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Specific miRNAs are associated with human cancer cachexia in an organ-specific manner

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Abstract

Background Cancer cachexia (CCx) is a complex and multi-organ wasting syndrome characterized by substantial weight loss and poor prognosis. An improved understanding of the mechanisms involved in the onset and progression of cancer cachexia is essential. How microRNAs contribute to the clinical manifestation and progression of CCx remains elusive. The aim of this study was to identify specific miRNAs related to organ-specific CCx and explore their functional role in humans.

Methods miRNA patterns in serum and in cachexia target organs (liver, muscle and adipose tissue) from weight stable ($N \leq 12$) and cachectic patients ($N \leq 23$) with gastrointestinal cancer were analysed. As a first step, a miRNA array (158 miRNAs) was performed in pooled serum samples. Identified miRNAs were validated in serum and corresponding tissue samples. Using *in silico* prediction, related genes were identified and evaluated. The findings were confirmed *in vitro* by siRNA knock-down experiments in human visceral preadipocytes and C2C12 myoblast cells and consecutive gene expression analyses.

Results Validating the results of the array, a 2-fold down-regulation of miR-122-5p ($P = 0.0396$) and a 4.5-fold down-regulation of miR-194-5p ($P < 0.0001$) in serum of CCx patients in comparison with healthy controls were detected. Only miR-122-5p correlated with weight loss and CCx status ($P = 0.0367$). Analysing corresponding tissues six muscle and eight visceral adipose tissue (VAT) cachexia-associated miRNAs were identified. miR-27b-3p, miR-375 and miR-424-5p were the most consistently affected miRNAs in tissues of CCx patients correlating negatively with the severity of body weight loss ($P = 0.0386$, $P = 0.0112$ and $P = 0.0075$, respectively). We identified numerous putative target genes of the miRNAs in association with muscle atrophy and lipolysis pathways. Knock-down experiments in C2C12 myoblast cells revealed an association of miR-27b-3p and the *in silico* predicted atrophy-related target genes *IL-15* and *TRIM63*. Both were up-regulated in miR-27b-3p knock-down cells ($P < 0.05$). Concordantly, in muscle tissue of CCx individuals, significant higher expression levels of *IL-15* ($P = 0.0237$) and *TRIM63* ($P = 0.0442$) were detected. miR-424-5p was identified to regulate the expression of lipase genes. Knock-down experiments in human visceral preadipocytes revealed an inverse association of miR-424-5p with its predicted target genes *LIPE, PNPLA2, MGLL* and *LPL* ($P < 0.01$).

Conclusions The identified miRNAs, in particular miR-122-5p, miR-27b-3p, miR-375 and miR-424-5p, represent features of human CCx and may contribute to tissue wasting and skeletal muscle atrophy through the regulation of catabolic signals. Further studies are needed to explore the potential of the identified miRNAs as a screening tool for early detection of cancer cachexia.

Keywords Cancer cachexia; Weight loss; miRNAs; Pathway genes

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Introduction

Cancer cachexia (CCx) is a multifactorial and multi-organ wasting syndrome that leads to substantial weight loss, primarily due to the loss of skeletal muscle and body fat.[1] Typical features of CCx include anorexia, systemic inflammation, hypermetabolism, resistance to anabolic signals, and a general catabolic state. The prevalence of CCx is estimated to be around 35% in all cancer patients, but can reach up to 80–90% in gastric or pancreatic cancer. CCx implies a poor prognosis and thereby may indirectly be responsible for 20% of cancer-related deaths.[2] The severity of CCx usually depends on tumour stage and progression and varies by tumour type. Despite considerable efforts to find efficient therapeutic options no effective standard treatment strategy has been established yet.[3] With advanced cachexia even parenteral nutrition and other intensive dietary treatments, show only limited efficacy. In addition, there is a lack of effective pharmacological treatments, for example, to reduce inflammation or to increase appetite.[4]

miRNAs have recently been implicated in health and disease and are bioactive molecules involved in metabolic regulation.[5] They constitute a class of single-stranded, endogenous, small non-coding RNAs (up to 22 nucleotides) that regulate 30–80% of gene expression of the human genome.[6] They post-transcriptionally control mRNA expression by forming an RNA-induced silencing complex (RISC) and promote gene silencing by inhibition of translation and/or by affecting mRNA stability and degradation.[7] Dysregulation of miRNAs is associated with many diseases including cancer.[8] However, there is still a lack of studies focusing on miRNA alterations in human CCx patients. So far, only a few studies addressed myogenic miRNA expression in different tissues in the context of human CCx.[9,10] In a study by Narasimhan et al. differentially expressed miRNAs, that is, let-7d-3p, miR-199a-3p, miR-345-5p, miR-423-3/5p, miR-532-5p, miR-1296-5p and miR-3184-3p, were identified to be relevant for CCx. These miRNAs were significantly up-regulated in muscle from CCx patients and showed both predictive and prognostic potential.[9]

Analysing subcutaneous adipose tissue of human CCx patients, Kulyte et al. identified miR-23a, miR-99b, miR-483-5p and miR-744 to be down-regulated, whereas miR-378 was significantly up-regulated. miR-378 was found to play an important role as a regulator of key lipolytic genes, thereby enhancing adipocyte lipolysis.[11] Okugawa et al. showed that colorectal cancer patients with a low psoas muscle index had high miR-21 and miR-203 serum levels. Thereby, miR-203 levels could have prognostic value in myopenia.[12]

To our knowledge, no detailed miRNA analysis comparing serum, muscle, liver and adipose tissue of human CCx patients has been conducted so far. The aim of this study was to identify miRNA signatures associated with CCx in several tissues. A microarray and broad evaluation of miRNAs in serum, liver, muscle and adipose tissue from cachectic and weight stable cancer patients was performed. In addition, we were interested to identify putative target genes and molecular pathways involved in organ-specific CCx.

