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Determination of serum miRNAs biomarkers of lung cancer by quantitative PCR (qPCR) and by droplet digital PCR (ddPCR)

Determinazione dei livelli sierici dei miRNA come biomarcatori del cancro al polmone mediante PCR quantitativa (qPCR) e droplet digital PCR (ddPCR)

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Summary

Lung cancer (LC) is often diagnosed at an advanced stage, when therapeutic options are limited; patients would greatly benefit from an early diagnosis. A 20% reduction of LC mortality has been shown in high risk individuals undergoing chest computed tomography screening for early diagnosis of LC. However, high radiation exposure, cost/benefit ratio and false positive rates still represent concerns about large scale use of this screening technique. Minimally invasive methods, allowing identification of subjects with early LC risk are urgently needed. MicroRNAs (miRNAs) are short, single-stranded, non-coding RNAs detectable in biological fluids. Levels of specific, circulating cell-free miRNAs have been shown to correlate with disease states, including LC. This suggested their potential application as circulating biomarkers of LC. We hypothesized that the serum level of specific miRNAs could discriminate between subjects with and without LC. Based on a systematic review of the literature, we selected a panel of ten miRNAs (miR-15b-5p, miR-21-5p, miR-27b-3p, miR-126-3p, miR-155-5p, miR-210-3p, miR-221-3p, miR-320a, miR-486-5p, let-7a-5p) and tested if these could discriminate between normal subjects and LC patients.

First, we compared the performance of three methods, namely relative qPCR, absolute qPCR and droplet digital PCR (ddPCR), in accurately measuring the levels of

circulating miRNAs. We found that although all three methods are suitable to this aim, ddPCR provided greater precision and higher throughput of analysis than the other qPCR methods, at a similar cost-per-sample. Moreover, ddPCR does not rely on the use of reference genes or external calibrators.

We then started to characterize the applicability of our miRNA panel to the clinic. Based on sensitivity to hemolysis, we excluded two miRNAs (miR-486 and miR-155). The remaining eight miRNAs were measured by ddPCR in 85 patients with early LC (stage I and II) and 83 controls. Four out of the eight analyzed miRNAs showed significant differences in serum levels between LC patients and controls (let-7a, miR-210, miR-320a: $p<0.0001$; miR-221: $p=0.0119$). For each of these four miRNAs, the Receiver Operating Characteristic (ROC) curve was constructed and the Area Under the Curve (AUC) calculated. AUCs showed fair accuracy in identifying early LC cases (about 0.7 for each miRNA).

In conclusion, ddPCR proved to be a robust method for absolute quantification of miRNA serum levels in LC patients. For four of our miRNAs, putative biomarkers of LC, the AUC showed fair accuracy in identifying early LC cases. Taken together, a combination of these, and possibly additional miRNAs, may aid to identify subjects

who need further investigation for the presence of early LC.

Introduction

Lung cancer epidemiology

Lung cancer is the leading cause of cancer related deaths worldwide [Jemal et al, 2011] with about 1.8 million new cases in 2012 [Globocan, 2012]. In Italy, more than 38,000 new lung cancers are annually diagnosed [AIRTUM, 2015], that heavily impact on the health-care system. The survival rate of lung cancer remains low (10-15% at 5 years from diagnosis), despite recent advances in management and treatment of the disease; advanced stage at diagnosis in about ¾ of lung cancer cases prevents effective treatment and long-term survival. Incidence and mortality are higher in men than in women, although in recent years this gap is gradually closing due to increasing diffusion of smoking habit among women. It is well known that lung cancer incidence parallels the use of tobacco, as cigarette smoking is the most important risk factor for lung cancer. Generally, the diseases is clinically diagnosed in patients 50 to 75 years of age, with over ⅓ of cases diagnosed in people over 70 years old.

Diagnosis of lung cancer sometimes occurs incidentally, with sputum cytology or chest-X-rays carried out for other reasons. However, these exams, when used for lung cancer screening purposes, have demonstrated limited sensitivity and their use has not produced a marked reduction in mortality [Flehinger and Melamed, 1994; Dominioni et al, 2013].

On the other hand, several studies, conducted in high-risk patients (older than 50 years and heavy smokers), using spiral computerized axial tomography (CAT), have reported promising results in identifying lung cancer in asymptomatic patients, when the tumor is small in size and at an early stage: 20% reduction in death risk [The National Lung Screening Trial Research Team, 2011]. Nevertheless, CAT scans present disadvantages, including exposure to radiation, high rate of false positive findings and risk of over-diagnosis. Currently the cost/benefit ratio of CAT screening for lung cancer is debated, therefore the routine use of CAT screening is still an open issue [Strauss and Dominioni, 2013].

Novel, sensitive and non-invasive methods for screening of lung cancer in high risk individuals are greatly needed, so that lung cancer may be identified at an early stage, when the chances of cure are higher.

Lung cancer etiology

Exogenous risk factors

Development of lung cancer results from synergy between individual factors and environmental exposures. Lung cancer has a primary etiologic factor, tobacco smoke, the major toxic agents of which are nicotine, carbon

monoxide, hydrogen cyanide, nitrogen oxides, volatile aldehydes, alkenes, and aromatic hydrocarbons [Hoffmann et al, 2001]. The risk of developing lung cancer is proportional to the duration of the habit and the average number of cigarettes smoked per day. The latter two parameters are combined to give a numerical value called "pack years" that considers lifetime exposure to tobacco smoking.

Conversely, smoking cessation may result in a reduced risk of cancer, which, however, remains relevant for 10-15 years after smoking cessation and still remains higher than in non-smokers, even after 40 years [Alberg et al, 2003]. After 10 years of abstinence, the risk of lung cancer is 30% to 50% lower than that of continuing smokers [Patterson et al, 2008]. This is explained by cellular changes in gene expression, such as the expression of growth factors, induced by prolonged inflammatory smoke stimulus, that determine an hyperproliferation of respiratory epithelium. Smoking cessation will not lead to reversion of existing cellular genetic modifications, such as DNA mutations induced by exposure to mutagens contained in tobacco smoke, but it may delay the development of cancer by avoiding the accumulation of further mutations and epigenetic changes [Fraser et al, 2011].

Endogenous risk factors

Endogenous factors that play a role in the etiology of lung cancer are genetic factors [Chen et al, 2014] and a previous history of lung disease [Alberg and Samet, 2003].

Common genes associated with lung cancer susceptibility are *TP53*, EGFR, and the RAS family. *TP53* is the tumor-suppressor gene most frequently affected by mutations in human cancers. Its protein product is a transcription factor that has been named "guardian of the genome", due to its ability to respond to various types of stress (DNA damage, ROS, hypoxia, etc), by inducing cell cycle arrest, apoptosis and DNA repair, thus preventing accumulation of DNA mutations and eliminating cells with a heavily damaged genome [Williams and Schumacher, 2016; Chen, 2016].

Prevalence of p53 mutations increases from in situ lesions to metastatic carcinomas [Travis et al, 2015].

EGFR is a transmembrane growth factor receptor with tyrosine kinase (TK) activity. Intracellular signaling by EGFR is mediated through different signal transduction pathways, i.e. the RAS-RAF-MEK-MAPK pathway, the PI3K-PTEN-AKT pathway and the STAT pathway. Downstream, EGFR signaling leads to increased proliferation, angiogenesis, metastasis and decreased apoptosis. Mutations in the TK domain result in constitutive and oncogenic activation of EGFR signaling

[da Cunha Santos et al, 2011] and are present in about 10% of NSCLC patients have EGFR mutations [Paez et al, 2004; Lynch et al, 2004].
Mutations in the RAS gene family lead to constitutive activation of the MAPK signal transduction pathway. This in turn promotes cell motility and, consequently, invasiveness and metastatic potential [Campbell and Der, 2014]. Approximately 15-25% of patients with adenocarcinoma have tumor *KRAS* mutations in their tumors, whereas this lesion is uncommon in squamous cell carcinoma [Chen et al 2014; Brose et al 2002].

A positive past medical history of pulmonary disorders, such as diffuse interstitial pulmonary fibrosis and chronic obstructive pulmonary disease (COPD), is associated with an increased susceptibility to lung cancer. In the case of diffuse interstitial pulmonary fibrosis, the increased risk of lung cancer development is linked to the higher proliferative activity of metaplastic tissue and focal hyperplasia present in association with the areas of fibrosis. Instead, the cause for the increased risk for cancer in COPD is likely due to the fact that COPD and lung cancer share a common etiological factor, chronic oxidative stress, suffered by airway walls during the course of chronic pulmonary disease [Spiro, 1997; Alberg and Samet, 2003; Fraser et al, 2011].

Lung cancer subtypes

Lung cancer is a heterogeneous disease both at the histological and at the molecular level. Based on morphology, two main types of lung cancer are identifiable: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) [Travis et al, 2015]: NSCLC comprises more than 80% of cases [Davidson et al, 2013; Langer et al, 2010; Chen et al, 2014] and it can be further subdivided into three subtypes: adenocarcinoma (ADC) (50%), squamous cell carcinoma (SQCC) (40%) and large cell carcinoma (LCC) (10%).

Small Cell Lung Cancer

SCLC consists of small cells with ill-defined cell borders, scant cytoplasm, finely granular nuclear chromatin and absent or inconspicuous nucleoli. The cells are round, oval or spindle-shaped. Nuclear molding is prominent. Necrosis is typically extensive and the mitotic count is high [Travis et al, 2015]. At the time of diagnosis, approximately 30% of patients with SCLC will have tumors confined to the mediastinum or the supraclavicular lymph nodes and they are designated as having limited-stage disease. Patients with tumors that have spread beyond the supraclavicular areas are said to have extensive-stage disease. SCLC is more responsive to chemotherapy and radiotherapy than

other types of lung cancer; however cure is difficult because SCLC is often disseminated by the time of diagnosis [National Cancer Institute: PDQ® Small Cell Lung Cancer Treatment].

Non-Small Cell Lung Cancer

NSCLC is any type of epithelial lung cancer other than SCLC. It is a heterogeneous group of diseases that comprises three major histological subtypes: ADC, SQCC and LCC.

ADC is a malignant epithelial tumor with glandular differentiation or mucin production, showing acinar, papillary, bronchioloalveolar or solid with mucin growth patterns or a mixture of these patterns [Travis et al, 2015; Travis et al, 2011] with different classification for resected tumors. The lesion is, in general, peripheral and of smaller size compared to other subtypes. It accounts for about 50% of NSCLC cases [Chen et al, 2014].

