

# A novel circular RNA biomarker signature to facilitate an improved diagnosis of pleural mesothelioma

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## Article

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## Additional Declarations:

No competing interests reported.

Table 4 is available in the Supplementary Files section.

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## **Abstract**

**Background:** Pleural mesothelioma (PM) is an aggressive asbestos-related cancer with poor prognosis. A definitive PM diagnosis currently relies on an invasive tissue biopsy procedure, highlighting the urgent need for minimally-invasive biomarkers to facilitate an accurate and early diagnosis. This study evaluated the diagnostic potential of eight novel circular RNAs (circRNAs) in PM-derived cell lines compared to non-PM controls.

**Methods:** A microarray analysis was performed on PM and non-malignant cell lines to identify PM-specific upregulated circRNAs. Twenty seven PM, 18 other cancer and five non-malignant cell lines were analysed by droplet digital PCR (ddPCR) to quantify the expression levels of eight circRNA candidates. An area under the receiver operating characteristic curve (AUC), was performed to evaluate diagnostic performance.

**Results:** The microarray identified 294 circRNAs upregulated in PM cells and subsequent ddPCR analysis validated seven significantly overexpressed circRNAs in PM cells compared with other cancer types, and six compared with non-malignant controls. The circRNAs, hsa\_circ\_0001829 and hsa\_circ\_0006646, distinguished cancer from non-malignant cells (AUC=0.937), whilst hsa\_circ\_0007386, hsa\_circ\_0006294, hsa\_circ\_0002346 and hsa\_circ\_0002348, effectively differentiated PM from other cancer types (AUC=0.921).

**Conclusion:** Combinational circRNA biomarker signatures have potential diagnostic capacity to distinguish PM from other non-PM conditions, providing rationale for their further validation in patient-derived samples.

**Keywords:** Biomarker, Circular RNA, Diagnosis, Droplet Digital PCR, Pleural Mesothelioma,

## 66 **Introduction**

67 Pleural mesothelioma (PM) is an aggressive thoracic malignancy primarily caused by asbestos  
68 exposure. There are limited treatment options available to patients [1-4] and consequently PM  
69 prognosis is poor, with a median survival of 14-18 months and 5-year survival below 10% [5-7].  
70 Early detection remains challenging due to the absence of sensitive and specific clinical  
71 biomarkers, non-specific symptoms that resemble other lung conditions, and a long latency  
72 period of 20 to 40 years following exposure [8]. Typical imaging findings include pleural  
73 thickening, pleural effusion, and increased 18F-FDG uptake on PET scans [9]. A definitive  
74 diagnosis usually requires an invasive biopsy and immunohistochemical (IHC) assessment using  
75 biomarkers such as calretinin, Wilms' tumor gene (WT1), cytokeratin 5/6 (CK5/6) and D2-40 [9,  
76 10]. However, these procedures are labor-intensive, costly, and may be unsuitable for patients  
77 with advanced disease. There is a critical need for minimally-invasive, blood-based biomarkers  
78 capable of supporting an earlier and more accurate PM diagnosis.

79 Several circulating biomarkers, including mesothelin, osteopontin, fibulin-3, high-mobility group  
80 box 1 (HMGB1), angiogenic factors, microRNAs (miRNAs), circulating tumour DNA (ctDNA),  
81 circulating tumour cells (CTCs) and exosomes have been investigated for PM diagnosis [9, 11,  
82 12], however their clinical utility has been limited by suboptimal sensitivity and specificity [13-  
83 15]. To date, soluble mesothelin-related peptide (SMRP) is the only FDA-approved blood-based  
84 biomarker for PM diagnosis and treatment monitoring, although its performance is compromised  
85 by low specificity and frequent false positive results [16-18].

86 Circular RNAs (circRNAs), a class of covalently closed non-coding RNAs, represent a potential  
87 alternative biomarker source [19, 20]. CircRNAs are abundant in whole blood, buffy coat and  
88 plasma [21], and are characterised by their robust stability in circulation, extended half-life and  
89 disease-specificity [22-25]. Although circRNA biomarker research is still emerging, several  
90 studies have demonstrated their potential diagnostic value for cancer detection, such as acute  
91 myeloid leukaemia and lung adenocarcinoma [25, 26]. Advances in divergent primer-based  
92 qPCR assays have further enabled accurate circRNA quantification in biological samples [27],  
93 facilitating the discovery of candidate biomarkers across multiple malignancies, such as lung,  
94 colorectal, gastric, prostate and breast cancer [28-32].

95 In this study, we identified and validated eight circRNAs overexpressed in PM cell lines using  
96 droplet digital PCR (ddPCR) [33]. Compared with conventional qPCR methods, ddPCR provides  
97 absolute quantification with improved sensitivity and reproducibility, reducing variability in  
98 circRNA measurement [34-36]. We identified a two-circRNA signature capable of distinguishing  
99 PM from non-malignant controls, and a four-circRNA signature capable of differentiating PM

from other cancer types. These findings support future investigation of these circRNAs as potential diagnostic biomarkers in biospecimens from PM patients and asbestos-exposed individuals.

## **Methods**

### **General chemicals and reagents**

TRIzol® (Life Technologies), chloroform and 2-propanol (Sigma-Aldrich), absolute ethanol (100%) (Point of Care Diagnostics) and molecular biology grade Glycogen (Roche Diagnostics) were used for this study.

### **Cell culture**

Human PM (n=27), lung cancer (n=6), breast cancer (n=3), colon cancer (n=4), pancreatic cancer (n=4), gastric cancer (n=1) and non-malignant mesothelial and fibroblast (n=5) cell lines were sourced from ATCC or collaborating institutions (Supplementary Material1: Table S1). PM cell lines included both epithelioid (n=14) and non-epithelioid (n=11) histological subtypes (Supplementary Material 1: Table S1). Cells were cultured in RPMI (Life Technologies, Paisley, UK) or EMEM (Life Technologies, Paisley, UK) supplemented with 10% fetal calf serum at 37 °C, with 95% humidity and 5% CO<sub>2</sub>.

### **Microarray circRNA expression analysis**

Total RNA was extracted from 10 PM and 2 non-malignant mesothelial cell lines (n=3 biological replicates of each) using the TRIzol® reagent. RNA samples were analysed on the Arraystar® Human circRNA Array v2 (8x15K), which profiles ~13,617 circRNAs using probes specific to circRNA back-splice junctions, including multiple circRNAs originating from the same gene. In total, 5,968 unique host genes were represented. Fold-change values comparing differentially expressed circRNAs between PM and non-malignant cells were generated using proprietary software. From the top 20 significantly overexpressed circRNAs (fold-change  $\geq 1.5$ ,  $p \leq 0.05$ ), eight biomarker candidates were selected for further analysis.

### **Microarray circRNA pathway analysis**

Gene Set Enrichment Analysis (GSEA) was performed using microarray-derived circRNAs associate with a known host gene. CircRNAs lacking an annotated host gene were excluded. For genes linked to multiple circRNA, the highest fold-change value was used. All 5,968 genes assigned to the 12,328 different circRNAs were ranked by fold-change to generate GSEA input.