Materials and methods

Study approval

As part of a cachexia study (project number 409/16 S, '*Deutsches Register Klinischer Studien*' DRKS00017285), patients with either benign or malignant diseases of the gastrointestinal tract who underwent surgery at the Department of Surgery, Klinikum rechts der Isar (University Hospital of the TUM) were recruited. In total 35 patients with cancer were included in the present analysis. The group size varies according to the sample material examined. On average, patients were 66 years old and suffered from pancreatic ductal adenocarcinoma or colorectal cancer. CCx was defined according to a modified classification of Fearon et al. with a weight loss of at least 5% during the last 6 months before surgery.[13] Skeletal muscle area index (SMAI) was evaluated as described before to detect sarcopenia.[14] Small tissues biopsies of liver, musculus rectus abdominis, visceral and subcutaneous adipose tissue were collected during surgery and snap-frozen on dry ice. Blood samples were collected in the fasting state before surgery. Serum obtained by centrifugation (4500 rcf, 10 min, RT) was frozen on dry ice. Detailed weight monitoring was conducted in the cachexia study which included the report of body weight 6 months before surgery, on the day of surgery, and at defined time points during a 12 month follow-up. Patients from the following studies served as control patients without cancer: The FREECE study (Effect of the *FTO*-Genotype on resting energy expenditure after defined cold exposure,[15] project number 236/16) as cancer-free control group for serum analysis of miRNAs, the MOBB cohort (Munich obesity biobank, project number 5716/13) as control group for the analysis of miRNAs in visceral adipose tissue as well as the IPBS study (intestinal permeability before and after bariatric surgery, '*Deutsches Register Klinischer Studien*' DRKS00009008 and DRKS00006210,[16]

project number 387114 s) as control group for serum miR-122-5p analysis before and after bariatric surgery. The study protocols were approved by the ethical review committee of the Faculty of Medicine of the Technical University of Munich. All participants provided written informed consent. All procedures were conducted according to the principles of the 2013 Declaration of Helsinki.

mRNA/miRNA extraction

serum samples stored at −80°C were thawed on ice and centrifuged at 16 000× *g* for 5 min at 4°C. miRNA was extracted from 200 μL serum using TRIzol™ Reagent (Thermo Fisher Scientific, USA) and the miRNeasy Serum/Plasma Advanced Kit, according to the manufacturer's recommendations (Qiagen, Germany). Cell and tissue RNA including miRNAs was isolated using TRIzol™ Reagent (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The amount and integrity of isolated miRNA was assessed using a Bioanalyzer device (Small RNA Chip, Agilent Technologies, USA).

miRNAs array analysis

We profiled miRNA spectra from four pooled serum samples, including pools of 2 men ± CCx and pools of 2 women ± CCx, to identify regulated miRNAs. In total, 169 different miRNAs were profiled by the human miRCURY LNA miRNA Focus PCR Panels YAHS-106YG-2 (Qiagen, Germany). Expression levels were determined by the cycle number via Real-Time quantitative PCR (RT-qPCR). Levels were normalized to internal reference genes using the $2^{-\Delta\Delta Ct}$ method. Finally, the fold-change in cachectic versus non-cachectic cancer samples was calculated.

miRNA expression analysis

The set of circulating miRNAs that was selected based on the previous array results and taken from the literature were analysed in three cohorts: cancer patients with and without CCx as well as in age- and BMI-matched healthy and weight-stable controls (FREECE/MOBB). To exclude hemolytic serum samples, the levels of miR-451a were assessed.

Reverse transcription and qPCR analysis

The isolated miRNA as well as the spike-in control UniSp6 to consider enzyme efficiency was reversely transcribed into cDNA using the miRCURY LNA RT Kit (Qiagen, Germany). The reaction was incubated at 42°C for 60 min and then heat-inactivated at 95°C for 5 min; 20-fold diluted cDNA samples were stored at −20°C. RT-qPCR was conducted using custom 384 well panels (4titude, UK). For RT-qPCR analysis, each reaction contained 4 μL of cDNA and 6 μL of mastermix using miRCURY Sybr Green Kit and LNA-enhanced miRNA primers (Qiagen, Hilden, Germany). PCR conditions were as following: 95°C for 2 min, 50 cycles of denaturation (95°C, 10 s) and annealing (56°C, 60 s), and an additional melting curve analysis on a LC480 Real-Time PCR system (Roche, Basel, Switzerland). For analysing the expression levels of related genes, cDNA was synthesized from up to 1 μg total RNA using the High capacity cDNA RT Kit (Applied Biosystems, USA). RT-qPCR was performed using 20 ng cDNA with the Maxima SYBR Green Master Mix (Thermo Fisher Scientific, USA) and specific primers (Table S2). For normalization, relevant endogenous controls were used as described in Table S2.

Cell culture C2C12 cells

Mouse C2C12 cells are a subclone from a myoblast line established from normal adult C3H mouse leg muscle. Cells were grown in DMEM high glucose medium (Gibco, USA) supplemented with 10% fetal calf serum gold (PAA Laboratories GmbH, Germany) and 1% Penicillin/Streptomycin (Pen/Strep) (Sigma, Germany). Differentiation was initiated at 80% confluency. Differentiation medium consisted of DMEM with 2% horse serum (Sigma, Germany) and 1% Pen/Strep. All culture and differentiation media were changed every day and cells were cultured at 37°C under 10% CO_2.

Isolation, culture and differentiation of human preadipocytes

All methods for the isolation, culture and differentiation of human preadipocytes (PACs) derived from adipose tissue material are described in detail elsewhere.[17] Briefly, PACs were isolated from adipose tissue by collagenase digestion, grown until confluency in T-75 flasks (Falcon, Corning, USA) and cryopreserved. For differentiation experiments, cells were thawed, grown until confluency in T-25 flasks (Falcon) and splitted on to 6-well plates (Falcon). At confluency, differentiation was induced, and cells were cultured for 14 days to allow differentiation and accumulation of lipids.

siRNA transfection

For transfection of anti-miR-27b-3p oligonucleotides in C2C12 cells, cells were seeded and cultured to a density of 60–70%. Medium was changed and cells were transfected with miRCURY LNA Inhibitor or Inhibitor Control (Qiagen, Germany) for 72 h with a final concentration of 30 nM. Cells

were transfected before initiation of differentiation. RNA was harvested on d0, d4 and d7.

Visceral PACs were seeded in 6-well plates. At a density of 80%, medium was changed, and cells were transfected with miRCURY LNA Inhibitor containing appropriate siRNA or Inhibitor Control (Qiagen, Germany) for 72 h with a final concentration of 30 nM. Then, cells were differentiated for 14 days as described. Cells were harvested and media collected on d0, d3, d7, d10 and d14.

Expression levels of miR-27b-3p, miR-375, miR-424-5p and related genes caused by miRNA depletion were analysed by RT-qPCR (all primers are listed in Table S2).

Oil red O staining

Visceral PACs were fixed with 4% paraformaldehyde for 20 min at room temperature. After one washing step with PBS, an Oil Red-O solution (0.3% in isopropanol and H_2O 3:2) was added for 60 min and rinsed off with PBS. For a better contrast, cells were counterstained with HE. Stained adipocytes were assessed under a light microscope (VHX series, Keyence, Osaka, Japan).

HE staining

Visceral PACs and C2C12 cells were fixed with 4% paraformaldehyde for 20 min at room temperature. After one washing step with PBS, haematoxylin solution (Haemalum (Mayer's) Gurr for microscopy, VWR, Germany) was added for 4 min and rinsed off with PBS. Eosin solution (2% in acetic EtOH) was added for 2 min. After rinsing with PBS, stained cells were assessed under a light microscope (VHX series, Keyence, Japan).