SQCC originates predominantly in proximal airways and is more strongly associated with smoking and chronic inflammation than ADC. It is characterized by keratinization that may take the form of squamous pearls or by individual cells with markedly eosinophilic dense cytoplasm [Chen et al, 2014; Travis et al, 2015]. It accounts for about 40% of all NSCLCs [Chen et al, 2014].

LCC is the less frequent tumor histotype among NSCLCs and it is diagnosed after ruling out the presence of cells or biomarkers characteristic of ADC, SQCC or SCLC. LCC is an undifferentiated NSCLC that lacks the cytological and architectural features of small cell carcinoma and glandular or squamous differentiation. Its cells typically have large nuclei, prominent nucleoli and a moderate amount of cytoplasm [Chen et al, 2014; Travis et al, 2015].

Lung cancer circulating biomarkers

Biomarkers are defined as molecules that can discriminate between a specific condition and normal status. Cancer biomarkers are biomolecules of various nature (proteins, genetic material, oligosaccharides, lipids, metabolites) that can be used for different medical purposes: diagnosis, prediction/staging, prognosis, treatment [I and Cho, 2015].

Determining the expression of a lung cancer biomarker in body fluids can be a convenient and non-invasive method for screening and diagnosis of a certain disease. Currently, selected serum biomarkers are used in clinical practice as ancillary methods for lung cancer detection but their clinical utility is hampered by limited sensitivity and/or specificity. Examples of commonly available lung

cancer biomarkers are: Cytokeratin 19 Fragment (CYFRA 21-1) [Schneider et al, 2000; Xu et al, 2015], Carcinoembryonic Antigen (CEA) [Schneider et al, 2000; Wang XB et al, 2014], Squamous Cell Carcinoma Antigen (SCC-Ag) [Schneider et al, 2000; Yu et al, 2013], Neuron-Specific Enolase (NSE) [Schneider et al, 2000; Wang B et al, 2014], Progastrin-Releasing Peptide (ProGRP) [Kim et al, 2011] and Epidermal Growth Factor Receptor (EGFR) [Romero-Ventosa et al, 2015].

CYFRA 21-1 is a cytokeratin 19 fragment found in epithelial and bronchial tree cancer and it is typically associated with SQCC.

CEA is an oncofetal protein normally produced in the gastrointestinal tissue during fetal development but not expressed in adult tissues. The levels of this protein in lung cancer are elevated and inversely correlated with response to cancer therapy.

SCC-Ag is a cytoplasmic structural protein that is elevated in NSCLC patients, particularly in patients whose tumors have high metastatic potential.

NSE is an isoenzyme of the glycolytic enzyme enolase that is present in cells with neuroendocrine differentiation. Indeed, its levels are frequently increased in SQCLC patients.

ProGRP is a precursor of GRP, which is produced by the neuroendocrine cells of SCLC; for this reason, its levels are higher in SCLC than in NSCLC.

Finally, EGFR mutations can be used as markers to predict the efficacy of treatments targeting EGFR. Mutations in the *EGFR* gene are routinely tested to identify patients who can benefit from treatment with tyrosine kinase inhibitors.

However, the cited biomarkers are not specific for the diagnosis of lung cancer; their altered levels only suggest the possible presence of cancer.

MicroRNAs

MicroRNAs (miRNAs) are endogenous, 19-24 nucleotides long, non-coding, single-stranded, RNA molecules. They mediate post-transcriptional gene silencing by inducing mRNA degradation or by suppressing translation initiation [Garzon et al, 2010; Krol et al, 2010]. MiRNA genes are transcribed into primary transcripts (pri-miRNA) by RNA polymerase II [Lee et al, 2004], or, in some cases, by RNA polymerase III [Borchert et al, 2006]. Pri-miRNA are processed by the RNase III enzyme Drosha to form a miRNA precursor (pre-miRNA) and then pre-miRNA are transported in the cytosol and processed by another RNase (Dicer) to form a mature miRNA. Mature miRNAs are incorporated into RNA-induced silencing complex (RISC) that can regulate the gene expression through different mecchanisms (Fig. 1).

Several studies demonstrated that miRNA targeting genes involved in cell cycle progression and differentiation are often down-regulated within tumor cells, while others, regulating the expression of genes involved in cell cycle progression and resistance to apoptosis, are up-regulated [Iorio et al, 2012 (a)]. MiRNAs are also present in blood and other biofluids. Circulating miRNAs are either stored in microparticles (exosomes, microvesicles and apoptotic bodies) [Zernecke et al, 2009; Valadi et al, 2007] or are associated with RNA-binding proteins [Arroyo et al, 2011] or lipoproteins [Vickers et al, 2011] that prevent their degradation. The abundance and variety of circulating miRNAs suggest a role in cell-cell communication [Zhang et al, 2010]. Circulating cell-free miRNAs have been proposed as a promising class of biomarkers due to their stability in biofluids; because miRNA levels are often altered in various diseases, including cancer, a potential application of miRNAs in disease diagnosis and prognosis has been proposed. [Esquela-Kerscher et al, 2006; Lujambio and Lowe, 2012; Iorio et al, 2012 (b); Negrini et al, 2014; Schetter et al, 2008; Pritchard et al, 2012 (a)].

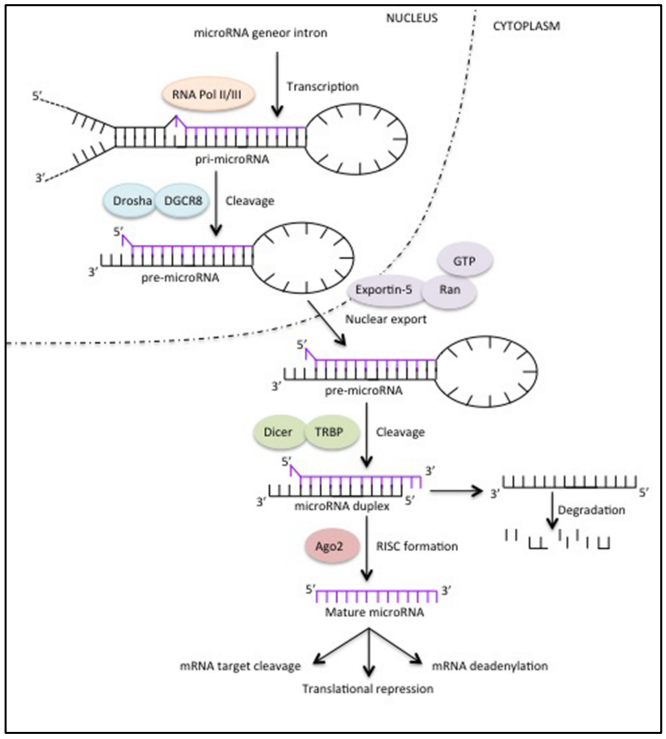

Figure 1. MiRNA biogenesis and mechanism of function. (Modified from Winter et al, 2009).

MiRNAs role in lung cancer

MiRNA loci map to genomic regions commonly amplified or deleted in human cancers [Gaur et al, 2007]. Furthermore, miRNAs control several biological processes including cell proliferation, apoptosis and differentiation [Calin and Croce, 2006].

Uncontrolled proliferation is a crucial step in cancer progression and, for example, it has been shown that miR-192 overexpression in NSCLC cell lines A549 and

H460 inhibits cell proliferation and carcinogenesis *in vivo* [Feng et al, 2011]. Importantly, miRNAs play a different role depending on the cellular context; indeed miR-34a has been correlated to prostate cancer inhibition [Liu et al, 2011], but it did not influence SCLC cells [Lee et al, 2011].

Defects in programmed cell death (apoptosis) are an important causal factor of development and progression of cancer. There are two apoptosis activation mechanisms: the extrinsic pathway and the intrinsic pathway (also called the mitochondrial pathway). The extrinsic pathway is mediated by death receptors that are activated after binding ligands. TNF-α is one of these ligands and it is target for miR-19a overexpressed in different tumors, including lung cancer [Liu et al, 2011]. The intrinsic pathway is characterized by release of cytochrome c from the mitochondrial intermembrane space into the cytosol. There are miRNAs that regulate pro- or antiapoptotic protein (Bcl-2 proteins family); for example, miR-503 decreases antiapoptotic Bcl-2 protein in NSCLC A549 cells [Qiu et al, 2013]. MiRNAs can also affect expression and activation of effector caspases; for example, miR-1 in A549 cells enhances activation of caspase-3 and 7 [Nasser et al, 2008].

During tumor growth, the center of the mass tends to be hypoperfused, and the reduction in oxygen levels reduction promotes angiogenesis. Let-7b and miR-126

levels are decreased in lung tumor tissue, and an antiangiogenic role in lung cancer has been proposed for these miRNAs [Jusufovic et al, 2012]; overexpression of miR-16 has been shown *in vitro* to reduce the ability of endothelial cells to form blood vessels [Chamorro-Jorganes et al, 2011].

MiRNAs as biomarkers of lung cancer

Several studies have reported that miRNAs are aberrantly expressed in circulating blood, leading to consider them as potential biomarkers [Ono et al, 2015; Ulivi et al, 2014]. Unfortunately the results of these studies are highly discordant and there is no overlap in the miRNA profiles proposed by various authors for the diagnosis of early lung cancer, even in studies using similar biological material [Ono et al, 2015; Ulivi et al, 2014].

Despite promising initial results, miRNA profiling of lung cancer proved to be more challenging than expected, as many pre- and post-analytical variables heavily impact on the findings. Thus, the discrepancy in results published in different studies may be attributable to:

- differences in sample material preparation and in the methodology applied for miRNA determination (microarray, RT-qPCR, droplet digital-PCR) [Ono et al, 2015];

- differences in human populations investigated (prevalence of Chinese studies compared to studies in western countries);
- differences in patient cohorts (lung cancer stage distribution) [Gyoba et al, 2016; Ulivi et al, 2014];
- small sample size [Gyoba et al, 2016; Ulivi et al, 2014].

The use of circulating, cell-free, miRNAs as biomarkers for early diagnosis of lung cancer may reasonably be proposed under three conditions: 1) an accurate and reproducible method for miRNA quantification is available; 2) the biomarkers are detectable in the specified sample material in the early stage of the disease; 3) the proposed miRNA signature of lung cancer is confirmed by external validation studies.

