Research Paper

MiR-21-mediated Metabolic Alteration of Cancer-associated Fibroblasts and Its Effect on Pancreatic Cancer Cell Behavior

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Abstract

In this study, we investigated whether the metabolic alteration of cancer-associated fibroblasts (CAFs) occurs via miR-21 remodeling and the effect of this alteration on pancreatic cancer cells. CAFs and normal fibroblasts (NFs) were isolated and cultured. Glucose consumption and lactic acid production were tested, and lactate dehydrogenase (LDHA), pyruvate kinase m2 (PKM2), and miR-21 expression were examined. The level of glycolysis in CAFs was determined after treatment with a miR-21 inhibitor. Primary miR-21-NC CAFs and miR-21-inhibitor CAFs were indirectly co-cultured with BxPc-3 in vitro, and the invasion capacity of these cells was determined. The aerobic oxidation index of cancer cells and the expression of succinmodehydrogenase (SDH) and fumarate hydratase (FH) were assessed. Compared with NFs, CAFs showed enhanced glucose uptake capacity, lactic acid production, and elevated LDHA, PKM2, and miR-21 expression. After miR-21 inhibitor treatment, the extent of glycolysis in CAFs was reduced. After indirect co-culture with CAFs, oxidative phosphorylation and SDH, FH, and MCT expression increased in BxPc-3 cells. After co-culture with miR-21-inhibitor-CAFs, oxidative phosphorylation and invasion ability of the pancreatic cancer cells decreased. MiR-21 was involved in metabolic alteration of CAFs and affected the development of cancer cells. This metabolic alteration may be an important mechanism by which the microenvironment promotes tumor progression in a nonvascular manner.

Key words: miR-21; cancer-associated fibroblasts; glycolysis; metabolic alteration

Background

Pancreatic cancer is the fourth leading cause of cancer-related death worldwide[1] due to difficulty in diagnosis, early tumor metastasis and tumor resistance to current therapies[2]. In 2015, the incidence of pancreatic cancer in China was approximately 90,100 patients, and the mortality was 79,400 patients[3], while the 5-year overall survival (OS) rate remains approximately 4.1%[4]. Therefore, it is important to determine the mechanism of pancreatic cancer progression and develop new treatments.

Aerobic glycolysis (Warburg effect) is a metabolic feature of tumor cells[5]. Recent studies have shown that fibroblasts are also subject to metabolic alterations related to glycolysis, a phenomenon known as the "reverse Warburg effect", since the metabolic alteration occurs in stromal rather than tumor cells[6]. This metabolic change may be
involved in tumor progression[7]. The literature shows that co-culturing cancer cells and fibroblasts significantly enhances the quality of mitochondria in tumor cells compared to those in cancer cell monocultures[8-10]. Additionally, co-culture with fibroblasts reflects the actual tumor microenvironment. Therefore, the Warburg effect exhibited by tumor cells during in vitro experiments might be misleading. Treating tumor cells with lactate also significantly improved mitochondrial quality, indicating the existence of a parasitic relationship between tumor cells and fibroblasts in which the tumor cells are the "parasites" and the stromal cells are forced to undergo glycolysis after the transformation-mediated aerobic oxidation occurred in tumor cells. In our preliminary work, we observed metabolic coupling between cancer cells and cancer-associated fibroblasts(CAFs) in the pancreatic cancer microenvironment[11]. After co-culture, a significant degree of aerobic oxidation occurred in tumor cells, and invasion and metastasis accelerated[11]. However, the specific mechanism responsible was not clear.

In recent years, metabolic reprogramming of tumor cells has been a hot topic of research, particularly the miRNA regulatory network associated with tumor development, and has attracted significant attention. Several studies have shown that miRNAs can directly or indirectly regulate metabolic enzymes, oncogenes, and tumor suppressor genes at multiple levels and multiple pathways, such as glucose and lipid metabolism and amino acid biosynthesis, to affect tumor metabolism. For example, miR-378 can indirectly regulate the metabolic turnover in breast cancer cells through a transduction signaling pathway, reduce the expression of tricarboxylic acid enzyme genes and oxygen consumption, and enhance the production of lactate[12]. miR-320, miR-123a, miR-422, miR-506, and miR-136 are indirectly involved in the p53, c-Myc, AMPK, and AKT signaling pathways and affect tumor metabolism[13-15]. Studies have also found that miRNA can affect the metabolism of tumor stromal cells, thus affecting tumor progression[16-18]. Tang et al. showed that high expression levels of miR-320 in stromal cells upregulated glycolysis[18]. The literature, as well as our own work, revealed that miR-21 is upregulated in the stromal cells of pancreatic cancer and is closely associated with the biological characteristics of pancreatic cancer[19]. It is not clear whether the reverse Warburg effect in CAFs is mediated by miR-21. This study aimed to explore the mechanism of the effect of CAFs on the progression of pancreatic cancer.