Single cell data

Dotplots of white adipose tissue single-cell RNA expression were retrieved from the Single Cell Portal (study no. SCP1376).[18]

Bioinformatic predictions

To identify relevant human target genes, we first employed the miRNA search tool MultiMiR in R, compiling nearly 50 million records from 14 different databases, including miRTarBase, TarBase, miRanda, miRDB and Targetscan.[19,20] We then investigated the functional interaction between the list of predicted targets with a comprehensive list of genes associated with muscle atrophy or triglycerides lipase activity (gene ontology terms 0014889/0004806). To generate maps of target genes, the String DB tool (basic settings: full STRING network, confidence, highest confidence (0.900) and advanced settings: hide disconnected nodes) and the program cytoscape were used.[21]

Statistical analyses

Statistical analysis was performed using GraphPad Prism 9 (Graph Pad Software, USA). Normality was tested based on Shapiro–Wilk tests. All data showed a non-Gaussian distribution. Statistical tests were two sided. Results are given (after age and BMI adjustment as well as logarithmic transformation) as heatmaps, dot plots or violin blots with mean and standard error of the mean (± SEM). Unpaired Mann–Whitney tests were performed to compare two conditions, whereas unpaired one-way ANOVA or Kruskal–Wallis tests were performed to determine differences between several groups. For testing the association between two variables, linear regression was used. To compare data from transfected versus non-transfected cells a two-tailed Wilcoxon signed-rank test was used. $P < 0.05$ was taken as a minimum level of significance.

Results

Patients with CCx were defined by a body weight loss greater than 5% during 6 months before surgery. Upon recruitment, patients' clinical characteristics were collected through standardized questionnaires and routine clinical chemistry (Tables 1 and 2).

Identification of differentially expressed miRNAs in serum from cancer patients with cachexia and controls using a miRNA array

For the miRNA array, we used a pooled miRNA sample from four female patients (two with and two without CCx). An additional run combined serum miRNA samples from males (two with and two without CCx). We captured 152 miRNAs for females and 103 miRNAs for males, while 49 residual miRNAs were not detectable. Among the most abundant miRNAs in serum, five miRNAs, including miR-19b-3p, miR-

Table 1 Demographical characteristics of the donors of the samples used in the serum array

	Cachexia and cancer	No-cachexia cancer
Patients (*N*)	4	4
Age (years), mean	67	56
Gender (f:m)	2:2	2:2
Body mass index mean (kg/m^2), ± SEM	22.50 ± 5.51	26.00 ± 2.06

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Table 2 Demographical characteristics of the donors of the samples used in the assay

	Serum			
	Cachexia and cancer	No-cachexia cancer	Control group I = FREECE	Control group II = IPBS
Patients (*N*)	23	12	18	22
Age (years), mean	70	63	32	42
Gender (f:m)	12:11	6:6	9:9	19:3
BW loss (%), ± SEM	10.93 ± 4.16	1.84 ± 1 59	0	26 ± 6.05
Body mass index mean (kg/m^2), ± SEM	24.48 ± 4.24	25.50 ± 3.48	22.33 ± 1.55	53.10 ± 8.40
	Muscle			
	Cachexia and cancer	No-cachexia cancer	Control group (no cachexia and no cancer)	
Patients (*N*)	20	11	5	
Age (years), mean	70	63	52	
Gender (f:m)	10:10	6:5	4:1	
BW loss (%), ± SEM	10.93 ± 4.16	1.84 ± 1 59	0	
Body mass index mean (kg/m^2), ± SEM	24.48 ± 4.24	25.50 ± 3.48	24.00 ± 4.47	
Sarcopenia (yes:no:missing values)	9:9:2	4:4:3	-	
SMAI (cm^2/m)	45.57	45.87	-	
	Liver			
	Cachexia and cancer	No-cachexia cancer	Control group (no cachexia and no cancer)	
Patients (*N*)	23	12	6	
Age (years), mean	70	63	52	
Gender (f:m)	12:11	6:6	5:1	
BW loss (%), ± SEM	10.93 ± 4.16	1.84 ± 1 59	0	
Body mass index mean (kg/m^2), ± SEM	24.48 ± 4.24	25.50 ± 3.48	24.00 ± 4.47	
	Adipose tissue			
	Cachexia and cancer	No-cachexia cancer	Control group (no cachexia and no cancer) (CCx & MOBB)	
Patients (*N*)	23	12	11	
Age (years), mean	70	63	56	
Gender (f:m)	12:11	6:6	8:3	
BW loss (%), ± SEM	10.93 ± 4.16	1.65 ± 1.52	0	
Body mass index mean (kg/m^2), ± SEM	24.48 ± 4.24	25.50 ± 3.48	25.02 ± 4.14	

Abbreviations: BMI, body mass index, BW, body weight, SMAI, skeletal muscle area index.

122-5p, miR-142-5p, miR-194-5p and miR-486-5p showed significant differences between the two conditions (Figure 1A). The changes of each of these miRNAs are listed in Table S1. For validation in tissue, the five miRNAs of the discovery array as well as miRNAs previously reported to be related to CCx, namely miR-27b-3p,[22–24] miR-103a-3p,[25] miR-199a-3p,[9,24] miR-375[26] and miR-424-5p[27] were analysed. The validation samples comprised patients with and without CCx and participants of the FREECE study representing a healthy control group.[15] All selected miRNAs were analysed in serum, corresponding visceral (VAT) and subcutaneous (SAT) adipose tissue as well as liver and muscle tissue.

miRNA-122-5p reflects cancer cachexia status in serum samples and muscle

Among the selected miRNAs, miR-122-5p and miR-194-5p showed significantly lower expression levels in serum from patients with CCx compared with the healthy control group from the FREECE study (P = 0.0396 and $P < 0.0001$, Figure 1B).

A lower, but not significant expression of miR-122-5p was also detectable in the CCx group in muscle tissue (Figure 1C). Furthermore, expression levels of miR-122-5p allowed a separation between CCx and non-CCx patients by using an area under curve (AUC) > 0.70 (P = 0.0367, Figure 1d). Correlations of miR-122-5p expression levels and the percentage of weight loss over 6 months before surgery revealed a significant negative relationship (Spearman correlation $r = -0.45$, P = 0.031; Figure 1E, left). To evaluate if this effect is exclusively CCx associated we analysed the expression of miR-122-5p in serum from patients who lost weight after a bariatric surgery (IPBS cohort,[16] mean body weight loss: 26%) (Figure 1E, right). No correlation was detectable, therefore, changes of miR-122-5p expression levels are associated with CCx rather than with weight loss per se.

