LiKidMiRs: A ddPCR-Based Panel of 4 Circulating miRNAs for Detection of Renal Cell Carcinoma

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Simple Summary: Early detection of renal cell carcinoma (RCC) significantly increases the likelihood of curative treatment, avoiding the need of adjuvant therapies, associated side effects and comorbidities. Thus, we aimed to discover circulating microRNAs that might aid in early, minimally invasive, RCC detection/diagnosis.

Abstract: Background: Decreased renal cell cancer-related mortality is an important societal goal, embodied by efforts to develop effective biomarkers enabling early detection and increasing the likelihood of curative treatment. Herein, we sought to develop a new biomarker for early and minimally invasive detection of renal cell carcinoma (RCC) based on a microRNA panel assessed by ddPCR. Methods: Plasma samples from patients with RCC (n = 21) or oncocytomas (n = 15), and 64 healthy donors, were selected. Hsa-miR-21-5p, hsa-miR-126-3p, hsa-miR-155-5p and hsa-miR-200b-3p levels were evaluated using a ddPCR protocol. Results: RCC patients disclosed significantly higher circulating levels of hsa-miR-155-5p compared to healthy donors, whereas the opposite was observed for hsa-miR-21-5p levels. Furthermore, hsa-miR-21-5p and hsa-miR-155-5p panels detected RCC with high sensitivity (82.66%) and specificity (71.89%). The hsa-miR-126-3p/hsa-miR-200b-3p panel identified the most common RCC subtype (clear cell, cRCC) with 74.78% sensitivity. Conclusion: Variable combinations of plasma miR levels assessed by ddPCR enable accurate detection of RCC in general, and of cRCC. These findings, if confirmed in larger studies, provide evidence for a novel ancillary tool which might aid in early detection of RCC.
1. Introduction

Renal cancer remains one of the leading urologic cancers worldwide, being listed as one of the twenty most common and deadly cancers, especially among men (1.5:1) [1,2]. Renal cell tumors (RCTs) correspond to a set of benign and malignant neoplasms, with extensive diversity at epigenetic, molecular, and clinical levels [3,4]. Among them, about 10% correspond to benign tumors, with oncocytomas constituting the most common benign tumor [1,4]. Concerning malignant RCTs, clear-cell renal cell carcinoma (ccRCC) is the most common subtype (65–75% of all RCCs) [5], followed by papillary renal cell carcinomas (pRCC, ~16%) and chromophobe renal cell carcinomas (chRCC, ~7%) [5]. RCCs derive from nephron epithelial cells [1,6,7] and are characterized by their heterogeneity, both morphological and molecular. Whereas localized RCC is mostly cured by surgery, locally advanced or systemic disease constitute major therapeutic challenges, entailing the need for development not only of biomarkers for early detection, but also novel therapies [8].

In recent years, several studies have been published concerning the use of circulating microRNAs (miRNAs) for early and minimally invasive detection of RCC [1,9]. MiRNAs are small non-coding RNAs involved in cell differentiation, growth, apoptosis, and proliferation, and have been implicated in suppressing gene expression after translation [10,11]. MicroRNA dysregulation has been extensively described in various cancers, including RCC [4,10–13]. Frequently, miRNA levels differ between cancerous and normal tissues, representing an opportunity for biomarker development, both in tissue samples and in liquid biopsies [10,11]. Nonetheless, the biomarker performance of most candidate miRNAs remains suboptimal, and concerns remain as to the most adequate methods for assessment and normalization [14,15]. Indeed, all published studies on the assessment of miRNAs in the liquid biopsies of RCC patients have used qRT-PCR [1,9], a technique which provides relative quantification, thus requiring normalization of the results. Although miR-16 should be the preferential normalizer due to its stability in RCC [15–18], many of the published studies used RNU44, U6, or other similar RNA species instead, which are unstable in liquid biopsies, eventually leading to biased results [14,19–27]. This problem might be solved using a different technology, droplet digital PCR (ddPCR), as it obviates the need for normalization and preamplification. DdPCR is a recent technology that appears to improve miRNA detection, as it is based on sample partitioning before the PCR reaction and on the Poisson distribution, allowing for absolute quantification, in a time-cost effective and reliable manner [28,29]. Furthermore, the time point of data acquisition increases the precision and robustness of the method [28,29].

