicated that the *KRAS*-variant is indeed a genetic marker of increased risk of developing breast cancer, in particular the triple negative subtype (ER, PR, HER2/*neu* negative) in younger women, in whom it was almost 5 times more prevalent compared to controls (OR = 4.78, CI = 1.71-13.38, p = 0.015). These results are of great clinical significance, as few genetic risk factors are known for this subtype of breast cancer, and those that are known account for only a minority of cases. Because the triple negative subtype carries the poorest prognosis, predicting the risk for this particular subtype is of utmost importance, to allow early screening and intervention for these patients. Furthermore, identifying the genetic causes of triple negative breast cancer are the first steps towards fully elucidating the biology of this disease in order to develop targeted effective therapies for these patients.

7.2.2 MiRNA expression in obesity and the metabolic syndrome

The genetic factors contributing to obesity and associated metabolic diseases are largely unknown. Deciphering the genetic regulators and molecular mechanisms involved in these disorders will reveal novel targets for therapeutic intervention and biomarkers to aid in prognostication and management challenges; for example, the appropriate selection of patients for surgical intervention. Given that miRNAs have also been shown to regulate a variety of metabolic processes, we undertook a miRNA profiling experiment on human obese and non-obese adipose tissue, to identify if miRNA expression was dysregulated in obese fat.

A further aim was to establish if circulating miRNAs have potential as biomarkers for selecting appropriate management strategies for the obese.

Results from this work demonstrate that metabolic miRNAs are dysregulated in obese adipose tissue and also have potential to serve as novel non-invasive biomarkers for obesity and related metabolic conditions. In addition they represent novel therapeutic targets for these common diseases. M*iR-17-5p* and *miR-132* expression were observed to be significantly decreased in visceral adipose tissue from obese compared to non-obese patients (19-fold and 25-fold respectively).

Circulating miRNA profiles reflected adipose tissue miRNA expression and correlated with existing clinical metabolic parameters such as BMI. We also demonstrated that omental and subcutaneous fat depots display significantly different miRNA profiles, with largely no correlation in gene expression between these two fat types. Finally levels of two systemic miRNAs (*miR-132* and *miR-145*) were found to be predictive of a favourable metabolic response to bariatric surgery. These results provide strong evidence to support miRNAs as key players in the regulation of complex metabolic pathways. Additionally, the dysregulation of miRNAs in adipose tissue and in the circulation of obese individuals highlights the potential for manipulating miRNAs as a novel therapeutic strategy for the management of obesity and the metabolic syndrome. Further dedicated, focused research in this field is imperative to ascertain the full potential of miRNAs as novel metabolic biomarkers and therapeutic agents against obesity.

7.3 Potential clinical applications

The goal of all translational research is to bring scientific discoveries into clinical application. The emergence of miRNAs as modulators of gene expression, coupled with findings from this study indicating their unique biomarker properties in various diseases, identifies miRNAs as obvious novel diagnostic and prognostic indicators, with further potential as therapeutic targets. In breast cancer, analogous to the derivation of intrinsic subtypes from gene expression profiles and the estimation of risk of disease recurrence from multi-gene assays such as *Oncotype DX*, it is predicted that tumour or circulating miRNA signatures could serve as diagnostic and prognostic tools with even greater accuracy than mRNA markers. One such future clinical application is the potential to develop a molecular diagnostics platform containing a panel of breast cancer-specific miRNA probes, to which would be applied a sample of whole blood at the point-of-care. This technology could be used in a breast cancer screening setting as an adjunct to mammography, with further potential in breast cancer clinics as a tool to stratify patients into prognostic groups and even predict response to various treatments. Such advancement would greatly improve the management of breast cancer and bring clinicians and patients nearer the goal of 'individualized treatment'. It has the potential to improve diagnostic capacity, and facilitate specifically tailoring therapeutic regimens thus sparing patients from adverse effects of unnecessary treatments.

A further breast cancer related clinical application of this work stems from the finding that an inherited variation in the *let-7* miRNA binding site within the 3'UTR of the *KRAS* oncogene greatly increases one's risk of developing the aggressive triple negative subtype of breast cancer. Screening young women with a family history of breast cancer for this *KRAS*-variant would be a cost-effective intervention. If women with the *KRAS*-variant were identified prior to development of a breast cancer, they could be offered early screening or even prophylactic treatment. This could lower the incidence of triple negative breast cancer, which carries a dismal prognosis despite the use of costly adjuvant therapies.

Another exciting clinical utilisation of the genetic profile of a patient is the ability to predict response to particular therapies based on specific genotypes [254]. One such example is the use of Poly ADP-Ribose Polymerase (PARP) inhibitors for *BRCA* mutation carriers. PARP is a nuclear enzyme functioning in base excision repair. Inhibition of this enzyme increases the number of cells with DNA lesions which would normally be repaired by homologous recombination in conjunction with *BRCA1* and *BRCA2*. In cells deficient of *BRCA1* and *BRCA2,* PARP inhibition exploits the faulty DNA repair mechanism of these cells and results in chromosome instability, cell cycle arrest and cell death. Already, certain miRNAs have been found to predict sensitivity to anticancer treatment[255]: *miR-30c, miR-130a* and *miR-335* are downregulated in various chemoresistant cancer cell lines, *let-7g* and *miR-181b* are strongly associated with response to the 5-fluorouracil-based antimetabolite S-1. In addition, several miRNAs have been shown to influence sensitivity to chemo- or radiotherapy: miRNAs of the *let-7* family induce radiosensitivity *in vitro* and *in vivo*, inhibition of *miR-21* and *miR-200b* increase sensitivity to gemcitabine in cholangiocarcinoma cell lines, and restoration of *miR-34* in p53-deficient human gastric cancer cells induces chemosensitisation. With specific regard to breast cancer therapeutics, Kovalchuk *et al* identified 137 differentially regulated miRNAs (63 upregulated and 75 downregulated) when comparing doxorubicin-resistant and -sensitive breast cancer cell lines [256]. Similarly Miller *et al* identified 8 miRNAs which were over-expressed in a tamoxifen-resistant breast cancer cell line (*miR-221, miR-222, miR-181, miR-375, miR-32, miR-171, miR-213, miR-203*) and another 7 miRNAs (*miR-342, miR-489, miR-21, miR-24, miR-27, miR-23, miR-200*), which were downregulated. It is obvious that the ability to personalize treatment based on a patients (tumour or systemic) miRNA expression is a very real and imminent possibility.

Findings to date illustrating the critical role of miRNA in breast tumour development and progression also provides a strong rationale for miRNA- based therapeutic strategies for breast cancer. There are two possible approaches for their use as cancer therapies, in acknowledgment of their dual role in carcinogenesis; firstly through antisense-mediated inhibition of oncogenic miRNAs (eg. knockdown of *miR-21* and the *miR-17-92* cluster), and secondly through 'replacement' of under-expressed tumour suppressor miRNAs (eg. *let-7a*) with either miRNA mimetics or viral vector-encoded miRNAs. Early in-vitro studies in breast cancer cell lines shows great promise for these potential therapeutic strategies [73].

In the field of obesity and metabolic disorders; the association between aberrant miRNA expression and abnormalities in glucose homeostasis and adipogenesis illustrates the feasibility of using these molecules as targets for therapeutic intervention. The dysregulation of miRNAs in adipose tissue and in the circulation of obese individuals highlights the potential for manipulating miRNAs as a novel therapeutic strategy for the management of obesity and the metabolic syndrome. One such approach is to antagonise miRNAs known to be over-expressed in metabolic diseases, such as *miR-122*. Indeed studies in model organisms, and more recently in primates, have already investigated the effects of systemic *miR-122* antagonism using locked nucleic acid (LNA) modified DNA oligonucleotides (LNA-anti-miRs). This resulted in effective silencing of endogenous *miR-122* thus inhibiting HCV replication in HuH-7 cells harboring the HCV-N replicon NNeo/C-5B [257-258]. Silencing of *miR-122* by systemic administration of high affinity LNA anti-miRs has resulted in dose dependent lowering of plasma cholesterol in mice and non-human primates (monkeys), after only three intravenous doses of 3 mg/kg. Additionally, this was achieved without significant adverse sequelae or hepatic toxicity [74]. These findings have unveiled the imminent potential of miRNAs as novel therapeutic strategies for metabolic diseases. Indeed, a phase I safety and pharmacokinetic study of systemic *miR-122* antagonism in humans using an LNA-based antisense molecule against miR-122 (SPC3649), led by Santaris Pharma, has just been completed on 48 healthy volunteers and results are eagerly anticipated.

Converse to miRNA antagonism as a therapeutic approach; where miRNA expression is known to be under-expressed in obese adipose tissue (e.g. *miR-17-5p and miR-132)* induction of miRNA expression using viral or liposomal delivery of tissue-specific miRNAs could potentially result in restoration of catabolic activity to the tissue. This concept of 'miRNA replacement therapy' has yet to be extrapolated in this setting. Further studies are necessary to examine the efficacy and safety of these novel therapeutic approaches however evidence to date is encouraging. Another clinical application for miRNAs in the field of obesity is in appropriate patient selection for bariatric surgery where valid outcome predictors are currently lacking. This work identified two miRNAs in circulation of obese patients which have potential as predictive factors for diabetes resolution following bariatric procedures.

7.4 Future perspective

The transition of miRNA applications from bench to bedside, as cancer biomarkers and as therapeutic agents, necessitates addressing several challenges through further investigations. As biomarkers, various issues regarding miRNA measurement and quantification, particularly in the circulation, need refining. Firstly we need to gain a better understanding of the exact mechanisms by which miRNAs are released into the circulation and if freely circulating miRNA molecules have any functional role in addition to reflecting the presence and pathological features of disease. Secondly there is no consensus on the most appropriate endogenous control for systemic miRNA analysis. In order to obtain reliable and reproducible results, there is a need to determine suitable normalization methods for blood-based miRNA investigations. Thirdly larger validation studies are urgently needed to support the preliminary findings from case-control cohort studies that have proposed miRNAs as novel biomarkers for cancer. These studies must report miRNA levels in blood from several hundred healthy individuals representing both genders, all ethnicities and age groups so that appropriate conclusions can be drawn from results of systemic miRNA profiling in specific disease cohorts.

Furthermore as additional short non-coding RNAs are continuously identified through biomarker discovery programmes, the available profiling technologies must adapt their platforms to incorporate newer potentially relevant targets. Functional validation of all miRNAs reported to be dysregulated in cancer, and the identification of their target genes and pathways is also important.

With regard to therapeutics whilst progress in this field is rapid and laudable, many obstacles must be overcome for miRNA based therapies to become a reality in management of common cancers. A significant amount of functional work remains to be done to fully elucidate the mechanisms by which miRNAs contribute to tumourigenesis, and establish a better understanding of the complexity of gene expression regulation by miRNAs. Pharmacological difficulties include developing safe, effective, site specific delivery mechanisms for miRNA directed therapies.

Despite these challenges, the remarkable potential of miRNAs as cancer biomarkers and therapeutics cannot be under-estimated. Ongoing and future studies emanating from this thesis include investigations to clarify the issues mentioned above, and validate the preliminary findings described throughout this work. Specific examples of planned studies which will further assess the potential of circulating miRNAs as breast tumour markers include investigating systemic miRNA levels serially in patients undergoing neoadjuvant chemotherapy for primary breast cancer, or upfront chemotherapy for recurrent or metastatic disease. The ability of systemic miRNA signatures to accurately classify breast cancer according to intrinsic subtype, predict response to treatments, and determine prognosis for patients, is also under investigation at present as is an effort to expand the panel of breast cancer specific circulating miRNAs identified through this work.

A parallel series of investigations to further clarify the role of miRNAs in obesity and the metabolic syndrome is also necessary. Additionally, the proposed link between breast cancer and obesity will be explored through further investigation of dysregulated miRNA pathways common to both diseases.

7.5 Conclusion

Overall this project has contributed significantly to our evolving knowledge of the genetics and molecular biology of common diseases such as cancer and obesity. If the current momentum in miRNA translational research can be maintained, this will bring an exciting new dimension to the field of diagnostics and therapeutics for these diseases, and has the potential to transform current practice to the ideal of individualized care for patients in the near future.

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Appendices

Appendix 1. Department of Surgery Biobank Forms

1.1 Breast Unit Patient Consent Form

BREAST UNIT, UNIVERSITY COLLEGE HOSPITAL GALWAY

INFORMED CONSENT FORM

PATIENT INFORMATION

Introduction

We would like to invite you to participate in the research projects of the Breast Unit in UCHG. You are under no obligation to take part and if, when you have the information below, you would prefer not to participate, we will accept your decision without question.

Approximately 1700 women are diagnosed with breast cancer every year in Ireland. Although major advances have been made in the management of breast cancer, many aspects of the disease are not fully understood. It is hoped that our understanding of the disease will be improved through research. Our Breast Unit is actively involved in research that aims to identify markers that will predict how a cancer develops, progresses and responds to a variety of treatments. This type of work requires the use of tissue and blood samples. It is hoped that it will eventually lead to improvements in the diagnosis, treatment and outcome for those who have breast cancer. Although this study may have no direct benefit to you, it is hoped that the results may benefit patients like you in the future.

Your Involvement

If you volunteer to participate in our research, there will be no additional risks to you outside those of your standard investigation and treatment. Your identity will remain confidential. Your name will not be published or disclosed to anyone outside the study group. All research is covered by standard institutional indemnity insurance and is approved by a Research Ethics Committee that ensures the ethical nature of the research. Nothing in this document restricts or curtails your rights. You may withdraw your consent at any time. If you decide not to participate, or if you withdraw your consent, your standard of treatment will not be affected in any way.

Procedure

We invite all patients who are undergoing treatment / investigation for breast disorders to participate. All samples for research will be taken at the time you are attending the hospital for routine diagnostic tests.

(i)   **Tissue Samples**

By participating, you give us consent to retain small pieces of your tissue obtained at the time of surgery. These samples will be stored and used in the future for research. This will not affect your diagnosis in any way.

(ii)   **Blood Samples**

By participating, you give us consent to take an extra 2 x 10ml sample (equivalent of 4 teaspoonfuls) of blood at the same time that your blood is being taken for routine tests. These samples will be stored and used in the future for research.

(iii)   **Clinical Information**

By participating, you give us consent to store information relating to your diagnosis and treatment on a breast cancer database. This information is only accessed by personnel directly involved in research within the Breast Unit.

Further Information

If you would like further information about our project, your participation and your rights, please contact the Surgical Professorial Unit (Tel: 091 524390).

Thank-you in anticipation of your assistance. Please read and sign the Consent section.

I have read the attached information sheet on the above project, dated ....

Please Initial Box

1

Appendix 1.1: Continued – Consent form Page 2

PARTICIPANT DECLARATION

I have read, or had read to me, this consent form. I have had the opportunity to ask questions and all my questions have been answered to my satisfaction. I freely and voluntarily agree to be part of this research study, though without prejudice to my legal and ethical rights. I have received a copy of this agreement and I understand that, if there is a sponsoring company, a signed copy will be sent to that sponsor. I understand I may withdraw from the study at any time.

(Name of sponsor :) ....

PARTICIPANT'S NAME: ....

CONTACT DETAILS: ....

PARTICIPANT'S SIGNATURE: ....

DATE: ....

Where the participant is incapable of comprehending the nature, significance and scope of the consent required, the form must be signed by a person competent to give consent to his or her participation in the research study (other than a person who applied to undertake or conduct the study). If the participant is a minor (under 18 years old) the signature of parent or guardian must be obtained:

NAME OF CONSENTER, PARENT, OR GUARDIAN: ....

SIGNATURE: ....

RELATION TO PARTICIPANT: ....

DECLARATION OF INVESTIGATOR'S RESPONSIBILITY

I have explained the nature and purpose of this research study, the procedures to be undertaken and any risks that may be involved. I have offered to answer any questions and fully answered such questions. I believe that the participant understands my explanation and has freely given informed consent.

NAME: ....

SIGNATURE: ....

DATE: ....

(Keep the original of this form in the investigator's file, give one copy to the participant, and send one copy to the sponsor (if there is a sponsor).

2

1.2 **Obesity Patient Consent Form**

GALWAY UNIVERSITY HOSPITALS - BIOBANK INFORMED CONSENT

Dear Sir/Madam,

We would like to invite you to participate in a clinical research study at Galway University Hospitals. The purpose of this study is to investigate the role of gene expression in metabolism and obesity. We ask that you consent to donating a small blood sample today, for the purpose of investigating the levels of genes in your blood.

You are under no obligation to take part and if you would prefer not to participate, we will accept your decision without question.

The mechanisms underlying obesity is currently not fully understood. It is hoped that our understanding of this disease will be improved through research. Galway University Hospitals are actively involved in research that aims to identify markers that will predict how diseases such as obesity develop, progresses and responds to a variety of treatments (medical and surgical) . This type of work requires the use of tissue and blood samples. It is hoped that it will eventually lead to improvements in the treatment and outcome for those who are obese and who suffer its metabolic complications. Although this study may have no direct benefit to you, it is hoped that the results may benefit patients like you in the future.

PARTICIPANT DECLARATION

I have read, or had read to me, this consent form. I have had the opportunity to ask questions and all my questions have been answered to my satisfaction. I freely and voluntarily agree to be part of this research study, though without prejudice to my legal and ethical rights. I understand I may withdraw from the study at any time.

PARTICIPANT'S NAME: ………………………………………………………

CONTACT DETAILS: …………………………………………………

PARTICIPANT'S SIGNATURE: …………………………………………………

DATE: ………………………………………………….

1.2 *continued* - **Obesity Patient Consent Form**

Where the participant is incapable of comprehending the nature, significance and scope of the consent required, the form must be signed by a person competent to give consent to his or her participation in the research study (other than a person who applied to undertake or conduct the study). If the participant is a minor (under 18 years old) the signature of parent or guardian must be obtained:

NAME OF CONSENTER, PARENT, OR GUARDIAN: …………….……...………………….………….

SIGNATURE: ………………….………….………………………….

RELATION TO PARTICIPANT: ………….……………….…...………………….…………………

DECLARATION OF INVESTIGATOR'S RESPONSIBILITY

I have explained the nature and purpose of this research study, the procedures to be undertaken and any risks that may be involved. I have offered to answer any questions and fully answered such questions. I believe that the participant understands my explanation and has freely given informed consent.

NAME: ……………….…………………………………….

SIGNATURE: ……………….…………………………………….

DATE: ……………….…………….…..…….…………….

CONSULTANT: ……………….……………...…….…………….

1.3 **Specimen Request Form**

Breast Genetics Study Dept of Surgery
Form must be completed & accompany specimen *Version 1 04/07/06*

Please affix Patient
Addressograph Label
Here if available

Surname:	First Name:	Reason For Request:	Breast Cancer ☐ Non Breast Cancer Control ☐	
Board Number	Hospital Number	Requesting Consultant:		Request Date:
DOB:	Sex:	Diagnosis:		

Blood Specimen Purple 10ml EDTA ☐

- This Patient has consented to being a **Test Patient**
  for Breast Cancer Genetics Research Study
- I x 10ml EDTA sample is required along with this request form
- Sample to be placed in tray provided in Phlebotomy Room, OPD.

Laboratory Use Only	Written Consent	
Lab Number:	Accompanying Specimen ☐	Contact Tommie Bleep735
Date Received:		Niall Bleep 392
Signature:	In Chart ☐	With any queries

Appendix 2. Suppliers of reagents and equipment used in experiments described in Chapter 2 (Materials and Methods)

Agilent Technologies, Waldbronn, Germany and Palo Alto, CA, USA
      Agilent 2100 Bioanalyzer System
      Agilent 2100 Expert Software (Version B.02.03)
      RNA 6000 Nano LabChip Series II Assay
      Small RNA Assay (cat. no. 5067-1548)
      Agilent Small RNA kit guide (manual part no. G2938-90093)

Ambion, Woodward, Austin, TX, USA
      RNase Inhibitor (cat. no. 2682)

Applied Biosystems, Foster City, CA, USA
      MultiScribe™ Reverse Transcriptase (cat. no. 4311235)
      TaqMan® MiRNA Assays
      TaqMan® Fast Universal PCR Mastermix (No AmpErase® UNG)
         (cat. no. 4352042)
      TaqMan® Genotyping Mastermix (cat. no. 4371357)
      Custom TaqMan® SNP Genotyping Assay (X40)
      Fast Optical 96-well Reaction Plate with Barcode, 0.1mL (cat. no. 4366932)
      7900HT Fast Real-Time PCR System
      ABI Prism 7900 Sequence Detection System

Becton Dickinson, New Jersey, USA
      Vacutainer Serum Separator Tubes II

Biogazelle, Ghent, Belgium
      QbasePLUS software

Eppendorf UK Ltd, Cambridge CB24 9ZR, UK
      Eppendorf 5417C MicroCentrifuge
      Eppendorf Microtubes

Exiqon, Vedbaek, Denmark
      Dig-labeled LNA oligonucleotide (60 nM) probes
      Hybridization buffer

Grenier Bio-one, St. Gallen, Switzerland
      Vacuette EDTA K3E blood bottles

Kinematica AG, Littau-Luzem, Switzerland
      Bench-top homogeniser (Polytron® PT1600E)

Lab Centraal, B.V. 2000 AH Haarlem, Netherlands
      Faster BHA-48 Class IIa biohazard cabinet

Molecular BioProducts, Inc., San Diego, CA, USA
      ART® Aerosol Resistant Tips (10, 20, 100, 200, 1000)

Molecular Research Centre, Inc, Cincinnati, OH, USA
Tri-Reagent® BD (cat. no. TB 126)
BAN Phase Separation Reagent 4-Bromoanisole (cat. no. BN 191)

Nanodrop Technologies, Wilmington, DE, USA
NanoDrop™ 1000 spectrophotometer

Qiagen, Crawley, West Sussex, RH10 9NQ, UK
QIAzol lysis reagent (cat. no. 79306)
RNeasy® Tissue Mini Kit (cat. no. 74804)
RNeasy® Plus Mini Kit (cat. no. 74106)
RNeasy MinElute® Cleanup Kit (cat. no. 74202)
RNase-free DNase kit (cat. no. 79254)

Sarstedt Ltd, Wexford, Ireland
Screw cap Microtubes (0.5 mL, 1.5 mL, 2 mL)

Sigma-Aldrich Corp., St. Louis, MO, USA
Isopropanol (P/N 190764)

SPSS Inc., IBM, Chicago, IL
SPSS Statistical Software for Windows (Version 17.0)
Systems Bioscience, CA, USA
MicroRNA qPCR array

Vector Laboratories, Burlingame, CA, USA
Nuclear Fast Red

VWR, West Chester, PA, USA
Eukitt

Appendix 3: Names and abbreviations of genes implicated in obesity and the metabolic syndrome

PANK1	Pantothenate kinase 1
MAPK7	Mitogen-activated protein kinase 7
CREB	cAMP response element-binding protein
RBL2	Retinoblastoma-like 2 (p130)
IGF1R	Insulin-like growth factor 1 receptor
CYP26B1	Cytochrome P450, family 26, subfamily B, polypeptide 1
INSIG1	Insulin induced gene 1
CAV2	Caveolin 2
BCKDHA	Branched chain keto acid dehydrogenase E1, alpha polypeptide
PMVK	Phosphomevalonate kinase
TRPV6	Transient receptor potential cation channel, subfamily V, member 6
CCNG1	cyclin G1
BCL2L2	BCL2-like 2
HMGCR	3-hydroxy-3-methylglutaryl–coenzyme A-reductase
IRS1	Insulin receptor substrate 1
MTPN	Myotrophin
USP1	Ubiquitin specific peptidase 1
JAK2	Janus kinase 2
ADIPOR2	Adiponectin receptor 2
FOXA2	Forkhead box A2
RAB27A	RAB27A, member RAS oncogene family
ONECUT2	One cut homeobox 2
KCNH2	Potassium voltage-gated channel, subfamily H (eag-related), member 2
RHOA	Ras homolog gene family, member A
CDC42	Cell division cycle 42
WHSC2	Wolf-Hirschhorn syndrome candidate 2
SIP1	Survival of motor neuron protein interacting protein 1

Publications

Circulating microRNAs as Novel Minimally Invasive Biomarkers for Breast Cancer

Helen M. Heneghan, MD,* Nicola Miller, PhD,* Aoife J. Lowery, MD,* Karl J. Sweeney, MD,†
John Newell, PhD,‡ and Michael J. Kerin, MD*

Objective: The development of clinically validated biomarkers for cancer has remained an insurmountable task despite other advances in the field of cancer molecular biology. Mi(cro)RNAs have many characteristics of an ideal biomarker most notably their inherent stability and resilience. Recent blood-based miRNA profiling studies, reporting their presence in serum and plasma, have generated the concept that circulating miRNAs hold much potential as novel noninvasive biomarkers for cancer and other disease processes. The objective of this study was to investigate the potential of circulating microRNAs as novel breast cancer biomarkers.

Methods: Using a novel approach to extract miRNAs from the circulation followed by real-time quantitative polymerase chain reaction analysis, levels of a panel of 7 candidate miRNAs were quantified in tissue and blood specimens of 148 patients with breast cancer and 44 age-matched and disease free control individuals.

Results: We report that cancer-specific miRNAs were detected and significantly altered in the circulation of breast cancer patients, and that increased systemic *miR-195* levels in breast cancer patients were reflected in breast tumors. Furthermore, we identified that circulating levels of *miR-195* and *let-7a* decreased in cancer patients postoperatively, to levels comparable with control subjects, following curative tumor resection. Finally, we found that specific circulating miRNAs correlated with certain clinicopathological variables, namely nodal status and estrogen receptor status.

Conclusion: These findings suggest that systemic miRNAs have potential use as novel breast cancer biomarkers and may prove useful in clinical management during the perioperative period.

(*Ann Surg* 2010;251: 499–505)

Current challenges in the management of breast cancer include a continuing search for sensitive and specific minimally invasive biomarkers that can be exploited to detect early neoplastic changes, thus facilitating the detection of breast cancer at an early stage, as well as for monitoring the progress of patients with breast cancer and their response to treatments. Existing diagnostic tools and biomarkers for breast cancer have many inherent deficiencies. Mammography is currently the gold standard diagnostic tool; however, it is not without limitations, including its use of ionizing radiation and a false positive rate of 8% to 10%.[1] To date, only 2 markers are established in the routine evaluation of breast tumors: estrogen receptor (ER) (for predicting response to endocrine therapies) and HER2/*neu* (for predicting response to Trastuzumab).[2] Although these markers assessed routinely, ER and HER2/*neu* assessment is far from perfect.[3] A number of circulating tumor markers (eg, carcinoembryonic antigen and carbohydrate antigen 15–3) are widely used in the management of breast cancer, but the sensitivity of these markers is low,[4–6] and so they are not useful as screening tools although they have long been in clinical use as prognostic markers and to monitor for disease progression or recurrence. The ideal biomarker should be easily accessible such that it can be sampled relatively noninvasively, sensitive enough to detect early presence of tumors in almost all patients and absent or minimal in healthy tumor free individuals.

Mi(cro)RNAs are short RNA molecules that regulate gene expression across a wide spectrum of biologic and pathologic processes. The recent discovery that miRNA expression is frequently dysregulated in cancer[7] has uncovered a new repertoire of molecular factors, upstream of gene expression, which warrants investigation to further elucidate their precise role in malignancy. The miRNA expression studies in breast cancer indicate their importance and potential use as disease classifiers and prognostic tools in this field.[8,9] A relevant and important feature of miRNAs is their remarkable stability. They are known to be well preserved in tissue samples even after years of formalin-fixation and paraffin-embedding, and can be efficiently extracted from and quantified in such specimens.[10] Investigation of cancer-specific miRNAs in the circulation is an emerging and exciting field of study. It is hypothesized that if miRNAs are present in the circulation of cancer patients, their unique stability and resilience should allow their detection and quantification to be practicable. The first report of circulating miRNAs, by Lawrie et al,[11] described elevated serum levels of *miR-21* in patients with diffuse large B-cell lymphoma. Subsequently, circulating miRNAs have been postulated as novel biomarkers for cancer, and other disease processes.[12–16] However, this concept needs investigation to validate the theory. To date, there has been no report on the role of circulating miRNAs in breast cancer. We hypothesized that levels of specific cancer-associated miRNAs in circulation would differ between breast cancer patients and healthy individuals. If this hypothesis held truth, it would signify a major breakthrough in breast cancer management, bringing us ever closer to finding a novel, sensitive, and noninvasive biomarker for this common disease.

The primary aim of this study was to investigate whether cancer-specific miRNAs are detectable and altered in the circulation of breast cancer patients compared with age-matched healthy controls, and if so, whether significantly altered systemic miRNAs reflected the tumor miRNA expression profile. We also aimed to identify the circulating medium which best represented miRNA levels (serum, plasma, or whole blood). Previous studies, although few in number, have reported discrepancies between serum and plasma miRNA expression levels, or the investigators have chosen either medium alone for use in their studies.[12,13,16] Finally, a potential relationship between circulating miRNA levels and existing clinicopathological features of breast cancer, such as tumor subtype, stage of disease, nodal status, or hormone receptor status, was investigated.

MATERIALS AND METHODS

Study Cohort

Following ethical approval and written informed consent, blood samples (whole blood, serum, and plasma) were collected prospectively from 127 females, including 83 consecutive breast cancer patients and 44 healthy age-matched female volunteers who served as controls for this study. All patients had histologically confirmed breast cancer and their relevant demographic and clinicopathological details were obtained from our prospectively maintained breast cancer database. The histologic tumor profile of patients in this study reflects that of a typical breast cancer cohort, inclusive of a 10% to 15% proportion with in situ disease, with the majority of invasive tumors being of ductal type, and Luminal A epithelial subtype (Table 1). In addition, repeat blood samples were collected from a subset of this cohort, at their initial clinical review 2 weeks postoperatively (n = 29). The control blood samples were collected from healthy women with no current or previous malignancy, or inflammatory condition. A similar cohort of age- and stage-matched breast cancer patients (n = 65) were identified from whom tumor and tumor-associated normal (TAN) tissues were prospectively collected (Table 1).

Blood Collection

Whole blood was collected in Vacuette EDTA K3E blood bottles (Grenier Bio-one); one processed for plasma, another unprocessed, and a third sample collected in Vacutainer Serum Separator Tubes II (Becton Dickinson) for serum. Samples for serum collection were left to clot at room temperature for 30 minutes and then all samples destined for serum and plasma collection were centrifuged at 2000 rpm @ 4°C for 10 minutes. Plasma/serum was removed, aliquoted, and stored at −20°C until required. The unprocessed whole blood sample was stored at 4°C until required.

miRNA Targets

The expression of a panel of 7 cancer associated miRNAs was chosen on the basis of their reported relevance to breast cancer (Table 2).[8,9,12,17,18]

RNA Isolation

Total RNA was extracted from 1 mL of blood/serum/plasma, respectively, using TRI Reagent BD technique (Molecular Research Centre, Inc.). RNA was extracted from breast tissue as described previously.[18] RNA concentration and integrity were determined using by NanoDrop spectrophotometry (NanoDrop ND-1000 Technologies Inc., DE, USA) and an Agilent Bioanalyzer (Agilent Technologies, Germany), respectively.

Analysis of miRNA Gene Expression

RNA samples were reverse transcribed using primers specific to each miRNA target, and real-time quantitative polymerase chain

TABLE 1. Summary of Clinical Details of Breast Cancer Patients Used for miRNA Analysis

Total Number Breast Cancer Patients: N = 148		
	Breast Cancer Blood Cohort (n = 83) n (%)	Breast Cancer Tissue Cohort (n = 65) n (%)
Mean age, yr	55.1	55.58
Range	30–88	33–92
Stage		
In situ	10 (12)	0 (0)
I	14 (17)	13 (20)
II	35 (42)	29 (45)
III	18 (22)	17 (26)
IV	6 (7)	6 (9)
Invasive tumor type		
Ductal	59 (71)	53 (81)
Lobular	7 (8)	9 (14)
Inflammatory	3 (4)	0 (0)
Other	4 (5)	3 (5)
n/a (in situ disease)	10 (12)	
Epithelial subtype		
Luminal A	53 (63)	34 (52)
Luminal B	8 (10)	13 (20)
Basal	9 (11)	9 (14)
HER2/*neu*	3 (4)	9 (14)
n/a (in situ disease)	10 (12)	
Nodal status		
Positive	38 (46)	31 (48)
Negative	45 (54)	34 (52)
Estrogen receptor status		
Positive	68 (82)	42 (65)
Negative	15 (18)	23 (35)
HER2/*neu* status		
Positive	11 (13)	22 (34)
Negative	62 (75)	43 (66)
n/a	10 (12)	—-

reaction (RQ-PCR) was carried out using TaqMan miRNA primers and probes as described by the manufacturer (Applied Biosystems). Briefly, 100 ng of total RNA was reverse transcribed using Multi-Scribe followed by RQ-PCR on a 7900 HT Fast Real-Time PCR System (Applied Biosystems). Triplicate samples, validated endogenous controls, and interassay controls were used throughout. miRNA expression levels were calculated using QbasePlus software.

Statistical Analysis

Data were analyzed using the software package SPSS 15.0 for Windows. Due to the magnitude and range of relative miRNA expression levels observed, results data were log transformed for analysis. Data are presented as mean ± SD. There was no evidence against normality for the log transformed data as confirmed using the Kolmogorov-Smirnov test. The 2-sample t test was used for all 2 sample comparisons and ANOVA, followed by Tukey HSD post hoc test, to compare the mean response between the levels of the between subject factors of interest. All tests were 2-tailed and results with a $P < 0.05$ were considered statistically significant.

RESULTS

Detection of miRNA in the Circulation of Breast Cancer Patients

Expression of 7 miRNAs, chosen for their established relevance to breast cancer, (Table 2: *miR-10b, miR-21, miR-145, miR-155, miR-195, miR-16,* and *let-7a*) were detectable in whole blood, serum, and plasma samples from breast cancer patients (n = 83) as well as healthy controls (n = 44) (Table 3). *miR-16* expression was stable and reproducible across all 127 participants and was used to normalize RQ-PCR data (Fig. 1, online only, supplemental digital content 2, available at: http://links.lww.com/SLA/A24). Analysis of miRNA expression levels in whole blood, serum, and plasma from a random sample of patients indicated that higher yields of miRNAs by RQ-PCR were obtained from whole blood, compared with either serum or plasma (Table 1, online only, supplemental digital content 3, available at: http://links.lww.com/SLA/A25). There was no significant difference between cancer patients and controls with regard to their mean white cell count (SD): 6.9 (2.11) versus 7.61 (2.14), respectively, $P = 0.122$, hemoglobin levels 13.02 (0.95) versus 13.24 (1.03), respectively, $P = 0.272$ or hematocrit levels 0.39 (0.024) versus 0.40 (0.025), respectively, $P = 0.617$. Based on these findings, whole blood was identified as the preferred medium for investigation of miRNAs in circulation.

Expression Profiles of Blood miRNAs in Breast Cancer Patients

To explore the potential of using circulating miRNAs as novel biomarkers for breast cancer, we investigated the levels of 6 target miRNAs in the circulation of 83 consecutive breast cancer patients and compared with those of 44 normal subjects. The levels of 2 tumor-associated miRNAs (*miR-195* and *let-7a*) were significantly higher, on average, in the breast cancer cohort than in healthy controls ($P < 0.001$ and $P < 0.001$), corresponding to an average fold-change of 19.25 and 11.20, respectively (Table 3, Fig. 1). Furthermore, blood levels of *miR-195* could detect individuals with breast cancer with 85.5% sensitivity and 100% specificity; while blood *let-7a* levels could detect breast cancer with 77.6% sensitivity and 100% specificity (Fig. 2). Within this breast cancer cohort, a subset of patients had postoperative blood samples collected (n = 29) to assess the effect of curative tumor resection on circulating miRNA levels. Thus, it was found that *miR-195* and *let-7a* expression in the blood had decreased significantly to levels comparable with control subjects ($P < 0.001$, Fig. 1). Expression of preoperative circulating *miR-10b, miR-21, miR-145,* and *miR-155* did not differ significantly between the breast cancer cohort and controls ($P = 0.449, 0.606, 0.062, 0.280$, respectively, Table 3).

Relationship of Systemic and Tumor miRNA Profiles

Given that circulating *miR-195* was so significantly elevated in breast cancer patients (19.25 fold), we proceeded to investigate *miR-195* expression in a similar cohort of stage and age-matched invasive breast tumors (n = 65), and in a cohort of TAN controls (n = 18). Tumor expression of *miR-195* was significantly higher compared with that in TAN: 1.23(0.43) versus 0.49(0.37), $P < 0.001$ (Fig. 3). Tumor *miR-195* expression was also significantly higher in Stage IV compared with Stages I and II tumors ($P = 0.006$ and 0.039, respectively ANOVA and Tukey post hoc analysis). There was no significant correlation between mean circulating *miR-195* expression and the stages of disease, however a similar trend of increasing systemic *miR-195* expression with advancing stage of breast cancer was observed (Fig. 4). Controlling for age and stage of disease, *miR-195* expression in tumor tissue showed a significant positive correlation with circulating *miR-195* levels (Pearson correlation coefficient, 0.326; $P = 0.021$) (Fig. 2, online only, supplemental digital content 4, available at: http://links.lww.com/SLA/A26).