### Materials and Methods

#### Materials

The glucose detection checkerboard and lactic acid checkerboard were obtained from the Jiancheng Institute of Biological Engineering (Nanjing, China). RIPA cracking liquid kits were from Biyuntian Biological Co., Ltd. (Shanghai, China). Dimethyl sulfoxide was from Sigma Co., Ltd. (Beijing, China). Dulbecco’s modified Eagle medium (DMEM) and fetal bovine serum were from HyClone (Logan, UT, USA). Transwell chambers were from Millipore (Burlington, MA, USA). Matrigel and the One-Step RT-PCR kit were from BD Biosciences (Franklin Lakes, NJ, USA). LDHA, α-SMA, vimentin, PKM2, SDH, FH, MCT1, and β-actin antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Lipofectamine 2000 Transfection Reagent and TRizol reagent were from Invitrogen Biotechnologies (Carlsbad, CA, USA). MiR-21 and the miR-21 inhibitor were designed and synthesized by Sangon Biotech (Shanghai, China).

#### Cell cultures and treatments

Human pancreatic cancer cells (BxPc-3 and Panc-1, obtained from the American Tissue Type Collection, Manassas, VA, USA) were maintained in DMEM supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL), 0.1 mM nonessential amino acids, 0.2 mM glutamine, 1 mM pyruvate, and 10% heat-inactivated fetal bovine serum and incubated in a 5% CO₂ humidified atmosphere at 37°C. Cells were grown to 80% confluence. In the invasion and migration experiments, the cells were cultured in DMEM without fetal bovine serum.

#### CAF cell separation, culture, and purification

Paired NF and CAF cells were derived from patients with pancreatic cancer and pancreatic trauma from the Second Affiliated Hospital of Xi’an Jiaotong University (Xi’an, China). All patients were newly diagnosed and had not received any relevant treatment prior to surgery. Informed consent was obtained from all patients before specimen collection. Fibroblast isolation was conducted as described previously[20]. First, the tissue was trimmed to 1 × 1 × 1 mm and washed gently with PBS 3 times (5 min each). Next, the tissues were washed once with the medium and placed in fresh cell cultural medium containing 15% fetal bovine serum, 2 mM l-glutamine, and 10% penicillin. The tissue was cut with a sterile scalpel blade, and sections of cells were gently scraped with a blunt blade. The cells were cultured in an incubator for 3–5 days at 37°C in 5% CO₂. The medium was replaced once and every 3 days.
were performed using the 2−ΔΔCt method. Each experiment was performed in triplicate.

Glucose uptake assay

According to Fischer et al., the glucose uptake rate is reflected by the amount of [3H]-2DG taken up by the cells. After 24 h in serum-free culture, the medium was changed to low-sugar DMEM, 37 kBq/m [3H]-2DG was added, and the cells were cultured for another 24 h. After digestion, the small fraction of cells remaining was counted, and other cells were lysed in 0.5 M NaOH for 15 min; the same volume of 0.5 M hydrochloric acid was added for neutralization. The dpm value in the cell lysate solution was examined using a microplate reader. Cellular [3H]-2DG uptake was calculated as follows: total cellular radioactivity − non-specific binding radioactivity)/24 h.

Lactic acid detection in cell culture medium

Cells in the 12-well plate were washed once with PBS, the medium was replaced with phenol-free red medium, and the cells were cultured for 20 h at a density of 1.5 × 10^5 cells/mL. The supernatant was collected according to the instructions of the lactic acid detection kit. Lactic acid content was examined using a DRY-CHEM FDC3500 analyzer (Fuji, Tokyo, Japan); additionally, digested cells were counted.