Thus, in this study, taking advantage of the performance of ddPCR in liquid biopsies, we sought to evaluate, for the first time, the ability of a microRNA panel (hsa-miR-21-5p, hsa-miR-126-3p, hsa-miR-155-5p, and hsa-miR-200b-3p), previously assessed in tissue samples [13,30] to detect RCC using plasma samples.

2. Materials and Methods

2.1. Samples

A total of five plasma samples were included in the technical optimization phase of the study, in which the ddPCR methodology was tested: one oncocytoma, one stage I pRCC, one stage I ccRCC, one stage I chRCC, and one healthy adult blood donor.

After optimizing the ddPCR pipeline, a cohort of 203 plasma samples was assessed, comprising 139 samples collected from RCT patients at the time of diagnosis and 64 healthy blood donors. Regarding RCT patients, 87 corresponded to ccRCC, 22 to chRCC and 15 to pRCC, whereas oncocytoma was diagnosed in the remaining 15. All patients were treated at IPO Porto by the same multidisciplinary team between 2015 and 2021. After peripheral blood collection into EDTA-containing tubes, plasma was separated by centrifugation at
2500 g for 30 min at 4 °C, and subsequently stored at −80 °C in the institutional biobank until further use. All blood samples were processed within 4 h from the time of collection. Relevant clinical and pathological data were analyzed from clinical charts and grouped in an anonymized database specifically constructed for the analysis.

2.2. RNA Extraction and cDNA Synthesis

Total RNA was extracted from 100 µL plasma using a MagMAX mirVana Total RNA Isolation kit (Thermo Fisher, Waltham, MA, USA, A27828), according to the manufacturer’s protocol. As a technical control, a non-human synthetic spike-in, ath-miR-159a (0.2 µL per sample of a stock solution at 0.2 nM), was added to the lysis buffer in all samples. The final 50 µL of RNA was collected to a 1.5 mL RNase-free tube. All steps were performed at room temperature, and extracted RNA was stored at −80 °C until further use.

Using TaqMan microRNA reverse transcription kit (Thermo Fisher, 4366596) according to the manufacturer’s protocol, five microliters of previously isolated RNA were reversely transcribed in a Veriti thermal cycler (Applied Biosystems™, Waltham, MA, USA) for the miRNAs of interest and the spike-in (ath-miR-159a, hsa-miR-21-5p, hsa-miR-126-3p, hsa-miR-155-5p, hsa-miR-200b-3p).

2.3. Droplet Digital PCR (ddPCR): DigiMir Pipeline

DdPCR reactions were prepared according to the optimizations performed: the volumes of cDNA input [2 µL (ath-miR-159a, hsa-miR-21-5p, hsa-miR-126-3p), 5 µL (hsa-miR-155-5p and hsa-miR-200b-3p)], 11 µL ddPCR Supermix for the probes (Bio-Rad, Hercules, CA, USA, #1863010), and 1 µL TaqMan hsa-miRNA Assay (20×). The volumes of bidistilled water were 8 µL (ath-miR-159a, hsa-miR-21-5p, hsa-miR-126-3p) and 5 µL (hsa-miR-155-5p and hsa-miR-200b-3p); assays: ath-miR-159a—000338, FAM; hsa-miR-21-5p—000397, FAM; hsa-miR-126-3p—002228, VIC; hsa-miR-155-5p—002623, FAM; hsa-miR-200b-3p—002251, FAM. Droplets were generated on the droplet generator QX200 (Bio-Rad, Hercules, CA, USA). The PCR run was set as follows: 95 °C for 10 min, 50 cycles of 94 °C for 30 s, and “Annealing Temperature optimized” for 1 min—ramp rate 2 °C/s—and 98 °C for 10 min. The Annealing Temperature was set at 56 °C for ath-miR-159a and at 55 °C for the other four miRNAs. After PCR reaction, plate was read on the QX200 Droplet Reader (Bio-Rad, Hercules, CA, USA).

The limit of the blank (LOB) and the limit of detection (LOD) were calculated for each target miRNA according to Armsbruster et al. [2]. Additionally, the limit of quantification (LOQ) for the five miRNAs was assessed by performing a 2-fold dilution series of an RCT sample.