Enrichment analyses were conducted in R (v4.5.1) [37] using fgsea [38] with pathways databases obtained from msigdb [39] KEGG MEDICUS [40], Gene Ontology Biological Processes [41], and Reactome [42] (msigdb v2025.1.Hs). Differentially up-/down-regulated pathways were identified as nominally significant ( $p$ -value < 0.05) or significant after multiple hypothesis testing correction (FDR < 0.05) using Benjamini-Hochberg correction [43, 44]).

#### **CircRNA-specific divergent primer and probe design**

Exon-exon junction sequences for each circRNA candidate were obtained from the National CircInteractome database (<http://circinteractome.nia.nih.gov>) [45]. Divergent circRNA primers and probes (Supplementary Material 1: Table S2) were designed using Roche Probe Design software and synthesised by Integrated DNA Technologies (Coralville, Iowa, USA). Probes were labelled with 6-FAM (target circRNA) or HEX (reference circRNA). GAPDH primer-probe sets were similarly designed for use as a linear RNA digestion control (Supplementary Material 1: Table S2).

#### **Total RNA extraction**

RNA was isolated from 27 PM, 18 other cancer and five non-malignant cell lines (n=3 biological replicates of each) (Table S1). Approximately  $1 \times 10^6$  cells were harvested from T75 flask using 0.05% Trypsin-EDTA, pelleted and extracted using TRIzol®. RNA pellets were solubilised at 60 °C for 10 min and assessed for purity using a NanoPhotometer®. Samples with A260/A280 ratios of 1.9-2.0 and A260/A230 ratios of 2.0-2.2 were used for downstream analysis.

#### **RNase R treatment and RNA purification**

Total RNA (2 µg) was subjected to RNase R digestion (RNase R +) with RNase R (Applied Biological Materials Inc., Richmond, BC, CA) to enrich circRNAs by degrading linear RNA. Undigested samples (RNase R -) served as controls. After 3 hr incubation at 37°C, RNA was column purified using an RNeasy MinElute® Cleanup kit (Qiagen) and re-assessed for purity.

#### **cDNA synthesis**

First-strand cDNA synthesis was performed using 10 µL of RNase R+ or RNase R- RNA with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA). Reactions contained RT buffer, dNTPs, random hexamers, MultiScribe® reverse transcriptase, and RNase inhibitor in a 20 µL final reaction volume and were subjected to thermocycling at 16°C for 30 min, 42°C for 30 min, and 85°C for 5 min in a MultiGene® thermocycler (Pacific Laboratory Products, Blackburn, VIC, Australia).

### **Divergent primer specificity validation by PCR and agarose gel electrophoresis**

Primer specificity was confirmed by PCR using cDNA from a representative PM cell line (not RNase R digested). Reactions contained AmpliTaq® Gold 360 reagents (Applied Biosystems, Waltham, MA, USA) and 0.4 µM primers, and were subjected to thermocycling at 95 °C for 10 min, 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, and a final elongation of 72 °C for 10 min in a MultiGene® thermocycler (Pacific Laboratory Products, Blackburn, VIC, Australia). PCR products were resolved on 2% agarose gel, imaged, excised and purified with a QIAquick® extraction kit (Qiagen), and verified by Sanger Sequencing (Ramaciotti Centre for Genomics, Sydney, NSW, Australia).

### **CircRNA detection using ddPCR**

Diluted cDNA was analysed using probe-based ddPCR. For each circRNA candidate, a primer-probe pre-mix was prepared containing 4.68 µM of forward and reverse primers for the target and reference circRNAs, as well as 1.62 µM of FAM- and HEX-labelled probes for the target and reference circRNAs, respectively (Table S2). A GAPDH-specific pre-mix, consisting of GAPDH-specific primers and FAM-labelled probe (Table S2) was also included to assess RNase R digestion efficiency. All ddPCR reactions contained Bio-Rad Probe Supermix (no dUTP), cDNA, and primer-probe pre-mix in a 20 µL final reaction volume. Droplets (15,000 to 20,000 per well) were generated using a droplet generator (Bio-Rad QX200) and subjected to thermocycling at 95 °C for 30 s, 60 °C for 30 s, 72 °C in a T1000 thermocycler (Bio-Rad). CircRNA copy number data was acquired using a QX200 droplet-reader (Bio-Rad). No template controls (NTCs) were included in all runs. CircRNA expression was quantified as a ratio of target to reference circRNA copy numbers per 20 µL of sample. The reference circRNA, hsa\_circ\_0000284, served as the internal control due to its stable expression profile across different cell types [46].

### **CircRNA biomarker specificity and sensitivity analysis**

RNase R-treated ddPCR data (n=3 biological replicates per cell line) were used to train logistic regression models evaluating (i) cancer vs non-cancer and (ii) PM vs other cancer classifications for individual and combinational circRNAs. Diagnostic performance; including specificity, sensitivity, receiver operating characteristic (ROC), AUC, and confidence intervals, were assessed using R v.4.3.1 with custom scripts and ggplot2 for visualisation [47].

### **PCA-Based Visualisation of circRNA-Dependent Cell Type Variation**

Principal component analysis (PCA) was performed to visualise circRNA expression-based

variance among PM, other cancer and non-malignant cell types. Normalised ddPCR copy-number data (n=3 biological replicated per cell line) were analysed using prcomp in R v.4.5.0 with centring and scaling enabled (Scale=TRUE). Scree plots and biplots were generated using the factoextra R package to assess the proportion of explained variance and variable contributions.

## Results

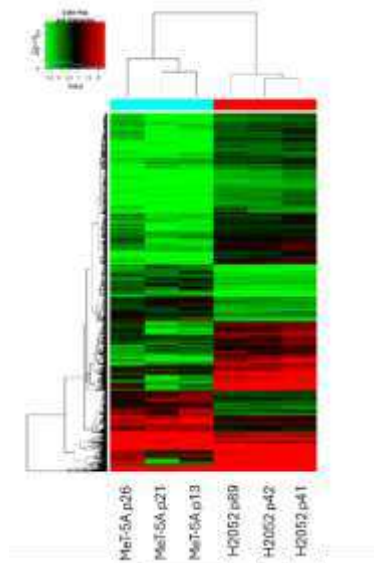
### Biological pathway analyses for differentially expressed circRNAs

The Arraystar® microarray identified 5,951 differentially expressed circRNAs in PM cells compared to the non-malignant mesothelial controls. A large proportion of circRNAs were found to be upregulated in the PM cells compared to the non-malignant controls (294 upregulated, 142 downregulated; fold-change  $\geq 1.5$  (upregulated) or  $\leq 0.5$  (downregulated),  $p$ -value  $\leq 0.05$ ) (Fig. 1 A-D). The majority of upregulated circRNAs originated from protein coding exons (Fig. 1E), with elevated representation across all chromosomes (Fig. 1F). The gene set enrichment analysis (GSEA) of the upregulated circRNA host genes revealed involvement in multiple biological processes, including regulation of cell adhesion, microtubule polymerization and retinoic acid receptor signalling (Fig. 2). Transcriptional regulation of cell cycle genes by *TP53*, was identified as the most enriched cellular pathway associated with the upregulated circRNA host genes (Fig. 2). The complete GSEA data of biological processes and cellular pathways of the upregulated circRNA host genes is provided in Supplementary Material 2.

