



**UNIVERSIDADE ESTADUAL PAULISTA
“JÚLIO DE MESQUITA FILHO”
FACULDADE DE MEDICINA**

Natália Bertoni

**Perfil de expressão global de microRNAs em
plasma e tumor de pacientes com carcinomas
de pâncreas.**

Tese apresentada à Faculdade de Medicina, Universidade Estadual Paulista “Júlio de Mesquita Filho”, Câmpus de Botucatu, para obtenção do título de Doutora em Cirurgia e Medicina Translacional. Área de Concentração: Genética.

Orientadora: Profa. Dra. Patricia Pintor dos Reis.

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***“Diga-me eu esquecerei,
Ensina-me e eu poderei lembrar,
Envolva-me e eu aprenderei.”
Benjamin Franklin***

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***“Aqueles que passam por nós não vão sós.
Deixam um pouco de si e levam um pouco de nós.”
Antoine de Saint-Exupéry***

Resumo

Os carcinomas de pâncreas são carcinomas agressivos e estão entre as neoplasias de alta complexidade no diagnóstico, geralmente detectados em estágios avançados da doença. Estes carcinomas estão associados a altas taxas de mortalidade, levando a óbito mais de 432 mil pacientes a cada ano, mundialmente. Uma vez que o diagnóstico precoce é essencial para melhor sobrevida dos pacientes, a identificação de biomarcadores circulantes e relacionados aos tumores provavelmente fornecerão informações úteis sobre o desenvolvimento da doença, a progressão e impacto na sobrevida. O papel dos microRNAs (miRNAs) na regulação de importantes processos celulares desde o desenvolvimento sugere sua aplicabilidade clínica como biomarcadores robustos. miRNAs são altamente estáveis e podem ser detectados nos fluídos corporais. A detecção de miRNAs no plasma e soro tem sido demonstrada útil no diagnóstico precoce e na predição de prognóstico de pacientes com neoplasias malignas. Portanto, nossos objetivos foram identificar o perfil global de expressão de miRNAs e os mRNAs-alvos regulados por miRNAs em tumor e plasma de pacientes com carcinomas de pâncreas e identificar vias moleculares reguladas por miRNAs. O miR-378h apresentou expressão significativamente aumentada (*fold change* ≥ 2 ; $p < 0,05$; FDR $< 0,05$) no tumor e no plasma de pacientes com adenocarcinoma ductal pancreático. Este miRNA atua como modulador de vias de crescimento celular e de resposta imune. Em adenocarcinoma de papila de Vater, o miR-5000-5p apresentou expressão significativamente diminuída (*fold change* ≥ 2 ; $p < 0,05$; FDR $< 0,05$) no tumor e no plasma, modulando vias envolvidas na sinalização Wnt e na proliferação celular. Este estudo contribuiu para a identificação de miRNAs circulantes e relacionados ao tumor como potenciais biomarcadores tumorais específicos importantes no processo de oncogênese pancreática.

Abstract

Pancreatic carcinomas are aggressive carcinomas and are among the tumors with high complexity at diagnosis, usually diagnosed in advanced stages. Pancreatic carcinomas are associated with high mortality rates, with more than 432 thousand deaths every year, worldwide. Since early disease diagnosis is essential for better patient survival, the identification of blood-based and tumor-related biomarkers will likely provide useful information on disease development, progression and impact on survival. Of note, the role of microRNAs (miRNAs) in regulating important cellular processes since early development suggests their clinical applicability as robust biomarkers. miRNAs are highly stable and can be detected in the body fluids. Detection of miRNAs in plasma and serum has been shown useful for early diagnosis and to predict prognosis of patients with cancer. Therefore, our aims were to identify the miRNA global expression profile in tumor and plasma from patients with pancreatic carcinomas and to elucidate molecular pathways regulated by miRNA target-genes. miR-378h was significantly upregulated ($FC \geq 2$ and $p < 0.05$ and $FDR < 0.05$) in plasma and tumor from patients with PDAC. This miRNA acts as a modulator of cell growth and immune response pathways. In AMP cancers, miR-5000-5p was significantly downregulated ($FC \geq 2$ and $p < 0.05$ and $FDR < 0.05$) in plasma and tumor, modulating pathways involved in Wnt signaling and cell proliferation. The identification of specific circulating plasma and tumor-related miRNAs deregulated in patients with PDAC and AMP may indicate these molecules as valuable biomarkers to assist in early diagnosis.

Lista de abreviaturas e siglas

AGO	Argonauta
Ago1	Argonauta 1
Ago2	Argonauta 2
AMP	<i>Adenocarcinoma of Vater ampulla</i> ou Adenocarcinoma de papila de Vater
AUC	<i>Area under the curve</i>
Avidin-PE	Avidina-Peroxidase
BD	Bem diferenciado
CAAE	Certificado de Apresentação para Apreciação Ética
DGCR8	<i>Di George syndrome critical region 8</i>
DNA	Ácido Desoxirribonucleico
dsRNA	<i>double-stranded RNA</i> (microRNA de fita dupla)
EDTA	<i>Ethylenediamine tetraacetic acid</i>
ELOSA	<i>Enzyme Linked Oligosorbent Assay</i>
FC	<i>Fold Change</i>
FDR	<i>False Discovery Rate</i>
FGF	<i>Fibroblast Growth Factor</i>
FIE-HRMS	<i>Flow Injection Electrospray High Resolution Mass Spectrometry</i>
GO	<i>Gene Ontology</i>
GSK-3	<i>Glycogen synthase kinase 3</i>
HCB	Hospital das Clínicas de Botucatu
HDL	<i>High Density Lipoprotein</i>
IQR	<i>Interquantile Range</i>
KEGG	<i>Kyoto Encyclopedia of Genes and Genomes</i>
MD	<i>Moderately differentiated</i> ou Moderadamente diferenciado
min	Minutos
mirDIP	<i>microRNA Data Integration Portal</i>
miR(s) ou miRNA(s)	Micro ácido ribonucleico

mRNA	Ácido ribonucléico mensageiro
MSEA	<i>Metabolite Set Enrichment Analysis</i>
NAD⁺	<i>Nicotinamide adenine dinucleotide</i>
NADP⁺	<i>Nicotinamide adenine dinucleotide phosphate</i>
PDAC	<i>Pancreatic ductal adenocarcinoma</i> ou adenocarcinoma ductal pancreático
PCA	<i>Principal Component Analysis</i>
PD	<i>Poorly differentiated</i> ou Pouco diferenciado
Pitx2	<i>Pituitary homeobox 2</i>
PLS-DA	<i>Partial Least Squares-Discriminant Analysis</i>
PPARα	<i>Peroxisome proliferator-activated receptor alpha</i>
pré-miRNA	miRNA precursor em forma de grampo (<i>hairpin</i>)
pri-miRNA	Transcrito longo primário de miRNA
RPM	Rotação por minuto (unidade de rotação em centrífuga)
RISC	<i>RNA-Induced Silencing Complex</i>
RNA	Ácido Ribonucleico
s	Segundos
SPRY	<i>Sprouty proteins</i>
TCA	<i>tricarboxylic acid cycle</i>
TCLE	Termo de Consentimento Livre e Esclarecido
TCR	<i>T cell antigen receptor</i>
TRBP	<i>transactivating response RNA-binding protein</i>
UHPLC	<i>ultra-high-performance liquid chromatography</i>
UTR	<i>Untranslated region</i> (região não traduzida)
WD	<i>Well differentiated</i>

Lista de símbolos

~	Aproximadamente
g	Força gravitacional
°C	Grau Celsius
kDa	<i>kilodaltons</i>
®	Marca registrada
≥	Maior ou igual a
<	Menor que
≤	Menor ou igual a
μL	Microlitro
mL	Mililitro
mg	Miligrama
<i>m/z</i>	<i>Mass to charge</i>
ng	Nanograma
%	Porcentagem
vs	<i>Versus</i>

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1. Capítulo I

Este capítulo refere-se ao projeto intitulado “Perfil de expressão global de microRNAs em plasma e tumor de pacientes com carcinomas de pâncreas”. Os resultados correspondentes ao projeto estão apresentados adiante no formato de manuscrito científico (**item 1.5**).

1.1. Introdução

1.1.1. Carcinomas de pâncreas

Os carcinomas do pâncreas representam a sétima causa mais frequente de morte por câncer em homens e mulheres, levando a óbito mais de 432 mil pacientes a cada ano, mundialmente (1). As estatísticas globais mostraram esses carcinomas como o 11º câncer mais comum, com mais de 459 mil novos casos no ano de 2018 (1). No Brasil, o carcinoma de pâncreas é responsável por aproximadamente 2% de todos os cânceres, com a ocorrência de mais de 12.000 casos novos a cada ano, e 4% do total de mortes por câncer, sendo a sexta causa mais frequente de morte câncer em homens e mulheres. Em 2018 foram registradas 11.858 mortes pela doença (2, 3). O desenvolvimento dos carcinomas de pâncreas está relacionado a fatores de risco como etilismo, tabagismo, obesidade, diabetes e pancreatite crônica (4).

Um dos principais problemas com o carcinoma pancreático é a dificuldade de detecção e diagnóstico destes tumores, estando associada ao diagnóstico tardio e, consequentemente, uma alta taxa de mortalidade (5). O tratamento padrão disponível é a cirurgia, porém apenas 10-20% dos pacientes sem doença metastática são elegíveis para a ressecção do tumor (6). Os pacientes que apresentam doença metastática e não são indicados à cirurgia (cerca de 50-60%) podem ser submetidos à quimioterapia ou quimioterapia combinada com radioterapia a fim de prolongar a sobrevida e aliviar os sintomas clínicos (7). Existem ainda os pacientes que apresentam tumores com bordas ressecáveis,

chamados de *borderlines*: ao momento do diagnóstico eles não são elegíveis à ressecção cirúrgica devido ao envolvimento do tumor com a vasculatura próxima, porém são tratados primeiramente com quimioterapia para redução do tamanho do tumor e são posteriormente submetidos à cirurgia (8).

O tipo histológico adenocarcinoma ductal pancreático (PDAC) compreende cerca de 96% dos casos e os outros tumores pancreáticos incluem carcinomas periampulares (9). Em relação aos carcinomas periampulares, ~12% surgem do complexo ampular (distal ao encontro dos ductos biliar e pancreático) e são classificados como adenocarcinoma de papila de Vater (AMP) (10). O principal tratamento também é a ressecção cirúrgica do tumor, porém ainda existe grande dificuldade em diferenciá-lo dos demais tipos periampulares no pré-operatório (10). No entanto, o adenocarcinoma de papila de Vater apresenta melhor prognóstico (com uma taxa de sobrevida em 5 anos > 45%) e detecção precoce quando comparado ao adenocarcinoma ductal pancreático (10).

Entender as diferenças e estudar as particularidades moleculares dos tipos histológicos adenocarcinoma ductal pancreático e adenocarcinoma de papila de Vater podem ajudar na detecção precoce e no direcionamento do tratamento destes pacientes.

1.1.2. microRNAs como biomarcadores no câncer

microRNAs (miRNAs) são RNAs pequenos, de 18-22 nucleotídeos de comprimento, não codantes e que desempenham um papel importante como reguladores da expressão gênica (11). A biogênese miRNAs através da via canônica se inicia com um transcrito longo primário (pri-miRNA) com centenas de pares de bases em forma de *stem-loop* sintetizado pela enzima RNA polimerase II, a partir da sequência de DNA. O pri-miRNA é então clivado pela ribonuclease RNase III Drosha, formando um complexo de ~650KDa acoplado ao co-fator DGCR8 (*Di George syndrome critical region 8*), o que resulta em uma estrutura precursora (pré-miRNA) em forma de grampo (*hairpin*) de 60-100 nucleotídeos de comprimento (12). A molécula precursora é exportada, por intermédio da proteína Exportina 5,

do núcleo para o citoplasma, onde é clivada pela enzima RNase III Dicer. A estrutura de *haipin* é desdobrada em uma estrutura madura de miRNA fita dupla de ~18-22 nucleotídeos (dsRNA), composto pelo miRNA maduro guia e pela fita complementar passageira. Tais sequências são nomeadas de acordo com a fita precursora, uma proveniente da fita 5' e uma da fita 3' (por exemplo, miR-125a-5p e miR-125a-3p) (11, 13).

A fita de miRNA maduro com mais ligações de afinidade/estabilidade para a proteína Argonauta é incorporada ao complexo de silenciamento induzido por RNA (em inglês RISC, *RNA-Induced Silencing Complex*), composto por uma família de proteínas chamada Argonauta (Ago 1 e Ago 2) e pela proteína de ligação ao RNA (em inglês TRBP, *transactivating response RNA-binding protein*) (14). O complexo é responsável por orientar o miRNA para o RNA mensageiro (mRNA) alvo. A outra fita de miRNA é geralmente degradada; entretanto, em alguns casos, ambas as fitas podem ser funcionais (15).

Em humanos, a regulação da expressão gênica ocorre predominantemente por meio do pareamento de bases das extremidades 5' do miRNA maduro com a extremidade 3' não traduzida (3' UTR) do mRNA alvo, levando, na maioria dos casos, à inibição da tradução ou à degradação do mRNA (11, 16). Na posição dos nucleotídeos de 2-7 da região 5' do miRNA está situada a sequência *seed*, região essencial para o pareamento e que permite aos miRNAs regularem um grande número de genes (11, 17). A predição de alvos regulados por miRNAs ocorre através da identificação de mRNA com complementaridade conservada à sequência *seed* do miRNA (17). Até o momento, existem 2.654 miRNAs maduros anotados e mapeados no genoma humano (<http://www.mirbase.org/cgi-bin/browse.pl?org=hsa>).

Os miRNAs estão envolvidos em muitos processos biológicos importantes, tais como o desenvolvimento embrionário, diferenciação, apoptose e proliferação celular (18-20) e em mecanismos de oncogênese (21-23). A descoberta dessas moléculas como importantes reguladores da expressão gênica e seu papel na carcinogênese humana têm os indicado como candidatos ideais a biomarcadores

diagnósticos, prognósticos e como alvos terapêuticos potenciais no câncer (24-30). Além disso, miRNAs são mais estáveis do que mRNAs, sua expressão é tecido-específica e são facilmente detectados utilizando pequenas quantidades de RNA.

Amostras biológicas obtidas através de biópsia de tecido são frequentemente utilizadas para caracterização de tumores, porém existem restrições na frequência de amostragem e uma representação incompleta do tamanho e características do tumor (31). Dessa maneira, amostras extraídas de fluídos corporais, como plasma, obtidas através de técnica minimamente invasiva (conhecida como biópsia líquida), apresentam componentes tumorais que podem auxiliar no diagnóstico precoce, monitoramento e progressão do câncer.

Os principais componentes presentes no sangue periférico que podem fornecer informações sobre os tumores compreendem células tumorais circulantes, DNA circulante livre de célula, DNA tumoral circulante, RNA circulante livre de célula (que contém principalmente pequenos RNAs e RNAs mensageiros), vesículas extracelulares circulantes, plaquetas, proteínas e metabólitos (31). De fato, estudos recentes têm demonstrado a utilidade de miRNAs como biomarcadores minimamente invasivos na detecção de células tumorais circulantes (32-35). Dessa maneira, a análise global de expressão de miRNAs, específicos ao tecido do tumor e circulantes (livres no plasma), integrada à identificação de mRNAs-alvo regulados por miRNAs, deve levar à elucidação de vias moleculares desreguladas e associadas à patogênese dos carcinomas de pâncreas.

1.1.3. microRNAs em carcinomas de pâncreas

Alguns estudos têm mostrado a expressão de miRNAs em carcinomas de pâncreas, porém ainda são necessárias mais informações a respeito dos mecanismos pelos quais os miRNAs podem regular vias moleculares potencialmente envolvidas na tumorigênese, progressão e metástase de carcinomas de pâncreas. Um estudo de expressão global de miRNAs comparou 10 casos de adenocarcinoma ductal pancreático, 7 casos de pancreatite crônica e 7 tecidos normais como controle e mostrou que os níveis de expressão de um grupo de 20

miRNAs foram capazes de diferenciar esses três tipos de tecido. Além disso, os autores identificaram os miRNAs miR-216 e miR-217 expressos em tecido pancreático normal e com perda de expressão em tecido pancreático tumoral, sendo que a expressão desses dois miRNAs sugere uma característica do tecido pancreático normal (36).

Outro estudo comparou 65 amostras de adenocarcinoma ductal pancreático, 65 amostras pareadas de tecido normal adjacente e 42 amostras de pancreatite crônica (37). Nessas amostras foram identificados dois grupos de miRNAs diferencialmente expressos, sendo um capaz de discriminar adenocarcinoma ductal pancreático de pancreatite crônica e pâncreas normal (miR-21, miR-221, miR-222, miR-181, miR-181b, miR-181d e miR-155) e outro com poder de predição de sobrevida a curto e a longo prazos (miR-452, miR-105, miR-127, miR-518a, miR-187 e miR-30a-3p) (37).

Os altos níveis de expressão dos miRNAs miR-155, miR-203, miR-210 e miR-222 em amostras de adenocarcinoma pancreático também foram relacionados com uma pior sobrevida (38). Outro estudo analisou 17 pares de amostras de carcinomas pancreáticos e tecidos histologicamente normais e identificou oito miRNAs desregulados (miR-196a, miR-190, miR-186, miR-221, miR-222, miR-200b, miR-15b e miR-95), os quais desempenham importantes funções e compartilham vias moleculares durante a patogênese desse carcinoma (39).

1.1.4. microRNAs circulantes

Tem sido demonstrado que miRNAs extracelulares circulam pelo sangue de pacientes com câncer, podendo assim, servir como novos marcadores diagnósticos. A primeira evidência que se tem registro a respeito de miRNAs circulantes foi sobre os níveis altos de expressão do miR-21 no soro de pacientes com linfoma difuso de grandes células B, significativamente relacionado com melhora da sobrevida livre de recidiva (40).

Os miRNAs circulantes podem estar envoltos por complexos lipídicos ou lipoproteicos (microvesículas), como exossomos, sendo assim altamente estáveis

na circulação sanguínea (34). A incorporação dos miRNAs ao plasma ocorre através desses complexos extracelulares ou junto a proteínas de ligação ao RNA, como proteínas da família AGO ou HDL (41). Apesar da presença e atividade da ribonuclease RNase no plasma, foi demonstrado que os miRNAs circulantes são resistentes à degradação pois, por meio de centrifugação diferencial e cromatografia de exclusão por tamanho, foram identificadas duas populações de miRNAs circulantes: cerca de 90% na forma livre de vesículas, ou seja, acoplados aos complexos AGO2 e uma pequena porção (~10% do total) encapsulados em vesículas (35, 42, 43). Dessa maneira, estratégias de análise de expressão de miRNAs circulantes na forma livre se faz importante na busca por potenciais biomarcadores em carcinomas.

Estudos têm apresentado o crescente papel dos miRNAs circulantes como biomarcadores em pacientes com carcinomas de pâncreas. Por exemplo, foi observada uma alta expressão dos miRNAs miR-21, miR-210, miR-155 e miR-196a no plasma de pacientes com adenocarcinoma ductal pancreático quando comparados com indivíduos sem a doença (44). A expressão do miR-196a no soro demonstra o potencial valor desse miRNA como marcador de pior prognóstico para pacientes com adenocarcinoma ductal pancreático (45). Amostras de plasma de pacientes com câncer de pâncreas também apresentaram níveis significativamente elevados do miR-210 quando comparadas com amostras de indivíduos saudáveis (46). O miR-18a circulante no plasma de pacientes com câncer de pâncreas pode ser um biomarcador útil para rastreio e monitoramento do tumor (47) e uma assinatura de 7 miRNAs (miR-20a, miR-21, miR-24, miR-25, miR-99a, miR-185 e miR-191) foi demonstrada útil para diferenciar pacientes com pancreatite crônica daqueles com câncer de pâncreas (48).

Apesar destes e outros estudos, ainda há necessidade de integração de dados de expressão de miRNAs e mRNAs-alvo regulados por miRNAs, fundamental para a elucidação de vias moleculares em carcinomas de pâncreas. As evidências da literatura indicam que miRNAs circulantes podem ser excelentes biomarcadores para monitorar o desenvolvimento e progressão do tumor, pois

podem ser detectados nos fluídos corporais mesmo após longos períodos de armazenamento das amostras (49). Da mesma forma, miRNAs circulantes podem representar biomarcadores com valor potencial para melhorar a detecção da doença, determinação do prognóstico/sobrevida ou predição de resposta ao tratamento em carcinomas de pâncreas. Considerando que a desregulação de miRNAs pode promover a iniciação e progressão tumoral, tais moléculas podem ser biomarcadores robustos para o desenvolvimento de estratégias diagnósticas para pacientes com carcinomas de pâncreas.

1.2. Justificativa

Apesar dos avanços nas informações sobre a biologia tumoral dos carcinomas de pâncreas, a detecção precoce e o prognóstico dos pacientes ainda são deficientes. Estratégias de tratamento vigentes para esses pacientes têm falhado em atenuar a agressividade da doença ou melhorar a sobrevida. O insucesso da quimioterapia convencional pode ser parcialmente atribuído à heterogeneidade genética e comportamento biológico tumoral que diferem entre os pacientes. A identificação de alterações moleculares em tumores e no plasma de pacientes com câncer pode contribuir para o desenvolvimento de estratégias de diagnóstico precoce e tratamento direcionado, constituindo um avanço importante para melhorar a sobrevida dos pacientes. Uma estratégia potencial para atingir essa meta é por meio da identificação de miRNAs e seus mRNAs-alvo como biomarcadores associados à oncogênese. Tal identificação poderá levar à caracterização de vias moleculares desreguladas em carcinomas de pâncreas. Portanto, estudos como o aqui proposto podem conduzir a avanços na identificação de biomarcadores clinicamente aplicáveis, para melhorar o diagnóstico e prognóstico/sobrevida de pacientes com carcinomas de pâncreas.

1.3. Objetivos

1.3.1. Identificar o perfil de expressão global de miRNAs em plasma e tumor de pacientes com carcinomas pâncreas (adenocarcinoma ductal pancreático e adenocarcinoma de papila de Vater);

1.3.2. Identificar, *in silico*, os mRNAs-alvo regulados por miRNAs;

1.3.3. Identificar vias moleculares biologicamente relevantes na tumorigênese dos carcinomas pancreáticos.

1.4. Materiais e Métodos

1.4.1. Aspectos éticos

O presente projeto foi aprovado pelo Comitê de Ética em Pesquisa da Faculdade de Medicina de Botucatu (CAAE: 61303516.5.0000.5411, **Anexo 5.1**). O Termo de Consentimento Livre e Esclarecido (TCLE) foi aplicado aos pacientes elegíveis ao estudo.

1.4.2. Critérios de inclusão e exclusão de pacientes

Este estudo incluiu amostras de sangue e de tecido de pacientes atendidos e tratados no Hospital das Clínicas de Botucatu (HCB), da Faculdade de Medicina de Botucatu, UNESP.

Os critérios de inclusão dos pacientes foram os seguintes: indivíduos maiores de 18 anos e capazes de fornecer consentimento informado, diagnóstico de carcinoma pancreático, de qualquer estadiamento e grau histológico, pacientes submetidos à cirurgia para retirada do tumor.

Os critérios de exclusão foram: pacientes submetidos a radio e/ou quimioterapia anteriormente à coleta de amostras e diagnosticados com doença neoplásica não primária.

1.4.3 Amostras

O perfil de expressão global de miRNAs foi determinado em amostras de tecido tumoral (miRNAs associados ao tumor) e em amostras de plasma (miRNAs circulantes) coletadas dos mesmos pacientes. As amostras de pacientes e de indivíduos voluntários saudáveis/sem doença (referenciais para as amostras de plasma) foram coletadas no HCB.

Entre as amostras de tecido e de plasma de pacientes com carcinomas de pâncreas coletadas pelo grupo de pesquisa, foram selecionadas aquelas que atenderam aos critérios de inclusão. Além disso, foi utilizado como critério de seleção de amostras aquelas que estivessem disponíveis plasma, tecido tumoral e

tecido histologicamente normal adjacente ao tumor do mesmo paciente. As amostras de tecido tumoral e de tecido histologicamente normal adjacente foram submetidas à análise de congelação para confirmar, por meio da histologia, a presença de tumor ou de células normais em pelo menos 90% dos tecidos. Nessa fase também foi analisado e confirmado o diagnóstico histopatológico dos tumores dos pacientes. Essa etapa foi importante para identificar as amostras de pacientes com adenocarcinoma ductal pancreático e adenocarcinoma de papila de Vater para análise molecular subsequente.

Foram coletadas amostras pareadas (tecido tumoral e tecido histologicamente normal correspondente) e plasma de 12 pacientes com carcinoma de pâncreas, sendo do subtipo histológico adenocarcinoma ductal pancreático (N=7) e adenocarcinoma de papila de Vater (N=5).

As amostras de tecido histologicamente normal correspondentes foram utilizadas como referencial para comparação com as amostras de tecido tumoral. Além disso, foram agrupadas em *pools* de acordo com o tipo histopatológico correspondente: *pool* normal de referência para adenocarcinoma ductal pancreático (N=7) e *pool* normal de referência para adenocarcinoma de papila de Vater (N=5). Para as amostras de plasma foram utilizadas como referencial para comparação amostras de plasma de indivíduos voluntários, sem carcinomas, sem comorbidades e não fumantes (N=2).

Os dados clínicos e histopatológicos dos pacientes estão apresentados na **Tabela 1**. O delineamento experimental e as amostras são mostrados adiante (**Figura 1**).

Tabela 1. Características clínicas e histopatológicas dos 12 casos.

Variáveis	Adenocarcinoma ductal pancreático (N=7)	N (%)	Adenocarcinoma de papila de Vater (N=5)	N (%)
Idade (anos)				
Média	61,2 ± 4,8		64,8 ± 5,7	
Intervalo	(52-70)		(55-73)	
Sexo				
Masculino	2	28,6	3	60
Feminino	5	71,4	2	40
Grau histológico				
BD	5	71,4	3	60
MD	1	14,3	1	20
PD	1	14,3	1	20
Tamanho do tumor (T)				
T1-T2	0	0	2	40
T3-T4	7	100	3	60
Características dos linfonodos (N)				
Positivo (N1, N2)	5	71,4	3	60
Negativo (N0)	1	14,3	1	20
Indeterminado (Nx)	1	14,3	1	20
Metástase à distância (M)				
Positivo (M1)	1	14,3	0	
Negativo (M0)	0	0	1	20
Indeterminado (Mx)	6	85,7	4	80
Estadiamento				
Ia	0	0	1	20
Ib	0	0	0	0
IIa	1	14,3	1	20
IIb	3	42,8	2	40
III	2	28,6	1	20
IV	1	14,3	0	0
Fumo				
Sim	2	28,6	3	60
Não	5	71,4	2	40
Álcool				
Sim	0	0	2	40
Não	7	100	3	60
Diabetes				
Sim	4	57,2	0	0
Não	3	42,8	5	100
Obesidade				
Sim	1	14,3	0	0
Não	6	85,7	5	100
Status do paciente				
Vivo com doença	5	71,4	2	40
Óbito pela doença	2	28,6	3	60

BD: bem diferenciado

MD: moderadamente diferenciado

PD: pouco diferenciado

1.4.3.1. Coleta e armazenamento de tecido

As amostras de tecido tumoral e de tecido histologicamente normal adjacente foram coletadas dos pacientes durante cirurgia e imediatamente congeladas em nitrogênio líquido, e então armazenadas em freezer, a -80°C até utilização.

1.4.3.2. Coleta, separação e armazenamento de plasma

Foram coletados dos pacientes (ao momento do diagnóstico, antes de qualquer tratamento) e dos indivíduos voluntários 4 mL de sangue periférico em tubo BD Vacutainer® (Becton Dickinson, NJ, EUA) com EDTA. O sangue foi mantido em gelo a ~4°C até ser processado para obtenção do plasma livre de células, por centrifugação a 1.200 rpm, por 15 minutos a 4°C. A fração plasmática (fase superior) foi aliquoteada e transferida para novos tubos *ependorf* de 1,5 mL e então armazenada em freezer, a -80°C até utilização.

1.4.4. Análise molecular

1.4.4.1. Extração de RNA

Amostras de tecido: para identificar os miRNAs associados ao tumor, o RNA foi extraído utilizando o *miRNeasy Mini Kit* (Qiagen, Valencia, CA), o qual também é otimizado para a extração de pequenos RNAs (incluindo miRNAs) presentes nos tecidos, segundo protocolo do fabricante.