Transfection of miR-21 inhibitor

MiR-21-specific small interfering RNA (miR-21-siRNA) was designed and synthesized as the following oligonucleotides: siRNA #1 sense strand: 5′-AAAACCTTCGGAAACCGCCCTGTCTC-3′ and antisense strand: 5′-AAGGCCGTGTTCGAGAGTTCTGCTC-3′; siRNA #2 sense strand: 5′-AAGAGCGAGCAGTGAAAGACATTGCTC-3′ and antisense strand: 5′-AACATCTTCTACCGTCTC-3′. The sequence of the non-specific control siRNA (NS-siRNA) was 5′-GGUC UCACUCCCCAUAGAGTT-3′. The cells were inoculated into 6-well plates at a density of 1 × 10^5 cells/mL 24 h prior to transfection. Before transfection, the cells were washed with serum-free, antibiotic-free DMEM/F12 medium. Next, the cells were transfected using the liposome-mediated method. According to the reagent instruction manual, the miR-21 inhibitor or control NS-siRNA and the Lipofectamine 2000 Transfection Reagent were diluted with serum-free, antibiotic-free DMEM/F12 medium. The two dilutions were mixed to form a complex that was used to transfect the cells. The experimental groups included normal cultures of CAFs and NFs (control group), NS-siRNA-transfected CAFs (miR-NC group), and miR-21-inhibitor-transfected CAFs (miR-21-inhibitor group). Five samples were prepared for each group, and further experiments were conducted after culturing the cells for 48 h.

Indirect co-culture model of CAFs and pancreatic cancer cells

CAF cultures were added to Petri dishes at a density of 1.5 × 10^5 cells/mL, gently washed after 24 h with PBS, and then cultured in serum-free medium for 48 h. CAF-culture medium (CAF-CM) was gathered from centrifuged cell culture fluid and stored at 4°C.
NF-CM, miR-21-NC CAF-CM, and miR-21-inhibitor CAF-CM were gathered in the same way.

BxPc-3 pancreatic cancer cells were added to Petri dishes at a density of $1.5 \times 10^5$ cells/mL; after 24 h, CAF-CM and medium (volume 1:2) were added, and the cells were cultured for 48 h. Cells in PBS or serum-free medium were used as controls. An inverted phase-contrast microscope was used to observe the morphology and growth of pancreatic cancer cells in each Petri dish. Proteins were extracted from the cells.

**Cell migration assay**

Cell migration capability was evaluated in a scratch test. First, $10 \times 10^5$ BxPc-3 and Panc-1 cells were seeded into 1.5 mL of medium in each well of a 24-well plate. The cells were grown to a confluent layer (48 h), and then a scratch was made in each well using a pipette tip. Subsequently, the cells were washed gently with PBS, and then NF-CM, miR-21-NC CAF-CM, or miR-21-inhibitor CAF-CM (0.5 mL) and 1.0 mL medium were added to the respective wells. A photograph was taken at time point 0. The cells were then incubated at 37°C in 5% CO₂, and photographs were acquired after 24 h. The 24-h time point was chosen to decrease the potential impact of proliferation on the closing of the scratch. ImagePro Plus 5.0 (NIH, Bethesda, MD, USA) was used to standardize the results.

**Mitochondrial activity detection**

After culturing the NF and CAF cells for 24 h, the solution was used to culture pancreatic cancer cells for 24 h. Cells grown in PBS or serum-free medium were used as blank controls. Fresh DMEM containing mitochondrial fluorescent probes (1:200) was added at 500 μL/well and incubated for an additional 30 min. The cells were stored and produced in a darkroom and immediately observed using an inverted fluorescence microscope.

**Western blotting**

In total, $5 \times 10^5$ cells in the logarithmic growth phase were added to 0.5 mL of pre-chilled cell lysis buffer and incubated on ice for 30 min. After centrifugation, the supernatant was collected, and its protein contents were measured. The proteins were separated by 10% SDS-PAGE and blotted onto a nitrocellulose membrane by semi-dry transfer. Next, the membrane was immersed in Tris-buffered saline with Tween 20 containing 5% skim milk for blocking, followed by overnight incubation with the primary antibody at 4°C. On the following day, the membrane was incubated with the secondary antibody conjugated to horseradish peroxidase at 1:2000 dilution (Santa Cruz Biotechnology) at room temperature for 2 h, and then an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Amersham, UK) was used for staining. The membrane was photographed, and the results were analyzed.