2.4. Quality Control Steps

All plasma samples were inspected for hemolysis as previously reported by others [31,32]. Hence, from 238 initial samples, 35 samples that presented absorbance higher than 0.25 at 414 nm were excluded. Appropriate engineering and manual controls were used to prevent contaminations—including a master mix made using a clean hood, clean gloves, PCR reagents and consumables—and reactions were performed in separate dedicated labs. RNA previously extracted from RCC cell lines (HKC8 was obtained from Expasy and Caki-1, 769-P, Caki-2, ACHN, A-498, HEK-293, 786-O were from ATCC), and a pool of them was used as positive control for the four candidate miRNAs. A no-template control (NTC) and no-enzyme control (NEC) were included in all cDNA synthesis and ddPCR stages as negative controls. For ddPCR pipeline optimization, further negative controls (“no-cDNA control” and “no-Supermix control”) were included, as recommended [33]. All samples were run in a single reaction for each target.
2.5. Statistical Analysis

Non-parametric tests were performed to compare levels of each miRNA among histologic subtypes and to evaluate associations with clinicopathological features. A Spearman test was used for correlation analyses between two variables. A Mann-Whitney U test was used for comparisons between two groups, whereas a Kruskal-Wallis test was used for multiple groups, followed by a Mann-Whitney U test with Bonferroni’s correction for pairwise comparisons. A result was considered statistically significant when the $p$-value < 0.05.

For each miRNA, samples were categorized as positive or negative based on the cut-off values established using Youden’s J index [34,35] (value combining the highest sensitivity and specificity), through Receiver-Operating Characteristic (ROC) curve analysis. Validity estimates (sensitivity, specificity, and accuracy) were determined to assess the detection biomarker performance. To improve the detection performance of the selected miRNAs, panels were constructed considering a positive result whenever at least one target miRNA was plotted as positive in an individual analysis.

A two-tailed $p$-value calculation and ROC curve analyses (without resampling analysis) were performed using SPSS 27.0 software for Windows (IBM-SPSS Inc., Chicago, IL, USA). All graphics were assembled using GraphPad Prism 8.0 software for Windows (GraphPad Software Inc., LA Jolla, CA, USA). To increase the statistical power through a resampling analysis, multiple ROC curves were constructed to calculate validity estimates for the best miRNA panels, as previously described [36,37]. In brief, samples were randomly divided into training (70%) and validation (30%) sets. Subsequently, the cut-off value was estimated in the training set considering the highest sensitivity and specificity and using this calculated cut-off, validity estimates were calculated in the validation set. The procedure was repeated 1000 times and the mean of the parameters (sensitivity and specificity) were calculated. These calculations were performed using R v3.4.4.

3. Results

3.1. Patients’ Cohort Characterization

The relevant clinical-pathological features of optimization and validation cohorts are depicted in Table 1. According to clinical-demographic factors, a significant, although weak, correlation was found between age and circulating levels of each miRNA—hsa-miR-21-5p, hsa-miR-126-3p and hsa-miR-200b-3p levels ($R^2 = 0.080$ and $p$-value < 0.001, $R^2 = 0.030$ and $p$-value = 0.023, $R^2 = 0.020$ and $p$-value = 0.032, respectively).

3.2. Distribution of Circulating miRNA Levels and Biomarkers Performance for Detection of Malignancy

Initially, target miRNA levels were compared between oncocytoma (a benign tumor) and healthy donor samples, and no significant differences between these groups were found for any of the tested hsa-miRNAs, except for hsa-miR-155-5p ($p$-value = 0.037).

Due to the clinical relevance of discriminating malignant disease (RCC) from healthy individuals, this comparison was subsequently performed. Interestingly, circulating levels of hsa-miR-21-5p and hsa-miR-155-5p significantly differed between these two groups ($p$-value < 0.001 and $p$-value = 0.013, respectively) (Figure 1). Circulating levels of hsa-miR-21-5p disclosed the highest accuracy for identifying malignant tumors, although hsa-miR-155-5p depicted the best specificity (90.63%). Remarkably, a panel comprising hsa-miR-21-5p/hsa-miR-155-5p detected about 83% of the three major RCC subtypes, with 71.89% accuracy (Table 2). Importantly, the same two hsa-miRNAs could discriminate RCTs from healthy individuals (Figures S1 and S2 and Table S1).
Table 1. Clinicopathological data of the technical optimization cohort (5 samples) and LiKidMiRs cohort (composed of 139 Renal Cell Tumors and 64 Healthy donors’ samples) used in this study.