Aim of the study

The aims of this PhD thesis are:

1) to find a reliable method for quantification of serum levels of selected miRNAs of interest for human lung cancer. For this purpose three different techniques of miRNA measurement were compared: relative qPCR, absolute qPCR and droplet digital PCR (ddPCR).
2) to quantify and evaluate miRNAs of interest in a prospective study comparing miRNA levels in patients with early NSCLC and in controls (age-matched smokers with no evidence of cancer).

Materials and methods

Study design

The initial approach to this study was the identification of a panel of miRNAs that may help discriminating lung cancer patients from smokers with no evidence of this disease. For this purpose, as a preliminary step, two systematic reviews of the pertinent miRNA literature were performed, the first on January 30, 2014 and the second on March 19, 2015, using the following search terms: diagnosis AND lung AND neoplasm [mesh] AND (circulating OR serum OR blood OR plasma) AND (microRNA* OR miR-*). After completion of this literature review on miRNA aberrantly expressed in lung cancer patients' biofluids, 10 miRNAs were initially chosen to be quantified in this study, based on high-quality papers reporting their potential use as biomarkers of lung cancer: **hsa-miR-15b-5p** [Boeri et al, 2011; Hennessy et al, 2012], **hsa-miR-21-5p** [Boeri et al, 2011; Geng et al, 2014; Hu et al, 2010; Le et al, 2012; Ma et al, 2013; Markou et al, 2013; Mozzoni et al, 2013; Qi et al, 2014; Shen et al, 2011 (a), Shen et al, 2011 (b); Tang et al, 2013], **hsa-miR-27b-3p** [Hennessey et al, 2012], **hsa-miR-126-3p** [Bianchi et al, 2011; Markou et al, 2013; Sanfiorenzo et al, 2013; Shen et al, 2011 (a)], **hsa-miR-155-5p** [Heegard et al, 2012; Sanfiorenzo et al, 2013; Zheng et al, 2011], **hsa-miR-210-3p** [Boeri et al, 2011; Shen et al, 2011 (a), Shen et al, 2011 (b)], **hsa-miR-**

221-3p [Boeri et al, 2011; Chen et al, 2012; Geng et al, 2014; Heegard et al, 2012], **hsa-miR-320a** [Chen et al, 2012; Sanfiorenzo et al, 2013], **hsa-miR-486-5p** [Bianchi et al, 2011; Boeri et al, 2011; Hu et al, 2010; Mozzoni et al, 2013; Shen et al, 2011 (a), Shen et al, 2011 (b)], **hsa-let-7a-5p** [Bianchi et al, 2011; Hu et al, 2010; Jeong et al, 2011; Heegard et al, 2012; Kang et al, 2013].

The study design developed in two parts:

Part 1. Comparison of reproducibility and precision of methods for miRNA quantification.

A methodological study was carried out to compare the reproducibility and precision of three different methods currently available for quantification of miRNAs in serum samples: 1) relative quantification by qPCR [Marabita et al, 2016]; 2) absolute quantification by qPCR [Hindson et al, 2013]; 3) absolute quantification by ddPCR [Hindson et al, 2013; Ferracin et al, 2015].

Part 2. Case-control study

A case-control experiment was performed to compare the level of a panel of selected circulating miRNAs in patients with early stage NSCLC and in controls.

Ethics statement

The Varese University Hospital Ethics Committee approved this study (Protocol approval n. 37527). All participants were volunteers and provided informed consent to use their samples for research purposes. Research was carried out in compliance with the Helsinki Declaration.

Samples and miRNAs used in this study

Peripheral blood samples (5 mL) were obtained by venipuncture from 168 volunteer adult subjects: 85 therapy-naïve patients with early NSCLC (stage I and II) [Rami-Porta et al, 2009] of both genders (mean age, 68±9 SD years; male/female ratio 3.3:1), and 83 controls (asymptomatic smokers undergoing check-up evaluation; mean age, 62±6.8 SD years; male/female ratio 2.3:1). The samples, both patients and controls, were collected between 2014 and 2016. Sample size calculation [MedCalc Statistical Software V.13.3.3 (MedCalc Software bvba, Ostend, Belgium)] was based on our preliminary experiments of measurements of serum levels of let-7a and miR-21, that are among the most frequently measured miRNAs. Accordingly, a sample size of at least 63 lung cancers and 63 controls was calculated as being required, based on α-error of 0.05, a power of 80% and

considering a difference of 15 copies/uL between mean values of lung cancer cases and controls to be relevant [assuming standard deviation of 30 copies/uL (twice the difference between mean values of lung cancer cases and controls)]. The planned sample size of 63 lung cancers and 63 controls was cautiously increased to 85 lung cases and 83 controls, considering possible errors and underestimated intra-group variability.

Serum preparation and RNA extraction

Peripheral blood was collected using sterile tubes without anticoagulant, with clot activator and gel for serum separation (BD Vacutainer®, Milan, Italy), and left at room temperature (R.T.) to coagulate, from a minimum of 30 to a maximum of 60 minutes; then serum was separated by centrifugation at 800 g for 8 min at R.T. Serum was divided in 500 µL aliquots and stored at -80°C until further processing.

Purification of total RNA, including miRNAs, was performed using the miRNeasy serum/plasma kit (Qiagen, Milan, Italy), starting from 200 µL of serum and following manufacturer's instructions. One µg of MS2 phage carrier RNA (Roche, Monza, Italy) and 1 µL of a mix of UniSp2, UniSp4 and UniSp5 spike-ins (Exiqon, Euroclone, Milan, Italy) were added in the Qiazol reagent just before the

purification process, to assess the efficiency of RNA purification and the presence of possible PCR inhibitors. RNA was eluted from the column with 14 µL of nuclease-free water and stored at -80°C. As the low amounts of RNA extracted from serum samples make the measurement of RNA concentrations to be used for retrotranscription and quantification of specific miRNAs unreliable, we conducted our analysis working with constant volumes for the whole procedure (blood, serum, RNA, RT and PCR).

Reverse Transcription

Two µL of RNA extracted from each sample was reverse transcribed into cDNA in 10 µL total reaction, using the Universal cDNA synthesis kit II, part of the miRCURY LNA™ Universal RT microRNA PCR system (Exiqon, Euroclone, Milan, Italy), according to the manufacturer's instructions. When assembling the reactions, 0.5 µL of UniSp6 and cel-miR-39-3p spike-ins were added for subsequent evaluation of efficiency of the reverse transcription step. Four µL of the cDNA were prediluted 4-fold and stored at -20°C until use.

The quality of extraction and retrotranscription were monitored by evaluation of two spike-ins: UniSp5 and UniSp6.

RT-qPCR

The four-fold diluted cDNAs were diluted a further 10 fold and 4 µL were used in each 10 µL qPCR reaction, completed with the addition of 6 µL of reaction mixture, composed of 1 µL of the specific miRCURY LNA PCR primer set and 5 µL of ExiLENT SYBR Green master mix (both from Exiqon-Euroclone, Milan, Italy). All reactions were performed in triplicate. A CFX96 realtime PCR instrument (Biorad, Milan, Italy) was used, following the manufacturer's instructions for cycling conditions [95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s and 60°C for 1 min (1.6 °C/s ramp rate)].

Evaluation of hemolysis

The samples were checked for the risk of hemolysis, a process that is known to affect determination of specific miRNAs [Pritchard et al, 2012 (b)]. As indicated by the Exiqon guidelines, we calculated the difference between the Cq values of hsa-miR-23a-3p and hsa-miR-451a, where hsa-miR-451a is a miRNA highly expressed in red blood cells whereas hsa-miR-23a-3p is a free miRNA unaffected by hemolysis [Exiqon guidelines: Profiling of microRNA in serum/plasma and other biofluids]. Samples were considered at risk of hemolysis when their ΔCq (miR-

23a - miR-451a) was > 5 and in this case they were not included in analysis.

Relative quantification of miRNAs by RT-qPCR

Throughout this study the "sample maximization approach" was used, that is the analysis of few miRNAs and several samples at a time. Each plate included a "no template control" (NTC) for each mix and an interplate calibrator (IPC). The latter was used to correct Cq values for inter-run variations [$Cqnorm = CqGOI - \frac{1}{m}\sum_{j=1}^{n}(CqIPCj - \frac{1}{n}\sum_{i=1}^{n} CqIPCi)$] where GOI is the gene of interest, m is the number of interplate calibrators in run "m", and n is the total number of interplate calibrators [Exiqon guidelines: Profiling of microRNA in serum/plasma and other biofluids]. Data normalization for miRNA analysis should be performed using endogenous reference miRNAs, whose levels are not affected by the samples and conditions. However, while this is easily performed when analyzing intracellular miRNAs, it is more difficult in the case of serum samples, as circulating miRNAs likely derive from several, heterogeneous sources and there is a lack validated and reliable reference miRNA.

In preliminary experiments we attempted to normalize the data using the Cqs of three putative endogenous

reference miRNAs (hsa-miR-103a-3p, hsa-miR-423-5p, hsa-miR-191-5p). Unfortunately, these miRNAs turned out to be well expressed yet quite variable from sample to sample. Therefore, the normalization was performed using the UniSp5 spike-in and values were expressed as 2^-DCq (Cq miRNA of interest, corrected for IPC – Cq UniSp5, corrected for IPC).

Standard curve construction and miRNAs absolute quantification with RT-qPCR

For the purpose of standard curve construction, three unmodified oligoribonucleotides corresponding to hsa-miR-21-5p (UAGCUUAUCAGACUGAUGUUGA), hsa-miR-126-3p (UCGUACCGUGAGUAAUAAUGCG) and hsa-let-7a-5p (UGAGGUAGUAGGUUGUAUAGUU) were synthesized and provided by Eurofins Genomics (Milan, Italy). Different dilutions of oligoribonucleotides in RNase-free water were prepared and the appropriate dilution (6 x 10^4 copies) was reverse transcribed to cDNA in 10 µL total reaction, using the Universal cDNA synthesis kit II, as part of the miRCURY LNA™ Universal RT microRNA PCR system (Exiqon, Euroclone, Milan, Italy). A two-fold dilution series over nine points were prepared from the cDNA, starting from a dilution at 2000 copies/µL, then the nine dilutions were used as templates for qPCR. Each point was

performed in triplicate. The standard curve was constructed by plotting Cq values against the logarithmic concentration of the calibrator oligoribonucleotides. The amount of an unknown sample was quantified by interpolating the Cq values in the standard curve.

MiRNA absolute quantification by ddPCR

The ddPCR method was applied using the QX200™ Droplet Digital™ PCR System (Biorad, Milan, Italy), as described in detail by Campomenosi et al. [Campomenosi et al, 2016].