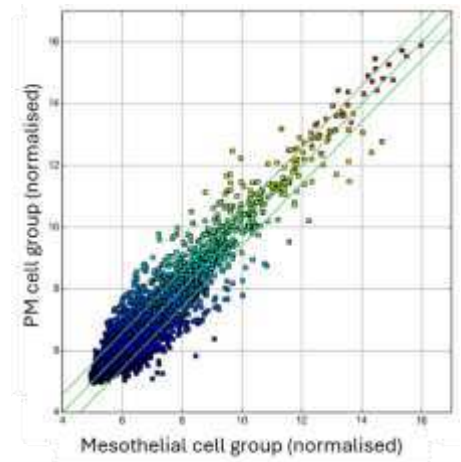
### Selection of highly overexpressed circRNAs as biomarker candidates

Eight circRNAs (hsa\_circ\_0084021, hsa\_circ\_0006349, hsa\_circ\_0007386, hsa\_circ\_0002346, hsa\_circ\_0002348, hsa\_circ\_0006646 and hsa\_circ\_0001829) were selected for ddPCR validation based on their statistically significant 2- to 6-fold increase in expression in PM cells and derivation from protein coding exons (Table 1). Notably, hsa\_circ\_0007386, hsa\_circ\_0002346 and hsa\_circ\_0002348 shared the *CRIM1* host gene on chromosome 2 (Table 1), while the remaining (hsa\_circ\_0084021, hsa\_circ\_0006349, hsa\_circ\_0006646 and hsa\_circ\_0001829) were derived from distinct genes (*PLEKHA2*, *TMEM39B*, *PTK2* and *SLC45A4*) (Table 1). The complete microarray dataset of differential circRNA expression and their associated host genes is provided in Supplementary Material 3.

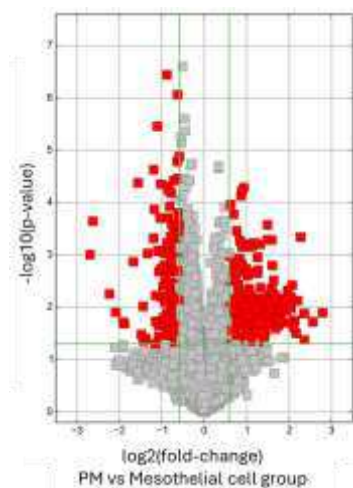
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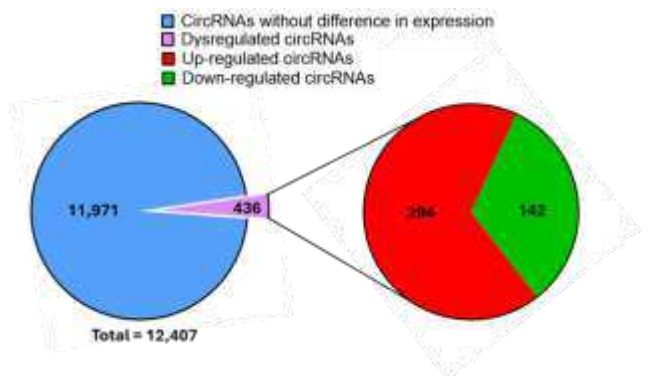
b



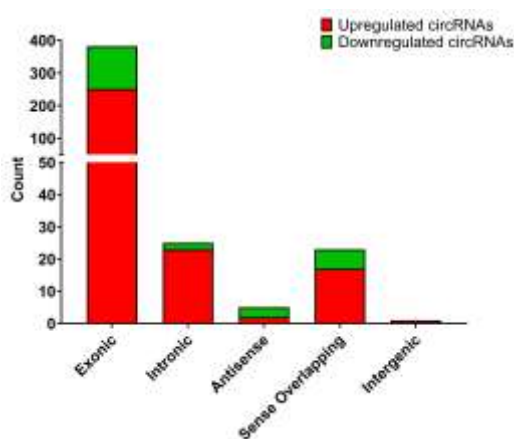
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e