Relationship of Circulating miRNAs to Clinicopathological Parameters

In addition to assessing the relationship of breast tumor and systemic miRNA profiles to the stage of disease, other relevant biopathologic associations of circulating miRNAs were investigated. Lymph node positive patients were found to have significantly lower

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TABLE 2. Candidate miRNAs for Investigation in the Circulation of Breast Cancer Patients

miRNA of interest	Previous association with breast cancer
miR-10b	Decreased expression in breast tumor tissue compared to normal breast tissue.[8,9]
miR-21	Increased expression in breast tumor tissue compared to normal breast tissue. Also increased in other solid cancers: colorectal, pancreas, gastric, lymphomas[17]
miR-145	Decreased expression in breast tumor tissue compared to normal breast tissue[8]
miR-155	Increased expression in breast tumor tissue compared to normal breast tissue[8]
miR-195	Reported by *Mattie et al* to be associated with hormone receptor status, as part of a "miRNA signature"[9]
let-7a	Reliable endogenous controls for analysis of miRNA by RQ-PCR in human breast tissue, with *miR-16*[18]
miR-16	Reliable endogenous controls for analysis of miRNA by RQ-PCR in human breast tissue, with *let-7a*[18] and used as a single endogenous control for investigating serum miRNA levels in recent studies.[12]

TABLE 3. Mean RQ Expression Levels (SD) of Target miRNAs in Blood From Breast Cancer Patients Compared With Blood From Healthy Controls

Target miRNA	Breast Cancer Blood Samples (n = 83)	Control Blood Samples (n = 44)	Mean Fold Change in miRNA Expression in Breast Cancer Blood Compared to Controls	*P*
miR-10b	1.05 (3.03)	0.83 (0.83)	1.27	0.449
miR-21	3.52 (10.30)	2.69 (7.47)	1.31	0.606
miR-145	3.58 (7.29)	1.65 (4.14)	2.17	0.062
miR-155	2.92 (6.23)	1.77 (4.48)	1.65	0.280
miR-195	6.91 (12.17)	0.36 (0.43)	19.25	<0.001
let-7a	5.05 (24.33)	0.45 (0.9)	11.20	<0.001

FIGURE 1. Expression levels of *miR-195* (**A**) and *let-7a* (**B**) in preoperative (n = 83) and postoperative (n = 29) blood samples from breast cancer patients and controls (n = 44). At 2 weeks postoperatively, a significant decrease in mean circulating *miR-195* and *let-7a* levels was observed, reaching levels comparable with control subjects .
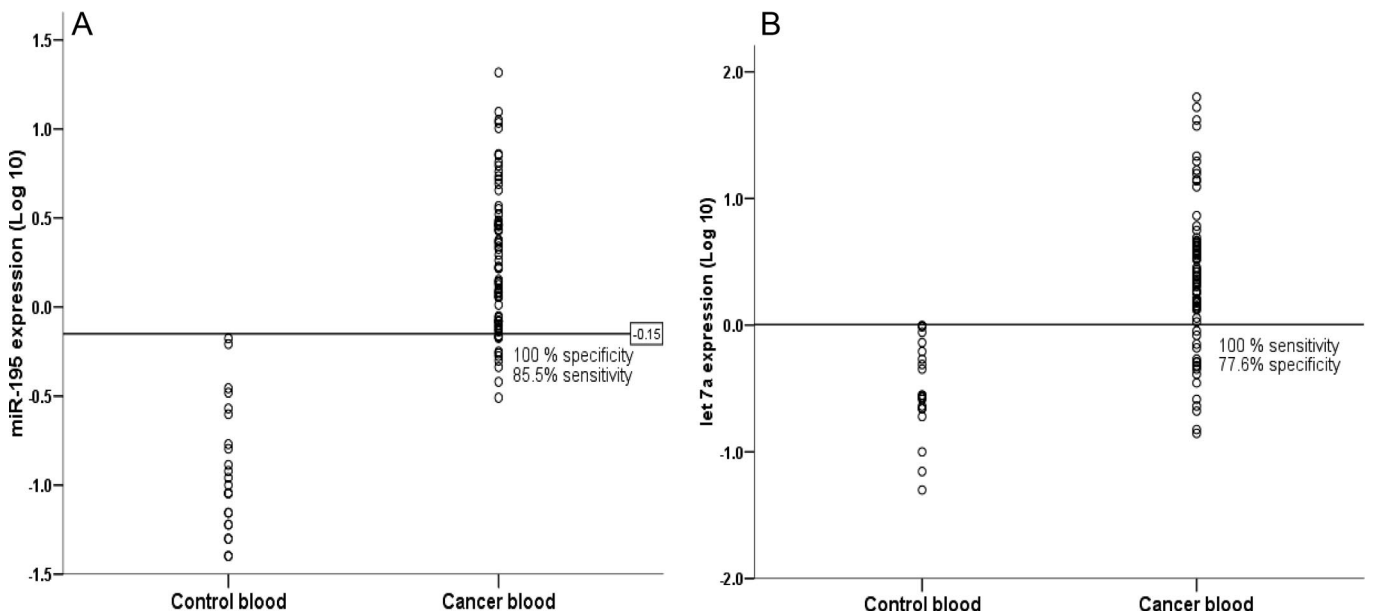
FIGURE 2. Blood *miR-195* (**A**) and *let-7a* (**B**) levels discriminate patients with breast cancer from healthy age-matched controls. The horizontal lines indicate a 100% specificity threshold. Specificities of 85.5% and 77.6% were observed for blood *miR-195* and *let-7a*, respectively.

levels, on average, of circulating *let-7a* compared with those with node negative disease (n = 38 and n = 45, respectively, $P = 0.002$).

Higher circulating levels of *miR-10b* and *miR-21* were observed in patients with ER negative disease (n = 15), compared with those with ER positive breast cancer (n = 68), ($P = 0.028$ and $P = 0.004$, respectively). A potential relationship between circulating miRNA levels, type of disease (in situ vs. invasive), intrinsic subtype, and HER2/*neu* status was also investigated, but no statistically significant difference was identified for any of these parameters.

DISCUSSION

This observational study is the first report of circulating miRNAs in breast cancer patients and our results demonstrate that
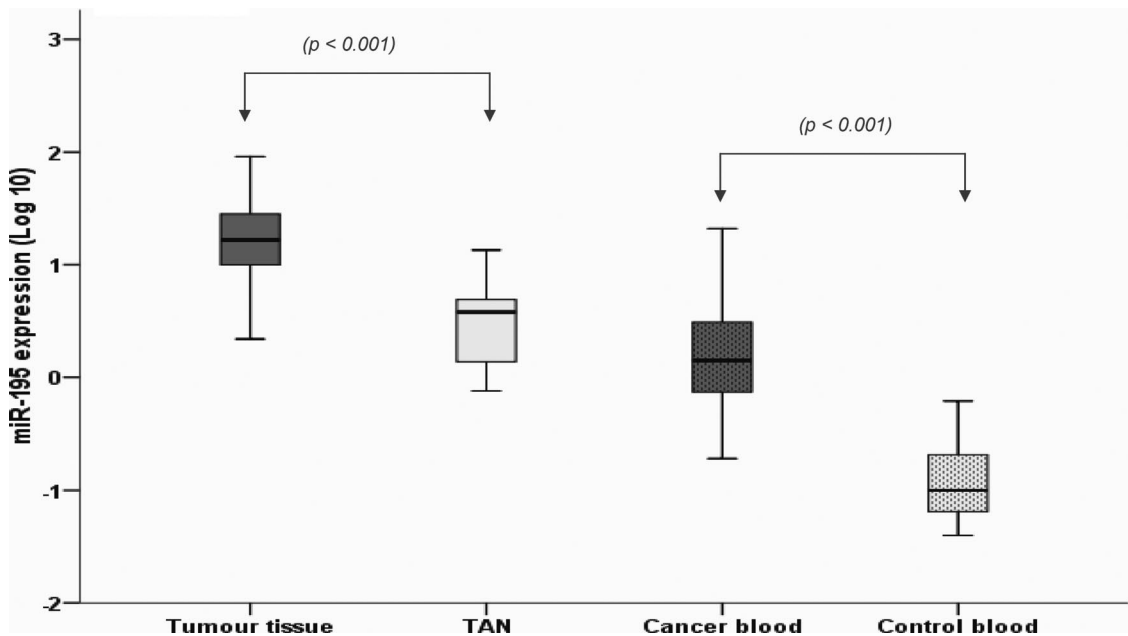
FIGURE 3. The *miR-195* expression in breast cancer tissues (n = 65), tumor associated normal (n = 18), preoperative invasive breast cancer blood samples (n = 73), and healthy control bloods (n = 44). The *miR-195* expression in tumor tissue differed significantly to TAN ($P < 0.001$) and similarly its expression in blood from breast cancer patients differed significantly to healthy control blood ($P < 0.001$).
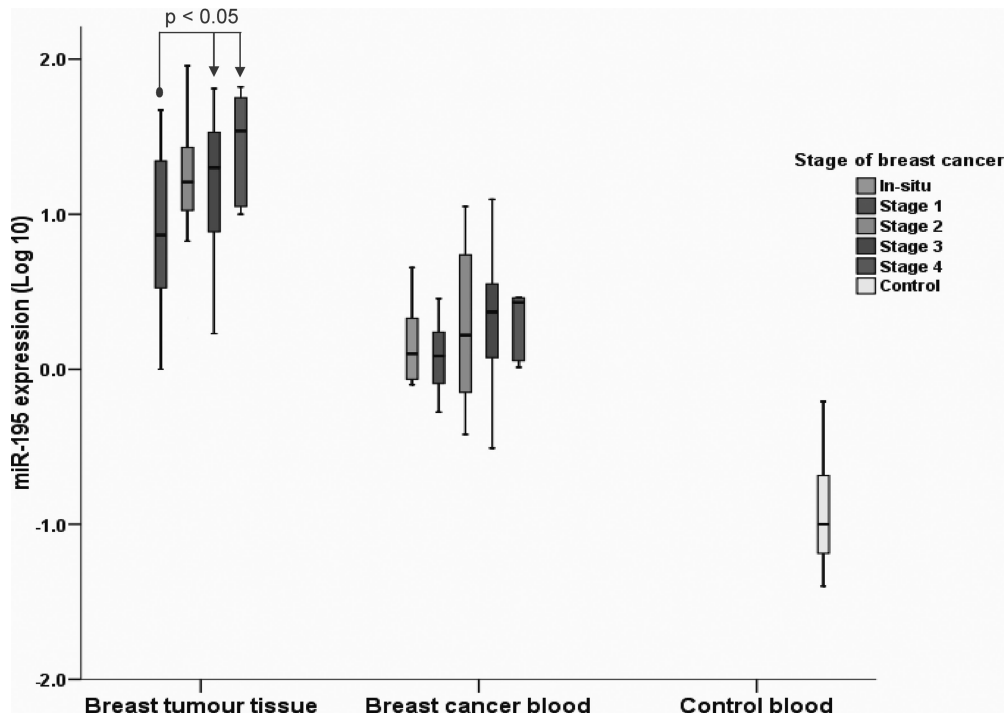
FIGURE 4. The *miR-195* expression according to stage of disease, in invasive breast cancer tissue specimens (n = 65) and invasive breast cancer preoperative blood sample (n = 73) and healthy control blood (n = 44). Tumor *miR-195* expression was significantly higher in Stage IV compared with Stages I and II tumors ($P = 0.006$ and 0.039, respectively, ANOVA and Tukey post hoc analysis). While there was no significant difference between stage of disease and *miR-195* expression in blood from breast cancer patients, there was a similar trend for increasing *miR-195* levels with advancing stage of disease.

cancer-associated miRNAs in blood can potentially serve as novel noninvasive biomarkers for breast cancer. We demonstrate that *miR-195* and *let-7a* are significantly increased in the blood of breast cancer patients in comparison to disease-free control subjects (19.25-fold and 11.20-fold changes, respectively), and can discriminate breast cancer patients from healthy controls with high specificity and sensitivity (Fig. 2). The *miR-195* expression in a similar cohort of breast tumors and TAN specimens shows a similar significant increase in tumor tissue over TAN. When profiling tumor and systemic *miR-195* levels according to the stages of breast cancer, we demonstrate similar profiles in both tissue types. Interestingly, circulating miRNAs were also detectable in patients with *in situ* disease, at levels comparable with early stage breast cancer patients (Fig. 4). In addition, this is the first report on the use of whole blood in preference to plasma or serum for miRNA detection and quantification. Whole blood samples from patient and control subjects were comparable for white cell counts, hemoglobin and hematocrit levels, thereby eliminating potential bias due to cellular and protein components.

The 2 miRNAs found to be significantly increased in the blood of breast cancer patients, *miR-195* and *let-7a*, have previously been described in breast cancer miRNA studies. The *miR-195* was reported by Mattie *et al* to be significantly higher in HER2/*neu* positive compared with HER2/*neu* negative breast cancers,[9] a finding that was also true for the cohort of 65 invasive breast tumors analyzed for *miR-195* expression in this study (HER2/*neu* positive n = 22, HER2/*neu* negative n = 43, P = 0.002, Fig. 3, online only, supplemental digital content 5, available at: http://links.lww.com/SLA/A27). Furthermore, we observed a significant increase in tumor *miR-195* levels in metastatic breast cancers, compared with early stage tumors. This pattern was reflected in the circulation, although to a lesser (nonsignificant) extent. Interestingly circulating levels of *miR-195* decreased significantly by 2 weeks following tumor resection (Fig. 1) albeit in a small subset of patients. Such observations support the concept of utilizing systemic miRNA profiling as a novel and noninvasive biomarker for breast cancer.

The finding that *let-7a* was increased over 5-fold in breast cancer patients was unexpected. *let-7a* is well described as having a functional role as a tumor suppressor[19] and has been shown to be downregulated in many solid organ cancers, including lung, colorectal, and gastric cancer.[20,21] In relation to breast cancer, *let-7a* in conjunction with *miR-16* has been described as a reliable endogenous control for analysis of miRNAs by RQ-PCR in human breast tissue.[18] As endogenous control genes are tissue- and organ-specific, it is acceptable that a house-keeping gene for one tissue type can be investigated as a target gene in another. The finding that *let-7a* was greatly increased in the blood of breast cancer patients raises an interesting question concerning the origin of circulating miRNAs. While recent blood based miRNA reports, including the present study, clearly show that malignancy alters miRNA levels in the circulation, it is still unknown how tumor associated miRNAs make their way into the bloodstream. Slack *et al* raised 2 hypotheses in a recent report,[22] firstly that tumor miRNAs may be present in circulation as a result of tumor cell death and lyses, or alternatively that tumor cells release miRNAs into the tumor microenvironment, where they enter newly formed blood vessels, and thereby make their way into the circulation. Our findings fit generally with the first hypothesis; however, it is clear that further studies are needed to gain greater insight into the origin of circulating miRNAs.

We identified higher circulating levels of *miR-21* and *miR-10b* in patients with ER negative disease. *MiR-21* has been described as an oncomiR, and is up-regulated in many solid and hematological cancers. In relation to breast cancer, higher levels of *miR-21* have been shown to be associated with advanced disease, poorer prognosis, and lymph node metastasis.[23–25] However, the relationship of tumor *miR-21* level to ER status has been inconsistently described; Mattie *et al* found higher *miR-21* levels to be associated with ER positive breast cancer in their study of 20 breast tumor biopsies, 11 of which were ER positive.[9] More recently, Qian *et al* showed high *miR-21* levels to be associated with estrogen receptor negative disease in a much larger study of 344 breast tumors, 218 of which were known to be ER positive and 120 ER negative.[26] Our findings in blood correspond to those of Qian *et al* in relation to breast tissue. Functional studies have shown that in vitro manipulation of *miR-21* expression can alter the responsiveness of ER negative cell lines to hormonal therapies. This further highlights the importance of *miR-21* expression in human breast cancer. Although this study did not find circulating *miR-21* to differ significantly between breast cancer patients and controls, its association with clinicopathological parameters such as ER status indicates that circulating *miR-21* may serve as a prognostic molecular marker for breast cancer and disease progression.

The role of *miR-10b* in breast cancer has also been addressed with varying conclusions on its precise function. Early studies collectively found *miR-10b* to be down-regulated in breast tumor compared with normal breast tissue.[8,9] More recently, Ma *et al* contested these findings, and reported that *miR-10b* played a part specifically in the metastatic process but not in primary tumor formation, having found this miRNA to be highly expressed in metastatic breast cancer cells.[25] To our knowledge, this is the first report of a significant association between *miR-10b* and the hormonal status of breast cancers. Given that hormone receptor negative status is considered a poor prognostic factor for breast cancer,[27] our observation that circulating *miR-10b* is higher in ER negative disease is in keeping with the findings of Ma *et al*.

CONCLUSION

Inherent characteristics of miRNAs such as their lower complexity, tissue-specific expression profiles, stability, and ease with which they are amplified and quantified, make these molecules ideal candidates as biomarkers to reflect various physiological and pathologic states. The results presented here showing significantly altered circulating miRNA levels in breast cancer patients compared with healthy individuals, with similar profile for *miR-195* in breast tumor tissues compared with TAN, and the associations of particular circulating miRNAs with commonly used prognostic indicators, highlights the potential of these molecules as novel noninvasive biomarkers for breast cancer. Circulating tumor associated miRNAs have the potential to detect breast cancer even in its earliest stages, and can differentiate tumors according to histologic features such as hormone receptor and lymph node status. Further prospective evaluation of blood-based miRNAs, in breast and other cancers is needed, to validate these findings and to further explore the exciting potential of circulating miRNAs to emerge as clinically useful novel biomarkers for cancer.

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Cancer Diagnostics and Molecular Pathology

Systemic *miRNA-195* Differentiates Breast Cancer from Other Malignancies and Is a Potential Biomarker for Detecting Noninvasive and Early Stage Disease

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ABSTRACT

Purpose. The potential of microRNAs (miRNAs) as novel tumor markers has been the focus of recent scrutiny because of their tissue specificity, stability, and association with clinicopathological parameters. Data have emerged documenting altered systemic miRNA expression across a spectrum of cancers; however, it remains uncertain as to whether circulating miRNAs are tumor specific. Our aim was to assess a panel of cancer-associated miRNAs in the circulation of patients with various malignancies, to determine whether these "oncomirs" were tumor specific, and thus to establish whether systemic miRNA analysis has utility in cancer diagnosis.

  Patients and Methods. Whole blood samples were prospectively collected from preoperative cancer patients (breast, prostate, colon, and renal cancer and melanoma; $n = 163$) and healthy age- and sex-matched controls ($n = 63$). Total RNA was isolated, and a panel of seven miRNAs was quantified by real-time quantitative polymerase chain reaction in each sample.

  Results. Differential expression of the general oncomirs *let 7a, miR-10b,* and *miR-155,* was observed in the majority of cancer patients in a nonspecific manner. Significantly, elevated circulating *miR-195* was found to be breast cancer specific and could differentiate breast cancer from other cancers and from controls with a sensitivity of 88% at a specificity of 91%. A combination of three circulating miRNAs, including *miR-195,* further enhanced the discriminative power of this test for breast cancer to 94%.

  Conclusion. These findings suggest that individual cancers display specific systemic miRNA profiles, which could aid in discriminating among cancer types. This finding is of notable clinical consequence because it illustrates the potential of systemic miRNAs as sensitive, specific, noninvasive cancer biomarkers. *The Oncologist* 2010;15:000–000

INTRODUCTION

Early diagnosis of cancer remains a compelling challenge for clinicians; it is the ultimate goal in order to minimize treatment-associated morbidity and mortality and achieve maximal long-term survival. Currently, the concept of individualized therapeutic regimens for cancer patients is in vogue, as clinicians and translational researchers attempt to tailor treatment regimens in order that each patient receives maximal benefit in the neoadjuvant and adjuvant settings. The discovery of novel classes of molecular markers in cancer has provided exciting, potentially viable biomarkers that may have utility in early cancer detection. These biomarkers may facilitate accurate tumor stratification, predict response to treatments, predict the risk for disease recurrence or progression, or even represent novel therapeutic targets. One notable example in recent years was the exciting discovery by Slamon and colleagues, in 1985, that the human epidermal growth factor receptor (HER)-2/*neu* oncogene was overexpressed in 20%–30% of human breast cancers, and that the prognosis for patients whose tumors overexpress HER-2/*neu* is poor [1, 2]. This finding led to the development of a specific recombinant monoclonal antibody against HER-2/*neu*, trastuzumab, to treat patients whose tumors overexpress this oncogene. Subsequent trials evaluating this antibody, involving >10,000 women, established that adjuvant trastuzumab therapy halves the recurrence rate and reduces mortality by 30% [3–5]. Currently, HER-2/*neu* expression in breast tumor tissue is routinely evaluated, and when overexpressed, trastuzumab is considered for inclusion in individual adjuvant therapy regimens.

Despite stellar efforts in probing the molecular biology of common cancers, progress similar to that observed in breast cancer has not been mirrored; there remains an enormous dearth of knowledge regarding the molecular taxonomy and complex pathways of other malignancies. Currently, there are few known or validated biomarkers for early detection, treatment planning, follow-up, or targeted therapy of cancer. The utility of currently available tumor markers is limited by disappointing sensitivities and specificities, even in the case of prostate-specific antigen (PSA), which is widely used in routine clinical practice for the screening and management of prostate cancer patients (Table 1).

MicroRNAs (miRNAs) are a contemporary class of very short RNAs that control gene expression by targeting messenger RNAs and triggering either translational repression or RNA degradation [6, 7]. Aberrant miRNA expression underpins a variety of pathological processes, including carcinogenesis, and a number of miRNAs are known to be dysregulated in tumor tissues [8, 9] (supplemental online Table S1). The potential of miRNAs as novel tumor markers has been the focus of much recent attention because of their tissue specificity and unique ability to predict clinicopathological parameters with accuracy superior to that of mRNA expression profiling [10]. This recognition led to the exploration of these tiny molecules in the circulation in the hope that, if present, systemic miRNA analysis could herald a breakthrough in clinical practice, where the quest for sensitive and specific noninvasive cancer biomarkers persists. Recent reports have documented altered

Table 1. Existing noninvasive tumor markers that are in routine clinical use for common cancers

Cancer	Tumor marker	Sensitivity	Specificity	Study
Breast cancer	CEA	29%–53%[a]	70%–99%	Harris et al. [31]
	CA15-3	54%–90%[a]	86%–99%	
Prostate cancer	PSA (>4 ng/ml)	20%–72%	90%–94%	Thompson et al. [32]
				Mettlin et al. [33]
Lung cancer	None	–	–	Arenberg [34]
Colon cancer	CEA (>5 ng/ml)	26%	72%	Moertel et al. [35]
	CA19-9	18%	89%	Herszényi et al. [36]
Uterine cancer	CA125	34.6%	90%	Moore et al. [37]
Melanoma	S-100 protein	15%–65%*	97%	Wollina et al. [38]
Non-Hodgkin's lymphoma	None	–	–	
Ovarian cancer	CA125	71%[a]	98%	Skates et al. [39]
Bladder cancer	Urine cytology	71%	97%	Stonehill et al. [40]
Renal cancer	None	–	–	de la Taille et al. [41]
Pancreas cancer	CA19-9	69%–93%	78%–98%	Tian et al. [42]

[a]Patients with metastatic disease only.
Abbreviations: CA, cancer antigen; CEA, carcinoembryonic antigen; PSA, prostate-specific antigen.

Table 2. Study participants demographic and clinicopathological details

Characteristic	Total n of study participants: 226					
	Breast cancer ($n = 83$) n (%)	Colon cancer ($n = 30$) n (%)	Prostate cancer ($n = 20$) n (%)	Renal cancer ($n = 20$) n (%)	Melanoma ($n = 10$) n (%)	Controls ($n = 63$) n (%)
Mean age, yrs (range)	55.1 (30–88)	68.8 (45–88)	60.6 (50–68)	64.5 (31–78)	52.9 (17–78)	52.1 (24–80)
Sex						
Male	0 (0)	19 (63)	20 (100)	11 (55)	5 (50)	19 (30)
Female	83 (100)	11 (37)	0 (0)	9 (45)	5 (50)	44 (70)
Stage						
In situ	10 (12)	0 (0)	0 (0)	0 (0)	0 (0)	NA
I	14 (17)	7 (24)	1 (5)	6 (30)	3 (30)	NA
II	35 (42)	13 (43)	10 (50)	6 (30)	5 (50)	NA
III	18 (22)	9 (30)	9 (45)	6 (30)	1 (10)	NA
IV	6 (7)	1 (3)	0 (0)	2 (10)	1 (10)	NA

Abbreviation: NA, not applicable.

serum or plasma miRNAs in a variety of cancers, including prostate cancer, colon cancer, lung cancer, and, most recently, breast cancer [11–16]. However, it is unknown whether the specific miRNAs reported to be altered in these studies are disease specific or a global cancer phenomenon. The aim of this study was to investigate the utility of a panel of circulating miRNAs (*miR-10b, miR-21, miR-145, miR-155, miR-195*, and *let 7a*) as potential cancer biomarkers, in particular for early-stage disease. These target miRNAs have previously been reported to be dysregulated in various malignancies and have been identified to play key regulatory roles through their functional interactions with critical cancer-associated genes. Our recently reported findings that circulating *miR-195* and *let-7a* are significantly elevated in breast cancer patients raised the obvious question as to whether elevated levels of these markers are specific to breast cancer patients or a general cancer phenomenon.

MATERIALS AND METHODS

Study Cohort

Following ethical approval and written informed consent, whole blood samples were collected prospectively from 226 participants, including 83 consecutive breast cancer patients, 30 colon cancer patients, 20 prostate cancer patients, 20 renal cell carcinoma patients, and 10 individuals with malignant melanoma. The control group comprised 63 healthy age-matched individuals from the community (female, 44; male, 19). All 163 cancer patients presented to the tertiary referral cancer center in the west of Ireland for management of their malignancy. Each case had a histologically confirmed diagnosis and the histological tumor profiles re-

flect those of typical cohorts for the respective malignancies (Table 2). Breast cancer cases were predominantly of the ductal type (71%), luminal A epithelial subtype (63%), and almost three quarters of the cohort had early-stage disease (71%), inclusive of a 12% proportion with in situ disease. The prostate cancer cohort comprised 20 males with adenocarcinoma of the prostate, 55% of whom had early, stage I and stage II, disease; the average Gleason score was 7 ($n = 13$), with a range of 6–9, and the mean PSA level at diagnosis was 7.67 (range, 3.1–17.5). Colon cancer cases ($n = 30$; male, 19) were all infiltrating adenocarcinomas, were predominantly left sided (73%), and 67% were early, stage I and stage II disease. The renal cell carcinoma cohort ($n = 20$; male, 11) had an average tumor size of 5.9 cm (range, 2–12 cm); 65% had Fuhrman nuclear grade II disease, 23% had extracapsular invasion, and 29% had node-positive disease. Of the 10 malignant melanoma cases (male, 5), seven patients presented with Clarkes level IV or V lesions; the mean Breslow's thickness was 2.3 mm (range, 0.9–5.0) and two patients had a positive sentinel node at presentation. All patients' demographic and clinicopathological details were entered in a prospectively maintained cancer database. The control blood samples were collected from age-matched healthy men and women residing in the same catchment area from which cases originated, and were collected on a contemporaneous basis with cases so as to minimize potential bias because of differential seasonal or environmental exposures. Control individuals were interviewed by a clinician prior to being enrolled in this study to ensure they had no current or previous malignancy, or concurrent inflammatory condition.

Blood Collection

We recently reported that systemic miRNA analysis is optimally performed on unclotted whole blood samples, versus serum or plasma [16]. Venous blood samples (nonfasting) were collected from each participant as follows: whole blood was collected in a Vacuette EDTA K3E blood bottle (Grenier Bio-One International AG, Kremsmünster, Austria). Unprocessed whole blood samples were stored at 4°C until required.

RNA Isolation: Copurification of Total RNA Using Trizol

Total RNA was extracted from 1 ml of whole blood using an adaptation of the TRI Reagent® BD technique (Molecular Research Center, Inc., Cincinnati, OH), as previously described. The RNA concentration was determined using a NanoDrop® spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). The wavelength-dependent extinction coefficient "33" was taken to represent the microcomponent of all RNA in solution. In general, concentrations in the range of 30–300 ng/μl of miRNA were obtained per sample. Integrity was assessed using RNA 6000 Nano Lab-Chip Series II Assays (for small RNA) on a 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany).

Each 1 ml of whole blood yielded 60 μl of total RNA, with yields in the range of 30–300 ng/μl of small RNA, which was then transferred to storage tubes prior to storage at −80°C.

Analysis of miRNA Gene Expression

We chose to study a panel of seven miRNAs in the circulation of all cancer patients (*miR-10b, miR-21, miR-145, miR-155, miR-195, let 7a,* and *miR-16*). These were chosen based on their previously documented associations with malignancies [17, 18] or for their potential as endogenous controls in the circulation [14–16].

Real-time quantitative polymerase chain reaction (RQ-PCR) quantification of miRNA expression was performed using TaqMan MicroRNA® Assays (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Total RNA was reverse transcribed using the Multi-Scribe™-based High-Capacity cDNA Archive kit (Applied Biosystems). RT⁻ controls were included in each batch of reactions. PCR reactions were carried out in final volumes of 10 μl using an ABI 7900 HT Fast Real-Time PCR System (Applied Biosystems). Briefly, reactions consisted of 0.7 μl cDNA, 5 μl TaqMan® Universal PCR Fast Master Mix, and 0.2 μM TaqMan® primer–probe mix (Applied Biosystems). Reactions were initiated with a 10-minute incubation at 95°C followed by 40 cycles at 95°C for 15 seconds and 60°C for 60 seconds. An interassay control

derived from a breast cancer cell line (ZR-75-1) was included on each plate and all reactions were performed in triplicate. *miR-16* was used as an endogenous control to standardize miRNA expression. The threshold standard deviation (SD) for intra-assay and interassay replicates was 0.3. The percentage PCR amplification efficiencies (*E*) for each assay were calculated using the slope of the semilog regression plot of cycle threshold versus log input of cDNA (10-fold dilution series of five points), with the following equation, and a threshold of 10% above or below 100% efficiency was applied: $E = (10^{-1/\text{slope}} - 1) \times 100$.

The relative quantity of miRNA expression was calculated using the comparative cycle threshold ($\Delta\Delta$Ct) method [19], normalized to *miR-16* levels, and the lowest expressed sample was used as a calibrator.

Statistical Analysis

Data were analyzed using the software package SPSS 17.0 for Windows (SPSS Inc., Chicago, IL). Because of the magnitude and range of relative miRNA expression levels observed, results data were log transformed for analysis. There was no evidence against normality for the log-transformed data as confirmed using the Kolmogorov–Smirnov test and so data are presented as the mean ± SD. Analysis of variance (ANOVA), followed by the Tukey honestly significant difference post hoc test, was used to compare the mean response between the levels of the between-subject factors of interest whereas the two-sample *t*-test was used for any two-sample comparisons. All tests were two tailed and results with a $p < .05$ were considered statistically significant. Receiver operating characteristic (ROC) curves were constructed and the area under the curve (AUC) was calculated to assess the ability of each miRNA to differentiate between cancer cases and controls, by computing sensitivity and specificity for each possible cutoff point of the individual miRNAs. This was performed univariately for each individual miRNA, and multivariately for combinations of the six target miRNAs in our panel via logistic regression analysis.

RESULTS

Dysregulated Expression of miRNAs in the Circulation of Cancer Patients

Expression of seven miRNAs, selected for their established relevance to cancer (*miR-10b, miR-21, miR-145, miR-155, miR-195, miR-16,* and *let-7a*), was detectable at variable levels in the circulation of all 226 study participants (163 cancer patients and 63 healthy age-matched controls) (Table 3). *miR-16* expression was stable and reproducible across all 226 participants' peripheral blood samples (sup-

Table 3. Mean logged real-time quantitative expression level (standard deviation) of target microRNAs in blood from all cancer patients compared with blood from healthy controls

Target miRNA	Breast cancer (n = 83)	Colon cancer (n = 30)	Prostate cancer (n = 20)	Renal cancer (n = 20)	Melanoma (n = 10)	Controls (n = 63)
miR-10b	3.0 (0.8)	1.9 (0.6)[a]	2.6 (0.8)	2.0 (0.4)[a]	1.0 (0.4)[a]	3.1 (0.6)
miR-21	3.3 (0.5)	2.3 (1.2)	2.9 (0.8)	2.6 (0.6)	1.5 (0.3)	2.9 (0.6)
miR-145	4.0 (0.4)	2.7 (0.8)[a]	3.0 (0.6)[a]	3.5 (0.7)	1.6 (0.9)[a]	3.7 (0.7)
miR-155	3.1 (0.6)	2.1 (0.5)[a]	1.3 (0.5)[a]	1.9 (0.5)[a]	1.4 (0.7)[a]	2.9 (0.7)
miR-195	4.2 (0.6)[a]	2.6 (0.6)	2.7 (0.9)	3.2 (0.4)	1.9 (0.3)	3.0 (0.4)
let 7a	3.4 (0.7)[a]	3.1 (0.9)[a]	3.3 (1.1)[a]	3.5 (0.7)[a]	2.3 (0.5)	2.2 (0.8)

[a]Significantly different ($p < .01$) from control microRNA levels.
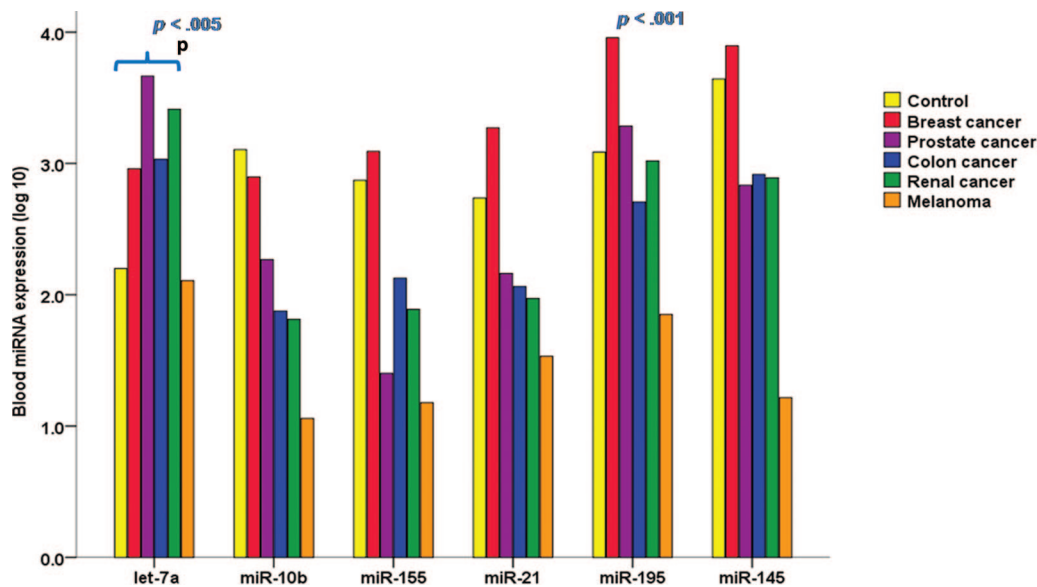
Figure 1. Circulating micro-RNA (miRNA) expression in early-stage cancers. Comparing early-stage cancers (TNM stages, in situ, I, and II; $n = 110$) with controls ($n = 63$), miR-195 expression was observed to be significantly elevated only in breast cancer patients ($p < .001$). Circulating let-7a levels were significantly elevated in patients with several early-stage visceral malignancies.
 Abbreviation: TNM, tumor–node–metastasis.

plemental online Fig. S1) and was therefore used to normalize RQ-PCR data. To explore the potential of using this panel of miRNAs as specific biomarkers for breast cancer, we compared levels of the six target miRNAs in the circulation of 83 consecutive breast cancer patients with those of 80 other cancer patients and 63 healthy control subjects. Circulating levels of five cancer-associated miRNAs (let-7a, miR-10b, miR-145, miR-155, and miR-21) were generally dysregulated in the presence of several cancers, with no specific one of these five markers denoting a particular malignancy. By contrast, an elevated level of systemic miR-195 was unique to the breast cancer cohort ($p < .001$), indicating that it may be a breast cancer–specific marker. This expression pattern held true for cancer patients with early-stage disease specifically (TNM stage, in situ, I, and II) (Fig. 1).

Generic "Oncomirs"

Let-7a levels were observed to be significantly higher in the circulation of patients with several visceral malignancies (breast, prostate, colon, and renal cancers) than in controls (ANOVA $p < 0.001$). Systemic let-7a levels were similarly higher in patients with these malignancies, with no difference among the various cancer patient types (Fig. 2A). Patients with malignant melanoma were not observed to have altered circulating let-7a levels ($p = 1.00$).

miR-10b, a prometastatic miRNA [20], was found to be significantly lower, on average, in blood from colon and renal cancer patients as well as melanoma patients (all stages of disease). Levels of circulating miR-10b did not differ significantly according to stage of disease in these patients. Systemic miR-10b levels were within the normal range in patients with breast and prostate cancers (Fig. 2B).
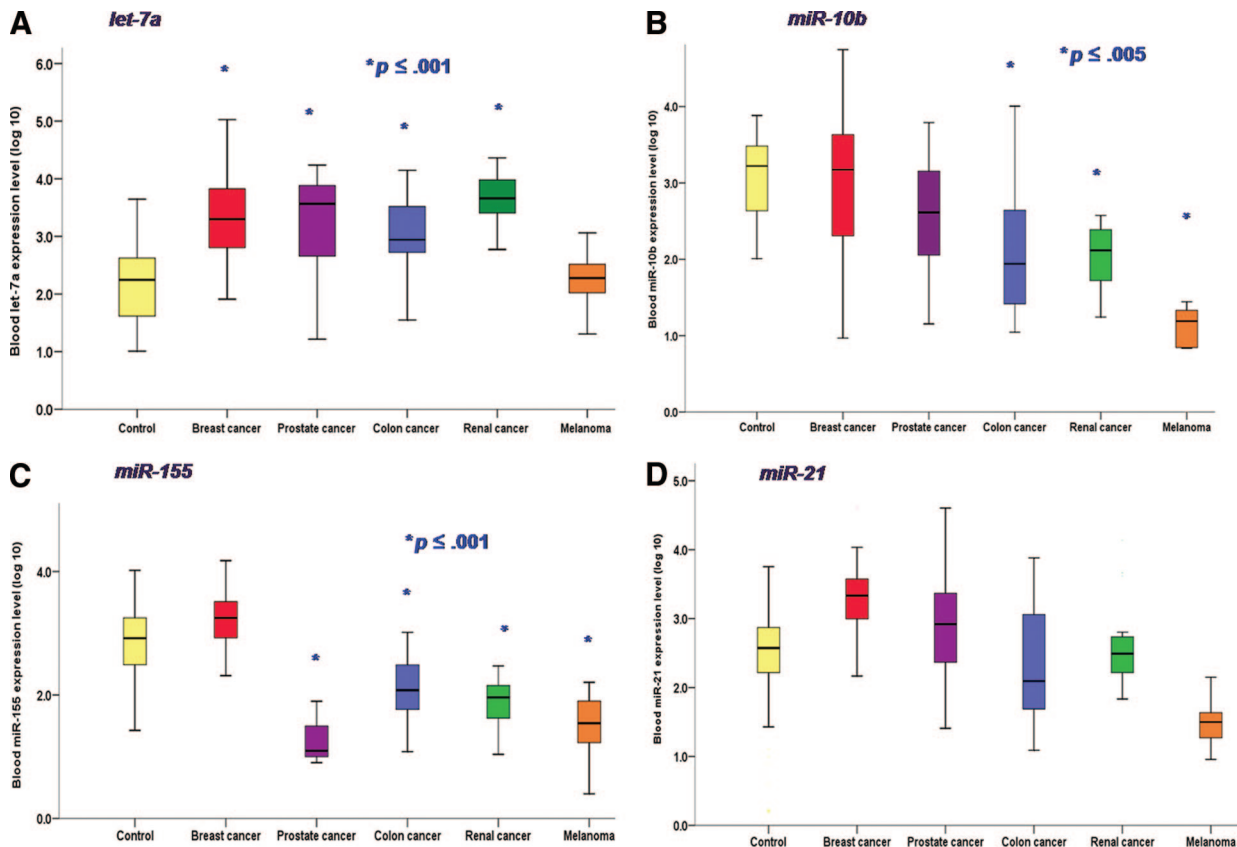
Figure 2. Nonspecific dysregulation of common "oncomirs" in the circulation. **(A):** *Let-7a* levels were significantly elevated in patients with a variety of visceral malignancies. **(B):** Circulating *miR-10b* expression levels in cancer patients. **(C):** Circulating *miR-155* expression levels in cancer patients. **(D):** *miR-21* expression was not significantly different among any of the cancer subgroups or when compared with controls.