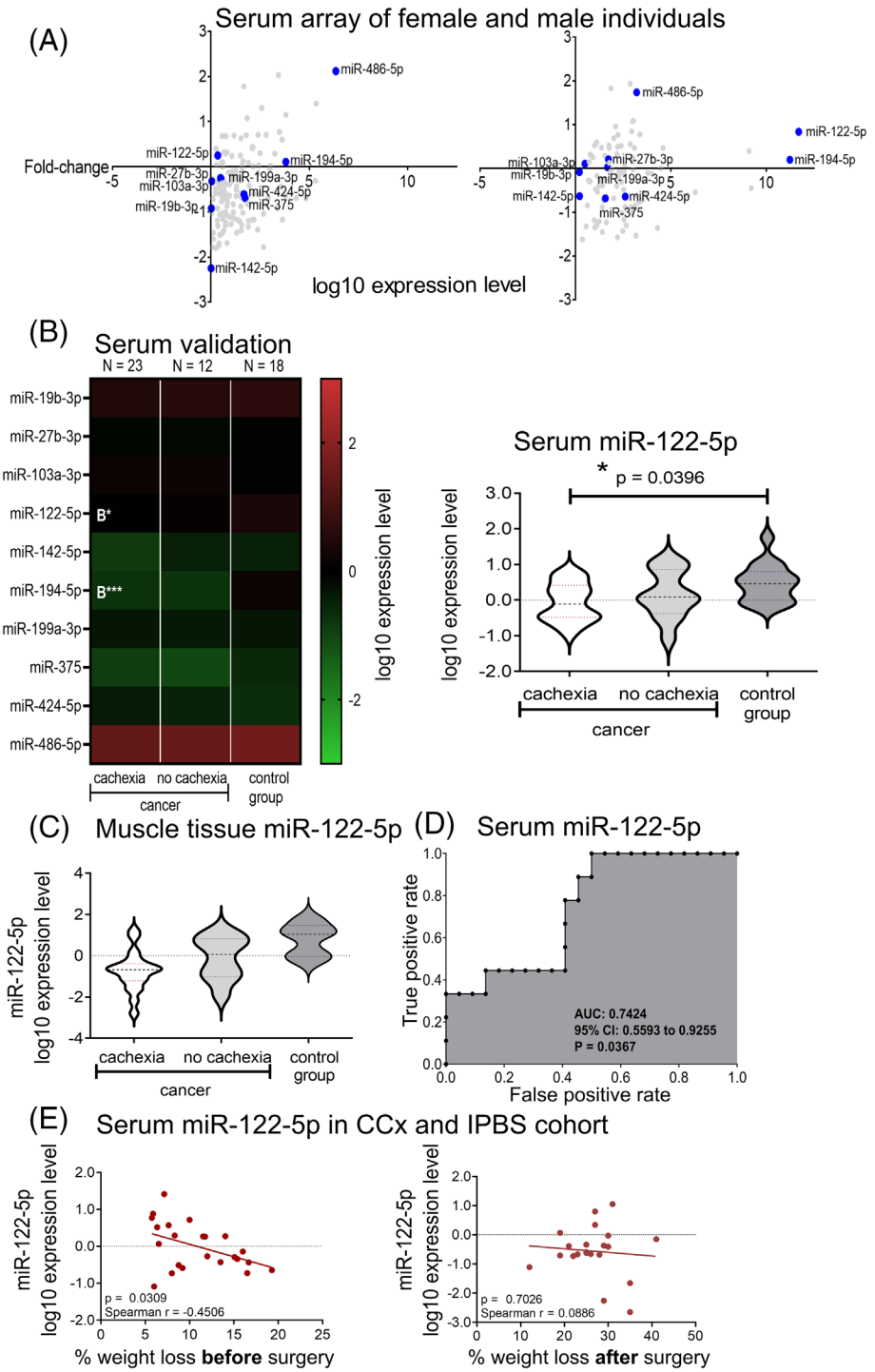

Figure 1 miRNA expression pattern in serum of CCx patients. (A) Scatter plots of logarithmic expression levels (C_q) and fold-change of miRNAs in female and male CCx versus non-CCx patients measured *via* microarray. Chosen miRNAs for validation analysis are labelled in blue. (B) Serum sample analysis of individuals of each group revealed significant down-regulation of miR-122-5p and miR-194-5p in comparison with the control group (indicated by letter B and significance level). Detailed expression levels of miR-122-5p in serum and of CCx in comparison with non-CCx patients and the control group. (C) Expression levels of miR-122-5p in muscle tissue of CCx in comparison with non-CCx patients and the control group. (D) Receiver operator characteristics of mir-122-5p derived from CCx versus non-CCx patients. (E) Correlation between miR-122-5p and weight loss over 6 months of CCx and the IPBS cohort. Data are normalized to relevant housekeeping genes, age and BMI adjusted, log-transformed and shown as mean ± SEM. Statistical analyses were performed using unpaired one-way ANOVA or Kruskal–Wallis tests with Bonferroni or Dunn's post-hoc tests (B, C) and linear regression (E). Statistical significances were defined as following: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

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Liver tissue of cancer cachexia patients revealed no changes of the miRNA expression pattern

Analysing the 10 identified miRNAs in liver samples of the patients with and without CCx, no differences in expression levels were detected (Figure S1a).

Changes of the miRNA pattern were muscle tissue associated

Significant changes in miRNA expression were seen in muscle tissue. Four miRNAs (miR-122-5p, miR142-5p, miR-199a-3p and miR-424-5p) were identified to be expressed at lower levels in patients with CCx compared with the control group (Figure 2A, labelled with B). miR-19b-3p, miR-27b-3p, miR-103a-3p, miR-142-5p, miR-199a-3p and miR-424-5p were lower expressed in individuals with CCx compared with study participants with cancer but without cachexia ($P = <0.05$, Figure 2A, labelled with A). miR-142-5p, miR-199a-3p and miR-424-5p expression was significantly different between all three groups (Figure 2A). For validation, the miRNA expression levels were correlated with weight loss during 6 months before surgery. Thereby, only miR-27b-3p resulted in a significantly negative relationship ($r = -0.408$, $P = 0.0386$, respectively Figure 2B). miR-27b-3p expression levels displayed a significantly 0.5-fold lower expression in CCx patients in comparison with the cancer patients without cachexia ($P = 0.0019$, Figure 2A). This reduced expression suggests an involvement in muscle wasting in the context of CCx. Expression levels of miR-27b-3p allowed a separation between CCx and cancer patients without CCx with an AUC > 0.8400 ($P = 0.0028$, Figure 2C). The role of miR-27b-3p and potential target genes was further investigated *in vitro*.

miR-27b-3p target genes are involved in muscle atrophy

Using *in silico* prediction, we identified putative target genes of miR-27b-3p and the GO:0014732 network (skeletal muscle atrophy) (display of all target genes in Figure S2a). Namely actinin alpha 3 (ACTN3), interleukin 15 (IL-15), myostatin (MSTN), peroxisome proliferator activated receptor gamma coactivator 1α (PPARGC1A), myogenin (MYOG) and tripartite motif containing 63 (TRIM63) were predicted (Figure 2D). These genes are part of the muscle atrophy pathway. To further study this association, C2C12 cells were differentiated and expression levels of miR-27b-3p were analysed. A continuous increase of miR-27b-3p during the differentiation process up to day seven was seen (Figure 2E). Transfecting cells with anti-miR-27b-3p resulted in a significant knock-down up to day four followed by an increase on day seven (Figure 2E). During transfection no changes in the phenotype of the cells were observed (Figure S3). Analysis of the putative target genes showed significant changes for Il-15 and Trim63 *in vitro* ($P = 0.0286$, $P = 0.0286$, respectively Figure 2E). Evaluating IL-15 and TRIM63 mRNA in muscle tissue of CCx patients, both genes were significantly up-regulated under CCx in comparison with the non-CCx group ($P = 0.0237$ and $P = 0.0442$, respectively, Figure 2F).