### Technical Optimization Cohort (n = 5 Samples)

| Cases                              | Description                  |
|------------------------------------|------------------------------|
| Sample #1                          | 66 years, Oncocytoma         |
| Sample #2                          | 53 years, pRCC, Stage I      |
| Sample #3                          | 57 years, ccRCC, Stage I     |
| Sample #4                          | 46 years, chRCC, stage I     |
| Sample #5                          | 45 years, healthy blood donor |

### LiKidMiRs Cohort (n = 203 samples)

| Renal cell tumor samples            | 139                          |
| Healthy blood donors                | 64                           |

### Renal cell tumor patients—clinicopathological features

| Age [years (median, interquartile range)] | 64 (17.0)                        |
| Gender                                 |                               |
| Male                                   | 96/139 (69.1)                  |
| Female                                 | 43/139 (30.9)                  |
| Size of tumor mass [cm (median, interquartile range)] | 4.50 (4.3)                     |
| Histology [n, (%)]                     |                               |
| ccRCC                                  | 87/139 (62.6)                  |
| pRCC                                   | 15/139 (10.8)                  |
| chRCC                                  | 22/139 (15.8)                  |
| Oncocytoma                             | 15/139 (10.8)                  |
| Stage [n, (%)]                         |                               |
| I                                      | 59/124 (47.6)                  |
| II                                     | 8/124 (6.5)                    |
| III                                    | 45/124 (36.3)                  |
| IV                                     | 12/124 (9.7)                   |
| ISUP nuclear grade [n, (%)]            |                               |
| 1                                      | 7/88 (8.0)                     |
| 2                                      | 47/88 (53.4)                   |
| 3                                      | 24/88 (27.3)                   |
| 4                                      | 10/88 (11.4)                   |
| Vital status                           |                               |
| Alive with disease                     | 6/139 (4.3)                    |
| Alive without disease                  | 120/139 (86.3)                 |
| Death from the disease                 | 13/139 (9.4)                   |

### Healthy Blood Donors—clinicopathological features

| Age [years (median, interquartile range)] | 46 (4.75)                      |
| Gender                                 |                               |
| Male                                   | 36/64 (56.3)                   |
| Female                                 | 28/64 (43.8)                   |
Figure 1. Violin plots with miRNA levels in Healthy Donors (HD) and Renal Cell Carcinoma (RCC) samples of hsa-miR-21-5p (A) and hsa-miR-155-5p (B), and respective Receiver-Operating Characteristic Curve (without resampling analysis) (C,D). In violin plots, dashed lines indicate the interquartile range and horizontal line the median of miR levels. In ROC curves, red line indicates the reference line and blue line the identity line for each miRNA. Abbreviations: AUC—Area Under the Curve; CI—Confidence Interval, HD—Healthy Donors, RCC—Renal Cell Carcinoma, *—p-value < 0.05, ***—p-value < 0.0001.

Table 2. Performance of miRNAs as biomarkers for detection of Renal Cell Carcinoma.

| miRNAs | SE%  | SP%  | PPV%  | NPV%  | Accuracy% |
|--------|------|------|-------|-------|-----------|
| hsa-miR-21-5p    | 62.90  | 64.06  | 77.23  | 47.13  | 63.30      |
| hsa-miR-155-5p   | 39.52  | 90.63  | 89.09  | 43.61  | 56.91      |
| hsa-miR-21-5p/hsa-miR-155-5p | 89.52  | 54.69  | 79.29  | 72.92  | 77.66      |
| Multiple ROC Curve (hsa-miR-21-5p/hsa-miR-155-5p) | 82.66  | 51.13  | 77.22  | 61.76  | 71.89      |

Abbreviations: SE—Sensitivity; SP—Specificity; PPV—Positive Predictive Value; NPV—Negative Predictive Value; ROC—Receiver-Operating Characteristic.
When the analysis was restricted to early-stage disease (patients with an organ-confined tumor) and healthy donor samples, hsa-miR-21-5p and hsa-miR-155-5p, but not the other miRNAs, retained statistical difference \((p\text{-value} < 0.01\) for both miRNAs) between these two groups (Figure 2A,B). Hence, these two miRNAs were able to detect small RCC (tumors limited to the kidney, without regional lymph node metastasis) with 89.04% sensitivity and high negative predictive value (NPV) (77.68%) (Table 3). Remarkably, the AUC for both miRNAs was higher than 65.00% (Figure 2C,D).