Anteriormente à extração das moléculas de interesse é necessário preparar as amostras e o processo consiste em romper os tecidos com um homogeneizador. Para isso foram utilizados ~10 mg dos tecidos de interesse, adicionados à tubos contendo *beads* de cerâmica e 700 µL de reagente de lise (fornecido pelo kit da Qiagen) e processados no equipamento Precellys® (Bertin Instruments, Montigny-le-Bretonneux, França) em ciclos de 6500 rpm por 10 segundos, até completa homogeneização e incubadas a temperatura ambiente por 5 minutos.

Logo após foram adicionados 140 μL de clorofórmio a cada amostra e incubadas por 3 minutos a temperatura ambiente e em seguida centrifugadas a 12.000 x g , por 15 minutos a 4°C. A centrifugação permite que cada amostra seja separada em fases orgânicas e aquosa, sendo que a fase inferior contém proteínas, a interfase contém DNA e a fase superior (aquosa) contém RNA. A fase aquosa contendo o RNA de cada amostra foi transferida para novos tubos *eppendorf* e então foi adicionado 500 μL de etanol 100% a cada amostra, para fornecer condições de ligação adequadas para todas as moléculas de RNA que contenham a partir de ~ 18 nucleotídeos. As próximas etapas consistiram na purificação do RNA através de colunas com filtro, onde o RNA total (incluindo os miRNAs e pequenos RNAs) adere à membrana e os contaminantes são descartados no material filtrado. A fase aquosa contendo etanol de cada amostra foi transferida para as colunas com filtro que foram centrifugadas a 8.000 x g , por 15 segundos a 25°C e o filtrado foi desprezado. Adicionou-se 700 μL da solução de lavagem *Buffer RWT* (fornecida pelo kit), seguida por centrifugação a 8.000 x g , por 15 segundos a 25°C e o filtrado foi desprezado. Adicionou-se 500 μL da solução de lavagem *Buffer RPE* (fornecida pelo kit), seguida por centrifugação a 8.000 x g , por 15 segundos a 25°C e o filtrado foi desprezado. Adicionou-se 500 μL solução de lavagem *Buffer RPE* (fornecida pelo kit), seguida por centrifugação a 8.000 x g , por 2 minutos a 25°C e o filtrado foi desprezado. Uma nova centrifugação foi realizada para secagem das colunas na velocidade máxima, por 1 minuto a 25°C. A coluna com filtro foi então transferida para um novo tubo coletor fornecido pelo kit para a eluição do RNA. Foram adicionados 50 μL de água estéril e livre de RNase (fornecida pelo kit) no centro do filtro e centrifugado por 1 minuto a 8.000 x g . O RNA total obtido (enriquecido com miRNAs) foi então armazenado em freezer, a -80°C, até sua utilização.

Amostras de plasma: para identificar os miRNAs circulantes livres expressos no plasma, o RNA foi extraído utilizando o *miRNeasy plasma/serum isolation kit* (Qiagen, Valencia, CA), o qual é otimizado para extração de miRNAs circulantes presentes em fluidos corporais, segundo protocolo do fabricante.

Um tubo *eppendorf* de 1,5 mL de plasma de cada amostra foi descongelado em gelo (por ~30 min.). De cada tubo, foi aliquoteado 200 μ L de plasma para extração do RNA. Foram adicionados 1000 μ L de reagente de lise (fornecido pelo kit) a cada amostra, as quais foram incubadas por 5 minutos a temperatura ambiente, a fim de desnaturar complexos de proteínas e RNases e remover grande parte do DNA residual. Logo após foram adicionados 200 μ L de clorofórmio a cada amostra e incubadas por 3 minutos a temperatura ambiente e em seguida centrifugadas a 14.000 rpm, por 15 minutos a 4°C. A centrifugação permite que cada amostra seja separada em fases orgânicas e aquosa, sendo que a fase inferior contém proteínas, a interfase contém DNA e a fase superior (aquosa) contém RNA. A fase aquosa contendo o RNA de cada amostra foi transferida para novos tubos *eppendorf* e então foi adicionado 900 μ L de etanol 100% a cada amostra, para fornecer condições de ligação adequadas para todas as moléculas de RNA que contenham a partir de ~18 nucleotídeos. As próximas etapas consistiram na purificação do RNA através de colunas com filtro, onde o RNA total (incluindo os miRNAs e pequenos RNAs) adere à membrana e os contaminantes são descartados no material filtrado. A fase aquosa contendo etanol de cada amostra foi transferida para as colunas com filtro que foram centrifugadas a 10.000 rpm, por 15 segundos a 25°C e o filtrado foi desprezado. Adicionou-se 700 μ L da solução de lavagem *Buffer RWT* (fornecida pelo kit), seguida por centrifugação a 10.000 rpm, por 15 segundos a 25°C e o filtrado foi desprezado. Adicionou-se 500 μ L da solução de lavagem *Buffer RPE* (fornecida pelo kit), seguida por centrifugação a 10.000 rpm, por 15 segundos a 25°C e o filtrado foi desprezado. Adicionou-se 500 μ L de etanol 80%, seguida por centrifugação a 10.000 rpm, por 2 minutos a 25°C e o filtrado foi desprezado. Uma nova centrifugação foi realizada para secagem das colunas a 14.000 rpm, por 5 minutos a 25°C. A coluna com filtro foi então transferida para um novo tubo coletor fornecido pelo kit para a eluição do RNA que foi realizada em duas etapas. Primeiro foram adicionados 7 μ L de água estéril e livre de RNase (Sigma-Aldrich, St. Louis, MO, EUA) no centro do filtro, incubado por 1 minuto em temperatura ambiente, seguido de centrifugação por 1 minuto a 14.000

rpm a 25°C. Essa etapa foi repetida uma vez até a obtenção do RNA. O RNA total obtido (enriquecido com miRNAs) foi então armazenado em freezer, a -80°C, até sua utilização.

1.4.4.2. Quantificação e qualidade do RNA

Todas as amostras de RNA (isolados dos tecidos e do plasma) foram avaliadas quanto à qualidade e pureza através do equipamento *NanoDrop 8000* (Thermo Fisher Scientific), que permite quantificar o RNA total contido em cada amostra e verificar se há contaminação por proteínas e/ou resíduos de solventes orgânicos.

1.4.4.3. Quantificação de expressão de microRNAs

A análise da expressão de miRNAs foi realizada utilizando a plataforma GeneChip® miRNA 4.0 Array (Thermo Fisher Scientific), que contém sondas para todos os 2.588 miRNAs mapeados no genoma humano e anotados no banco de dados de miRNAs miRBase (<http://www.mirbase.org/>). Portanto, essa plataforma é útil para a quantificação global da expressão de miRNAs no tecido tumoral e plasma dos pacientes. Sondas de miRNAs endógenos (controles internos) estão incluídas na plataforma e foram utilizadas para normalização dos dados. Os experimentos seguiram o protocolo do fabricante.

Primeiramente a concentração do RNA de todas as amostras (plasma e tecido) foi ajustada para 17 ng/μL. As amostras de RNA foram então submetidas a incorporação da cauda poli-A à extremidade 3', seguido por um segundo passo de reação de ligação de molécula de biotina à cauda 3' poli-A do RNA, processos necessários para a marcação e posterior identificação dos miRNAs. A fim de confirmar se o processo de incorporação de biotina funcionou adequadamente, foi realizado o ensaio colorimétrico ELOSA (*Enzyme Linked Oligosorbent Assay*) e a marcação bem sucedida detecta os RNAs controles incluídos em todas as reações de marcação com a biotina.

Uma vez confirmada a marcação do RNA com as moléculas de biotina, o passo seguinte consiste em hibridizar o RNA no cartucho GeneChip® miRNA 4.0 Array, para cada amostra, e então detectado com o conjugado Avidina-Peroxidase (*Avidin-PE*), que se liga ao RNA marcado com biotina com forte afinidade.

Os cartuchos de *microarray* de miRNA com as amostras são então colocados nas bandejas do forno de hibridização e incubados a 48°C a 60 rpm por 16 a 18 horas. Após este período, é retirado o conjugado de hibridização de cada cartucho de *microarray* e cada um é preenchido com tampão que permite atingir a temperatura ambiente antes da lavagem e coloração. Neste momento também é preparada a estação de fluídos, onde é realizado o procedimento de lavagem dos cartuchos com os reagentes fornecidos no kit. Os passos de lavagem e coloração são então realizados utilizando o roteiro de fluídos apropriado para os cartuchos. A última etapa ocorre na estação de trabalho do scanner e consiste na leitura dos cartuchos.

1.4.5. Análise de dados

1.4.5.1. Processamento de dados de expressão de microRNAs

Os dados gerados pela plataforma GeneChip® miRNA 4.0 Array foram exportados para o *software* Transcriptome Analysis Console 4.0 (Thermo Fisher Scientific) para análise, normalização e controle de qualidade.

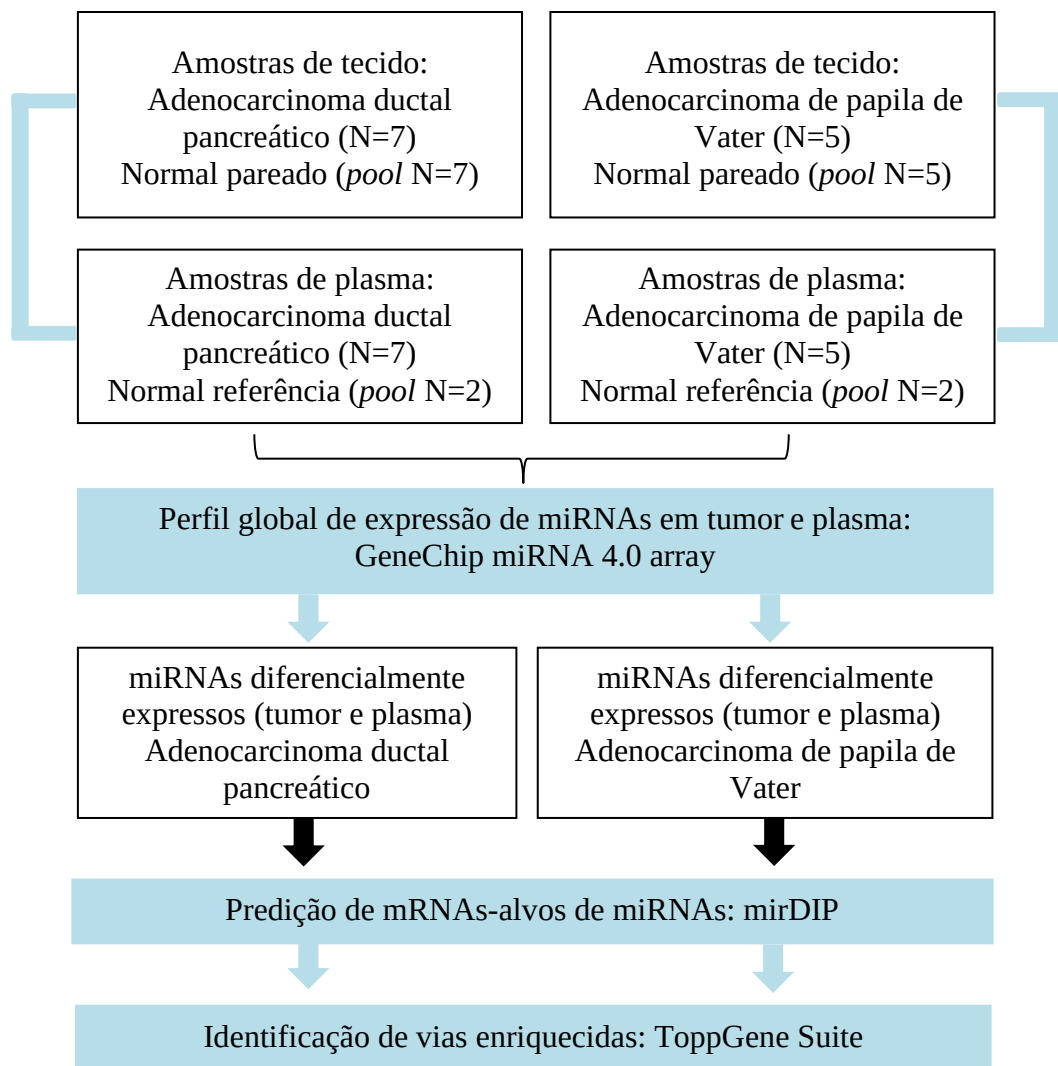
Para determinação de miRNAs diferencialmente expressos tem sido amplamente aceito aplicar o valor de *fold change* ≥ 2 ; valor de $p < 0,05$; FDR $< 0,05$ como valores de corte (*cutoff*) para significância estatística.

1.4.5.2. Predição de alvos de microRNAs

Os miRNAs identificados como diferencialmente expressos nas amostras de tumor e plasma foram utilizados para análises subsequentes de bioinformática, para predição dos mRNAs-alvo potencialmente regulados por esses miRNAs. Esta análise foi realizada utilizando a ferramenta *microRNA Data*

Integration Portal (<http://ophid.utoronto.ca/mirDIP/>) (50), a qual é útil para a predição global de mRNAs-alvo regulados por miRNAs, pois integra os principais bancos de dados de miRNAs como o DIANA-microT (51, 52), microRNA.org (<http://www.microrna.org/microrna/miRanda>) (53, 54), picTar (<https://pictar.mdc-berlin.de/>), PITA (https://genie.weizmann.ac.il/pubs/mir07/mir07_dyn_data.html), RNA22 microRNA target detection (<https://cm.jefferson.edu/rna22/Interactive/>) e TargetScan (17, 55). A combinação destes bancos de dados oferece uma maior precisão na predição de mRNAs-alvo regulados pelos miRNAs. Os algoritmos de predição procuram principalmente por alto grau de complementaridade entre as extremidades 3' da sequência alvo do mRNA e a extremidade 5' do miRNA, com maior atenção à sequência *seed* do miRNA (56). Durante as buscas foi utilizada as pontuações “*very high*” (top 1%) e “*high*” (top 5%) para probabilidade de interação como critério de seleção dos resultados. Adicionalmente, para a identificação de vias biológicas estatisticamente enriquecidas foi utilizado o ToppGene Suite (<https://toppgene.cchmc.org/>) (57).

Figura 1. Fluxograma do delineamento experimental e amostras do projeto de pesquisa.



1.5. Manuscrito.

Este item se refere aos resultados do **Capítulo I** e está apresentado no formato de manuscrito científico.

microRNA global expression profile of liquid and tissue biopsies from patients with pancreatic carcinomas.

Natália Bertoni^{a,b}; Tainara F Felix^b; Márcio Carvalho^c; Claudia N Hasimoto^a; Juan Carlos Llanos^a; Rogério A Oliveira^d; Luis A J Mur^e; Patricia P Reis^{a,b*}.

^a Dept. of Surgery and Orthopedics, Faculty of Medicine, São Paulo State University (UNESP), Botucatu, SP, Brazil.

^b Experimental Research Unity (UNIPEX), Faculty of Medicine, São Paulo State University (UNESP), Botucatu, SP, Brazil.

^c Dept. of Veterinary Clinic, Faculty of Veterinary Medicine and Animal Science, São Paulo State University (UNESP), Botucatu, SP, Brazil.

^d Dept. of Biostatistics, Institute of Biosciences, São Paulo State University (UNESP), Botucatu, SP, Brazil.

^e Institute of Biological, Environmental and Rural Sciences (IBERS), Aberystwyth University, Ceredigion, United Kingdom.

***Corresponding author:**

Patricia P Reis, Ph.D.
Dept. of Surgery and Orthopedics
Faculty of Medicine
São Paulo State University
Botucatu, SP, Brazil
CEP: 18618-970
patricia.reis@unesp.br

Abstract

Pancreatic carcinomas are usually aggressive and diagnosed in advanced disease stages, being associated with high mortality rates, with over 432,000 deaths every year, worldwide. Early disease diagnosis is of utmost importance to increase patient survival. The identification of blood-based and tumor-related biomarkers provides information on disease development and progression, and may potentially contribute for the development of molecular diagnostic strategies to aid in diagnosis and treatment. microRNAs (miRNAs) may be optimal biomarkers, as they are highly stable and detectable as abundant molecules in body fluids such as blood plasma. Our study aims were to identify specific miRNAs deregulated in both tumor and plasma from patients with pancreatic ductal adenocarcinoma (PDAC) and adenocarcinoma of Vater ampulla (AMP), as well as to correlate miRNAs with target genes and disease pathways. Global miRNA expression was performed using GeneChip® miRNA 4.0 Array (Thermo Fisher Scientific) and computational tools were used to predict miRNA target genes and pathways. miR-378h was significantly upregulated ($FC \geq 2$ and $p < 0.05$ and $FDR < 0.05$) in plasma and tumor from patients with PDAC. This miRNA acts as a modulator of cell growth and immune response pathways. In AMP cancers, miR-5000-5p was significantly downregulated ($FC \geq 2$ and $p < 0.05$ and $FDR < 0.05$) in plasma and tumor, modulating pathways involved in Wnt signaling and cell proliferation. The identification of specific circulating plasma and tumor-related miRNAs deregulated in patients with PDAC and AMP may indicate these molecules as valuable biomarkers to assist in early diagnosis.

Keywords: pancreatic carcinoma, pancreatic ductal adenocarcinoma, adenocarcinoma of Vater ampulla, circulating microRNA, biomarkers, metabolic pathways, liquid biopsy.

1. Introduction

Pancreatic carcinomas represent the seventh most frequent cause of cancer death for both sexes leading to more than 432 thousands deaths every year, worldwide (1). Global statistics showed the pancreatic carcinomas as the eleventh most common cancer with ~458 thousand new cases in 2018 (1). In Brazil, this disease comprises ~2% of cancer incidence with more than 9,000 new cases each year and ~4% of cancer deaths (2). Risk factors include tobacco use and alcohol consumption, diabetes, obesity and chronic pancreatitis (3). Pancreatic carcinomas are aggressive and have rapid tumor growth, with high complexity at diagnosis, usually detected in advanced stages (4). Most patients have unresectable tumors at diagnosis (5). The most frequent type of pancreatic cancer (~96%) is pancreatic ductal adenocarcinoma (PDAC) and other subtypes include periampullary tumors (6). Of these, adenocarcinoma of Vater ampulla (AMP) represents ~12% of the cases and it is difficult to diagnose AMP from other periampullary tumors, preoperatively (7). AMP patients better prognosis (>45% 5-year survival rate) compared to PDAC patients (7).

The identification of blood-based and tumor-related biomarkers is a helpful strategy for early disease diagnosis. miRNAs are small non-coding RNAs that play important roles in gene expression regulation (8) and are involved in several biological processes of embryonic development, cellular differentiation and proliferation, apoptosis (9, 10) and oncogenesis (11-13). miRNAs have been demonstrated as circulating biomarkers for cancer detection (14-17). Studies have shown that circulating miRNAs can be identified in plasma or serum with deregulated expression (e.g. from pancreatic cancer patients) when compared to healthy controls (18-22).

miRNAs are highly stable and detectable as circulating molecules in body fluids being carried by exosomes (16), or associated with protein complexes such as Argonaute-2 or HDL particles (23). Extracellular miRNAs can be donated to recipient cells by receptor-mediate capture, endocytosis or fusion of the plasma membrane from receiving cells with exosomes. The transfer of circulating miRNAs

can alter gene expression of recipient cells and represents an important communication mode (23, 24).

Here we aimed to perform global miRNA expression analysis, as a means to identify specific miRNAs commonly deregulated in tumor and plasma from patients diagnosed with PDAC and AMP tumors. Our study contributes with knowledge on the differences between circulating, tumor-derived miRNAs in PDAC and AMP, which may help in the development of novel early detection and treatment strategies for patients.

2. Material and methods

2.1. Ethics statement

The present study was performed with approval of Research Ethics Committee of the Faculty of Medicine, São Paulo State University (UNESP), Botucatu, São Paulo, Brazil (Protocol #61303516.5.0000.5411). All eligible patients signed informed consent before sample collection.

2.2. Patient samples

We analysed plasma and tissue samples (paired tumors and histologically normal) from twelve patients diagnosed with primary PDAC (N = 7) or AMP (N = 5), which had surgical removal of the tumor as the primary treatment. Surgically resected tumor and normal tissues were subject to histological frozen section analysis to ensure at least 90% presence of tumor or normal cells. Peripheral blood was collected in BD Vacutainer® EDTA tube (Becton Dickinson, NJ, USA) and plasma samples were stored at -80°C until miRNA extraction. Clinical and histopathological data of patients are shown in **Table 1**.

Histologically normal tissue samples were used as a reference for comparison with tumor tissue samples. They were pooled according to the corresponding histopathological type: normal reference pool for PDAC (N = 7) and normal reference pool for AMP (N = 5). The reference pool for comparison with

plasma samples comprised samples from healthy volunteers (N = 2).

2.3 RNA extraction

miRNA isolation from solid tumor and histologically normal tissues was performed using the miRNeasy® Mini Kit (Qiagen, Valencia, CA). In addition, the miRNeasy® Serum/Plasma Kit (Qiagen, Valencia, CA) was used to purify circulating plasma miRNAs. We followed the manufacturer protocols. Isolated RNA samples were further assessed for quantity and quality using *NanoDrop 8000* (Thermo Fisher Scientific, Waltham, MA, USA).

2.4 miRNA expression quantification and data analysis

Quantification of miRNA expression was performed using the GeneChip® miRNA 4.0 Arrays (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions, as previously described (25). Data was exported to Transcriptome Analysis Console® 4.0 (Thermo Fisher Scientific) for analysis, normalization and quality control.

Our analysis strategy was performed to identify differentially expressed miRNAs in tumor tissue and plasma (fold change, $FC \geq 2.0$ and $p < 0.05$ and FDR < 0.05). Solid tumor samples from PDAC and AMP patients (N = 12) were compared to their paired, histologically normal tissues. Plasma samples from PDAC and AMP patients were compared to the reference plasma from healthy volunteers (N=2).

2.5 miRNA target prediction and pathway enrichment analyses

microRNA Data Integration Portal tool (<http://ophid.utoronto.ca/mirDIP/>) (26) was used to predict miRNA target genes for the deregulated miRNAs. The criteria for target selection considered results with “very high” (top 1%), “high” (top 5%) and “medium” (top 1/3) score classes for interaction probability. Additionally, the ToppGene Suite (<https://toppgene.cchmc.org/>) (27) was used to identify statistically enriched

pathways.

3. Results and Discussion

In this study, we identified miRNA expression profiles in tumor and plasma samples from patients with pancreatic carcinomas. Our analyses showed that 84 miRNAs were differentially expressed (81 up- and 3 down-regulated) in solid tumor samples (PDAC and AMP) compared to their matched normal pancreatic tissue ($FC \geq 2.0$ and $p < 0.05$ and $FDR < 0.05$) (**Table 2**). In plasma samples, quantification of miRNA expression showed that 29 miRNAs were commonly upregulated in plasma from PDAC and AMP patients compared to normal reference plasma from healthy volunteers (**Table 3**). Interestingly, miR-877-5p and miR-378h were significantly upregulated in solid tumor and plasma from pancreatic cancer patients (bolded in Tables 2 and 3).

Considering the difficulty in early detection of pancreatic cancer, the identification of distinct miRNA expression profiles associated with the different tumor subtypes may contribute to improve differential diagnosis and tailored treatment. Therefore, we stratified our data on miRNA deregulation according to tumor subtype. This analysis showed 46 deregulated miRNAs (43 up- and 3 down-regulated) in solid tumor (**Table 4**) and 12 upregulated miRNAs in plasma from PDAC patients (**Table 5**). miR-378h was upregulated both in tumor and plasma from PDAC patients (bolded in **Tables 4** and **5**).

To the best of our knowledge, there are no previous reports on the up-regulation of miR-378h detected in tumor and plasma from PDAC patients. Most of the previous reports show general data for miR-378 and other miR-378 family members, not specifically miR-378h. A previous study showed that estrogen is capable of up-regulating miR-378h levels in human endothelial cells, suggesting that miR-378h expression may be linked to estradiol exposure in endothelial cells through estrogen receptors (28).

miR-378 family members have been shown as potential biomarkers of liver pathogenesis and therapeutic targets for liver disease (29, 30). Overexpression

of miR-378 was suggested as a marker for anti-angiogenic therapy in ovarian cancer, since this miRNA modulates genes that regulate angiogenesis, apoptosis and cell cycle (31). Similar to our results, Li and colleagues identified serum circulating miRNAs that can distinguish patients with pancreatic cancer from healthy controls, including miR-378 (32). They also measured the miRNA expression levels in solid tissue samples from PDAC and pancreatic duct cells and showed increased levels of miR-378 (32). Our findings show a specific isoform of miR-378 (miR-378h), as a circulating molecule over-expressed in both PDAC tumor and patient-derived plasma. This miRNA may be a candidate biomarker (upon validation in a larger patient cohort) for early detection of PDAC.

Analyses of AMP identified 112 tumor-related miRNAs (99 up- and 13 down-regulated miRNAs) and 57 miRNAs deregulated in plasma (47 up- and 10 down-regulated miRNAs) (**Tables 6 and 7**, respectively). miR-5000-5p was commonly down-regulated in plasma and solid tumor from AMP patients (bolded in **Tables 6 and 7**). There are few studies in the literature about miR-5000. Schotte and colleagues identified, through high-throughput sequencing, novel miRNAs potentially related to lymphoblastic leukemia in children, including miR-5000 (33). A subset of up-regulated miRNAs (miR-5000-3p, miR-5009-3p and miR-552) showed a potential role as biomarkers of side population colorectal cancer cells (34).

The following analyses considered the miRNAs both deregulated in tumor and plasma from PDAC (miR-378h) and AMP (miR-5000-5p) patients. Deregulated miRNAs were subject to target prediction analysis and revealed 323 miRNA-target genes of miR-378h (**Supplementary Table S1**) and 333 miRNA-target genes of miR-5000-5p (**Supplementary Table S2**), filtered for “very high” and “high” interaction probability.

miRNA-target genes were then subjected to enrichment analyses to gain further information about their biological roles. Gene Ontology (GO) categories were categorized into three hierarchically structured groups, to identify proteins associated with molecular functions, biological processes and cellular component. **Supplementary Table S3** and **Supplementary Table S4** show the top 5

statistically significant enriched GO terms of differentially expressed target-genes of miR-378h and miR-5000-5p, respectively. ToppGene analysis identified enriched pathways potentially associated with PDAC and AMP cancers (**Table 8**).

The main pathways identified and potentially associated with PDAC were: nicotinate/nicotinamide metabolic, TCR signalling and Spry regulation of FGF signalling. Nicotinate and nicotinamide are precursors for NAD⁺ and NADP⁺ coenzymes, important for metabolic pathways including glycolysis, TCA cycle and fatty acids biosynthesis, which is important to ensure cell survival (35). FGF are signalling proteins that play roles in cell growth, organogenesis and also in tissue maintenance and repair. The regulation of FGF signalling is provided by several proteins including SPRY proteins. Deregulation of FGF signalling and SPRY function can lead to cancer (36). The activation of T Cell Receptor (TCR) has a central role in the adaptive immune response and promotes signalling cascades involved in cell proliferation and differentiation and cell survival (37). A previous study from our group also showed the role of miRNAs in modulating networks of adaptive and innate immune response in PDAC (25). Therefore, we have shown that different miRNAs potentially modulate genes involved in biological pathways of immune response; our data may contribute for validation studies towards the development of immunotherapy strategies for PDAC patients.

The main pathways identified and potentially associated with AMP include altered canonical Wnt signalling, mechanism of gene regulation by peroxisome proliferators via PPAR α (alpha), inactivation of GSK-3 by AKT, multi-step regulation of transcription by Pitx2 and axon guidance mediated by netrin. Wnt signalling is a well-known, important mechanism that regulates morphogenesis during embryogenesis; alterations in Wnt signalling have been extensively associated with several cancers (38, 39). A possible therapeutic intervention targets aberrant Wnt signalling in cancer (40). The peroxisome proliferator-activated receptor alpha (PPAR α) is a transcription factor that regulates processes of inflammation, nutrient metabolism and energy homeostasis (41). Glycogen synthase kinase 3 (GSK-3) is involved in biological processes of metabolism, gene

expression, proliferation, cell fate determination and survival and can be inhibited by AKT, resulting in cell proliferation and also in tumorigenesis processes (42). The transcription factor Pitx2 is induced by Wnt/b-catenin pathway when cell proliferation is required, activating genes that regulate cell growth (43). Axon guidance have been demonstrated regulating biological processes including cancer and the netrins family members of axon guidance factors have been linked to survival and invasion-promoting factors (44).