Subcutaneous adipose tissue revealed only changes of miR-142-5p

Analysing the miRNA panel in SAT, a significantly lower expression was only seen for miR-142-5p in CCx patients in comparison with the control group without cancer (CCx/MOBB cohorts) (Figure S1b).

miRNA pattern in visceral adipose tissue reflects cachexia

VAT showed strong CCx associated changes in its miRNA pattern. With the exception of miR-122-5p and miR-486-5p, the expression of miR-19b-3p, miR-27b-3p, miR-103a-3p, miR-142-5p, miR-194-5p, miR-199a-3p, miR-375 and miR-424-5p was significantly lower in VAT of CCx patients in comparison with the cancer group without CCx ($P < 0.05$, Figure 3A). For validation, miRNA expression levels were correlated with percentage weight loss over 6 months. Thereby, an association of miR-375 and miR-424-5p expression levels with weight loss was detectable (Figure 3B). Additionally, miR-375 displayed a significantly 3.3-fold lower expression in CCx patients in comparison with the non-CCx group as well as a significantly lower expression in comparison with the healthy control group ($P = 0.0096$ and $P = 0.0003$, respectively, Figure 3A). A significant 7.5-fold lower expression of miR-424-5p was detected in patients with CCx in comparison with the non-CCx group ($P = 0.0010$, Figure 3A), while no difference was found compared with the control group. Both miRNAs, allowed to distinguish between CCx and cancer patients without CCx with an AUC > 0.83 ($P = 0.0013$ and $P = 0.0005$, respectively, Figure 3C). Based on these observations, the effects of the two miRNAs were further investigated with *in vitro* experiments using visceral PACs.

miR-375 and miR-424-5p target genes are involved in triglyceride lipase activity

Using *in silico* prediction, we found a partial overlapping of putative target genes of miR-375 and miR-424-5p and the GO:004806 network (TG lipase activity) (display of all target genes in Figure S2b). Among the candidate genes, the CCx-associated genes hormone-sensitive lipase (LIPE), adipo-