f

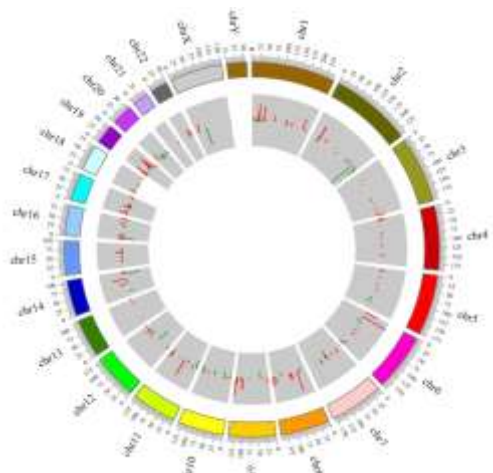


Figure 1

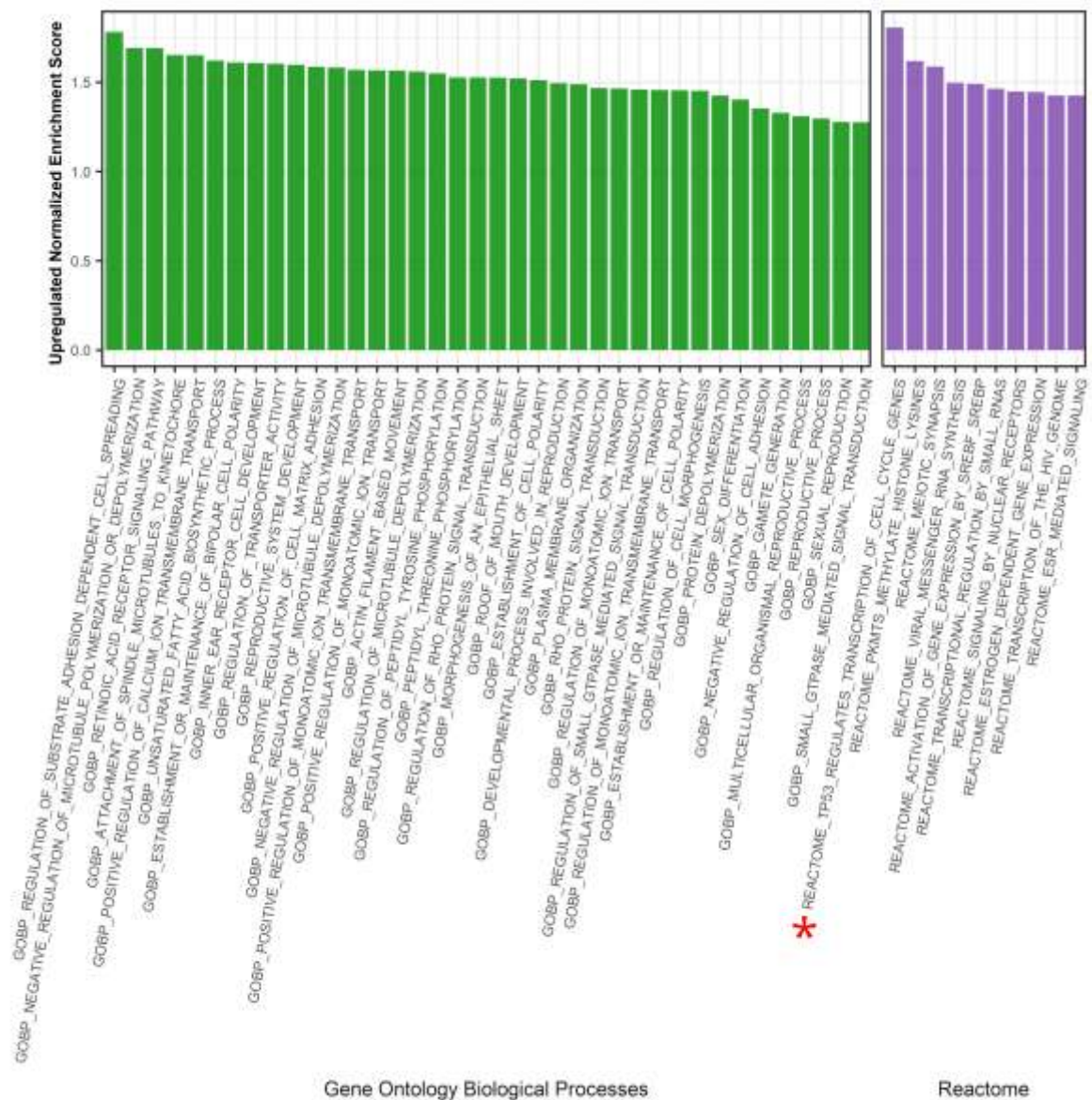


Figure 2

| CircRNA ID       | FC   | P-value | Chromosome | CircRNA Type | Gene Name      |
|------------------|------|---------|------------|--------------|----------------|
| hsa_circ_0084021 | 5.88 | 0.0190  | chr8       | exonic       | <i>PLEKHA2</i> |
| hsa_circ_0006349 | 4.82 | 0.0005  | chr1       | exonic       | <i>TMEM39B</i> |
| hsa_circ_0007386 | 4.39 | 0.0176  | chr2       | exonic       | <i>CRIM1</i>   |
| hsa_circ_0002346 | 4.10 | 0.0104  | chr2       | exonic       | <i>CRIM1</i>   |
| hsa_circ_0002348 | 4.02 | 0.0070  | chr2       | exonic       | <i>CRIM1</i>   |
| hsa_circ_0006646 | 2.63 | 0.0144  | chr8       | exonic       | <i>PTK2</i>    |
| hsa_circ_0001829 | 1.77 | 0.0452  | chr8       | exonic       | <i>SLC45A4</i> |

**Table 1.** List of eight upregulated circRNA candidates selected for biomarker validation. FC refers to fold-change in circRNA expression in the PM cell group with respect to the non-malignant mesothelial cell control group.

### The circRNA biomarker candidates are overexpressed in PM cell lines compared to non-PM cells

All eight circRNA primer sets produced distinct PCR products (88-128 bp), consistent with their expected sizes (Supplementary Material 1: Table S2; Supplementary Material 4: Fig. S1 A). Sanger Sequencing confirmed concordance with the known exon-exon junction sequences sourced from the CircInteractome database (Supplementary Material 4: Fig. S1 B). RNase R treatment effectively degraded linear RNA (GAPDH) (Supplementary Material 4: Fig. S1 C), while enriching circRNAs by ~ 10-fold for most candidates, except hsa\_circ\_0002348 (Supplementary Material 4: Fig. S1 D). Therefore, the RNase R treatment was applied to all subsequent samples prior to the cDNA synthesis and ddPCR.

Six circRNAs (hsa\_circ\_0007386, hsa\_circ\_0006294, hsa\_circ\_0002346, hsa\_circ\_0084021, hsa\_circ\_0001829 and hsa\_circ\_0006646) showed significantly elevated expression in most PM cell lines compared to the non-malignant controls (Fig. 3A-C, E-G, respectively). Hsa\_circ\_0001829 and hsa\_circ\_0006646 exhibited the highest fold-change (~ 4- to 5-fold,  $p$ -value < 0.00005) (Table 2) and were also highly expressed in other cancer cell lines (8-fold and 3-fold, respectively,  $p$ -value < 0.00005) (Table 2). Hsa\_circ\_0084021 showed moderate elevation (~ 3-fold) in PM cells compared to the other cancer type and non-malignant controls, without significant difference between the other cancer and non-malignant groups (Table 2). Hsa\_circ\_0002348 and hsa\_circ\_0006349 did not show significant differential expression between the PM and non-malignant cell groups (Fig. 3 D, H; Table 2).

All eight circRNAs were significantly upregulated in PM cells compared to the other cancer types (Fig. 3A-H). Hsa\_circ\_0007386, hsa\_circ\_0006294, hsa\_circ\_0002346 and hsa\_circ\_0002348 displayed the greatest fold-change expression in PM cells compared to the other cancer cell group (> 3-fold,  $p$ -value < 0.00005) (Table 2). These four circRNAs exhibited > 26-fold and > 6-fold

elevated expression levels ( $p$ -value  $<0.00005$ ) in the PM cells compared to the gastric cancer cell line and colorectal cancer cells, respectively (Table 3). Conversely, hsa\_circ\_0001829 was consistently significantly under-expressed in the PM cells compared to the colorectal and gastric cancer cell lines (Table 3). Hsa\_circ\_0006646, hsa\_circ\_0084021 and hsa\_circ\_0006349, exhibited modest PM-specific elevation compared to the other cancer types, though not uniformly statistically significant across all cancer cell types (Table 3).