The level of *miR-145*, a tumor suppressor miRNA [21], was significantly lower in blood from colon cancer, prostate cancer, and melanoma patients than in control patients ($p = .001$), although levels in breast cancer patients did not differ from those of the control group ($p = .162$).

The level of *miR-155*, a miRNA associated with a variety of malignant tumors [22], was observed to be significantly lower systemically in patients with all malignancies ($p < .001$) except breast cancer, for whom levels were similar to those of the control group ($p = .38$) (Fig. 2C).

miR-21, a well-described oncogenic miRNA [23], was observed to be expressed at generally higher levels in the circulation of cancer patients, with the exception of melanoma cases. However this difference did not reach statistical significance in this study cohort (Fig. 2D).

Breast Cancer–Specific Tumor Marker

Only one of our panel of miRNAs, *miR-195*, was exclusively overexpressed in a specific cancer population. *miR-195* was observed to be significantly overexpressed only in blood from breast cancer patients ($p < .001$), with levels in

other cancer patients largely comparable with those of healthy controls (Fig. 3A). On average, levels of *miR-195* in breast cancer patients were 25-fold (unlogged fold change) higher than levels in control subjects. Circulating *miR-195* levels correlated significantly with tumor size (Pearson's correlation coefficient, 0.446; $p < .001$), and patients with all types of tumors, irrespective of size, expressed significantly higher levels of *miR-195* in the circulation than did the control cohort (Fig. 3B). We observed significant incrementally higher systemic miR-195 levels between small tumors (T1 and T2) and both the T3 and T4 tumor sizes ($p = .002$ and $p < .001$, respectively; ANOVA and Tukey post-hoc analysis) (Fig. 3C). *miR-195* was also detectable in patients with noninvasive disease, at levels significantly higher than in the control group (Fig. 3B, 3C). ROC analysis determined the optimal cutoff value for *miR-195* to differentiate breast cancer cases from controls, and from this analysis the sensitivity of circulating *miR-195* alone was determined to be 87.7%, at a specificity of 91%, with an area under the ROC curve of 0.937 (Fig. 3D). A panel of three circulating miRNAs further increased the sensitivity over individual markers in isolation. The combination of
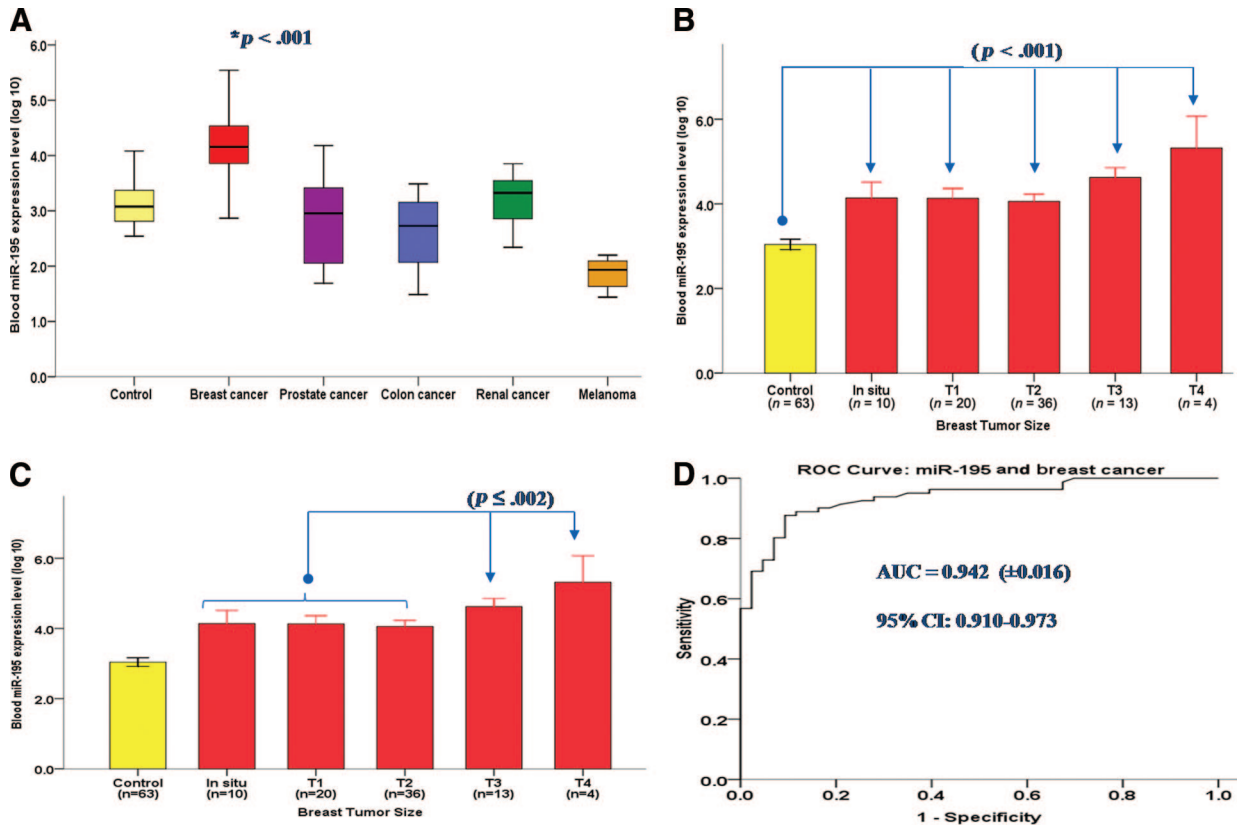
Figure 3. Circulating *miR-195*: A breast cancer–specific tumor marker. **(A):** Circulating *miR-195* was significantly higher in patients with breast cancer than in control patients and patients with other cancers. **(B, C):** Circulating *miR-195* according to breast tumor size. **(D):** ROC curve of the breast cancer sample set analyzed for systemic miR-195 expression.

Tumor size is documented as per the AJCC tumor–node–metastasis system: Tis, carcinoma in situ; T1, tumor $\leq$2 cm; T2, tumor 2.1–4.9 cm; T3, tumor $\geq$5 cm; T4, tumor of any size penetrating the skin or chest wall.

Abbreviations: AJCC, American Joint Committee on Cancer; AUC, area under the curve; CI, confidence interval; ROC, receiver operating characteristic.

circulating levels of *miR-195, let-7a*, and *miR-155* increased the sensitivity for differentiating breast cancer cases from controls to 94% (logistic regression analysis, $p < .001$). Circulating *miR-195* levels did not correlate with levels of the existing breast tumor marker cancer antigen 15.3 (Pearson's correlation coefficient, 0.138; $p = .346$).

DISCUSSION

Our results demonstrate that cancer-associated miRNAs are generally dysregulated in the circulation of patients with visceral malignancy; this dysregulation appears to be relatively nonspecific. This is somewhat predictable, given that the majority of miRNAs investigated in this study have been associated with a variety of common cancers and are known to be involved in multiple critical stages of carcinogenesis. However, a growing body of evidence, based on high-throughput miRNA microarray studies in many cancer types, has identified other miRNAs that are specific for a given tissue type [10]. Our data for circulating *miR-195*, which was observed to be significantly elevated only in

breast cancer patients, support the hypothesis that certain miRNAs are site specific. *miR-195* had previously been demonstrated to be overexpressed in primary breast cancer tissue, which prompted its inclusion in the panel of miRNAs investigated in this study. Mattie et al. [24] identified *miR-195* levels to be significantly higher in HER-2/*neu*$^+$ than in HER-2/*neu*$^-$ breast cancers. Subsequently, Zhang et al. [25] identified *miR-195* to be significantly elevated in breast tumor compared with normal breast tissue, a finding that was reproducible in our breast cancer cohort, thus validating these results [16].

Several findings from this, and previously published data from our group [16], provide convincing evidence to support circulating *miR-195* as a breast cancer–specific tumor marker. *miR-195* expression is higher in breast tumors than in normal breast tissue, a finding mirrored in the circulation, wherein *miR-195* levels are considerably higher (19-fold) in breast cancer patients than in healthy controls. Two weeks following curative resection, systemic levels decreased to a basal level, comparable with those of the

control group. Furthermore, both tumor and circulating levels correlated with disease burden (tumor size and stage of disease). Finally, *miR-195* was not elevated in blood from patients with other malignancies (prostate, colon, and renal cancer or melanoma).

let-7a, one of the well-established cancer-associated miRNAs, was observed to be significantly elevated in almost all of our cancer patients, with the exception of those with malignant melanoma. Our findings support published data that implicate *let-7a* as a protagonist in many cancers, particularly lung, breast, colon, gastric, and ovarian cancer. However, we observed a paradoxical effect in the circulation (i.e., significantly higher systemic *let-7a* levels in cancer patients than in controls), compared with that described previously at the tumor tissue level, where *let-7a* is most commonly found to be underexpressed in tumors, compared with normal tissue, for each specific cancer [26]. Although unexpected, this finding may be explained by the interaction of *let-7a* with its target mRNA, the *KRAS* oncogene, at the cellular level. Recent evidence proposes that dysfunctional interaction between *let-7* and KRAS, resulting from a single nucleotide polymorphism in the *let-7* complementary site in the KRAS 3′ untranslated region, prevents *let-7* from binding and exerting its tumor suppressor effect, resulting in overexpression of the oncogene [27]. A plausible hypothesis is that this particular failure of miRNA and mRNA to bind could lead to lower expression levels of *let-7a* in tumor tissues [28], and a reciprocal increase in free *let-7a* sequences entering the tumor microenvironment, and subsequently, the circulation. This hypothesis warrants further elucidation in order to define the precise mechanism by which miRNAs enter the circulation.

miR-155 levels were observed to be significantly lower in the circulation of all cancer patients, with the exception of breast cancer patients, in contrast to expression levels observed in primary breast tumor tissue [22]. Volinia et al. [29] identified *miR-155* as an oncogenic miRNA overexpressed in several solid tumors, including colon cancer, lung cancer, lymphoma, and breast cancer. Subsequent functional studies have further defined its critical role in carcinogenesis [30]; it is known to promote cell migration and invasion by targeting *RhoA*, a gene involved in cell junction formation and stabilization. *miR-155* also mediates transforming growth factor β–induced epithelial-to-mesenchymal transition—a remarkable process central to the development of tumor invasion and metastasis that involves the dissolution of epithelial tight junctions, intonation of adherens junctions, remodeling of the cytoskeleton, and loss of apical–basal polarity. Our cohort of prostate cancer, renal cancer, colon cancer, and malignant mela-

noma patients had a greater proportion of advanced cancers (TNM stages 2–4) than our breast cancer subgroup, 29% of whom had in situ or stage I disease. This may have contributed to the disparity in circulating *miR-155* expression levels between breast cancer patients and patients with other cancers. However, our data suggest that *miR-155* does have specific value as a biomarker for breast cancer. In our breast cancer subgroup, circulating levels of *miR-155* in combination with *miR-195* and *let-7a* did increase the ability of these miRNAs to discriminate cancer cases from controls, above the sensitivity of either miRNA alone. The analysis of this panel of miRNAs in blood could achieve a sensitivity of 94%.

Although our findings demonstrate the potential utility of circulating miRNAs as cancer-specific biomarkers, it is important to acknowledge that the sample size of cancers evaluated in this study is relatively small and that the panel of miRNAs selected for evaluation was biased toward our search for breast cancer–specific markers. Nonetheless, these data suggest that sustained effort toward developing circulating miRNAs as cancer-specific biomarkers is warranted.

CONCLUSION

The unique properties of miRNAs, including their remarkable stability, tissue-specific expression profiles, and the ease with which they are quantified, herald these molecules as ideal cancer biomarkers. Our data demonstrate the specificity of elevated circulating *miR-195* for breast cancer, and the remarkably high sensitivity of *miR-195* in combination with the general oncomirs *let-7a* and *miR-155* for discriminating breast cancer cases from controls, thus prompting their potential utility as unique, noninvasive breast tumor markers. Further evaluation of blood-based miRNAs in larger cancer cohorts is necessary to validate these findings, and to further elucidate the feasibility of developing circulating miRNA assays specific for individual cancers as clinically useful tools to detect even early-stage malignancy.

AUTHOR CONTRIBUTIONS

Conception/Design: Helen M. Heneghan, Nicola Miller, Michael J. Kerin
Provision of study material or patients: Helen M. Heneghan, Michael J. Kerin
Collection and/or assembly of data: Helen M. Heneghan, Ronan Kelly

Data analysis and interpretation: Helen M. Heneghan, Nicola Miller, John Newell
Manuscript writing: Helen M. Heneghan, Michael J. Kerin
Final approval of manuscript: Helen M. Heneghan, Nicola Miller, Ronan Kelly, John Newell, Michael J. Kerin

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Beginning with the January 2008 issue of *The Oncologist*, we are publishing a brief synopsis of each article in the online Table of Contents. Please confirm if the following is a suitable synopsis for your manuscript:

The study investigates the use of seven different micro-RNAs as novel tumor markers in breast cancer, prostate cancer, colon cancer, renal cancer, and melanoma patients and finds miR-195 to be useful in differentiating breast cancer from the other cancer types.

A 3'-untranslated region *KRAS* variant and triple-negative breast cancer: a case-control and genetic analysis

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Summary

Background We previously identified a functional variant in a *let-7* microRNA (miRNA) complementary site in the 3′-untranslated region of the *KRAS* oncogene (rs61764370) which is associated with cancer. We aimed to investigate the association of this *KRAS* variant with breast cancer and tumour biology.

Methods We assessed frequency distributions of the *KRAS* variant in 415 patients with histologically confirmed breast cancer and 457 controls from Connecticut, USA (study group 1) and association of this variant with breast-cancer subtypes in 690 Irish women with known oestrogen receptor (ER), progesterone receptor (PR), and HER2 statuses, and 360 controls (study group 2). We pooled data for study groups 1 and 2 with a cohort of 140 women with triple-negative breast cancer and 113 controls to assess the association of the *KRAS* variant with triple-negative breast cancer risk, and genome-wide mRNA and specific miRNA expression in patients with triple-negative breast cancer.

Findings Although frequency distributions of the *KRAS* variant in study group 1 did not differ between all genotyped individuals, eight (33%) of 24 premenopausal women with ER/PR-negative cancer had the *KRAS* variant, compared with 27 (13%) of 201 premenopausal controls (p=0·015). In study group 2, the *KRAS* variant was significantly enriched in women with triple-negative breast cancer (19 [21%] of 90 cases) compared with 64 (13%) of 478 for luminal A, 13 (15%) of 87 for luminal B, and two (6%) of 35 for HER2-positive subgroups (p=0·044). Multivariate analysis in the pooled study groups showed that the *KRAS* variant was associated with triple-negative breast cancer in premenopausal women (odds ratio 2·307, 95% CI 1·261–4·219, p=0·0067). Gene-expression analysis of triple-negative breast-cancer tumours suggested that *KRAS*-variant positive tumours have significantly altered gene expression, and are enriched for the luminal progenitor and *BRCA1* deficiency signatures. miRNA analysis suggested reduced levels of *let-7* miRNA species in *KRAS*-variant tumours.

Interpretation The *KRAS* variant might be a genetic marker for development of triple-negative breast cancer in premenopausal women, and altered gene and miRNA expression signatures should enable molecular and biological stratification of patients with this subgroup of breast cancer.

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Introduction

The heterogeneity of breast cancer is shown in the variable risk factors, treatment responses, and outcomes of patients. Breast tumours are classified into oestrogen-receptor (ER) positive and/or progesterone-receptor (PR) positive, *HER2* (*ERBB2*) amplified, and triple-negative tumours (ie, ER/PR negative and HER2 negative).[1] Gene expression and receptor profiling further classifies breast cancer into four biological subgroups: luminal A (ER and/or PR receptor positive, HER2 negative), luminal B (ER and/or PR receptor positive, HER2 positive), HER2 positive (ER/PR negative, HER2 positive), and basal-like tumours (triple-negative breast cancer).[1]

Triple-negative breast cancer is the most aggressive subgroup, with the poorest cause-specific survival at 5 years.[2] Transcriptional profiling studies suggest there is further heterogeneity within triple-negative breast cancers and these tumours can be categorised into two broad subgroups: triple-negative tumours that express epidermal growth factor receptor (EGFR) or cytokeratin (CK) 5/6 and are therefore termed basal-like, and triple-negative tumours that do not express EGFR or CK5/6. Basal-like triple-negative tumours are marked by a younger age of onset than are non-basal-like forms and low expression of *BRCA1*; the basal-like phenotype is common in carriers of the *BRCA1* mutation.[3] An aberrant luminal progenitor cell population (that might be ER positive) could be the target for transformation in *BRCA1*-associated basal tumours.[4] Although prognostic gene-expression markers are highly divergent, several modules such as DNA repair deficiency, signatures of immune response, or transition from epithelium to mesenchyme are commonly noted in a subset of these tumours.[5] Identification of the drivers of these transcriptional modules is a promising approach for discovery of specific and personalised therapies.

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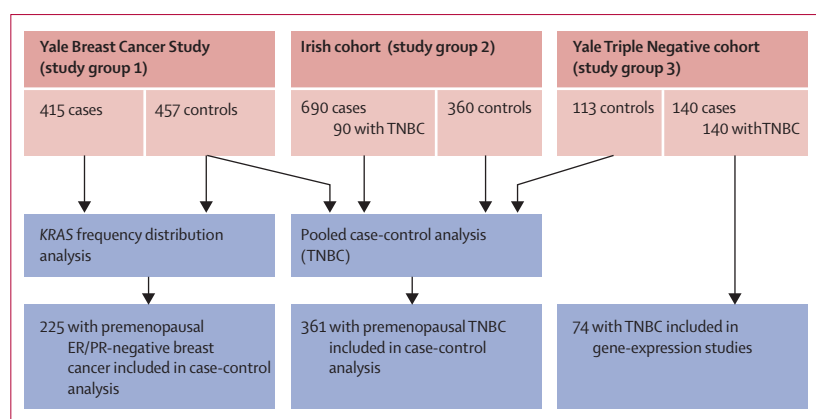
Figure 1: Study groups
TNBC=triple-negative breast cancer. ER=oestrogen receptor. PR=progesterone receptor.

Association of the triple-negative breast cancer phenotype with young age of onset and an absence of association with known risks or reproductive factors[6] supports the notion that there are genetic risks for development of this cancer.[7] Unfortunately, few genetic markers of such increased risk exist. Although *BRCA1* mutations are often associated with triple-negative tumours, these mutations are rare and account for only 10–15% of patients with triple-negative breast cancer, dependent on ethnic background and family history.[8,9]

MicroRNAs (miRNAs) are a novel class of small non-coding RNAs that regulate gene expression by base pairing with sequences within the 3′-untranslated region (UTR), 5′-UTR, and coding sequence regions of target mRNAs, causing mRNA cleavage or translational repression.[10,11] miRNAs are misregulated in every cancer studied so far including breast cancer, in which certain miRNA changes (specifically reduced *let-7*) are found in breast tumour-initiating cells, suggesting that low *let-7* expression allows self-renewal and proliferation of these cells[12] and probably increases risk of breast cancer.

Because miRNAs are global gene regulators, inherited variations in miRNAs are associated with increased cancer risk. Evidence is accumulating that polymorphisms disrupting miRNA coding sequences[13] or 3′-UTR miRNA binding sites are strong predictors of cancer risk, including breast cancer.[14,15] However, none of the previously identified miRNA-altering polymorphisms has been associated with triple-negative breast cancer, or with altered gene or miRNA expression in tumours.

We previously identified a novel germline polymorphism (*rs61764370*) in a *let-7* miRNA complementary site within the 3′-UTR of the *KRAS* oncogene, which is referred to here as the *KRAS* variant. We showed that the *KRAS* variant is associated with low concentrations of *let-7* in tumours and altered *KRAS* regulation in lung cancer.[16] Other groups reported that the *KRAS* variant predicts poor cancer specific outcome in head and neck

cancer[17] and altered drug response in colon cancer,[18,19] suggesting that this variant has biological relevance. Recently we showed that the *KRAS* variant is enriched in ovarian cancer and is most frequently associated with patients from families with hereditary breast and ovarian cancer.[20] On the basis of this evidence, we aimed to assess the role of the *KRAS* variant in breast-cancer risk and tumour biology.

Methods
Study populations
In this case-control study and genetic analysis, we assessed data from four cohorts (figure 1). To assess frequency distributions of the *KRAS*-variant genotype, we assessed individuals from the Yale Breast Cancer Study (study group 1), who were enrolled in a breast cancer case-control study in Connecticut, USA; the study was approved by the Yale institutional review board as previously described.[13] Briefly, patients were aged 30–80 years and had incident, histologically confirmed breast cancer and no history of cancer (other than non-melanoma skin cancer). ER and PR statuses were established for all cases but HER2 statuses were not known and not obtainable. Controls were recruited either from Yale–New Haven Hospital (New Haven, CT, USA) or Tolland County, CT, USA. Controls from the Yale–New Haven Hospital underwent breast-related surgery for histologically confirmed benign breast diseases. Controls from Tolland County were identified either through random-digit dialling (for individuals aged <65 years) or through the Health Care Finance Administration files (≥65 years). Informed consent and data for family histories of cancer, reproductive history, demographic factors, and blood sample were obtained from all participants. 415 cases and 457 controls had DNA samples available for this study, which were obtained between 1990 and 1999.

To define the association of the *KRAS* variant with receptor status and breast cancer subtype, we assessed a cohort of 690 Irish women diagnosed with breast cancer with complete receptor status and subtype classification. Patients from this cohort (study group 2) had histologically confirmed breast cancer and were recruited from the west of Ireland after appropriate ethical approval from the Galway University Hospital (Galway, Ireland) ethics committee. Informed consent and a detailed family history of breast cancer or ovarian cancer, and a blood sample were obtained from all cases. We included 710 cases of breast cancer of all stages and histological types, apart from preinvasive carcinomas. ER, PR, and HER2 statuses were established for all samples by use of standard histopathological analysis and immunohistochemistry, and confirmed by fluorescence in-situ hybridisation for *HER2* positivity. Although gene-expression analysis was not done, these samples were classified as luminal A, luminal B, HER2, or triple-negative breast

	Controls	All		ER and/or PR positive		ER/PR negative		$P_{interaction}$
		Cases	Odds ratio (95% CI)*	Cases	Odds ratio (95% CI)*	Cases	Odds ratio (95% CI)*	
All ages								
Non-variant (T/T)	391	347	Reference	145	Reference	62	Reference	..
Variant (T/G or G/G)	79	68	0·95 (0·67–1·36)	28	0·93 (0·58–1·49)	18	1·59 (0·88–2·86)	0·118
Premenopausal								
Non-variant (T/T)	174	84	Reference	40	Reference	16	Reference	..
Variant (T/G or G/G)	27	16	1·64 (0·79–3·43)	4	0·87 (0·28–2·75)	8	4·78 (1·71–13·38)	0·015
Postmenopausal								
Non-variant (T/T)	217	263	Reference	105	Reference	46	Reference	..
Variant (T/G or G/G)	52	52	0·77 (0·51–1·16)	24	0·90 (0·53–1·53)	10	0·90 (0·43–1·90)	0·991

Data are number or odds ratio (95% CI), unless otherwise stated. ER=oestrogen receptor. PR=progesterone receptor. *Age, ethnic origin, and menopausal status were adjusted in monomial unconditional logistic regression. G/G phenotype occurs in less than 5% of cases and controls and was combined with the G/T phenotype. Minor allele frequency (controls) 0·087, p for Hardy-Weinberg equilibrium 0·783.

Table 1: Association of the *KRAS*-variant with ER/PR-positive versus ER/PR-negative breast cancer in women in study group 1

cancer by receptor status (see webappendix p 1). 690 of 710 patients had complete information and were assessed in this study. The 360 controls in this cohort were healthy women from the same geographical area, and were mainly older than 60 years, with no selfreported personal history of any cancer and no family history of breast cancer or ovarian cancer. Cases and controls were mainly recruited from July, 2006, to July, 2010.

To establish whether the *KRAS* variant predicted an increased risk of development of triple-negative breast cancer, we did a pooled analysis of a cohort of patients with triple-negative breast cancer and controls from Yale (study group 3) and patients with triple-negative breast cancer and controls from study group 2 and controls from study group 1. Patients in study group 3 were receiving treatment either at Yale–New Haven Hospital or at the Bridgeport Hospital (Bridgeport, CT, USA). After approval by the Yale Human Investigation Committee, tissue or saliva specimens were obtained from 156 patients. Complete data were available for 140 patients who were diagnosed in 1990–2007 and were included in this study. 130 cases of triple-negative breast cancer had samples of tumour available before any treatment for gene and miRNA-expression analysis, 78 of whom were also genotyped for the *KRAS* variant. 113 controls in this cohort were healthy women who presented to the Yale–New Haven Hospital and who had no personal history of cancer apart from non-melanoma skin cancer and were recruited between 2000 and 2007. We obtained clinical information, age, ethnic origin, and family history for all cases and controls. Webappendix p 2 summarises basic information for the aforementioned three cohorts.

To assess association of the *KRAS* variant with *BRCA* mutations in ER-negative tumours, we analysed *BRCA1*-mutation carriers with breast cancer and known *KRAS*-variant status from our previous study of the Rotterdam population. The Rotterdam population has been

described[21] but, briefly, consisted of Dutch patients with breast cancer and documented *BRCA1* mutations who were identified by investigators at the Erasmus University through the Rotterdam Family Clinic (Rotterdam, Netherlands).

Procedures

For *KRAS*-variant genotyping assays, we genotyped DNA from all samples for the *KRAS* variant with a custom TaqMan SNP genotyping assay (Applied Biosystems, Carlsbad, CA, USA). On the basis of a previous study,[16] we regarded samples that were heterozygous or homozygous for the variant G allele as positive for the *KRAS* variant.

For gene-expression analysis, we measured genome-wide mRNA expression in 78 patients from the Yale triple-negative cohort who were also tested for the *KRAS* variant. We isolated total RNA from tissue specimen with the RecoverAll total nucleic acid isolation kit (Applied Biosystems) and hybridised to the whole-genome DASL assay (HumanRef-8 version 3.0, Illumina, San Diego, CA, USA). Data preprocessing and statistical analysis were done with the lumi package in Bioconductor/R software. Gene-expression data from three whole-genome DASL runs were combined and processed together. Samples with less than 30% detectable probes and probes that were detectable in less than 10% of the samples were discarded before quantile-normalisation. 74 samples and 18345 probes remained after filtering.

For miRNA analysis, we produced arrays with the Multiplex RT and TaqMan low density array human miRNA panel–real-time PCR system (Applied Biosystems) as per the manufacturer's protocol.[22] We examined expression levels of miRNAs of interest.

Statistical analysis

Genotype distributions of all cases and controls were tested for Hardy-Weinberg equilibrium and were found to
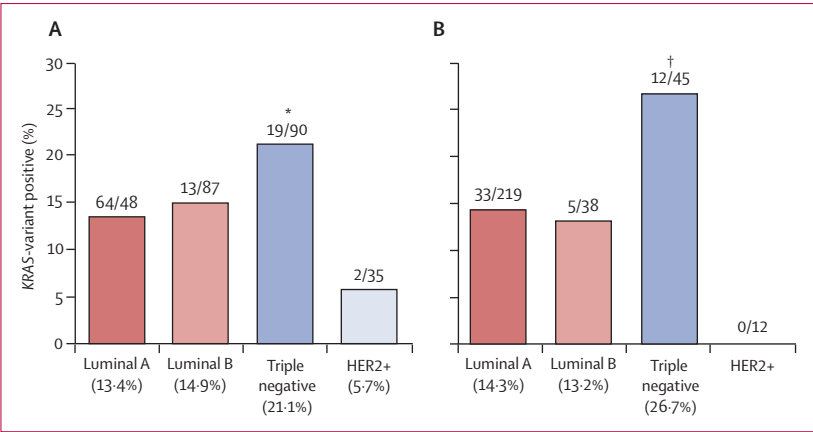
Figure 2: Distribution of the *KRAS* variant in breast-cancer subtypes in all women (A) and premenopausal (≤51 years) women (B) from study group 2
Data are numbers of cases diagnosed with breast-cancer subtype/numbers of patients tested for the *KRAS* variant. *p=0·044 versus all other subtypes. †p=0·033 versus all other subtypes.

	Odds ratio (95% CI)	p value
All ages		
Univariate analysis		
KRAS variant	1·162 (0·797–1·694)	0·4363
Multivariate analysis		
KRAS variant	1·352 (0·901–2·028)	0·1455
Age	0·913 (0·942–0·967)	<0·0001
Ethnic origin	2·536 (2·784–5·999)	<0·0001
Premenopausal women		
Univariate analysis		
KRAS variant	1·879 (1·067–3·310)	0·029
Multivariate analysis		
KRAS variant	2·307 (1·261–4·219)	0·0067
Age	0·913 (0·871–0·956)	0·0001
Ethnic origin	2·536 (1·582–4·067)	0·0001

Age, ethnic origin, menopausal status, and study site were adjusted in a logistic regression model. G/G phenotype occurs in less than 5% of cases and controls and was combined with the G/T phenotype.

Table 2: Association of the *KRAS*-variant in 230 patients with triple-negative breast cancer compared with 930 controls from pooled analysis of study groups 1–3

be in equilibrium. We did unconditional logistic regression to estimate the relative risk associated with every genotype. Controls were adjusted for age (continuous) and ethnic origin (white, black, Hispanic, or other). The population was stratified by menopausal status (estimated by age ≤51 years or >51 years), and separate risk estimates were obtained by ER and PR statuses with multinomial logistic regression with a three-level outcome variable coded as 0 for controls, 1 for cases with ER-positive and/or PR-positive tumours, and 2 for ER/PR-negative tumours. We did tests for interaction with a Wald χ^2, comparing the parameter estimates obtained for every genotype in cases of ER-positive and/or PR-positive disease compared with ER/PR-negative disease.

Patients in study group 2 were stratified according to subtypes of breast cancer and a χ^2 test was done with GraphPad Prism4 software to calculate p values, odds ratios (ORs), and 95% CI. The dominant model was used for all genetic association analysis because of the low frequency of the *KRAS* variant.

We compared categorical variables (eg, ethnic origin, stage, and study site) between study groups with a χ^2 test or two-sided Fisher's exact test, and continuous variables (eg, age) with a *t* test. We calculated ORs and 95% CI for the *KRAS* variant in controls and cases of triple-negative breast cancer with an unconditional logistic regression model with a binary outcome variable. Multivariate logistic regression analyses with a binary outcome variable coded as controls and cases included variables such as *KRAS*-variant status, age, ethnic origin, and study site. The population was also stratified by age group, and separate logistic regression analyses were done for patients aged 51 years or younger (premenopausal group) or older than 51 years (postmenopausal group). Statistical analyses were done with SAS version 9.1.3.

Pathway activation was measured as correspondence with previously published expression signatures and axes derived from principal component analysis of the expression set. Principal component analysis was used to separate biological from technical sources of information in the gene-expression dataset. Every component was characterised by correspondence to RNA quality, the structure of a batch effect, and biological annotations of the contributing probes (ie, probes with expression profiles that have high absolute projection values for the specified component). Signatures of gene expression are provided as lists of genes and their changes in expression in a specific condition. Such signatures are especially valuable for noisy data because they require coordinated differential expression of multiple probes, typically in the order of 100. Because mRNA was extracted from formalin-fixed, paraffin-embedded (FFPE) blocks that were up to 20 years old, analysis of the data set with a signature approach was justified.[23] We calculated signature scores as Pearson correlation between the respective signature vector of gene contributions and a sample's expression profile for these genes. Association of the *KRAS* variant with the outcomes described by the respective signature was analysed by a paired Kolmogorov-Smirnov test between signatures scores of *KRAS* variant and wild-type samples. Differential gene expression was assessed with a linear model, taking into account technical batch artifacts as an offset. Model fitting and empirical Bayesian error moderation of the fold changes were performed with the LIMMA package for R.[24]

We analysed miRNA expression in eight batches of 46 miRNAs and two endogenous controls. miRNA expression was normalised on the basis of the geometric mean of all expressed samples: a miRNA was judged to

have been expressed if threshold fluorescence was detected after fewer than 35 cycles and when the geometric mean cycle number of all expressed miRNAs was subtracted. miRNAs that were not expressed in more than two thirds of all samples were removed, followed by scale-normalisation in all remaining threshold-cycle values.

Role of the funding source

There was no funding source for this study. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Overall, frequency distributions of the *KRAS*-variant genotype did not differ between cases and controls who were genotyped from study group 1 (figure 1, table 1). However, the *KRAS* variant was significantly associated with breast cancer in premenopausal patients with ER/PR-negative tumours (table 1). This association was not observed for postmenopausal women. Eight (33%) of 24 premenopausal women with ER/PR-negative cancer had the *KRAS* variant, compared with 27 (13%) of 201 controls and four (9%) of 44 premenopausal women with cancer that was positive for ER and/or PR (webappendix p 10). Thus, the *KRAS* variant might be a genetic marker of increased risk of development of receptor-negative breast cancer for premenopausal women.

In study group 2, 478 women had luminal A breast cancer, 87 had luminal B disease, 90 had triple-negative disease, and 35 had HER2-positive disease. 98 (14%) of 690 breast-cancer cases from this cohort had the *KRAS* variant, but prevalence varied between the breast cancer subtypes: the *KRAS* variant was significantly enriched in women with triple-negative breast cancer (19 [21%] of 90 cases) compared with 64 (13%) of 478 for luminal A, 13 (15%) of 87 for luminal B, and two (6%) of 35 for HER2-positive subgroups (p=0·044; figure 2). This association with triple-negative breast cancer was also noted in women younger than 51 years (p=0·033, figure 2).

By comparison of cases of triple-negative breast cancer from groups 2 and 3 and controls across all three cohorts (n=1160), we did not note a significant difference between cases or between controls for the prevalence of the *KRAS* variant (webappendix p 3). However, there were significantly more non-white women in the controls from study groups 1 and 3 than there were in the study group 2, which allowed assessment of the association of the *KRAS* variant in non-white women with triple-negative breast cancer in the multivariate analysis. After controlling for age, ethnic origin, and study site, the *KRAS* variant did not predict an increased risk of development of triple-negative breast cancer for all women in multivariate analysis (table 2, webappendix p 4). However, the *KRAS* variant was
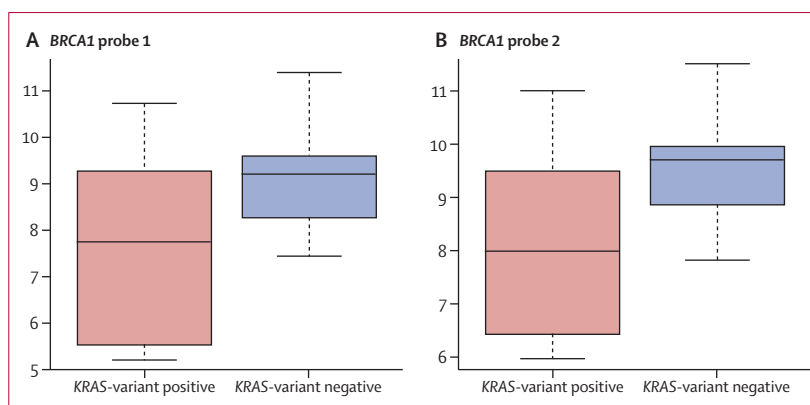
Figure 3: **BRCA1 gene expression among the *KRAS*-variant positive and *KRAS*-variant negative cases of triple-negative breast cancer**
Y-axes are in arbitrary units. (A) *BRCA1* probe 1, p=0·06. (B) *BRCA1* probe 2, p=0·01.