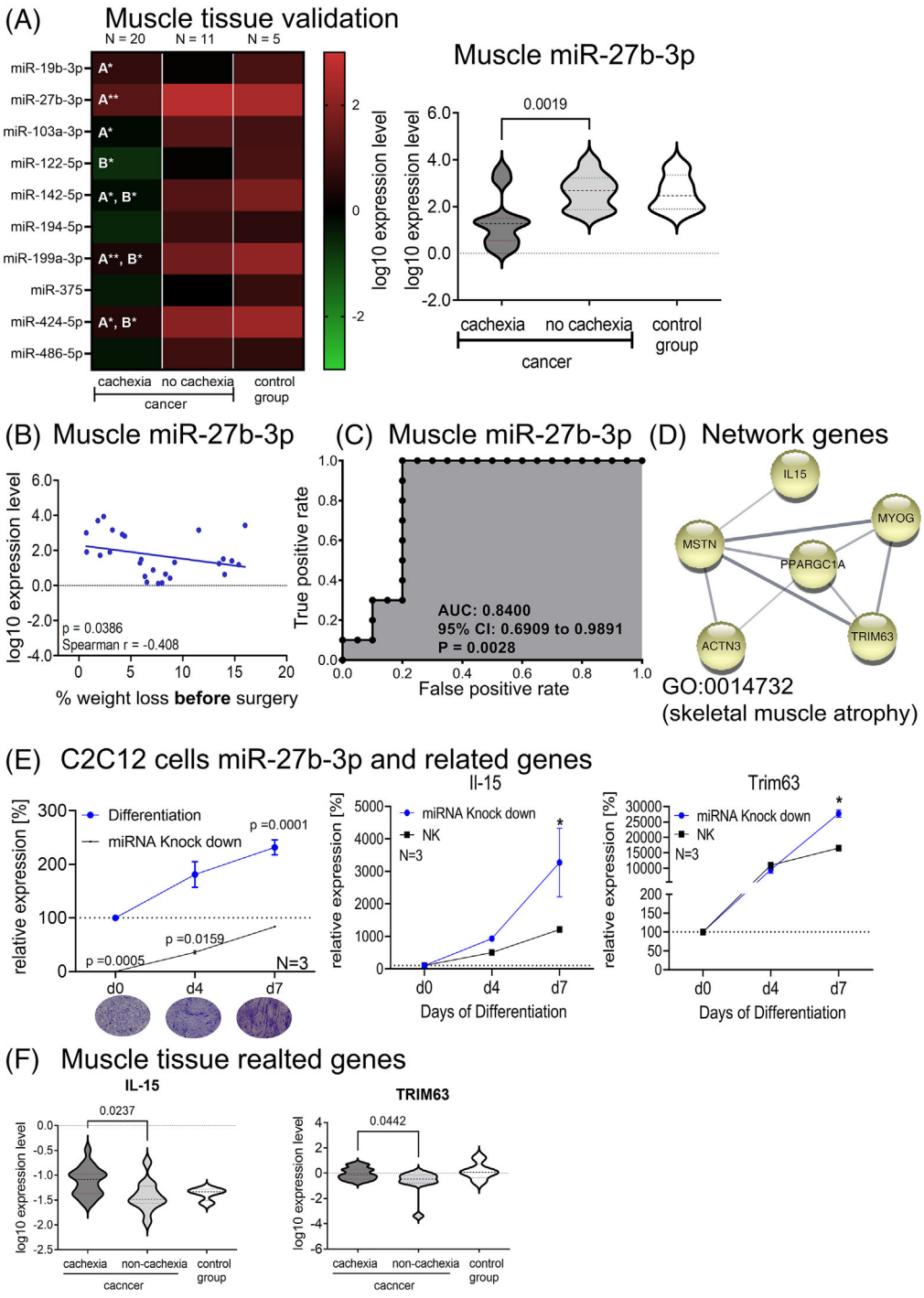

Figure 2 miRNA expression pattern in muscle tissue of CCx patients. (A) Muscle tissue expression levels of miR-19b-3p, miR-27b-3p, miR-103a-3p, miR-142-5p, miR-199a-3p and miR-424-5p. White (A) indicates significant differences between the CCx and no-CCx cancer groups. White (B) indicates significant differences between the CCx and the control group. Detailed expression levels of miR-27b-3p in muscle tissue of CCx in comparison with non-CCx patients and the control group. (B) Correlation between miR-27b-3p levels and cachexia induced weight loss 6 months before surgery. (C) Receiver operator characteristics of the prediction model derived from CCx to non-CCx patients. (D) Network of miR-27b-3p targets and predicted target genes involved in skeletal muscle atrophy. (E) miRNA expression levels, knock-down efficiency, corresponding HE staining's as well as the expression levels of Il-15 and Trim63 of anti-miR-27b-3p transfected cells during the differentiation of C2C12 cells up to day 7. (F) Expression levels of skeletal muscle atrophy genes IL-15 and TRIM63 in muscle tissue. Data are normalized to relevant housekeeping genes, age and BMI adjusted, log-transformed and shown as mean ± SEM. Statistical analyses were performed using unpaired one-way ANOVA or Kruskal–Wallis tests with Bonferroni or Dunn's post-hoc tests (A, F) and linear regression (B) and unpaired Mann–Whitney tests (E). N = number of independent experiments, statistical significances were defined as followed: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

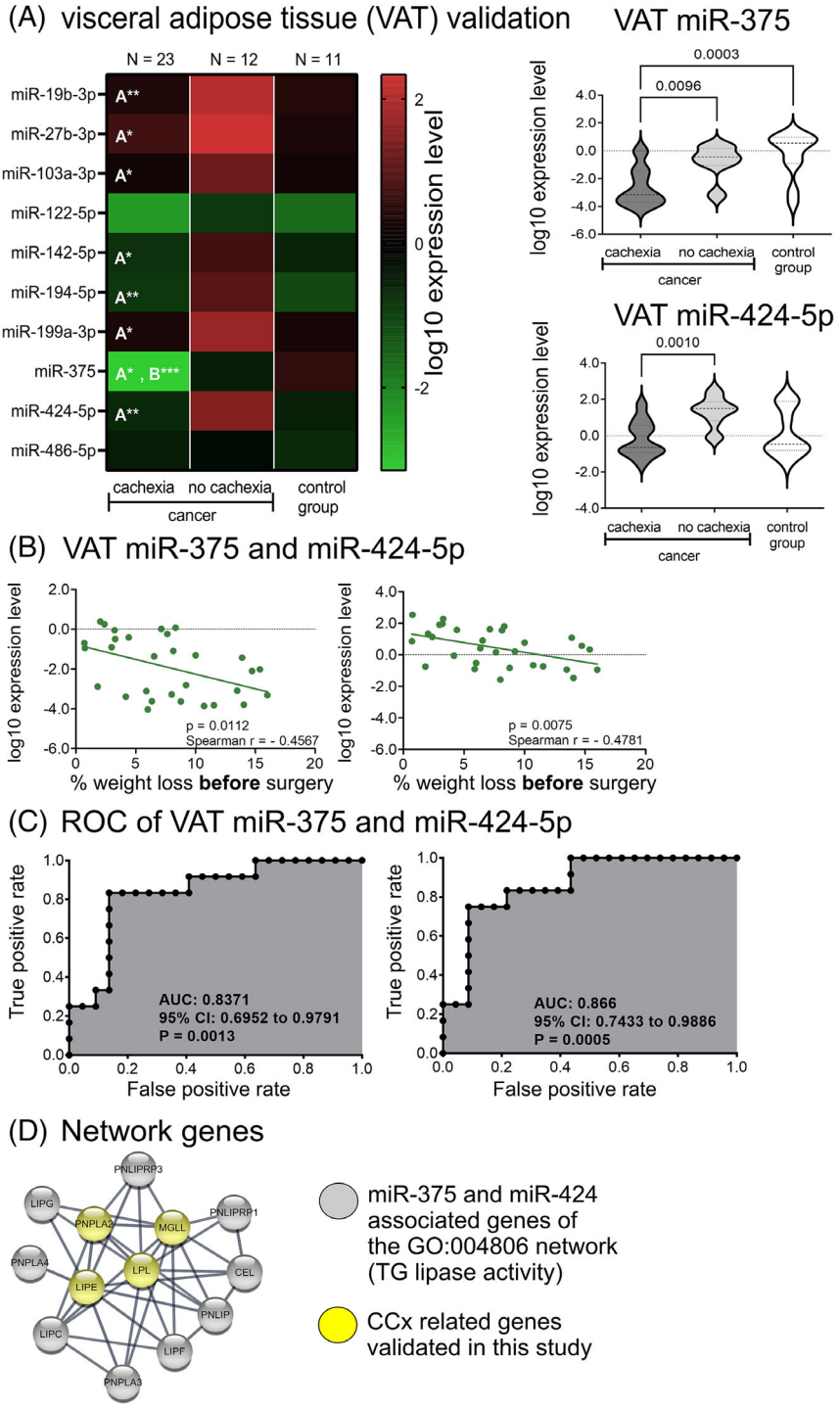

Figure 3 miRNA expression pattern in VAT of CCx patients. (A) Visceral adipose tissue (VAT) expression levels of miRNAs miR-19b-3p, miR-27b-3p, miR-103a-3p, miR-142-5p, miR-199a-3p, miR-375 and miR-424-5p in patients with CCx, CCx-free cancer and in the control group. Detailed expression levels of miR-375 and miR-424-5p in VAT of the three groups. White (A) indicates significant differences between the CCx and no-CCx cancer groups. White (B) indicates significant differences between the CCx and the control group. (B) Correlation analysis revealed an association between miR-375 and miR-424-5p expression levels and weight loss 6 months before surgery. (C) Receiver operator characteristics of the prediction model derived from CCx versus non-CCx patients. (D) Overlapping network of miR-375 and miR-424-5p targets and predicted target genes involved in triglyceride lipase activity pathway. Yellow labelled genes were validated in this study. Data are normalized to relevant housekeeping genes, age and BMI adjusted, log-transformed and shown as mean ± SEM. Statistical analyses were performed using unpaired one-way ANOVA or Kruskal–Wallis tests with Bonferroni or Dunn's post-hoc tests (A) and linear regression (B). N = number of independent experiments; statistical significances were defined as following: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

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cyte-triglyceride-lipase (PNPLA2), lipoprotein lipase (LPL) and monoglyceride lipase (MGLL) were further analysed in cell culture (Figure 3D).

miR-424-5p regulates triglyceride lipase gene expression of visceral preadipocytes

Because of the marked changes of miRNAs in VAT, human PACs isolated from this tissue were used for all following experiments. To evaluate the influence of the two miRNAs on lipolysis-related target gene expression, PACs were differentiated and expression levels of miR-375 and miR-424-5p were assessed (Figure 4A). Thereby, miR-375 showed an increasing expression over the differentiation process. In contrast, miR-424-5p revealed a decrease during differentiation. In parallel, lipid accumulation was assessed by Oil red-O staining. As both miRNAs were expressed during early preadipocyte differentiation, we decided to knock-down the miRNAs on d3 before the induction of differentiation. In this experiment, a complete knock-down of miR-424-5p over the differentiation time was detected (Figure 4B). In addition, during knock-down a significant depletion of miR-375 over the differentiation time was observed. During transfection no changes in the phenotype of the cells were observed (Figure S4).