Here we identified the miRNA global expression profile of tissue and liquid biopsies and we were able to highlight miRNAs expressed both in tumor and plasma from patients with pancreatic carcinomas (PDAC and AMP), that modulates genes involved in metabolic pathways. Furthermore, our work contributes entering novel information into databases. The present study paves new roads for additional analyses to validate potential biomarkers for early diagnosis in pancreatic cancer.

4. Conclusions

Deregulated expression of circulating plasma and tumor-related miRNAs in patients with PDAC and AMP cancers may indicate specific miRNAs as valuable biomarkers to aid in early diagnosis. Despite the limitation regarding sample size, the main innovation of our study was the investigation of miRNAs expressed both in plasma and tumor from the same pancreatic cancer patients, suggesting that these circulating miRNAs may be derived from the tumor. Considering that miRNAs are important gene expression regulators, they may be associated with roles in tumor development and progression. Data integration and identification of miRNA-regulated pathways followed by validation in a large number of cases may contribute to the development of new strategies for early detection of pancreatic carcinoma.

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Table 1. Clinical and histopathological characteristics of PDAC and AMP patients.

Variables	Pancreatic ductal adenocarcinoma (N = 7)	N (%)	Adenocarcinoma of Vater ampulla (N = 5)	N (%)
Age (years)				
Median	61.2 ± 4.8		64,8 ± 5,7	
Average (range)	61 (52-70)		64 (55-73)	
Gender				
Male	2	28,6	3	60
Female	5	71,4	2	40
Tumor grade				
WD	5	71,4	3	60
MD	1	14,3	1	20
PD	1	14,3	1	20
Tumor size (T)				
T1-T2	0	0	2	40
T3-T4	7	100	3	60
Nodal status (N)				
Positive (N1, N2)	5	71,4	3	60
Negative (N0)	1	14,3	1	20
Undetermined (Nx)	1	14,3	1	20
Distant metastasis (M)				
Positive (M1)	1	14,3	0	
Negative (M0)	0	0	1	20
Undetermined (Mx)	6	85,7	4	80
Stage				
Ia	0	0	1	20
Ib	0	0	0	0
IIa	1	14,3	1	20
IIb	3	42,8	2	40
III	2	28,6	1	20
IV	1	14,3	0	0
Tobacco use				
Sim	2	28,6	3	60
Não	5	71,4	2	40
Alcohol use				
Sim	0	0	2	40
Não	7	100	3	60
Diabetes				
Sim	4	57,2	0	0
Não	3	42,8	5	100
Obesity				
Sim	1	14,3	0	0
Não	6	85,7	5	100
Patient status				
Alive with disease	5	71,4	2	40
Dead of disease	2	28,6	3	60

WD: well differentiated

MD: moderately differentiated

PD: poorly differentiated

Table 2. Deregulated miRNAs in solid tumor from twelve patients with pancreatic carcinomas (PDAC and AMP) vs. matched normal pancreatic tissue. miRNAs are listed according to statistical significance of *p*-value, from the most significant to the least significant (*cont.*).

miRNA	Fold change (FC)	<i>p</i>-value
Down-regulated		
hsa-miR-216a-5p	-27,45	0,0002
hsa-miR-216b-5p	-34,28	0,0006
hsa-miR-217	-53,47	0,0007
Up-regulated		
hsa-miR-574-3p	13,51	0,0031
hsa-miR-877-5p	4,37	0,0046
hsa-mir-6836	8,18	0,0049
hsa-miR-222-3p	20,91	0,0063
hsa-miR-500a-3p	7,4	0,0069
hsa-miR-1973	11,71	0,0079
hsa-let-7e-5p	4,71	0,0081
hsa-miR-532-3p	4,65	0,0088
hsa-miR-3157-3p	2,93	0,0091
hsa-miR-4750-3p	2,2	0,0093
hsa-miR-151b	7,91	0,0097
hsa-miR-502-3p	5,5	0,0101
hsa-miR-378h	8,11	0,0102
hsa-miR-423-3p	9,64	0,0114
hsa-miR-15b-5p	19,88	0,0118
hsa-miR-125a-5p	10,31	0,0121
hsa-mir-4417	2,5	0,0132
hsa-miR-10a-5p	11,93	0,0134
hsa-mir-320d-2	2,48	0,0134
hsa-miR-27a-3p	18,2	0,0135
hsa-miR-24-3p	6,18	0,0145
hsa-miR-17-5p	9,37	0,0153
hsa-miR-6511b-5p	3,57	0,0159
hsa-miR-3619-5p	5,23	0,0162
hsa-mir-1260a	2,32	0,0167
hsa-miR-221-3p	23,37	0,0167
hsa-miR-200b-5p	10,03	0,0169
hsa-miR-151a-5p	5,28	0,0176
hsa-miR-24-2-5p	3,49	0,0185
hsa-miR-23b-3p	6,78	0,0186
hsa-mir-6722	2,77	0,0189
hsa-mir-92b	3,63	0,019

miRNA	Fold change (FC)	p-value
Up-regulated		
hsa-let-7d-5p	6,45	0,0205
hsa-mir-548a-1	2,1	0,0205
hsa-mir-4479	2,34	0,0209
hsa-miR-501-3p	5,16	0,0209
hsa-miR-497-5p	18,64	0,0215
hsa-miR-2277-5p	3,04	0,0217
hsa-miR-4743-5p	4	0,0231
hsa-miR-194-5p	20,82	0,0234
hsa-miR-20a-5p	14,23	0,024
hsa-miR-143-3p	10,37	0,0253
hsa-miR-548ac	3,7	0,0255
hsa-miR-197-3p	6,19	0,0262
hsa-miR-532-5p	6,57	0,0263
hsa-miR-23a-3p	7,13	0,0271
hsa-miR-106a-5p	6,93	0,0274
hsa-miR-6793-3p	2,03	0,0282
hsa-miR-6124	5,39	0,0288
hsa-miR-6893-5p	3,68	0,0292
hsa-mir-940	2,1	0,0295
hsa-let-7f-5p	19,76	0,0298
hsa-miR-652-3p	8,41	0,0299
hsa-miR-342-3p	9,36	0,0301
hsa-miR-494-3p	6,9	0,0301
hsa-miR-27b-3p	8,46	0,0306
hsa-miR-28-5p	11,53	0,0317
hsa-miR-6511b-3p	2,06	0,0326
hsa-miR-671-5p	5,29	0,0333
hsa-mir-6776	4,45	0,0342
hsa-mir-6511a-1	2,12	0,0359
hsa-mir-6511a-2	2,12	0,0359
hsa-mir-6511a-3	2,12	0,0359
hsa-mir-6511a-4	2,12	0,0359
hsa-mir-6511b-1	2,12	0,0359
hsa-mir-6511b-2	2,12	0,0359
hsa-miR-31-5p	49,01	0,036
hsa-miR-212-3p	2,15	0,0363
hsa-miR-500b-3p	2,11	0,0364
hsa-mir-320a	2,78	0,0368
hsa-let-7a-5p	3,85	0,039
hsa-miR-503-5p	3,52	0,0405

miRNA	Fold change (FC)	<i>p</i>-value
	Up-regulated	
hsa-miR-92b-3p	7,6	0,0407
hsa-mir-320e	3,91	0,0414
hsa-mir-4657	2,24	0,0438
hsa-miR-29a-3p	7,94	0,0444
hsa-miR-192-5p	13,11	0,0452
hsa-miR-155-5p	7,01	0,0454
hsa-miR-92a-3p	6,01	0,0472
hsa-miR-107	6,42	0,0479
hsa-miR-210-3p	8,07	0,0482

Table 3. Deregulated miRNAs in plasma from twelve patients with pancreatic carcinomas (PDAC and AMP) vs. normal reference plasma from healthy volunteers. miRNAs are listed according to statistical significance of *p*-value, from the most significant to the least significant.

miRNA	Fold change (FC)	<i>p</i>-value
Up-regulated		
hsa-miR-5787	16,55	0,0037
hsa-miR-378h	5,05	0,0071
hsa-miR-4487	14,94	0,0111
hsa-mir-550a-1	3,99	0,0112
hsa-mir-550a-2	3,99	0,0112
hsa-mir-550a-3	3,99	0,0112
hsa-miR-6732-5p	3,83	0,0124
hsa-miR-4706	5,11	0,0127
hsa-miR-1469	12,5	0,0137
hsa-miR-4281	59,68	0,0197
hsa-mir-6800	11,96	0,0205
hsa-miR-8075	3,66	0,0222
hsa-mir-6800	9,07	0,0234
hsa-miR-6858-5p	8,84	0,0261
hsa-miR-877-5p	3,44	0,0271
hsa-miR-6821-5p	10,41	0,029
hsa-miR-6791-5p	20,6	0,0298
hsa-miR-4270	11,96	0,0317
hsa-miR-149-3p	19	0,0349
hsa-miR-6756-5p	8,05	0,036
hsa-miR-3620-5p	9,63	0,0361
hsa-miR-4516	38,18	0,0375
hsa-miR-6752-5p	29,73	0,0387
hsa-miR-6775-5p	9,08	0,039
hsa-miR-638	6,48	0,042
hsa-miR-1207-5p	6,1	0,0436
hsa-miR-5001-5p	7,1	0,0456
hsa-miR-4687-3p	18,47	0,0459

Table 4. Deregulated miRNAs in solid tumor from PDAC patients vs. matched normal pancreatic tissue. miRNAs are listed according to statistical significance of *p*-value, from the most significant to the least significant (*cont.*).

miRNA	Fold change (FC)	<i>p</i>-value
Down-regulated		
hsa-miR-216a-5p	-39,64	0,0092
hsa-miR-217	-76,99	0,0184
hsa-miR-216b-5p	-50,3	0,0216
Up-regulated		
hsa-miR-7113-3p	2,02	0,0015
hsa-miR-7108-3p	2,15	0,0022
hsa-miR-371b-3p	2,11	0,003
hsa-mir-6776	8,93	0,0032
hsa-miR-4258	3,33	0,0034
hsa-mir-6891	2,56	0,005
hsa-miR-3157-3p	4,97	0,0063
hsa-miR-6752-3p	2,35	0,0074
hsa-miR-548y	2,01	0,008
hsa-miR-6812-3p	2,11	0,0084
hsa-miR-378h	7,15	0,0101
hsa-mir-4276	2,2	0,0105
hsa-miR-6785-3p	2,46	0,0127
hsa-miR-6889-3p	2,29	0,0131
hsa-miR-6729-3p	2,03	0,0143
hsa-miR-6784-3p	2,11	0,016
hsa-mir-5585	2,04	0,0179
hsa-miR-6730-3p	2,2	0,0183
hsa-let-7a-1	2,27	0,0185
hsa-let-7a-1	2,27	0,0188
hsa-mir-6722	2,99	0,019
hsa-miR-6511a-3p	2,56	0,0193
hsa-mir-424	2,01	0,021
hsa-mir-6787	2,1	0,0225
hsa-miR-1180-5p	2,4	0,0242
hsa-mir-320c-1	2,16	0,0256
hsa-mir-4647	2,03	0,0264
hsa-miR-1914-5p	2,17	0,0269
hsa-miR-1260a	2,25	0,0285
hsa-mir-320e	2,97	0,0302
hsa-miR-6803-3p	2,24	0,0321
hsa-mir-3605	2,11	0,0348
hsa-miR-7114-3p	2,13	0,0365

miRNA	Fold change (FC)	<i>p</i>-value
	Up-regulated	
hsa-miR-874-5p	2,29	0,0372
hsa-miR-6511b-5p	3,01	0,038
hsa-miR-5094	2,1	0,0395
hsa-miR-1224-3p	2,26	0,0412
hsa-miR-4750-3p	2,69	0,0412
hsa-miR-181c-5p	2,29	0,0469
hsa-miR-6798-3p	2,4	0,0476
hsa-miR-1973	9,91	0,0479
hsa-mir-548g	2,18	0,049
hsa-mir-3153	2,12	0,0496

Table 5. Deregulated miRNAs in plasma from PDAC patients vs. normal reference plasma from healthy volunteers. miRNAs are listed according to statistical significance of p-value, from the most significant to the least significant.

miRNA	Fold change (FC)	p-value
Up-regulated		
hsa-miR-6732-5p	3,69	0,0095
hsa-miR-5787	19,51	0,0159
hsa-miR-4487	13,21	0,0182
hsa-miR-378h	4,28	0,022
hsa-mir-6800	16,59	0,0349
hsa-miR-8075	3,41	0,0352
hsa-miR-1469	16,99	0,0362
hsa-miR-4707-5p	11,62	0,0417
hsa-miR-4505	6,18	0,0464
hsa-miR-6756-5p	12,92	0,047
hsa-miR-6789-5p	25,33	0,0474
hsa-miR-6749-5p	23,11	0,0491

Table 6. Deregulated miRNAs in solid tumor from AMP vs. matched normal pancreatic tissue. miRNAs are listed according to statistical significance of *p*-value, from the most significant to the least significant (*cont.*).

miRNA	Fold change (FC)	<i>p</i>-value
Down-regulated		
hsa-miR-216a-5p	-15,06	0,00000563
hsa-miR-4757-3p	-2,58	0,0002
hsa-miR-548ak	-2,44	0,0006
hsa-miR-217	-40,4	0,0019
hsa-miR-216b-5p	-23,84	0,0029
hsa-mir-544b	-2,23	0,0032
hsa-miR-7153-3p	-3,74	0,0066
hsa-miR-6808-3p	-2,07	0,0074
hsa-miR-520h	-2,11	0,0101
hsa-miR-204-5p	-2,4	0,0129
hsa-miR-5692a	-2,03	0,0143
hsa-miR-5701	-2,89	0,0179
hsa-miR-5000-5p	-2,33	0,023
Up-regulated		
hsa-miR-194-5p	154,36	0,0009
hsa-miR-145-5p	18,69	0,001
hsa-miR-143-3p	32,72	0,0021
hsa-miR-222-3p	96,87	0,0033
hsa-miR-28-5p	27,2	0,0034
hsa-miR-28-3p	15,41	0,004
hsa-miR-221-3p	144,66	0,0044
hsa-miR-378c	29,44	0,0045
hsa-miR-342-3p	72,4	0,0063
hsa-miR-4485	30,71	0,0066
hsa-miR-192-5p	125,97	0,0066
hsa-miR-10a-5p	56,32	0,0073
hsa-miR-20a-5p	141,1	0,0073
hsa-miR-320d	6,52	0,0076
hsa-mir-28	5,2	0,0078
hsa-miR-17-5p	102,04	0,0084
hsa-mir-361	8,34	0,0085
hsa-miR-125a-5p	45,45	0,009
hsa-mir-4485	6,91	0,0095
hsa-mir-93	2,18	0,0101
hsa-miR-361-5p	18,35	0,0102

miRNA	Fold change (FC)	p-value
Up-regulated		
hsa-miR-182-5p	27,14	0,0102
hsa-miR-6855-5p	2,92	0,0108
hsa-miR-106a-5p	38,31	0,0112
hsa-miR-151b	26,47	0,0113
hsa-miR-200a-3p	137,78	0,0113
hsa-miR-574-3p	30,12	0,012
hsa-miR-26a-5p	12,72	0,0124
hsa-miR-3135b	40,75	0,0126
hsa-miR-423-3p	11,48	0,0134
hsa-miR-671-5p	13,11	0,0134
hsa-miR-15b-5p	119,22	0,0135
hsa-miR-130a-3p	13,12	0,0137
hsa-miR-107	25,41	0,0137
hsa-miR-500a-3p	9,05	0,0141
hsa-miR-151a-5p	15,85	0,0149
hsa-miR-191-5p	15	0,016
hsa-miR-532-3p	7,71	0,0166
hsa-miR-92a-3p	25,96	0,0174
hsa-miR-320c	5,14	0,0178
hsa-miR-195-5p	21,32	0,0179
hsa-miR-27b-3p	47,74	0,018
hsa-miR-22-3p	18,89	0,0183
hsa-miR-23b-3p	21,58	0,0192
hsa-miR-150-5p	49,27	0,0192
hsa-miR-422a	17,17	0,02
hsa-miR-497-5p	25,72	0,0202
hsa-let-7e-5p	10,26	0,0204
hsa-miR-30d-5p	30,23	0,0205
hsa-miR-151a-3p	34,16	0,0207
hsa-miR-25-3p	44,33	0,0208
hsa-miR-27a-3p	122,61	0,021
hsa-miR-181b-5p	46,06	0,0215
hsa-miR-199a-5p	31,45	0,022
hsa-mir-3937	2,06	0,0235
hsa-miR-30c-5p	14,25	0,0239
hsa-mir-92b	8,77	0,0241
hsa-miR-30a-5p	25,03	0,0245
hsa-miR-23a-3p	28,97	0,0247

miRNA	Fold change (FC)	p-value
Up-regulated		
hsa-miR-29a-3p	39,39	0,0251
hsa-miR-103a-3p	17,18	0,0253
hsa-miR-200b-5p	19,88	0,0257
hsa-miR-320b	4,65	0,0258
hsa-miR-24-3p	18,37	0,0267
hsa-miR-425-5p	22,07	0,0267
hsa-miR-4532	12,03	0,027
hsa-miR-99b-5p	8,48	0,0273
hsa-miR-320a	4,5	0,0279
hsa-miR-6068	46,74	0,0293
hsa-let-7g-5p	33,37	0,0297
hsa-mir-663b	4,86	0,0299
hsa-let-7i-5p	25,12	0,0303
hsa-let-7d-5p	22,68	0,0304
hsa-miR-215-5p	130,66	0,0304
hsa-mir-423	5,94	0,0307
hsa-mir-663b	4,9	0,0309
hsa-miR-106b-5p	43,02	0,0329
hsa-miR-625-5p	6	0,0341
hsa-miR-200c-3p	10,57	0,0346
hsa-let-7f-5p	42,92	0,0356
hsa-mir-6125	2	0,0366
hsa-miR-93-5p	21,58	0,0371
hsa-miR-494-3p	11,55	0,0374
hsa-miR-146a-5p	124,84	0,0376
hsa-mir-4479	3,73	0,0397
hsa-let-7a-5p	10,41	0,0397
hsa-miR-652-3p	21,13	0,0404
hsa-miR-378a-3p	9,28	0,042
hsa-mir-6768	2,11	0,0423
hsa-miR-140-3p	7,31	0,0425
hsa-miR-181a-5p	22,83	0,0429
hsa-miR-16-5p	28,24	0,044
hsa-mir-663a	2,81	0,045
hsa-miR-4429	18,05	0,0456
hsa-miR-365b-5p	4,33	0,0463
hsa-miR-23a-5p	3,17	0,0477
hsa-miR-595	2,18	0,0489

miRNA	Fold change (FC)	<i>p</i>-value
	Up-regulated	
hsa-mir-6836	22,92	0,0493
hsa-miR-2277-5p	5,85	0,0498

Table 7. Deregulated miRNAs in plasma from AMP patients vs. normal reference plasma from healthy volunteers. miRNAs are listed according to statistical significance of *p*-value, from the most significant to the least significant (*cont.*).

miRNA	Fold change (FC)	<i>p</i>-value
Down-regulated		
hsa-miR-6080	-2,45	0,0005
hsa-miR-3157-3p	-3,09	0,0007
hsa-miR-939-3p	-2,08	0,003
hsa-miR-486-3p	-3,4	0,013
hsa-miR-6778-3p	-3,43	0,0233
hsa-miR-1273g-3p	-9,36	0,0271
hsa-miR-5000-5p	-7,7	0,0324
hsa-miR-3689a-5p	-3,86	0,0336
hsa-miR-3689b-5p	-3,86	0,0336
hsa-miR-3689e	-3,86	0,0336
Up-regulated		
hsa-miR-4281	33,46	0,00000454
hsa-miR-6775-5p	6,1	0,0000931
hsa-miR-4687-3p	8,18	0,0002
hsa-mir-6800	5,43	0,0007
hsa-miR-3141	4,61	0,001
hsa-miR-149-3p	10,66	0,0014
hsa-miR-1228-5p	6,5	0,0017
hsa-miR-4516	19,39	0,0018
hsa-miR-6791-5p	12,32	0,002
hsa-miR-5787	6,53	0,0021
hsa-miR-4270	9,11	0,0025
hsa-mir-6800	4,27	0,0028
hsa-miR-4695-5p	2,04	0,0031
hsa-miR-3620-5p	4,88	0,0032
hsa-miR-6794-5p	4,94	0,005
hsa-miR-4463	6,07	0,0059
hsa-miR-1207-5p	4,22	0,0098
hsa-miR-1908-5p	4,03	0,0106
hsa-miR-1343-5p	6,23	0,011
hsa-miR-3656	4,69	0,0115
hsa-miR-6803-5p	3,43	0,0121
hsa-miR-6743-5p	5,11	0,0126
hsa-miR-6782-5p	2,69	0,0127
hsa-miR-6858-5p	4,27	0,0135
hsa-miR-6850-5p	3,7	0,0164
hsa-miR-4632-5p	2,36	0,0172

miRNA	Fold change (FC)	<i>p</i>-value
Up-regulated		
hsa-miR-4508	3,26	0,0185
hsa-mir-6089-1	5,57	0,0197
hsa-mir-6089-2	5,57	0,0197
hsa-miR-4459	3,98	0,0199
hsa-miR-6786-5p	4,1	0,0203
hsa-miR-6821-5p	8,07	0,0222
hsa-miR-6891-5p	2,93	0,0228
hsa-miR-328-5p	6,8	0,0233
hsa-miR-6752-5p	12,16	0,0236
hsa-miR-1469	4,47	0,0239
hsa-miR-4467	2,59	0,0284
hsa-miR-1268a	2,92	0,0293
hsa-miR-8069	3,98	0,0333
hsa-miR-6787-5p	2,44	0,0336
hsa-miR-6087	3,22	0,0351
hsa-miR-4707-5p	2,41	0,0378
hsa-miR-6756-5p	5,68	0,0403
hsa-miR-6765-5p	4,59	0,0428
hsa-miR-4505	2,96	0,0454
hsa-miR-4651	4,71	0,0479
hsa-miR-4758-5p	2,1	0,0493

Table 8. Enriched pathways identified and potentially associated with pancreatic carcinomas.

Pancreatic ductal adenocarcinoma		
Pathway		
Name		<i>p</i>-value
nicotinate and nicotinamide metabolic		0,03007
TCR signalling in naive CD4+ T cells		0,04974
Spry regulation of FGF signalling		0,1051
TCR signalling in naive CD8+ T cells		0,1178
Adenocarcinoma of Vater ampulla		
Pathway		
Name		<i>p</i>-value
Altered canonical Wnt signalling		0,04814
Mechanism of Gene Regulation by Peroxisome Proliferators via PPARa(alpha)		0,05575
Inactivation of Gsk3 by AKT causes accumulation of b-catenin in Alveolar Macrophages		0,08306
Multi-step Regulation of Transcription by Pitx2		0,09805
Axon guidance mediated by netrin		0,1406

Supplementary Table S1. List of predicted targets of commonly upregulated miR-378h in tumor and plasma from pancreatic ductal adenocarcinoma patients (*cont.*).

miRNA	Gene	Number of Sources	Score Class
hsa-miR-378h	KIAA1522	11	Very High
hsa-miR-378h	FOXG1	9	Very High
hsa-miR-378h	RAB10	9	Very High
hsa-miR-378h	ZFPM2	10	Very High
hsa-miR-378h	DYNC1LI2	10	Very High
hsa-miR-378h	HSPA12A	11	Very High
hsa-miR-378h	KDSR	10	Very High
hsa-miR-378h	DSCAM	10	Very High
hsa-miR-378h	TOGARAM1	6	Very High
hsa-miR-378h	ZFP36L2	9	Very High
hsa-miR-378h	NEUROD1	7	Very High
hsa-miR-378h	PEF1	10	Very High
hsa-miR-378h	CREBRF	9	Very High
hsa-miR-378h	VANGL1	12	Very High
hsa-miR-378h	PAPD5	10	Very High
hsa-miR-378h	CHIT1	10	Very High
hsa-miR-378h	DYRK1A	9	Very High
hsa-miR-378h	TMEM245	11	Very High
hsa-miR-378h	SERINC1	8	Very High
hsa-miR-378h	CTDP1	10	Very High
hsa-miR-378h	EIF4G3	9	Very High
hsa-miR-378h	TSPAN17	9	Very High
hsa-miR-378h	METTL4	9	Very High
hsa-miR-378h	PAPOLG	10	Very High
hsa-miR-378h	ACOD1	6	Very High
hsa-miR-378h	SDAD1	10	Very High
hsa-miR-378h	PDIA4	7	Very High
hsa-miR-378h	TLK2	10	Very High
hsa-miR-378h	ATG12	10	Very High
hsa-miR-378h	GOLT1A	7	Very High
hsa-miR-378h	IL6ST	9	Very High
hsa-miR-378h	CD226	10	Very High
hsa-miR-378h	TRAF3	10	High
hsa-miR-378h	BMP2	8	High
hsa-miR-378h	TOB2	7	High
hsa-miR-378h	QSER1	10	High

miRNA	Gene	Number of Sources	Score Class
hsa-miR-378h	MED19	8	High
hsa-miR-378h	WWC2	11	High
hsa-miR-378h	KPNA6	10	High
hsa-miR-378h	SLC38A1	11	High
hsa-miR-378h	IGF1R	10	High
hsa-miR-378h	GRSF1	9	High
hsa-miR-378h	AGO1	10	High
hsa-miR-378h	RBMS1	8	High
hsa-miR-378h	C16orf72	10	High
hsa-miR-378h	MKL2	11	High
hsa-miR-378h	DCBLD2	9	High
hsa-miR-378h	TMED7-TICAM2	6	High
hsa-miR-378h	MAPK1	6	High
hsa-miR-378h	FANCA	10	High
hsa-miR-378h	IPO9	8	High
hsa-miR-378h	SBDS	8	High
hsa-miR-378h	RIT1	9	High
hsa-miR-378h	PLAGL2	8	High
hsa-miR-378h	SGCZ	7	High
hsa-miR-378h	CSNK1G2	7	High
hsa-miR-378h	GRB2	8	High
hsa-miR-378h	JADE2	10	High
hsa-miR-378h	KLK4	8	High
hsa-miR-378h	SOX7	10	High
hsa-miR-378h	DACT1	8	High
hsa-miR-378h	NPAS4	6	High
hsa-miR-378h	KCND1	7	High
hsa-miR-378h	BCL2L2	7	High
hsa-miR-378h	OTUB2	10	High
hsa-miR-378h	UBE2W	10	High
hsa-miR-378h	ZDHHC9	8	High
hsa-miR-378h	DCAF12	9	High
hsa-miR-378h	CALN1	8	High
hsa-miR-378h	ELAC1	8	High
hsa-miR-378h	VIRMA	5	High
hsa-miR-378h	CAMKK2	7	High
hsa-miR-378h	RBMS3	9	High
hsa-miR-378h	VAT1L	7	High
hsa-miR-378h	ALS2	5	High

miRNA	Gene	Number of Sources	Score Class
hsa-miR-378h	PIWIL2	6	High
hsa-miR-378h	H3F3B	7	High
hsa-miR-378h	SKP2	8	High
hsa-miR-378h	RSPH4A	7	High
hsa-miR-378h	KIF2A	8	High
hsa-miR-378h	C1orf21	9	High
hsa-miR-378h	VAT1	8	High
hsa-miR-378h	HNRNPA1	7	High
hsa-miR-378h	IMPG1	6	High
hsa-miR-378h	PDIK1L	9	High
hsa-miR-378h	SOBP	9	High
hsa-miR-378h	PTPRT	8	High
hsa-miR-378h	DCX	7	High
hsa-miR-378h	MROH8	5	High
hsa-miR-378h	NSFL1C	8	High
hsa-miR-378h	SOX6	8	High
hsa-miR-378h	PAX8	8	High
hsa-miR-378h	ADD2	10	High
hsa-miR-378h	C11orf44	6	High
hsa-miR-378h	UCK1	7	High
hsa-miR-378h	PITPNA	8	High
hsa-miR-378h	NUP58	10	High
hsa-miR-378h	FRMPD4	7	High
hsa-miR-378h	PAG1	7	High
hsa-miR-378h	ARL10	6	High
hsa-miR-378h	PHF7	6	High
hsa-miR-378h	MEF2D	10	High
hsa-miR-378h	CRLF3	8	High
hsa-miR-378h	C2orf88	9	High
hsa-miR-378h	RCC2	10	High
hsa-miR-378h	WDR97	4	High
hsa-miR-378h	SFT2D3	7	High
hsa-miR-378h	CBL	10	High
hsa-miR-378h	ZKSCAN1	6	High
hsa-miR-378h	SRSF10	7	High
hsa-miR-378h	RASGRF1	9	High
hsa-miR-378h	CSRNP3	8	High
hsa-miR-378h	HDDC2	4	High
hsa-miR-378h	RIMS4	9	High