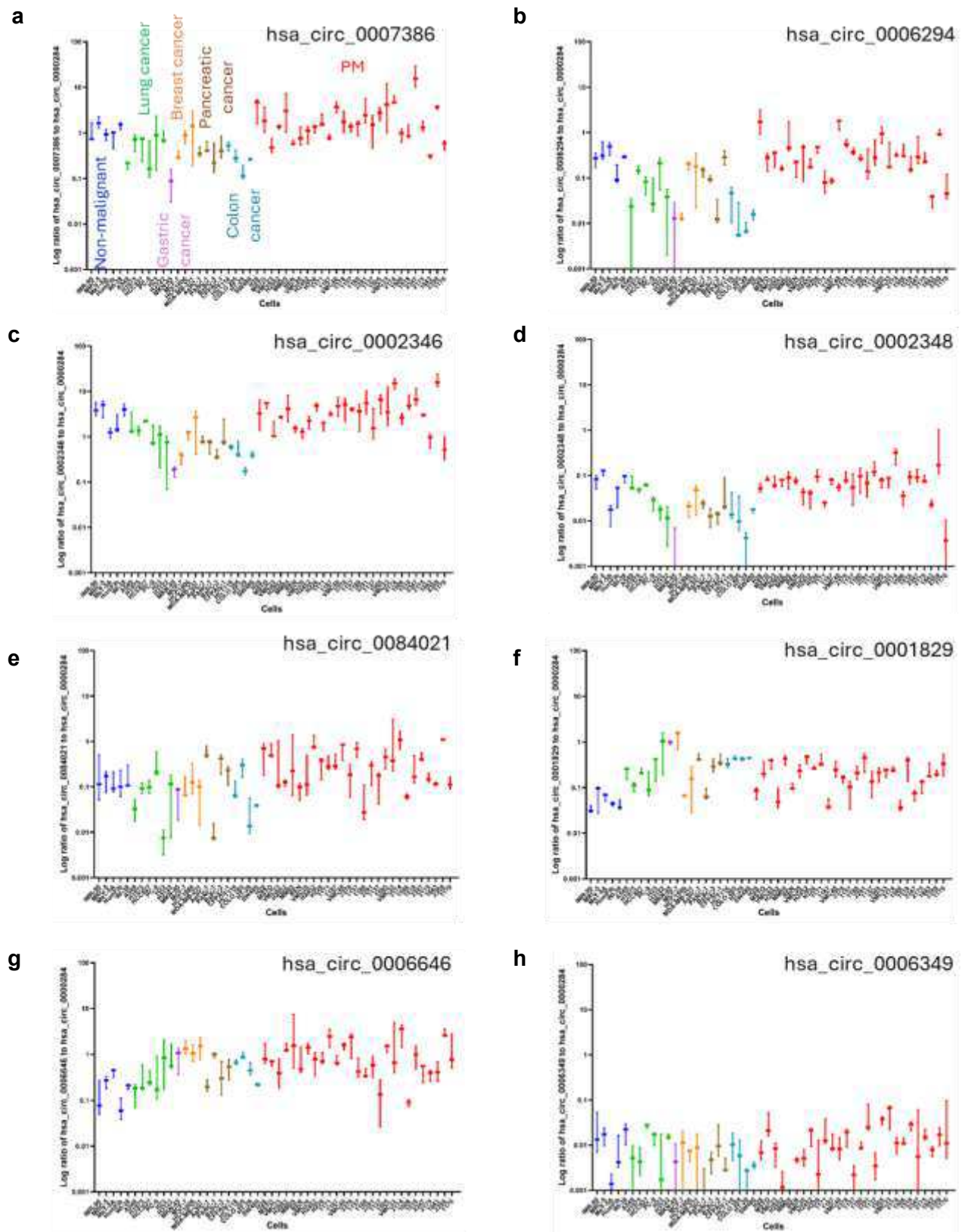


**Figure 3**

| CircRNA ID       | PM vs Non-malignant<br>FC (mean $\pm$ SD) | PM vs Other Cancer FC<br>(mean $\pm$ SD) | Other Cancer vs Non-<br>malignant FC (mean $\pm$<br>SD) |
|------------------|-------------------------------------------|------------------------------------------|---------------------------------------------------------|
| hsa_circ_0007386 | 2.20 $\pm$ 3.30 **                        | 5.04 $\pm$ 7.57 ****                     | 0.44 $\pm$ 0.44 ****                                    |
| hsa_circ_0006294 | 1.47 $\pm$ 1.66 *                         | 5.33 $\pm$ 6.01 ****                     | 0.28 $\pm$ 0.31 ****                                    |
| hsa_circ_0002346 | 1.43 $\pm$ 1.35 *                         | 4.79 $\pm$ 4.50 ****                     | 0.30 $\pm$ 0.26 ***                                     |
| hsa_circ_0002348 | 1.32 $\pm$ 1.77 NS                        | 3.68 $\pm$ 4.94 ****                     | 0.36 $\pm$ 0.33 ***                                     |
| hsa_circ_0006349 | 1.13 $\pm$ 1.30 NS                        | 1.92 $\pm$ 2.21 **                       | 0.59 $\pm$ 0.55 NS                                      |
| hsa_circ_0084021 | 2.69 $\pm$ 2.95 ****                      | 2.89 $\pm$ 3.17 ****                     | 0.93 $\pm$ 1.06 NS                                      |
| hsa_circ_0001829 | 4.29 $\pm$ 2.72 ****                      | 0.54 $\pm$ 0.34 ***                      | 7.96 $\pm$ 7.42 ****                                    |
| hsa_circ_0006646 | 5.53 $\pm$ 5.72 ****                      | 1.78 $\pm$ 1.83 **                       | 3.12 $\pm$ 2.50 ****                                    |

**Table 2.** Summary of differential circRNA expression between PM, other cancer and non-malignant cell groups. FC refers to the fold-change in circRNA expression. \*, \*\*, \*\*\*, \*\*\*\* represent a *p*-value <0.05, <0.005, <0.0005 and <0.00005, respectively. NS depicts no statistical significance.