**Figure 2.** Violin plots of miRNAs levels in Healthy Donor (HD) and early stages of Renal Cell Carcinoma (Stage I and II) samples of hsa-miR-21-5p (A) and hsa-miR-155-5p (B), and respective Receiver-Operating Characteristic Curve (without resampling analysis) (C,D). In violin plots, dashed lines indicate the interquartile range and horizontal line the median of miR levels. In ROC curves, red line indicates the reference line and blue line the identity line for each miRNA. Abbreviations: AUC—Area Under the Curve; CI—Confidence Interval; HD—Healthy Donors, **—\(p\text{-value} < 0.001.\)
3.3. MiRNA Levels and Clinicopathological Features

Among RCC subtypes (ccRCC, pRCC, and chRCC), significant differences were found for all four miRNAs (hsa-miR-126-3p, hsa-miR-155-5p, and hsa-miR-200b-3p, p-value < 0.01; hsa-miR-21-3p, p-value = 0.045, Figure 3). Furthermore, all four hsa-miRs circulating levels significantly differed between the two major RCC subtypes, ccRCC and pRCC (hsa-miR-126-3p, p-value < 0.001; hsa-miR-155-5p and hsa-miR-200b-3p, p-value < 0.01; hsa-miR-21-3p, p-value = 0.039, Figure 3). Nonetheless, no statistical differences were found between pRCC and chRCC or between ccRCC and chRCC for the tested circulating miRNAs.

Due to the poorer outcome and higher incidence of ccRCC, comparisons in circulating hsa-miRNAs were performed between this subtype and the other two RCC subtypes (Figure 4). Interestingly, ccRCC patients displayed significantly lower circulating levels of all hsa-miRs compared to patients diagnosed with the other malignant subtypes (p-value = 0.048 for hsa-miR-21-5p and p-value < 0.01 for hsa-miR-126-3p, hsa-miR-155-5p and hsa-miR-200b-3p—Figure 4).

Table 3. Performance of miRNAs as biomarkers for identification of early stages Renal Cell Carcinomas.

| miRNAs                        | SE%   | SP%   | PPV% | NPV%  | Accuracy% |
|-------------------------------|-------|-------|------|-------|-----------|
| hsa-miR-21-5p                 | 81.82 | 43.75 | 60.00| 70.00 | 63.08     |
| hsa-miR-155-5p               | 48.48 | 90.63 | 84.21| 63.04 | 69.23     |
| hsa-miR-21-5p/hsa-miR-155-5p | 92.42 | 34.38 | 59.22| 81.48 | 63.85     |
| Multiple ROC Curve (hsa-miR-21-5p/hsa-miR-155-5p) | 89.04 | 36.23 | 59.28| 77.68 | 62.88     |

Abbreviations: SE—Sensitivity; SP—Specificity; PPV—Positive Predictive Value; NPV—Negative Predictive Value; ROC—Receiver-Operating Characteristic.

Figure 3. Violin plots of hsa-miR-21-5p (A), hsa-miR-126-3p (B), hsa-miR-155-5p (C) and hsa-miR-200b-3p (D) levels in the malignant subtypes (ccRCC, pRCC and chRCC). Dashed lines indicate the interquartile range and horizontal line the median of miR levels. Abbreviations: ccRCC—Clear-Cell Renal Cell Carcinoma; chRCC—Chromophobe Renal Cell Carcinoma; pRCC—Papillary Renal Cell Carcinoma; n.s.—not significant, *—p-value < 0.05, **—p-value < 0.001, ***—p-value < 0.0001.
Moreover, circulating hsa-miR-126-3p and hsa-miR-200b-3p levels discriminated ccRCC from other RCC subtypes with 74.78% sensitivity and 52.95% specificity (Figure 5).

Figure 5. Receiver-Operating Characteristic Curves (without resampling analysis) of hsa-miR-21-5p (A), hsa-miR-126-3p (B), hsa-miR-155-5p (C) and hsa-miR-200b-3p (D) in ccRCC and other RCCs (pRCC and chRCC). Red line indicates the reference line and blue line the identity line for each miRNA. Abbreviations: AUC—Area Under the Curve; CI—Confidence Interval.