miRNA	Gene	Number of Sources	Score Class
hsa-miR-378h	ZNF507	9	High
hsa-miR-378h	TMCO1	9	High
hsa-miR-378h	PEX2	9	High
hsa-miR-378h	SUFU	7	High
hsa-miR-378h	FZD5	8	High
hsa-miR-378h	CNTN5	9	High
hsa-miR-378h	ZNF652	7	High
hsa-miR-378h	RAB11A	8	High
hsa-miR-378h	CDC20B	5	High
hsa-miR-378h	PDE1B	6	High
hsa-miR-378h	WDFY3	8	High
hsa-miR-378h	SULF1	10	High
hsa-miR-378h	JADE3	8	High
hsa-miR-378h	CDC42BPA	10	High
hsa-miR-378h	BEND4	11	High
hsa-miR-378h	REST	8	High
hsa-miR-378h	PLEKHA2	10	High
hsa-miR-378h	FKBP5	7	High
hsa-miR-378h	LENG9	6	High
hsa-miR-378h	MOCS1	10	High
hsa-miR-378h	DDI2	9	High
hsa-miR-378h	UBQLN4	7	High
hsa-miR-378h	IL1RAPL1	6	High
hsa-miR-378h	ITPRIPL2	10	High
hsa-miR-378h	MED12L	9	High
hsa-miR-378h	SAMD1	5	High
hsa-miR-378h	PER2	5	High
hsa-miR-378h	IQSEC2	9	High
hsa-miR-378h	WBP2	7	High
hsa-miR-378h	ANKRD52	6	High
hsa-miR-378h	ST5	7	High
hsa-miR-378h	NFRKB	6	High
hsa-miR-378h	RAB15	7	High
hsa-miR-378h	CBX5	9	High
hsa-miR-378h	LDLRAD2	8	High
hsa-miR-378h	ZNF335	6	High
hsa-miR-378h	COQ10B	7	High
hsa-miR-378h	SSPN	9	High
hsa-miR-378h	GNA13	9	High

miRNA	Gene	Number of Sources	Score Class
hsa-miR-378h	GFPT1	9	High
hsa-miR-378h	GSTM4	7	High
hsa-miR-378h	ALPK3	7	High
hsa-miR-378h	SLC30A8	8	High
hsa-miR-378h	WDR37	7	High
hsa-miR-378h	TAF1	9	High
hsa-miR-378h	PAPOLA	8	High
hsa-miR-378h	TOLLIP	8	High
hsa-miR-378h	SCN5A	8	High
hsa-miR-378h	FOXQ1	7	High
hsa-miR-378h	KIF26B	7	High
hsa-miR-378h	LAS1L	6	High
hsa-miR-378h	UMPS	9	High
hsa-miR-378h	PTCD1	6	High
hsa-miR-378h	IGF1	7	High
hsa-miR-378h	NUFIP2	8	High
hsa-miR-378h	RTN2	7	High
hsa-miR-378h	SAR1A	7	High
hsa-miR-378h	NKX3-1	8	High
hsa-miR-378h	GPM6B	9	High
hsa-miR-378h	AP4B1	8	High
hsa-miR-378h	SCAMP2	4	High
hsa-miR-378h	PHC3	9	High
hsa-miR-378h	ZHX1	9	High
hsa-miR-378h	ELK4	9	High
hsa-miR-378h	AGK	9	High
hsa-miR-378h	DMXL2	9	High
hsa-miR-378h	YY1	6	High
hsa-miR-378h	PDPK1	8	High
hsa-miR-378h	RNF144B	5	High
hsa-miR-378h	CUEDC1	6	High
hsa-miR-378h	PPP1R12B	10	High
hsa-miR-378h	CARF	9	High
hsa-miR-378h	SHANK3	7	High
hsa-miR-378h	GRIK3	7	High
hsa-miR-378h	FBXL20	6	High
hsa-miR-378h	SARM1	8	High
hsa-miR-378h	PAPPA	7	High
hsa-miR-378h	TRIM44	7	High

miRNA	Gene	Number of Sources	Score Class
hsa-miR-378h	ARL8B	8	High
hsa-miR-378h	TRIB2	9	High
hsa-miR-378h	HNRNPLL	9	High
hsa-miR-378h	GPR156	9	High
hsa-miR-378h	NUP155	6	High
hsa-miR-378h	TFCP2L1	6	High
hsa-miR-378h	PEBP1	8	High
hsa-miR-378h	SRSF3	7	High
hsa-miR-378h	CD3G	5	High
hsa-miR-378h	DNAJC6	9	High
hsa-miR-378h	KSR1	7	High
hsa-miR-378h	ATP5J2-PTCD1	6	High
hsa-miR-378h	DUSP8	6	High
hsa-miR-378h	PTGES3	10	High
hsa-miR-378h	GRIK2	10	High
hsa-miR-378h	PSD3	7	High
hsa-miR-378h	KBTBD7	8	High
hsa-miR-378h	SLC7A6	8	High
hsa-miR-378h	MAP2K6	7	High
hsa-miR-378h	KCNIP2	8	High
hsa-miR-378h	CDC40	9	High
hsa-miR-378h	AP1AR	5	High
hsa-miR-378h	TLR8	5	High
hsa-miR-378h	AAK1	7	High
hsa-miR-378h	CAB39	9	High
hsa-miR-378h	ARID5B	9	High
hsa-miR-378h	DAZAP2	9	High
hsa-miR-378h	PRIM2	3	High
hsa-miR-378h	MNAT1	4	High
hsa-miR-378h	SIX5	4	High
hsa-miR-378h	NXPE3	7	High
hsa-miR-378h	C4orf46	9	High
hsa-miR-378h	PRPSAP2	9	High
hsa-miR-378h	MARCH6	7	High
hsa-miR-378h	NR3C1	9	High
hsa-miR-378h	SHE	10	High
hsa-miR-378h	VPS35	9	High
hsa-miR-378h	XPO5	6	High
hsa-miR-378h	PLCXD2	8	High

miRNA	Gene	Number of Sources	Score Class
hsa-miR-378h	MSI2	9	High
hsa-miR-378h	CDCA7L	6	High
hsa-miR-378h	PSMA5	8	High
hsa-miR-378h	RFXAP	8	High
hsa-miR-378h	PODXL	9	High
hsa-miR-378h	MAPK1IP1L	7	High
hsa-miR-378h	ABI2	6	High
hsa-miR-378h	NCBP2	9	High
hsa-miR-378h	SPATA2	8	High
hsa-miR-378h	PLA2G12A	9	High
hsa-miR-378h	TBC1D24	9	High
hsa-miR-378h	BMP8B	6	High
hsa-miR-378h	MPP3	8	High
hsa-miR-378h	GOLPH3L	8	High
hsa-miR-378h	TRAF6	10	High
hsa-miR-378h	PRR23C	9	High
hsa-miR-378h	OR7D2	7	High
hsa-miR-378h	SLC2A3	6	High
hsa-miR-378h	FCRLB	5	High
hsa-miR-378h	TBX6	6	High
hsa-miR-378h	ARHGEF10	9	High
hsa-miR-378h	GPT2	8	High
hsa-miR-378h	GNAT1	5	High
hsa-miR-378h	CLOCK	8	High
hsa-miR-378h	COMMD10	8	High
hsa-miR-378h	NT5E	9	High
hsa-miR-378h	SLC2A14	6	High
hsa-miR-378h	CSNK1G1	5	High
hsa-miR-378h	SYT12	5	High
hsa-miR-378h	ZNF776	8	High
hsa-miR-378h	TRABD2B	6	High
hsa-miR-378h	GPR180	8	High
hsa-miR-378h	MBNL2	9	High
hsa-miR-378h	SAMD8	5	High
hsa-miR-378h	DHX36	7	High
hsa-miR-378h	WDR31	7	High
hsa-miR-378h	FOSL2	3	High
hsa-miR-378h	CADM4	5	High
hsa-miR-378h	USP6NL	8	High

miRNA	Gene	Number of Sources	Score Class
hsa-miR-378h	VCPIP1	8	High
hsa-miR-378h	PTCHD1	6	High
hsa-miR-378h	SMOC1	8	High
hsa-miR-378h	RPN2	7	High
hsa-miR-378h	TRDMT1	9	High
hsa-miR-378h	CHAMP1	8	High
hsa-miR-378h	FAM234B	8	High
hsa-miR-378h	FAHD2A	4	High
hsa-miR-378h	HIPK3	8	High
hsa-miR-378h	NR2C2	6	High
hsa-miR-378h	CHRNA4	9	High
hsa-miR-378h	PROK2	8	High
hsa-miR-378h	PLCB1	9	High
hsa-miR-378h	RAP1GDS1	9	High
hsa-miR-378h	C6orf89	4	High
hsa-miR-378h	ADAR	9	High
hsa-miR-378h	PTPN11	9	High
hsa-miR-378h	GABRG2	9	High
hsa-miR-378h	FBXO40	8	High
hsa-miR-378h	INO80E	8	High
hsa-miR-378h	ZNF514	2	High
hsa-miR-378h	ACOT13	9	High
hsa-miR-378h	ZNF250	9	High
hsa-miR-378h	SEPSECS	8	High
hsa-miR-378h	NOVA2	4	High
hsa-miR-378h	HLTF	8	High
hsa-miR-378h	PRDM9	8	High
hsa-miR-378h	CFAP44	9	High
hsa-miR-378h	FOXN3	8	High
hsa-miR-378h	ZC3H12C	8	High
hsa-miR-378h	LRRC58	7	High
hsa-miR-378h	TAF9B	7	High
hsa-miR-378h	TMCC3	8	High
hsa-miR-378h	TCF12	8	High
hsa-miR-378h	CALD1	8	High
hsa-miR-378h	ADAMDEC1	8	High
hsa-miR-378h	RBBP9	8	High
hsa-miR-378h	POU2F1	10	High
hsa-miR-378h	AHSA1	7	High

miRNA	Gene	Number of Sources	Score Class
hsa-miR-378h	KCNJ10	8	High
hsa-miR-378h	PRR20A	7	High
hsa-miR-378h	FSIP1	8	High
hsa-miR-378h	TINF2	8	High
hsa-miR-378h	ZXDB	6	High
hsa-miR-378h	BMP8A	5	High
hsa-miR-378h	PRR20E	6	High
hsa-miR-378h	LNPEP	4	High
hsa-miR-378h	RPL23A	4	High
hsa-miR-378h	ZBTB20	5	High
hsa-miR-378h	ZNF260	8	High
hsa-miR-378h	CRLS1	7	High
hsa-miR-378h	SRGAP2	9	High
hsa-miR-378h	NDFIP2	8	High

Supplementary Table S2. List of predicted targets of commonly downregulated miR-5000-5p in tumor and plasma from adenocarcinoma of Vater ampulla patients (*cont.*).

miRNA	Gene	Number of Sources	Score Class
hsa-miR-5000-5p	SSBP2	9	Very High
hsa-miR-5000-5p	NOVA1	9	Very High
hsa-miR-5000-5p	NFAT5	9	Very High
hsa-miR-5000-5p	LMTK3	9	High
hsa-miR-5000-5p	ARL8B	10	High
hsa-miR-5000-5p	NR5A2	11	High
hsa-miR-5000-5p	SETD2	10	High
hsa-miR-5000-5p	EBF3	10	High
hsa-miR-5000-5p	CHKA	10	High
hsa-miR-5000-5p	ERCC4	9	High
hsa-miR-5000-5p	RUNX1T1	9	High
hsa-miR-5000-5p	CHD9	9	High
hsa-miR-5000-5p	HBEGF	10	High
hsa-miR-5000-5p	CDK13	8	High
hsa-miR-5000-5p	ERO1B	9	High
hsa-miR-5000-5p	FUBP3	8	High
hsa-miR-5000-5p	WNK1	9	High
hsa-miR-5000-5p	CSNK1G3	9	High
hsa-miR-5000-5p	DENND6A	8	High
hsa-miR-5000-5p	CTDSPL2	10	High
hsa-miR-5000-5p	TRDN	10	High
hsa-miR-5000-5p	DNAJC21	9	High
hsa-miR-5000-5p	NIPA1	11	High
hsa-miR-5000-5p	FXR1	10	High
hsa-miR-5000-5p	CPEB4	9	High
hsa-miR-5000-5p	EFR3A	8	High
hsa-miR-5000-5p	NANOS1	8	High
hsa-miR-5000-5p	TATDN3	9	High
hsa-miR-5000-5p	G6PC2	9	High
hsa-miR-5000-5p	BCAP29	11	High
hsa-miR-5000-5p	ITCH	8	High
hsa-miR-5000-5p	ICK	8	High
hsa-miR-5000-5p	ESR1	9	High
hsa-miR-5000-5p	SEPT11	8	High
hsa-miR-5000-5p	IRS1	9	High
hsa-miR-5000-5p	SAMHD1	6	High

miRNA	Gene	Number of Sources	Score Class
hsa-miR-5000-5p	LBH	9	High
hsa-miR-5000-5p	SLC38A2	8	High
hsa-miR-5000-5p	CAPZA2	8	High
hsa-miR-5000-5p	ZBTB34	8	High
hsa-miR-5000-5p	MDM1	10	High
hsa-miR-5000-5p	IGDCC3	9	High
hsa-miR-5000-5p	HSPG2	6	High
hsa-miR-5000-5p	CELF2	8	High
hsa-miR-5000-5p	KMT2D	9	High
hsa-miR-5000-5p	FAM122A	9	High
hsa-miR-5000-5p	PPARA	10	High
hsa-miR-5000-5p	DDX3X	9	High
hsa-miR-5000-5p	AXIN2	9	High
hsa-miR-5000-5p	SHANK2	9	High
hsa-miR-5000-5p	NR2F2	9	High
hsa-miR-5000-5p	CREBRF	10	High
hsa-miR-5000-5p	KALRN	8	High
hsa-miR-5000-5p	GDAP2	10	High
hsa-miR-5000-5p	RELA	8	High
hsa-miR-5000-5p	EGR1	9	High
hsa-miR-5000-5p	NACC2	8	High
hsa-miR-5000-5p	CA5B	8	High
hsa-miR-5000-5p	GRHL2	9	High
hsa-miR-5000-5p	C15orf41	8	High
hsa-miR-5000-5p	ANKS1A	8	High
hsa-miR-5000-5p	PRPF4	9	High
hsa-miR-5000-5p	MTX3	9	High
hsa-miR-5000-5p	BTBD3	9	High
hsa-miR-5000-5p	UBE2H	8	High
hsa-miR-5000-5p	ZBTB6	7	High
hsa-miR-5000-5p	MEIS2	9	High
hsa-miR-5000-5p	CA10	9	High
hsa-miR-5000-5p	CRYZ	10	High
hsa-miR-5000-5p	ITPKB	9	High
hsa-miR-5000-5p	INA	9	High
hsa-miR-5000-5p	MAB21L1	8	High
hsa-miR-5000-5p	PGAP1	9	High
hsa-miR-5000-5p	RHOQ	9	High
hsa-miR-5000-5p	TNRC6A	9	High

miRNA	Gene	Number of Sources	Score Class
hsa-miR-5000-5p	SDC2	8	High
hsa-miR-5000-5p	CRK	11	High
hsa-miR-5000-5p	EXOC5	9	High
hsa-miR-5000-5p	FBXW7	8	High
hsa-miR-5000-5p	CCDC6	8	High
hsa-miR-5000-5p	SUMO1	10	High
hsa-miR-5000-5p	VCPIP1	8	High
hsa-miR-5000-5p	TTN	10	High
hsa-miR-5000-5p	RBMS1	9	High
hsa-miR-5000-5p	RRP1B	7	High
hsa-miR-5000-5p	PGM2L1	7	High
hsa-miR-5000-5p	LONRF1	9	High
hsa-miR-5000-5p	POGZ	9	High
hsa-miR-5000-5p	C4orf46	8	High
hsa-miR-5000-5p	ZNF131	7	High
hsa-miR-5000-5p	ZFHX4	8	High
hsa-miR-5000-5p	DST	8	High
hsa-miR-5000-5p	NAA50	6	High
hsa-miR-5000-5p	THSD4	10	High
hsa-miR-5000-5p	UBL3	8	High
hsa-miR-5000-5p	NEUROG1	8	High
hsa-miR-5000-5p	VAPB	10	High
hsa-miR-5000-5p	OGT	7	High
hsa-miR-5000-5p	EIF1AD	9	High
hsa-miR-5000-5p	MAK16	8	High
hsa-miR-5000-5p	SKP1	9	High
hsa-miR-5000-5p	USP15	8	High
hsa-miR-5000-5p	NREP	10	High
hsa-miR-5000-5p	XPO4	9	High
hsa-miR-5000-5p	TMEM169	9	High
hsa-miR-5000-5p	ERBB4	8	High
hsa-miR-5000-5p	NUDT4	9	High
hsa-miR-5000-5p	DENND5B	9	High
hsa-miR-5000-5p	ASXL1	8	High
hsa-miR-5000-5p	MTM1	7	High
hsa-miR-5000-5p	NRIP1	8	High
hsa-miR-5000-5p	PHTF2	9	High
hsa-miR-5000-5p	PDE5A	10	High
hsa-miR-5000-5p	APP	9	High

miRNA	Gene	Number of Sources	Score Class
hsa-miR-5000-5p	HEY1	9	High
hsa-miR-5000-5p	ESRRG	9	High
hsa-miR-5000-5p	APC	9	High
hsa-miR-5000-5p	NTNG1	9	High
hsa-miR-5000-5p	ARL13B	10	High
hsa-miR-5000-5p	DCAF7	8	High
hsa-miR-5000-5p	PRR14L	9	High
hsa-miR-5000-5p	CXXC4	7	High
hsa-miR-5000-5p	MAP3K5	8	High
hsa-miR-5000-5p	TCF4	9	High
hsa-miR-5000-5p	ARHGEF7	9	High
hsa-miR-5000-5p	SH3D19	7	High
hsa-miR-5000-5p	MLEC	8	High
hsa-miR-5000-5p	GOLT1B	8	High
hsa-miR-5000-5p	CPEB3	7	High
hsa-miR-5000-5p	RYK	9	High
hsa-miR-5000-5p	NUAK1	8	High
hsa-miR-5000-5p	SREK1IP1	8	High
hsa-miR-5000-5p	WWC2	9	High
hsa-miR-5000-5p	DGKE	9	High
hsa-miR-5000-5p	CLCN4	8	High
hsa-miR-5000-5p	CDO1	8	High
hsa-miR-5000-5p	TMED2	8	High
hsa-miR-5000-5p	PAQR8	7	High
hsa-miR-5000-5p	UBXN7	10	High
hsa-miR-5000-5p	BPTF	7	High
hsa-miR-5000-5p	NUCKS1	8	High
hsa-miR-5000-5p	LUZP2	8	High
hsa-miR-5000-5p	NKAIN2	9	High
hsa-miR-5000-5p	DDX58	10	High
hsa-miR-5000-5p	NDUFA4	8	High
hsa-miR-5000-5p	NRP1	10	High
hsa-miR-5000-5p	NLGN4Y	8	High
hsa-miR-5000-5p	NSL1	9	High
hsa-miR-5000-5p	BMT2	8	High
hsa-miR-5000-5p	XRN1	8	High
hsa-miR-5000-5p	XKRX	9	High
hsa-miR-5000-5p	BLOC1S5	8	High
hsa-miR-5000-5p	TP53BP1	9	High

miRNA	Gene	Number of Sources	Score Class
hsa-miR-5000-5p	LEPROTL1	8	High
hsa-miR-5000-5p	NT5C2	8	High
hsa-miR-5000-5p	PAPOLG	9	High
hsa-miR-5000-5p	RAB3GAP2	9	High
hsa-miR-5000-5p	ENY2	8	High
hsa-miR-5000-5p	ZBTB1	8	High
hsa-miR-5000-5p	USP45	9	High
hsa-miR-5000-5p	BHLHE22	8	High
hsa-miR-5000-5p	MTF2	9	High
hsa-miR-5000-5p	UNC5D	8	High
hsa-miR-5000-5p	NCBP3	8	High
hsa-miR-5000-5p	VPS36	8	High
hsa-miR-5000-5p	ZC3H6	8	High
hsa-miR-5000-5p	PHKA1	8	High
hsa-miR-5000-5p	ZBTB8A	8	High
hsa-miR-5000-5p	FLRT3	8	High
hsa-miR-5000-5p	FBXL15	8	High
hsa-miR-5000-5p	DUSP1	8	High
hsa-miR-5000-5p	TFDP2	10	High
hsa-miR-5000-5p	JARID2	8	High
hsa-miR-5000-5p	TRIM66	8	High
hsa-miR-5000-5p	UCHL5	9	High
hsa-miR-5000-5p	MIER3	9	High
hsa-miR-5000-5p	SNRPF	9	High
hsa-miR-5000-5p	GID4	8	High
hsa-miR-5000-5p	AMOTL1	8	High
hsa-miR-5000-5p	UBE2Q1	8	High
hsa-miR-5000-5p	XRN2	8	High
hsa-miR-5000-5p	INO80	8	High
hsa-miR-5000-5p	RALGAPA1	8	High
hsa-miR-5000-5p	ERMN	8	High
hsa-miR-5000-5p	MAD2L1	9	High
hsa-miR-5000-5p	FBXO32	7	High
hsa-miR-5000-5p	LPGAT1	10	High
hsa-miR-5000-5p	PEX5L	8	High
hsa-miR-5000-5p	SNAP23	8	High
hsa-miR-5000-5p	NOD2	8	High
hsa-miR-5000-5p	LYRM2	9	High
hsa-miR-5000-5p	CD81	7	High

miRNA	Gene	Number of Sources	Score Class
hsa-miR-5000-5p	RAD52	7	High
hsa-miR-5000-5p	COPS7B	8	High
hsa-miR-5000-5p	PLXDC1	9	High
hsa-miR-5000-5p	LIN28A	8	High
hsa-miR-5000-5p	RALGAPB	9	High
hsa-miR-5000-5p	SLC6A15	6	High
hsa-miR-5000-5p	ENTPD5	9	High
hsa-miR-5000-5p	TLN2	10	High
hsa-miR-5000-5p	SRSF3	8	High
hsa-miR-5000-5p	GNAI1	8	High
hsa-miR-5000-5p	BNIP1L	9	High
hsa-miR-5000-5p	TSPAN2	9	High
hsa-miR-5000-5p	RAC1	9	High
hsa-miR-5000-5p	ADGRL4	9	High
hsa-miR-5000-5p	MMP14	7	High
hsa-miR-5000-5p	CPD	9	High
hsa-miR-5000-5p	RETREG1	8	High
hsa-miR-5000-5p	COX6C	8	High
hsa-miR-5000-5p	RNF13	7	High
hsa-miR-5000-5p	KCNA1	8	High
hsa-miR-5000-5p	PTPRB	9	High
hsa-miR-5000-5p	IP6K1	9	High
hsa-miR-5000-5p	CCR9	9	High
hsa-miR-5000-5p	GABRA4	8	High
hsa-miR-5000-5p	RC3H1	8	High
hsa-miR-5000-5p	PRSS23	7	High
hsa-miR-5000-5p	ZBTB38	8	High
hsa-miR-5000-5p	PMEPA1	8	High
hsa-miR-5000-5p	KRIT1	9	High
hsa-miR-5000-5p	BACE2	8	High
hsa-miR-5000-5p	STEAP4	8	High
hsa-miR-5000-5p	DICER1	9	High
hsa-miR-5000-5p	ITGA6	9	High
hsa-miR-5000-5p	GOSR2	6	High
hsa-miR-5000-5p	RRAGD	8	High
hsa-miR-5000-5p	SELENOI	7	High
hsa-miR-5000-5p	SHOX2	9	High
hsa-miR-5000-5p	PPP1R2	8	High
hsa-miR-5000-5p	UBE2W	7	High

miRNA	Gene	Number of Sources	Score Class
hsa-miR-5000-5p	NFATC1	9	High
hsa-miR-5000-5p	BCL2L15	9	High
hsa-miR-5000-5p	MIS18A	6	High
hsa-miR-5000-5p	EIF5AL1	7	High
hsa-miR-5000-5p	DNAJB5	8	High
hsa-miR-5000-5p	DTX4	7	High
hsa-miR-5000-5p	ECHDC1	8	High
hsa-miR-5000-5p	CTNNB1	8	High
hsa-miR-5000-5p	SHOC2	8	High
hsa-miR-5000-5p	RABEP1	9	High
hsa-miR-5000-5p	ANXA4	8	High
hsa-miR-5000-5p	ALS2	8	High
hsa-miR-5000-5p	DLG2	10	High
hsa-miR-5000-5p	PRTG	9	High
hsa-miR-5000-5p	MITF	9	High
hsa-miR-5000-5p	TTC33	8	High
hsa-miR-5000-5p	CFAP44	9	High
hsa-miR-5000-5p	TBX15	9	High
hsa-miR-5000-5p	PLEKHA5	7	High
hsa-miR-5000-5p	NOX4	9	High
hsa-miR-5000-5p	PILRB	8	High
hsa-miR-5000-5p	CHURC1	8	High
hsa-miR-5000-5p	TAP2	7	High
hsa-miR-5000-5p	MED1	9	High
hsa-miR-5000-5p	HMGN4	7	High
hsa-miR-5000-5p	CPS1	7	High
hsa-miR-5000-5p	RCOR3	8	High
hsa-miR-5000-5p	ATL2	7	High
hsa-miR-5000-5p	AJUBA	7	High
hsa-miR-5000-5p	PAX9	8	High
hsa-miR-5000-5p	ATXN10	8	High
hsa-miR-5000-5p	TCEA3	8	High
hsa-miR-5000-5p	PARP8	7	High
hsa-miR-5000-5p	HOXD3	7	High
hsa-miR-5000-5p	NFIB	8	High
hsa-miR-5000-5p	PRLR	7	High
hsa-miR-5000-5p	NFAM1	8	High
hsa-miR-5000-5p	TACR1	8	High
hsa-miR-5000-5p	LRIT3	9	High

miRNA	Gene	Number of Sources	Score Class
hsa-miR-5000-5p	CITED2	8	High
hsa-miR-5000-5p	NFX1	8	High
hsa-miR-5000-5p	SLC22A23	9	High
hsa-miR-5000-5p	ARAP2	8	High
hsa-miR-5000-5p	KMT2C	8	High
hsa-miR-5000-5p	SLCO3A1	9	High
hsa-miR-5000-5p	CREB3L2	9	High
hsa-miR-5000-5p	ZNF384	8	High
hsa-miR-5000-5p	B3GALT5	9	High
hsa-miR-5000-5p	FAM83C	8	High
hsa-miR-5000-5p	RGS5	9	High
hsa-miR-5000-5p	NMUR1	7	High
hsa-miR-5000-5p	BACH1	8	High
hsa-miR-5000-5p	GEMIN8	8	High
hsa-miR-5000-5p	MAP3K2	8	High
hsa-miR-5000-5p	RFC3	8	High
hsa-miR-5000-5p	PIK3CD	9	High
hsa-miR-5000-5p	NSUN3	6	High
hsa-miR-5000-5p	TM2D3	9	High
hsa-miR-5000-5p	RUNX2	9	High
hsa-miR-5000-5p	RASSF2	7	High
hsa-miR-5000-5p	PIP5K1B	8	High
hsa-miR-5000-5p	LUC7L2	8	High
hsa-miR-5000-5p	GNE	8	High
hsa-miR-5000-5p	OTULIN	8	High
hsa-miR-5000-5p	USO1	8	High
hsa-miR-5000-5p	SLC35B4	8	High
hsa-miR-5000-5p	FZD1	7	High
hsa-miR-5000-5p	C3orf70	8	High
hsa-miR-5000-5p	PTAR1	8	High
hsa-miR-5000-5p	KLHL24	9	High
hsa-miR-5000-5p	PAN3	7	High
hsa-miR-5000-5p	NRK	8	High
hsa-miR-5000-5p	TMEM131	8	High
hsa-miR-5000-5p	RABGAP1L	8	High
hsa-miR-5000-5p	HTR2C	9	High
hsa-miR-5000-5p	FCGR1A	8	High
hsa-miR-5000-5p	GPR160	8	High
hsa-miR-5000-5p	PARVA	6	High

miRNA	Gene	Number of Sources	Score Class
hsa-miR-5000-5p	FAM76A	9	High
hsa-miR-5000-5p	ABCA9	7	High
hsa-miR-5000-5p	ZHX1	8	High
hsa-miR-5000-5p	RP1	7	High
hsa-miR-5000-5p	RAB3IP	9	High
hsa-miR-5000-5p	SLC1A2	7	High
hsa-miR-5000-5p	EMC1	8	High
hsa-miR-5000-5p	MOB1B	7	High
hsa-miR-5000-5p	ADARB1	9	High
hsa-miR-5000-5p	GOLGA7B	7	High
hsa-miR-5000-5p	TMPRSS11F	8	High
hsa-miR-5000-5p	SLC7A11	7	High
hsa-miR-5000-5p	AFDN	8	High
hsa-miR-5000-5p	RCBTB1	7	High
hsa-miR-5000-5p	CUL2	8	High
hsa-miR-5000-5p	CHRNA5	9	High
hsa-miR-5000-5p	KCNJ13	8	High
hsa-miR-5000-5p	SNTB2	8	High
hsa-miR-5000-5p	SFPQ	8	High
hsa-miR-5000-5p	MAX	8	High
hsa-miR-5000-5p	ITPR2	8	High
hsa-miR-5000-5p	SSR1	7	High
hsa-miR-5000-5p	LGSN	8	High
hsa-miR-5000-5p	PIK3CG	7	High

Supplementary Table S3. Top 5 enriched Gene Ontology terms of miR-378h target-genes, potentially associated with PDAC.