The four-fold diluted cDNAs were diluted further 10 fold and were used in each ddPCR reaction, adding the desired miRCURY LNA PCR primer set at the appropriate dilution (experimentally determined by testing two different volumes of cDNAs and primers) (Table 1), 10 µL of QX200 EvaGreen ddPCR Supermix (Biorad, Milan, Italy) and nuclease-free water up to 20 µL. Each 20 µl ddPCR reaction was loaded into an 8-channel droplet generation cartridge (Biorad, Milan, Italy); 70 µL of QX200 Droplet generation oil (Biorad, Milan, Italy) were added into the appropriate wells and the cartridge was loaded in the QX200™ Droplet Generator (Biorad, Milan, Italy) to generate the emulsion. The resulting droplets were transferred to a 96-well plate (Eppendorf) with a Rainin

multichannel pipette, the plate sealed with Pierceable foil (Biorad, Milan, Italy) and amplified by standard PCR using a T100™ Thermal Cycler (Biorad, Milan, Italy). Cycling conditions were: 95°C for 5 min, followed by 40 cycles of 95°C for 30 s and 60°C for 1 min, followed by signal stabilization steps (4°C for 5 min, 90°C for 5 min) and final hold at 4°C. The ramp rate was 2°C/s. After PCR, plates were loaded into QX200™ Droplet Reader (Biorad, Milan, Italy) for detection.

Table 1. Conditions for ddPCR

Candidate miRNAs under study	Primer volume	cDNA volume
hsa-miR-15b-5p	1 µL	2.5 µL
hsa-miR-21-5p	1 µL	2.5 µL
hsa-miR-27b-3p	1 µL	2.5 µL
hsa-miR-126-3p	1 µL	2.5 µL
hsa-miR155-5p	1 µL	5 µL
hsa-miR-210-3p	0.5 µL	5 µL
hsa-miR-221-3p	0.5 µL	2.5 µL
hsa-miR-320a	0.5 µL	2.5 µL
hsa-miR-486-5p	1 µL	5 µL
hsa-let-7a-5p	1 µL	2.5 µL

Statistical analyses

Correlation between the qPCR (absolute and relative) and the ddPCR output analyses was tested by linear regression model. The precision of miRNA measurements was estimated with the Coefficient of Variation [CV=(SD/mean)*100] of quadruplicate measures for each sample, for both qPCR (each point was the mean of three technical replicates) and ddPCR. The CVs of the two assays were compared by t-test for paired data. Differences between control and lung cancer patients were tested by Mann-Whitney test, the Receiver Operating Characteristic (ROC) curve was constructed and the Area Under the Curve (AUC) calculated. A p value <0.05 was considered statistically significant. Data were analyzed with SPSS 10.6 software (Illinois, USA).

Reproducibility and precision of miRNA assays by ddPCR

We performed a panel of experiments to verify the reproducibility and precision of miRNA measurements performed by ddPCR. In particular, we tested:

- reproducibility of assay, starting from RNA (repeating retrotranscription) (four samples, six miRNAs);
- reproducibility of assay, starting from the same cDNA (multiple freeze-thaw cycles) (eight samples, eight miRNAs);
- precision of ddPCR replicate assays (ten samples, eight miRNAs).

Results

REPRODUCIBILITY AND PRECISION OF MIRNA QUANTIFICATION METHODS

To assess the reproducibility and precision of miRNA quantification approaches, two experiments were carried out. In the first (Fig. 2A), 15 serum samples (7 from patients; 8 from control individuals) were used to compare the precision of miRNA measurements performed with "relative" qPCR, "absolute" qPCR and droplet digital PCR (ddPCR), and to assess the correlation of measurements obtained with the three methods. In the second experiment (Fig. 2B) we investigated the correlation between the three methods of miRNA analysis in a larger number of samples (70 samples: 35 from patients, 35 from controls).

For comparison of the different quantification methods listed above, we used three miRNAs that, according to the literature, are aberrantly expressed in lung cancer patients: hsa-miR-21-5p, hsa-miR-126-3p and hsa-let-7a-5p [Campomenosi et al, 2016].

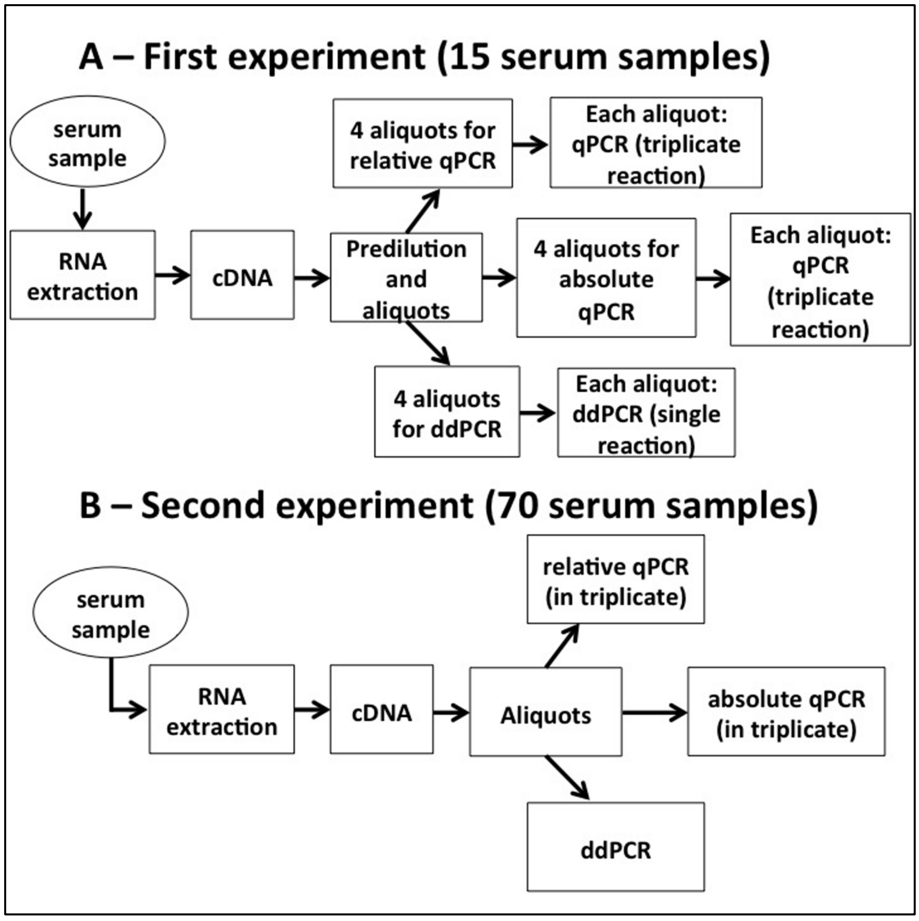

Figure 2. Workflow of the experiments.

A) In the first experiment, for determination of each miRNA of interest (miR-21, miR-126 and let-7a) in each of 15 serum samples, we performed 4 independent "relative" qPCRs, 4 independent "absolute" qPCRs and 4 independent ddPCRs. B) In the second experiment, the miRNAs of interest were measured with "relative" qPCR, "absolute" qPCR and ddPCR in 70 serum samples. All qPCRs were run in triplicate; ddPCRs were run as single reaction.

Comparison between relative RT-qPCR and ddPCR

In the first experiment (Fig. 2A), each of the four analyses in qPCR was done in triplicate, for a total of 180 amplifications. We found that the trend of expression of miRNAs under study was similar with that found with ddPCR, as can be seen by comparative inspection of scatter plots in Fig. 3; however, the dispersion of the values was higher for qPCR.

Indeed the precision, as measured by the Coefficient of Variation (CV), was significantly greater for ddPCR compared to qPCR when analyzing miR-21 ($p=0.047$), while there was no difference in precision between the two techniques for miR-126 ($p=0.072$) and let-7a ($p=0.079$) (Table 2).

The correlation of qPCR and ddPCR values was statistically significant (Fig. 4). In detail, R-squared values were 0.980 for miR-21, 0.983 for miR-126 and 0.978 for let-7a ($p<0.0001$ for all analyses). Notably, the time needed for a complete set of analyses was about 4-fold shorter with the ddPCR system compared to "relative" qPCR.

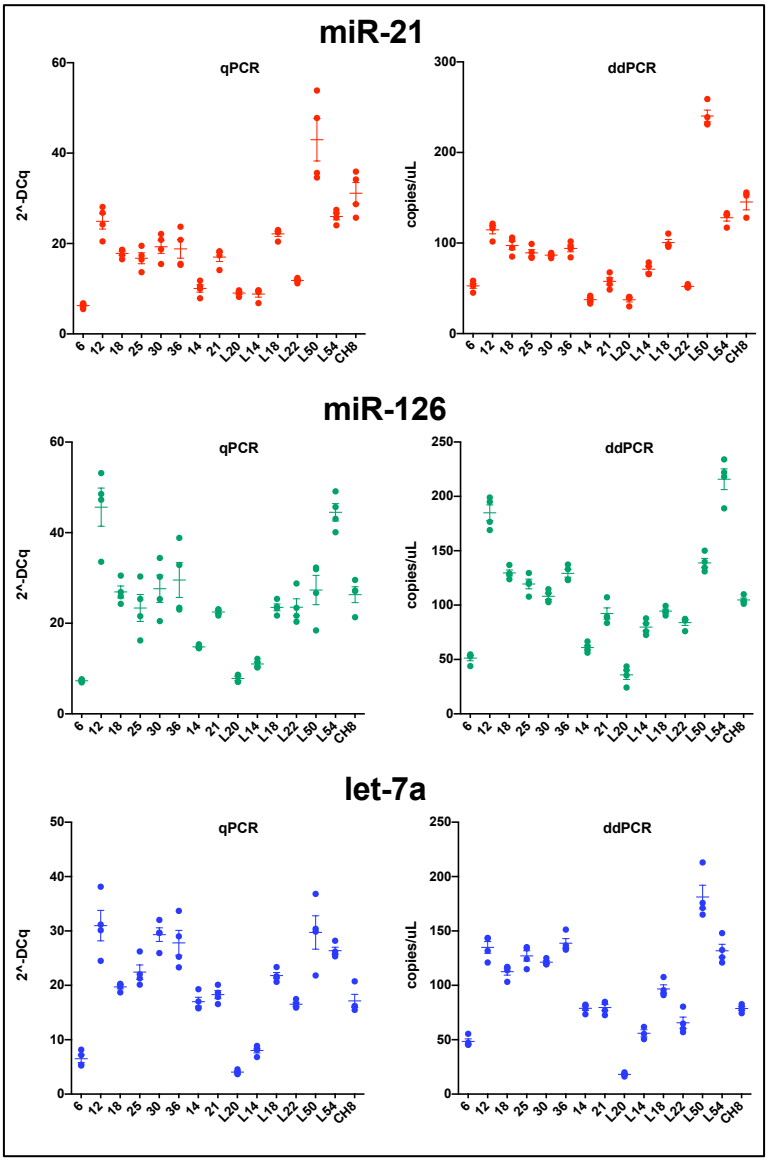

Figure 3. Scatter plots showing the distribution of expression of the selected miRNAs by "relative" qPCR (panels on the left) and ddPCR (panels on the right). For each of the fifteen samples the results of the four analyses is reported, together with the mean and standard deviation. (2^-DCq=(Cq miRNA of interest, corrected for IPC – Cq UniSp5, corrected for IPC))

Table 2. Mean and standard deviation of Coefficients of Variation of miR-21, miR-126 and let-7a determinations with "relative" qPCR and ddPCR.