**Statistical analysis**

Each experiment was repeated at least three times. The data were expressed as the mean ± standard deviation ($\bar{x} \pm s$) and analyzed using Student’s t-test. $P < 0.05$ indicates a statistically significant difference.

**Results**

**Preliminary identification of pancreas CAFs and NFs**

After 10 days, cell morphology was observed using an inverted phase contrast microscope, and NFs and CAFs both showed a spindle shape (Fig. 1). The CAFs were spindle- or fusiform-shaped, had inconsistent sizes, showed dense growth, and exhibited a disordered arrangement. NFs showed multiple flat stellate shapes with similar cell sizes and were arranged radially outward in the same direction. Immunohistochemical analysis of CAFs showed that vimentin and α-smooth muscle actin expression were upregulated. In NFs, vimentin expression increased, whereas α-smooth muscle actin expression decreased.

**CAF glycolytic metabolism**

According to previous studies, CAF cells exhibit the Warburg effect, similar to tumor cells; lactate is produced, secreted, and absorbed by tumor cells to synthesize anabolic biomass and to facilitate rapid proliferation. To determine whether glycolysis occurs in CAFs, we examined lactate levels and glucose uptake in the culture medium. The results demonstrated that glucose uptake and lactate production were significantly increased in CAFs compared to NFs (Fig. 2A, B). To further verify the change in glycolysis in CAFs, RT-PCR was conducted to detect the mRNA expression of the CAF glycolytic enzymes lactate dehydrogenase (LDHA) and pyruvate kinase m2 (PKM2). The results showed that LDHA and PKM2 were highly expressed in CAFs (Fig. 2C, E). The Western blotting and PCR test results were consistent: LDHA and PKM2 protein expression was higher in CAFs than in the control group (Fig. 2D, F). These results further confirm that glycolysis occurs in CAFs in vitro.
Figure 1. Preliminary identification of pancreas CAFs and NFs. **A**: CAFs showed dense growth and were disorderly arranged. NFs were arranged radially outward in the same direction. **B, C**: Immunohistochemical analysis of CAFs showed that vimentin and α-smooth muscle actin expression increased. In NFs, vimentin expression increased, while α-smooth muscle actin expression decreased.

Figure 2. CAF glycolytic metabolism. **A, B**: Glucose uptake and lactate production were significantly increased in CAFs compared to NFs. **C, D**: PCR and Western blotting results showed that LDHA and PKM2 mRNA and protein were highly expressed in CAFs. **E, F**: Results of statistical analysis. The results relative to control cells are presented as a standard ratio. *indicates \( P < 0.05 \) by t-test.
miR-21-mediated reverse Warburg effect phenomenon in CAFs

Reports have shown that pancreatic stellate cells exhibit high expression of miR-21, which plays an important role in tumor cells. To test whether miR-21 was involved in the reverse Warburg effect process in CAFs, the status of miR-21 in CAFs was first examined. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) results showed that the expression of miR-21 was significantly higher in CAFs than in NFs (Fig. 3). After transfection with the miR-21 inhibitor, the relative expression of miR-21 in CAFs decreased significantly compared to the expression in cells transfected with blank control (Fig. 3). The expression of glycolytic enzyme mRNAs in CAFs was measured again using qRT-PCR after transfection. The results showed that the mRNA expression in the miR-21-inhibitor group was significantly lower than that in the blank transfection group (Fig. 4A, B). The Western blot results were consistent with those of PCR; the corresponding protein expression levels in the miR-21-inhibitor group were significantly lower than those in the blank transfection group (Fig. 4C, D). Sugar uptake and lactate production in the miR-21-inhibitor group were significantly lower than those in CAFs (Fig. 4E, F). The above results further confirmed that miR-21 was involved in the reverse Warburg effect metabolic alteration of CAFs.