GO: Molecular Function	
Name	<i>p</i>-value
kinase binding	$3,25 \times 10^{-3}$
transcription regulatory region DNA binding	$5,48 \times 10^{-3}$
regulatory region nucleic acid binding	$6,12 \times 10^{-3}$
RNA polymerase II regulatory region sequence-specific DNA binding	$1,02 \times 10^{-2}$
RNA polymerase II regulatory region DNA binding	$1,23 \times 10^{-2}$
GO: Biological Processes	
Name	<i>p</i>-value
regulation of steroid hormone biosynthetic process	$7,88 \times 10^{-3}$
positive regulation of RNA metabolic process	$1,88 \times 10^{-2}$
regulation of hormone metabolic process	$2,23 \times 10^{-2}$
positive regulation of nuclear division	$2,53 \times 10^{-2}$
peptidyl-threonine phosphorylation	$4,41 \times 10^{-2}$
GO: Cellular Component	
Name	<i>p</i>-value
synapse	$1,53 \times 10^{-2}$
synapse part	$2,54 \times 10^{-2}$
nucleoplasm part	$3,01 \times 10^{-2}$
transferase complex	$6,18 \times 10^{-2}$
nuclear chromatin	$2,29 \times 10^{-1}$

Supplementary Table S4. Top 5 enriched Gene Ontology terms of miR-5000-5p target-genes, potentially associated with AMP.

GO: Molecular Function	
Name	<i>p</i>-value
regulatory region nucleic acid binding	2,38x10 ⁻⁷
transcription regulatory region DNA binding	6,39x10 ⁻⁷
RNA polymerase II regulatory region sequence-specific DNA binding	6,31x10 ⁻⁶
RNA polymerase II regulatory region DNA binding	8,18x10 ⁻⁶
transcription regulatory region sequence-specific DNA binding	8,57x10 ⁻⁶
GO: Biological Processes	
Name	<i>p</i>-value
positive regulation of RNA metabolic process	3,72x10 ⁻⁹
positive regulation of transcription by RNA polymerase II	1,11x10 ⁻⁸
positive regulation of transcription, DNA-templated	1,86x10 ⁻⁸
negative regulation of cellular macromolecule biosynthetic process	1,44x10 ⁻⁷
positive regulation of nucleic acid-templated transcription	1,74x10 ⁻⁷
GO: Cellular Component	
Name	<i>p</i>-value
somatodendritic compartment	1,35x10 ⁻³
neuron projection	3,63x10 ⁻³
nuclear chromatin	1,09x10 ⁻²
dendrite	1,11x10 ⁻²
dendritic tree	1,11x10 ⁻²

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2. Capítulo II

Esse capítulo refere-se à etapa adicional do estudo desenvolvida durante o Programa de Doutorado Sanduíche no Exterior (PDSE – CAPES), realizado na Universidade de Aberystwyth, País de Gales, Reino Unido, sob supervisão do Professor Luis Alejandro Jose Mur.

Prof. Mur é diretor de pesquisa no tema de biologia e saúde e pertence ao IBERS (*Institute of Biological, Environmental and Rural Sciences* – Universidade de Aberystwyth), centro de pesquisa e ensino de grande prestígio, referência em estudos sobre respostas a desafios globais, incluindo a saúde pública. O grupo liderado pelo Prof. Mur possui reconhecimento internacional e uma das linhas de pesquisa abordam a metabolômica e microbiologia aplicadas ao câncer, com foco na identificação e validação de biomarcadores metabólitos e a relação dessas moléculas com os processos de tumorigênese. A metodologia e as análises realizadas durante o período da bolsa PDSE não estão disponíveis em nossa instituição (FMB UNESP Botucatu), porém têm sido amplamente utilizadas e otimizadas no laboratório do Prof. Mur, que possui domínio do conhecimento para conduzir tais experimentos.

Nesta etapa adicional foi analisado o perfil do metaboloma de amostras de plasma e tumor de pacientes com carcinomas de pâncreas, dos tipos adenocarcinoma ductal pancreático (N = 7) e adenocarcinoma de papila de Vater (N = 5), que correspondem ao mesmo conjunto amostral analisado no **Capítulo I**.

Este estudo, portanto, contribui para a identificação de alterações no metaboloma dos carcinomas de pâncreas e pode auxiliar em avanços na identificação de biomarcadores clinicamente aplicáveis, para melhorar o diagnóstico e prognóstico/sobrevida dos pacientes. Os resultados correspondentes a esse estudo estão apresentados adiante (**item 2.1**, abaixo), elaborados na língua inglesa, pois fazem parte do manuscrito científico que está em preparo e que será submetido para publicação após finalização.

Durante o período do meu doutorado, também foi possível colaborar em outros estudos sobre metaboloma tumoral, especificamente em carcinoma de

pulmão. Foram analisadas amostras de tecido tumoral e tecido histologicamente normal (pareadas) de oito pacientes com carcinoma de células escamosas de pulmão. Neste estudo, analisamos o perfil de metabólitos das amostras de câncer pulmonar pelo método *Flow Infusion Electrospray High Resolution Mass Spectrometry* (FIE-HRMS). Os resultados mostraram características não relatadas anteriormente, como alterações no metabolismo da creatina, serotonina e uma variedade de co-fatores enzimáticos, principalmente no complexo da vitamina B.

Essa abordagem aponta o potencial do método FIE-HRMS como uma ferramenta apropriada de auxílio ao entendimento do processo metabólico de crescimento e proliferação do carcinoma de células escamosas de pulmão. Os resultados obtidos mostraram que os metabólitos fazem parte de vias de metabolismo de nucleotídeos, metabolismo de aminoácidos e metabolismo energético, importantes vias que suprem o crescimento e proliferação dos tumores. Este estudo também retratou a capacidade do método FIE-HRMS em indicar novas características das células tumorais, ainda não relatadas na literatura. Os dados podem ser úteis no desenvolvimento de aplicações clínicas para melhorar as estratégias de tratamento dos pacientes com carcinoma de células escamosas pulmonar (Paes de Araújo R, **Bertoni N**, Seneda AL, Felix TF, Carvalho M, Lewis KE, Hasimoto EN, Beckmann M, Drigo SA, Reis PP, Mur LAJ. Defining Metabolic Rewiring in Lung Squamous Cell Carcinoma. *Metabolites*. 2019;9(3). Disponível em: <https://www.mdpi.com/2218-1989/9/3/47/htm>).

Um segundo manuscrito científico, fruto da colaboração entre o nosso grupo de pesquisa e do Prof. Mur, também está em fase final de elaboração. Neste trabalho adicional, utilizamos as mesmas oito amostras de carcinoma de células escamosas de pulmão e incluímos amostras de plasma dos mesmos pacientes, para análise de expressão de miRNAs na plataforma GeneChip® miRNA 4.0 Array (Thermo Fisher Scientific). Identificamos miRNAs diferencialmente expressos e genes-alvo dos miRNAs. Os dados foram integrados com as alterações metabólicas previamente identificadas nas mesmas amostras. Os resultados dessa análise contribuem para a identificação de novos mecanismos metabólicos regulados por

miRNAs em carcinoma de células escamosas pulmonar. O manuscrito está anexado no **item 5.2**, em Anexos.

2.1. Manuscrito.

Biopsy and blood plasma metabolomics can distinguish between different types of pancreatic cancer.

Natália Bertoni^{1,2}; Rachel Paes de Araújo³; Tainara F Felix^{1,2}; Márcio Carvalho⁴; Claudia N Hasimoto²; Manfred Beckmann³; Sandra A. Drigo^{1,2}; Patricia P Reis^{1,2*}; Luis A J Mur^{3*}.

¹São Paulo State University (UNESP), Faculty of Medicine, Dept. of Surgery and Orthopedics, Botucatu, SP, Brazil.

²São Paulo State University (UNESP), Experimental Research Unity (UNIPEX), Botucatu, SP, Brazil.

³Aberystwyth University, Institute of Biological, Environmental and Rural Sciences (IBERS), Ceredigion, United Kingdom.

⁴São Paulo State University (UNESP), School of Veterinary Medicine and Animal Science, Dept. of Veterinary Clinic, Botucatu, SP, Brazil.

*Correspondence: Luis Alejandro Jose Mur, lum@aber.ac.uk, tel.: + 44 (0)1970622981; Patricia P. Reis, patricia.reis@unesp.br, tel.: +55 (14) 38801451

Abstract:

Pancreatic ductal adenocarcinoma (PDAC) comprise the majority of malignant tumours affecting the pancreas whilst the less common adenocarcinoma of Vater ampulla (AMP) shows better prognosis following earlier diagnosis due to symptomatic disease. We initiated a pilot metabolomic study aiming to discriminate between histologically normal (NOR), PDAC (n=7) and AMP (n=5) pancreatic biopsies and to define specific changes in plasma. Metabolites were profiled by FIE-HRMS and assessed using t-test and principal component analysis (PCA). Differentially accumulating metabolites were mapped to pathways using the mummichog algorithm in R, and biologically meaningful patterns were indicated by Metabolite Set Enrichment Analysis (MSEA). Analyses readily distinguished between NOR and cancer biopsies, based on alterations that may be related to changes including sphingolipid, arachidonic, steroid and phospholipid processing. Discrete metabolite differences were seen between AMP and PDAC; which indicated that sphingolipid metabolism in particular was a feature of PDAC (providing potential metabolite biomarkers with AUC > 0.8) but nucleotide and phosphatidylethanolamine pathways were prominent in AMP biopsies. Plasma analysis from the same patients did not show the same features as biopsies. However, plasma metabolites (AUC > 0.9) could distinguish between AMP and PDAC. With PDAC these were linked to well characterised effects on bile acids and progesterone, as well as a glucose effect which likely reflect diabetic status. With AMP, distinctive change in 3-oxalomalic acid and pantothenic acid were observed in the plasma metabolome. Our data indicates that PDAC and AMP tumours are metabolomically distinct and are linked to different changes in plasma which, upon validation, could serve as diagnostic biomarkers.

Introduction

Cancers of the pancreas have the lowest survival rate and represents the seventh most frequent cause of cancer death in both males and females (1). The statistics of 2018 showed the pancreatic cancer as the 11th most common cancer in the world (1). Moreover, pancreatic cancer is usually diagnosed in advanced stage and approximately 90% of patients have unresectable tumours at the time of diagnosis (2).

Pancreatic ductal adenocarcinomas (PDAC) comprise the majority of malignant tumours affecting the pancreas (~96 %) and other pancreatic tumours include periampullary carcinomas (3). Considering the periampullary tumours, adenocarcinoma of Vater ampulla (AMP) represents approximately 12% of periampullary carcinomas and there is a difficult to distinguish it from other periampullary cancers preoperatively (4). Nevertheless, AMP shows better prognosis (5-year survival rate of > 45%) and early detection when compared to PDAC (4).

There is a need to understand the differences in disease subtypes of patients with pancreatic cancer and study the particularities in metabolism could help to guide the therapy and to manage the treatments. Metabolites are defined as substrates and products of metabolism that can be profiled from samples, contributing to functional insights and to biomarker exploration studies (5).

Here we aimed to elucidate the differences between PDAC and AMP using untargeted metabolomics by flow infusion electrospray ionization high resolution mass spectrometry (FIE-HRMS) as a method that provides a comprehensive characterization of physiological and pathological status of living cells. Our study shows that PDAC and AMP can be distinguish by metabolomics both in tissue and plasma samples, which reflect distinct metabolites interconnected in pathways that could be potential targets for future clinical applications and could be useful in preoperative diagnosis and treatment response monitoring.

Materials and Methods

Patient samples

The present study was conducted with approval of Research Ethics Committee of the Faculty of Medicine, São Paulo State University (UNESP), Botucatu, São Paulo, Brazil (Protocol #61303516.5.0000.5411). The suitable patients signed informed consent and did not receive treatment before surgery and sample collection. Tissue samples (paired tumour and histologically normal) from patients with primary PDAC (N=7) and AMP (N=5) were subjected to frozen section histology analysis to certify the presence of tumour or normal cells in at least 90% of tissues. Plasma sample was also obtained from the 12 patients and all the samples were collected before surgery. The tissue samples were snap-frozen in liquid nitrogen and the plasma samples were stored at -80°C until metabolite extraction. **Table 1** shows clinical and histopathological data.

Sample preparation and metabolites extraction

The sample preparation and metabolites extraction from tumour and normal tissue samples was performed as described by Paes de Araújo *et al* (6). For plasma samples, these were placed on ice for ~1h to ensure complete thawing and were centrifuged at 4° C at 13.000 rpm for 10 minutes. A 50 µL aliquot of each plasma sample was diluted with 450 µL methanol + 50 µL ddH₂O and mixed thoroughly by vortexing for 5 seconds. Samples were then placed on ice for 30 minutes to allow precipitation, and centrifuged at 4°C at 13.000 rpm for 10 minutes. The supernatant was decanted and sample was dried in vacuum concentrator, at ~4°C for 3h. Sample end processing was performed by adding 250 µL of 70% aqueous methanol and mixing by vortexing for 5 seconds. A quantity of 100 µL from each sample (normal and tumour tissues and plasma) was added into a glass vials and sealed, separately, and sent to Flow Infusion Electrospray Ionization High Resolution Mass Spectrometry (FIE-HRMS).

The FIE-HRMS was carried out using Exactive mass analyser coupled with and Accela UHPLC system (Thermo Scientific) generating untargeted metabolite fingerprints in positive and negative mode in a single run, as detailed by

Baptista *et al* (7).

Metabolomic data analysis

MetaboAnalyst 4.0 was used to carry out the statistical analyses. Data filtering was applied in order to identify and remove variables that were not used based on the interquartile range (IQR) and then data were normalised to percentage total ion count, log transformed and auto scaled (8,9). The significance was set to $p\text{-value} < 0.1$ based on univariate analysis (t-test or ANOVA) and allowed the identification of significant metabolites. Differences among the sample groups were analysed with unsupervised principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA). Unsupervised hierarchical clustering was used to show major sources of variation in a heatmap.

Metabolites that did not show significance differences among groups were not analysed. Compounds were identified according to mass to charge (m/z), p -values and t -scores used to query the Kyoto Encyclopedia of Genes and Genomes (KEGG) and then the annotation was made using 3 ppm tolerance in their accurate mass. The Metabolite Set Enrichment Analysis (MSEA) was carried out to identify biologically patterns (10).

Results

Metabolites are deregulated in pancreatic cancer and distinguish tumour from normal tissues.

We were able to identify overall distinct metabolomic profiles in tumour and normal tissue samples from patients with pancreatic cancer (**Figure 1A** and **Figure 1B**) and these data was confirmed by unsupervised hierarchical clustering analysis, which stratify tumour and normal tissues according to metabolite expression (**Figure 1C**). A Metabolite Set Enrichment Analysis (MSEA) based on Over Representation Analysis (ORA) was used to show significantly enhanced pathways (**Figure 2**). These indicated pancreatic cancer linked to sphingolipid and arachidonic acid metabolism (**Supplementary Figure S1**) and metabolites mainly

linked to nervous tissue (**Supplementary Figure S2**).

Pancreatic cancer subtypes have distinct metabolomic profiles

Metabolite expression in the two different histological subtypes of pancreatic cancer (pancreatic ductal adenocarcinoma and pancreatic cancer of Vater ampulla) showed clusters and consistent results when compared to histologically normal tissues. **Figure 3** presents the PCA and unsupervised hierarchical clustering data of tissue samples, which separates the groups. We also performed analyses of the normal tissues separately that showed differences between them as well (**Supplementary Figure S3**). **Supplementary Figure S4** shows change in tissue specific metabolites on carcinogenesis. **Figures 4** and **Figure 5** shows metabolomic profile of AMP and PDAC, respectively. These data indicate that sphingolipid metabolism is a feature of PDAC, providing potential metabolite biomarkers with $AUC > 0.8$ (**Supplementary Figure S5** **Supplementary Figure S6**).

We also performed analyses between AMP and PDAC tumour tissue samples as showed in **Figure 6**, which confirms that these two subtypes have different metabolomic profiles.

Pancreatic ductal adenocarcinoma and pancreatic cancer of Vater ampulla also have metabolomic differences in plasma

Plasma analyses from the same patients did not show the same features as biopsies (**Figure 7**). However, plasma metabolites ($AUC > 0.9$) could distinguish between AMP and PDAC (**Figure 8**). The enhanced pathways are bile acid biosynthesis and purine metabolism (**Figure 8A**). Further, five individual metabolites exhibited AUC accuracies of >0.8 (9,11 alpha-Epoxy pregn-4-ene-3,20-dione (Progesterone): $AUC = 0.943$, CI 0.771-1; Pantothenic acid: $AUC = 0.943$, CI 0.771-1; Cholic acid: $AUC = 0.943$, CI 0.686-1; 3-Oxalomalic acid: $AUC = 0.886$, CI 0.585-1; D-Glucose: $AUC = 1$, CI 0.941-1) (**Figure 8B**).

In PDAC, metabolite profiles were linked to well characterized effects on bile acids and progesterone as well as a glucose effect, which likely reflect

diabetic status. In AMP, distinctive changes in 3-oxalomalic acid and pantothenic acid were identified. PDAC and AMP tumours have distinct metabolic profiles. Circulating plasma metabolites may be explored as potential biomarkers for early disease detection and patient management associated with specific pancreatic cancer subtypes.

Table 1. Clinical and histopathological characteristics from the 12 cases.

Variables	Pancreatic ductal adenocarcinoma (N=7)	N (%)	Adenocarcinoma of Vater ampulla (N=5)	N (%)
Age (years)				
Median	61.2 ± 4.8		64,8 ± 5,7	
Average (range)	61 (52-70)		64 (55-73)	
Gender				
Male	2	28,6	3	60
Female	5	71,4	2	40
Tumor grade				
WD	5	71,4	3	60
MD	1	14,3	1	20
PD	1	14,3	1	20
Tumor size (T)				
T1-T2	0	0	2	40
T3-T4	7	100	3	60
Nodal status (N)				
Positive (N1, N2)	5	71,4	3	60
Negative (N0)	1	14,3	1	20
Undetermined (Nx)	1	14,3	1	20
Distant metastasis (M)				
Positive (M1)	1	14,3	0	
Negative (M0)	0	0	1	20
Undetermined (Mx)	6	85,7	4	80
Stage				
Ia	0	0	1	20
Ib	0	0	0	0
IIa	1	14,3	1	20
IIb	3	42,8	2	40
III	2	28,6	1	20
IV	1	14,3	0	0
Tobacco use				
Sim	2	28,6	3	60
Não	5	71,4	2	40
Alcohol use				
Sim	0	0	2	40
Não	7	100	3	60
Diabetes				
Sim	4	57,2	0	0
Não	3	42,8	5	100
Obesity				
Sim	1	14,3	0	0
Não	6	85,7	5	100
Patient status				
Alive with disease	5	71,4	2	40
Dead of disease	2	28,6	3	60

WD: well differentiated

MD: moderately differentiated

PD: poorly differentiated

LIST OF FIGURES

Figure 1. Principle Component Analysis (PCA) plot showing differentially expressed metabolomic profiles in tissues from patients with pancreatic cancer (CAN) compared to matched histologically normal (NOR). **(A)** the negative ionization mode and **(B)** the positive ionization mode; **(C)** unsupervised hierarchical clustering analysis showing that metabolomic expression profiles are able to distinguish tumour and normal pancreatic tissues.

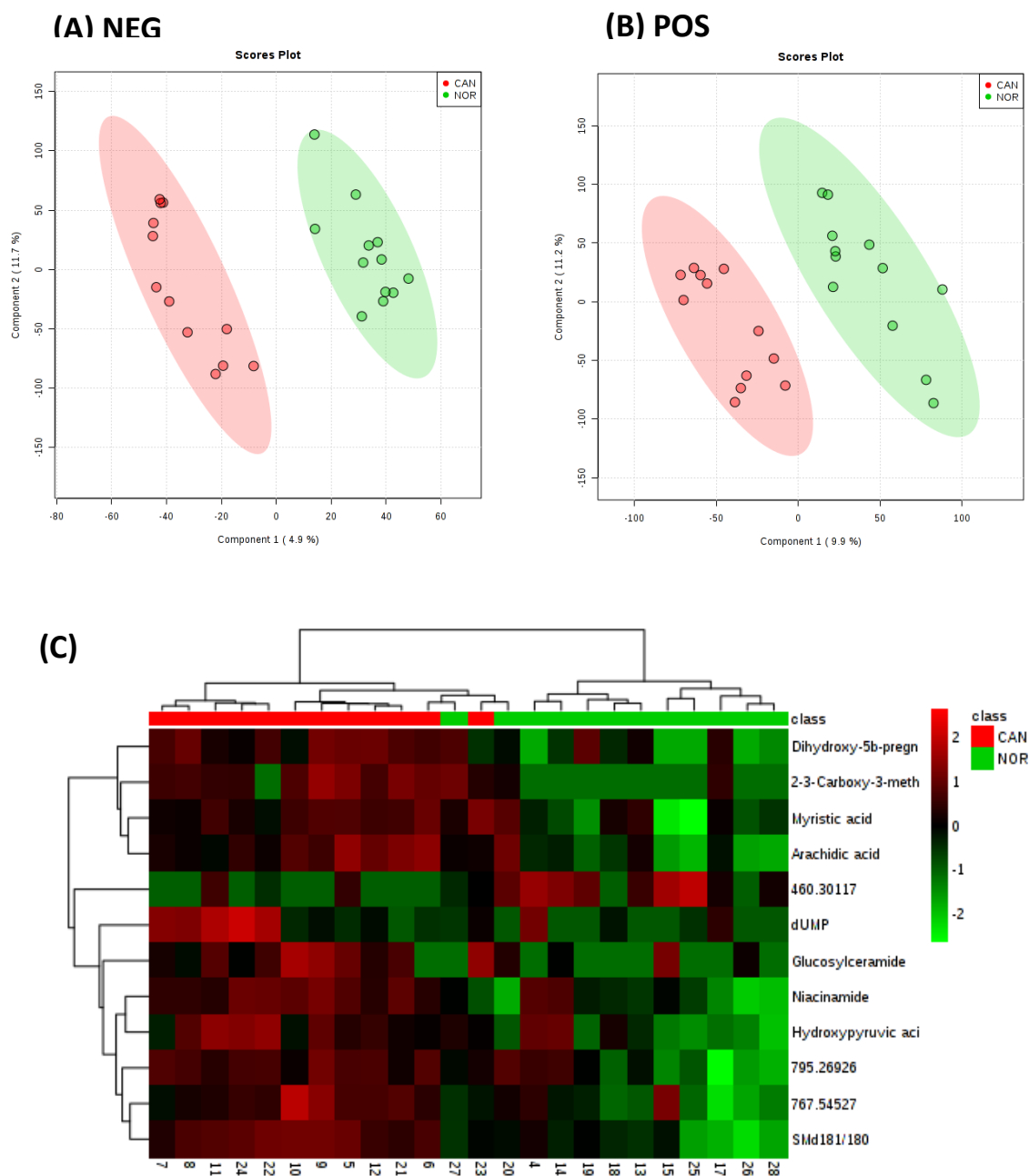


Figure 2. Significantly enhanced pathways in pancreatic tissue compared to normal tissue. **(A)** Enriched pathways and **(B)** enriched metabolites linked to tissue type.

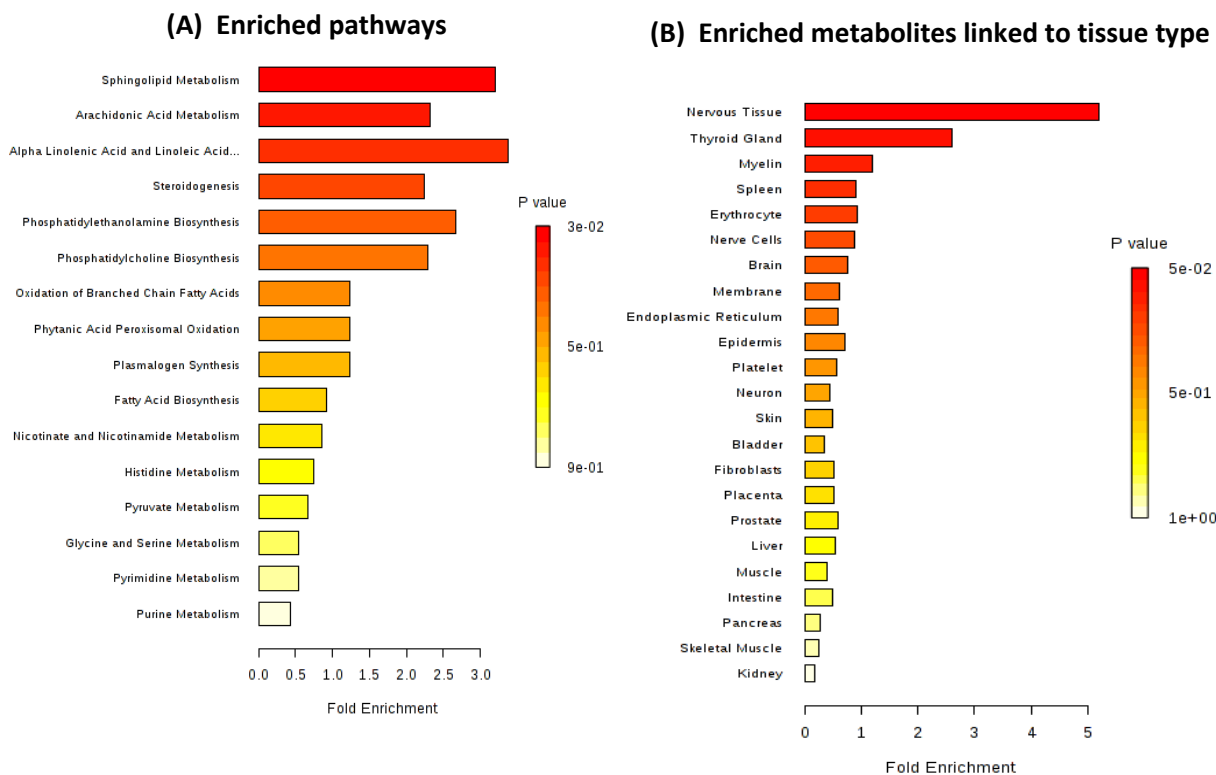


Figure 3. Principle Component Analysis (PCA) plot showing metabolomic profiles generated in tissue samples from patients with pancreatic ductal adenocarcinoma (PDAC) and pancreatic cancer of Vater ampulla (AMP) compared to matched histologically normal (N) tissue samples. **(A)** Is the negative ionization mode and **(B)** is the positive ionization mode; **(C)** hierarchical clustering analyses with top 25 differentially accumulated metabolites.