		N	Coefficient of Variation		p*
			Mean	Std. Deviation	
Pair 1	miR-21 qPCR	15	12.129	5.827	0.047
	miR-21 ddPCR	15	8.319	3.221	
Pair 2	miR-126 qPCR	15	13.221	8.187	0.072
	miR-126 ddPCR	15	7.948	4.859	
Pair 3	let-7a qPCR	15	11.198	5.871	0.079
	let-7a ddPCR	15	7.992	3.265	

* paired samples t-test

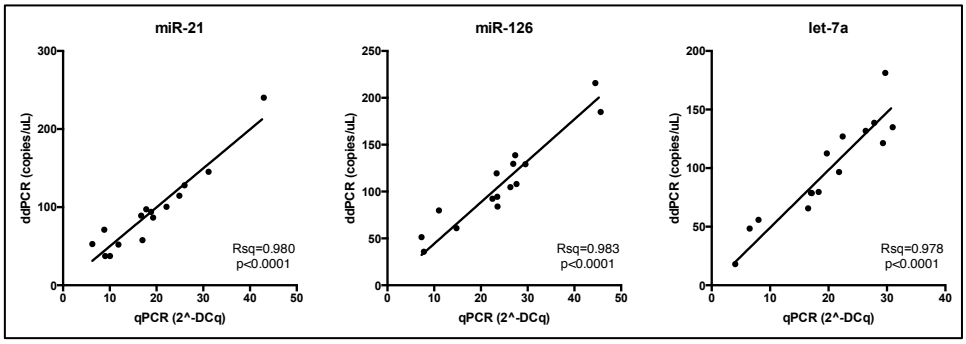

Figure 4. Correlation analysis between "relative" qPCR and ddPCR data for the first experimental set, consisting of 15 samples analyzed 4 times with each technique.

In the second set of experiments (described in figure 2B) the correlation between the output data of the two

methods ("relative" qPCR and ddPCR) was still significant (see fig. 5), although the R-squared were lower than in the previous set of experiments (Fig. 5). Indeed, R-squared values were 0.536, 0.631 and 0.597 for miR-21, miR-126, let-7a, respectively.

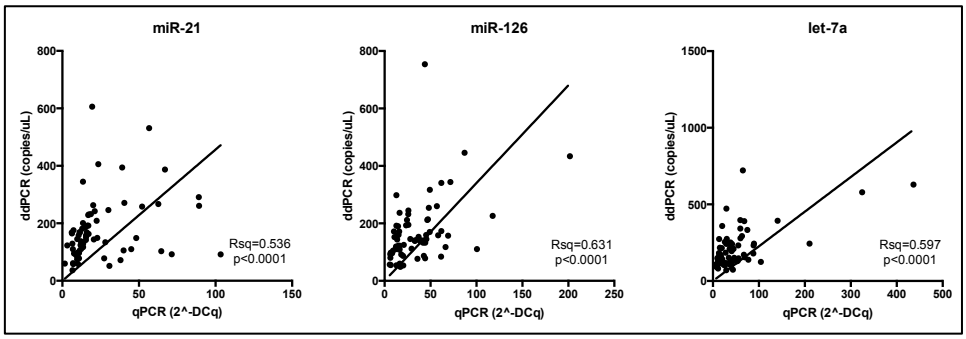

Figure 5. Correlation analysis between "relative" qPCR and ddPCR data for the second experimental set, consisting of 70 samples analyzed with each technique.

Comparison between absolute RT-qPCR and ddPCR

As for the comparison between "relative" qPCR and ddPCR, we found that the trend of miRNAs expression was similar also when comparing "absolute" qPCR and ddPCR. Notably, for miR-126 and let-7a, the samples showed higher values in qPCR then in ddPCR: the estimated copy numbers in qPCR were approximately 2.4 and 3.9 fold greater, respectively, than in ddPCR (Fig. 6).

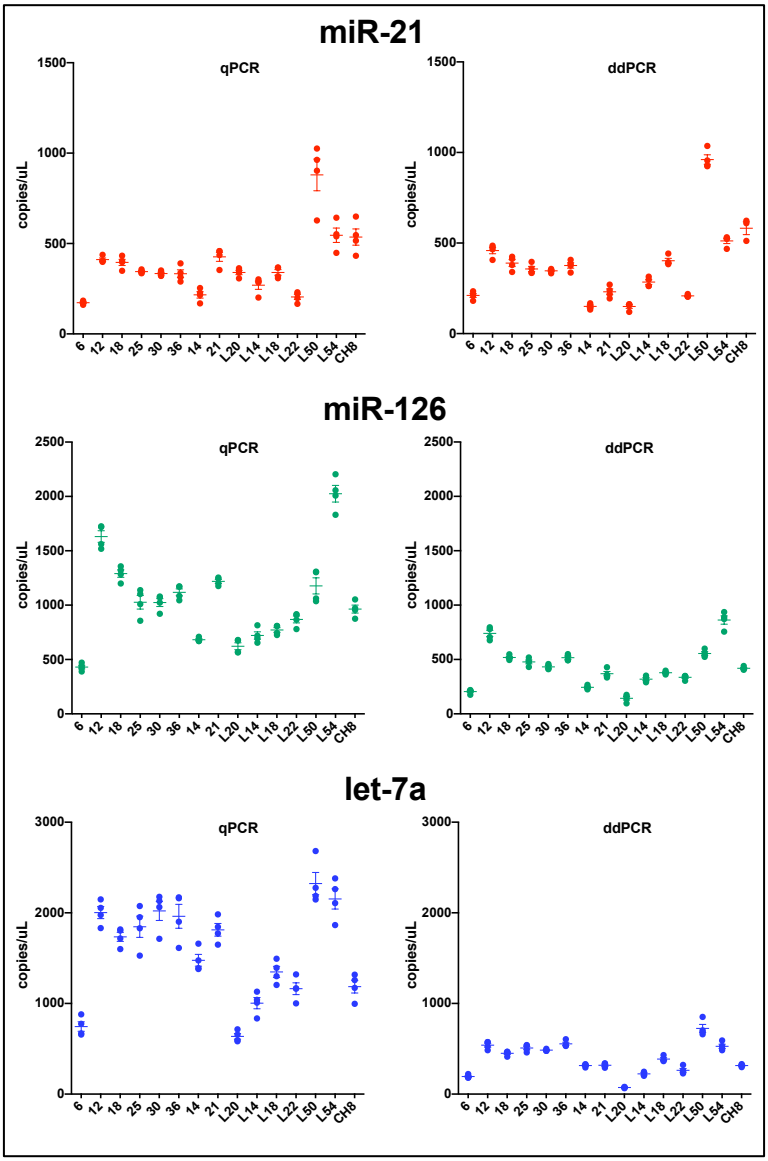

Figure 6. Scatter plots showing the distribution of expression of the selected miRNAs by "absolute" qPCR (panels on the left) and ddPCR (panels on the right). For each of the fifteen samples the results of the four analyses is reported, together with the mean and standard deviation.

(Modified from Campomenosi et al, 2016)

In the first experiment of comparison between "absolute" qPCR and ddPCR (Figure 2A), the precision of miRNA quantification, as measured by the CV, was significantly better for ddPCR compared to qPCR for let-7a (p=0.028), while it was not significantly different for miR-21 and miR-126 (Table 3).

Table 3. Mean and standard deviation of Coefficients of Variation of miR-21, miR-126 and let-7a determinations with "absolute" qPCR and ddPCR.
(Modified from Campomenosi et al, 2016)

		N	Coefficient of Variation		p*
			Mean	Std. Deviation	
Pair 1	miR-21 qPCR	15	11.040	5.4387	0.123
	miR-21 ddPCR	15	8.319	3.2207	
Pair 2	miR-126 qPCR	15	7.386	2.8837	0.675
	miR-126 ddPCR	15	7.944	4.8446	
Pair 3	let-7a qPCR	15	10.298	2.4077	0.028
	let-7a ddPCR	15	7.992	3.2646	

* paired samples t-test

Linear regression analysis indicated a significant correlation between "absolute" qPCR and ddPCR values (Fig. 7). R-squared values were 0.963 for miR-21, 0.984 for miR-126 and 0.978 for let-7a (p<0.0001 for all

regressions). However, the slope (b) of the regression line for miR-126 (b=0.420 [CI95% 0.391-0.452]) and let-7a (b=0.2561 [CI95% 0.234-0.278]) was significantly lower than one, due to the lower number of copies measured by ddPCR as compared to what estimated with the external calibrator in qPCR.

These systematic differences, characterized by higher values of "absolute" qPCR compare to ddPCR measurements of miRNAs will be addressed in the discussion of the thesis.