	Signature expression	Kolmogorov-Smirnov p value
NRAS	Upregulated	0·02
BRCA mutant-like	Upregulated	0·04
Luminal progenitor	Upregulated	0·04
MAPK (Creighton)	Upregulated	0·06
PCA oestrogen	Downregulated	0·04

Signature scores were computed as Pearson correlation between the signature vector of gene contributions and each sample's expression profile for these genes. The Kolmogorov-Smirnov test was used to analyse the association of the *KRAS*-variant with signature activation.

Table 3: **Association of the *KRAS*-variant with pathway signatures in tumours of patients with triple-negative breast cancer and positive *KRAS*-variant status**

associated with a significantly increased risk of development of triple-negative breast cancer in the 361 premenopausal women in this pooled group in multivariate analysis (table 2, webappendix pp 5–6).

Because *BRCA1* coding sequence mutations are associated with risk of triple-negative breast cancer, and because we noted an apparent enrichment of the *KRAS* variant in *BRCA1* mutation-carriers with breast cancer,[21] we aimed to establish whether the association of the *KRAS* variant with premenopausal triple-negative breast cancer was due only to its association with carriers of *BRCA1* mutation. Of 36 women with triple-negative breast cancer from cohort 2 and 3 who were *BRCA* tested, 25 (69%) were *BRCA* negative and 11 (31%) were *BRCA* positive. Of these patients, eight (32%) *BRCA*-negative women harboured the *KRAS* variant compared with three (27%) women who were *BRCA* positive. These findings suggest that the *KRAS* variant is associated with an independent group of patients with triple-negative breast cancer without *BRCA* mutations.

Although we did not note an association between *KRAS*-variant status and ER or PR negative statuses in
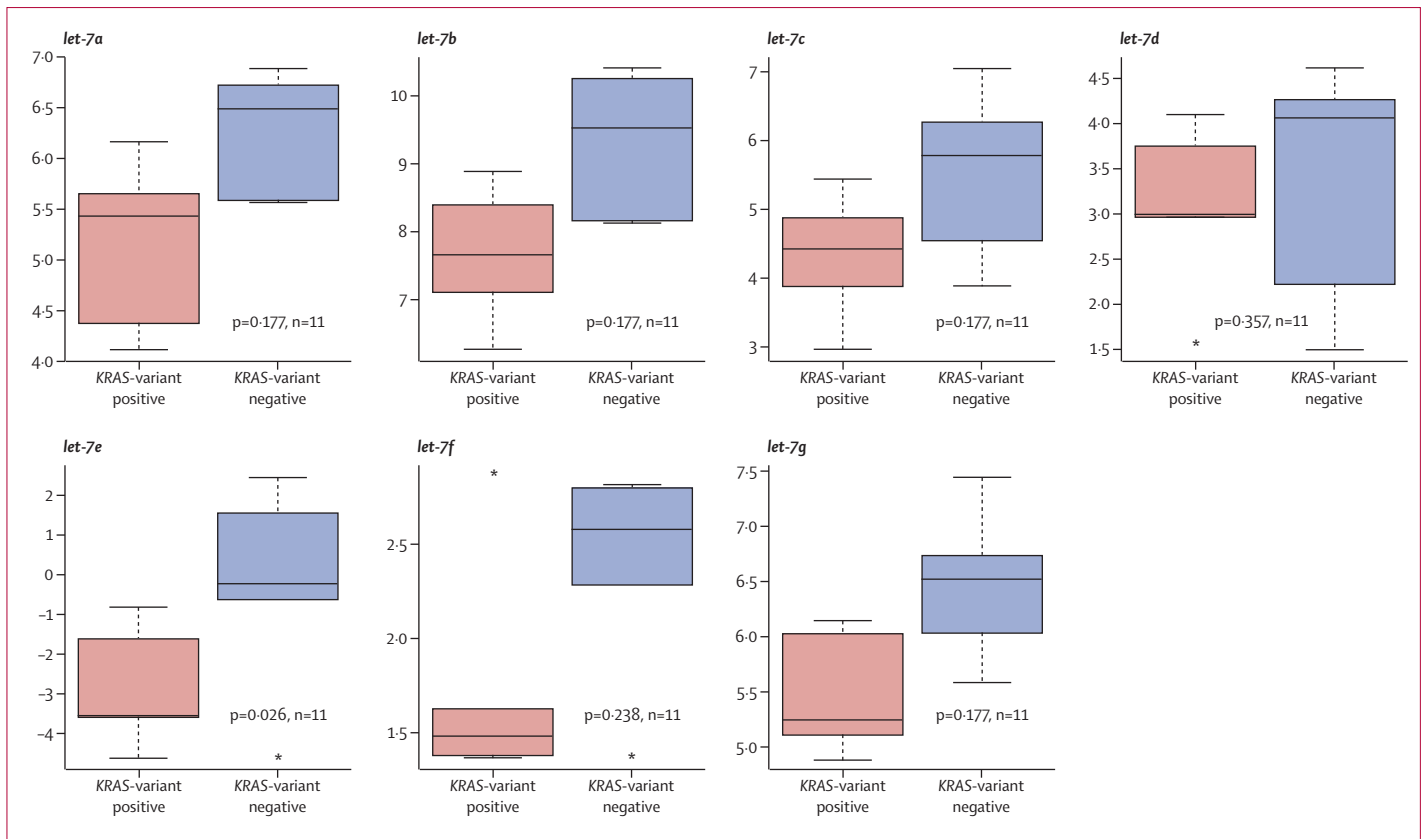
Figure 4: Expression of let-7 family of microRNAs in the *KRAS*-variant positive versus *KRAS*-variant negative cases of triple-negative breast cancer
Y-axes are in arbitrary units.

the Rotterdam population cohort,[21,23] we had not considered menopausal status. In this study, we did not note an enrichment of the *KRAS* variant in 126 premenopausal *BRCA1*-mutation carriers who had ER/PR-negative breast cancer compared with all 268 *BRCA1*-mutation-carriers from the Rotterdam cohort (21·8% *vs* 23·5%, p=0·95). These findings again support the notion that association of the *KRAS* variant with premenopausal triple-negative breast cancer is independent of its association with *BRCA1* mutations.

However, to further assess potential biological interaction between the *KRAS* variant and altered *BRCA1* expression in triple-negative disease, we appraised *BRCA1* expression levels in 74 triple-negative tumours from study group 3 (figure 1). We noted that those patients with the *KRAS* variant had significantly reduced *BRCA1* expression compared with *KRAS*-variant-negative triple-negative tumours (p=0·06 for probe 1 [ILMN_2311089] and p=0·01 for probe 2 [ILMN_1738027], figure 3). Furthermore, the *KRAS* variant was significantly associated with a gene expression signature of decreased *BRCA1* activity (p=0·04).[25] These findings suggest that, although the *KRAS* variant is not restricted to patients with triple-negative breast cancer with known *BRCA1* mutations,

there might be some biological interaction between the *KRAS* variant, altered *BRCA1* expression or functionality, and development of triple-negative breast cancer.

We compared signalling pathways in triple-negative breast-cancer tumours that were *KRAS*-variant positive with those that were *KRAS*-variant negative from patients in study group 3. Although analysis of *KRAS* mRNA did not vary by *KRAS*-variant status, this finding agrees with the other publications about the effect of miRNA binding to the *KRAS* 3′-UTR.[16,26] However, we noted an increase in both an *NRAS* mutation[27] and a MAP-kinase activation signature[28] (table 3) in tumours with the *KRAS* variant. This supports the notion that the *KRAS* variant alters gene expression of canonical RAS pathways, and is to our knowledge the first in-vivo evidence that the *KRAS* variant leads to continued altered downstream gene expression in tumours with which it is associated.

Because we had previously noted altered concentrations of *let-7* miRNA in lung tumours with the *KRAS* variant, we examined *let-7* concentrations in triple-negative breast cancer tumours with the *KRAS* variant. Consistent with our previous findings, we noted lower concentrations of all *let-7* miRNA family members in *KRAS*-variant-associated tumours (figure 4).
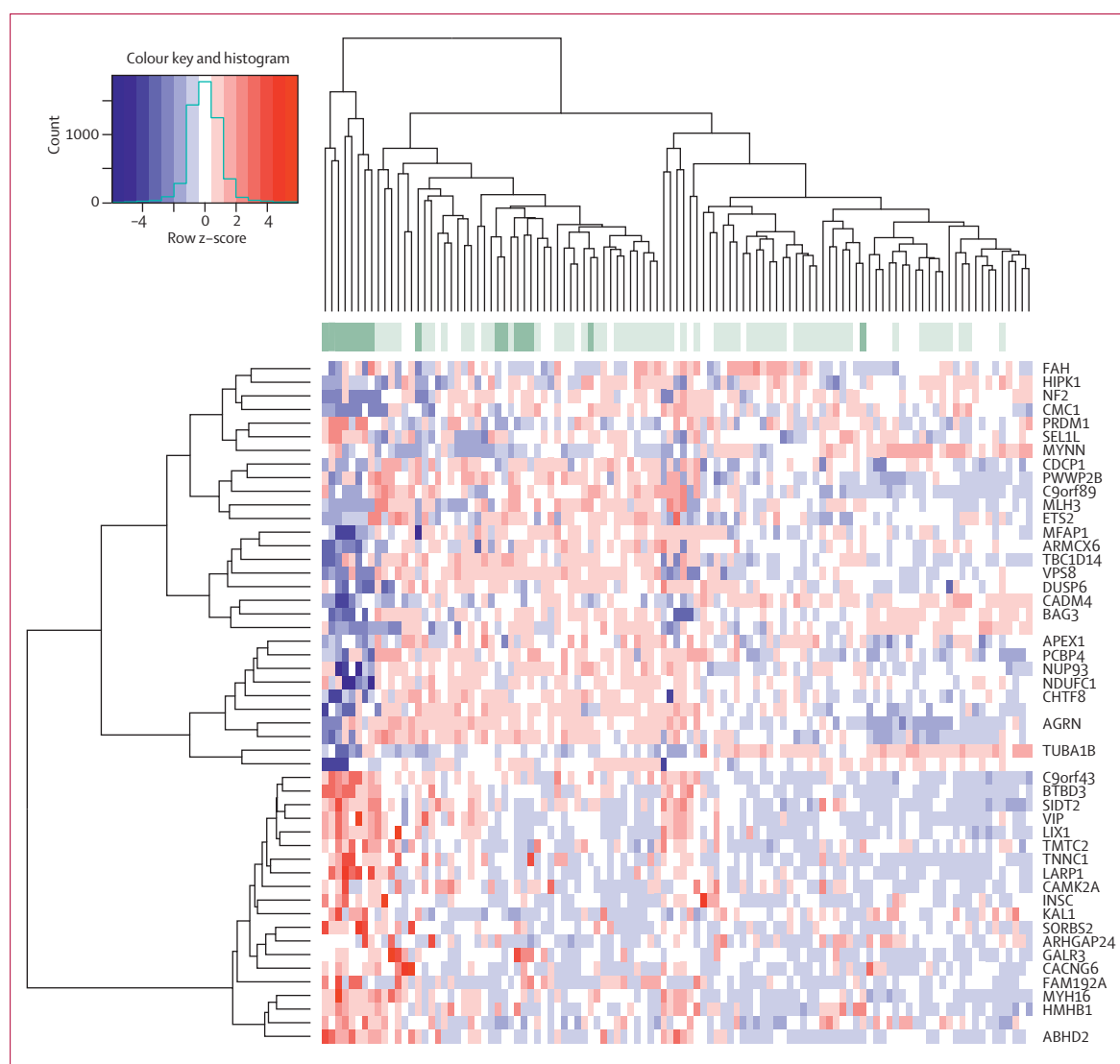
Figure 5: Heat map of *KRAS* variant differentially expressed genes in patients with triple-negative breast cancer, analysed by LIMMA model
The 50 most significant genes were used for the clustering; p<0·0001 for clustering. *KRAS*-variant samples are dark green, wild-type samples are light green. White have unknown *KRAS*-variant status.

To establish how the *KRAS* variant integrates with known gene-expression signatures of triple-negative breast cancer, we assessed known signatures that are differentially expressed in such tumours. We found that *KRAS*-variant tumours have several features of triple-negative and basal-like tumour biology, including decreased oestrogen signalling in a main component derived from our expression set (p=0·04). Furthermore, *KRAS*-variant tumours have a luminal progenitor signature (p=0·04), which has been suggested[4] as a candidate progenitor for basal-like breast cancer (table 3, webappendix p 11). Within the luminal progenitor and the *BRCA* mutation-like signatures, markers of cell adhesion, tissue invasion, proliferation, and angiogenesis (such as α5 integrin, DUSP6, and

aurora kinase B) were differentially regulated (webappendix p 7). This finding is in agreement with the slight enrichment by functional annotations that we noted in three of 41 genes for wound healing (p=0·02), three of 151 genes for glycan expression (p=0·05), and four of 148 genes for MEK activation (p=0·009) on the basis of the differentially expressed genes in a linear model comparing *KRAS* variant versus non-variant for the dataset (figure 5, webappendix pp 8–9).

Discussion

Our data suggest that a germline polymorphism in the *KRAS* 3′-UTR (the *KRAS* variant) is a genetic marker of increased risk of development of triple-negative breast cancer in premenopausal women. Because study

Panel: Research in context

Systematic review

Examination of inherited variants in microRNAs (miRNA) and miRNA binding sites that predict cancer risk is a new and rapidly growing area of research. However, the effect of these miRNA disrupting variants on tumour biology has not been assessed. Because other investigators have shown the potential of the *KRAS* variant to act as a biomarker of poor outcome or poor response to targeted chemotherapy agents, we postulated that this altered biology may be noted in gene and miRNA differences in tumours. Our aim was to understand if miRNA disrupting variants, such as the *KRAS* variant, could both be associated with tumour risk and tumour biology as notable in differences in gene and miRNA expression.

Interpretation

Our study shows that altered tumour gene-expression patterns can be partly accounted for by inherited variants that disrupt miRNAs binding sites. This finding could explain how such variants can act as biomarkers of cancer outcome and response to therapy, and suggests that such variants might be a simple way to subclassify tumours into biologically relevant subgroups. Our conclusions provide evidence that baseline genetic differences between patients can predict genetic differences in their tumours, which is an exciting direction of study in oncology.

group 1 was small and only assessed patients with known ER and PR statuses, we validated this association in larger case-control groups with full receptor status. Most importantly, we show that patients with triple-negative breast cancer who have the *KRAS* variant have tumours with distinct gene-expression patterns compared with patients without this variant, suggesting that the mutation might drive specific pathways that influence tumour biology and could modify tumour development. The *KRAS* variant could ultimately be of value in subclassifying tumours into meaningful biological subgroups to both predict prognosis and help to direct treatment in the future (panel).

The finding of reduced *let-7* concentrations in triple-negative breast cancer tumours that are associated with the *KRAS* variant, as has been reported in lung cancer, is notable. Studies suggest that *KRAS* overexpression, through NFκB, can lead to induction of *LIN-28* (a negative regulator of *let-7*) and lowering of *let-7* expression.[29–31] These conclusions suggest a potential mechanism whereby *let-7* is lowered in premalignant tissue and, ultimately, tumours associated with the *KRAS* variant. Furthermore, *let-7* regulates proliferation of breast-like stem cells,[12] and low *let-7* concentrations could allow expansion of this group of cells, potentially increasing breast-cancer risk in women with the *KRAS* variant. The association we noted of the *KRAS* variant

with triple-negative breast cancer risk only in premenopausal women suggests a meaningful interaction between the *KRAS* variant and hormonal exposure. Such associations and potential mechanisms need additional validation in large cohorts and tumour-initiation models.

Although more than half of breast tumours that carriers of the *BRCA1* mutation develop are triple-negative subtype,[32] *BRCA1* mutations are rare and thus only account for about 10–15% of all cases of triple-negative disease.[8,9] Up to 23% of premenopausal patients with triple-negative breast cancer have the *KRAS* variant, without an apparent significant enrichment in *BRCA* mutation carriers in these cohorts or in young ER/PR-negative *BRCA1*-mutation carriers.[23] However, the *KRAS* variant is associated with a *BRCA1* mutation-like gene-expression signature, supporting the notion that there might be increased oncogenic risk in the presence of the *KRAS* variant and high *KRAS* expression and low *BRCA1* expression, either through mutation or other mechanisms.

We previously showed the *KRAS* variant affects the regulation of KRAS expression in vitro, promoting high KRAS concentrations.[16] The *KRAS* oncogene is an important upstream mediator of the MAPK pathway, and its overexpression can lead to increased activation of the RAF/MEK/MAPK pathway, thereby promoting tumorigenesis. We showed here that patients with the *KRAS* variant and triple-negative breast cancer show activation of the MAPK pathway (table 3). Oh and colleagues[33] reported that hyperactivation of MAPK in breast cancer cells decreases ERα expression leading to a negative phenotype, which is in agreement with our finding that the *KRAS* variant is associated with even lower oestrogen signalling in these histologically ER-negative tumours. MAPK activation has been implicated in oestrogen-independent tumour growth and insensitivity to anti-oestrogen treatment,[34] and might be a mechanism by which the *KRAS* variant drives the development of triple-negative breast cancer more than other breast cancer subtypes. The role of the *KRAS* variant in tumorigenesis and its specific association with triple-negative breast cancer remains to be delineated.

The *KRAS* variant is a biomarker of poor outcome in several cancers, including head and neck cancer,[17] and is a biomarker of poor response to targeted therapies in colon cancer.[18] Our finding that patients with the *KRAS* variant and triple-negative breast cancer have a luminal progenitor signature and differential expression of angiogenic and metastatic markers within the signature suggests that tumours harbouring the *KRAS* variant might be an aggressive subgroup of this cancer. Follow-up studies will be necessary to establish the effect of the *KRAS* variant on outcome in patients with triple-negative breast cancer and patients with breast cancer in general.

Our study suggests that the *KRAS* variant is associated with tumours that maintain unique gene-expression patterns. Although investigations remain to be done to establish the mechanisms of development of triple-negative breast cancer in women who are *KRAS*-variant positive, our findings give insight into crucial steps and pathways required for transformation and tumour development in these women. We believe our results are meaningful steps towards understanding of the mechanisms of gain of function miRNA-disrupting polymorphisms in cancer biology, which seem to be distinct in function from previously discovered genetic markers of cancer risk.

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Breast Cancer
R E S E A R C H

LETTER

Circulating microRNAs: promising breast cancer biomarkers

Helen M Heneghan, Nicola Miller* and Michael J Kerin

See related research by Roth *et al.*, http://breast-cancer-research.com/content/12/6/R90 and related letter by Roth *et al.*, http://breast-cancer-research.com/content/13/1/403

We read with interest the recent article by Roth and colleagues [1] reporting the findings of altered tumor-specific microRNAs (miRNAs) in sera of breast cancer patients. This report further substantiates emerging data suggesting that blood-based miRNAs have immense potential as novel non-invasive cancer biomarkers. However, we have several concerns regarding the authors' study.

Roth and colleagues claim this article to be the first evidence that circulating miRNAs have potential as breast cancer biomarkers, yet refer to previous reports of similar findings [2,3]. This aside, other claims in the study are unsubstantiated. Firstly, the finding that total RNA levels were significantly higher in M0 patients compared to controls and M1 breast cancer patients most likely reflects the quality of RNA extraction techniques and is not clinically relevant. We have previously demonstrated that total RNA levels differ significantly depending on the RNA isolation method and starting blood medium [2]. The authors have not adequately discussed their finding that patients with metastatic disease had significantly lower total RNA levels compared to M0 patients; if their claim that total RNA concentration indicated tumour progression held truth, then one would expect a sequential increase in total RNA concentration from controls, to M0 and M1 patients.

The four candidate miRNAs (*miR-10b*, *miR-34a*, *miR-141*, and *miR-155*) selected by Roth and colleagues for analysis is contentious. Evidence demonstrating consistent differential expression of this miRNA panel in breast tumours, and functionality in breast tumour genesis and progression, is lacking. Given that currently 1,212 mature human miRNAs have been identified (miRBase, release 16 September 2010 [4]), and others

strongly associated with breast cancer, there are more appropriate miRNAs worthy of consideration as breast cancer biomarkers.

Another concerning issue is the time-point at which serum samples were obtained from patients. Our group has previously demonstrated that circulating miRNAs that are elevated in breast cancer patients when the tumour is *in situ* (*miR-195* and *let-7a*) decrease to basal levels by 2 weeks post-tumour resection. Whilst the half-life of tumour-associated miRNAs in blood is undefined, ours and other studies would suggest that it is less than 14 days [5]. The authors' samples were obtained from M0 patients as late as 4 weeks post-operatively, at which time there would have been no disease, or at most microscopic foci, remaining. As the authors' primary aim was to evaluate the feasibility of using circulating miRNAs for detection and staging of breast cancer, it would have been prudent to obtain blood samples pre-operatively.

We do believe that blood-based miRNA analysis has imminent clinical utility as tumour markers. However, if this concept is to translate readily from bench to bedside, then supporting data demonstrating feasibility and validity of this novel approach must stem from carefully planned and well-designed studies. If the current momentum in miRNA translational research can be maintained, then an era of non-invasive rapid diagnostics and individualized care for breast cancer patients is rapidly forthcoming.

Abbreviations
miRNA, microRNA.

Competing interests
The authors have applied for a patent regarding the detection and quantification of miRNAs in the circulation and the use of circulating miRNAs as biomarkers for cancer. This has not yet been granted.

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Systemic microRNAs: novel biomarkers for colorectal and other cancers?

Helen M Heneghan, Nicola Miller and Michael J Kerin

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BOOK REVIEWS

Functional pain syndromes: presentation and pathophysiology

An excellent book that will become essential reading for anybody wishing to have an integrated understanding of functional pain and its treatment. The growing number of books like this one, which recognise and link similar 'functional' problems together, are to be welcomed and supported. This book echoes current clinical experience and research evidence in pointing to the fact that these functional problems are crucially affected by central sensitisation and not merely a reflection of a number of different peripheral end organ problems. The end organs are mainly the site of symptom production. The authors elegantly explain, illustrate and link these issues together in a concise and authoritative manner. The editors have done a fine job of bringing together a wide scope of pain syndromes, while at the same time underpinning and linking these issues together. The possibility that we may be dealing with one 'genotype' (neural sensitisation) with multiple 'phenotypes' (ie, fibromyalgia, irritable bowel syndrome, vulvodynia, etc.) is intriguing but deserves further study. We agree with the review by Albert Ray (IASP website) that books like this compel us to look for treatments that are directed at the neuroplastic capabilities of the nervous system and not only at the end organ sites. At the very least, effective treatments would entail a multi-discplinary approach. We highly recommend this book and appreciate its publication.

Qasim Aziz, Claude Botha

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Competing interests None.

ABC of practical procedures

It is 2033. Medical trainees and practitioners, from first year undergraduates to consultant specialists and senior general practitioners no longer need textbooks. All their learning requirements are found on the virtual web. Practical procedures and clinical examination skills are practiced on 'virtual patients' with instant feedback and grading of competency. Only trainees who attain a grade of 'highly competent' or 'expert' on the World Council of Medicine's virtual assessment programme are allowed to perform and hone their skills on 'real patients, in real clinical areas'.

For those of you that feel that this may be a drug fuelled Orwellian nightmare of things to come, welcome to the teaching and learning of clinical skills in the 21st century.[1] Whether this is the correct method to learn skills remains to be seen but this is what our trainees expect. High tech, complex, computer driven simulation, allowing students at all levels of training and competency to practice and receive instant feedback on their performance on many standard (and higher) clinical procedures and tasks. No longer 'See one, do one, teach one', but 'Observe multiple video examples (with playback option), practice multiple times in the safe environment of the simulator, and receive video evidence of your performance to reflect on, and place into your on-line e.portfolio'. Not so snappy perhaps but coming to a training programme near you soon.

Even a short trawl on YouTube will net multiple videos (admittedly of highly variable content and quality) demonstrating clinical procedures, examination skills, and even patients with clinical signs. The North American Medical school universities have invested heavily in information technology. Their efforts are easily accessible and filled with beautiful, well produced learning materials. Likewise the large medical journals, led by the *New England Journal of Medicine*, have produced multiple e.learning materials, including a whole section on clinical procedures.[2]

Into this challenging environment comes a new book on practical procedures edited by Tim Nutbeam and Ron Daniels. It is a well researched, well written textbook, containing multiple, high definition photographs of the procedures it covers. The book itself is divided into six sections: an introductory phase covering the generics of all practical procedures, followed by five more focused sections on 'Sampling', 'Access', 'Therapeutic interventions', 'Monitoring' and 'Specials' (covering suturing and joint aspiration, paediatrics, and obstetrics and gynaecology). I really wanted to like this book but came away with an intense sense of disappointment. This is a book for my own 'swap shop' generation and not the MTV, iPhone generation of today.

In light of the GMC's Tomorrow's Doctors (2009) and the ever changing MTAS application process, there are several quite important omissions, insertion of a nasogastric tube being the most obvious. These would have lent themselves easily to the target interprofessional audience, and are recognised as problematic by medical undergraduates and foundation doctors alike.

While the majority of the photographs are well used and augment the text, some of the hand drawn illustrations look very clumsy and old fashioned. The illustration 'how to percuss for ascites' was perhaps the nadir; why a man with a rippling six-pack abdomen would need to be assessed for ascites was obviously the author's attempt at demonstrating their own shifting dullness.

In 1990 this book would have been a wonderful addition to any healthcare professional's book collection. Where such books fit into the new high tech learning environment remains unclear but today's trainees demand learning materials that are interactive, with high levels of production, and self assessment. 'All singing, all dancing' is a minimum requirement that this book fails to deliver. With the obvious amount of work and energy that has been put into this project I do hope it finds a place among its target audience. Perhaps if the publishers and editors are looking for a follow-up project they may think about an e.learning package to accompany this text. Without it I am concerned that this book has already had its day.

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Competing interests None.

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LETTERS

Systemic microRNAs: novel biomarkers for colorectal and other cancers?

We read with interest the recent article by Ng *et al* which concludes that plasma microRNAs (miRNAs) hold potential as non-invasive screening tools for colorectal cancer (CRC).[1] Investigation of miRNAs in the circulation has recently gained momentum, upon recognising their potential as novel tumour markers.[2] In our institution we have recently identified that circulating
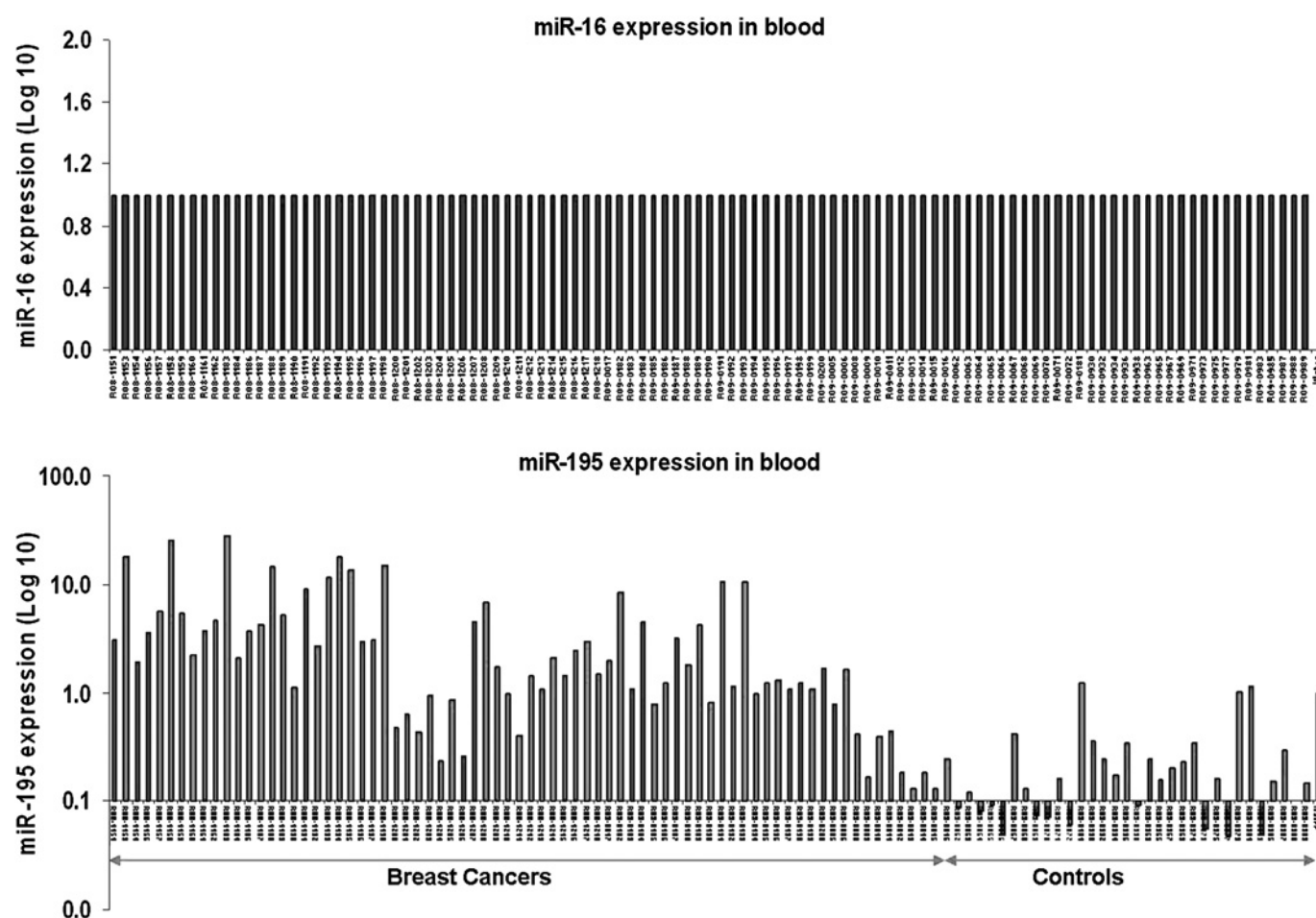
Figure 1 MiR-16 expression as determined by reverse tanscription—PCR (RT—PCR), across 127 whole blood RNA samples (83 samples from patients with breast cancer, 44 samples from healthy females). This illustrates the stability of miR-16 in blood samples, even in the presence of malignancy. In contrast, the expression of miR-195 is highly variable in our blood samples, being significantly higher in breast cancer cases compared with healthy age-matched controls.

miR-195 and let-7a are significantly elevated in blood from patients with breast cancer (n=83) compared with controls (n=44). Similar to Ng and colleagues, we observed a postoperative decrease in systemic miRNA levels, and a significant correlation between systemic and tumour miRNA levels, and stage of disease.[3] Whilst we agree with Ng et al that plasma miRNAs may be useful CRC biomarkers, we have several comments in relation to their article.

First, the use of plasma above serum or whole blood is questionable. There is as yet no consensus on the optimal circulating medium or miRNA isolation technique, from which to quantify systemic miRNAs. Like Ng and colleagues, we found plasma samples to yield low miRNA concentrations when standard column-based methodology was used for RNA isolation (range 4.8—23.9 ng/µl).[1] However we observed that miRNA concentrations from plasma, and indeed serum, may be increased if a co-purification technique is adopted for RNA isolation from blood derivatives. Furthermore, we have observed that unclotted whole blood yields consistently higher miRNA concentrations compared with

serum and plasma (range 20.3—221.6 ng/µl, for 127 samples), irrespective of the RNA isolation method. Whole blood holds other advantages as a biomarker specimen; most notably that no additional processing of the sample is required prior to RNA extraction and therefore is less labour intensive to work with. In this regard it holds potential as a point-of-care test, which would be attractive in determining an ideal cancer screening tool.

The choice of endogenous controls for blood-based miRNA studies is a contentious yet critical issue. Currently there is no strong evidence to suggest an ideal normalisation control in this context. Ng et al use U6 snRNA (small nuclear RNA) as their normaliser, on the grounds that U6 is readily detectable in all plasma samples. There is evidence to suggest, however, that U6 expression is rather variable in a variety of tissue types, including colon tissue, and no evidence at all to support its stable expression in blood.[4] Alternatively miR-16 is abundantly expressed in blood and many solid tissues, and has been shown to be stably expressed in tumour and normal specimens by several authors. We observed miR-16 to show very little variability across 127 blood

samples, and thus we used it to normalise data for our circulating miRNA studies (figure 1).

We are optimistic that circulating miRNAs have immense potential as clinically useful biomarkers, but it is perhaps premature to suggest systemic miR-92 or miR-17-3p as a CRC screening tool. The cohort of patients in the primary investigations of Ng et al included only small numbers with advanced CRC. Additionally their validation cohort is inclusive of a majority (55%) with stage III and IV disease. Thus the systemic miRNAs discussed were not studied in a screening population.

Finally, this is the first association of miR-92 and miR-17-3p specifically with CRC, despite several miRNA microarray studies on CRC tissues over the last few years, and other miRNAs documented to be dysregulated in CRC based on array analyses (eg, miR-10a, miR-31, miR-143 and miR-145). This raises the question as to whether elevated levels of these miRNAs in plasma reflect a general cancer phenomenon, or a true CRC occurrence. If not the latter, then surely this brings the value of miR-92 and miR-17-5p as screening tools further into question.

PostScript

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Authors' response

We sincerely appreciate the interest in our recent article published in Gut[1] and the comments raised. The comments by Heneghan et al[2] raised some important questions concerning the emerging circulating microRNA (miRNA) aspects of cancer diagnostics. These comments include: (1) the choice of circulating medium; (2) the choice of endogenous control; (3) premature for colorectal cancer (CRC) screening; and (4) whether elevated miRNAs in plasma reflect a general cancer phenomenon, or a true CRC occurrence.

In response to comment 1, based on our experience and commercial kit recommendation, total RNA <50 ng is recommended for quantitative PCR (qPCR) of miRNA. A large amount of RNA cannot improve qPCR results and so is unnecessary. Although we agree with the authors that total RNA extracted from whole blood generates a higher yield than that from plasma or serum because a high percentage of RNA/miRNA is derived from the cellular portion in whole blood, one concern about using whole blood for cancer diagnosis is whether the elevated miRNAs identified are primarily derived from the tumour itself or are simply a secondary response of blood cells during tumourigenesis. If the elevated miRNAs are mainly due to the response of blood cells, those miRNAs may not reflect the patient's cancer phenomenon and so lower the testing accuracy. Heneghan et al recently showed that miR-195 and let-7a are elevated in blood from patients with breast cancer. However, a previous study by the same group of authors demonstrated that let-7a is suitable as an endogenous control for qPCR in breast cancer.[3] So, this raises the issue that let-7a elevation in blood is probably due to a secondary phenomenon such as inflammation from blood cells. Accordingly, using whole blood for this diagnostic purpose is questionable.

In response to comment 2, ideally an absolute quantitation approach with standard curve calibration is recommended to be used for qPCR in the field of diagnostics. For relative quantitation, there is still no consensus on the use of an internal normalisation control in plasma. Downregulation of miR-16 has been reported in several cancers including leukaemia, pituitary adenomas, prostate carcinoma and lung cancer.[4—6] In our laboratory, we also found that miR-16 in plasma was aberrantly expressed in patients with breast cancer (unpublished data). Thus, the use of miR-16 as an internal normalisation control in whole blood is still questionable. Furthermore, it was surprising that the same group of authors previously recommended let-7a as one reliable endogenous control in breast cancer.[3] Accordingly, let-7a is not likely to be breast cancer specific and so it raises the issue as to whether let-7a should be used as an endogenous control or diagnostic marker for breast cancer. Thus, an internal normalisation control is still a critical issue for debate. From our point of view, we should eventually switch to an absolute quantitation approach to eliminate the use of an endogenous control.

With regard to comment 3, we agree with the authors that it is premature to apply plasma miR-92 for CRC screening. Larger scale validations are underway, as mentioned in the Discussion section of our original paper.

In response to comment 4, in our paper we showed that elevation of plasma miR-92 and miR-17-3p levels is likely to be derived from CRC. Firstly, miR-92 and miR-17-3p had been selected for further marker validation because of their elevated levels in both plasma and corresponding tumour of patients with CRC. Secondly, their plasma levels were significantly reduced after surgical removal of the tumours. Thirdly, elevation of these miRNAs in plasma due to inflammation, such as inflammatory bowel disease, has been ruled out. Finally, our recent data showed that plasma levels did not increase in other cancer types including breast and gastric cancer. Collectively, miR-92 and miR-17-3p are very likely to be CRC specific.

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Gastric retention and wireless capsule endoscopy in adults: a modified technique for direct duodenal deployment

We read with interest the recent multicentre European study by Fritscher-Ravens et al on the feasibility of wireless capsule endoscopy (WCE) in a paediatric population.[1] In this study a large proportion of the study population needed direct deployment of WCE using various devices. However, the authors encountered significant complications with the use of a polyp retrieval net. Complications included traumatic oesophageal intubation and the cumbersome nature of release of the capsule in the duodenum.

While swallowing is not a major issue in adults, gastric retention of the capsule remains a noticeable complication in ~5% of individuals.[2] Various techniques including body position, motility medications, or a special capsule delivery system used in conjunction with an over tube, a polypectomy snare and a polyp retrieval net have been used to overcome this issue.[3] These techniques, including the commonly employed use of a polyp retrieval net, can be associated with significant technical and traumatic complications in both paediatric and adult populations.[1 3 4]

JOURNAL OF CLINICAL ONCOLOGY | CORRESPONDENCE

Circulating miRNA Signatures: Promising Prognostic Tools for Cancer

TO THE EDITOR: We read with interest the recent article by Hu et al,[1] which reports the novel finding of a four-microRNA (miRNA) signature in serum that has prognostic and predictive value for non–small-cell lung cancer (NSCLC). The emergence of miRNAs as modulators of gene expression and their established role in carcinogenesis identifies them as obvious candidate diagnostic and prognostic indicators for malignancy.[2] The recent surge of reports documenting altered miRNAs in the circulation of cancer patients has given momentum to their putative role as noninvasive cancer biomarkers.[3] However, several issues must be addressed before the proposed serum miRNA signature is accepted as a prognostic biomarker for NSCLC.