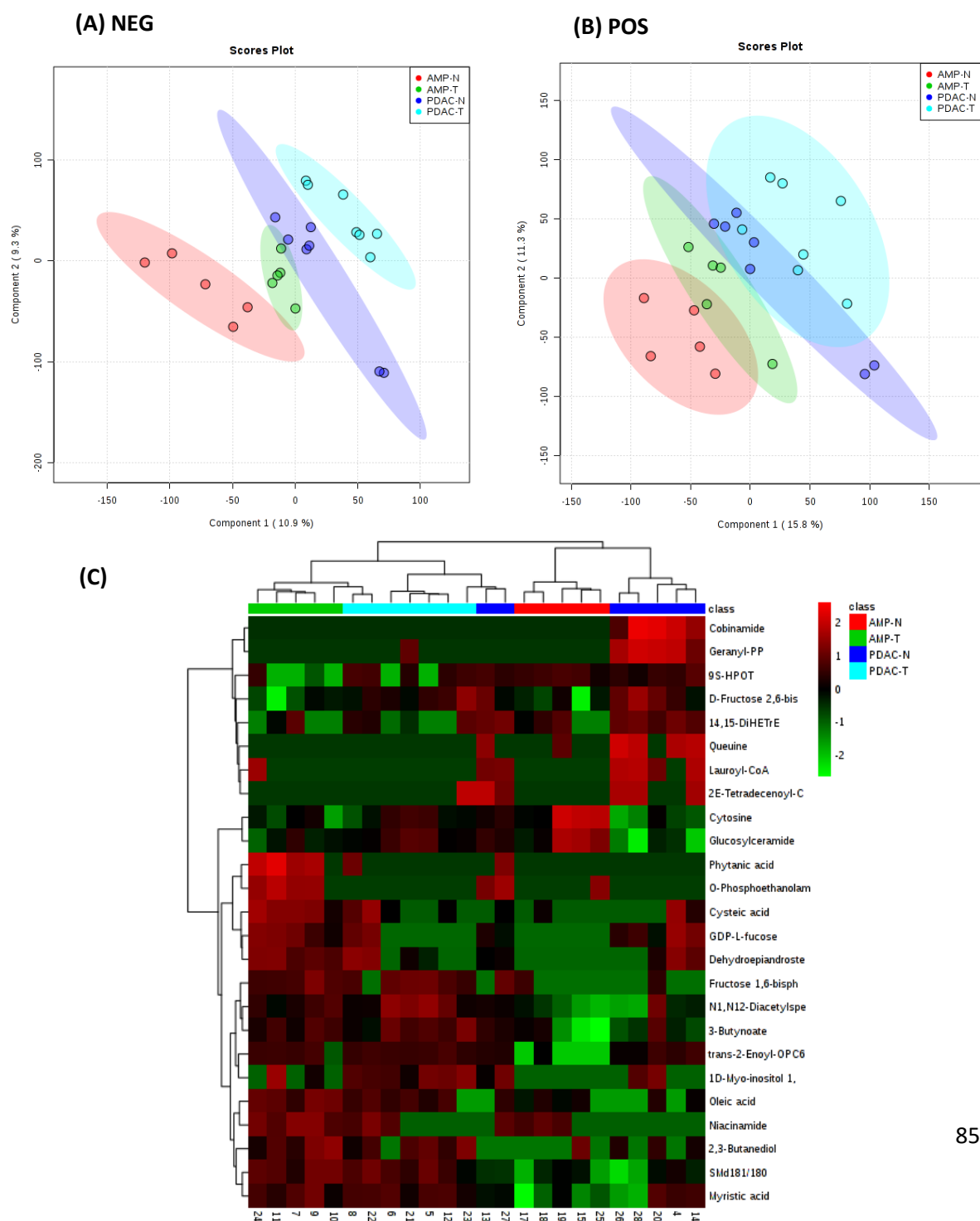


Figure 4. Metabolite variation linked to cancer formation in pancreatic cancer of Vater ampulla (AMP). Identified nucleotide metabolites for each sample were extracted from FIE-HRMS derived matrix and assessed by (A) principal component analyses and (B) hierarchical cluster analysis. (C) Significantly enriched pathways in AMP tumour and matched histologically normal tissues.

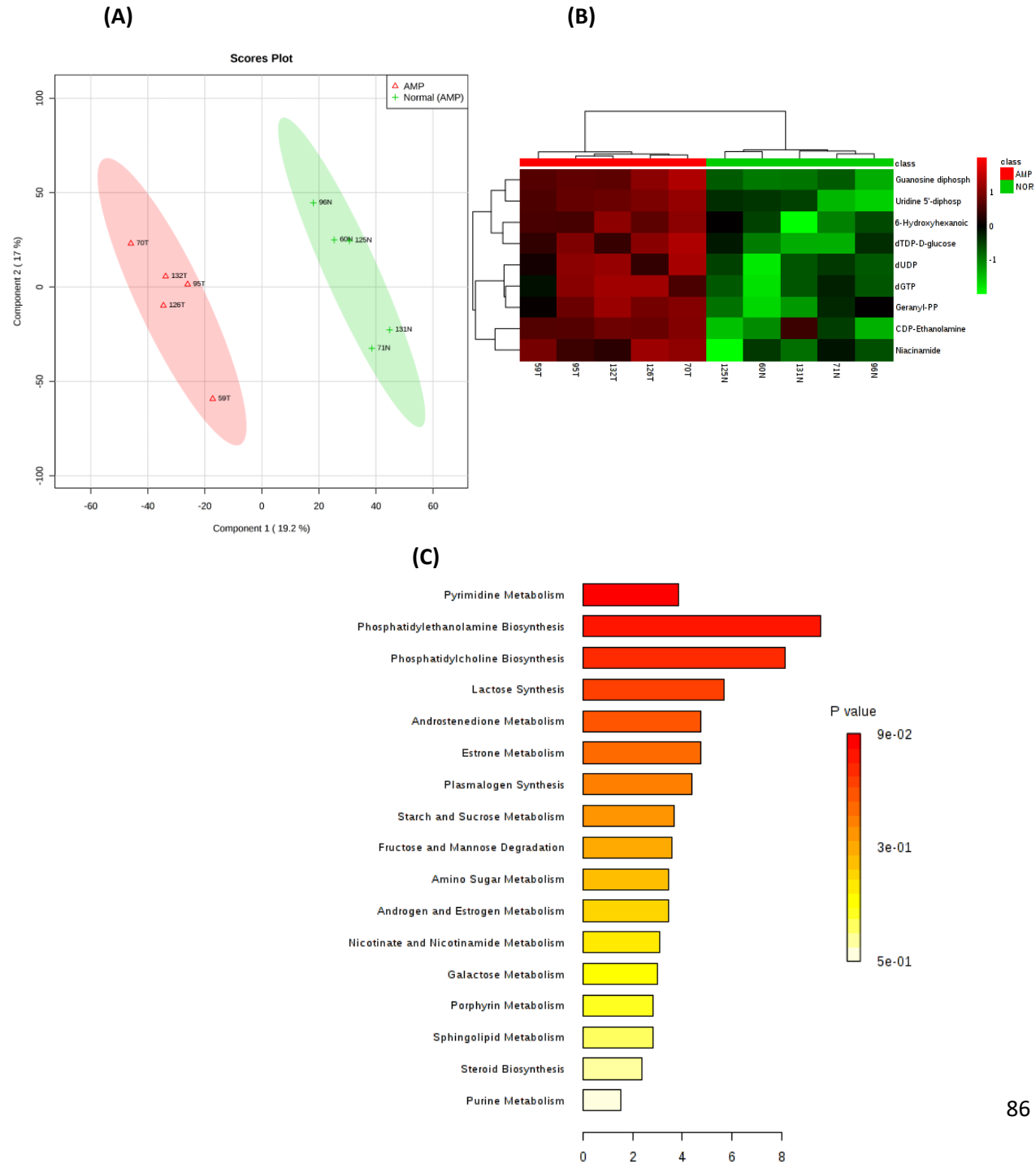
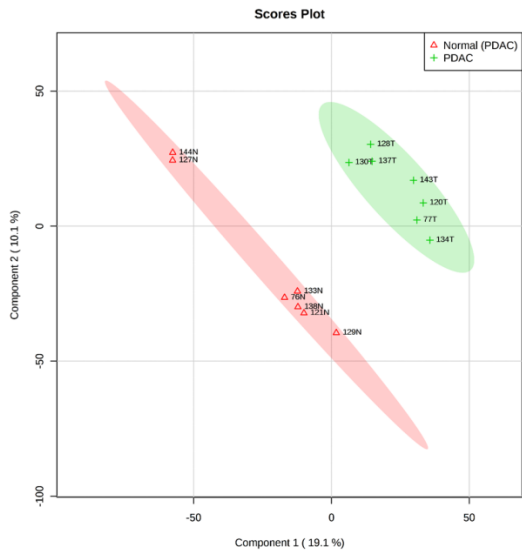


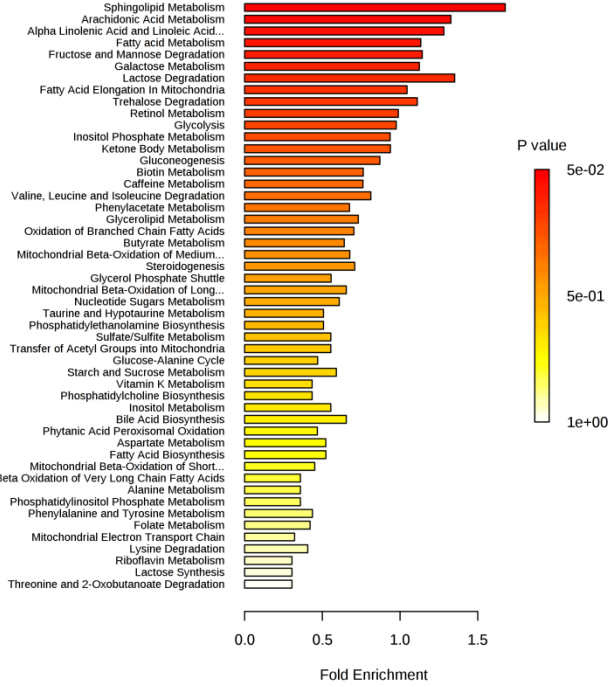
Figure 5. Metabolite variation linked to cancer formation in pancreatic ductal adenocarcinoma (PDAC). Identified nucleotide metabolites for each sample were extracted from FIE-HRMS derived matrix and assessed by **(A)** principal component analyses and **(B)** hierarchical cluster analysis. **(C)** Significantly enriched pathways in PDAC tumour and matched histologically normal tissues.

(A)



(C)

Enrichment Overview (top 50)



(B)

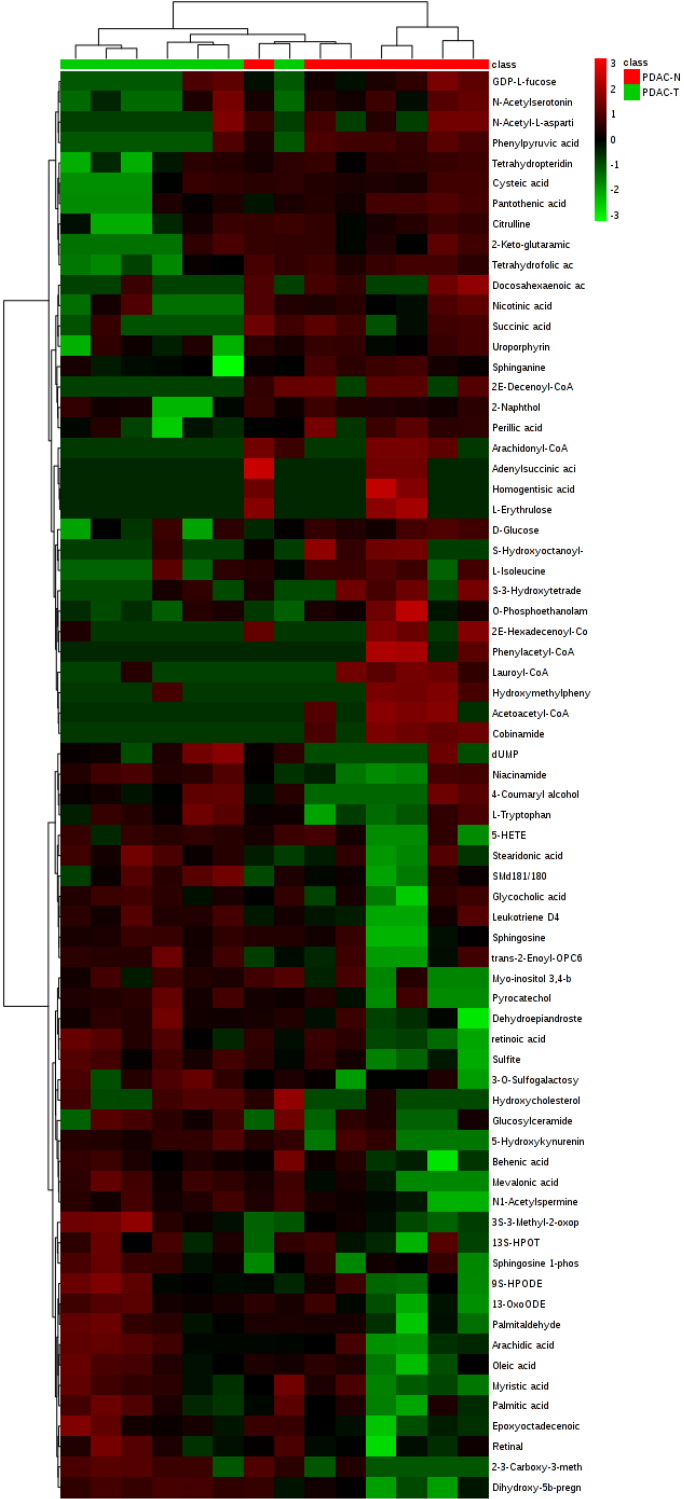


Figure 6. Principle Component Analysis and unsupervised hierarchical clustering analysis for the two cancer subtypes (AMP and PDAC) comparing only tumour tissue samples.

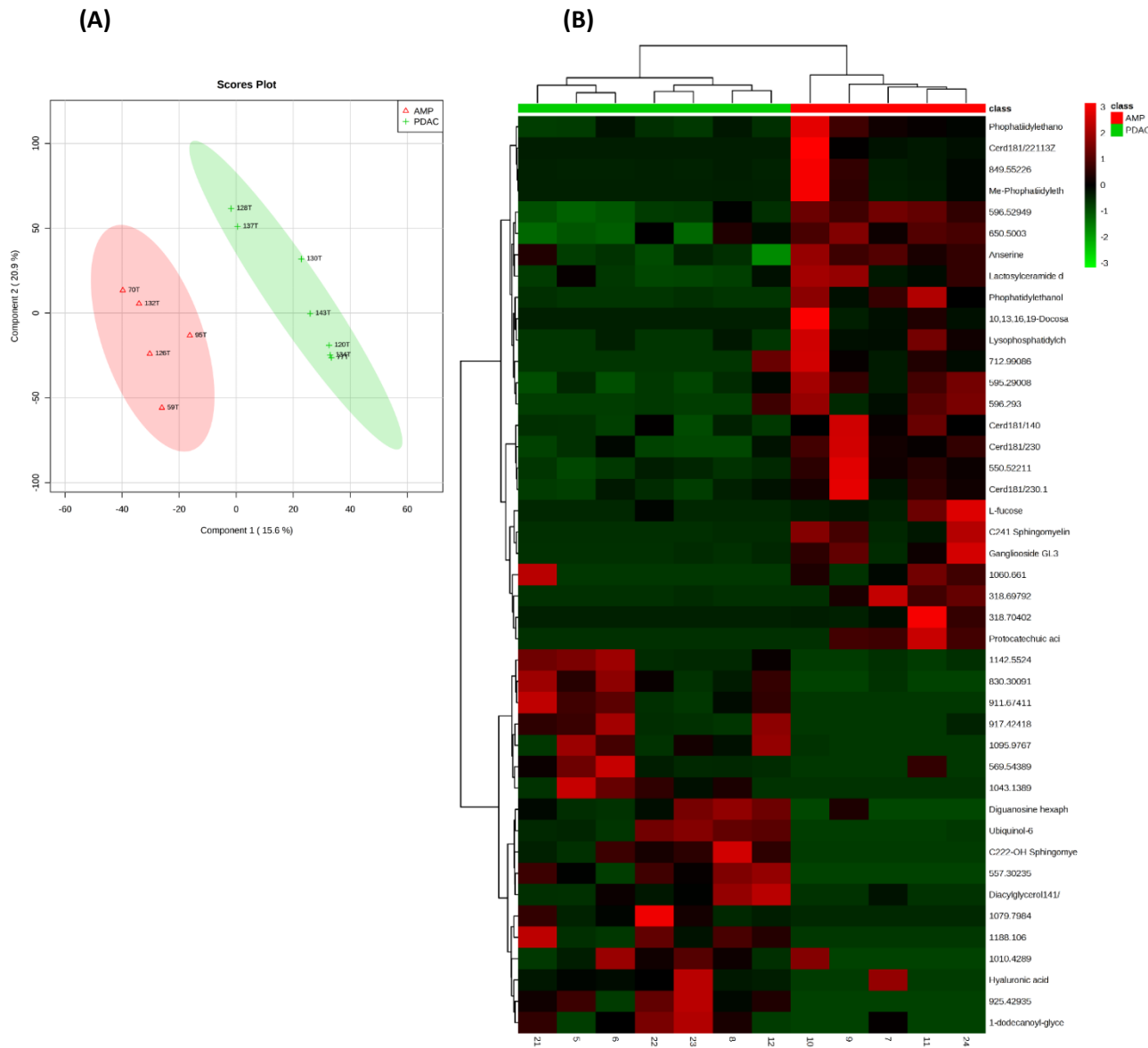
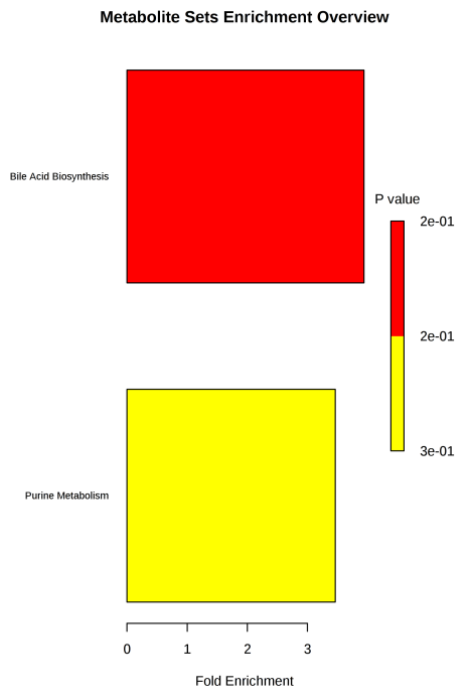
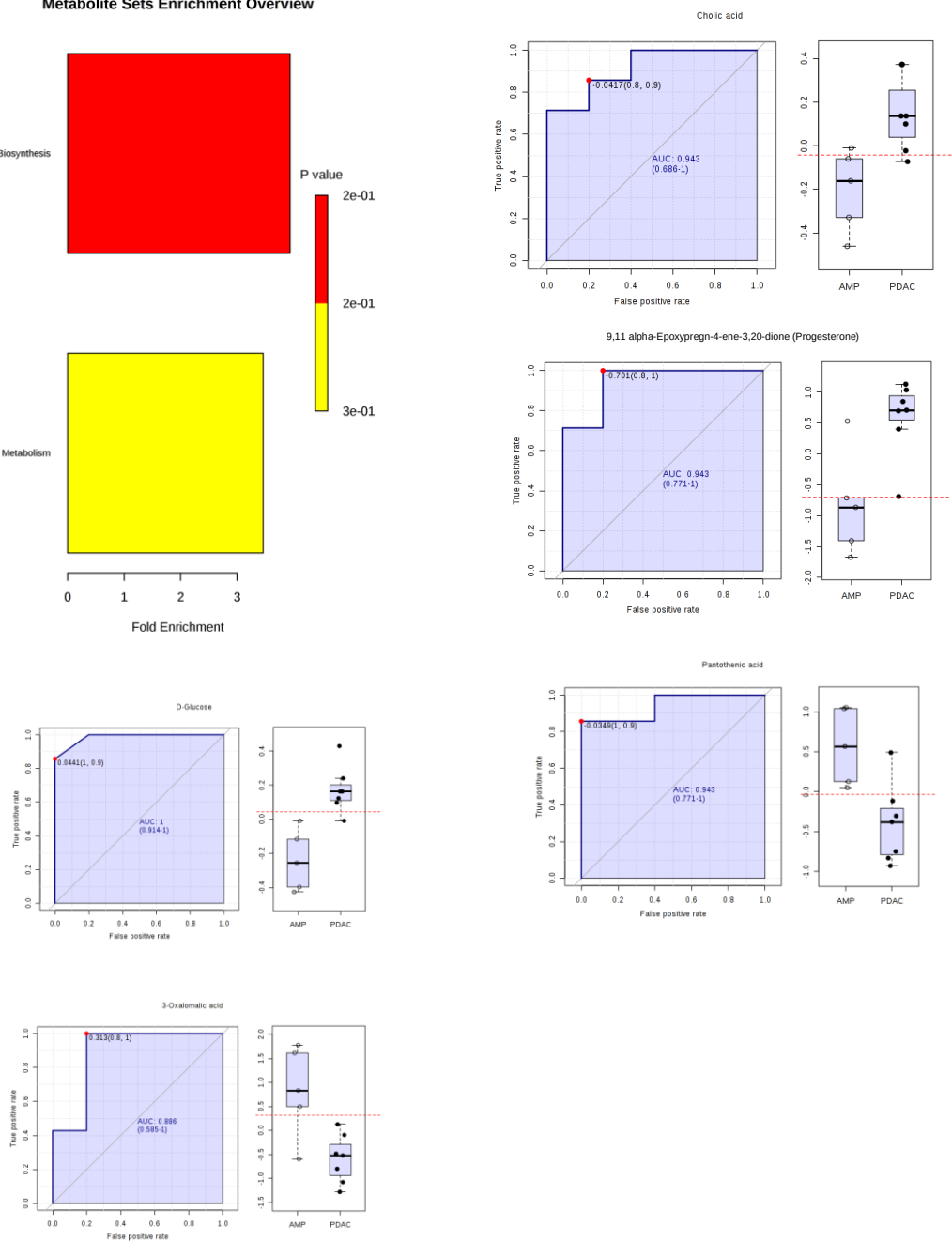


Figure 8. AMP and PDAC markers in plasma.

(A)

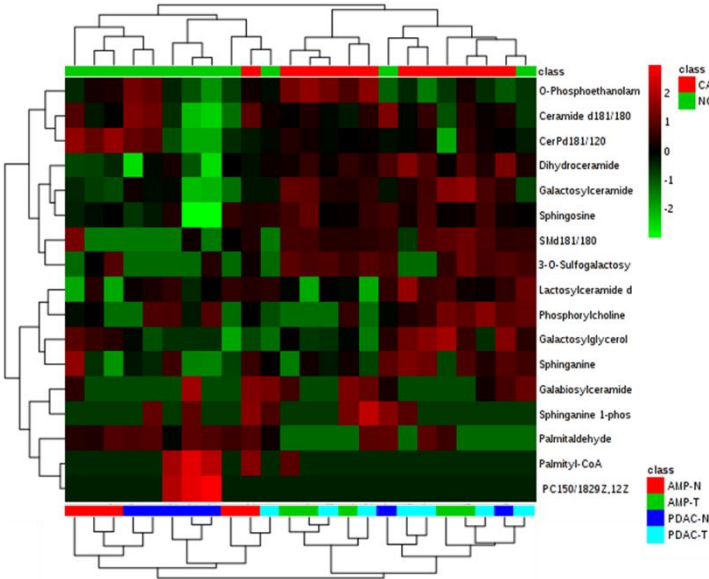


(B)

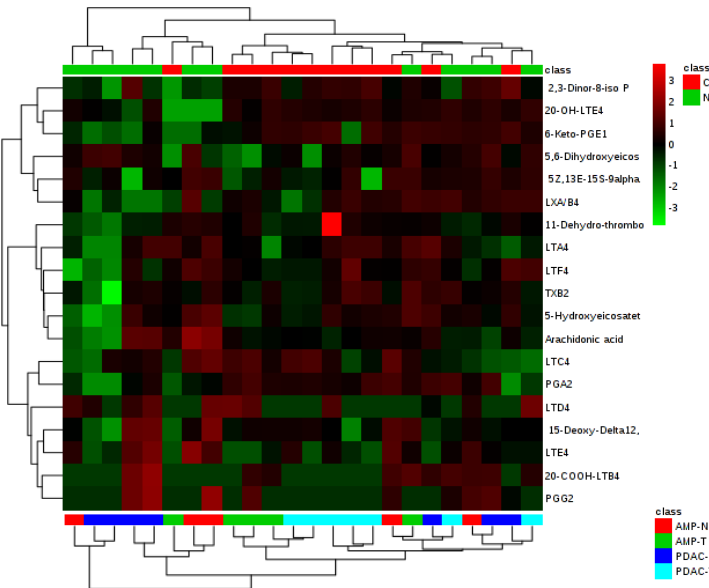


Supplementary Figure S1. Significantly enhanced pathways in pancreatic tissue compared to normal tissue samples. Identified **(A)** sphingolipid metabolism pathway and **(B)** arachidonic metabolism pathway metabolites for each group were extracted from FIE-HRMS derived matrix and evaluated by unsupervised hierarchical clustering analysis.

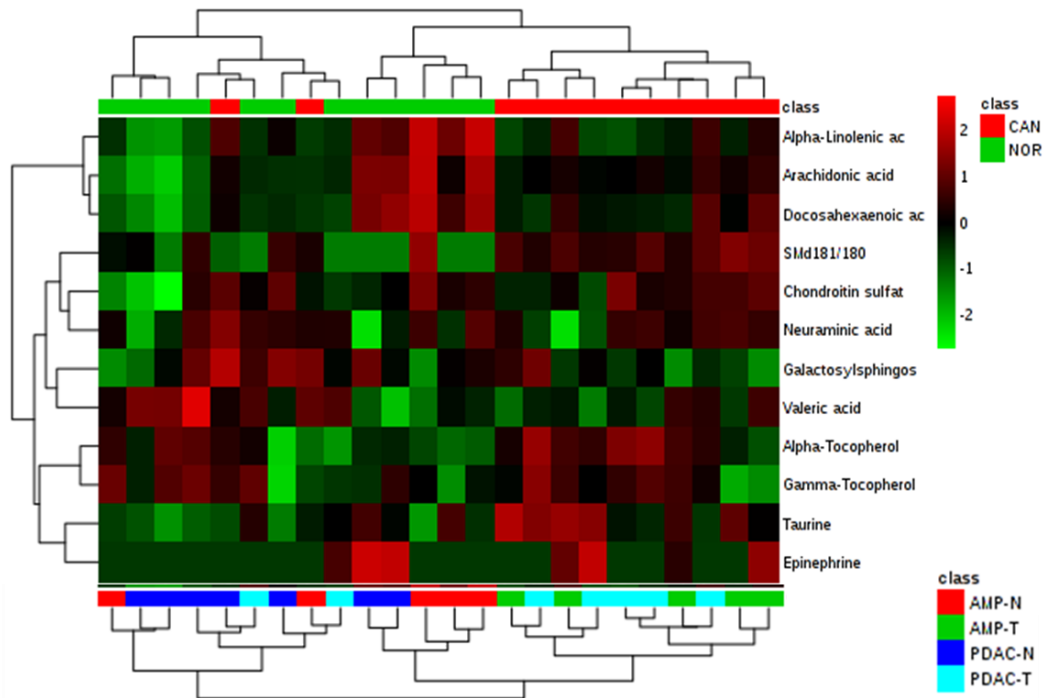
(A) Sphingolipid metabolism pathway



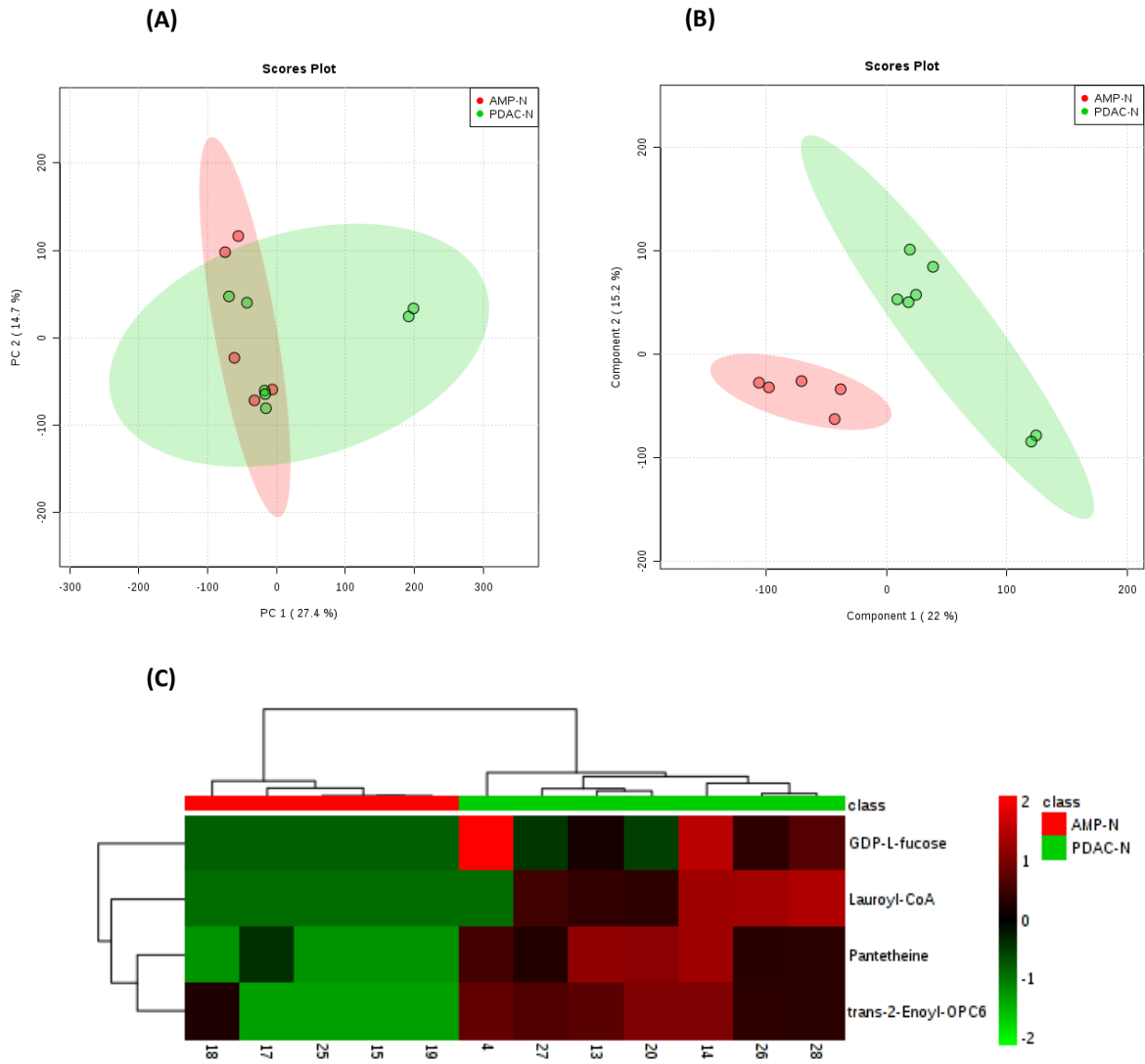
(B) Arachidonic metabolism pathway



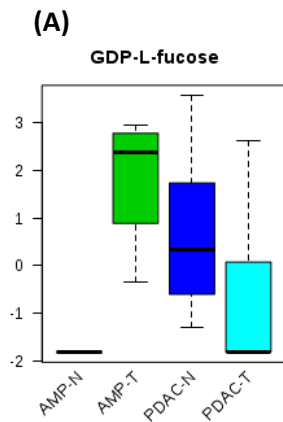
Supplementary Figure S2. Unsupervised hierarchical clustering analysis exploring organ type nerves related-metabolites in pancreatic tissue compared to normal tissue samples.