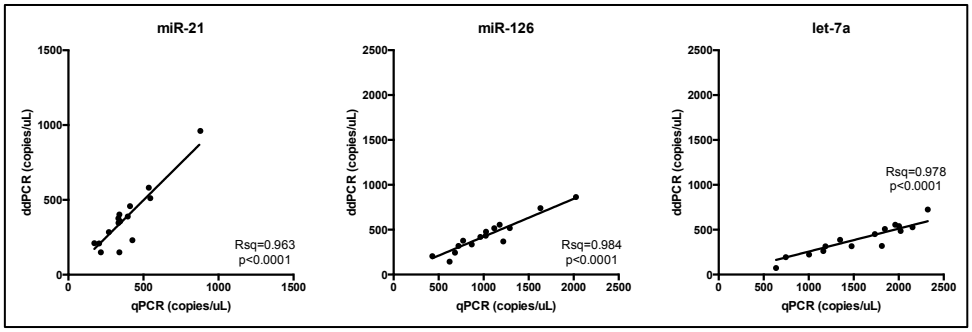

Figure 7. Correlation analysis between "absolute" qPCR and ddPCR data for the first experimental set, consisting of 15 samples analyzed 4 times with each technique. (Modified from Campomenosi et al, 2016)

In the second experiment (Figure 2B), repeated on the cohort of 35 samples from patients and 35 from matched controls to compare "absolute" qPCR to ddPCR, again we found significant correlation between "absolute" qPCR and

ddPCR values (R-squared=0.948 for miR-21, 0.954 for miR-126 and 0.949 for let-7a; p<0.0001 for all regressions) (Fig. 8). Consistently, the slopes for miR-126 and let-7a were confirmed to be significantly lower than 1 (b=0.695 [CI95% 0.658-0.731] and b=0.347 [CI95% 0.328-0.366], respectively) (Fig. 8).

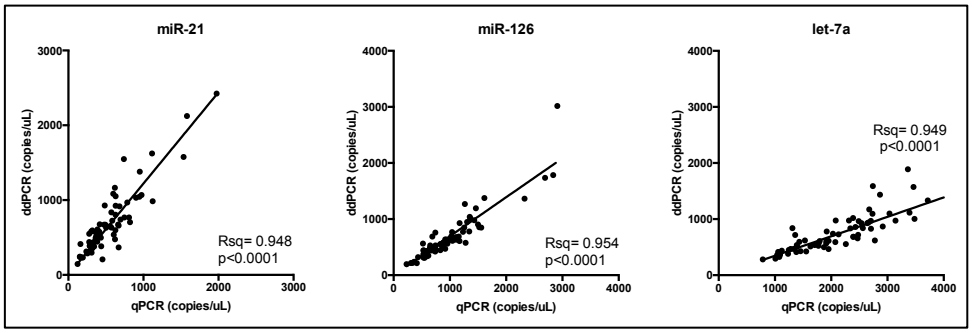

Figure 8. Correlation analysis between "absolute" qPCR and ddPCR data for the second experimental set, consisting of 70 samples analyzed with each technique.
(Modified from Campomenosi et al, 2016)

Refinement of miRNA panel based on sensitivity to hemolysis

A good biomarker needs to be "robust" (i.e. it should not be influenced by confounding factors, one of which is hemolysis), when serum samples are used as starting material. Indeed, the levels of some miRNAs have been shown to change in the presence of hemolysis, as some

miRNAs are released by lysed red blood cells [Pritchard et al, 2012 (b)]. Therefore, we decided to perform preliminary experiments aimed at identifying the sensitivity to hemolysis of the miRNAs selected. In this preliminary experiment, we found that among the 10 miRNAs examined (hsa-miR-15b-5p, hsa-miR-21-5p, hsa-miR-27b-3p, hsa-miR-126-3p, hsa-miR-155-5p, hsa-miR-210-3p, hsa-miR-221-3p, hsa-miR-320a, hsa-miR-486-5p, hsa-let-7a-5p) the level of miR-486 and miR-155 significantly increased in presence of hemolysis. Notably, when the few hemolyzed samples were excluded from analysis, the levels of these two miRNAs were not different in the serum of NSCLC patients compared to controls. These observations led us to exclude miR-486 and miR-155 as possible biomarkers of lung cancer (Fig. 9). The remaining eight miRNAs underwent further analyses to identify those able to discriminate between lung cancer cases and controls.

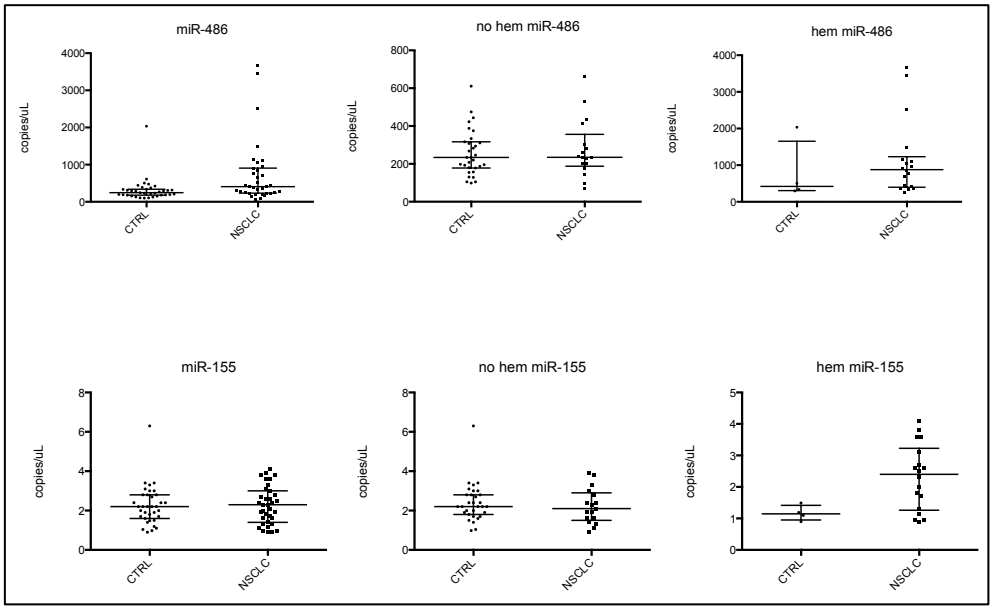

Figure 9. Scatter plots showing the concentration of miR-486 and miR-155 in controls (CTRL) and patients (NSCLC) considered globally (left panel), and either in absence (middle panel) or presence (right panel) of hemolysis. These miRNAs are influenced by hemolysis.

Reproducibility of miRNAs analyses performed by ddPCR

We performed various reproducibility tests. First, we tested miRNAs stability before and after multiple freeze-thaw cycles of the cDNAs between the two measures (Fig. 10). We analyzed eight samples and four miRNAs by ddPCR. We found a good correlation between the two measures and R-squared (Fig. 10), whose values

depended on the specific miRNA being analyzed, suggesting that each miRNA sequence has an intrinsic stability.

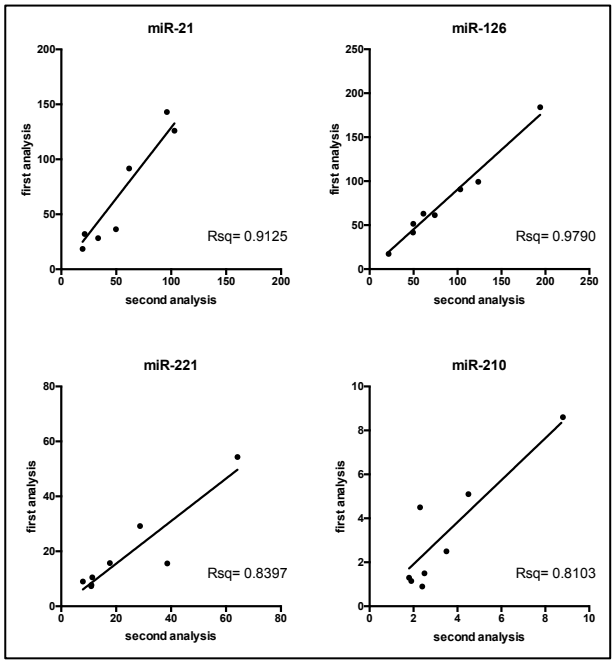

Figure 10. Correlation analysis between miRNA measures obtained from the same cDNAs before or after multiple freeze-thaw cycles, for four miRNAs under study. Axis numbers indicate copies of the specific miRNA per microliter of ddPCR reaction.

We also decided to test the correlation of the measures obtained from two independent retrotranscription reactions on four samples, tested with three miRNAs. The R-squared was about 0.9 for all miRNAs (Fig. 11).

Finally, we aimed to test reproducibility of two ddPCR reactions performed on the same ten samples, with eight

miRNAs. The R-squared values ranged from 0.9068 for miR-320a to 0.9970 for miR-126 (Fig. 12).

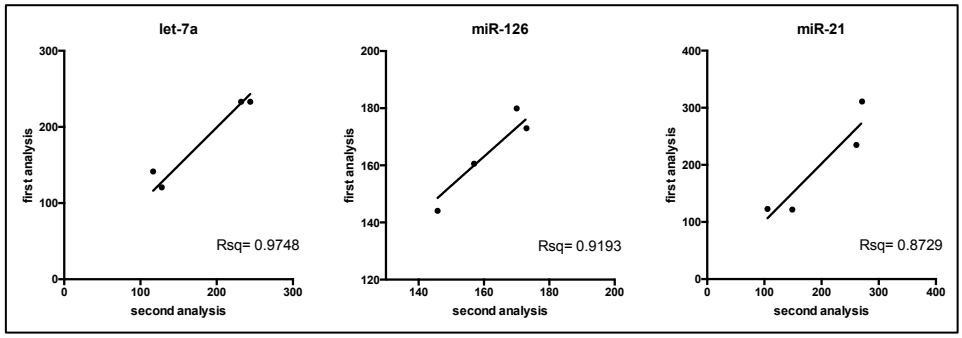

Figure 11. Regression lines showing the correlation between measures obtained on the same RNAs after two independent retrotranscriptions followed by ddPCR analysis, for three of the miRNAs under study.