Table 4. Performance of miRNAs as biomarkers for identification of Clear-Cell Renal Cell Carcinoma.

| miRNAs                | SE%  | SP%  | PPV%  | NPV%  | Accuracy% |
|-----------------------|------|------|-------|-------|-----------|
| hsa-miR-21-5p         | 60.92| 67.57| 81.54 | 42.37 | 62.90     |
| hsa-miR-126-3p        | 78.16| 56.76| 80.95 | 52.50 | 71.77     |
| hsa-miR-155-5p        | 66.67| 64.86| 81.69 | 45.28 | 66.13     |
| hsa-miR-200b-3p       | 60.92| 75.68| 85.48 | 45.16 | 65.32     |
| hsa-miR-126-3p/hsa-miR-200b-3p | 80.46| 56.76| 81.40 | 55.26 | 73.39     |
| Multiple ROC Curve    |      |      |       |       |           |
| (hsa-miR-126-3p/hsa-miR-200b-3p) | 74.78| 52.95| 79.49 | 47.46 | 68.28     |

Abbreviations: SE—Sensitivity; SP—Specificity; PPV—Positive Predictive Value; NPV—Negative Predictive Value.

4. Discussion

RCC remains a leading cause of cancer-related death worldwide. Alongside prostate and bladder cancers, RCC is one of the most common urological malignancies [38]. Early detection of RCC (ideally at stage I or II) significantly increases the likelihood of a cure through surgical treatment, with a 5-year survival rate of 98%, averting the need for...
subsequent therapies, which are not curative and often carry significant adverse side effects [15]. Nonetheless, 20–30% of patients display metastatic disease at diagnosis [38,39], and even following curative-intent nephrectomy, the standard of care for localized RCC, metastases develop in up to 20–40% of patients [39]. Notably, the response to medical treatment (mainly targeted therapy or immunotherapy) is rather limited, with a 5-year survival rate lower than 10%. Among RCCs, ccRCC, pRCC, and chRCC represent more than 90% of cases, emphasizing the importance of accurately detecting these tumor subtypes and discriminating them from benign conditions [39,40].

Circulating miRNAs are emergent cancer biomarkers which might be assessed using minimally invasive strategies, eventually constituting promising RCC biomarkers. Nevertheless, only a few studies have addressed this issue, mostly using conventional qPCR techniques [14,15,17–25,41,42]. Owing to the diversity of the results of those studies and the need to overcome the limitations of normalization, we assessed the clinical potential of a circulating miRNA-based panel for RCC detection using ddPCR.

Accurate identification of patients harboring RCC and discrimination from healthy individuals, as well as from carriers of benign renal lesions (including tumors), is pivotal to reliably establishing therapeutic vs. monitoring strategies. Thus, after a first analysis between oncocytomas and healthy donors, we compared healthy donors with RCC patients. Remarkably, two (hsa-miR-21-5p and hsa-miR-155-5p) out of the four candidate miRNAs disclosed statistically significant differences in plasma levels. Although hsa-miR-21-5p has been described to act as oncomiR, we observed lower circulating levels in RCC patients [20,43–45]. This might be due to the distinct miRNAs levels in the different clinical samples. Indeed, higher miRNA levels may be found in tissues compared to body fluid samples [46]. Importantly, increased hsa-miR-21-5p levels were also found in serum samples of RCC patients, further supporting that circulating miRNA levels in serum and plasma may be different [20]. Moreover, differences were also reported for hsa-miR-21-5p levels in serum and plasma among patients with Non-ST-elevation myocardial infarction, a non-cancer-related pathology [47]. Herein, higher hsa-miR-21-5p levels were found in serum when compared with respective control samples, whereas lower levels were observed in plasma samples from the same patients [47]. Of note, plasma has been reported to be the sample of election for translational studies [47–49], as red blood cell lysis during the coagulation process increases discharging of RNA and platelets to the serum, increasing the non-tumor derived circulating miRNAs present in each sample [48]. Importantly, hsa-miR-21-5p is expressed in platelets [47,50] and, thus, an increase of platelets in serum might explain the higher levels found for this miRNA. Furthermore, in breast cancer, lower hsa-miR-30b-5p levels were found in tissue compared with plasma, unveiling the disparities between these two sample sources [51]. Moreover, inadequate normalization and biased results may occur if the normalizer used is not the most suitable. Indeed, U6 is more prone to degradation by serum RNases [1]. Interestingly, in a previous study we found that hsa-miR-21-5p miRNA was significantly downregulated in tissue samples from RCT patients, discriminating RCT patients from healthy donors [13].