The analysis of genes of the TG lipolysis pathway showed significant changes only for miR-424-5p transfected cells (Figure 4C). Depletion of miR-424-5p resulted in significantly increased expression levels of LIPE, PNPLA2, MGLL and LPL (P = 0.0095, 0.0022, 0.0022, and 0.0095, respectively). Comparing cells transfected with anti-miR-oligonucleotides or the inhibitor control (NK), no influence of miR-375 on LIPE, PNPLA2, MGLL and LPL expression was detectable.

Evaluating LIPE, PNPLA2, MGLL and LPL expression in VAT no significant differences were seen between patients with CCx in comparison with cancer patients without CCx. To elucidate the discrepancy between the expression profiles in PACs and VAT genes, expression levels of the different cell types of adipose tissue were analysed. Thereby, the single-cell/nucleus RNA sequencing dataset, publicly available on the Single Cell Portal (study no. SCP137) was used. As expected, all four genes were predominantly expressed in adipocytes. In addition, gene expression of lipolysis-related genes was also detectable in macrophages and endothelial cells (Figure 4D).

Despite this discrepancy, the observed findings in VAT on miR-424-5p in the context of CCx suggest that miR-424-5p is involved in the intracellular regulation of lipolysis (Figure 4E).

Discussion

This study was dealing with the differential expression of miRNAs in patients with CCx in comparison with either cancer patients without evidence of CCx or cancer-free controls. For the first time, a combination of serum, liver, muscle, subcutaneous and visceral fat tissues was used to explore miRNA signatures in patients with CCx. Our results identified some miRNAs to be differentially expressed in CCx with consecutive changes in the expression of dependent genes. These genes are known to contribute to muscle and lipid loss, thereby indicating a functional role in cancer cachexia.

miRNAs have been widely studied concerning their potential use for diagnostic purpose.[28] Specific circulating miRNA profiles have been reported to play a role in various diseases including cancer[8] and metabolic diseases such as type 2 diabetes[29] and obesity.[30] We became interested to perform miRNA arrays in serum of cancer patients with and without CCx. For this we used gender specific pooled samples in order to consider biological variability. The single patient samples were used to further investigate the potential predictive value of the identified miRNAs in individual patients. Therefore, we analysed the expression of identified candidate miRNAs in tissues known to be affected by CCx such as muscle, liver and adipose tissue. In serum, we observed a significant down-regulation of miR-122-5p in CCx patients correlating with percentage weight loss over 6 months. In the corresponding muscle tissue miR-122-5p was additionally down-regulated in patients with CCx. Similarly, Chen et al. detected lower plasma miR-122 expression levels in gastric cancer patients in comparison with healthy controls.[31] miR-122 plasma levels effectively distinguished patients with metastasized gastric cancer from healthy controls. In addition, in cancer tissue of pancreatic ductal adenocarcinoma (PDAC) patients, miR-122-5p was lower expressed and related to metastatic stage, tumour size and lymph node metastasis. Overexpression of miR-122-5p suppressed the proliferation, migration and invasion of human pancreatic adenocarcinoma cell lines *in vitro* and inhibited tumorigenesis *in vivo*.[32] Although miR-122 is considered as a liver-specific miRNA[33] and its decreased expression is associated with tumorigenesis,[34] such expression differences could not be seen in our liver samples. The role of this miRNA needs to be examined in larger cohorts to elucidate its role in organ-specific CCx.

Through the establishment of our CCx biobank, we were able to examine miRNA expression profiles of organs involved in cachexia in addition to serum. The corresponding measurements in muscle tissue revealed miRNA-27b-3p to be strongly associated with CCx. Decreased levels of miR-27b-3p have already been reported in colorectal cancer patients and shown inhibition effects on cell proliferation, migration and invasion in colon tissue.[35] Additionally, miR-27b regulates the myogenic paired box gene three (Pax3) gene, known to interfere with muscle cell differentiation.[36] In the case of CCx, the lower miR-27b-3p expression seems to be associated with the muscle atrophy status seen in cachectic individuals. This is supported by the up-regulation of TRIM63 in muscle tissue of CCx patients. TRIM63 is one of the members of the so-