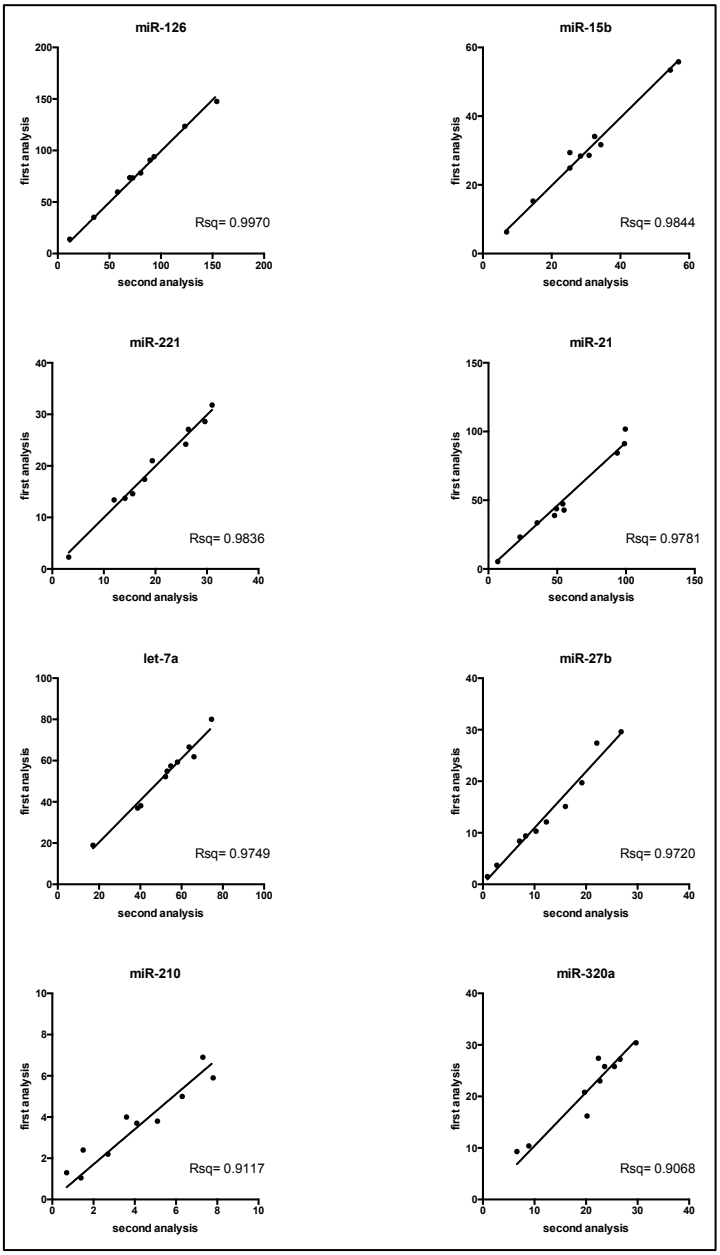

Figure 12. Correlation analysis between two ddPCR replicates performed on ten samples and eight miRNAs.

In summary, from Part 1 of this study we can conclude that ddPCR appears to be the most robust of the three techniques tested ("relative" qPCR, "absolute" qPCR, ddPCR), in terms of reproducibility, precision and throughput.
Therefore, ddPCR was the technique chosen for our case-control experiment in Part 2 of this study, to quantify the selected panel of miRNAs in serum samples.

CASE-CONTROL EXPERIMENT (TRAINING SET)

ddPCR analyses of serum miRNAs to identify putative biomarkers for screening of early lung cancer

The panel of eight miRNAs selected based on our preliminary review of the literature, and after the refinement based on sensitivity to hemolysis (from the 10 miRNAs initially considered, we excluded miR-486 and miR-155), was measured in our training set, composed of 85 therapy-naïve patients with stage I and II NSCLC, of both genders, and 83 matched controls (see Materials and Methods on page 30). As illustrated in Fig. 13, four out of the eight tested miRNAs showed significant differences in

serum levels between lung cancer patients and controls: let-7a, miR-320a, miR-210 ($p<0.0001$) and miR-221 ($p=0.0119$). For each of these four "predictive miRNAs", the Receiver Operating Characteristic (ROC) curve was constructed and the Area Under the Curve (AUC) calculated (Fig. 14). AUC indicated fair accuracy in identifying early non-small cell lung cancer cases (0.745 for miR-210; 0.730 for let-7a; 0.708 for miR-320a; 0.612 for miR-221). By applying the cut-off values indicated in Table 5, we found a sensitivity of 75.3% for miR-210, 70.6% for let-7a, 80% for miR-320a and 63.5% for miR-221 (Table 4).

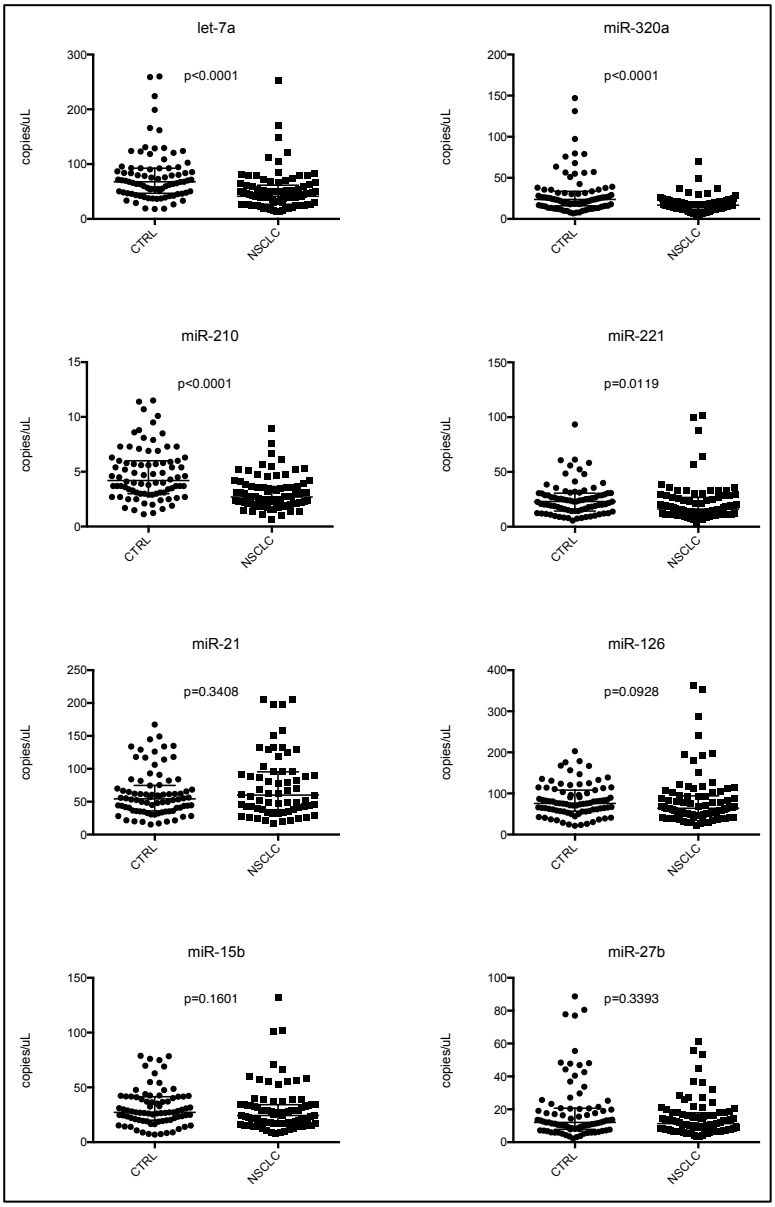

Figure 13. Scatter plots showing the concentration (in copies/ µL) of the eight selected miRNAs in controls (CTRL) and in patients with stage I-II (NSCLC), evaluated by ddPCR (significance of difference assessed by Mann-Whitney test).

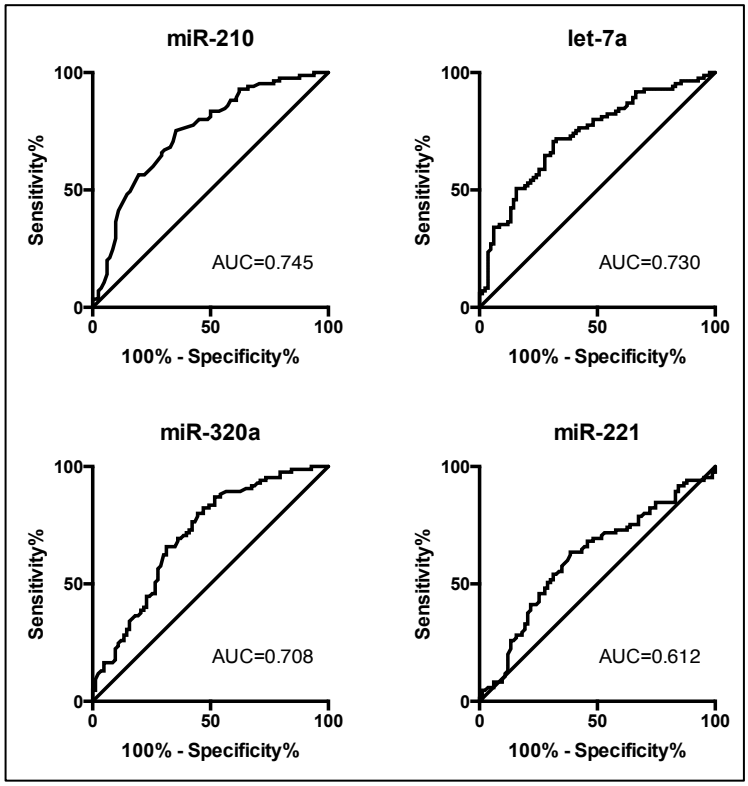

Figure 14. Graphs showing ROC and AUC values for four putative miRNA biomarkers

Table 4. AUC of the ROC curves, cut-off, sensitivity and specifity of four predictive miRNAs biomarkers.

miRNA	**AUC**	**Cut-off copies/µL**	**Sensitivity %**	**Specificity %**
miR-210	0.745	≤3.6	75.3 (64.7-84)	64.6 (53.3-74.9)
let-7a	0.730	≤53.1	70.6 (59.7-80)	68.7 (57.6-78.4)
miR-320a	0.708	≤21.9	80 (69.9-87.9)	55.4 (44.1-66.3)
miR-221	0.612	≤19.5	63.5 (52.4-73.7)	61.5 (50.1-71.9)

Discussion

Lung cancer is the main cause of cancer-related mortality in the developed world and the identification of sensitive and specific biomarkers for lung cancer screening may lead to early diagnosis and thus improve survival rates. These biomarkers should be ideally measured using non-invasive, reliable, widely available and low cost techniques. Circulating miRNAs are considered good candidates as lung cancer biomarkers [Markou et al, 2013]. However, several methodological problems have been highlighted in the quantification of miRNAs in biofluids and currently there is no consensus on which method should be used (microarray; relative/absolute qPCR; ddPCR) to quantify circulating miRNAs [Ono et al, 2015; Ferracin et al, 2015].

We compared the performance of three miRNA quantification methods: relative qPCR, absolute qPCR and ddPCR.