Concerning hsa-miR-155-5p, upregulation of this circulating hsa-miR was found in RCC patients, and a panel comprising hsa-miR-155-5p and hsa-miR-21-5p could identify 82.66% of RCC patients with 71.89% accuracy. Interestingly, hsa-miR-155-5p was shown to be upregulated in tissue [13,52] and ccRCC serum samples [18], and is also associated with cancer development [52]. Moreover, an hsa-miR-21-5p/hsa-miR-155-5p panel depicted high sensitivity (89.04%) for identifying organ-confined carcinomas, which might allow for reducing false-negative results and increase the likelihood of curative-intent treatment. To the best of our knowledge, this is the first study that evaluated the biomarker performance of plasma circulating hsa-miRs to detect early-stage RCC. Previously, Wang and colleagues described a 5-miRNA panel (miR-193a-3p, miR-362, miR-572, miR-378, and miR-28-5p) that was able to identify early-stage RCC, albeit in serum samples [22]. Furthermore, our panel achieved a higher NPV than that reported by Wang et al. [22].
We further evaluated whether circulating hsa-miRNAs might also convey relevant information to discriminate ccRCC from the remainder RCC subtypes. Indeed, all four miRNAs were able to differentiate this major RCC subtype from the others. The panel constituted by hsa-miR-126-3p and hsa-miR-200b-3p disclosed the best performance, with 74.78% sensitivity and 52.95% specificity. Since ccRCC is an aggressive RCC subtype, early detection is of major importance, and its accurate identification might improve patient outcomes [20,53]. Although stratification by stage was not performed due to a limited number of cases with advanced stages, for early stages, hsa-miR-126-3p and hsa-miR-200b-3p levels also differed significantly between ccRCC and the remainder RCC subtypes. Considering that various studies have reported other strategies for RCC identification (including imaging and epigenetic biomarkers), our results seem to offer the best sensitivity for RCC detection [9,54]. Indeed, the methodology we developed uses a lower initial sample volume [15,17,20,22,25,41,42], which is more cost-effective, and the procedure to obtain the sample is better tolerated by patients. Molecular imaging such as 18F-fluorodeoxyglucose (FDG) positron emission tomography/computed tomography (PET/CT) was reported to detect localized RCC, but it discloses lower sensitivity (only 22%) [54,55]. Despite the superior specificity (85.9%) of 124I-cG250 PET for RCC detection, when compared to our hsa-miR-21-5p/hsa-miR-155-5p panel (51.13%), this monoclonal antibody has a half-life of several days, constituting a significant disadvantage in relation to the protocol reported by us [56]. Moreover, diffusion magnetic resonance imaging was reported to characterize malignant lesions with similar sensitivity (86%) to our panel but with higher specificity (78%) [57]. Nevertheless, it should be noted that despite the better performance, these imaging biomarkers are more costly and less well-tolerated by the patient compared to liquid biopsies [54].

The intense exploration of circulating epigenetic markers such as DNA methylation, miRNAs, and IncRNAs is well illustrated by the more than 60 articles published in this field since 2003 [9]. So far, 10 DNA methylation-based studies (e.g., using VHL, RASSF1A, P16, P14, RARB, TIMP3, GSTP1, APC) for RCC detection have been published [58–67] and only 33.33% of these had an RCC cohort with more than 50 patients [60,63,64]. Compared with those studies, our results provide higher sensitivity (6–83%). However, DNA methylation-based markers displayed high specificity (53–100%). This was also observed in three IncRNAs studies (e.g., GIHCG, LINC00887) [68–70], in which the diagnostic performance was generally lower than in our study (67.1–87.0%), but the specificity reached values >80% for all biomarkers. Although our biomarker panels disclosed high sensitivity, their specificity is limited. Thus, in an envisaged routine setting, they would ideally be used in first-line screening, requiring complementary use of more specific biomarkers in cases deemed positive. In liquid biopsies, DNA methylation-based markers such as VHL, RASSF1A, TIMP3, SFRP1, SFRP2, SFRP4, SFRP5, PCDH17, and TCF21 are highly specific (100%) [58,59,61,62,65–67] and, thus, constitute good candidates as second-line tests, in this setting.