The four miRNAs (*miR-1, miR-30d, miR-286, miR-499*) that were significantly altered in lung cancer patients with longer survival compared with patients with poorer outcome have not previously been demonstrated to have functional roles in the etiology or progression of lung cancers, with the exception of *miR-1,* which is reported to be aberrantly expressed in a number of cancers and contributes to carcinogenesis by its regulation of several genes implicated in cell cycle progression and apoptosis including the *MET* oncogene, *FoxP1,* and *HDAC4.* The authors have not provided any evidence that these miRNAs are also abnormal in matched tumor specimens from the same patient cohorts. The expression of these miRNAs in serum from healthy individuals or indeed from patients with benign or infectious lung disease is also not considered. Important characteristics of an ideal tumor marker are that it is produced exclusively by malignant tissue or by tissue predisposed to progressing to malignancy; systemic levels should also correlate with tumor expression, thereby reflecting biologic properties of the tumor. A simple association of altered marker levels among a single cohort of patients, as described in Hu et al,[1] prompts many questions regarding the true biologic significance of these findings.

The finding that the *let-7* family of miRNAs, which are well known to be of functional and prognostic value in lung tumor specimens,[4] were similarly expressed in serum from long- and short-term survival groups in this study is surprising. In the biomarker discovery phase of this study, the authors screened two pooled serum samples from 30 NSCLC patients with longer overall survival (mean, 49.54 months) and 30 patients with shorter overall survival (mean, 9.54 months), using high-throughput Solexa sequencing technology. While this approach is pioneering the next generation of deep sequencing techniques, it is not without limitations. When applied to a relatively heterogeneous group of lung cancer patients as in this study (which included patients with stages I to IIIA, adenocarcinomas, squamous cell carcinomas, post-treatment with surgery, and radiation or systemic chemotherapy), the contribution by a single patient to the genetic pool is minimal, and that contribution has numerous potential confounding factors. Bioinformatic analysis of the data produced

by this sequencing technique is critical to identifying the most significant findings.[5] The authors do not provide a description of how their genome-wide sequencing data were critically analyzed to ensure that the aforementioned four serum miRNAs are the most relevant in this setting. In their previous report on characterization of miRNAs in serum by Solexa sequencing, they identified *miR-25* and *miR-223* as the two miRNAs of most significance in differentiating NSCLC patients from controls, neither of which appeared as markers in their current study.[6]

The authors state that the individual contribution of each of the four miRNAs to predicting lung cancer survival is equivalent to the contribution of their combination, which suggests that there is little additional prognostic information gained by including three of these four markers in a predictive biomarker assay. This implies that other miRNAs remain to be identified in this setting, which could improve on the predictive power of an miRNA signature in NSCLC patients. Additionally, the fact that their serum miRNA signature did not associate with stage of disease or histology subtype calls into question the association of these four miRNAs with prognosis, given the well accepted correlation of advanced stage and higher tumor grade with poorer outcome.

The use of serum over plasma or even whole blood for systemic miRNA analysis is questionable. At present, there is no consensus on the optimal circulating medium or isolation technique from which to
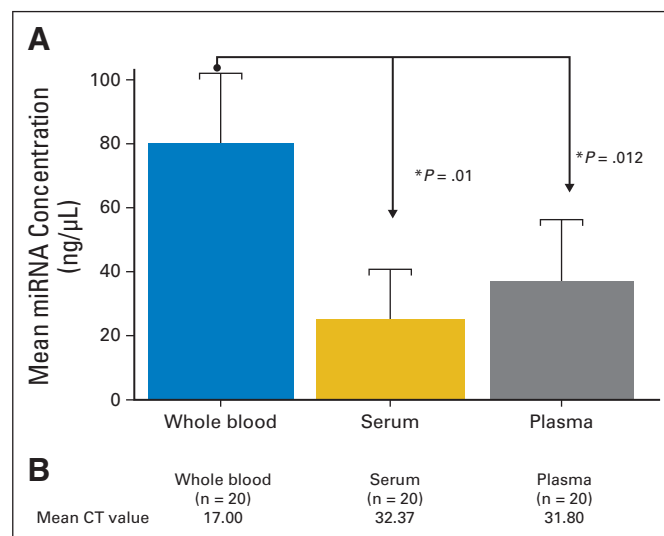
Fig 1. (A) Using the total RNA copurification Trizol-based isolation technique, higher yields of microRNAs (miRNAs; in nanograms per microliter) are consistently attainable from whole blood samples compared with matched serum or plasma samples (n = 20). The miRNA fraction was measured by NanoDrop spectrophotometry, taking the wavelength-dependent extinction coefficient '33' to represent the microcomponent of all RNA in solution. (*) Statistically significant. (B) Earlier amplification of miRNAs occurred in whole blood compared with matched serum and plasma samples when performing quantitative real time polymerase chain reaction, as illustrated here by expression of the abundant *miR-16* in all specimens. CT, cycle threshold.

quantify circulating miRNAs. We have previously found serum samples to yield low miRNA concentrations when a copurification technique is used for isolation of total RNA from serum samples (Fig 1). The range of miRNA obtained from individual serum samples can vary significantly, and thus the authors' use of serum as a starting point for subsequent quantitative real-time polymerase chain reaction (qRT-PCR) quantification of miRNAs is questionable. The initial concentration of miRNA in each PCR reaction, if variable for all samples, could seriously influence miRNA copy numbers detected.

Finally the choice of housekeeping miRNAs for blood-based qRT-PCR analysis is a contentious yet critical issue. Currently, there is no consensus on the ideal normalization control in this context. Hu et al[1] use a healthy donor sample, processed together with their test samples as a control, which would not take into account or control for variable miRNA expression between test samples. Evidence is accumulating to support *miR-16* as a potentially ideal normalizing miRNA gene in this setting. It is abundantly expressed in blood and many solid tissues and has been shown to be stably expressed in tumor and normal specimens by several authors.[7,8] However, further investigations are warranted to identify and validate an accurate normalization protocol for miRNA quantification in blood and its derivatives.

We are optimistic that systemic miRNA analysis has immense potential as a novel clinically useful biomarker for cancer. The authors' four-miRNA serum signature with predictive power for lung cancer patients represents a breakthrough in biomarker discovery for cancer management. However, further studies are warranted to fully investigate the mechanism by which miRNAs enter the circulation, to identify optimal quantification techniques and analytic approaches for quantify circulating miRNAs. We have previously found serum sam-

miRNAs identified by high-throughput sequencing, and to further elucidate the biologic significance of miRNAs in the bloodstream.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST
The author(s) indicated no potential conflicts of interest.

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■ ■ ■

B r i e f   R e p o r t — E n d o c r i n e   R e s e a r c h

Differential miRNA Expression in Omental Adipose Tissue and in the Circulation of Obese Patients Identifies Novel Metabolic Biomarkers

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Background: Omental fat accumulation is associated with development of the metabolic syndrome, although its molecular characteristics are poorly understood. Mi(cro)RNAs (miRNAs), a class of small noncoding RNAs, are known to regulate various metabolic processes, although their role in obesity and the metabolic syndrome is not clearly defined. This study sought to characterize the miRNA expression in omentum, sc fat and in the circulation of obese and nonobese individuals. Their potential as noninvasive metabolic biomarkers was also explored.

Methods: miRNA was extracted from paired omentum and sc fat tissues, and blood samples, from a total of 50 obese and nonobese patients. A miRNA microarray was performed and a panel of differentially expressed miRNAs validated using RQ-PCR.

Results: The miRNA expression profiles were unique for omentum and paired sc fat; no correlation in miRNA expression was observed between these two fat depots. Expression of two miRNAs (*miR-17–5p* and *miR-132*) differed significantly between obese and nonobese omental fat ($P =$ 0.048 and $P = 0.016$). This expression pattern was reflected in the circulation in which these same two miRNAs were also significantly dysregulated in blood from obese subjects. The miRNA expression in omental fat and blood from obese patients correlated significantly with body mass index, fasting blood glucose, and glycosylated hemoglobin.

Conclusion: This study demonstrates that candidate metabolic miRNAs are altered in adipose tissue and circulation of the obese. Omental fat tissue and systemic miRNA levels reflect components of the metabolic syndrome, highlighting their potential as novel biomarkers for this complex syndrome. (*J Clin Endocrinol Metab* **96: E0000–E0000, 2011**)

Obesity and the metabolic syndrome are major public health concerns and present a formidable therapeutic challenge. Undoubtedly the leading factors contributing to its development are dietary excesses and lifestyle issues. However, there is a growing body of evidence to suggest that aberrant genetic expression may play a significant predisposing and causative role in its pathogenesis (1). The metabolic syndrome (2) is also postulated to have underlying genetic determinants that are incompletely understood. Furthermore, the development of this syndrome is closely linked to depot-specific distribution of fat, with

central adiposity conferring greatest risk, and genetic factors are thought to predispose to this particular phenotype (3). The investigation of the molecular mechanisms and genetic abnormalities underpinning metabolic disorders may identify new pathways involved in this disease and influence future approaches to its treatment.

Mi(cro)RNAs (miRNAs) are a class of noncoding small RNA molecules that regulate gene expression by targeting mRNAs and triggering either translational repression or RNA degradation (4, 5). Profiling and functional investigations have identified miRNAs as critical regulators of a

Abbreviations: BMI, Body mass index; miRNA, mi(cro)RNA; RQ-PCR, real-time quantitative PCR.

variety of cellular processes including differentiation, proliferation, and apoptosis (6). Thus far, significant progress has been made in elucidating the role of miRNAs in a variety of diseases including cancers, diabetes mellitus, and infectious, inflammatory, and autoimmune conditions (7–13). Few studies have investigated miRNA expression in fat tissue as potential biomarkers for obesity and the metabolic syndrome. The primary aim of this pilot study was to characterize the expression of miRNAs in paired omental and sc fat in obese and nonobese individuals. We wanted to determine whether miRNA expression was dysregulated in obese adipose tissue and to identify whether any observed dysregulation was specific to either omental or sc fat depots. Finally, we wanted to establish whether circulating miRNAs reflected tissue expression in this setting.

Patients and Methods

This study was approved by the Galway University Hospitals Research Ethics Committee. Written informed consent was obtained from all participants for the use of their tissue and blood specimens.

Study design and patient samples

This pilot study was divided into three phases: phase I, adipose tissue marker discovery; phase II, marker selection and validation; and phase III, circulating biomarker evaluation.

Phase I: adipose tissue biomarker discovery

Paired omental and sc adipose tissue samples were obtained from five patients at the time of elective abdominal surgery; three of this cohort were morbidly obese [body mass index (BMI) > 40 kg/m^2] and underwent bariatric surgical procedures (sleeve gastrectomy). Two nonobese individuals (BMI < 25 kg/m^2) underwent elective laparoscopic Nissen's fundoplication. All patients had similar demographic characteristics. miRNA expression profiles were generated from all 10 adipose tissue samples. Analysis of these expression profiles form obese and nonobese adipose tissue permitted the establishment of two miRNA expression patterns, which were then compared. Dysregulated miRNAs in obese fat were identified for further analysis and validation.

Phase II: miRNA biomarker selection and validation in adipose tissue

Paired omental and sc adipose tissue samples were obtained from a larger cohort of 19 bariatric surgery patients (all with BMI > 40 kg/m^2, all underwent sleeve gastrectomy) and 10 control subjects (all with BMI < 25 kg/m^2) who underwent elective laparoscopic procedures (Nissen's fundoplication, cholecystectomy, paraesophageal hernia repairs). Demographic and baseline clinical details of these cohorts are illustrated in Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org. Sixty-eight percent of the obese cohort was deemed metabolically unhealthy based on the diagnosis of one or more metabolic comorbidities such as hyperlipidemia, hypertension, or diabetes mellitus (as defined in Supplemental Table 2). Candidate miRNA markers were identified from the profiling experiment in phase I and validated in this larger cohort of fat tissues.

Phase III: candidate miRNA evaluation as circulating metabolic biomarkers

Putative miRNA biomarkers identified in phase II were analyzed in the circulation of an expanded cohort of obese patients (n = 30) and nonobese controls (n = 20). The controls for this phase consisted of age-matched healthy volunteers with BMI less than 25 kg/m^2 as well as the nonobese surgical patients who donated a whole-blood sample in addition to omental and sc fat samples at the time of elective abdominal surgery.

Adipose tissue and blood collection

Paired omental fat and sc fat (from the periumbilical area) samples were harvested intraoperatively from each surgical patient and snap frozen in liquid nitrogen before storage at −80 C until required for analysis. A fasting venous blood sample (10 ml) was collected from each participant for miRNA quantification.

RNA isolation: copurification of total RNA from adipose tissue and blood using Trizol

Total RNA was prepared from all fat specimens (omental and sc) and whole blood using a copurification technique and its concentration determined by spectrophotometry (Supplemental Materials and Methods 1).

miRNA microarray profiling

miRNA expression profiling of 10 adipose tissue samples was performed using a customized real-time PCR-based miRNA array containing a panel of 95 miRNA assays (Systems Biosciences, Mountain View, CA; Supplemental Materials and Methods 2).

Validation of miRNA gene expression in adipose tissue and blood by real-time quantitative PCR (RQ-PCR)

RQ-PCR quantification of miRNA expression was performed using TaqMan microRNA assays (Applied Biosystems Inc., Foster City, CA) according to the manufacturer's protocol. One hundred nanograms of total RNA were reverse transcribed using the MultiScribe-based high-capacity cDNA archive kit (Applied Biosystems), and RQ-PCR was performed as described in Supplemental Materials and Methods 3. *MiR-16* was used as an endogenous control, given its stable expression in all samples in the profiling experiment.

Statistical analysis

The significance analysis of the microarrays was employed to analyze the data from the miRNA profiling experiment. The data are presented as median and interquartile ranges. The Mann-Whitney *U* test was used for the two-sample comparisons. The Kruskal-Wallis nonparametric one-way ANOVA, followed by the Mann-Whitney *U* test, was used to compare more than two independent samples. Results with a *P* < 0.05 were considered statistically significant.
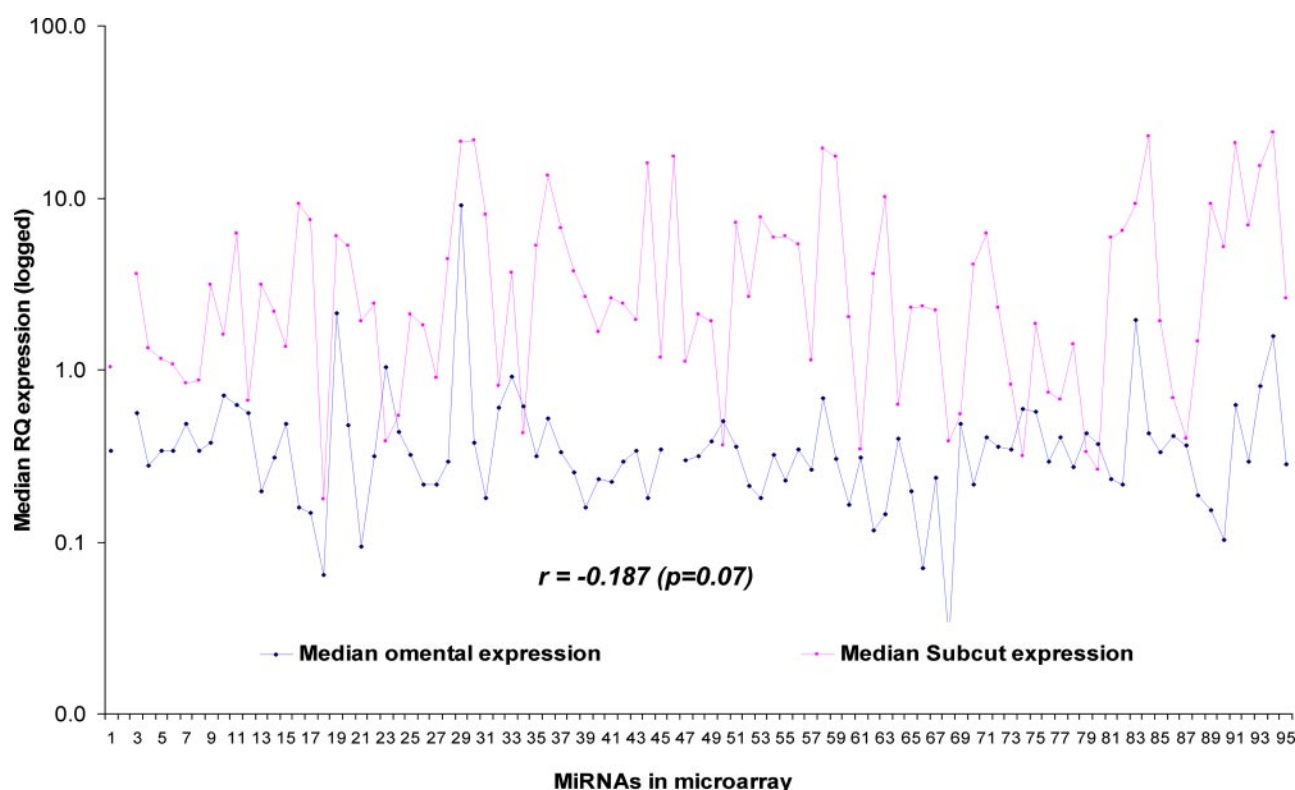
FIG. 1. This line graph demonstrates the median expression values of the 95 target miRNAs in the array platform, in both omental fat (n = 5, *blue line*) and sc fat (n = 5, *pink line*). Data are logged due to the magnitude of RQ-PCR expression values, although the distribution of the data were not normal and so analysis was performed using nonparametric tests for correlation (Spearman's rank correlation). Correlation analysis of miRNA expression in matched omental and sc fat samples yielded a Spearman's rank correlation coefficient of −0.187, which highlights the weak correlation in miRNA expression between these two fat types (P = 0.07).

Results

Identification of differentially expressed miRNAs in omentum *vs.* sc fat

Comparing omental and sc expression profiles from the microarray experiment revealed a poor correlation of miRNA expression in paired samples (Spearman's rank correlation coefficient −0.187, P = 0.07, Fig. 1). miRNA expression was largely underexpressed in omental fat compared with sc fat. In sc fat, there was no difference in expression of any miRNA on the array platform between the obese and nonobese individuals (P > 0.05 for all miRNA targets). In contrast, omental fat displayed differential expression of a number of miRNAs between obese and nonobese individuals. Using a 2-fold expression difference as a cutoff, five miRNAs were dysregulated in obese omentum (*miR-122, miR-17–5p, miR-132, miR-143, miR-145,* Supplemental Table 3). These markers were selected for validation in a larger cohort of fat specimens (paired omental and sc specimens, from 19 morbidly obese patients and 10 healthy nonobese controls). A further three miRNAs, not included in the array platform, were selected for investigation in this larger cohort based on evidence from literature that they played potential roles in adipogenesis, insulin sensitiv-

ity, and appetite regulation (*miR-34a, miR-99a,* and *miR-195* (14–16).

In the validation experiment, the expression of these eight candidate miRNAs in sc fat was again similar between obese and nonobese subjects (Table 1). In omental fat, however, two of the eight miRNA targets (*miR-17–5p* and *miR-132*) were significantly underexpressed in obese omentum compared with controls (P = 0.048 and P = 0.016, respectively; Table 1).

Circulating miRNAs reflect expression in omental fat tissue

Upon investigating expression of these eight miRNAs in the circulation of a larger cohort of obese (n = 30) and nonobese individuals (n = 20), the systemic levels of *miR-17–5p* and *miR-132* were observed to be significantly underexpressed in obese compared with controls (P = 0.024 and P = 0.029, respectively), reflecting the same expression pattern as was observed in omentum. Expression of *miR-17–5p* displayed a significant inverse correlation with the BMI in both adipose tissue and blood samples (Spearman's rank correlation coefficients −0.419 and −0.346, P = 0.037 and P = 0.023, respectively). The expression of the other six miRNAs

TABLE 1. Median RQ expression levels (IQR) of target miRNAs in specimens from obese *vs.* nonobese individuals[a]

Target miRNA	Obese (n = 19)	Nonobese (n = 10)	Relationship between obese *vs.* nonobese	*P* value
Subcutaneous fat				
miR-17-5p	0.6 (0.3–2.8)	0.9 (0.6–7.4)	↓	0.396
miR-132	85 (17–11,056)	1,175 (585–1,346,597)	↓	0.072
miR-34a	25 (8–104)	186 (124–20,783)	↓	0.053
miR-99a	42 (4–1,788)	389 (208–619)	↓	0.090
miR-122	45,095 (7,174–127,281)	177,828 (21,380–7,943,282)	↓	0.364
miR-143	79,433 (457–912,011)	977,237 (495,363–9,177,623)	↓	0.253
miR-145	143,354 (3,109–523,100)	630,957 (398,458–6,834,649)	↓	0.073
miR-195	55,069 (450–111,563)	7,592 (4,486–10,698)	↑	1.000
Omental fat				
miR-17-5p	0.6 (0.1–0.8)	1.1 (0.7–2)	↓	**0.048**
miR-132	140 (0.2–528)	2,369 (708–7,312)	↓	**0.016**
miR-34a	138 (53–485)	460 (212–653)	↓	0.265
miR-99a	62 (6–432)	230 (90–584)	↓	0.205
miR-122	144,582 (21,973–2,911,100)	28,866 (9,790–47,402)	↑	0.148
miR-143	181,970 (3,179–24,448,529)	20,893 (10,958–25,339)	↑	0.664
miR-145	223,932 (93,111–931,390)	2,286,965 (753,506–3,743,909)	↓	0.172
miR-195	575 (335–355,014)	38,019 (32,499–39,632)	↓	0.513
Blood				
miR-17-5p	0.04 (0.03–0.13)	0.3 (0.03–0.6)	↓	**0.024**
miR-132	2.2 (0.6–6.1)	5.2 (3–21)	↓	**0.029**
miR-34a	31 (20–102)	48 (20–168)	↓	0.312
miR-99a	0.1 (0.02–0.33)	0.2 (0.1–0.2)	↓	0.373
miR-122	562 (195–1072)	550 (195–1,072)	∼/↑	1.000
miR-143	0.4 (0.1–2.9)	0.7 (0.2–3)	↑	0.393
miR-145	404 (56–1001)	871 (222–3,071)	↓	0.059
miR-195	479 (138–5623)	2,692 (132–5,830)	↓	0.594

P values in *boldface* indicate statistically significant.

P values (*P* < 0.05). *P* values in *lightface* indicate nonsignificant *P* values.

[a] × 10³.

in circulation also reflected the same pattern as was observed in omental tissue (Table 1).

Discussion

This is the first report of miRNA expression in both adipose tissue and in the circulation of obese humans, and our results demonstrate that miRNAs can potentially serve as novel noninvasive biomarkers for obesity and related metabolic conditions. They may even represent novel therapeutic targets for these common diseases. It has long been recognized that omental and sc fat are anatomically and physiologically distinct tissues and presumed that intrinsic molecular features of omental fat explain why abdominal obesity is such a significant risk factor for metabolic and cardiovascular diseases. Our profiling of 95 miRNA targets in both fat tissues revealed that no miRNA was exclusively expressed in either fat depot, suggesting perhaps their common developmental origin. The observed poor correlation between miRNA expression in omental and sc fat in this study gives further impetus to the hypothesis that the omentum is a distinct tissue, with important functional consequences for obese individuals with extensive central adiposity.

The finding that omental and circulating levels of *miR-17–5p* and *miR-132* were significantly decreased in obese

individuals compared with nonobese individuals, and that *miR-17–5p* expression correlates inversely with BMI, is consistent with results reported by Klöting *et al.* (14). *In vitro* data indicate that this miRNA may induce acceleration of adipocyte differentiation by negatively regulating the key cell cycle regulator *Rb2/p130* (17). Known gene targets for *miR-132* include cAMP response element-binding protein, which has a role in glucose homeostasis (18), and brain-derived neurotrophic factor, which is implicated in appetite regulation and energy homeostasis (19).

The finding of aberrant miRNA expression in various disease processes has led to studies investigating miRNA manipulation as a potential therapeutic strategy. Where miRNA expression is known to be underexpressed, as in obese adipose tissue (*e.g. miR-17–5p* and *miR-132*), the induction of miRNA expression could potentially restore catabolic activity to the tissue. This concept of miRNA replacement therapy has been successful in other disease models. Restoration of *let-7* expression in lung cancers has been shown to restrain the growth of tumors by repressing multiple cell cycle and proliferation pathways together with *ras* and *MYC* suppression (20). This current study did not attempt to investigate the therapeutic potential of miRNAs in obesity; functional analyses and modulation

of miRNA *in vitro* is ongoing in our department, in addition to validation of the above findings in a larger cohort of obese patients. Other ongoing work stemming from the current study is an evaluation of the expression of metabolic miRNA gene targets, which will shed further light on the interaction between miRNA and mRNA involved in obesity and metabolic diseases.

Conclusion

The results from this pilot study provide evidence to support miRNAs as potentially important players in the regulation of complex metabolic pathways. Additionally, the dysregulation of miRNAs in omental fat and in the circulation of obese individuals highlights the potential for manipulating miRNAs as a novel therapeutic strategy for the management of obesity and the metabolic syndrome. Further dedicated, focused research in this field is imperative to ascertain the full potential of miRNAs as novel metabolic biomarkers and therapeutic agents against obesity.

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Review Article

MicroRNAs as Novel Biomarkers for Breast Cancer

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Breast cancer is a complex phenotypically diverse genetic disease, involving a variety of changes in gene expression and structure. Recent advances in molecular profiling technology have made great progress in unravelling the molecular taxonomy of breast cancer, which has shed new light on the aetiology of the disease and also heralded great potential for the development of novel biomarkers and therapeutic targets. Mi(cro)RNAs are a contemporary class of small noncoding endogenous RNA molecules, generating great excitement in the clinical and scientific communities. The recent discovery that miRNA expression is frequently dysregulated in cancer has uncovered an entirely new repertoire of molecular factors upstream of gene expression, which warrants extensive investigation to further elucidate their precise role in malignancy. We present a comprehensive and timely review of the role of miRNAs in cancer: addressing miRNA function, their putative role as oncogenes or tumor suppressors, with a particular emphasis on breast cancer throughout. We discuss the recent discovery of quantifiable circulating cancer-associated miRNAs, which heralds immense potential for their use as novel minimally invasive biomarkers for breast and other cancers. Finally, we comment on the potential role of miRNAs in breast cancer management, particularly in improving current prognostic tools and achieving the goal of individualized cancer treatment.

1. Introduction

The molecular biology of malignancy is diverse, complex, and remains poorly understood. The incidence of malignancies such as breast cancer is increasing consistently, and breast cancer has now become the commonest form of female malignancy among women in almost all of Europe and North America. Each year more than 1.3 million women will be diagnosed with breast cancer worldwide and approximately 4 652 000 will die from the disease [1] despite the fact that breast cancer is highly curable if diagnosed and treated appropriately at an early stage. In Ireland alone, the annual incidence is currently over 2300 and rising [2]. The value of current histological prognostic indicators in predicting the course of the disease is weak and many of the molecular mechanisms underlying breast cancer progression remain poorly understood. This deficit has led to significant interest in the quest for novel predictive markers for breast cancer.

Mi(cro)RNAs are a contemporary class of tiny noncoding endogenous RNA molecules, only 18–25 nucleotides long.

Since their discovery in 1993, these small molecules have been shown to play critical regulatory roles in a wide range of biological and pathological processes. Elucidating their mechanisms of action is still in its infancy. Nonetheless, work in this area to date has demonstrated that miRNAs may regulate cellular gene expression at the transcriptional or posttranscriptional level; by suppressing translation of protein coding genes, or cleaving target mRNAs to induce their degradation, through imperfect pairing with target mRNAs of protein coding genes [3]. MiRNA biogenesis in the human cell is a multistep complex process. A simplified representation is shown in Figure 1 [4]. The specific region of miRNA importance for mRNA target recognition is located at the 5′ end of the mature miRNA sequence, from bases 2 to 8. This is often referred to as the "seed sequence" [5]. Computational target prediction algorithms have been developed to identify putative mRNA targets, and these place considerable importance on this seed sequence, using it to search for complementary sequences in the 3′-UTRs of known genes that exhibit conservation across species. These

algorithms predict that each miRNA may potentially bind to as many as 200 targets and estimate that miRNAs control the expression of at least one third of human mRNAs, further highlighting their crucial role as regulators of gene expression [6].

At the time of writing, 8 273 mature miRNA sequences have been described in primates, rodents, birds, fish, worms, flies, plants, and viruses [7]. This represents a growth of over 200 microRNAs in the last 2 years. In the human genome, over 600 mature miRNAs have been reported to date; however, computational prediction estimates that this could increase to >1000 [8]. It is obvious that the microRNA story is just beginning.

2. Experimental Techniques for miRNA Analysis

The explosion of interest in miRNAs over the past two years necessitates effective tools for detecting their presence, quantification, and functional analysis. High-throughput profiling techniques such as miRNA microarrays and bead-based miRNA profiling have facilitated miRNA expression profiling, that is, far superior to existing low through-put techniques such as Northern blotting and cloning, and is essential for validation of microarray data. Castoldi et al. [9] described a novel miRNA microarray platform using locked nucleic acid-modified capture probes. Locked nucleic acid modification improved probe thermostability and increased specificity, thus enabling miRNAs with single nucleotide differences to be discriminated—an important consideration as sequence-related family members may be involved in different physiologic functions [10]. An alternative high-throughput miRNA profiling technique is the bead-based flow cytometric approach developed by Lu et al. [11]; a method which offers high specificity for closely related miR-NAs because hybridization occurs in solution. Quantitative real-time PCR methodologies have been widely applied to miRNA research. To date, the most successful approach in terms of specificity and sensitivity is a two-step approach using looped miRNA-specific reverse transcription primers and TaqMan probes from Applied Biosystems [12].

To complement these miRNA profiling assays and to address functional questions necessitated the development of methods to manipulate miRNA expression. 2-*O*-Methyl anti-sense single-strand oligonucleotides and locked nucleic acid-modified oligonucleotides have been developed as miRNA inhibitors, making the suppression of endogenous miRNA activity and its downstream effect on mRNA expression achievable both in vitro and in vivo [13–16]. The effects of target miRNA knockdown on cell morphology and function can be determined using standard assays for processes such as cell proliferation, migration, invasion, and angiogenesis. MiRNA inhibition can be studied in animal models via transfection with tumor cells treated with miRNA inhibitors [17] or by the intravenous injection of "antagomirs" (2-*O*-methyl-modified nucleotides with a cholesterol moiety at the 3′-end [18]. The most recent development in the field of miRNA inhibition, led by Naldini and colleagues, describes techniques to manipulate miRNA expression in vivo by expressing decoy miRNA targets via lentiviral vectors [19]. This new approach to examine loss-of-function in vivo complements the results obtained by classic knockout technology as described above. It allows inhibition of specific miRNAs by building in multiple different decoys in the same miRNA inhibitor. This exciting new development should lead to answers for interesting functional questions with clinical or therapeutic relevance. For example, one could now potentially knock down the oncogenic proprieties of the miR-17-92-1 cluster which is well documented to be involved in human cancer [20]. This technique could also help one examine the let-7 microRNA family—a large, well-known tumor suppressor miRNA family [21] thereby providing insights into the functional consequence of knocking down all let-7 miRNAs [22].

MiRNA mimicry, a complementary technique to the aforementioned miRNA inhibition, has recently been used in vitro to identify the cellular processes and phenotypic changes associated with specific miRNAs transfected into cell lines [23]. Functional assays (e.g., proliferation, migration, invasion, and angiogenesis) then allow us to determine the effect of miRNA upregulation on tumorigenic or nontumorigenic cell populations. These revolutionary technologies will undoubtedly help us shed light on the functional roles of miRNAs and hold immense potential for application to the clinical arena as novel therapeutic targets.

2.1. MiRNA and Human Cancer. Early experimental work into the regulatory role of miRNAs uncovered their important role in various cellular processes such as differentiation, cell growth, and cell death. These processes are commonly dysregulated in cancer, implicating miRNAs in carcinogenesis. The first evidence of involvement of miRNAs in malignancy came from the identification of a translocation-induced deletion at chromosome 13q14.3 in B-cell chronic lymphocytic leukemia [24]. Loss of miR-15a and miR-16-1 from this locus results in increased expression of the anti-apoptotic gene BCL2. Intensifying research in this field, using a range of techniques including miRNA cloning, quantitative PCR, microarrays and bead-based flow cytometric miRNA expression profiling has resulted in the identification and confirmation of abnormal miRNA expression in a number of human malignancies including breast cancer (Table 1). MiRNA expression has been observed to be upregulated or downregulated in tumours compared with normal tissue, supporting their dual role in carcinogenesis as either "Oncomirs" or tumour suppressors respectively [11].

The ability to obtain miRNA expression profiles from human tumors has led to remarkable insight and knowledge regarding the developmental lineage and differentiation states of tumours. Even within a single developmental lineage it has been shown that distinct patterns of miRNA expression are observed, that reflect mechanisms of transformation, and further support the idea that miRNA expression patterns encode the developmental history of human cancers. In contrast to messenger RNA (mRNA) profiles it is possible also to successfully classify poorly differentiated tumours using these new miRNA expression profiles [24, 25]. This has exciting implications clinically, in that miRNA expression may accurately diagnose poorly differentiated tissue samples
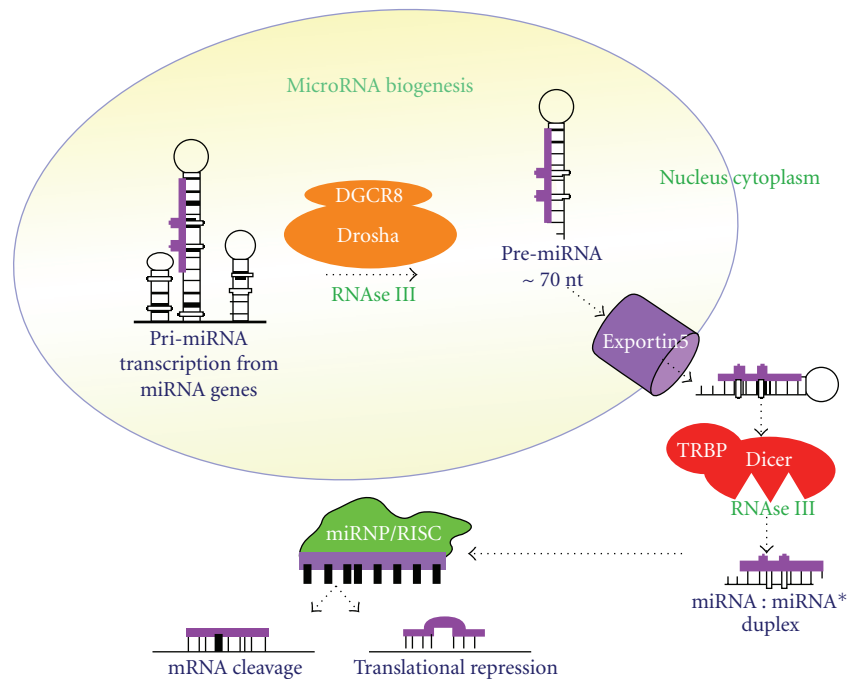
FIGURE 1: MiRNA biogenesis and processing in human cells: the multistep process begins in the nucleus where the RNase III enzyme Drosha, coupled with its binding partner DGCR8, cleaves nascent miRNA transcripts (pri-miRNA) into ~70 nucleotide precursors (pre-miRNA). These pre-miRNAs consist of an imperfect stem-loop structure. Pre-miRNAs are then exported from the nucleus into the cytoplasm by Exportin 5. In the cytoplasm, the hairpin precursors are cleaved by Dicer and its binding partner the transactivator RNA-binding protein TRBP into a small, imperfect dsRNA duplex (miRNA : miRNA*) that contains both the mature miRNA strand and its complementary strand. The miRNA strand is incorporated into the miRNP complex and targets complementary mRNA sequences, exerting its functionality via mRNA cleavage or translational repression.

which proved to be of uncertain histological origin thus facilitating treatment planning. Again in contrast to mRNA, Lu et al. showed that even a modest number of miRNAs are sufficient to classify human tumours and miRNAs remain largely intact in routinely collected, formalin-fixed, and paraffin-embedded clinical tissues [11]. Such information would eliminate the diagnostic uncertainty that previously existed in this setting and will be particularly useful for metastatic lesions of uncertain primary origin.

2.2. Breast Cancer and Genomic Signatures. Recent advances in phenotyping and molecular profiling of human cancers have greatly enhanced the diagnosis and biological classification of several tumors, in particular breast cancers where this technology has enhanced disease classification beyond single-gene markers. Prior to this a very limited armamentarium of prognostic markers beyond those offered by histopathological analysis was available in the clinical arena. Pioneering work by Sorlie et al. [33–35] identified microarray-generated gene expression signatures which stratified breast cancers into intrinsic subtypes largely based on their ER, progesterone (PR), and HER2/*neu* receptor status. Subtypes were designated *Luminal A*, which strongly expressed ER and/or PR, but were HER2/*neu* negative; *Luminal B*, which were ER, PR, and HER2/*neu* (triple) positive; *Basal* tumours which were ER, PR, and HER2/*neu* triple negative; and an *HER2* subset which was ER negative ER but

had high expression of several genes in the HER2/*neu* amplicon, including *HER2* and *GRB7*. Survival analyses showed significantly different outcome for patients depending on their tumour subtype, emphasising the clinical relevance of stratification by such molecular profiling. This novel method of disease stratification based on the molecular taxonomy of the breast tumour heralds the promise of improving and individualizing patients' treatment regimens [36]. Great scientific endeavours in this field of microarray-based gene expression profiling are ongoing and intensifying, with the aim of translating such technical advances to the clinical arena, in providing us with a new tool for accurate molecular diagnosis of breast cancer [37]. One such application recently has been the development by Paik et al. of a multigene assay predictive of recurrence of tamoxifen-treated, node-negative breast cancer (*Oncotype DX*) [38]. This, and other similarly novel genomic tests (e.g., *MammaPrint, Theros, MapQuant Dx*) prove the feasibility of accelerating the transition between empirical and molecular medicine. Analogous to the derivation of intrinsic subtypes of breast cancer from gene expression signatures, it is predicted that in the very near future miRNA signatures, which are currently showing capability of accurately classifying tumours according to currently available prognostic variables, will serve as novel biomarkers and prognostic indicators thus providing strong rationale for individualised treatment. Additionally it is thought that miRNAs have the potential to improve greatly

TABLE 1: MiRNAs with altered expression in malignancy.