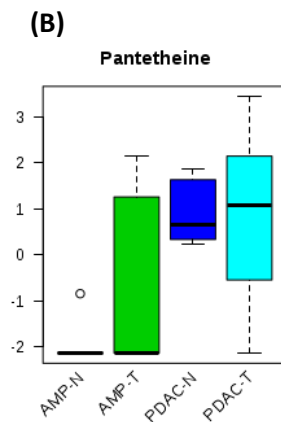
Supplementary Figure S3. Principle Component Analysis (PCA) plot showing metabolomic profiles generated in matched histologically normal samples from patients with PDAC and AMP. **(A)** is the negative ionization mode and **(B)** is the positive ionization mode. **(C)** Identified metabolites for each sample were extracted from FIE-HRMS derived matrix and evaluated by unsupervised hierarchical clustering analysis.



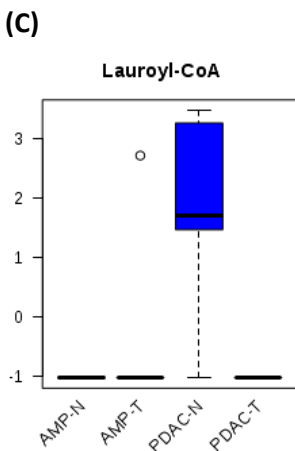
Supplementary Figure S4. Change in tissue specific metabolites on carcinogenesis. **(A)** GDP-L-fucose **(B)** Pantetheine **(C)** Lauroyl-CoA **(D)** trans-2-Enoyl-OPC6-CoA.



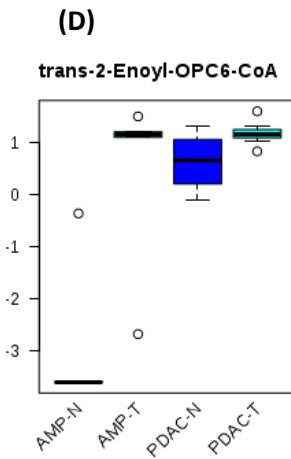
[map00051](#)
Fructose and mannose
metabolism
[Map00520](#)
Amino sugar and nucleotide sugar
metabolism



[map00770](#)
Coenzyme A Biosynthesis

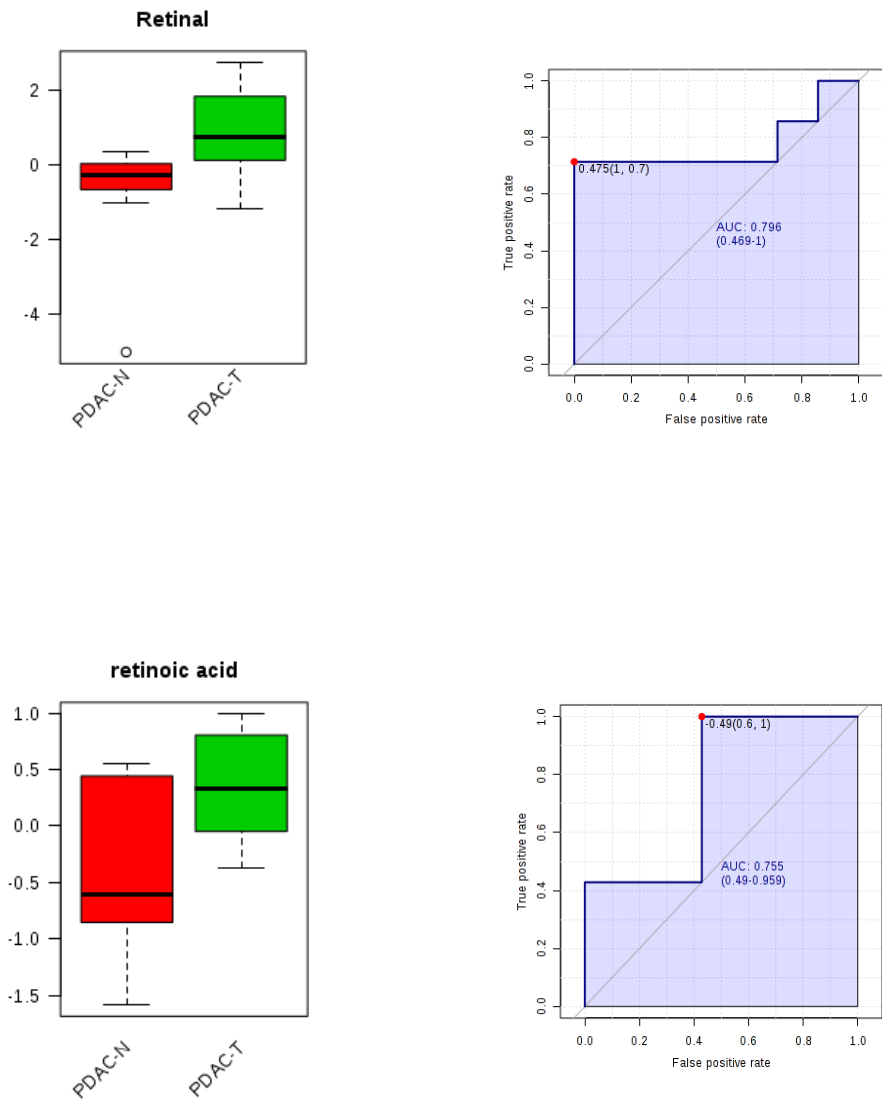


[map00062](#)
Fatty acid elongation

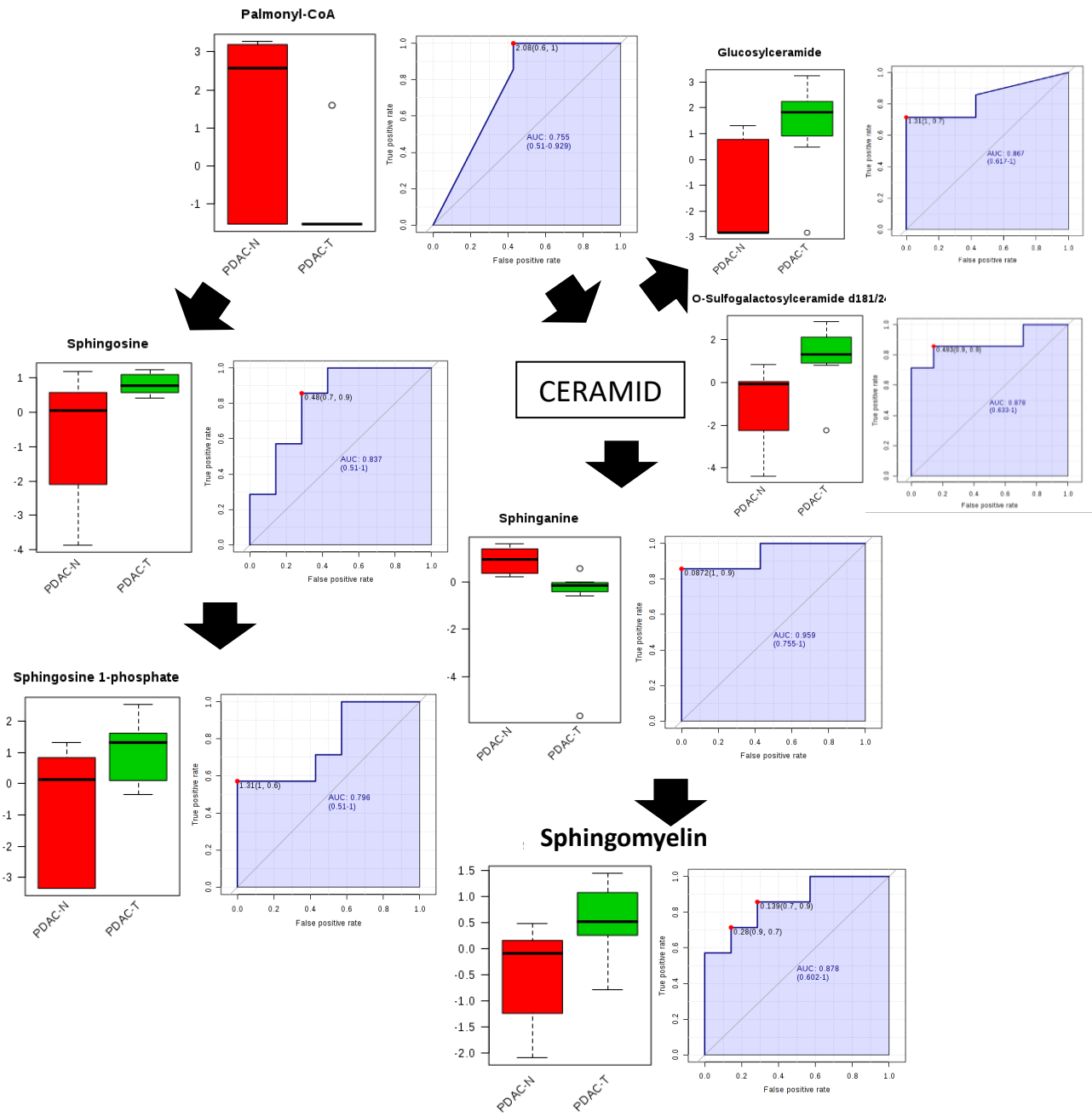


[map00592](#)
alpha-Linolenic acid
metabolism

Supplementary Figure S5. Sphingolipid metabolism in PDAC. Red colours represent normal tissue and green colours represent tumour tissue.



Supplementary Figure S6. Retinoic acid metabolism in PDAC. Red colours represent normal tissue and green colours represent tumour tissue.



References

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3. Conclusões

I. Um grande número de miRNAs estava significativamente desregulado no tumor e no plasma de pacientes com carcinomas de pâncreas comparado com os referenciais normais pareados, em cada subtipo histológico;

II. Dentre os miRNAs alterados, foi possível identificar miRNAs diferencialmente expressos no tumor e também no plasma de pacientes com adenocarcinoma ductal pancreático e adenocarcinoma de papila de Vater;

III. Os miRNAs identificados no tumor e no plasma desses pacientes regulam um grande número de genes envolvidos em vias biológicas relevantes de transcrição, sinalização e divisão celular, diferenciação e proliferação celular e de indução de morte celular;

IV. O aumento da expressão do miR-378h tanto no tumor quanto no plasma de pacientes com adenocarcinoma ductal pancreático pode ser de particular interesse para o desenvolvimento de aplicações como estratégias moleculares complementares no diagnóstico precoce. Os níveis elevados de expressão deste miRNA no plasma de pacientes e não no grupo de referência é uma estratégia de detecção eficaz em relação à quantificação de moléculas com expressão diminuída ou ausente. Além disso, a análise de predição para esse miRNA apresenta genes-alvo envolvidos em vias biológicas de resposta imune, o que sugere a imunoterapia como possível estratégia de tratamento para pacientes com adenocarcinoma ductal pancreático;

V. As análises do metaboloma mostraram que existe variação metabólica entre os dois tipos de carcinoma de pâncreas e que, apesar das amostras de plasma não apresentarem as mesmas características metabólicas das amostras de tecido, os metabólitos plasmáticos também podem distinguir adenocarcinoma ductal pancreático de adenocarcinoma de papila de Vater;

VI. Os resultados sugerem uma aplicação potencial: a amostra de sangue (biópsia líquida) pode ser obtida para diagnóstico molecular na ausência ou impossibilidade de obtenção de amostra de tecido sólido (biópsia sólida); a realização de diagnóstico

molecular utilizando amostra obtida por biópsia líquida pode ser mais vantajosa por se tratar de um método minimamente invasivo e de rápida realização, o que diminuiria a necessidade de um procedimento invasivo/internação do paciente, além de menores riscos associados à execução;

VII. Os subtipos de tumores (PDAC a AMP) apresentam perfis de expressão global de miRNAs e perfis de expressão do metaboloma distintos, o que propõe que os miRNAs e os metabólitos identificados possuem potencial como biomarcadores de detecção precoce;

VIII. miRNAs específicos identificados no tumor e no plasma regulam genes envolvidos em vias de metabolismo, o que sugere que devem desempenhar papel biológico no desenvolvimento e progressão destes dois subtipos (PDAC e AMP) de carcinomas de pâncreas.

4. Considerações finais

Este foi um estudo de identificação de alterações na expressão de miRNAs no plasma e no tumor de pacientes com carcinoma de pâncreas, com diagnóstico de adenocarcinoma ductal pancreático ou adenocarcinoma de papila de Vater, com etapa adicional de identificação da expressão do metaboloma nas mesmas amostras. Apesar do número limitado de amostras analisadas, os resultados enriqueceram com novas informações e levaram à identificação de miRNAs e de metabólitos diferencialmente expressos e as alterações puderam ser detectadas e quantificadas no plasma dos pacientes. Esses resultados reforçam dados da literatura no sentido de indicar a aplicação das biópsias líquidas como estratégia diagnóstica molecular. Além disso, a integração de dados moleculares e de metabolismo deste estudo, quando validadas, poderá contribuir para o desenvolvimento de potenciais biomarcadores para diagnóstico precoce de pacientes com carcinomas de pâncreas.

5. Anexos

5.1. Comprovante de aprovação do projeto de pesquisa pelo CEP



UNESP -FACULDADE DE
MEDICINA DE BOTUCATU



PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Expressão de microRNAs e mRNAs-alvo em plasma e tumor de pacientes com carcinomas de fígado, pâncreas e vias biliares.

Pesquisador: Natália Bertoni

Área Temática:

Versão: 1

CAAE: 61303516.5.0000.5411

Instituição Proponente: Faculdade de Medicina de Botucatu/UNESP

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.850.128

Apresentação do Projeto:

Carcinomas de fígado, pâncreas e vias biliares são neoplasias de incidência populacional importante e frequentemente associadas a mau prognóstico. Acredita-se que essas neoplasias tenham características em comum, uma vez que as estruturas que acometem tem sua origem a partir das mesmas células progenitoras. É portanto plausível supor que existam biomarcadores aplicáveis aos carcinomas acima descritos. A importância de identificar biomarcadores (especialmente aqueles presentes no sangue) está na possibilidade de diagnóstico precoce, determinação de prognóstico e até mesmo melhor compreensão dos fatores envolvidos na gênese dos tumores (conhecimento que pode colaborar para o desenvolvimento de estratégias terapêuticas).

Micro RNAs (miRNA) são pequenos fragmentos do ácido ribonucleico (RNA) que tem importante papel na regulação da expressão de genes. Vários miRNAs já foram associados a diversos tipos de neoplasias. Alguns deles são reconhecidos marcadores da tendência (ou não) para desenvolvimento de metástases. Os miRNAs podem ser detectados no tecido tumoral ou no sangue periférico.

O estudo proposto tem por objetivo identificar miRNAs associados a tumores de fígado, pâncreas e vias biliares, seu possível uso no diagnóstico, seu papel na gênese das neoplasias e sua

Endereço: Chácara Butignolli, s/n

Bairro: Rubião Junior

UF: SP

Município: BOTUCATU

Telefone: (14)3880-1608

CEP: 18.618-970

E-mail: capellup@fmb.unesp.br



associação com o prognóstico. Para tanto, pretendem incluir 79 sujeitos recrutados no Hospital das Clínicas da Faculdade de Medicina de Botucatu. Destes, alguns serão analisados retrospectivamente (através de amostras de tumor conservada em parafina) e outros prospectivamente (pela coleta de sangue e, quando viável, uso de excedente de fragmentos de tumor obtidos na cirurgia de rotina).

Resumidamente, será identificada e quantificada a expressão de miRNAs no soro e/ou tecidos. Esses miRNAs serão caracterizados e serão identificados seus genes alvos. Para cumprir essas etapas serão aplicados tanto testes moleculares quanto análises complexas de bioinformática. Os resultados serão testados para associação com características clínicas dos sujeitos de pesquisa.

Esse projeto destina-se a doutoramento da proponente, orientado pela Profa.Dra. Patricia Pintor dos Reis.

Objetivo da Pesquisa:

Identificar a expressão de micro-RNAs e seus alvos no plasma e em tecido tumoral de sujeitos com neoplasias de fígado, pâncreas e vias biliares.

Avaliação dos Riscos e Benefícios:

Trata-se de estudo complexo, com possibilidade de gerar conhecimento valioso sobre a gênese de tumores malignos e de encontrar caminhos para desenvolvimento de diagnóstico precoce e estratégias terapêuticas. Nesse sentido, traz benefícios, embora sejam esses indiretos. Os riscos são pequenos, e dizem respeito a: (a) confidencialidade ao lidar com dados clínicos dos sujeitos; (b) coleta de 20ml de sangue para estudos com plasma; (c) coleta de tecido tumoral. Note-se porém que a coleta de tecido será a habitualmente realizada nas cirurgias já indicadas para tratamento das neoplasias, sendo utilizado pequeno excedente de tecido no estudo.

Comentários e Considerações sobre a Pesquisa:

O projeto apresentado tem grande mérito e é factível nas condições propostas.

Considerações sobre os Termos de apresentação obrigatória:

Os documentos pertinentes estão apresentados. O Termo de Consentimento é escrito de forma clara, concisa, e explicita os riscos e a ausência de benefícios diretos aos sujeitos. A proponente solicita dispensa do TCLE para sujeitos incluídos retrospectivamente que tenham perdido seguimento ou ido a óbito. Estima em 23 o número desses sujeitos.

Recomendações:

Ao final apresentar Relatório Final de Atividades

Endereço: Chácara Butignolli, s/n

Bairro: Rubião Junior

CEP: 18.618-970

UF: SP

Município: BOTUCATU

Telefone: (14)3880-1608

E-mail: capellup@fmb.unesp.br

Continuação do Parecer: 1.850.128

Conclusões ou Pendências e Lista de Inadequações:

RECOMENDO APROVAÇÃO, sem necessidade de envio à CONEP.

Considerações Finais a critério do CEP:

Projeto de Pesquisa APROVADO, deliberado em reunião ORDINÁRIA do CEP de 05 de Dezembro de 2.016, sem necessidade de envio à CONEP.

O CEP, no entanto, solicita aos pesquisadores que após a execução do projeto em questão, seja enviado para análise o respectivo "Relatório Final de Atividades", o qual deverá ser enviado via Plataforma Brasil na forma de "NOTIFICAÇÃO".

OBS: LEMBRAMOS QUE A PRESENTE PESQUISA SOMENTE PODERÁ SER INICIADA APÓS DIA 05/12/2016 – DATA DA APROVAÇÃO DO CEP.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_788720.pdf	24/10/2016 14:10:01		Aceito
Outros	2572_Anuencia_Natalia_Bertoni.pdf	24/10/2016 14:09:35	Natália Bertoni	Aceito
Folha de Rosto	Plat_Brasil_2572_Natalia_Bertoni.pdf	24/10/2016 12:21:47	Natália Bertoni	Aceito
Projeto Detalhado / Brochura Investigador	Projeto_Doutorado_Natalia_Bertoni.pdf	08/09/2016 10:13:38	Natália Bertoni	Aceito
Outros	Pedido_dispenza_TCLE.jpg	06/09/2016 12:06:02	Natália Bertoni	Aceito
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TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_Projeto_Doutorado_Natalia_Bertoni.pdf	06/09/2016 11:38:46	Natália Bertoni	Aceito

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Não

Endereço: Chácara Butignolli, s/n

Bairro: Rubião Junior

UF: SP

Município: BOTUCATU

Telefone: (14)3880-1608

CEP: 18.618-970

E-mail: capellup@fmb.unesp.br

Continuação do Parecer: 1.850.128

BOTUCATU, 05 de Dezembro de 2016

Assinado por:
SILVANA ANDREA MOLINA LIMA
(Coordenador)

Endereço: Chácara Butignolli , s/n	CEP: 18.618-970
Bairro: Rubião Junior	
UF: SP Município: BOTUCATU	
Telefone: (14)3880-1608	E-mail: capellup@fmb.unesp.br

5.2. Manuscrito.

Predicting the metabolomic impact of deregulated microRNAs in tumour and plasma from patients with lung squamous cell carcinoma.

Natália Bertoni^{1,2}; Tainara F Felix²; Rachel Paes de Araújo³; Márcio Carvalho⁴; Ana Laura Seneda^{1,2}; Érica N Hasimoto¹; Daniele Cataneo¹; Antônio Cataneo¹; Keir E. Lewis⁵; Manfred Beckmann³; Bing Ji⁶; Sandra A Drigo^{1,2}; Patricia P Reis^{1,2*}; Luis A J Mur^{3*}.

¹ São Paulo State University (UNESP), Faculty of Medicine, Dept. of Surgery and Orthopedics, Botucatu, SP, Brazil.

² São Paulo State University (UNESP), Experimental Research Unity (UNIPEX), Botucatu, SP, Brazil.

³ Aberystwyth University, Institute of Biological, Environmental and Rural Sciences (IBERS), Ceredigion, United Kingdom.

⁴ São Paulo State University (UNESP), School of Veterinary Medicine and Animal Science, Dept. of Veterinary Clinic, Botucatu, SP, Brazil.

⁵ Department of Respiratory Medicine, Prince Phillip Hospital, Llanelli SA14 8LY, UK; School of Medicine, Swansea University, Swansea SA2 8PP, UK.

⁶ Shandong University, School of Control Science and Engineering, Jinan, China.

***Corresponding authors:**

Luis Alejandro Jose Mur, Ph.D
Aberystwyth University
Aberystwyth, Wales, UK.
SY23 3DA
Tele: + 44 (0)1970 622981
lum@aber.ac.uk

Patricia P. Reis, Ph.D.
São Paulo State University
Dept. Surgery and Ortopedics
Faculty of Medicine
Botucatu, SP, Brazil
CEP: 18618-970
patricia.reis@unesp.br

ABSTRACT

Lung Squamous Cell Carcinoma (SCC) is one of the predominant subtypes of Non-Small Cell Lung Cancer (NSCLC), which usually shows a poor prognosis. We have previously defined key metabolomic changes in lung SCC samples and matched histologically normal lung tissues from eight patients. Here we define microRNA (miRNA) changes in the same samples, and in an additional plasma sample set from these same patients. Three miRNAs (hsa-miR-6084, hsa-miR-4521 and hsa-miR-29b-1-5p) exhibited statistically significant reductions in their expression levels ($FC \geq 2$ and $p < 0.05$) in lung SCC and plasma compared to normal lung tissue from the same patients. A network analysis-based approach using OmicsNet was employed to relate miRNA target genes to previously identified metabolomic changes in the same lung SCC samples. The hsa-miR-6084 has not been previously associated with lung SCC and had the largest predicted impact on global metabolite expression. Particularly prominent regulatory nodes included ATP, amino acid metabolism, methyl-transfer, glutathione/glutathione *S* transferase, aldehyde dehydrogenase and lipid/steroid processing. The miRNAs hsa-miR-4521 and hsa-miR-29b-1-5p had overlapping but fewer nodes but particularly appeared to target glutamate metabolism, as well as to impact nucleotide biochemistry. Our networking strategy contributes to the identification of miRNA-regulated metabolomic changes associated with lung SCC.

Introduction

It is known that approximately 98.5% of the human genome consists of non-coding RNAs: molecules that play functional regulatory roles in many cellular processes including transcriptional and post-transcriptional modifications and can participate in disease context such as pathogenesis of human cancers (1, 2). In this scenario, microRNAs (miRNAs) are a category of non-coding RNAs (~18 – 25 nucleotides in length) (3) that regulate gene expression in important biological processes (4) and are associated with tumorigenesis (5).

miRNAs have been demonstrated to regulate tumor metabolism by modulating genes that encode proteins that either directly regulate or interact with the metabolome (6). Indeed, miRNAs play important roles in cell metabolism, including energy metabolism (7-9). Tumour cell metabolism is characterized by the Warburg effect, which allows their ability to survive, as tumour cells use a variety of metabolic pathways to sustain their high proliferation rates, mainly by anaerobic glycolysis (even in the presence of normal levels of oxygen) (10).

According to the International Agency for Research on Cancer, lung cancer is the most diagnosed cancer, for both sexes, and the leading the cause of cancer death, worldwide (11). Non-small cell lung cancer (NSCLC) represents the majority (~85%) of lung cancer cases. Among the most common NSCLC subtypes are adenocarcinoma (AD) and squamous cell carcinoma (SCC); the latter usually shows a poor prognosis (12).

Given the prevalence and poor prognosis associated with lung cancer, genomics, coding and non-coding transcriptomics (13), proteomics (14) and metabolomic approaches have been used to improve the disease diagnosis and patient prognosis (15).

We have recently provided a snap-shot of changes in the sputum metabolome linked to cancer (16) and in biopsies of lung SCC samples compared to adjacent histologically normal tissue (17). Using flow infusion electrospray ionization high resolution mass spectrometry (FIE-HRMS) we observed the well-characterised Warburg effect, perturbed lipid processing via β -oxidation and

glycolysis via glucose-6-phosphate to feed into nucleotide and therefore nucleic acid biosynthesis (17). Additionally, we showed changes in metabolism towards creatine, serotonin and a range of enzyme co-factors, most particularly, of the vitamin B complex (17).

Considering how such carcinogenic events are generated at the genomic level, deregulation of microRNAs (miRNAs) are now well-established as key features and are potential biomarkers for diagnosis, prognosis and treatment. miRNAs are small, non-coding RNAs of ~18-22 nucleotides in length, which bind to 3' untranslated region (3'UTR) of target mRNA to post-transcriptionally regulate gene expression (18). The regulatory power of miRNA is considerable with the expression of possibly as many as ~60% of mRNAs controlled by miRNAs (19). miRNAs play a role in tumorigenesis acting as tumour suppressors or oncogenes. In the context of lung cancer, down-regulated miRNAs include let7, miR-34 and miR-200 families and those upregulated miR-21, miR-17-92 cluster and miR-221/222 to influence mechanisms such as cell proliferation, cell death and metastasis (20). The diagnostic potential of miRNAs is highlighted by the stability of miRNAs in body fluids such as plasma, serum, urine and saliva. The employment of miRNA biomarkers is also attractive since their genetic targets, and therefore their mode of actions, are definable. However, understanding their biological roles is complicated by most miRNAs having multiple target RNAs and multiple miRNA targeting the same mRNA (21).