Relative qPCR is the most commonly used method reported in the literature for quantification of circulating miRNAs, in spite of the lack of reliable endogenous reference miRNAs in biofluids [Le et al, 2012]. Indeed, in our preliminary experiments we tested miR-103a-3p, miR-191-5p and miR-423-5p as endogenous reference miRNAs, as suggested by the Exiqon guidelines, but these miRNAs showed excessively high variability among samples, making their use as reference molecules unreliable. Therefore, we performed normalization of

relative qPCR results using the UniSp5 spike-in, a synthetic miRNA which is added by the operator at the beginning of RNA extraction, as suggested by several authors [Schwarzenbach et al, 2015; Roberts et al, 2014]. However, also UniSp5 spike-in is not exempt from criticisms, as this small molecule neither follows the endogenous miRNA processing nor is it complexed in the serum in the same way that endogenous miRNAs are. Overall it is generally agreed that normalization remains a major weakness of the relative qPCR technique, regardless of the molecule used for normalization [Marabita et al, 2016]. On the other hand, the absolute qPCR method estimates the number of target copies in unknown samples based on their fluorescence compared to that of a standard calibrator. Therefore, absolute qPCR depends on accurate quantification, dilution and downstream processing of the calibrator itself. In constructing the calibrator, errors are possible at several levels, including errouneous estimation of the initial concentration, suboptimal efficiency of retrotranscription [Bustin et al, 2015] that may contribute to erroneous quantification and low reproducibility of the calibration curve in different experiments. These problems, and the fact that efficiency in PCR amplification of synthetic standards may differ from that of complex samples, represent the major weaknesses of quantification by absolute qPCR [Lai et al, 2005].

In the ddPCR technique, PCR-positive and PCR-negative droplets are counted to directly provide absolute quantification of the target DNA in digital form [Hindson et al, 2011]. The output of the analyses is given in copies per microliter of reaction, with 95% confidence intervals. Thus, the ddPCR system allows measurement of miRNA expression levels with remarkable precision, averting the need for technical replicates [Hindson et al, 2011], because the sample is partitioned into thousands of micro-reactions. This, in turn, accelerates the quantification process, as more samples, or a higher number of targets, can be analyzed on a single 96-multiwell plate. Furthermore, reagents for quantification based on DNA binding dyes, like EvaGreen, have shown results comparable to hydrolysis probes in ddPCR when applied to the quantification of circulating miRNAs [Miotto et al, 2014].

In the present study, the results of miRNAs analyses with the different techniques significantly correlated. The correlation between relative qPCR and ddPCR suggests that the normalization by the UniSp5 spike-in is applicable to miRNA quantification whenever there is no availability of endogenous reference genes, such as in the serum. However, it is important to note that ddPCR showed less variability in replicate analyses than relative qPCR, in particular when four replicates of the same samples were analyzed with the two techniques (Fig. 3, 4 and Table 2).

When absolute qPCR and ddPCR were compared, the correlation was again significant. In this case, the correlation was similar either when comparison was made with four replicate analyses of the same samples (Fig. 6, 7 and Table 3) or with a larger number of sera (Fig. 8). However, we observed that for two of three miRNAs (miR-126 and let-7a), the ddPCR methods yielded approximately 2.4 and 3.9 fold lower values than absolute qPCR, respectively. We confirmed these systematic differences between absolute ddPCR and qPCR measurements in a separate experiment: we quantified by ddPCR the cDNAs of the specific calibrators used to build the calibration curves for qPCR and consistently found a lower concentration than the theoretical one used for calculations in qPCR. This systematic difference is further confirmed by lower levels of the miRNA measurements obtained by the absolute qPCR method, compared with those obtained by ddPCR, reported by other authors [Hindson et al, 2013]. It is likely that such discrepancy is due to sub-optimal efficiency of retrotranscription and/or amplification of standard template, leading to a defect in calibration curve construction.

The fact that ddPCR does not require a reference or a calibrator for quantification, represents one of the main advantages of this method. However, to make the results on the miRNAs more reliable we recently decided to introduce, for future analyses, a "normalization" step with

UniSp4. UniSp4 is one of the spike-ins introduced in serum samples right before extraction, thus it is present at all the different stages of the experiment. We calculated a corrective factor that takes into consideration the copies of UniSp4 introduced and the copies of UniSp4 detected by ddPCR. In this way the levels of the miRNAs of interest will be corrected by taking into consideration this correction factor, making the analysis more robust. The other strong point of ddPCR is that it is an end-point analysis; absolute quantification is based on the presence or absence of fluorescence in each droplet rather than on fluorescence levels during the reaction, making it less sensitive to the presence of potential PCR inhibitors [Rački et al, 2014].

Our results suggest that for the purpose of miRNA measurement in biofluids the ddPCR method is more robust compared to relative or absolute qPCR, with the important advantage of providing absolute quantification without the need to use calibrators and standard curves, also providing a higher throughput at a similar cost-per-reaction. The time needed to complete a set of analyses, including post-PCR processing data, was about 4-fold shorter with ddPCR than with relative qPCR and 2-fold shorter than with absolute qPCR. In our laboratory the estimated cost of the amplification step, for each miRNA determination in serum was about 3.33 € for qPCR (performed in triplicate) and about 3.66 € for ddPCR.

For these reasons we decided to use ddPCR as the technique of choice for quantification of circulating miRNAs as putative biomarkers of lung cancer.

The second part of the present work consisted of a case-control study in a cohort of 85 patients with stage I-II NSCLC and 83 controls. This cohort was used as training set for assessing the diagnostic accuracy of eight miRNAs (hsa-miR-15b-5p, hsa-miR-21-5p, hsa-miR-27b-3p, hsa-miR-126-3p, hsa-miR-210-3p, hsa-miR-221-3p, hsa-miR-320a, hsa-let-7a-5p) reported in the literature to be aberrantly expressed in the serum of patients, using the ddPCR technique [Ferracin et al, 2015].

Four out of eight miRNAs that we investigated showed significant differences between NSCLC cases and controls, namely let-7a, miR-320a, miR-210 ($p<0.0001$) and miR-221 ($p=0.0119$) (Fig. 13).
These findings are only partially concordant with the literature. While alterations in the levels of miR-21 are frequently reported in the serum of lung cancer patients (mainly with advanced stage disease) [Geng et al, 2014; Qi et al, 2014; Ma J et al, 2013; Markou et al, 2013; Le et al, 2012; Shen et al, 2011 (a); Shen et al, 2011 (b); Tang et al 2013], in our training set miR-21 levels were not affected in samples from early stage NSCLC patients as compared to controls. However, there are two

considerations to make: first, our cohort was exclusively composed of early-stage patients (stage I and II), therapy naïve, while the previously mentioned studies included mainly patients with advanced lung cancer. Moreover, most of these published works were performed in oriental populations (Chinese cohorts). At present, no data are available on differences in miRNA expression based on ethnic origins, but this potential explanation for the different results obtained in our study cannot be ruled out. In another independent Italian lung cancer patient cohort, no evidence of increase miR-21 levels was found [Ferracin et al, 2015] similar to our study.

We found decreased levels of miR-126 and let-7a in lung cancer patients compared to controls, in agreement with the literature [Bianchi et al, 2011; Sanfiorenzo et al, 2013; Markou et al, 2013; Shen et al, 2011 (a); Jeong et al, 2011; Heegard et al, 2012; Kang et al 2013]. These miRNAs have been described to possess oncosuppressive activity and their levels are decreased in lung tumors compared to normal tissues. MiR-126 has been shown to target molecules with a potential role in blood-vessel formation such as VEGFA, VCAM1, EGFL7 and PIK3R [Jusufovic et al, 2012; Zhu et al, 2011; Harris et al, 2008; Guo et al 2008], while the let-7 family targets the expression of proteins involved in lung cancer development like MYC and K-RAS [Johnson et al 2005; Kim et al 2009]. The decrease in let-7a serum levels in

lung cancer patients in our study was significant, suggesting a potential application of this miRNA as a biomarker for early NSCLC.

Although miR-221 was one of the miRNAs selected because its levels are altered in presence of lung cancer, there is no agreement on the direction of variation: Chen reported an increase in miR-221 in sera of lung cancer patients, whereas Heegard found a decrease of the same miRNA, in keeping with our results [Chen et al, 2012; Heegard et al, 2012]. The decrease in miR-221 is in agreement with its proposed role as an anti-angiogenic miRNA [Kuehbacher et al, 2008; Urbich et al, 2008]. Again, comparison of cohorts with different ethnicity would help to understand if this might be a possible explanation.

According to the literature, miR-320a and miR-210 appear to be up-regulated in the serum of lung cancer bearing patients, compared to control subjects [Chen et al, 2012; Shen et al, 2011 (a); Shen et al, 2011 (b)]. However, we found a significant down-regulation of these miRNAs. Again, published work refers mainly to Chinese cohorts.

Some of the published studies rely on the ratio or on the difference between two different miRNAs, rather than on increase or decrease of specific miRNAs to predict tumor risk. For example, Hennessey et al. described that the difference between miR-15b and miR-27b could be used as an indicator to discriminate NSCLC from healthy

controls [Hennessey et al, 2012]. Boeri et al found a signature of 16 miRNA ratios, discriminating between cancer patients and healthy controls, among which miR-15b was present as a ratio with miR-92a (miR-15b/miR-92a) [Boeri et al, 2011]. In our case we found a negligible variation of miR-15b and miR-27b serum level in lung cancer patients compared to controls.

For each of the four miRNAs (let-7a, miR-210, miR-320a and miR-221), for which we found significant differences in serum levels between lung cancer patients and controls, the ROC curve was constructed and the AUC calculated. These showed fair accuracy in identifying early lung cancer cases (about 0.7 for each of the four miRNA) (Fig. 14), suggesting their potential role as biomarkers of lung cancer.

In conclusion, ddPCR proved to be a robust method for absolute quantification of miRNA serum levels in patients with early NSCLC. The ddPCR approach has advantages, as it averts the search of a stable reference miRNA and it does not need construction of calibrator curve for the analysis. Also the miRCURY LNA assay (Exiqon) provided a greater specificity compared to other miRNA expression platforms [Mestdagh et al, 2014]. A major benefit is that it has a universal cDNA system so we can quantify any miRNA without making miRNA-specific reverse transcription [Miotto et al, 2014].

These results, obtained in the training set, need to be validated both internally and externally, to assess whether a combination of four miRNAs (let-7a, miR-210, miR-320a and miR-221) and possibly additional miRNAs may identify subjects who need further investigation for the presence of early lung cancer.

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