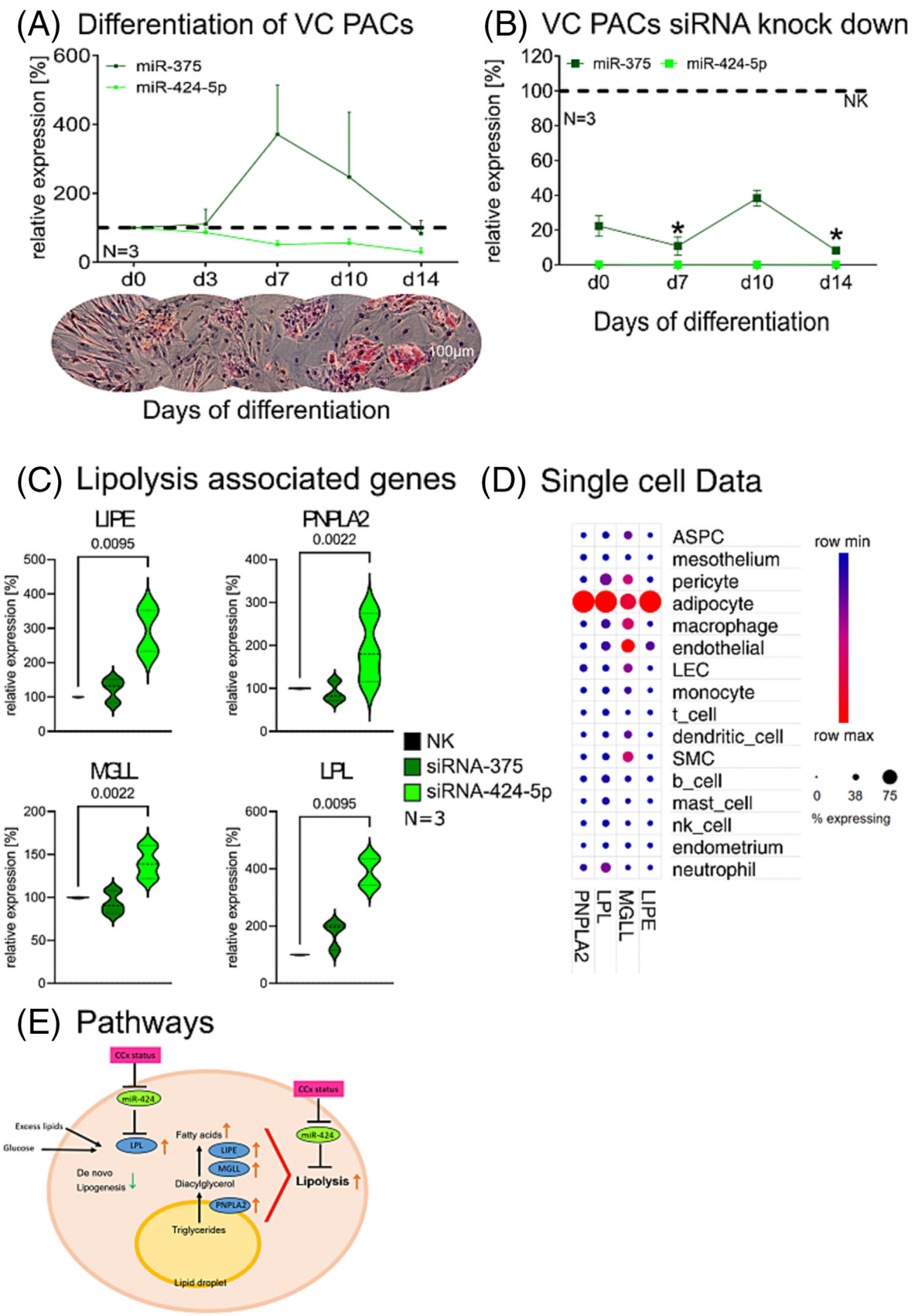

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Figure 4 Evaluation of the impact of miR-375 and miR-424-5p on visceral preadipocytes (PAC). (A) miR-375 and miR-424-5p expression levels and corresponding to Oil red-O/HE staining's over the differentiation of visceral PACs up to day 14. (B) Knock-down efficiency during the differentiation of visceral PACs cells up to day 14. (C) Transfection with miR-424-5p revealed significant changes of lipolysis genes LIPE, PNPLA2, LPL and MGLL ($N = 3$). (D) Single cell data of LIPE, PNPLA2, LPL and MGLL in different cell types. (E) Associated intra-cellular pathway influenced by miR-424-5p. Data are normalized to relevant housekeeping genes, log-transformed and shown as mean ± SEM. Statistical analyses were performed using unpaired one-way ANOVA or Kruskal–Wallis tests with Bonferroni or Dunn's post-hoc tests (A–C). N = number of independent experiments; statistical significances were defined as following: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

called 'atrophy-related genes' (atrogenes). It is expressed at low levels in adult muscle under normal conditions, but is highly over-expressed upon catabolic stimuli. Increased expression levels of Trim63 were also detected in RNA-sequencing analyses of cachectic mouse models and indicate an up-regulated proteasomal degradation pathway in muscle tissue.[37] CCx also goes along with a significant up-regulation of IL-15 in muscle. *Pistilli* and *Alway* showed that systemic administration of Il-15 results in apoptosis of skeletal muscle from rats.[38] As a cytokine, IL-15 influences the immune system: it stimulates T-cell and NK-cell proliferation.[39] Thereby, increasing systemic inflammation as it is present in CCx and tissue wasting may occur. However, IL-15 is also known to exert an anabolic effect on muscle proteins. Furthermore, other data suggests that IL-15 overexpression induces muscle hypertrophy and is involved in the inhibition of protein degradation.[40] Our data from cancer patients with cachexia revealed the targeting of miR-27b-3p on IL-15 and TRIM63 suggesting a role of this miRNA in the context of muscle atrophy.

In VAT, we identified the miRNAs miR-375 and miR-424-5p to be differentially expressed in CCx. Both miRNAs were reduced during CCx and we found evidence in human cell culture experiments that miR-424-5p inhibits genes of the TG lipase pathways. However, analysis of the expression of these genes in bulk VAT did not show differences between CCx and non-CCx patients. These findings may be due to the cellular heterogeneity of VAT.[18] Although mature adipocytes account for over 90% of the fat pad volume, lipid-loaden adipocytes only make up 20–40% of total cell numbers.[41] This suggests that other cells may also have an effect on lipolytic pathways in adipose tissue. An analysis of the single cell fractions of VAT would be necessary to obtain detailed information on the effect of certain miRNAs in a cell-type specific manner. Our findings of an association of miR-375 with CCx are underlined by some previously published data. miR-375 was detected to be differentially expressed in certain types of cancer such as gastric cancer, endometrial cancer or pancreatic ductal adenocarcinomas.[42] The systematic review by Shrestha et al. revealed miR-375 to be the most consistently reported down-regulated miRNA in gastric cancer. Thereby, miR-375, which has an anti-oncogene activity, was suggested as a candidate tumour suppressor.[43] Recently, our group could identify a connection between circulating miR-375 levels and response to cold exposure.[44] Furthermore, miR-375 may drive thermogenesis in visceral adipose tissue derived stem cells.

Our finding of miR-424-5p depletion in VAT from CCx patients was in contrast to a recent report by Connolly et al., who identified elevated miR-424-5p to be associated with muscle wasting via the inhibition of protein synthesis and loss of muscle mass.[45] Contrasting Similar results on miR-424-5p were reported in a recent study investigating muscle biopsies of non-small cell lung cancer patients with CCx and of age- and sex-matched healthy controls.[10] These differing results compared with our study are hard to explain, but may be related to a variety of differences between groups and tissues.

It is important to emphasize no directly CCx specific GO terms are available. To the best of our knowledge, we used the annotated GO terms 'TG lipase activity' and 'skeletal muscle atrophy', both associated with wasting syndromes. In addition, by increasing the confidence in the String DB tool, the number of target genes examined was limited.

A major limitation of our study was the cross-sectional design. Therefore, we can only present associations. To study cause-effect relationships and time-courses would require collecting samples and data prospectively.

Taken together, this study demonstrates specific miRNA species as potential markers of human CCx. Changes in several miRNAs, mainly miR-122-5p, miR-27b-3p, miR-375 and miR-424-5p, have been associated with CCx severity (i.e. body weight loss) and potentially contribute to the observed wasting syndrome by activating muscle atrophy and adipose tissue lipolysis pathways. However, there is still very limited knowledge on the role of miRNAs in CCx and additional prospective human studies are urgently needed to better elucidate these associations.

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Conflict of interest

The authors declare no competing interests in a financial or non-financial manner.

Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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