Tissue/tumor type	Increased expression	Decreased expression
Breast [26, 27]	miR-21, miR-29b-2	miR-125b, miR-145 miR-10b, miR-155, miR-17-5p, miR-27b
Ovarian [28, 29]	miR-141, miR-200(a-c), miR-221	let-7f, miR-140, miR-145, miR199a, miR-424
Endometrial [30–32]	miR-103, miR-107, miR-185, miR-205, miR-210, miR-449	miR-99b, miR-152, miR-193, miR-204, miR-221, let-7i
Glioblastoma [4, 25]	miR-221, miR-21	miR-181a, miR-181b, miR-181c
Chronic lymphocytic leukaemia [24]		miR-15, miR-16
Lymphoma [4, 11]	miR-155, miR-17-92cluster	miR-15a
Colorectal [4, 11, 25]	miR-10a, miR-17-92 cluster, miR-20a, miR-24-1, miR-29b-2, miR-31	miR-143, miR-145, let-7
Thyroid [4, 25]	miR-221, miR-222, miR-146, miR-181b, miR-197, miR-346	
Hepatocellular [4, 25]	miR-18, miR-224	miR-199a, miR-195, miR-200a, miR-125a
Testicular [11]	miR-372, miR-373	
Pancreatic [4, 11, 25]	miR-221, miR-376a, miR301, miR-21, miR-24-2, miR-100, miR-103-1,2, miR-107, miR-125b-1	miR-375
Cholangiocarcinoma [25]	miR-21, miR-141, miR-200b	
Prostate [11]	let-7d, miR-195, miR-203	miR-128a
Gastric [4, 11, 25]	miR-223, miR-21, miR-103-2	miR-218-2
Lung [4, 11, 25]	mir-17-92 cluster, miR-17-5p	let-7 family

the precision of the recently derived genomic signatures, given that miRNA profiles have superior accuracy to mRNA profiling in this regard [11]. A comprehensive interrogation of the breast cancer subclasses via miRNA expression profiling could further characterize the molecular basis underlying these subtypes, perhaps define more precise subsets of breast cancer, and provide opportunities for the identification of novel targets that can be exploited for targeted therapy.

2.3. *MiRNA and Breast Cancer.* Elucidation of the molecular mechanisms involved in breast cancer has been the subject of extensive research in recent years, yet several dilemmas and major challenges still prevail in the management of breast cancer patients including unpredictable response and development of resistance to adjuvant therapies. The emergence of miRNAs as regulators of gene expression identifies them as obvious novel candidate diagnostic and prognostic indicators, and potential therapeutic targets. Calin et al. [24] showed that half of the known mature human miRNAs are located in cancer-associated genomic regions, or fragile sites, thus potentiating their role in cancer. A specific example of this is the polycistron cluster miR-17-92 at the c13orf25 locus on chromosome 13q31. This locus is known to undergo loss of heterozygosity in a number of different cancer types, including breast cancer [39]. A number of other miRNAs (miR-196 and miR-10a) are located in homeobox clusters, which are known to be involved in the development of breast

cancer and associated with the malignant capacity of cancer cells [40].

MiRNA expression studies in breast cancer indicate their importance and potential use as disease classifiers and prognostic tools in this field. In their analysis of 76 breast tumour and 34 normal specimens, Iorio et al. [26] identified 29 miRNAs that were differentially expressed in breast cancer tissue compared to normal, and a further set of 15 miRNAs that could correctly discriminate between tumour and normal. In addition, miRNA expression correlated with biopathological features such as ER and PR expression (*miR-30*) and tumour stage (*miR-213* and *miR-203*). The differential expression of several *let-7* isoforms was associated with biopathologic features including PR status (*let-7c*), lymph node metastasis (*let-7f-1, let-7a-3, let-7a-2*), or high proliferation index (*let-7c, let-7d*) in tumour samples. Mattie et al. identified unique sets of miRNAs associated with breast cancers currently defined by their HER2/*neu* or ER/PR status [27]. Significantly, there was overlap between the miRNAs identified in both studies. In initial studies in our own Department, we have shown that the expression levels of miR-195 and mir-154 are negatively correlated with ER positivity in a cohort of early breast cancers [41]. In another recent publication we were the first to identify reliable endogenous controls for analysis of miRNA by RQ-PCR in human breast tissue [42], subsequent to our validation of a two-gene normaliser (MRPL19 and

PPIA) for analysis of gene expression in primary breast tissue [43].

3. Circulating microRNAs: Novel Minimally Invasive Biomarkers for Breast Cancer?

Current challenges in the management of breast cancer include a continuing search for sensitive minimally invasive markers that can be exploited to detect early neoplastic changes thus facilitating the detection of breast cancer at an early stage, as well as for monitoring the progress of patients with breast cancer and their response to treatments. Existing biomarkers for breast cancer have many inherent deficiencies. Mammography is currently the gold standard diagnostic tool however it is not without limitations, including its use of ionizing radiation and a false positive rate of 8–10% [44]. To date, only two markers have been established so far in the routine assessment of breast cancer: ER (for predicting response to endocrine therapies) and HER2 (for predicting response to Trastuzumab) [45]. Although these markers are currently available, ER and HER2 assessment is far from perfect [46]. A number of circulating tumour markers (e.g., carcinoembryonic antigen [CEA] and carbohydrate antigen 15-3 [CA 15-3]) are used clinically in the management of breast cancer, but the sensitivity of these markers is low, so that they are not useful as screening tools [47] though they have long been in clinical use as prognostic markers and to monitor for disease progression or recurrence. Despite their frequent use, CEA and Ca 15.3 remain poor markers for early stage disease with a documented preoperative sensitivity of only 9.11 and 5.36, respectively, as documented by Uehara et al. [48, 49].

The ideal biomarker should be easily accessible such that it can be sampled relatively noninvasively, sensitive enough to detect early presence of tumours in almost all patients and absent or minimal in healthy tumour-free individuals.

There is also great need for the identification of sensitive, reliable and acceptable markers of response to neoadjuvant and adjuvant therapies. MiRNAs have enormous potential to serve as an idea class of cancer biomarkers for the following reasons.

(1) MiRNA expression is known to be aberrant in cancer [11, 24].

(2) MiRNA expression profiles are pathognomonic, or tissue-specific [11].

(3) MiRNAs are remarkably stable molecules that have been shown to be well preserved in formalin fixed, paraffin embedded tissues as well as fresh snap frozen specimens [50, 51].

Acknowledging the exceptional stability of miRNAs in visceral tissue very recently instigated efforts to establish if miRNAs were also preserved, detectable, and quantifiable in the circulation and other bodily fluids (urine, saliva, etc.). This area of miRNA research is only now emerging, and is generating much excitement in clinical and scientific communities, such as its potential. MiRNA presence in serum was described for the first time in March 2008, in patients with diffuse large B-cell lymphoma [52]. Subsequent to this, a small number of studies have reported similarly, on the presence of miRNA in circulation and their potential for use as novel biomarkers for diseases and physiological states including malignancy, diabetes mellitus and pregnancy [53–55]. However these studies have been limited by small numbers and inconsistencies in methodologies [56]. This concept needs extensive investigation to validate the theory. To date no work has been published on the role of circulating miRNAs in breast cancer—an area where, if feasible, their use as novel minimally invasive biomarkers would be an incredible breakthrough in our management of this disease.

4. Therapeutic Potential

The association of aberrant miRNA expression with tumorigenesis and the functional analysis of specific miRNAs illustrate the feasibility of using miRNAs as targets of therapeutic intervention. Anti-miRNA 2-*O*-methyl or locked nucleic acid oligonucleotides used to inactivate oncomirs such as miR-21 in breast tumors may taper tumor growth [17]. Anti-miR-21-induced reduction in tumor growth, interestingly, was also shown by Si et al. to be potentiated by the addition of the chemotherapeutic agent topotecan, an inhibitor of DNA topoisomerase I. This suggests that suppression of the oncogenic miR-21 could sensitize tumor cells to anticancer therapy, which is an exciting prospect for patients exhibiting a poor response to primary chemotherapy. Conversely, the induction of tumor suppressor miRNA expression using viral or liposomal delivery of tissue-specific tumor suppressors to affected tissue may result in the prevention of progression, or even shrinking, of breast tumors. Tumor suppressor miRNA induction has also been shown to be subject to epigenetic control. Using chromatin remodelling drugs to simultaneously inhibit DNA methylation and histone deacetylation, epigenetic alterations in cancer and normal cells were manipulated by Saito et al. [57], who showed that certain miRNAs were upregulated in tumor cells but not in normal cells. MiR-127, which exhibited reduced expression in 75% of human cancer cells tested, was significantly upregulated after treatment. The induction of this miRNA was associated with downregulation of the proto-oncogene BCL6, suggesting a cancer-protective effect for miR-127 and a novel therapeutic strategy for the prevention and treatment of malignancy. This concept of inducing tumour suppressor miRNA expression has been termed "miRNA Replacement Therapy"; in anticipation of the promising clinical potential it holds.

5. Conclusion

The involvement of miRNAs in the initiation and progression of human malignancy holds great potential for new developments in current diagnostic and therapeutic strategies in the management of patients with breast cancer. Much of the work on microRNAs is still in its infancy and requires further exploration so that we may better understand their role in tumorigenesis. This scientific endeavour

will undoubtedly lead to exciting developments in the future management of breast cancer. As the functional roles of miRNAs in cancer biology are further uncovered we predict that; circulating miRNAs will serve as novel minimally invasive biomarkers for breast and other cancers, that improved methods of stratifying and subclassifying breast cancers will lead to tailored and individualized therapeutic regimens, thus sparing many patients from toxic effects of treatments from which they would derive no benefit. There is obviously great demand now for further intensive research into the identification of novel miRNAs, the elucidation of their mRNA targets, and an understanding of their functional effects, so as to improve our knowledge of the roles of these novel biomarkers in carcinogenesis and to expose their true potential as therapeutic agents.

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ELSEVIER

MiRNAs as biomarkers and therapeutic targets in cancer ☆

Helen M Heneghan, Nicola Miller and Michael J Kerin

The knowledge that miRNA expression is frequently dysregulated in cancer has uncovered an entirely new repertoire of molecular factors upstream of gene expression, with exciting potential as novel biomarkers and therapeutic targets in cancer. Exploiting the unique characteristics of these molecules including their stability, tissue specificity, ease of detection and manipulation, will bring clinicians ever closer to achieving the goal of individualized cancer treatment. We present a comprehensive and timely review of the role of miRNAs in cancer. Herein we address briefly miRNA biogenesis, the putative role of miRNAs as oncogenes or tumor suppressors, and their potential as sensitive and specific tumor markers with particular emphasis on the commonest cancers; breast, prostate, lung and colorectal. We also discuss circulating tumor-associated miRNAs which are emerging as clinically useful tools for early detection, prognostication and management of various cancers. Finally we explore their potential therapeutic applications in the field of cancer and highlight some of the potential challenges that need to be overcome in order to bring miRNAs from bench to bedside. Given the evidence to date, we envisage a pivotal role for miRNAs in the future individualized management of cancer patients.

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Introduction

With over 12 million new cases per annum worldwide, and a predicted 27 million annual diagnoses expected by the year 2050, cancer presents an epic health problem [1]. Although laudable progress has been made in unraveling and understanding cancer biology over the last decade, similar advances have not occurred in terms of early diagnosis, screening tests, or more targeted and less toxic cancer therapies. Sensitive and specific biomarkers are critical tools for early detection and monitoring of cancer, in addition to representing potential therapeutic targets. The identification of novel molecular tumor markers remains a high priority in order to reduce cancer-associated morbidity and mortality, and provide clinicians with new strategies for targeted therapy of individual malignancies. Mi(cro)RNAs have recently emerged as an exciting new class of disease biomarker with further potential as the next generation of cancer therapeutics. Since their discovery in 1993, these small, endogenous, non-coding RNAs have been shown to play important regulatory roles in governing gene expression and cellular processes, whilst aberrant expression of miRNAs has been observed in a diversity of pathological events. Importantly, they have been critically implicated in the pathogenesis of most human cancers. Although elucidating their mechanisms of action is still in its infancy, the discovery of miRNAs has uncovered an entirely new and exciting repertoire of molecular factors upstream of gene expression, with great potential for new developments in current diagnostic and therapeutic strategies in the management of cancer patients.

MiRNA biogenesis

MiRNA biogenesis in the human cell is a multistep complex process which begins in the nucleus where miRNA genes are transcribed by RNA polymerase II to form large capped and polyadenylated primary miRNA transcripts (pri-miRNAs). These nascent pri-miRNAs are cleaved by the RNase III enzyme Drosha, coupled with its binding partner DGCR8, into 70–90 nucleotide precursors (pre-miRNA) which consist of an imperfect stem-loop hairpin structure. Pre-miRNAs are then exported from the nucleus into the cytoplasm by Exportin 5. In the cytoplasm, the hairpin precursors are cleaved by Dicer and its binding partner, the transactivator RNA-binding protein TRBP, into a small dsRNA duplex (miRNA:-miRNA*) that contains both the mature miRNA strand and its complementary strand. This mature miRNA strand is preferentially incorporated into a miRNA-associated RNA-induced silencing complex (miRISC), and guides RISC to target mRNAs containing complementary sequences to the mature miRNA. A simplified representation is shown in Figure 1 [2].
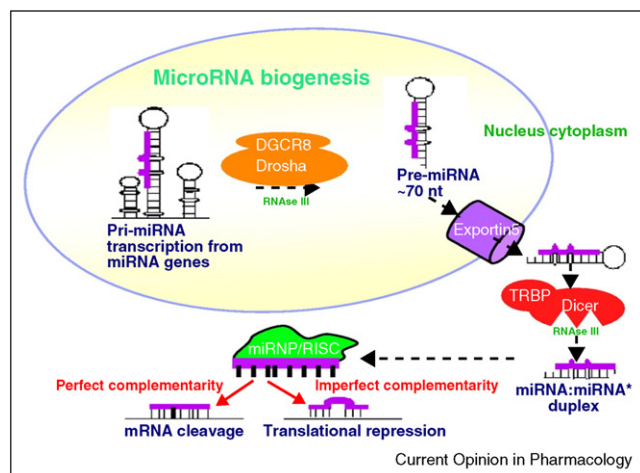
MiRNA function

MiRNAs exert their functionality via sequence-specific regulation of post-transcriptional gene expression and it is estimated that they regulate up to 30% of all

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MicroRNA biogenesis in the cell. The multistep process begins in the nucleus where the RNase III enzyme Drosha, coupled with its binding partner DGCR8, cleaves nascent miRNA transcripts (pri-miRNA) into ~70 nucleotide precursors (pre-miRNA). These pre-miRNAs consist of an imperfect stem-loop structure. Pre-miRNAs are then exported from the nucleus into the cytoplasm by Exportin 5. In the cytoplasm, the hairpin precursors are cleaved by Dicer and its binding partner the transactivator RNA-binding protein TRBP into a small, imperfect dsRNA duplex (miRNA:miRNA*) that contains both the mature miRNA strand and its complementary strand. The miRNA strand is incorporated into the miRNP complex and targets complementary mRNA sequences, exerting its functionality via mRNA cleavage or translational repression.

protein-coding genes [3]. The specific region important for mRNA target recognition is located in the 5′-end of the mature miRNA strand, from bases 2 to 8, often referred to as the 'seed-sequence' [4••]. Governance of gene expression and protein translation by these non-coding RNA molecules occurs largely through one of two mechanisms, dependent upon the complementarity of the miRNA seed sequence with its target mRNA. Firstly, binding of miRNA to protein-coding mRNA sequences with perfect base-pairing homology induces the RNA-mediated interference (RNAi) pathway leading to cleavage of mRNA by Argonaute in the RISC. The alternative and more common mechanism by which miRNAs regulate their target genes, is through imperfect binding to partially complementary sequences in the 3′ untranslated region (UTR) of downstream target coding mRNAs which leads to repression of protein translation [5]. Thus miRNAs can reduce protein levels of their target genes without significantly altering the mRNA levels, consistent with translational control.

MiRNAs and cancer
Early *in vitro* studies investigating the function of miRNAs uncovered their critical role in regulating various cellular processes such as differentiation, cell growth, proliferation and apoptosis. Similarly, it was repeatedly observed that dysregulated miRNA expression underpinned a variety of pathological events, including carcinogenesis. Intensifying research in this field, using a range of techniques including miRNA cloning, quantitative PCR, microarrays and bead-based flow cytometric miRNA expression profiling, has resulted in the identification and confirmation of abnormal miRNA expression in most human malignancies (Table 1). In fact more than half of these cancer-associated miRNAs are located in cancer 'hotspot' chromosomal regions, including fragile sites, regions of loss of heterozygosity, amplification or common breakpoint regions [6]. The first evidence of involvement of miRNAs in malignancy came from the identification of a translocation-induced deletion at chromosome 13q14.3 in B-cell chronic lymphocytic leukemia [7••]. Loss of *miR-15a* and *miR-16-1* from this locus results in increased expression of the anti-apoptotic gene BCL2. MiRNA expression in tumors has subsequently been observed to be up- or down-regulated compared with normal tissue, supporting their complex dual role as either 'oncomirs' or tumor suppressors, respectively [8••]. MiRNA expression profiling of a variety of human tumors has given remarkable insight into the developmental lineage and differentiation states of tumors. Even within a single developmental lineage it has been shown that distinct patterns of miRNA expression are observed that reflect mechanisms of transformation, and further support the idea that miRNA expression patterns encode the developmental history of human cancers. In contrast to mRNA profiles it is also possible to successfully classify poorly differentiated tumors using miRNA expression patterns [9]. Furthermore, miRNAs remain largely intact in routinely collected, formalin-fixed, paraffin-embedded clinical tissues [8••]. This has exciting implications clinically, in that miRNA expression may accurately diagnose poorly differentiated tumors which proved to be of uncertain histological origin thus facilitating treatment planning.

MiRNAs as biomarkers for cancer
The emergence of miRNAs as modulators of gene expression identifies them as obvious novel candidate diagnostic and prognostic indicators, and potential therapeutic targets. In addition to their tissue specificity, miRNAs hold other unique characteristics that herald them as ideal tumor markers including their stability, ease of detection and association with established clinicopathological prognostic parameters. Acknowledgment of the exceptional stability of miRNAs in visceral tissue, instigated efforts to establish if these tiny molecules were also preserved, detectable, and quantifiable in the circulation and other bodily fluids (e.g. urine, saliva, and sputum). The last two years have seen an accumulating body of evidence to support circulating miRNAs as non-invasive, sensitive biomarkers of disease states, particularly cancers (breast, lung, pancreas, ovarian, and prostate). This novel approach has immense potential to advance cancer

Table 1

MiRNAs with altered expression in malignancy

Tissue/tumor type	Increased expression	Decreased expression
Breast [10•,11]	miR-21, miR-29b-2	miR-125b, miR-145 miR-10b, miR-155, miR-17-5p, miR-27b
Ovarian [41]	miR-141, miR-200(a-c), miR-221	let-7f, miR-140, miR-145, miR199a, miR-424
Endometrial [41]	miR-103, miR-107, miR-185, miR-205, miR-210, miR-449	miR-99b, miR-152, miR-193, miR-204, miR-221, let-7i
Glioblastoma [2,9]	miR-221, miR-21	miR-181a, miR-181b, miR-181c
Chronic lymphocytic leukemia [7••]		miR-15, miR-16
Lymphoma [2,8••]	miR-155, miR-17-92cluster	miR-15a
Colorectal [2,8••,9]	miR-10a, miR-17-92 cluster, miR-20a, miR-24-1, miR-29b-2, miR-31	miR-143, miR-145, let-7
Thyroid [2,9]	miR-221, miR-222, miR-146, miR-181b, miR-197, miR-346	
Hepatocellular [2,9]	miR-18, miR-224	miR-199a, miR-195, miR-200a, miR-125a
Testicular [8••]	miR-372, miR-373	
Pancreatic [2,8••,9]	miR-221, miR-376a, miR301, miR-21, miR-24-2, miR-100, miR-103-1,2, miR-107, miR-125b-1	miR-375
Cholangiocarcinoma [9]	miR-21, miR-141, miR-200b	
Prostate [8••,41]	let-7d, miR-195, miR-203	miR-128a
Gastric [2,8••,9]	miR-223, miR-21, miR-103-2	miR-218-2
Lung [2,8••,9]	mir-17-92 cluster, miR-17-5p	let-7 family

diagnosis and stratification beyond currently available methods, particularly for those malignancies which still routinely present in advanced stages and for which there is no reliable tumor marker available at present.

MiRNA expression studies in tumor tissues and in the circulation have identified the following markers as key players in the respective cancers.

Breast cancer

The seminal report of aberrant miRNA expression in breast cancer by Iorio *et al.* in 2005 identified 29 miRNAs that were differentially expressed in breast cancer tissue compared to normal, a subset of which could correctly discriminate between tumor and normal with 100% accuracy [10•]. Among the leading miRNAs differentially expressed; *miR 10b, miR-125b* and *mR-145* were down-regulated whilst *miR-21* and *miR-155* were consistently over-expressed in breast tumor tissues. In addition, miRNA expression correlated with biopathological features such as ER and PR expression (*miR-30*) and tumor stage (*miR-213* and *miR-203*). Mattie *et al.* subsequently identified unique sets of miRNAs associated with breast tumors defined by their HER2/*neu* or ER/PR status [11]. Significantly, there was overlap between the miRNAs identified in both studies. Our group has described 3 miRNA signatures predictive of ER, PR and Her2/*neu* receptor status, respectively, which were identified by applying artificial neural network analysis to miRNA microarray expression data [12]. We have also identified a systemic miRNA profile diagnostic of breast cancer, based largely on circulating *miR-195* levels [13•]. Our results thus far highlight *miR-195* as a potentially ideal breast tumor marker; circulating levels reflect tumor miR-

195 levels, correlate with tumor size and stage of disease, decrease to basal level two weeks post-curative tumor resection, and *miR-195* is not elevated in blood from patients with other malignancies (prostate, colon, renal, melanoma — Heneghan H.M. et al., Manuscript under review). MiRNAs with prognostic value for breast cancer include *miR-10b, miR-21, miR-145, miR-9-3* and *let-7*; levels of these miRNAs correlate with tumor grade, degree of vascular invasion, lymph node metastases, or metastatic potential [14]. Analogous to the derivation of intrinsic subtypes from gene expression profiles, and the estimation of risk of disease recurrence from multi-gene assays such as *Oncotype DX*, it is predicted that tumor or circulating miRNA signatures could serve as novel bio-markers and prognostic indicators, and will provide strong rationale for individualized treatment for breast cancer.

Lung cancer

A number of miRNAs are known to be intimately involved in lung cancer initiation, progression and prognosis. Lung is one of the tissues with the most abundant expression of the let-7 family of miRNAs in its normal non-cancerous state [15]. Tumors have repeatedly been shown to under-express most of the transcripts of the let-7 family; consistent with its known tumor suppressor role. Let-7 regulates several oncogenic pathways, including the RAS pathway where it represses activity of the *KRAS* oncogene, mutations of which are commonly implicated in adenocarcinoma of the lung. Decreased let-7 levels have also been shown to correlate with prognosis of lung cancer patients, independent of disease stage [16]. Yanai-hara *et al.* have identified a 12 miRNA signature, diagnostic of non-small cell lung cancers (*miR-17-3p, miR-21, miR-106a, miR-146, miR-155, miR-191, miR-192, miR-203,*

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miR-205, miR-210, miR-212, and miR-214) [17]. Rabino-wits et al. proceeded to evaluate these 12 miRNAs in the circulation of 37 individuals, including 27 patients with lung adenocarcinoma and 9 healthy controls [18]. In doing so they elegantly illustrate that circulating exosomal miRNA signatures mirror those of the primary lung tumor, discriminate cancer cases from controls accurately and thus propose this type of analysis as a potential screening tool for the early detection of lung cancer.

Colorectal cancer

The role of miRNAs as biomarkers for colorectal cancer (CRC) is equally as promising. Schetter et al. have ident-ified miRNAs which can distinguish cancerous from normal colon tissue; in particular miR-21 was observed to be over-expressed in 87% of colon cancers [19•]. Subsequent mechanistic investigations provide evidence for miR-21's oncogenic role in CRC by demonstrating how it suppresses the cell cycle regulator CDC25A [20], and can also target and repress the tumor suppressor gene PDCD4 (programmed cell death 4) thus inducing inva-sion, intravasation and metastatic potential [21]. MiR-21 expression in colorectal tumors has also been shown to have prognostic value; higher miR-21 expression is associ-ated with advanced stage, invasion and poorer prognosis [19•]. Over-expression of the oncogenic miR-17-92 cluster is also implicated in the etiology of CRC, specifically in adenoma to adenocarcinoma progression. Ng et al. inves-tigated the potential of plasma miRNAs as biomarkers of early colorectal cancers. Using an unbiased array-based approach the authors identified 2 of the 6 miRNAs in the miR-17-92 cluster (miR-17-3p and miR-92) to be signifi-cantly elevated in the circulation of CRC patients, with reasonable ROC curve areas of 0.717 and 0.885, respect-ively. Furthermore these 2 markers could discriminate CRC from inflammatory bowel disease, gastric cancers and normal individuals in an independent validation cohort [22]. MiRNAs with tumor suppressor properties which are under-expressed in CRC specimens, and thus potentially function as tumor suppressors, include miR-31, miR-34a, miR-96, miR-143, miR-145, and let-7a [19•,23]. MiR-34a is a well described tumor suppressor miRNA which regulates the p53 pathway and when over-expressed induces apoptosis and acute senescence. Con-versely reduction of miR-34 expression and function attenuates p53-mediated cell death and is therefore implicated in tumorigenesis, including initiation of CRC [24,25]. It is postulated therefore that loss of mir-34a expression in colorectal biopsy specimens may be an early biomarker of CRC.

Prostate cancer

The rationale for miRNAs as biomarkers for prostate cancer is less well defined than for other common cancers given that their investigation in prostate cancer speci-mens is still in its infancy. Nonetheless emerging data suggest the miRNA expression is clearly dysregulated in

prostate tumors and of the 6 profiling studies in this field to date, decreased expression of miR-23b, miR-34a, miR-100, miR-145 and miR-205 is consistently reported in cancerous tissue compared to normal prostate. In 5 of these 6 miRNA profiling studies, miR-221 and miR-222 are also aberrantly expressed in tumor tissues [26]. Mitch-ell et al. have demonstrated the presence of tumor-derived miRNAs in the circulation of prostate cancer patients. Serum levels of miR-141, a miRNA expressed in a variety of epithelial cancers including prostate, breast, lung, and colon, were shown to distinguish cancer patients from healthy males with high accuracy [27•]. No relation-ship was made between levels of this tumor-related miRNA and clinicopathological parameters for prostate cancer, and it remains to be established whether miR-141 is similarly upregulated in prostate tumors. The role of miR-141 in initiation and progression of the disease also merits investigation. Nevertheless the growing body of evidence in this field supports miRNAs as promising biomarkers for prostate cancer.

Therapeutic potential of miRNAs

The association of aberrant miRNA expression with cancer, and functional analyses of specific miRNAs which has established their roles as tumor suppressors or onco-genes, illustrates the feasibility of manipulating miRNA expression as a therapeutic strategy for cancer. Indeed the potential clinical applications of miRNA either as drugs or drug targets, have not escaped the attention of pharma-ceutical and biotechnology industries; a number of major companies have active programmes focused on develop-ing novel miRNA-based therapeutics for cancer and other disease processes [28]. There are two possible approaches for their use as cancer therapies, in acknowledgment of their dual role in carcinogenesis; firstly through antisense-mediated inhibition of oncogenic miRNAs, and secondly through 'replacement' of under-expressed tumor sup-pressor miRNAs with either miRNA mimetics or viral vector-encoded miRNAs.

'OncomiR' knockdown

Inhibition of miRNA activity using synthetic oligonucleo-tides has been demonstrated in vitro using 2′-O-methyl modification of oligoribonucleotides, which are comp-lementary to the mature target miRNA sequence. Repli-cation of this effect in vivo has also been demonstrated using injection of a cholesterol-conjugated 2′-O-methyl-modified oligoribonucleotide [29••]. Administration of synthetic 2′-O-methyl anti-miRNA oligonucleotides (AMOs) targeting the oncogenic miR-21 has been shown to potently inhibit breast cancer cell growth in vitro, and taper tumor growth in an MCF-7 breast cancer xenograft mouse model [30]. One of the first in vivo applications of miRNA antagonism utilized modified AMOs conjugated with cholesterol and delivered systemically to target miR-122; a liver-specific miRNA implicated in the replication of HCV and in the etiology of hepatocellular carcinoma.

Krutzfeldt *et al.* demonstrate that this approach to antagonizing miR-122 results in significant inhibition of HCV replication with just a single injection resulting in miR-122 silencing for 23 days [29••]. Technical challenges presented by use of modified AMOs included the potential for instability and toxicity. Advancements on this approach led to the development of locked-nucleic acid (LNA) modified oligonucleotides to antagonize endogenous miRNAs *in vivo*. These LNA-antimiRs were shown to exhibit far superior thermal stability when hybridized to their target RNA molecule, as well as high stability and low toxicity in biological systems [31,32]. Elmen *et al.* elegantly demonstrate that saline-formulated LNA-antimiR-mediated antagonism of miR-122 in mice results in dose-dependent reduced expression of liver miR-122, and a coincidental reduction of plasma cholesterol levels. These effects were specific for miR-122, reversible and without additional toxicity [33••]. Such is the clinical potential for this approach to cancer therapeutics that Santaris Pharmaceuticals have already brought a miR-122 antagonist (SPC3649) through a Phase I clinical trial, and are now planning to investigate its efficacy as a treatment for Hepatitis C infection in a Phase II study later in 2010 [34].

The oncogenic miR-17-92 cluster presents another ideal target for miRNA-based cancer therapeutics given its established association with a variety of malignancies [9]. However, simultaneous targeting of the 6 miRNAs in this polycistronic cluster presents its own challenges. In an effort to address this issue Ebert *et al.* pioneered the concept of 'miRNA sponges'. These competitive miRNA inhibitors are transcripts expressed from strong promoters, containing multiple and tandem binding sites to target microRNAs. When vectors encoding these sponges are transiently expressed in cultured cells, they provide a means to sequester miRNAs *in vivo* such that a single sponge can be used to block ('soak up') an entire microRNA seed family [35••]. This approach would be ideal to target clustered miRNAs with oncogenic function, such as the miR-17-92 cluster.

MiRNA replacement therapy
Conversely, several studies suggest that the reintroduction of specific miRNAs known to be down-regulated in cancer cells could have a therapeutic benefit by halting or even reversing tumor growth. Restoring a cell's endogenous complement of miRNAs can be achieved by introducing a short synthetic duplex RNA molecule that is loaded into RISC, or by inducing expression of the stem-loop pre-miRNA in a liposomal delivery or viral vector expression system. Perhaps the most convincing evidence for miRNA replacement as a strategy for cancer therapy lies in the potential shown by manipulating *let-7* levels in models of lung cancer. The Slack laboratory has previously studied various murine models of human lung cancer, and demonstrated how loss of the tumor suppres-

sor *let-7* induces lung tumor formation and growth, through loss of its regulatory effect on the oncogenes *HMGA2* and *RAS* amongst others. Restoration of *let-7* expression in lung cancers restrained the growth of tumors by repressing multiple cell cycle and proliferation pathways together with *ras* and *MYC* suppression [36••]. Intranasal administration of *let-7* in a K-*ras* mutant mouse model of lung cancer effectively reduced growth of the tumor. Despite these encouraging results, the application of let-7 directly as a therapeutic agent for cancer is premature as yet, given that details of the immunogenic and cytotoxic effect of let-7 remain to be explored. Its ubiquitous expression and involvement in multiple cellular pathways imply that manipulation of its levels, for the purpose of treating one specific diseased tissue, is likely to have diverse off-target effects. The development of safe, effective, and tissue-specific delivery methods for let-7 requires further endeavor before this miRNA progresses as a cancer therapy. Nonetheless, several RNA-based therapeutics and diagnostic companies (Asuragen and Regulus) are actively involved in developing miRNA-based diagnostics and therapeutics for non-small cell lung cancer and other malignancies, with let-7 as a core focus. *MiR-34a* is implicated in prostate cancer development as a tumor suppressor, and therapeutic strategies aimed at its replacement in prostate cancer cells are also being pursued by pharmaceutical companies. Preliminary *in vitro* studies illustrate its potential herein. Ectopic *miR-34a* expression results in cell cycle arrest and growth inhibition, as well as attenuated chemoresistance to camptothecin by inducing apoptosis in PC-3 cells. These effects are mediated through repression of the deacetylase sirtuin SIRT1 and cyclin-dependent kinase 6 (CDK6) [37].

MiRNA manipulation to enhance response to standard cancer therapies
In addition to manipulating miRNAs directly for therapeutic effect, an alternative approach lies in using miRNA modulation to enhance traditional or standard treatments for cancer. Weidhaas *et al.* provide evidence that overexpressing members of the *let-7* family of miRNAs *in vitro* in lung cancer cells, and *in vivo* in a *C. elegans* model of radiation-induced cell death, result in increased sensitivity to radiation therapy whereas decreasing let-7 levels induce a state of radioresistance [38••]. Analogous experiments in breast cancer cell lines demonstrate that increasing *miR-34* levels protect these cells from radiation-induced cell death whilst knockdown of *miR-34* using antagomirs radiosensitized the cells [39]. Chemosensitivity of breast cancer cells is also regulated by miRNAs; Miller *et al.* provide evidence that *miRs-221/222* and *miR-181b* are over-expressed, and a panel of 7 miRNAs (*miR-342*, *miR-489*, *miR-21*, *miR-24*, *miR-27*, *miR-23*, and *miR-200*) under-expressed in tamoxifen-resistant breast cancer cells (OHT^R cells) in comparison to tamoxifen-sensitive MCF-7 cells. Similar dysregulation in

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Figure 2
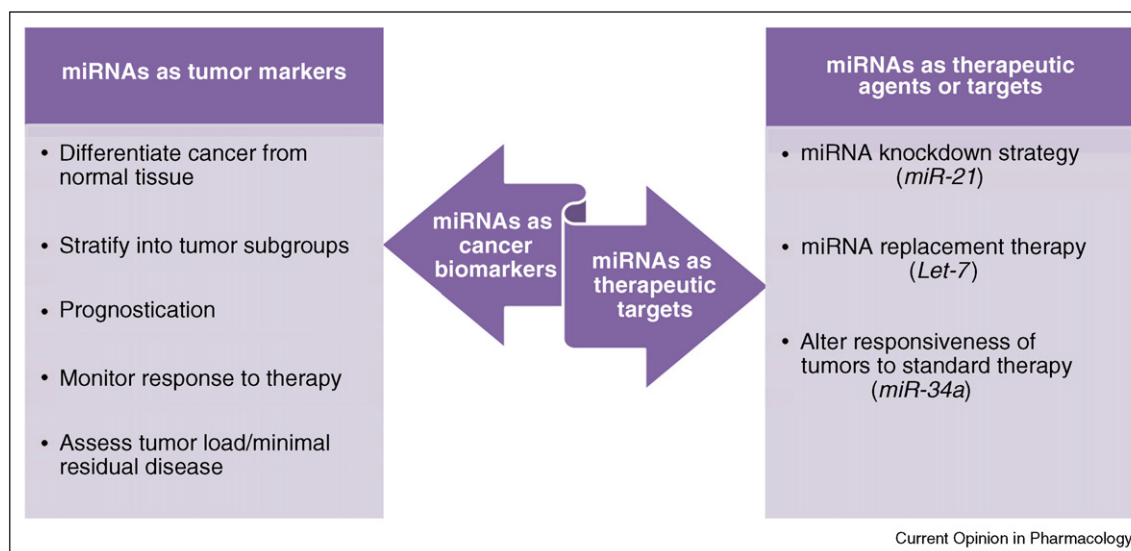
Potential applications of miRNAs as biomarkers, therapeutic agents and targets for cancer.

miRNA expression was also observed in primary breast cancer tissues, where *miRs-221/222* were again over-expressed in HER2/*neu*-positive tumor samples which were resistant to tamoxifen. These effects on tamoxifen sensitivity were shown to be mediated by the direct target of *miR-221* and *miR-222*, the cell cycle inhibitor p27^{Kip1}. Manipulating levels of p27^{Kip1} resensitized the OHT^{R} cells to tamoxifen treatment, thereby illustrating a potential role for *miR-221/222* antagonism as a means of increasing sensitivity of breast tumors to standard hormonal therapy [40].

Our perspective and conclusion

The transition of miRNA applications from bench to bedside, as cancer biomarkers and as therapeutic agents, necessitates addressing several challenges. As biomarkers, various issues regarding miRNA measurement and quantification, particularly in the circulation, need refining. Firstly we need to gain a better understanding of the exact mechanisms by which miRNAs are released into the circulation and if freely circulating miRNA molecules have any functional role in addition to reflecting the presence and pathological features of disease. Secondly there is no consensus on the most appropriate endogenous control for systemic miRNA analysis. In order to obtain reliable and reproducible results, there is a need to determine suitable normalization methods for blood-based miRNA investigations. Thirdly larger validation studies are urgently needed to support the preliminary findings from case–control cohort studies that have proposed miRNAs as novel biomarkers for cancer. These studies must report miRNA levels in blood from several hundred healthy individuals representing both genders, all ethnicities and age groups so that appropriate

conclusions can be drawn from results of systemic miRNA profiling in specific disease cohorts. Furthermore as additional short non-coding RNAs are continuously identified through biomarker discovery programmes, the available profiling technologies must adapt their platforms to incorporate newer potentially relevant targets. Functional validation of all miRNAs reported to be dysregulated in cancer, and the identification of their target genes and pathways is also important.