As previously reported, most circulating miRNA studies are based on blood-based liquid biopsies [1]. When compared with our protocol, only a few studies included more than 100 RCC patients, which might, at the least partially, explain the differences in results [9]. Additionally, the discrepant results might also be explained, as described above, by the biased normalization (e.g., spike-in as normalizer miRNA, U6, RNU48) [14,19,20,23,24]. Nevertheless, the sensitivity reported for the most widely studied serum miRNAs (miR-210, miR-1233, and miR-378) was generally lower than our plasma panel [14,17,25]. Indeed, using this less time-consuming and more cost-effective approach, we were able to detect RCC using a minimally invasive technique, with a lower initial quantity of plasma than serum-based studies (although detecting other miRNAs), and obtained similar or even better results, obviating the need for normalization and the associated bias (due to ddPCR absolute quantification) [15,17,20,22,25,41,42]. Hence, our results from multiple ROC curve analysis demonstrate a potential clinical application of this technology to identify RCC, and is the first study to quantify circulating miRNAs in these patients using ddPCR (Figure 6).
These results require validation in more extensive prospective studies. Overall, and notwithstanding our promising results for RCC detection, it should be acknowledged that the lack of long-term follow-up constitutes a significant limitation. Further studies using liquid biopsies should also be considered to further subtype RCC, namely, to distinguish oncocytomas from chRCCs, which will lead to a prioritization of treatments for patients with malignant tumors.

![Graphical representation of the potential clinical impact of LiKidMiRs. Created with BioRender.com.](image)

**Figure 6.** Graphical representation of the potential clinical impact of LiKidMiRs. Created with BioRender.com.

## 5. Conclusions

Our findings support the research question that a minimally invasive test can be developed to detect RCC, improving patient survival through increased diagnosis at earlier stages. This might help to reduce the morbidity and mortality associated with advanced disease, as well as the lack of curative treatment at those stages. Furthermore, and to the best of our knowledge, this work is the first to report a novel tool to quantify circulating miRNAs in plasma using ddPCR in RCC patients.

### Supplementary Materials:

The following are available online at https://www.mdpi.com/article/10.3390/cancers14040858/s1, Table S1: Performance of miRNAs as biomarkers for detection of Renal Cell Tumors., Figure S1: Violin plots with all points of miRNAs levels in Healthy Donors (HD) and Renal Cell Tumors (RCT) samples of hsa-miR-21-5p (A), hsa-miR-126-3p (B), hsa-miR-155-5p (C) and hsa-miR-200b-3p (D). Dashed lines indicate the interquartile range and horizontal line the median of miR levels. Abbreviations: HD—Healthy Donors; RCT—Renal Cell Tumors; n.s.—not significant, **—p-value < 0.001, ***—p-value < 0.0001., Figure S2: Receiver Operating Characteristic Curve (without resampling analysis) of hsa-miR-21-5p (A) and hsa-miR-155-5p (B). Red line indicates the reference line and blue line the identity line for each miRNA. Abbreviations: AUC—Area Under the Curve; CI—Confidence Interval.

### Author Contributions:

J.P.S. performed molecular analyses and wrote the manuscript; J.P.S. and J.L. collected the clinical data; J.P.S., V.C., S.S., D.B.-S. and J.R. analyzed the data; C.C.-M. processed clinical samples; I.B. provided clinical information about the patients; V.C., S.S., D.B.-S., J.L., R.H. and C.J. critically revised the manuscript; R.H. and C.J. supervised the work. All authors have read and agreed to the published version of the manuscript.

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### Institutional Review Board Statement:

This study was approved by the Ethics Committee (CES 518/2010) of the Portuguese Oncology Institute of Porto, Portugal. All procedures performed in tasks involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

### Informed Consent Statement:

Written informed consent was obtained from all subjects involved in the study.

### Data Availability Statement:

All data generated or analyzed during this study are included in this article.

### Conflicts of Interest:

The authors declare that they have no competing interests.
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