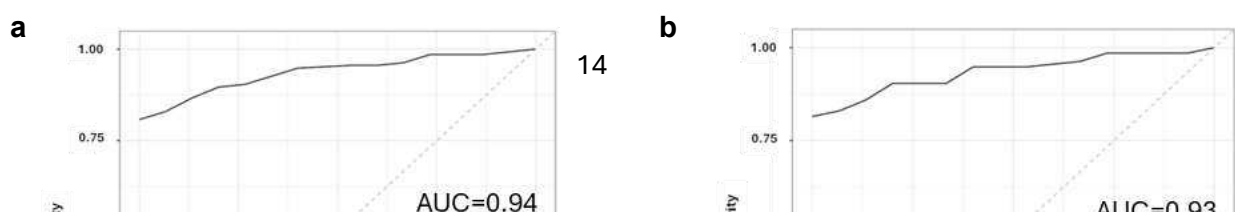
| CircRNA ID           | PM vs Lung<br>Cancer FC<br>(mean $\pm$ SD) | PM vs Breast<br>Cancer FC<br>(mean $\pm$ SD) | PM vs Colorec-<br>tal Cancer FC<br>(mean $\pm$ SD) | PM vs Pancre-<br>atic Cancer FC<br>(mean $\pm$ SD) | PM vs Gastric<br>Cancer FC<br>(mean $\pm$ SD) |
|----------------------|--------------------------------------------|----------------------------------------------|----------------------------------------------------|----------------------------------------------------|-----------------------------------------------|
| hsa_circ_000<br>7386 | 2.65 $\pm$ 3.98<br>****                    | 2.91 $\pm$ 4.4 **                            | 8.62 $\pm$ 12.94<br>****                           | 6.23 $\pm$ 9.37<br>****                            | 28.83 $\pm$ 43.32<br>****                     |
| hsa_circ_000<br>6294 | 5.36 $\pm$ 6.05<br>****                    | 3.50 $\pm$ 3.95<br>****                      | 23.82 $\pm$ 26.89<br>****                          | 3.33 $\pm$ 3.75<br>****                            | 32.71 $\pm$ 36.93<br>****                     |
| hsa_circ_000<br>2346 | 3.29 $\pm$ 3.09<br>****                    | 3.65 $\pm$ 3.44<br>****                      | 10.74 $\pm$ 10.11<br>****                          | 5.66 $\pm$ 5.33<br>****                            | 25.69 $\pm$ 24.17<br>****                     |
| hsa_circ_000<br>2348 | 2.37 $\pm$ 3.18<br>***                     | 4.58 $\pm$ 6.14<br>****                      | 6.11 $\pm$ 8.20 ****                               | 3.92 $\pm$ 5.26<br>****                            | 38.89 $\pm$ 52.18<br>****                     |
| hsa_circ_000<br>6349 | 1.31 $\pm$ 1.52<br>NS                      | 1.86 $\pm$ 2.15 *                            | 2.83 $\pm$ 3.26 ***                                | 2.73 $\pm$ 3.15 **                                 | 3.22 $\pm$ 3.71 *                             |
| hsa_circ_008<br>4021 | 3.89 $\pm$ 4.28<br>****                    | 3.59 $\pm$ 3.94<br>****                      | 3.92 $\pm$ 4.31 ****                               | 1.49 $\pm$ 1.64 NS                                 | 6.61 $\pm$ 7.26<br>****                       |
| hsa_circ_000<br>1829 | 0.68 $\pm$ 0.43<br>NS                      | 0.43 $\pm$ 0.27<br>NS                        | 0.53 $\pm$ 0.34 ****                               | 0.71 $\pm$ 0.45 NS                                 | 0.23 $\pm$ 0.14 **                            |
| hsa_circ_000<br>6646 | 2.30 $\pm$ 2.38<br>***                     | 0.98 $\pm$ 1.02<br>NS                        | 2.05 $\pm$ 2.12 ***                                | 2.27 $\pm$ 2.35<br>***                             | 1.27 $\pm$ 1.31 NS                            |

**Table 3.** Summary of differential circRNA expression between PM and specific other cancer type cell groups. FC refers to the fold-change in circRNA expression. \*, \*\*, \*\*\*, \*\*\*\* represent a *p*-value of <0.05, <0.005, <0.0005 and <0.00005, respectively. NS depicts no statistical significance.

## Unique combinations of circRNAs exhibit high specificity and sensitivity for PM discrimination

Receiver operating characteristic (ROC) analysis of ddPCR-measured circRNA copy numbers was used to evaluate the specificity and sensitivity of individual and combinatorial circRNA signatures. Two analyses were performed: (1) cancer cells (including PM) versus non-malignant controls, and (2) PM cells versus other cancer types. The top ten circRNA signatures

differentiating cancer from non-malignant cells exhibited a  $\geq 80\%$  sensitivity,  $\geq 87\%$  specificity, and AUC values of 0.91-0.94 (Table 4), with the top three circRNA signatures consisting of two to three circRNA combinations (Fig. 4A-C). Among these, a two-circRNA signature (hsa\_circ\_0001829 and hsa\_circ\_0006646) exhibited the strongest capacity to differentiate between cancer (including PM) and non-malignant cell types, with specificity and sensitivity values of 87% and an AUC of 0.94 (Table 4). The top 10 circRNA signatures differentiating PM from other cancer types exhibited sensitivity and specificity values  $\geq 79\%$  and  $\geq 85\%$ , respectively, and AUC values of 0.92 (Table 4), with the top three circRNA signatures consisting of four to five circRNA combinations (Fig. 4D-F). Among these, a four-circRNA signature (hsa\_circ\_0007386, hsa\_circ\_0006294, hsa\_circ\_0002346 and hsa\_circ\_0002348) showed the strongest capacity to differentiate PM from other cancer types, with sensitivity and specificity values of 80% and 87%, respectively, and an AUC of 0.92 (Table 4). A PCA depicted a distinct visual separation of different cell types based on the aforementioned circRNA signatures. The two-circRNA combination distinctly separated non-malignant cells from cancer types, while the four-circRNA combination effectively segregated PM cells from other cancers (Fig. 5A-B). These results support a two-step circRNA biomarker work-flow: firstly differentiating cancer from non-malignant cells, followed by a differentiation of PM from other cancer conditions (Fig. 5C).



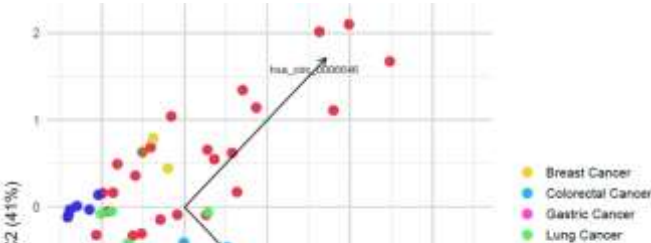
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**Figure 4.**

**Table 4.** Summary of the specificity, sensitivity and area under the curve (AUC) values of the top ten circRNA biomarker signatures

Note to Editor - Table 4 is too big to include here in the main text. It has been provided separately as in an Excel (.xlsx) file titled “Tables”.

**a**



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**Figure 5.**

**Discussion**

497 PM prognosis remains poor, partly due to the limited availability of effective blood-based  
 498 biomarkers capable of enabling an early and accurate PM diagnosis. Radiological screening is  
 499 generally ineffective for early-stage detection among asbestos-exposed individuals [11] and  
 500 differential diagnosis is complicated by other lung diseases with similar imaging characteristics  
 501 [48]. A definitive PM diagnosis typically relies on a tissue-based IHC analysis using markers such  
 502 as WT-1, calretinin, CK5/6 and podoplanin, which are unreliable for differential diagnosis [49,  
 503 50], especially for the sarcomatoid PM subtype, which often does not express them [48, 51].  
 504 Minimally-invasive biomarkers, including proteins, miRNAs, ctDNA, extracellular vesicles (EVs)  
 505 and CTCs, have been explored, but mesothelin remains the only FDA-approved blood-based  
 506 biomarker, primarily for disease monitoring post-surgery. Its limited sensitivity (19-68%) restricts  
 507 clinical utility for early-stage detection [52]. Implementing alternative biomarkers presents  
 508 challenges, including limited multi-centre validation, assay variability, low circulating biomarker  
 509 levels, and tumour heterogeneity. Overcoming these barriers requires harmonising and  
 510 standardising methodologies across varied clinical settings. This involves systematically  
 511 evaluating patient cohorts with differing disease severities and applying consistent approaches  
 512 across large, representative populations [9, 48, 53]. Importantly, this type of integration of  
 513 advanced technologies into real-world clinical environments will be vital for achieving reliable,  
 514 scalable, and sustainable implementation.