With regard to therapeutics whilst progress in this field is rapid and laudable, many obstacles must be overcome for miRNA-based therapies to become a reality in management of common cancers. A significant amount of functional work remains to be done to fully elucidate the mechanisms by which miRNAs contribute to tumorigenesis, and establish a better understanding of the complexity of gene expression regulation by miRNAs. Pharmacological difficulties include developing safe, effective, site-specific delivery mechanisms for miRNA directed therapies.

Despite these challenges, the remarkable potential of miRNAs as cancer biomarkers and therapeutics cannot be underestimated (Figure 2). If the current momentum in miRNA translational research can be maintained, this will bring an exciting new dimension to the field of diagnostics and therapeutics for cancer and has the potential to transform current practice to the ideal of individualized care for cancer patients in the near future.

Conflicts of interest

None to disclose.

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EXPERT REVIEW

The Therapeutic Potential of MicroRNAs: Disease Modulators and Drug Targets

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ABSTRACT MiRNAs are a class of small, naturally occurring RNA molecules that play critical roles in modulating numerous biological pathways by regulating gene expression. The knowledge that miRNA expression is dysregulated in many pathological disease processes, including cancer, has led to a rapidly expanding body of literature as we try to unveil their mechanism of action. Their putative role as oncogenes or tumour suppressor genes presents a wonderful opportunity to provide targeted cancer treatment strategies. Additionally, their documented function in a host of benign diseases broadens the potential market for miRNA-based therapeutics. The present review outlines the underlying rationales for considering mi (cro)RNAs as therapeutic agents or targets. We highlight the potential of manipulating miRNAs for the treatment of many common diseases, particularly cancers. Finally, we summarize the challenges that need to be overcome to fully harness the potential of miRNA-based therapies so they become the next generation of pharmaceutical products.

KEY WORDS miRNA manipulation · miRNA therapeutics · oncomirs · tumor supressors

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ABBREVIATIONS

AGO	argonaute
amiRNA	artificial microRNA
AML	acute myeloid leukemia
AMO	anti-miRNA oligonucleotide
ECM	extracellular matrix
ER	estrogen receptor
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HO-1	heme oxygenase-1
HSC	hepatic stellate cells
LNA	locked nucleic acids
MCL	mantle cell lymphoma
miRAGE	miRNA serial analysis of gene expression
miRISC	miRNA-associated RNA-induced silencing complex
miRNA	microRNA
mRNA	messenger RNA
NSCLC	non-small-cell lung cancer
PAMAM	polyamidoamine
PR	progesterone receptor
RAKE	RNA-primed array-based Klenow enzyme
SAGE	serial analysis of gene expression
SERM	selective estrogen receptor modulator
TGFß	transforming growth factor beta
UTR	untranslated region

INTRODUCTION

Mi(cro)RNAs are a class of small non-coding RNA fragments that have captured the attention of the scientific world since their discovery almost two decades ago. They have since been demonstrated to play critical roles in

almost all aspects of the cell cycle, and their expression is known to be dysregulated in various pathological conditions, including carcinogenesis (1). The functional roles of miRNAs in health and disease have been partly elucidated over the last 5 years; this process has unravelled their remarkable potential as disease biomarkers and therapeutic targets (2).

The association of aberrant miRNA expression with almost every cancer and common disease, along with functional analyses of specific miRNAs, has exposed the remarkable potential of manipulating miRNA expression as a therapeutic strategy for these conditions (Table I). The therapeutic application of miRNAs involves various strate-

gies: first, through antisense-mediated inhibition of over-expressed miRNAs; second, through replacement of under-expressed miRNAs with either miRNA mimetics or viral vector-encoded miRNAs; and third, by modulating miRNA expression to augment a patient's response to existing treatment modalities (2).

MIRNA BIOGENESIS

The biogenesis of human miRNA originates in the nucleus, where there is transcription of a large primary (pri-)

Table I MiRNAs Implicated as Therapeutic Targets in Common Diseases

Disease	miRNA	Expression level in disease state	Stage of Investigation (in vitro/in vivo)
Hepatitis B virus	miR-122, miR-31	n/a	In vivo (21)
Hepatitis C virus	miR-122	n/a	In vivo (24,26)
	miR-199a	n/a	In vitro (29)
Hepatic fibrosis	miR-27a, miR-27b	Over-expressed	In vitro (30)
	miR-29a , miR-29b	Under-expressed	In vivo (31)
Hepatocellular carcinoma	miR-122	Under-expressed	In vitro (34–36)
Lung cancer (NSCLC)	Let 7 family	Under-expressed	In vivo (42–44)
	miR-21	Over-expressed	In vivo (47)
Pulmonary arterial hypertension	miR-204	Under-expressed	In vivo (48)
Breast cancer:			
Inhibition of metastases	miR-10b	Over-expressed[a]	In vivo (54)
	miR-21	Over-expressed	In vivo (55)
	miR-1258	Under-expressed	In vitro (56)
Breast Cancer:			
Response to adjuvant therapy	miR-21	Over-expressed	In vivo (58)
	miR-205	Under-expressed	In vitro (59)
	miR-128a	Over-expressed	In vitro (60)
	miR-125b	Over-expressed	In vitro (61)
	miR-155	Over-expressed	In vitro (62)
	miR-34a	Over-expressed	In vitro (63)
	miR-342	Under-expressed	In vitro (64)
Haematology:			
Leukaemia (B-CLL)	miR-15, miR-16	Under-expressed	In vitro (66)
AML	miR-29b	Under-expressed	In vitro (69)
Lymphoma	miR-17-92	Over-expressed	Tumour: In vivo (71)
	cluster		Radiotherapy: In vitro (72)
Prostate cancer	miR-34a	Under-expressed	In vivo (73)
	miR-16	Under-expressed	In vivo (74)
	miR-143	Under-expressed	In vitro (75)
Bladder cancer	miR-203	Under-expressed	In vivo (76)
Cardiac hypertrophy induced arrhythmia	miR-1, miR-133	Over-expressed	In vivo (77)
	miR-208	Over-expressed	In vivo (78)
	miR-100	Over-expressed	In vitro (79)
	miR-29	Under-expressed	In vivo (80)
Glioblastoma	miR-21	Over-expressed	In vitro (105)

[a] conflicting results reported by different studies

miRNA by RNA polymerase II or III. Seventy percent of human miRNAs are transcribed from introns and/or exons, suggesting that regulation of this process is under gene promoter control. The remaining 30% of pri-miRNAs are located in intergenic regions, and so have independent promoters (3,4). Pri-miRNAs are several hundred or thousand nucleotides in length and contain at least one miRNA stem loop. This single unit may contain up to six precursor (pre-) miRNAs, which are produced by the cleaving action of the RNase III enzyme Drosha, combined with the microprocessor complex subunit DGCR8. Pre-miRNAs range from 70 to 90 nucleotides in length and contain a hairpin structure that is critical for their transport to the cell cytoplasm by the energy-dependent Exportin-5 (5). Once in the cytoplasm this hairpin can then be cropped by the RNAase III enzyme Dicer, to produce a double-stranded structure, miRNA:miRNA*, consisting of the miRNA and its complement. This multi-step process culminates in the mature miRNA strand being incorporated into a miRNA-associated RNA-induced silencing complex (miRISC). It is in

this formation that miRISC interacts with its target mRNA and exhibits its cellular effects (6) (Fig. 1).

MIRNA FUNCTION

MiRNAs have been implicated in almost every part of the cell cycle. They exhibit their function by sequence-specific modulation of gene expression at a post-transcriptional level. It is estimated that miRNAs govern over 30% of protein coding genes in this way (7). An understanding of their mechanism of action is crucial for their application in a therapeutic setting. The seed-sequence, the short region of importance in miRNA target recognition, extends from bases 2 to 8 on the 5′ tail of the mature miRNA strand (4). Each miRNA has two possible mechanisms of action, determined by the degree of complementarity between the miRNA seed sequence and its mRNA target, which is governed by Watson and Crick base pairing. First, if the target mRNA and miRISC have perfect base pairing

Fig. 1 MiRNA biogenesis and processing. Simplified representation of the steps involved in miRNA biogenesis and processing in human cells. This multi-step process begins in the nucleus of the cell, where there is transcription of a large primary (pri-) miRNA by RNA polymerase II. This large pri-miRNA is then cleaved by the RNase III enzyme Drosha and coupled with the microprocessor complex subunit DGCR8 to produce pre-miRNA. Pre-miRNAs range from 70 to 90 nucleotides in length and contain a stem loop structure for their transport to the cell cytoplasm by Exportin-5 (5). Once in the cytoplasm, this hairpin structure is cropped off by the RNase III enzyme, Dicer, producing the double-stranded miRNA:miRNA* duplex. This process culminates in the mature miRNA strand being incorporated into a miRNA-associated RNA-induced silencing complex (miRISC). It is in this formation that miRISC targets complementary mRNA sequences and exerts its cellular effects, via transcriptional cleavage or transcriptional repression.
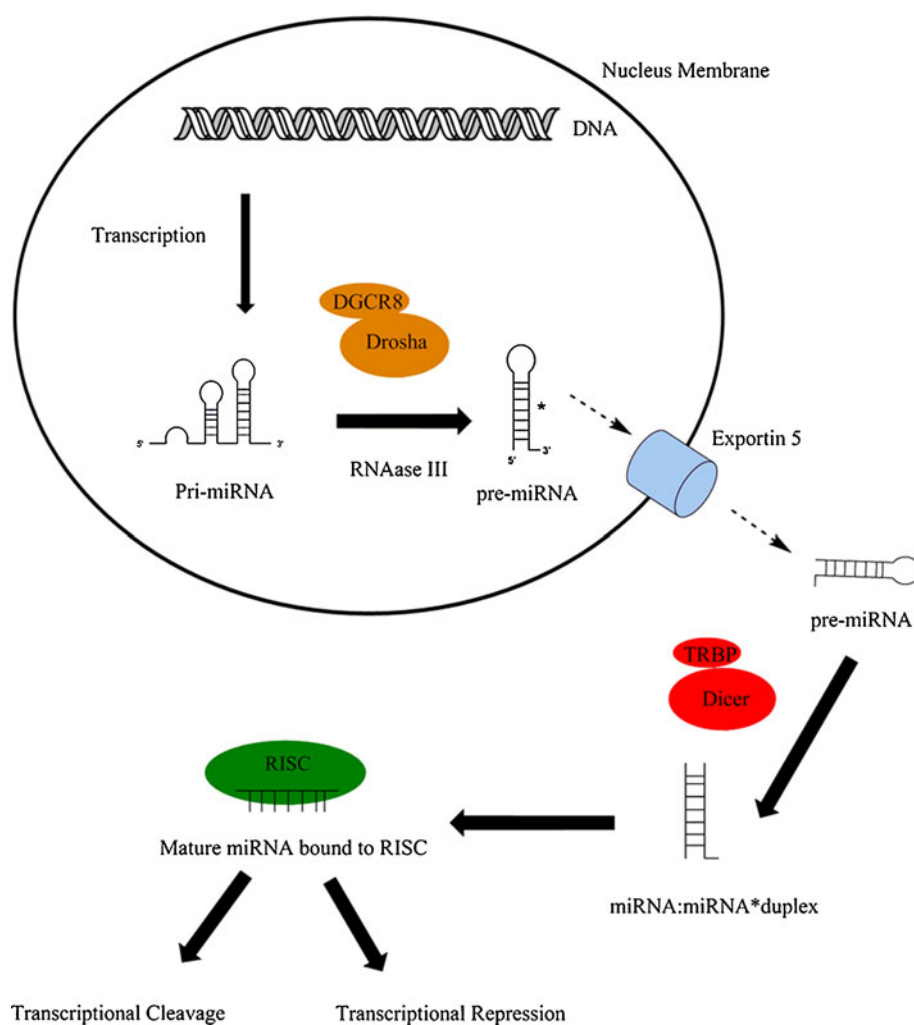
Fig. 2 Typical pattern of base pairing, with imperfect complementarity, between a miRNA and its target mRNA. Typically, the miRNA binds to a specific site or sites within the 3′UTR region of the mRNA sequence. According to thermodynamic analysis, some degree of complex formation occurs along the entire miRNA-mRNA duplexed region. Base pairing is particularly weak in the central region due to mismatched 'bulges' in the miRNA sequence (**a**), and particula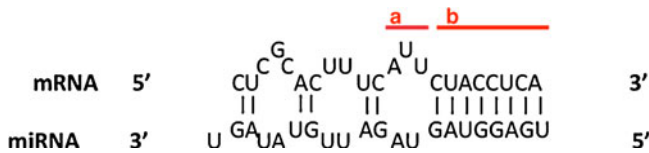rly strong at the 5′ end (seed region) of the miRNA (**b**). Base pairing between *let*-7 miRNA and *hbl-1* mRNA in *C. elegans* is shown as an example (Lin et al., 2003).

homology, the mRNA is cleaved and degraded through activation of the RNA-mediated interference pathway. Second, and more commonly, miRNAs modulate their gene targets by repression of protein translation. MiRNAs exhibit this effect by imperfectly binding to partially complementary sequences located often in the 3′ untranslated region (UTR) of target mRNAs, although miRNAs can also bind to the coding region and 5′UTR of target genes (8). The proposed mechanism by which imperfect pairing between a miRNA and its target results in translation inhibition or repression is that efficiency of translation is reduced consequent to various mismatched 'bulges' in the central region, or to a lesser extent the 3′end, of the miRNA (Fig. 2). These bulges appear to affect the strength with which the miRNA binds to its mRNA target

and can affect the Argonaute (AGO)-mediated endonucleolytic cleavage of mRNA (9). Thus far, over 1,000 human miRNAs have been identified (10), each with the capacity to influence several mRNA targets through imperfect base pair homology (Fig. 3).

MIRNA PROFILING AND IDENTIFICATION OF DISEASE-SPECIFIC MIRNAS

MiRNA expression profiling of a variety of human tissues, both healthy and pathological, has given remarkable insight into the developmental stages of many diseases. It has been shown that distinct patterns of miRNA expression are observed in individual tissues and in different disease states. These tissue- and disease-specific expression patterns reflect mechanisms of cellular transformation and further support the idea that miRNA expression patterns encode the developmental history of human disease. In contrast to mRNA expression profiles, it is even possible to successfully classify poorly differentiated tumors using miRNA expression patterns (11,12). A number of different techniques are available for miRNA expression profiling. Oligonucleotide microarray-based miRNA analysis was first described in 2004 and has since become the most commonly used method for detecting cancer-specific miRNA expression profiles involving large numbers of samples (13). Bead-based flow cytometric technology is a highly specific high-throughput method of miRNA expression profiling, devel-
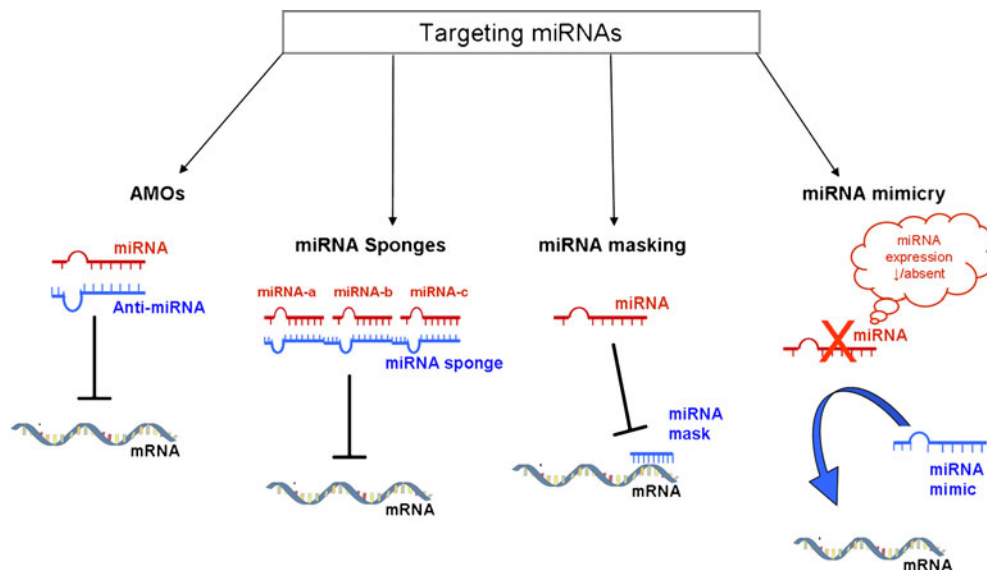
Fig. 3 Strategies of miRNA manipulation and potential miRNA therapeutic strategies. The effects of oncogenic miRNAs can be down-regulated by anti-miRNA AMOs (anti-miRNA oligonucleotide), miRNA sponges, and miRNA-masking. AMOs can bind to complementary miRNAs and induce either duplex formation or miRNA degradation. MiRNA sponges exhibit multiple miRNA binding sites, resulting in the ability to simultaneously sequester multiple miRNAs. MiRNA masks are complementary to the 3′UTR of the target miRNA, resulting in competitive inhibition of the downstream target effects. The downstream effects of tumour suppressor miRNAs can be restored by introducing synthetic miRNAs (miRNA mimicry).

oped by Lu *et al.* (12). While microarray-based miRNA profiling experiments are technically more challenging to perform, bead-based flow cytometry provides a higher specificity. Other technologies in this realm include tag-based sequencing methods such as miRNA serial analysis of gene expression (miRAGE) (14) and the high-throughput RNA-primed array-based Klenow enzyme (RAKE) assay, which is an enzymatic on-chip-labeling technique (15). However, laborious and costly cloning and sequencing steps have limited the use of SAGE, and widespread use of the RAKE assay has been hindered primarily by the fact that a large amount of starting RNA is required (16). More recently, the introduction of platforms that permit large-scale parallel analysis of genome-wide sequences have advanced miRNA identification and analysis even further. Deep sequencing technology is one such platform which enables the simultaneous sequencing of millions of different RNA molecules in a single sample. Deep sequencing overcomes many of the limitations of microarray-based profiling. The latter is susceptible to cross-hybridization and measures only the relative abundance of miRNAs that have already been identified. In contrast, deep sequencing is not dependent on any prior sequence information. Instead, it provides unbiased information about all RNA species in a given sample, thus allowing for discovery of novel and disease-specific miRNAs or other types of small RNAs that have eluded previous cloning and standard sequencing efforts. In conjunction with the evolution of next-generation sequencing technologies, advanced bioinformatic tools have had to evolve simultaneously in order to analyze the massive amounts of data generated (17–20). As these highly sophisticated techniques continue to develop, the extent and significance of miRNA regulation of gene expression will become even more evident. The future of miRNA expression profiling may lie in techniques which can be applied to profile miRNA expression *in vivo*, and not just in archived specimens. Molecular imaging of miRNAs presents a non-invasive method of monitoring miRNA biogenesis and function based on reporter and fluorescent beacon imaging approaches. Molecular imaging is superior to traditional miRNA expression profiling methods, as it can be applied to living cells and provides further insight into potential disease altering miRNAs for consideration in therapeutic modalities (21). This exciting development could be invaluable in the clinical setting, allowing individual response to treatment to be evaluated at a cellular level.

MIRNAS AND THERAPEUTICS

The rapidly expanding body of knowledge on miRNA expression and function is ideal for exploiting as the next generation of disease therapeutics. The fact that these tiny RNA fragments are implicitly involved in many pathological states and that they mediate potent and specific gene silencing makes them attractive therapeutic targets. To date, the greatest efforts in this setting have been in exploring the potential application of miRNA therapeutics for various cancers. In the cancer state, miRNAs have been demonstrated to play a dual role, that of an oncogene or a tumor suppressor. Gain or loss of function of individual miRNAs has been reported in almost every solid and hematological cancer, with pathological roles in tumor cell proliferation, progression of tumors and the metastatic process (1,22). Early *in vitro* work involving miRNA manipulation in cancer cell lines demonstrated the remarkable therapeutic potential of this strategy. A number of different molecular and pharmacological strategies may be employed to help realize this potential.

MiRNAs with oncogenic capacity can be deactivated or silenced by several RNA interference-type strategies, namely miRNA-specific knockdown by anti-miRNA oligonucleotides (AMOs), miRNA sponges and miRNA masking. AMOs are synthetic antisense oligonucleotides that competitively inhibit the interaction between miRNAs and their mRNA targets. The most widely employed types of AMOs are 2′-O-methyl AMOs, 2′-O-methoxyethyl AMOs and locked nucleic acids (LNAs) (23). Locked nucleic acids (LNA) are modified oligonucleotides with many advantages over traditional AMOs, including the fact that they do not require a vector and have superior thermal stability and lower toxicity (24). These latter molecules are being utilized in the majority of current *in vivo* studies in this field. As a potential therapeutic approach, however, AMOs have several inherent weaknesses, such as their transient duration of action and inability to target more than one miRNA at a time.

Given that miRNAs have been observed to function not in isolation but often in clusters in pathological processes, knockdown of multiple over-expressed miRNAs presents a therapeutic challenge. The unique concept of 'miRNA sponges' holds great appeal in this context. These competitive miRNA inhibitors are transcripts expressed from strong promoters that display numerous and tandem binding sites for the miRNAs of interest. Sponges, which may be located in non-protein coding RNA or in the 3′-UTR of a reporter gene, are frequently under the control of potent promoters, such as CMV, to ensure large quantities of the transcript are produced (25). Ebert *et al.* demonstrated the efficacy of these miRNA inhibitors *in vitro* by transiently transfecting cultured cells with vectors encoding miRNA sponges. This resulted in a reduction in the level of miRNA targets to at least that attainable with AMOs (12,26). A single sponge bearing a heptameric seed sequence can target families of over-expressed miRNAs which share this seed. In doing so, the sponge can effectively manipulate abnormal expression levels, thereby

preventing their binding with endogenous mRNA targets (26). Drosphilia miR-SP is a dynamic technology that allows transgenic miRNA silencing, with precise *in vivo* spatial resolution (27). This advanced miRNA-sponge technology aims to overcome the lack of tissue specificity associated with traditional miRNA-sponges, while providing insight into interactions between miRNAs and other genes. Transgenic miRNA sponges (miR-SPs) are synthesised by locating modified miRNA complementary oligonucleotides downstream of repetitive upstream activation sequences (UAS).

MiRNA masking is an alternative miRNA knockdown strategy to the AMO approach, with the advantage of targeting miRNAs in a gene-specific manner (28). A miR-mask is synthesized as a single-stranded 2′-O-methyl-modified oligoribonucleotide, which has perfect complementarity to an endogenous miRNA binding site in the 3′ UTR of a protein coding mRNA gene. Unlike an AMO, which binds to the target miRNA directly, a miR-mask binds with high affinity to the target miRNA's binding site in the 3'UTR of its mRNA target. This specific mechanism avoids off-target effects. The miR-mask technology has already been validated *in vivo*, thereby highlighting its potential clinical utility. Using a zebrafish model, Choi *et al.* successfully inhibited the repressive action of *miR-430* on transforming growth factor beta (TGFß) using a miR-mask, which was complementary to the *miR-430* binding site in its target mRNAs *squint* (*sqt*) and *lft2* (29).

With regard to tumor suppressor miRNAs or those with decreased expression in benign disease states, the fundamental principle in miRNA-based treatment strategies is to restore their expression level to normal. This can be achieved through miRNA mimicry or viral vector-encoded miRNA replacement. MiRNA mimics are small chemically altered double-stranded RNA molecules that imitate endogenous miRNAs (30), or the precursor pre-miRNA molecules. The viability of this approach has been demonstrated in numerous *in vitro* and *in vivo* settings, the details of which will be discussed later in this review. Gene therapy in the form of viral vectors is another approach for the therapeutic replacement of miRNAs. Adenoviral and lentiviral vectors encoding miRNAs have been investigated as miRNA delivery vehicles in this context, with encouraging results (31,32). In fact, adenoviral vector-encoded miRNA replacement strategies have already been studied *in vivo* (33) and have attracted interest from miRNA therapeutics companies such as Mirna Therapeutics and Asuragen. These studies reported transduction efficiency and minimal toxicity. However, Grimm *et al.* highlighted the potential for serious toxicity to occur with this miRNA replacement strategy. Systemic administration of short RNAs was achieved in adult mice using a delivery vector based on duplex-DNA-containing adeno-associated virus type 8 (AAV8), resulting in down-regulation of critical liver-derived miRNAs, resulting in morbidity and even fatalities (34). The authors postulated that mortality in this instance was consequent to oversaturation of endogenous miRNA pathways. Their experience is important to consider in bringing this strategy from bench to bedside. We will now discuss the rationale and evidence for miRNA therapeutic applications in many common diseases.

LIVER DISEASES

The seminal advances with respect to miRNA therapeutics have been in the field of liver disorders: hepatitis, hepatic fibrosis, and hepatocellular carcinoma (HCC). HCC is one of the most common cancers worldwide and among the leading causes of cancer-related deaths (35). It usually arises in the setting of pre-existing chronic liver disease, which is caused by viral hepatitis (B or C) in 80% of cases worldwide (36). The role of miRNAs in viral hepatic diseases is particularly complex. In addition to miRNA-mediated RNA-silencing pathways influencing viral-host cell interactions (37), viruses not only exploit the hosts cellular miRNAs, but also encode their own miRNAs (38).

Viral Hepatitis B

There is compelling evidence to suggest that miRNAs participate in the development of and host reponse to hepatitis B viral infection (39). Using computational analysis, Jin *et al.* identified that HBV putatively encodes only one candidate pre-miRNA and that viral miRNA only targeted viral mRNA, not host cellular transcripts. The authors proposed that HBV had evolved to use viral miRNAs as a means to regulate its own gene expression to its benefit (40). This hypothesis was confirmed *in vitro* when vector-based artificial miRNA (amiRNA) successfully inhibited HBV replication and expression (41). Ely *et al.* confirmed the *in vivo* viability of this potential therapeutic approach to HBV by employing RNA polymerase II promoter cassettes that transcribes anti-HBV primary miRNA shuttles, specifically pri-mi-122 and pri-mi-31, with a resulting decreases in HBV expression (42).

Viral Hepatitis C

Relative to HBV, there is less evidence to support the involvement of viral miRNAs in the replication of hepatitis C virus (HCV). However, HCV replication appears to be subject to the regulatory miRNAs of the human host cell (39). *MiR-122* was the first liver-specific cellular miRNA identified and constitutes over 70% of miRNAs in the liver. It is known to have two potential binding sites for HCV and enhances the replication of HCV by targeting the viral 5′

non-coding region. Within hepatic tissue, *miR-122* is only detected in the HuH-7 human hepatoma cell line, which is interesting, as HCV can only replicate in these cells. When *miR-122* is inactivated *in vitro* by transfection with 2′-O-methylated RNA oligonucleotide with exact complementarity to *miR-122*, HCV replication in these cells decreases by over 80% (43). This confirms that *miR-122* plays an important direct role in HCV translation by targeting the 5′ untranslated region and enhancing the association of ribosomes at an early stage (44). Krutzfeld *et al.* provided the first report of successful miRNA antagonism *in vivo* when *antagomir-122* was conjugated with cholesterol and delivered intravenously, resulting in *miR-122* knockdown for 23 days (45). However, this method of antagmiR delivery, employing synthetic 2′-O-methyl anti-miRNA oligonucleotides (AMOs), raises concerns regarding their stability and toxicity. Locked nucleic acid (LNA)-modified oligonucleotides, as mentioned, present significant advantages for sequence-specific antagonism of miRNAs; they display advanced thermal stability when combined with their target RNA and have a low toxicity profile in mammals (24,46). The use of LNAs *in vivo* as a mechanism of delivering miRNAs for therapeutic purposes was demonstrated by Elmen *et al.* In a murine model, this group delivered unconjugated LNA-antimiR oligonucleotide complementary to the 5′end of *miR-122,* and observed specific dose-dependent *miR-122* silencing without hepatotoxicity (47). It has also been documented that *miR-122* is an indirect facilitator of HCV replication; Heme Oxygenase-1 (HO-1) is capable of inhibiting HCV replication, and *miR-122* down-regulates this pathway. The combination of *miR-122* down-regulation, with up-regulation of HO-1, is a potential new strategy for antiviral therapies directed towards HCV (48).

MiR-199a is another liver-specific miRNA that has been associated with HCV replication. *In vitro* studies have demonstrated that over-expression of *miR-199a* results in inhibition of HCV replication, independent of the interferon pathway, while inactivation of *miR-199a* induces accelerated viral replication (49).

Hepatic Fibrosis

Liver fibrosis is a largely irreversible condition that occurs in association with most chronic liver diseases. Hepatic stellate cells (HSCs) become activated in response to repeated injury and exposure to inflammatory mediators. They subsequently lose their lipid droplets and migrate to the injured area, where they secrete large amounts of extracellular matrix (ECM), resulting in fibrosis (50). This process can result from chronic hepatitis, and ultimately leads to liver cirrhosis and potentially hepatocellular carcinoma. Many miRNAs have been implicated in the

pathogenesis of hepatic fibrosis. *MiR-27a* and *miR-27b* have recently been studied in rat HSCs *in vitro*. They are normally over-expressed in the inflammatory state, and down-regulation of both miRNAs resulted in the HSCs returning to a more quiescent state, with decreased proliferation and restored lipid droplets (51). *MiR-29a* and *miR-29b* are also of interest in the setting of hepatic fibrosis. A recent microarray conducted on murine livers identified the *miR-29* family as being significantly down-regulated in fibrotic liver tissue (52). In this elegant study by Roderburg *et al.*, *miR-29* was shown to play a regulatory role in pathways involving the genes TGF-β and NF-κB. The authors also found that over-expression of *miR-29b* resulted in down-regulation of collagen expression in murine HSCs (52). These data illustrate the future potential for *miR-29b* as an antifibrotic agent.

Hepatocellular Carcinoma

MiR-122 is one of the most extensively investigated miRNAs; it is now known that its function extends far beyond virus replication and infection of the liver. Computational tools and *in vitro* expression data suggest that *miR-122* also has a role in the cellular stress response (53) and hepatocellular carcinogenesis (54). Converse to the major positive role of *miR-122* in HCV replication, it has a negative role in hepatic tumorigenesis and in fact is a tumor suppressor in the liver. MiRNA expression profiling has revealed that *miR-122* is down-regulated by at least 50% in human HCC tissue compared to normal or non-cirrhotic liver.

Transfection of HCC cell lines with *miR-122* has been shown to induce cellular apoptosis and reduce cell viability (55,56). This presents a novel chemotherapeutic strategy in HCC, a disease with a typically poor prognosis for which there are limited treatment options. An increase in *miR-122* expression in malignant cells could result in targeted cell death. Young *et al.* devised a mechanism to test this theory by developing small molecule modifiers of *miR-122* function (57). These miRNA modifiers (1–3) act at the transcriptional level to either up- or down-regulate *miR-122* expression. More specifically, the authors observed that small molecule *miR-122* inhibitor 2 inhibited HCV replication, while small molecule *miR-122* inhibitor 3 induced an increased expression of the pro-apoptotic *miR-122* in the HCC cell line HepG2, leading to *caspase* activation and reduced cell viability. This study highlights the remarkable potential of miRNA manipulation as a plausible novel therapeutic strategy.

LUNG DISEASES

Lung cancer is the leading cause of cancer death worldwide, with non-small-cell lung cancer (NSCLC) accounting

for 80–85% of lung cancer cases. To date, over 40 miRNAs are known to be dysregulated in NSCLC. Various miRNA signatures, derived from lung tumor tissue or even plasma/serum samples, have been proposed as biomarkers of this disease with utility in diagnosis and prediction of overall survival (58,59). With regard to miRNA therapeutic strategies for lung cancer, much of the work to date has focused on harnessing the tumor suppressor properties of the *let-7* family for this purpose. *Let-7* is stably expressed in normal adult lung; however, expression profiling of NSCLCs has revealed that various members of the *let-7* family are decreased in tumor tissue compared to normal lung. *Let-7a*, among other miRNAs, has been shown to have prognostic value in that low levels correlate with poor survival (60). Functional work has identified and defined the regulatory roles of the *Let-7* family in several oncogenic pathways, including the RAS pathway, where it represses activity of the *KRAS* oncogene, mutations of which are commonly implicated in adenocarcinoma of the lung (61,62).

Esquela-Kerscher and Slack *et al.* pioneered many of the early *in vitro* and *in vivo* investigations into the role of let-7 miRNAs in NSCLC. They identified that the tumor suppressor effect of *let-7* was transient and that replacement of let-7 through gain-of-function techniques could reduce cell proliferation in various human lung cancer cell lines. In a murine model of human lung cancer, this group demonstrated how loss of *let-7* induced lung tumor formation and growth, through loss of its regulatory effect on the oncogenes *RAS* and *HMGA2* amongst others. Restoration of *let-7* expression in lung cancers, using intranasal delivery techniques, restrained the growth of tumors by repressing multiple cell cycle and proliferation pathways together with *ras* and *MYC* suppression (63,64). This work paved the way for further investigations into the therapeutic feasibility of miRNAs in the clinical treatment of lung cancer.

Although promising, the application of *let-7* as a therapeutic agent for cancer is premature as yet, given that details of the immunogenic and cytotoxic effect of *let-7* administration remain to be determined. Its ubiquitous expression and involvement in multiple cellular pathways imply that manipulation of its levels is likely to have diverse off-target effects. The development of safe, effective, and tissue-specific delivery methods for *let-7* requires further effort before this strategy advances as a cancer therapy.

Kumar *et al.* have demonstrated similar tumor suppressor effects of the *let-7* miRNAs on lung cancers *in vivo* (65). Using a lentiviral system, they first transfected murine *KRAS*-expressing lung adenocarcinoma cells (LKR 13) with a *let-7 g* miRNA duplex; this resulted in decreased cell proliferation and induction of cell death. In tumor xenografts, the authors observed significant reduction of both murine and human non-small-cell lung tumors when

let-7 g was over-expressed using these lentiviral vectors. Furthermore, they found that *let-7 g*-mediated tumor suppression was more pronounced in lung cancer cell lines harboring oncogenic K-Ras mutations than in lines with other mutations. The potential of *let-7* in the treatment of lung cancer extends beyond its direct effects on the tumor. Preliminary *in vitro* data suggest that there is potential to use miRNA modulation to enhance standard treatments for cancer, such as radiotherapy for lung tumors. Weidhaas *et al.* provide evidence that over-expressing members of the *let-7* family in lung cancer cells and in a *C. elegans* model of radiation-induced cell death results in increased sensitivity to radiation therapy, whereas decreasing *let-7* levels induces a state of radioresistance (66). These effects were mediated through altered *RAS* signalling.

The potential role of miRNAs in treating lung disorders is not confined to *let-7*. Blower *et al.* showed that altering expression levels of *let-7i, miR-16* and *miR-21* in a lung cancer cell line (A549) altered the potency of chemotherapeutic agents up to four-fold (67). Manipulation of the oncogenic *miR-21* in NSCLC has also been investigated as a possible therapeutic strategy. Using transgenic mice, Hatley *et al.* demonstrated that over-expression of *miR-21* was associated with cell proliferation and tumor growth, whilst genetic deletion of *miR-21* partially protected against tumor formation (68). Inhibiting *miR-21* increased tumor sensitivity to DNA-damaging chemotherapeutic agents and could potentially restore the activity of multiple tumor suppressors acting at various critical points of tumorigenesis.

Pulmonary arterial hypertension has also been associated with aberrant miRNA expression and function. Decreased *miR-204* levels in affected lungs have been shown to correlate with disease severity in both animal and human studies (69). Targeted delivery of synthetic *miR-204* to the lungs of affected animals resulted in a significant reduction in disease severity. This illustrates another potential application of miRNA therapeutics.

BREAST CANCER

Since Iorio *et al.* first reported dysregulated miRNA expression in breast tumors in 2005, evidence has accumulated implicating miRNAs as key players in breast tumorigenesis, progression, and metastases and in determining tumor response to existing treatments (70,71). As in other cancers, miRNAs play dual roles as oncogenes or tumor suppressors in this prevalent disease. In a therapeutic capacity, there have been two predominant objectives and approaches to manipulating miRNA expression in breast tumors thus far: knockdown of candidate breast cancer-related 'oncomirs' to suppress tumor growth and inhibit or prevent distant metastases, and modulation of miRNA expression with the

intent of augmenting or altering tumor responsiveness to adjuvant chemotherapeutic or hormonal agents.

'OncomiR' Knockdown

It is widely accepted that metastases are responsible for most cancer-related deaths. However, targeting or interrupting the metastatic process with therapeutics has been largely unsuccessful as a result of our limited understanding of this pathological process (72). Recent endeavours to explore the role of miRNAs in the metastatic cascade have identified potentially key pathways in this process and novel therapeutic targets. Accumulating data have proven that miRNAs exert their effects at multiple steps in the metastatic cascade by influencing cancer cell adherence, migration, invasion, motility, and angiogenesis (73). In their miRNA microarray analysis of paired tumor tissues and metastatic lymph nodes, Baffa *et al.* identified a metastatic cancer miRNA signature inclusive of *miR-10b, miR-21, miR-30a, miR-30e, miR-125b, miR-141, miR-200b, miR-200c,* and *miR-205. MiR-10b* is implicated in many cancers, including breast cancer, and is thought to promote tumor invasion and metastasis by inhibiting translation of the *HOXD10* gene, thereby resulting in increased expression of the prometastatic gene, *RHOC* (74). Weinberg's group has also recently reported exciting findings from work on antagonizing *miR-10b* in metastatic breast cancer cell lines (MDA-MB-231 cells). Silencing *miR-10b* with antisense oligonucleotides was found to inhibit *Twist*-mediated cell migration and invasion. They observed similar anti-metastatic effects after systemic *miR-10b* antagonism in a murine model (75).