Such complexity lends itself to network analyses where molecular components within a cell as shown as nodes and their direct or indirect interactions as links. These networks can depict very large numbers and different type of variables such as gene transcripts and proteins (22), based on their known relationships for visual and functional integration. This approach has been useful in providing new understanding of cancer biology (23). Software for visualising miRNAs (24) and their transcriptional targets are widely used and are being continually improved (25), for instance the derivation of DimiG where miRNA, their mRNA targets, tissue-specific expression pathways and disease may be

visualised. DimiG models were validated by comparison with the literature for in a range of cancers, including lung (26). Integration of the metabolome into miRNA regulatory networks has been rarely attempted and usually with subsets of metabolites (27). We here use the recently launched “OmicsNet” platform (28) to model the interaction of the targets of miRNA differentially expressed (DE) in lung SCC samples compared to adjacent histologically normal tissues and their corresponding metabolomes. This predicted our DE miRNA were influencing major aspects of cancer metabolism such as ATP generation, the generation of methyl groups and amino acid biosynthesis. To our knowledge, the possible impact of differential miRNA expression on a lung cancer metabolome has not been previously assessed.

Materials and Methods

Ethics statement

This study was approved by the Research Ethics Board of the Faculty of Medicine, São Paulo State University (UNESP), Botucatu, São Paulo, Brazil.

Patient samples

We have analyzed lung squamous cell carcinoma samples, matched histologically normal lung tissues and plasma from 8 patients. Here we integrate our previously published metabolomic data (17) with original, global microRNA expression profiling data generated in the same patient samples, as well as in an additional set of plasma samples obtained from these patients.

RNA extraction

Solid tissues (tumour and matched histologically normal lung)

RNA from solid, fresh frozen lung tumour and normal tissues were extracted using the TRIzol® Reagent protocol (Invitrogen/Thermo Fisher Scientific) followed by purification in the RNeasy® Mini Kit (Qiagen). All steps were performed according to standard protocols, as recommended by the

manufacturers.

Plasma samples

200µL of plasma from lung SCC patients was used for RNA extraction with the miRNeasy® Serum/Plasma Kit (Qiagen), following the manufacturer's protocol. All RNA samples were assessed for quantity and quality using NanoDrop 8000 (Thermo Fisher Scientific).

microRNA expression analysis

Quantification of miRNA expression was performed using the GeneChip® miRNA 4.0 Array (Thermo Fisher Scientific), which contains probes for all the 2,588 miRNAs mapped in the human genome and annotated in miRbase. Experiments followed the manufacturer instructions, as previously described (29). Data were exported for quality control, normalization and miRNA quantification in Transcriptome Analysis Console software (Applied Biosystems).

We have profiled patient tumour, plasma and control samples (paired histologically normal tissue) for comparison. A subset of miRNAs was commonly, significantly deregulated in plasma and tumour samples and was identified using ANOVA test, compared to miRNA expression in control samples. The eBayes (empirical Bayes method), was used to correct the variance of ANOVA. This method uses the information of all probability sets to improved estimator the variance. The relative expression of the targeted miRNA in plasma was also determined.

Metabolomic analyses

Metabolomic data for these samples were described by Paes de Araújo *et al.* (17), including the methods of metabolite isolation, data analysis and interpretation.

Bioinformatic prediction analyses

miRNA target prediction analysis

Commonly deregulated miRNAs were used for target gene prediction analysis in microRNA Data Integration Portal (<http://ophid.utoronto.ca/mirDIP/>) (30). We used stringent criteria for target selection, by considering results with “very high” (top 1%), “high” (top 5%) and “medium” (top 1/3) score classes for interaction probability of miRNAs with target genes.

Network analyses

Predicted miRNA target genes were integrated to previously identified metabolites in the same lung SCC samples using OmicsNet platform (<https://www.omicsnet.ca/>) and networks were created (28). The networks were based on the protein-metabolite interactions as defined by the KEGG metabolic database (31). The derived networks were project as force-directed layout where node sizes were proportional to being hubs or terminal nodes. For publication, the derived 3D models are presented in 2D format. In the models, miRNA target genes were indicated in blue and differentially accumulating metabolites in pink. Within the networks o that are more tightly connected to each other than the rest of nodes as highlighted using the WalkTrap (32) algorithm in OmicNet.

Results

Deregulated miRNA expression in lung squamous cell carcinoma.

A subset of 12 miRNAs was identified as commonly, significantly deregulated ($FC \geq 2$ and $p \leq 0.05$ and $FDR \leq 0.05$) in tumour and plasma samples, compared to histologically normal lung tissues (all samples from the same patients) (**Table 1**). Three miRNAs exhibited significant reductions among lung SCC, plasma and normal tissue; hsa-miR-6084, hsa-miR-4521 and hsa-miR-29b-1-5p which are highlighted in grey in **Table 1**.

The mirDIP analysis with these 12 miRNAs identified a total of 18,344 interactions. These interactions were filtered for “medium”, “high” or “very high” interaction probability: we selected only the “very high” interactions and, if not

possible, we selected only the “high” or only the “medium” interactions. This filtering lead to a total 9,413 genes were predicted to be regulated by the miRNAs .

Network analyses of deregulated miRNA expression and metabolomic pathways in lung squamous cell carcinoma.

Interaction networks derived for each of the deregulated miRNA target genes and the previously defined metabolite changes (17).

Integrated analysis for deregulated hsa-miR-6084 target genes and metabolites is shown in **Figure 1**. This suggested that miR-6084 was influencing large numbers of metabolite-gene nodes the characteristics of which are described in **Supplementary Table 2**. Therefore, the OmicsNet WalkTrap algorithm was used to indicate some of the key predicted nodes which are named in Figure 1 and some are highlighted in **Figure 2**.

The various components of the ATP node were extracted and are listed separately in **Supplementary Table 3** and **Figure 2A**. These were all receptor kinases and therefore dependent for the activity on the availability of ATP. Biochemically, NAD⁺/NADP⁺/NADPH node highlighted by WalkTrip can be linked to ATP generation and this node is also listed in **Supplementary Table 3**. Moving clockwise around the overall model in **Figure 1** is the aldehyde dehydrogenase (ALDH) node, the components of which are listed in **Supplementary Table 4**. **Supplementary Table 4** shows that the ALDH node is linked with retinoic acid (RA) metabolism. Components of the lipid and steroid processing node (**Supplementary Table 5** and **Figure 2B**) included HSD3B2 (Hydroxy-Delta-5-Steroid Dehydrogenase), CYP1B1 whose activities include arachidonic acid metabolism and CYP4F14. Examining the glutathione node (**Supplementary Table 6** and **Figure 2C**), this included a range of glutathionylated metabolites were all linked to a relatively small number of glutathione -S transferases [μ -types; GTSM2, GSTM3 GSTM4, GTSM5, microsomal Glutathione S-Transferase 3 and GSTP1 (Pi 1)]. The bioenergetic changes in lung SCC were further highlight by the prominence of the Acetyl Co A node (**Supplementary**

Table 7). The importance of de novo fatty acid processing is a well-established feature of cancer and CoA-acyl chains of varying lengths where linked to this node. These CoA-acyl chains were linked to acyl-CoA oxidases (ACOX1, ACOX3) which catalyze the desaturation of acyl-CoAs to 2-trans-enoyl-CoAs as part of the peroxisomal fatty acid beta-oxidation pathway. This node also contained Acyl-CoA dehydrogenases (ACADs) which catalyze the initial step in each cycle of fatty acid β -oxidation in the mitochondria. Thus, this node describes lipid processing to provide acetyl CoA to feed into the Krebs cycle. Another node highlights the methylation cycle with the prominence of S-adenosyl-L-methionine (SAM) and S-adenosyl-L-homocysteine (SAH) (**Supplementary Table 8**). SAM donates methyl groups to a range of compounds including nucleic acids, proteins and lipids to form SAH. However, we noted that the node included important DNA methyltransferases (DNMT).

The much simpler predicted hsa-miR-4521 network is represented in **Figure 3A** and showed the prominence of methyl, nucleotide and amino acid metabolisms. The S-adenosyl-L-homocysteine node was again indicated and clearly associated with a range of DNMTs as probable has-miR-4521 targets (**Figure 3B**). In the case of amino acid metabolism, the processing of glutamate appeared to be an important node (**Figure 3C**) which aligns with its role in the construction of a variety of macromolecular precursors required for proliferating cells.

The hsa-mir-29b-1-5p network exhibit considerable over-lap with that of has-miR-4521 in that the same key metabolites pathways; methyl, nucleotide and amino acid metabolisms, were targeted (**Figure 4**). However, in this case the network appeared to be linked to fewer miRNA regulated genes.

To further illustrate the link between the de-regulated miRNA and the key metabolites, their relative accumulation in SCC versus histologically normal tissue was correlated (**Figure 5**). Displaying the relative correlations (r^2) as a heat map indicated that the expression of each of the miRNA significantly correlated with the others. Relating the miRNA expression with metabolite levels, these were only significantly positively correlated adenosine and GMP. With each other key

metabolite there was a negative correlation with miRNA expression. This indicated that the down regulation of the three miRNA is associated with the increases in metabolites in the highlighted network nodes.

Discussion

As a highly heterogeneous disease, the characterization of cancer demands the consideration of multiple biological variables such as gene transcripts, protein and metabolites. It is known well-recognised that key variables act together to influence key carcinogenic mechanisms. Thus, network models for cancer are now well-established and may be used to visualize key regulatory circuits; for example p53 (e.g. re-activation of ATM in response to DNA damage) (33) or patterns in transcriptome (e.g. risk stratification model for lung adenocarcinoma) (34) or proteome (e.g. patient-specific pathway activities) (35) data. As such, models are based on pre-existing knowledge of biological relationships and interactions, they are inherently incomplete. However, they serve as a key role in illustrating complex relationships that may not be immediately obvious and thereby generate testable hypotheses. In building such networks, the roles of miRNA are particularly attractive as they represent specific regulatory elements that furthermore have been linked to many cancers (4). Indeed, network models with miRNA have been derived (e.g. disease-miRNA associations) (26) but in our case, we attempted to link miRNA deregulated in lung SCC plasma and tissue compared to histologically normal tissue to key metabolite changes which we had previously defined in the same tissue type.

Considering that miRNAs and metabolites have been demonstrated as valuable tools for classification, diagnosis and/or prognosis in cancer (36, 37), they may have potential use as combination biomarkers providing a better understanding of lung SCC. Twelve miRNA were initially targeted as being differentially regulated (Table 2). Subsequent more rigorous statistical assessments targeted three individuals as being significantly deregulated and, in each case, downregulated. Deregulation of hsa-miR-6084 has not previously been reported in lung cancer. Voellenkle and colleagues performed deep-sequencing techniques to profile

miRNAs population expressed by endothelial cells exposed to hypoxia (38). Novel miRNAs were detected as overexpressed, validated and associated with RISC immunoprecipitates (confirming the novelty) and these included hsa-miR-6084 (38). hsa-miR-6084 was also reported as overexpressed in patients with colorectal cancer (current smokers compared to never smokers, associated only in rectal tissue) (39).

hsa-miR-4521 was detected amongst a collection of potential cancer-related miRNAs isolated from urine samples of various cancer donors, (up-regulated in lung and liver cancer samples) (40). hsa-miR-4521 was also amongst the miRNA that were down-regulated in breast cancer cell lines following the imposition of hypoxia, suggesting its role in cell proliferation) (41). The cluster miR-4521/3676 is associated with tRNA sequences and this genomic region can synthesize tRNA-derived small RNAs (tsRNAs). Of particular interest are ts-4521 and ts-3676 which are downregulated and mutated in patients with chronic lymphocytic leukaemia and lung cancer. These two tsRNA can also act as piRNAs by interacting with Piwi-like protein 2 (42).

hsa-miR-29b-1-5p is an important tumour suppressor differentially expressed in human BRCA1 breast tumours or familial non-BRCA1 patients (43). It has been reported in metastatic breast cancer cells where it was linked with the promotion of cell migration, invasion and apoptotic resistance (44). Molecular effects of hsa-miR-29b-1-5p deregulation have been extensively characterised and include the aberrant expression of DNA methyltransferases (DNMT) 3a and 3b in a range of cancers (43). DNMT1 is vital in maintaining CpG methylation patterns following DNA replication with a preference for hemi-methylated DNA. DNMT3A/B are required for genome-wide de novo methylation and is essential for the establishment of DNA methylation patterns including non-CpG sites (45). Here, we identified miRNAs that may be important to lung carcinogenesis, in addition to hsa-miR-6084, which has not been previously associated with lung cancer.

To further predict the roles of the identified miRNAs in lung SCC, their assigned gene targets were integrated into a network which included previously

defined metabolite changes (17). The value of this approach was indicated by the important regulatory effects predicted for each miRNA. An important aspect was that each suppressed miRNA appeared to be targeting SAM/SAH as methyl donors and DNMTs involved in maintaining and establishing de novo DNA methylation patterns. All three differentially expressed miRNAs in lung SCC samples target this mechanism, suggesting that this increased DNA methylation is a crucial aspect of carcinogenesis. Such aligns with the hypermethylation of tumour suppressor gene promoters to suppress expression that is a feature of lung cancer (46). It is not appropriate to consider the known epigenetic changes linked to lung cancer but they have been reviewed here (47, 48).

A central feature of cancer metabolism is the “Warburg effect”, whereby ATP is inefficiently generated by aerobic glycolysis. With this phenomenon most of the cell’s glucose is converted to lactate but the diversion of glycolysis towards the pentose-phosphate pathways towards NADPH generation and nucleotide biosynthesis that is clearly beneficial to actively dividing cells. More NADPH is generated from the catabolism of glutamine into lactate via the activity of NADP⁺-specific malate dehydrogenase (49). The Warburg effect and associated metabolic changes were observed in the lung SCC samples investigated here (17). It was therefore of considerable relevance that hsa-miR-6084 to be targeting a metabolic node centering on ATP but this nodes contained ATP-dependent receptor kinases rather than enzymes linked to any Warberg effect. Wider Warburg effects are suggested by the importance of NAD⁺/NADH/NADP⁺/NADPH generation with hsa-miR-6084. In this context, the targeting by hsa-miR-29b-1-5p and hsa-miR-4521 of genes involved in regulating genes regulating glutamate/glutamine and nucleotide pathways was relevant and these can generate NAD(P)H. The demands of cancerous cells for nutrients is such that they are dependent on externally supplied nutrients i.e. are auxotrophic (50). In our case, the metabolic nodes targeted by hsa-miR-4521 and hsa-miR-29b-1 in lung SCC appeared to change in flux towards three phosphoenolpyruvate (PEP) derived amino acids; phenylalanine, tyrosine and tryptophan. Taking into account the glutamate nodes targeted by hsa-

miR-4521 and hsa-miR-29b-1-5p, glutamic-pyruvic transaminase 1 and 2 genes (GPT and GPT2) encode enzymes that play important roles in the intermediary metabolism of glucose and amino acids. GPT2 supplies cell survival and growth via TCA cycle and its degradation reduces proliferation and induce cell death of cancer cells (51). The hsa-miR-4521 glutamate node also interact with genes from gamma-glutamyltransferase family (GGT1, GGT5, GGT6 and GGT7). GGT is usually used as clinical indicator of liver tissue damage (e.g. hepatocellular carcinoma) (52). GGT also contributes to the cell with amino acids promoting cell proliferation (53) and was described as a promising candidate for rapid detection of metastatic lymph nodes in colorectal cancer (54) and a possible prognostic biomarker in advanced urothelial carcinoma (53).

NADH generation is also a feature of the Acetyl CoA node in the hsa-miR-6084 network is an important outcome of its suppression. Fatty acid biosynthesis is vital to cancer growth and controlled by the rate-limiting enzyme acetyl-CoA carboxylase (ACC) (55). However, this does not feature in the Acetyl CoA node, which instead features important enzymes and metabolite in fatty acid catalysis via β -oxidation. This represents an important bioenergetic pathway with each round of acyl-chain shortening of fatty acid generating a molecule of NADH, FADH₂ and acetyl CoA. NADH and FADH₂ enter the electron transport chain to produce ATP. In cancer cells this is likely to be as important as the classical Warburg effect in serving their bioenergetic demands (56). Mitochondrial-based β -oxidation is classically linked with processes on shorter chained fatty acid and peroxisome with long-chain and very-long-chain fatty acids (57). Therefore, the association of peroxisomal-located acyl-CoA oxidase 1 (ACOX1) and ACOX2 genes would argue that longer chained storage lipids would be utilized in lung SCC samples, activated through the suppression of hsa-miR-6084 expression.

Lipid and steroidal processing also features another highlighted node based on HSD3B2, CYP1B1 and CYP4F14 enzymes and a large number of associated arachidonate-linked, fatty acid and steroid metabolites. Upregulation of each enzyme has been associated with different forms of cancer. HSD3B2 is

involved with the synthesis and degradation of di-hydro-testosterone (58). Increased expression of HSD3B2 was seen in prostate cancers particularly where androgen receptor expression was also and is linked to aggressiveness (58, 59). CYP1B1 is a key enzyme involved in oestrogen metabolism and its overexpression of CYP1B1 may lead to the accumulation of oestrogen metabolites that can form adducts in DNA (60). CYP1B1 overexpression has been associated with an increased risk of breast cancer (61). CYP4F14 encodes a fatty acid ω hydroxylase P450 that metabolizes proinflammatory eicosanoids and long-chain fatty acids. It also plays a key role in vitamin E biosynthesis, a key anti-oxidant that suppresses lipid peroxidation (62). CYP4F14 is also involved in metabolism of endogenous polyunsaturated fatty acids (PUFAs) and CYP4B1 which is involved in synthesis of cholesterol, steroids and other lipids and is a risk factor in bladder cancer (63).

Considering other hsa-miR-6084 nodes, one was a glutathione- GST hub that was being targeted by hsa-miR-6084 in particular μ -class GSTs. Elevated glutathione levels are associated with increased proliferation via the maintenance of reduced glutaredoxin or thioredoxin, which are required for ribonucleotide reductase activity, the rate-limiting enzyme in DNA synthesis (64). GSTs catalyze the nucleophilic addition of glutathione to a wide variety of exogenous and endogenous chemicals which can be the products of oxidative stress or “environmental” chemicals such as carcinogens or drugs. As such GSTs have been linked to tumour-resistance against chemotherapeutic drugs (65). The human μ class GST is encoded by a 100-kb gene cluster ordered on chromosome 1p13.3 in the following order GSTM4-GSTM2-GSTM1-GSTM5-GSTM3 and it may be significant that most are likely to be targeted by hsa-miR-6084. Generally, the lack of GST-M expression may be linked to susceptibility to cancer or a poor prognosis (66, 67). Thus, genetic polymorphisms within GSTM1, as well as GSTP1 (also targeted by hsa-miR-6084) or GSTT1 increase the risk of lung cancer as they play a major role in the detoxification of tobacco carcinogens (68). Considering molecular mechanisms individual GSTs, GSTM2 has been associated with cell migration and actin disassembly in lung cancer cells (69). Genetics/epidemiological

studies have explored how far individual GST mutants or multiple mutations contribute to cancer with some suggestions that this could be the case with ambiguous conclusions (68, 70). Our data suggest that there is an inverse relationship between hsa-miR-6084 and GST expression, with overexpression phenotypes being more relevant. Clearly, GST overexpression is a response to increased oxidative stress, a known feature of cancer cells. However, overexpression of μ and π GSTs, targeted by hsa-miR-6084, appear to be particularly important in cancer. Indeed, GSTP1 has been suggested as a biomarker for early cancer detection (71). Mechanistically, μ and π GSTs regulate mitogen-activated protein (MAP) kinase pathways via protein-protein interactions with c-Jun N-terminal kinase 1 (JNK1) and ASK1 (apoptosis signal-regulating kinase) leading to cytokine- and stress-induced apoptosis. Additional interactions have been described for Protein Kinase C (PKC), Protein Kinase A (PKA) and Epidermal Growth Factor Receptor (EGFR). Thus, under normal conditions oxidative stress destabilizes a GST-JNK complex to initiate apoptosis. This destabilization trigger is perturbed if GSTs are overexpressed (65). A corollary of GST overexpression is chemotherapeutic drug resistance, and it is likely that suppression of hsa-miR-6084 to increase GSTP1 expression would lead to this phenomenon. This is suggested from a converse situation where the targeting and suppression of GSTP1 by miR-513a-3p increases the sensitivity to cisplatin in lung cancer adenocarcinoma cells (72).

As with GSTs, ALDH catalyze oxidation of aldehydes and are involved detoxification processes and can activate p53 and reduce intracellular reactive oxygen species (ROS) levels to protect cells from oxidative damage (73). In addition, as in the hsa-miR-6084 ALDH node, they are also associated with retinoic acid (RA) metabolism. Over-expression of a series of ALDHs (ALDH1A1, ALDH3A1, ALDH3B1, and ALDH6A1; with ALDH1A1 and ALDH6A1) has been shown in prostate cancer tissue (74) and has been suggested to be a prognostic marker (75). Further, ALDH activity has been linked to tumour reoccurrence in breast and esophageal cancer (76, 77). It may be that the ALDH node could be a

therapeutic target, given the association of this class of enzymes with cancer stem cells (CSCs) which tend to be quiescent and highly chemoresistant (78). Over-accumulation of RA could be one mechanism through which ALDH activity could promote carcinogenesis. This may appear to be counter-intuitive as drugs based on RA have been developed as a mean of treating primarily in leukemia but they also have relevance to lung cancer. Thus, RA inhibits cell proliferation through such as cyclin D1, human telomerase reverse transcriptase (hTERT), epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF) (79), p27 and the cell cycle regulator, Cdk5 (80). Equally, however, RA has been shown to activate cell survival and migration in the lung adenocarcinoma A549 line acting through ERK and blocking anti-cancer cells. Thus, RA effects could be dependent on the cellular context and tissue type (81). Our ALDH regulated node would be consistent with such a role in lung SCC at least.

In this study, we have sought to integrate differing types of data derived from the same lung SCC sample set to provide wider insights into major regulatory shifts as they impact in changes metabolism. Whilst the detailed changes have been either previously published (17) or presented here (Supplementary Table 1), our systems level view has suggested how a small number of deregulated miRNAs – indeed possibly as few as one in the case of hsa-miR-6084 - can contribute to key hallmarks of cancer metabolism.

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Table 1: Differentially expressed miRNAs in lung SCC

miRNA	Average of the normal control group (log2)	Average of the diseased group (log2)	FC	P-val	FDR P-val	Accession #
hsa-miR-6084	2.03	0.91	-2.16	1.71E-18	6.47E-16	MIMAT0023709
hsa-miR-4521	4.13	0.81	-9.99	3.52E-18	6.47E-16	MIMAT0019058
hsa-miR-29b-1-5p	1.92	0.83	-2.13	2.33E-17	8.06E-16	MIMAT0004514
hsa-miR-4440	5.08	6.84	3.38	0.0040	0.0538	MIMAT0018958
hsa-miR-29c-5p	2.36	0.47	-3.7	0.0180	0.2395	MIMAT0004673
hsa-miR-4646-5p	3.61	1.72	-3.71	0.0194	0.2577	MIMAT0019707
hsa-miR-4793-3p	4.94	6.68	3.35	0.0260	0.3437	MIMAT0019966
hsa-miR-21-5p	6.23	5.2	-2.04	0.0283	0.3700	MIMAT0000076
hsa-miR-6873-5p	1.87	0.65	-2.33	0.0285	0.3721	MIMAT0027646
hsa-miR-193a-3p	3.2	0.71	-5.62	0.0351	0.4571	MIMAT0000459
hsa-miR-363-3p	5.42	1.33	-17.09	0.0413	0.5361	MIMAT0000707
hsa-miR-4518	2.38	0.76	-3.08	0.0418	0.5393	MIMAT0019055

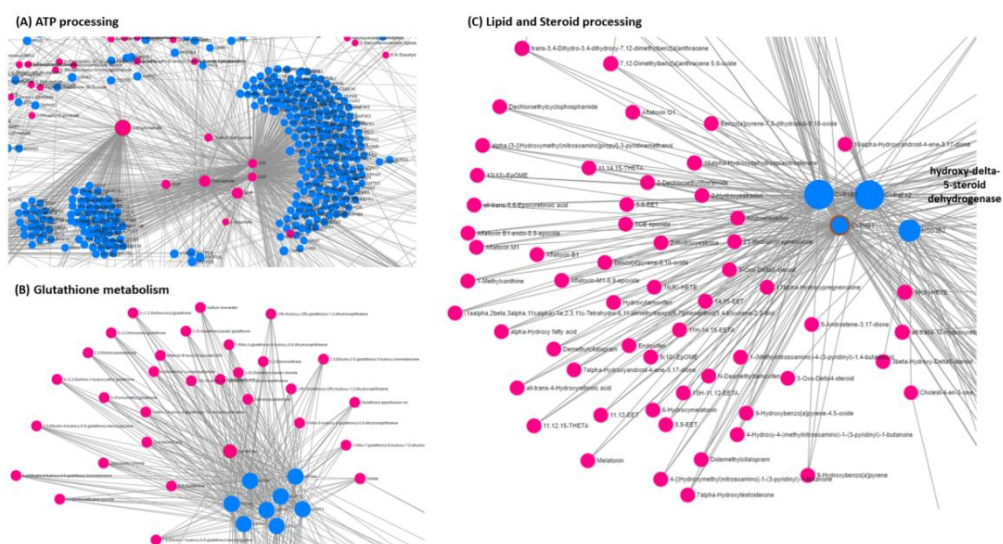


Figure 2. Highlights of hsa-miR-6084 metabolomic network. The keys nodes of hsa-miR-6084 metabolomic network are (A) ATP processing; (B) glutathione metabolism and (C) lipid and steroid processing. The pink nodes are the differentially accumulating metabolites and the blue nodes are the miRNA target genes. OmicsNet was used to generate target genes-metabolites interactions.

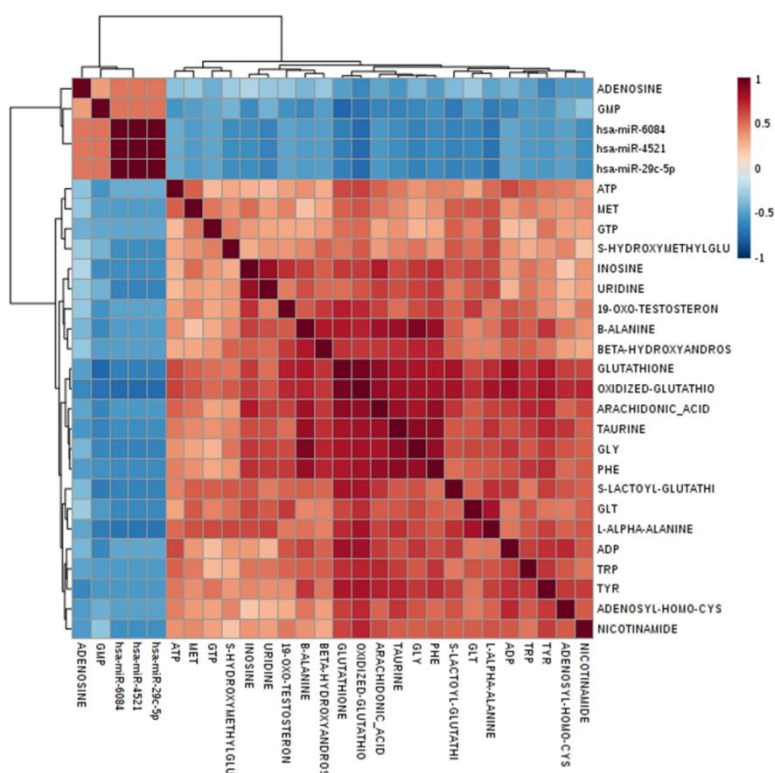


Figure 5. Correlation analysis of predicted key metabolite changes with miRNA expression. Fold changes between SCC and paired histologically normal tissue for miRNA and key significant metabolites as defined by Paes de Araujo *et al.*, (2019). Each variable was regressed (Pearsons r^2) with the result compared using a heatmap and dendrogram.

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