515 CircRNAs are promising liquid biopsy biomarkers due to their stability in circulation, disease  
 516 specificity, long half-life, and detectability via quantitative PCR methods [35, 54-56]. Although no  
 517 circRNA-based diagnostic tests have been clinically approved to date, ongoing trials in breast  
 518 (NCT05771337), colorectal (NCT07224789), liver (NCT06042842), and pancreatic  
 519 (NCT04584996) cancer support their clinical potential. In this study, we profiled circRNAs in a  
 520 large PM and non-PM cell line cohort to assess their diagnostic utility.

521 Consistent with other cancers [57-59] [60, 61], we observed an upregulation of circRNAs in PM  
 522 cell lines. The microarray analysis identified 294 significantly upregulated circRNAs in PM cells  
 523 compared to the normal mesothelial cell controls (Fig. 1A-D), with the GO and Reactome  
 524 pathway analyses revealed an association of their host genes with oncogenic cellular processes  
 525 such as, cell adhesion, cytoskeleton dynamics, retinoic acid receptor signalling, calcium  
 526 signalling, and TP53-mediated transcriptional regulation of cell cycle genes (Fig. 2) - all of which  
 527 have been implicated in PM and/or cancer onset and progression [62-72]. Transcriptional  
 528 regulation by small RNAs, such as microRNA (miRNA), was another notable overrepresented  
 529 pathway (Fig. 2), aligning with previous studies highlighting a dysregulation of tumour-suppressor  
 530 miRNAs in PM [73-75]. Host genes of the eight validated circRNA biomarker candidates included

531 *PLEKHA2*, *TMEM39B*, *CRIM1*, *PTK2*, and *SLC45A4* (Table 1) are associated with cancer  
 532 development and/or progression [76-80], supporting their selection as cancer-specific  
 533 biomarkers of PM.

534 The ddPCR validation revealed *hsa\_circ\_0001829* and *hsa\_circ\_0006646* to be highly  
 535 upregulated in the PM versus non-malignant cells (Fig. 3F-G, Table 2), with an AUC of 0.94, and  
 536 specificity and sensitivity of 87% (Fig. 4A, Table 4), comparable to combinational circRNA panels  
 537 reported in other cancers [81]. Concordant with our findings, a gastric cancer study has also  
 538 demonstrated the strong diagnostic potential of a two-circRNA biomarker signature  
 539 (*hsa\_circ\_0001017* and *hsa\_circ\_0061276*; 95.5% sensitivity, 95.7% specificity) for an accurate  
 540 differentiation of gastric cancer patients from healthy subjects as opposed to using a singular  
 541 circRNA biomarker (*hsa\_circ\_0001017*; 79.4% sensitivity, 81.1% specificity) [82]. Another study  
 542 identified a five-circRNA signature (*circPDLIM5*, *circSCAF8*, *circPLXDC2*, *circSCAMP1*, and  
 543 *circCCNT2*), detectable in EVs, with strong diagnostic capacity for a differentiation of high-grade  
 544 prostate cancer from benign prostatic hyperplasia [83]. The addition of this five-circRNA  
 545 signature with standard of care biomarkers (ERSPC and PCPT) yielded an average AUC from 0.8  
 546 to 0.85 [83]. The improvement in AUC from 0.80 to 0.85, while numerically modest, reflects a  
 547 clinically meaningful enhancement in discriminatory power. Moreover, the gain in AUC has the  
 548 potential to reduce misclassification rates and strengthen the potential utility of the biomarkers  
 549 in early detection and risk stratification, particularly in heterogeneous patient populations  
 550 observed in PM. This provides rationale for prospective studies aiming to assess the diagnostic  
 551 potential of circRNA biomarker signatures in combination with standard of care clinical  
 552 biomarkers of PM.

553 Notably, our study identified a four-circRNA signature (*hsa\_circ\_0007386*, *hsa\_circ\_0006294*,  
 554 *hsa\_circ\_0002346* and *hsa\_circ\_0002348* (Fig. 3A-D, Table 2-3) with capacity to distinguish PM  
 555 from other cancer types (AUC 0.92, specificity 87%, sensitivity 80%), demonstrating their  
 556 potential utility to facilitate a differential diagnosis of PM. This is particularly relevant, given that  
 557 PM shares radiological and symptomatic features with other pleural malignancies [84, 85]. A  
 558 previously developed SOMAscan® 13-protein biomarker test showed good accuracy for  
 559 distinguishing PM from healthy asbestos-exposed controls (AUC 0.86), but failed to differentiate  
 560 PM from other pleural malignancy within an asbestos-exposed validation cohort [86]. In contrast,  
 561 our current study demonstrated a two- to five-fold increase in circRNA expression in PM cells  
 562 compared with lung cancer cells for most circRNA biomarker candidates (with the exception of  
 563 *hsa\_circ\_0006349* and *hsa\_circ\_0001829*) (Table 3), indicating their potential utility for  
 564 differentiating PM from lung cancer. To definitively confirm the diagnostic value of this circRNA

signature, its expression must be validated in prospectively collated tissue and blood samples from patients with PM and lung cancer.

The use of a heterogeneous PM cell line cohort, comprising 27 epithelioid and non-epithelioid PM cell lines, together with 23 non-PM controls spanning both malignant and non-malignant cell types, constitutes a major strength of this study. This comprehensive cell panel enabled the detection of robust and statistically significant circRNA expression differences between the PM and non-PM cell groups. Importantly, this study provides the first evidence supporting novel combinational circRNA biomarker signatures with potential to improve differential PM diagnosis. These findings address the unmet need for the validation of multi-biomarker panels capable of supporting a more sensitive and reliable PM detection, whilst also providing strong rationale for prospective validation studies aiming to further validate combinational circRNA biomarker panels using clinically-relevant biospecimens; particularly blood samples.

A key limitation of this study is the reliance on conventional 2D cell lines, as biomarker expression has been shown in published literature [87, 88] to differ significantly in spheroid models that better capture tumour microenvironment. To strengthen the validity of the circRNA biomarkers identified in this study, parallel investigations in spheroid systems are warranted - specifically incorporating patient-derived tumour cells, which would add translational relevance. A further assessment of the diagnostic sensitivity and specificity of these circRNA candidates in patient blood or plasma would be particularly valuable for determining their suitability for liquid biopsy-based PM diagnostics. Furthermore, the non-invasive nature of liquid biopsy makes circRNA signatures a promising strategy for the diagnosis of PM. In instances where physical signs of PM are not detectable, the use of circRNAs for early diagnosis may be feasible. This is supported by evidence of their utility in the early detection of both non-malignant disease [89] and cancer [90], supporting the motivation to investigate their early detection capacity in PM. Given the long latency period of PM following initial asbestos exposure, this approach offers a significant clinical advantage.