Numerous other miRNAs have been implicated in the metastatic pathway. Yan *et al.* performed *in vitro* LNA silencing of *miR-21* in two breast cancer cell lines (MCF-7 and MDA-MB-231), which resulted in significantly reduced cell proliferation and migration. Their subsequent *in vivo* studies resulted in similar inhibition of breast tumor growth following *miR-21* knockdown with antimiRs (76). Zhang *et al.* have been first to report that *miR-1258* inhibits breast cancer brain metastases by negatively regulating the heparanase pathway (77). Again, these results strongly support the potential of miRNAs to be applied to the clinical setting for therapeutic gain.

Augmenting Response to Adjuvant Therapy

Chemotherapeutic drugs, radiotherapy and endocrine agents (aromatase inhibitors and selective oestrogen receptor modulators, SERMs) are the adjuvant therapies used in the routine management of women with breast cancer at present. Despite their success in improving disease-free and/or overall survival, a proportion of women derive no benefit from these treatments or develop resistance to these

agents over time (78). The basal subtype of breast cancer (classically ER, PR and HER2/*neu* negative) presents a specific therapeutic challenge, as there are no targeted therapies currently available. Preliminary studies suggest that miRNA modulation in tumor tissue can augment its response to systemic therapies. *MiR-21* is again one of the most studied miRNAs in this setting. Mei *et al.* combined taxol chemotherapy with *miR-21* inhibitor treatment, via a polyamidoamine (PAMAM) dendrimer vector, to evaluate the effects of combination therapy on suppression of breast cancer cells and found that cells treated with this combination demonstrated significantly reduced cell viability and invasiveness compared with cells treated with taxol alone, reflecting an enhanced chemotherapeutic effect of taxol in the presence of decreased *miR-21* levels (79).

There is also experimental evidence that manipulation of *miR-205* levels can improve breast tumors' response to anticancer agents. Based on computational target prediction algorithms, Iorio *et al.* hypothesised that *miR-205* was involved in regulation of the HER3 receptor, a kinase-inactive member of the HER family which plays an important and necessary function in HER2-mediated tumorigenesis. Indeed, their *in vitro* experiments demonstrated that *miR-205*, which is down-regulated in breast tumors compared with normal breast tissue, directly targeted the HER3 receptor and inhibited activation of the downstream mediator Akt (80). Furthermore, reintroduction of *miR-205* in SKBr3 cells was found to inhibit their clonogenic potential and increase the responsiveness of these cells to the tyrosine-kinase inhibitors Gefitinib and Lapatinib, thus overcoming HER3-mediated resistance and restoring proapoptotic activity.

Manipulation of several other miRNAs has been shown to have the potential to augment breast tumors' responsiveness to existing therapies. Inhibition of endogenous *miR-128a*, which is highly expressed in letrozole-resistant breast tumors, overcomes resistance to the aromatase inhibitor letrozole by modulating TGFß signalling (81). *MiR-125b, miR-155,* and *miR-342* have also been implicated in regulating chemosensitivity, whilst knockdown of *miR-34a* is associated with increasing cancer cells sensitivity to radiation (82–85).

HEMATOLOGY

Much of the initial data on miRNA expression profiling and function stemmed from studies of hematological malignancies. However, few experiments testing the therapeutic potential of miRNAs in this setting have been conducted.

Leukemia

The first evidence of involvement of miRNAs in malignancy came from the identification of a translocation-induced

deletion at chromosome 13q14.3 in B-cell chronic lymphocytic leukemia (86). Loss of *miR-15a* and *miR-16-1* from this locus results in increased expression of the anti-apoptotic gene *BCL2* (87). *BCL2* inhibition through replacement of these deficient miRNAs is therefore a plausible therapeutic strategy. Similar potential exists for miRNA-based therapeutics in the management of acute myeloid leukemia (AML), for which distinctive patterns of aberrant miRNA expression have been identified (88). Eyholzer *et al.* demonstrated that *miR-29b* expression is decreased in AML patients displaying either CEBPA deficiency or loss of chromosome 7q (89). Data from Calin and Croce's laboratory showed that restoration of *miR-29b* in AML cell lines and primary bone marrow or peripheral blood samples induced apoptosis and dramatically reduced tumorigenicity in a xenograft leukemia model (90).

Lymphoma

The *miR-17-92* cluster is located in a region frequently amplified in B-cell lymphoma (91). This cluster, located at chromosome 13q31-q32, is comprised of seven individual miRNAs that are transcribed as a polycistronic unit (*miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-19b, miR-20a,* and *miR-92*). He *et al.* clearly illustrated the oncogenic activity of *miR-17-92,* by demonstrating accelerated tumor development and reduced Myc-induced apoptosis in a Eu-Myc transgenic mouse model of human B-cell lymphoma (92). In theory, modulating this cluster of miRNAs could inhibit lymphoma progression. Targeting the *miR-17-92* cluster could also be used to augment response to radiotherapy in human mantle cell lymphoma (MCL). MCL cells over-expressing *miR-17-92* display increased cell survival and reduced cell death following radiotherapy. Knockdown of this miRNA cluster could increase the radiosensitivity of MCL cells, thereby improving prognosis for these patients (93). The miRNA sponge concept would be an ideal therapeutic strategy in this setting, as a single sponge molecule could target the entire miRNA cluster simultaneously.

MIRNA THERAPEUTICS IN OTHER COMMON CONDITIONS

In addition to cancers, miRNAs are known to be dysregulated in a wide range of other disease processes. From a therapeutic perspective, the most promising applications at present for miRNA-based treatments are in the settings of urological, cardiovascular and neurological diseases.

MiR-34a is known to be under-expressed in prostate cancer cells, which exhibit advanced proliferation and metastatic potential and express the adhesion molecule CD44. Liu *et al.* demonstrated that increasing the expression of *miR-34a* in these CD44(+) prostate cancer cells suppressed tumor progression and metastases and resulted in increased survival in a mouse model. Furthermore, inhibition of *miR-34a* in CD44(−) prostate cancer cells by administering *miR-34a* antagomiR contributed to increased tumor burden and metastases (94). This study provides evidence to support the suggestion that the negative regulatory effect of *miR-34a* on CD44 could be exploited for therapeutic benefits in prostate cancer. Several other miRNAs have been implicated as inhibitors of the metastatic process in prostate cancer, including *miR-16* and *miR-143* (95,96). Bladder cancer is also associated with aberrant expression of miRNAs, such as *miR-203*, which represent ideal therapeutic targets. *In vitro* data have identified pro-apoptotic effects of *miR-203* on bladder cancer cells through its down-regulatory effect on bcl-w, implying that gain-of-function modulation with *miR-203* mimetics has potential utility in the treatment of this malignancy (97).

Altered miRNA expression has also been demonstrated in various cardiovascular diseases, including heart failure, arrhythmias, and fibrosis, unveiling further opportunities for miRNA-targeted therapies. Cardiac hypertrophy and its associated arrhythmias may be suppressed by *miR-1* and *miR-133* over-expression through post-transcriptional repression of HCN2 and HCN4 genes (98). *MiR-208a* and *miR-100* have also been implicated as a modulator of cardiac hypertrophy and electrical conduction (99,100). Myocardial infarction can be complicated by fibrin deposition in the damaged muscular wall, the adverse consequences of which include stiffening of the ventricular walls, diminished contractility, and abnormalities in cardiac conductance. Increasing the expression of *miR-29b* in cardiac fibroblasts has been shown to decrease the expression of collagen transcripts in these cells, hence reducing collagen production (101). This knowledge highlights *miR-29b* as a potential therapeutic agent for fibrotic diseases.

An important role of miRNAs in neurological conditions has also been identified. This work has stimulated the expectation that miRNAs hold potential as therapeutic agents for the treatment of debilitating neurodegenerative conditions such as Huntington's, Parkinson's, and Alzheimer's diseases, for which no disease-modifying treatment strategies exist currently (102). Animal model studies have shown that loss of neural miRNAs may be involved in the development and progression of these neurodegenerative diseases. *In vitro* experiments provide further support for miRNAs as therapeutic agents in these conditions; they have been shown to partially preserve miRNA-deficient neurons when over-expressed in these cells. A serious limitation of many novel drugs in this setting to date has been their inability to cross the blood-

brain barrier. Indeed, this will remain one of the major challenges in developing miRNA-based therapeutic strategies for neurological diseases.

POTENTIAL CHALLENGES

The rush to identify novel miRNAs with a role in specific disease processes continues in an effort to expedite the transition of miRNA-based therapeutics from bench to bedside. While significant advances have been made in this field to date, various challenges remain to be overcome before miRNA therapies become a reality. The development of therapeutic strategies involving disease-specific miRNAs is subject to identification and validation of their multiple mRNA targets and to elucidating the complex pathways which they partly or wholly regulate. A significant amount of functional work remains to be performed in order to achieve this. Thus far, predicting gene targets of miRNAs has been largely computationally governed, and miRNA targets are predicted by sequence complementarity rather than in a gene-specific manner. This complex approach to target identification, along with the fact that individual miRNAs have multiple potential targets, leads to difficulty in predicting the spectrum of side effects and toxicity profiles which may be associated with miRNA-based therapeutics. Only *in vivo* investigations followed by carefully designed early phase clinical studies will identify these issues and help overcome them.

Another obstacle which must be surmounted before miRNA-based therapies become a reality is the issue of site-specific, safe, and effective delivery. The two main approaches at present for delivering miRNA therapies to target tissues, direct delivery of miRNA mimics or antigomirs and viral vector-encoded miRNA delivery, have specific limitations. The direct delivery approach, made possible by conjugating the oligonucleotide to cholesterol or coating it with liposomes or polycationic agents, avoids the immunogenic safety issues intrinsic to viral vector delivery. But it is challenged by the need for repeated dose delivery to achieve therapeutic effect. This becomes a critical issue if the route of delivery necessitates an invasive procedure. The viral vector-encoded miRNA delivery approach has the potential to simplify the delivery of multiple miRNA mimics/antagomirs in a single dose, due to the small size of the miRNA coding sequence, but is limited by its potential to trigger a host immune response, transient expression, and poor integration into the host genome (103,104). Future work must focus on developing more efficient delivery systems which minimize the number of healthy cells exposed to these therapies, promote good uptake/integration into the target tissue, and reduce the potential for off-target effects.

CONCLUSION

MiRNAs and their role in disease processes is a rapidly evolving field, as evidenced by the increasing body of literature. Their intricate involvement in the pathogenesis of many common diseases, including cancers, makes them ideal candidates for novel therapeutic strategies. In this review, we have highlighted the ways in which miRNAs can be used as disease modulators and drug targets. We have detailed the evidence to date that specific miRNAs can be associated with and manipulated for the treatment of liver disorders, diseases of the lung, breast cancer, and hematological malignancies. Given the increasing global interest in miRNAs, coupled with advances in molecular biochemistry and pharma technologies, miRNA-based therapeutic strategies look set to become the next generation of individualised targeted therapy adopted by the pharmaceutical and medical fields.

ACKNOWLEDGMENTS & DISCLOSURES

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Obesity Comorbidities

Role of microRNAs in obesity and the metabolic syndrome

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Summary

Obesity and the metabolic syndrome are major public health concerns, and present a formidable therapeutic challenge. Many patients remain recalcitrant to conventional lifestyle changes and medical therapies. Bariatric surgery has made laudable progress in the treatment of obesity and its related metabolic disorders, yet carries inherent risks. Unravelling the molecular mechanisms of metabolic disorders is essential in order to develop novel, valid therapeutic strategies. Mi(cro)RNAs play important regulatory roles in a variety of biological processes including adipocyte differentiation, metabolic integration, insulin resistance and appetite regulation. Investigation of these molecules and their genetic targets may potentially identify new pathways involved in complex metabolic disease processes, improving our understanding of metabolic disorders and influence future approaches to the treatment of obesity. This review discusses the role of miRNAs in obesity and related components of the metabolic syndrome, and highlights the potential of using miRNAs as novel biomarkers and therapeutic targets for these diseases.

Keywords: Adipogenesis, biomarkers, metabolic syndrome, microRNAs, obesity.

Introduction

Obesity and the metabolic syndrome are major public health concerns, and present a formidable therapeutic challenge. The incidence of this disease spectrum continues to rise and contributes significantly to global morbidity, mortality and socioeconomic burden. Current treatment modalities include lifestyle modification, diet and pharmacologic agents yet many patients remain recalcitrant to conventional medical therapy. Bariatric surgery has made laudable progress in the treatment of obesity and its related metabolic disorders, yet carries inherent risks. Scientists and clinicians must focus on improving understanding of the molecular mechanisms underpinning metabolic disorders in order to develop novel, valid therapeutic strategies. Mi(cro)RNAs play important regulatory roles in a variety

of biological processes including adipocyte differentiation, metabolic integration, insulin resistance and appetite regulation (1) (Fig. 1). Investigation of these tiny molecules and their genetic targets may potentially identify new pathways involved in complex metabolic disease processes, improving our understanding of metabolic disorders and influence future approaches to the treatment of obesity.

The purpose of this review is to discuss the role of miRNAs in obesity and related components of the metabolic syndrome, and to highlight the potential of using miRNAs as novel biomarkers and therapeutic targets for these diseases.

MiRNA biogenesis

Mi(cro)RNAs are a class of non-coding endogenous RNA molecules, only 18–25 nucleotides in length. Since their discovery in 1993, these molecules have been shown to play critical regulatory roles in a wide range of biological and
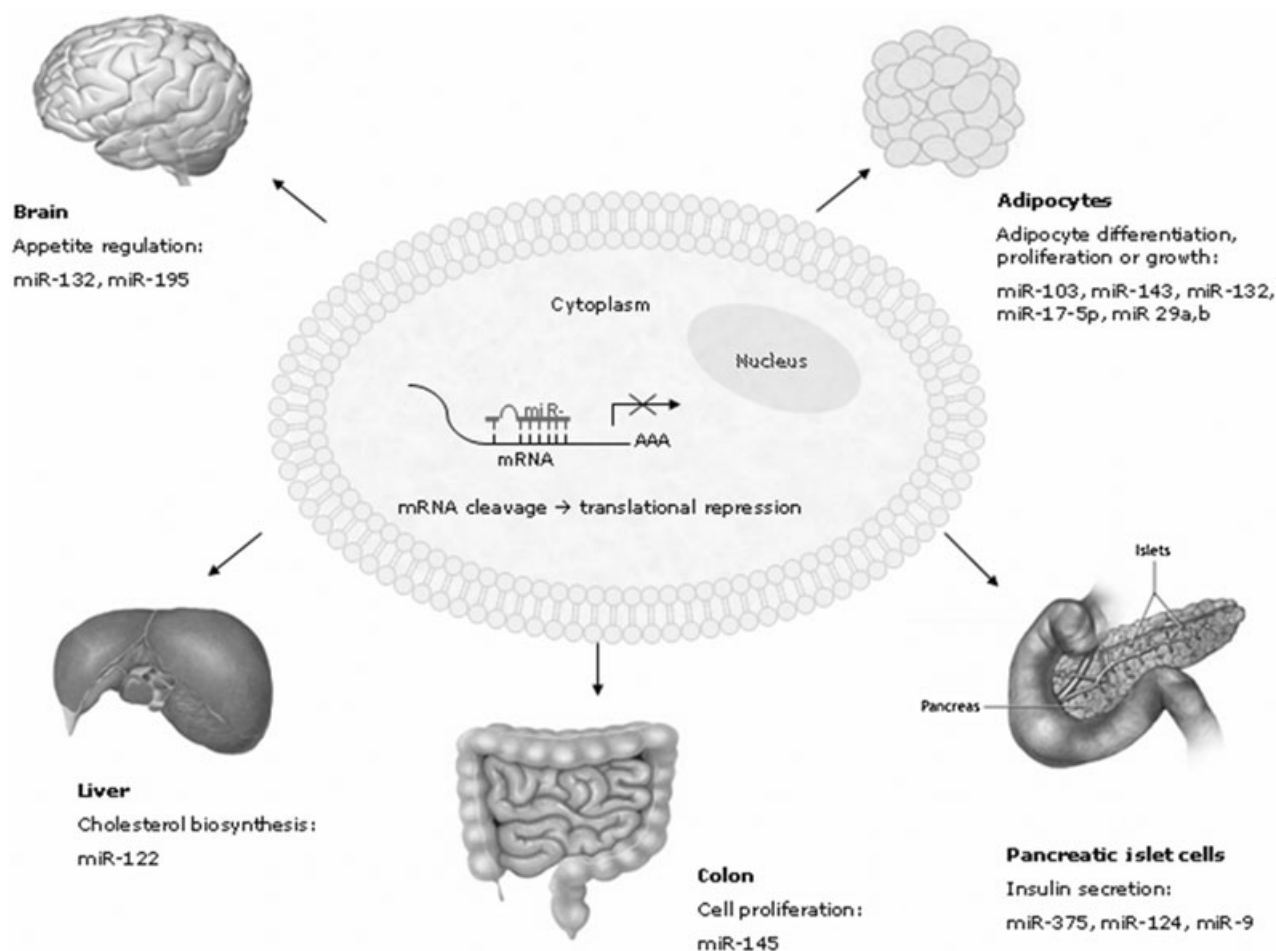
Figure 1 Known role of miRNAs in metabolically related tissues: various miRNAs are specific to certain tissues important in metabolism such as the brain, liver, muscle, adipocyte and the pancreatic islet.

pathological processes (Table 1 & Supporting information, Table S1). The definitive hypothesis of their mechanisms of action remains to be elucidated. Laterally, it has been demonstrated that miRNAs may regulate cellular gene expression at the transcriptional or post-transcriptional level, by suppressing translation of protein coding genes, or cleaving target mRNAs to induce their degradation, through imperfect pairing with target mRNAs (2). MiRNA biogenesis in the human cell is a complex process (Fig. 2) (3). The miRNA target region critical to their recognition is located at the 5′ end of the mature miRNA sequence, from bases 2 to 8, referred to as the 'seed sequence' (4). Computational target prediction algorithms have been developed to identify putative mRNA targets, and thus place considerable importance on this seed sequence, using it to search for complementary sequences in the 3′-UTRs of known genes that exhibit conservation across species. These algorithms predict that each miRNA may potentially bind to as many as 200 targets and estimate that miRNAs control the expression of at least one-third of human

mRNAs, further highlighting their crucial role as regulators of gene expression (5).

Currently 8273 mature miRNA sequences have been described in primates, rodents, birds, fish, worms, flies, plants and viruses (6). In the human genome, over 600 mature miRNAs have been reported to date; however, computational prediction estimates that this could increase to more than 1000 (7). Thus, the microRNA story is in the embryonic stage of expansion.

MiRNAs and glucose homeostasis

Maintaining appropriate blood glucose levels depends on the fine regulation of insulin release. Recently heralded as 'ribo-regulators' of glucose homeostasis, miRNAs play a principal role in the production and secretion of insulin, while simultaneously influencing the sensitivity or resistance of its target tissues (8–10).

The pancreatic islet-specific *miR-375* plays a key role in blood glucose homeostasis through its regulation of beta

Table 1 MicroRNAs with altered expression in obesity and the metabolic syndrome

MiRNA	Target tissue	Function	Target gene	Reference
miR-103	Adipose	Adipocyte differentiation	PANK1	(22,29)
miR-143	Adipose	(pre)Adipocyte differentiation	MAPK7	(21)
miR-132	Adipose	Adipocyte proliferation and growth, insulin resistance	CREB	(27)
miR-17-5p	Adipose	Adipocyte clonal expansion, insulin resistance	RBL2	(27,28)
miR-99a	Adipose, liver	Fatty acid metabolism, Cholesterol biogenesis	IGF1R, CYP26B1	(27)
miR-29a, b	Adipose, liver, kidney, muscle	Glucose transport, Amino acid metabolism, insulin resistance	INSIG1, CAV2, BCKHA	(9,35)
miR-122	Liver	Cholesterol biosynthesis, cellular stress response, Hepatitis C virus replication	PMVK, TRPV6, BCL2L2, CCNG1, HMGCR	(40,49)
miR-145	Colon	Cell proliferation	IRS1	(48)
miR-375	Pancreas	Insulin secretion, Pancreatic islet development	MTPN, USP1, JAK2, ADIPOR2	(8,10,11)
miR-124a	Pancreas	Pancreatic islet development	FOXA2, RAB27A	(13)
miR-9	Pancreas	Insulin secretion	ONECUT2	(17)
miR-133	Heart	Long QT syndrome, cardiac hypertrophy	HERG, RHOA, CDC42, WHSC2	(13)
miR-192	Kidney	Kidney and diabetic nephropathy development	SIP1	(14)
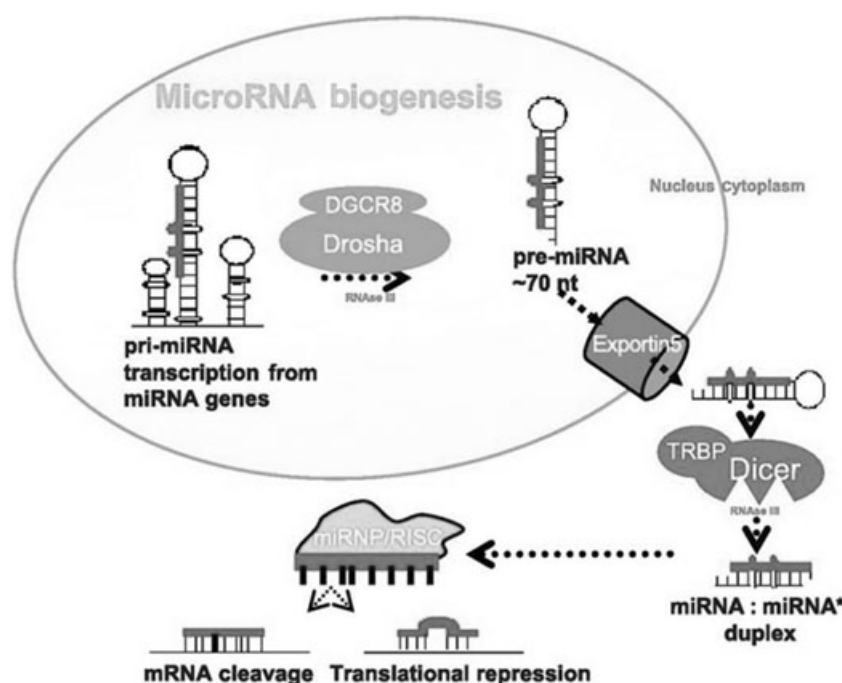
Figure 2 MiRNA biogenesis and processing in human cells: the multistep process begins in the nucleus where the RNase III enzyme Drosha, coupled with its binding partner DGCR8, cleaves nascent miRNA transcripts (pri-miRNA) into ~70 nucleotide precursors (pre-miRNA). These pre-miRNAs consist of an imperfect stem-loop structure. Pre-miRNAs are then exported from the nucleus into the cytoplasm by Exportin 5. In the cytoplasm, the hairpin precursors are cleaved by Dicer and its binding partner the transactivator RNA-binding protein TRBP into a small, imperfect dsRNA duplex (miRNA : miRNA*) that contains both the mature miRNA strand and its complementary strand. The miRNA strand is incorporated into the miRNP complex and targets complementary mRNA sequences, exerting its functionality via mRNA cleavage or translational repression.

cell function, particularly exocytosis of insulin-containing vesicles. Additionally, *miR-124a* and *let-7b*, both of which are also abundantly expressed in pancreatic islet cells are postulated to be important ribo-regulators of blood glucose (11). They concomitantly repress myotrophin, indicating that multiple miRNAs are likely to converge to affect translational control of a single target protein.

Tang *et al.* have shown that *miR-30d* influences insulin transcription (12). Using the pancreatic beta-cell line MIN6, *miR-30d* was found to be up-regulated by increased cell exposure to higher glucose concentrations, and that higher *miR-30d* levels were associated with increased insulin gene expression. Conversely, inhibition of *miR-30d* was shown to rescind glucose-stimulated insulin gene transcription. Based on these results, it is suggested that the putative target genes of *miR-30d* may be negative regulators of insulin gene expression. Thus, emerging data lead us to believe that the powerful miRNA regulatory mechanism is intimately involved in glucose homeostasis.

MiRNAs and diabetes

Since their discovery, miRNAs have been implicated as novel protagonists in the pathogenesis of diabetes, regulat-

ing insulin production, secretion and action (13). They also appear to play a role in the development of diabetic complications such as nephropathy and cardiac hypertrophy (13,14). Evidence supporting the importance of miRNAs in the pathogenesis and progression of diabetes is burgeoning. Poy *et al.* originally identified several miRNAs which were differentially expressed in pancreatic endocrine cell lines. *MiR-375* overexpression was found to reduce beta cell number and viability and thereby suppress glucose-stimulated insulin secretion. Conversely functional experiments showed that *miR-375* inhibition enhanced insulin secretion. These effects were shown to be mediated through *miR-375*'s gene targets which include myotrophin and PDK1 (15,16) and the results indicate that *miR-375* is a potentially important modulator of beta cell function.

Several proteins controlling insulin exocytosis have been identified; however, the factors regulating individual components of the secretory mechanism of beta-cells remain largely unknown. MiRNA research is now beginning to unearth, at least in part, some novel regulatory mechanisms central to this process. *MiR-9* has been shown to regulate insulin release by decreasing expression of the transcription factor Onecut-2, that in turn promotes granuphilin/Slp4, a negative regulator of secretin (17).

MiR-124a expression in beta cells, found to be increased at gestational age e18.5 compared with e14.5 in mice, is postulated to be crucial to pancreatic development and differentiation of pancreatic beta cells (18). In this study using pancreatic beta cell lines, Baroukh and colleagues demonstrated that *miR-124a* preferentially targets Foxa2 – a master regulator of pancreatic development and of genes involved in glucose metabolism and insulin secretion (Kir6.2 and Sur-1). They conclude that this miRNA is an important regulator of a key transcriptional protein network in beta-cells, and is responsible for modulating intracellular signalling.

MiRNAs and adipogenesis

Adipose tissue is not only a storage depot of triglycerides but also has a functional role in regulating energy homeostasis. However, it is well acknowledged that abnormal and excessive fat accumulation in obese patients is associated with adverse health outcomes including an increased risk of life threatening diseases such as Type II Diabetes Mellitus, cardiovascular and cerebrovascular disease, and malignancy (19). Crucial to the development of novel therapeutic strategies for obesity, and its associated metabolic syndromes, is a better understanding of the regulation of adipogenesis. While it is accepted that this complex process is tightly controlled by a combination of multiple transcription factors and extracellular hormones, little is known about the precise mechanisms of adipogenesis. Recently miRNAs have been recognized as a class of epigenetic

regulators of metabolism and energy homeostasis, primarily because the simultaneous regulation of a large number of target genes can be accomplished by a single miRNA. Emerging evidence suggests microRNAs play a key role in the pathological development of obesity by affecting adipocyte differentiation (20–22).

Existing data demonstrate that *miR-14* and *miR-278* in the body fat of *Drosphila* flies regulate lipid metabolism (23,24), *miR-122* in mouse liver controls triglyceride metabolism and cholesterol biosynthesis (25). Similarly, experimental *in vivo* studies using antisense oligonucleotides transfected into human preadipocytes suggest that *miR-143* is involved in adipocyte differentiation (21).

Takanabe *et al.* observed *miR-143* expression to be increased 3.3-fold in adipose tissue of obese mice, and they also report similarly altered levels of the adipocyte differentiation markers PPARγ and aP2 (26). Klöting *et al.* carried out the first miRNA expression profiling in human omental and subcutaneous adipose tissue and uncovered significant correlations between the expression of several fat depot specific miRNAs, adipose tissue morphology and key metabolic parameters such as BMI, lipid and hormone levels (27)

Wang *et al.* reported that the *miR-17-92* cluster is up-regulated twofold during the early clonal expansion stage of adipogenesis and that this family of miRNAs accelerate adipocyte differentiation by negatively regulating the key cell cycle regulator and tumour suppressor gene Rb2/p130 (28).

Xie *et al.* have recently provided the first experimental evidence for *miR-103* function in adipose biology (22). Using 3T3-L1cells (Mouse embryonic fibroblast – adipose like cell line), they demonstrated that expression of *miR-103* was induced approximately ninefold during adipogenesis and consequently down-regulated in adipose tissue harvested from obese mice. The accelerated *miR-103* differentiation during adipogenesis was accompanied by:

• increased expression of key transcription factors (*Pparγ2*);
• increased expression of key cell cycle regulators (G0/G1 switch 2 – *G0s2*);
• increased levels of molecules associated with lipid metabolism (*Fabp4*);
• increased levels of molecules associated with glucose homeostasis (*Glut4*);
• increased levels of molecules associated with endocrine function of adipocytes (adiponectin).

Computational studies predict that *miR-103* affects multiple mRNA targets in pathways that involve cellular acetyl-CoA and lipid metabolism (TargetScan v4.2; an online target prediction program [http://www.targetscan.org] that predicts biological targets of miRNAs by identifying the presence of conserved 8-mer and 7-mer sites that

match the seed region of each miRNA) (29). The inverse pattern of miRNA expression observed in differentiating adipocytes and obese tissue indicates that obesity leads to a loss of miRNAs that characterize fully differentiated and metabolically active adipocytes. Xie *et al.* postulate that these changes are likely due to the chronic inflammatory environment in obese adipose tissue, which has been well described previously (22,30). The authors then show that when differentiated 3T3-L1 adipocytes were treated with TNF-α (a macrophage produced cytokine involved in chronic inflammation, largely responsible for inducing insulin resistance in obese adipose tissue) for 24 h, levels of miR-103 and miR-143 reduced in the adipocytes, while levels of miR-221 and miR-222 were increased. They also observed similar miRNA expression patterns in adipose tissue from obese mice, as well as simultaneously increased levels of TNF-α (22). They concluded that these changes in miRNA expression observed in adipocytes were likely caused by the enhanced expression of TNF-α seen in obese fat tissue.

MiRNAs and neural factors promoting obesity

The brain, central and peripheral nervous systems have been implicated as key regulators of appetite, body fat content and glucose metabolism (31). Pardini *et al.* provide evidence that insulin circulates at levels proportionate to body fat mass, that circulating insulin is transported to the brain, and that insulin receptors are concentrated in brain areas involved in the control of food intake and autonomic function (32).

MiRNAs have recently been shown to be differentially expressed in brain tissue and have been linked to the regulation of neural factors specific to obesity, in particular the control of appetite, and in neural signalling to liver, muscle, pancreas and gastrointestinal tract, to influence metabolism. *MiR-132* has been shown to be highly expressed in brain tissue and neuronal cell types, and evidence exists to show that *miR-132* is involved in the regulation of cAMP response element-binding protein (CREB) which is also known to function in glucose homeostasis (33). Moreover, several miRNAs are commonly overexpressed in both brain and pancreatic beta cells suggesting an overlap in function (e.g. *miR-9*, *miR-124a*). Plaisance *et al.* showed that over-expression of miR-9 (previously thought to be a brain specific miRNA) in insulin secreting cells caused a reduction in insulin exocytosis by diminishing the expression of the transcription factor Onecut-2 and, in turn, by increasing the level of Granuphilin/Slp4, a Rab GTPase effector associated with β-cell secretory granules that exerts a negative control on insulin release (17). *MiR-124a* similarly was initially found to be overexpressed in brain and neural tissue, and subsequently has been found to be abundant in pancreatic beta cells (13,18,34). Further evidence to support *miR-124a* in pancreatic function lies in the knowledge that one of the established target genes for *miR-124a* is FoxA2 (forkhead box protein A2, also known as HNF3 beta), a transcription factor important for beta-cell differentiation, pancreatic development, glucose metabolism and insulin secretion (35).

Hypothalamic brain-derived neurotrophic factor (BDNF) is a key element in the regulation of energy balance and has been implicated in the development of obesity (36). This protein, encoded by the BDNF gene and secreted from the hypothalamus, is a member of the neurotrophin family of growth receptors and low expression levels have previously been linked to increased appetite and obesity (37). Han *et al.* studied children and adults with the rare genetic condition – WAGR syndrome (Wilms' tumor, aniridia, genitourinary anomalies and mental retardation) and found that many of the people with this syndrome (68%) lack the BDNF gene, and have correspondingly low blood levels of the protein. Consequently the BDNF deficient patients had unusually large appetite and a strong tendency towards obesity. Further support for the role of BDNF in appetite regulation was provided by Stanek *et al.* (38) who showed that serum BDNF levels inversely correlated with appetite and weight in otherwise healthy adults. Interestingly, evidence has emerged to implicate microRNAs in the regulation of brain BDNF secretion and action. Pyramidal neurons, the primary source of BDNF in cerebral cortex, express high levels of DICER, an RNAse III endoribonuclease and key molecule for miRNA biogenesis, as well as components of the RNA-induced silencing complex (RISC), such as eIF2c which is involved in the binding of a miRNA to its target mRNA (39,40). Both *miR-30a-5p* and *miR-195* have been shown to target specific sequences surrounding the proximal polyadenylation site within the BDNF 3′-untranslated region on chromosome 11p13. Furthermore, neuronal overexpression of *miR-30a-5p* and *miR-195*, miRNAs enriched in layer III pyramidal neurons, resulted in down-regulation of BDNF protein.

MiRNAs and liver biosynthesis of cholesterol

MiR-122 is a liver-specific miRNA implicated in cholesterol and lipid metabolism, and in hepatitis C virus replication (25,41). Krützfeldt *et al.* provide evidence to support *miR-122* as a key regulator of the cholesterol biosynthetic pathway; in particular they observed that the expression of at least 11 genes involved in cholesterol biosynthesis was decreased between 1.4-fold and 2.3-fold in antagomir-122-treated mice, including hydroxy-3-methylglutaryl-CoA-reductase (Hmgcr), a rate-limiting enzyme of endogenous cholesterol biosynthesis. Observational and functional studies of *miR-122* have highlighted this miRNA as a potential therapeutic target for the treatment of hypercholesterolemia and hepatitis C (25,41). Early antagonism of

miR-122, using locked nucleic acid (LNA) modified DNA oligonucleotides (LNA-anti-miRs), resulted in effective silencing thus inhibiting HCV replication in HuH-7 cells harbouring the HCV-N replicon NNeo/C-5B (42). Silencing of *miR-122* by systemic administration of high affinity LNA anti-miRs has resulted in dose dependent lowering of plasma cholesterol in mice and non-human primates (monkeys), after only three intravenous doses of 3 mg kg⁻¹. Additionally, this was achieved without significant adverse sequelae or hepatic toxicity (41). These findings have unveiled the impending potential of miRNAs as novel therapeutic strategies. Indeed, a phase I safety and pharmacokinetic study of systemic *miR-122* antagonism in humans using an LNA-based antisense molecule against *miR-122* (SPC3649), led by Santaris Pharma, has been completed on 48 healthy volunteers and results are eagerly anticipated (43).

Novel biomarkers of the metabolic syndrome

Current challenges in the management of obesity and its related disorders include a search for unique biomarkers that are reflective or predictive of metabolic health and disease. Metabolic profiling has long been used to facilitate detection of disease states; indeed some of the first attempts to determine biomarkers of disease by global metabolic profiling were applied to the study of inborn errors of metabolism, where the relationship between disease state, genetics and the metabolic biomarker is easily understood. However, in many other metabolic diseases, the relationship between disease, genetics and metabolic state is complex and not readily understood. MiRNAs have been heralded as potential novel biomarkers for many pathological states, consequent to their tissue specific expression and association with clinicopathologic variables (Heneghan HM, Miller N, Lowery AJ, Sweeney KJ, Kerin MJ. *J Oncol* 2009. – in press). Their recent discovery in the circulation has prompted further exploration of their potential use as novel minimally invasive biomarkers of disease (44,45). In a preliminary study investigating this concept, Chen *et al.* have characterized the serum miRNA profile of diabetic patients and found that it differed significantly compared with healthy controls (44). It is unknown how miRNAs make their way into the bloodstream; however, Slack *et al.* raised two hypotheses in a recent report relating to the potential use of circulating miRNAs as tumour markers (46); first that tissue miRNAs may be present in circulation as a result of cell death and lyses, or alternatively that tissue cells actively secrete miRNAs into their microenvironment, where they enter blood vessels, and thereby make their way into the circulation. Future studies in this emerging field of research will provide a better understanding of the mechanisms by which miRNAs are released into the circulation. Further investigations in this field are also warranted to explore the ability of metabolic miRNA profiling to provide non-invasive translational biomarkers to reflect the state and extent of metabolic flux.

Future potential

The association between aberrant miRNA expression and abnormalities in glucose homeostasis, adipogenesis and obesity, and functional analysis of specific miRNAs illustrates the feasibility of using these molecules as targets for therapeutic intervention (47). Antagomirs (cholesterol conjugated anti-sense oligonucleotides) are pioneering targets for miRNAs silencing, as evidenced by hepatic *miR-122* blockade *in vivo* (41). These preliminary data have prompted early clinical trials. Conversely, where miRNA expression is known to be under-expressed (e.g. *miR-103* in obese adipose tissue), induction of miRNA expression using viral or liposomal delivery of tissue-specific miRNAs to affected tissue could potentially result in restoration of catabolic activity to the tissue, although this concept of 'miRNA replacement therapy' has yet to be extrapolated in this setting. Further studies are necessary to examine the efficacy and safety of these novel therapeutic approaches, and to explore the potential for circulating miRNAs to aid in the management of patients with obesity and the metabolic syndrome; however, evidence to date is encouraging (44).

Conclusion

The documented involvement of miRNAs in glucose and lipid metabolism has provided strong evidence in support of their role as key players in the regulation of complex metabolic pathways. Additionally, it indicates potential for novel therapeutic strategies in the management of obesity and the metabolic syndrome. Further dedicated, focused research in this field is imperative to ascertain the full potential of miRNAs as novel metabolic biomarkers and potent therapeutic agents against obesity.

Potential conflicts of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Gene Names and abbreviations.

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