This study focused on the diagnostic capacity of circRNAs, but our group has previously demonstrated that a combinational miRNA signature (miR-Score) can predict long survival ( $\geq 20$  months) in PM patients undergoing extrapleural pneumonectomy (EPP) treatment [91]. The validated prognostic performance of the miR-Score, together with the emerging diagnostic potential of circRNA signatures, provides a strong rationale for developing an integrated biomarker pipeline, within the same research program, encompassing both miRNA and circRNA signatures. Such a unified framework would strengthen clinical stratification and support future

prospective validation studies aimed at implementing multi-RNA biomarker panels across diverse patient cohorts and real-world clinical settings.

This study provides the first evidence of dysregulated circRNA expression in PM and their association with oncogenic pathways implicated in disease onset and/or progression. A two-circRNA signature (hsa\_circ\_0001829 and hsa\_circ\_0006646) differentiates cancer from non-malignant cells, while a four-circRNA signature (hsa\_circ\_0007386, hsa\_circ\_0006294, hsa\_circ\_0002346 and hsa\_circ\_0002348) differentiates PM from other cancers. These findings support their potential utility as diagnostic biomarkers of PM. Further validation of these circRNA signatures in clinically-relevant patient tissue and blood samples will be essential to confirm their diagnostic utility.

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## Author contributions

B.W.J. wrote the manuscript, contributed to the conception and design of the study, performed the experiments and carried out the data analysis. E.M.R. carried out the biomarker specificity and sensitivity analysis, carried out bioinformatics and pathway data analyses and edited the manuscript. D.L.B., R.Z. and L.Z. contributed to the cell culture, RNA extraction, cDNA synthesis and ddPCR experiments. H.S. carried out the principle component analysis. S.A.F. contributed to the study conception. P.W., R.C.Y.L., S.G.G. and G.R. contributed to the conception and RNA sample preparation for the circRNA microarray analysis. J.M. cultured and provided the human fibroblast cells (HumFib). Z.K. and S.S. cultured and provided the other cancer cells (HCC1806,

COLO205, HT-29, AsPC-1, BxPC-3, PANC-1, CFPAC-1 and MDA-MB-231). Sonja K contributed to the study design. Steven K and A.L. reviewed the circRNA specificity and sensitivity data. E.H.B. contributed to the study design and provided supervision. Y.Y.C. contributed to acquisition of the study funding, study conception and design, and provided supervision. S.A.F., H.S., G.R., Steven K, A.L., R.C.Y.L, Sonja K and Y.Y.C provided critical revision to the manuscript. All authors reviewed the manuscript.

#### **Competing interests**

The authors declare that they have no competing interests.

#### **Data availability**

All relevant data generated are included in this article. Other data not relevant to the results presented here are available from the corresponding author, Dr. Johnson, upon reasonable request.

#### **Ethics declarations**

Not applicable.

#### **Consent for publication**

All authors give their consent for Scientific Reports to publish this manuscript.

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#### **Figure Legends**

**Figure 1.** Characterisation of differentially expressed circRNA in PM versus non-malignant cells. **(A)** Representative heat map of hierarchical clustering showing upregulated (red) and downregulated (green) circRNAs in PM cells (H2052) compared to the non-malignant mesothelial cell control group (MeT-5A). **(B)** Scatter plot of normalized circRNA expression (log<sub>2</sub> scaled) in both groups: middle green line indicates no change, flanking green lines represent  $\pm 1.5$ -fold changes. **(C)** Volcano plot of circRNAs showing log<sub>2</sub> fold-change versus -log<sub>10</sub> scaled *p*-value; Red data points indicate  $\geq 1.5$  fold-change and *p*-value  $\leq 0.05$ . **(D)** Pie charts depicting the proportion of up- and down-regulated circRNAs. **(E)** A bar graph showing distribution of circRNAs

by circRNA type (exonic, intronic, sense-overlapping, antisense, intergenic). **(F)** Chromosomal distribution of up- (red) and down-regulated (green) circRNAs.

**Figure 2.** GO and Reactome GSEA pathway enrichment of upregulated circRNAs host genes in PM cells. Green columns represent overrepresented GO biological processes (GOBP) and purple columns represent Reactome cellular pathways. The y-axis indicates normalised enrichment score for statistically significant processes and pathways ( $NES > 1$ ,  $p\text{-value} < 0.05$ ). GO terms and Reactome pathway significant after FDR correction (adjusted  $p\text{-value} < 0.05$ ) are marked with a red asterisk.

**Figure 3.** Expression of circRNA biomarker candidates quantified by ddPCR. Graphs show the expression of **(A)** hsa\_circ\_0007386, **(B)** hsa\_circ\_0006294, **(C)** hsa\_circ\_0002346, **(D)** hsa\_circ\_0002348, **(E)** hsa\_circ\_0084021, **(F)** hsa\_circ\_0001829, **(G)** hsa\_circ\_0006646 and **(H)** hsa\_circ\_0006349 across indicated cell types. Expression was normalised to the internal control circRNA (hsa\_circ\_0000284). Data represents the mean of  $n=3$  biological replicates per cell line. Error bars indicate standard deviation.

**Figure 4.** ROC curves for combinational circRNA biomarker signatures. The x-axis shows 1 – specificity and y-axis shows sensitivity. ROC curves for **(A)** hsa\_circ\_0001829, hsa\_circ\_0006646, **(B)** hsa\_circ\_0001829, hsa\_circ\_0006646, hsa\_circ\_0006349, and **(C)** hsa\_circ\_0001829, hsa\_circ\_0006646, hsa\_circ\_0002348 correspond to the top three circRNA biomarker signatures with capacity to differentiate between cancerous and non-malignant cell types. ROC curves for **(D)** hsa\_circ\_0007386, hsa\_circ\_0006294, hsa\_circ\_0002346, hsa\_circ\_0002348, **(E)** hsa\_circ\_0007386, hsa\_circ\_0006294, hsa\_circ\_0002346, hsa\_circ\_0002348, hsa\_circ\_0006349 and **(F)** hsa\_circ\_0007386, hsa\_circ\_0006294, hsa\_circ\_0002346, hsa\_circ\_0002348, hsa\_circ\_0084021 correspond to the top three circRNA biomarker signatures with capacity to differentiate between PM and other cancer cell types.

**Figure 5.** PCA analysis and two-step diagnostic workflow of circRNA biomarkers. **(A)** PCA plot showing separation of cancer cells (including PM) from non-malignant cells using the two-circRNA signature (hsa\_circ\_0001829 and hsa\_circ\_0006646). **(B)** PCA showing separation of PM cells from other cancers using the four-circRNA signature (hsa\_circ\_0007386, hsa\_circ\_0006294, hsa\_circ\_0002346 and hsa\_circ\_0002348). **(C)** Schematic of a proposed two-step circRNA biomarker work-flow for differential PM diagnosis.

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