Involvement of miR-21 in CAF-cancer cell metabolic coupling and the consequent promotion of tumor progression

Our previous studies showed that co-culturing cancer cells with CAFs enhanced their aerobic oxidation capacity, including the mitochondrial fluorescence intensity in cancer cells as observed by fluorescence microscopy (Fig. 5A). The results showed that the expressions of succinohydrogenase (SDH) and fumarate hydratase (FH) proteins were higher in the CAF co-culture group than in the NF and monoculture groups (Fig. 5B, C), suggesting metabolic coupling. To examine whether the metabolic coupling disappeared after silencing miR-21 in CAFs, the changes in the aerobic oxidation index were monitored after the indirect co-culture of miR-21 inhibitor with cancer cells. The results showed that the expression of proteins was significantly lower in cancer cells than in CAFs co-cultured with miR-21 inhibitor (Fig. 5D, E), suggesting the attenuation of the metabolic coupling phenomenon.
Figure 4. miR-21-mediated reverse Warburg effect phenomenon in CAFs. **A, C:** The results showed that mRNA expression in the miR-21 inhibitor group was significantly lower than that in the blank transfection group. **B, D:** The Western blot results were consistent with those of PCR. The corresponding protein expression levels were significantly lower in the miR-21 inhibitor group than in the blank transfection group. **E, F:** Sugar uptake and lactate production in the miR-21 inhibitor group were significantly lower than in CAFs.
Figure 5. Involvement of miR-21 in CAF-cancer cell metabolic coupling and the consequent promotion of tumor progression. **A**: Mitochondrial fluorescence intensity in cancer cells as observed by fluorescence microscopy. **B, C**: The expression of SDH and FH was higher in the CAF co-culture group than in the NF and monoculture groups. **D, E**: The expression of proteins was significantly lower in cancer cells than in CAFs co-cultured with miR-21 inhibitor, suggesting the attenuation of the metabolic coupling phenomenon.

Figure 6. Involvement of miR-21 in the indirect promotion of pancreatic cancer cell invasion by CAFs. The number of cells passing through was significantly higher in the CAF-CM group than in the phosphate-buffered saline (PBS) or NF-CM induction groups. After transfection with miR-21 inhibitor, the number of cells passing through was significantly lower in the miR-21 inhibitor group than in the CAF-CM induction or blank transfection groups (**A, C**: Panc-1; **B, D**: BxPc-3).
Discussion

CAFs have been shown to be involved in tumor progression[22]. However, the mechanism of its positive effect on tumors has not yet been fully elucidated. Most researchers believe that the release of a growth factor is involved. Many recent papers have discussed possible mechanisms from the perspective of energy metabolism[23-26]. It is believed that some of the activated CAFs "abandon" oxidative phosphorylation and switch to the glycolytic pathway. The metabolites, such as lactate, released by this enforced metabolic mode are taken up and used by tumor cells to maintain growth[6, 24]. This process is known as the reverse Warburg effect. Our previous studies showed that a similar process occurs in the pancreatic CAFs, in which the secretion of lactic acid was significantly increased, and the oxidation and invasion capacities of cancer cells were enhanced after co-culture. The invasive capacity of pancreatic cancer cells decreased if this coupling phenomenon was suppressed. This finding was consistent with results reported earlier[9, 27-29], further indicating that the metabolic alteration of stromal cells plays an important role in tumor progression. However, the specific mechanism is still not clear. This study showed that the expression level of miR-21 was significantly higher in pancreatic CAFs and that the co-culture of stromal cells decreased if this coupling phenomenon was suppressed. Therefore, miR-21 was involved in the metabolic alteration of stromal cells, playing an indirect role in promoting tumor progression. Thus, miR-21 might be a new target for CAF treatment.

miRNA is a class of single-stranded, non-coding RNA molecules composed of 18 to 23 nucleotides. These molecules are a newly discovered family of regulatory molecules associated with tumor development and progression[13, 15]. miRNAs do not encode proteins, but they bind to the 3’ end non-coding region of the target mRNA[15]. miRNAs regulate the function of target genes by degrading or inhibiting the translation of their mRNA[15]. MiR-21 is a highly conserved protein encoded by a single gene located on chromosome 17. There are numerous studies on the functional role of miR-21 in tumor progression that mainly focus on tumor parenchymal cells. Studies on the abnormal expression and function of miRNAs in stromal cells are relatively rare. Recently, miR-21 was found to be abnormally expressed in ovarian cancer fibroblasts[30]. This type of fibroblast with high expression of miR-21 has obvious characteristics of myofibroblasts, namely, upregulated α-SMA expression and high proliferative ability, and can indirectly promote the progression of tumor cells[30]. Transfection of LX-2 hepatic stellate cells increased the expression of α-SMA and collagen mRNA, and co-culture with hepatocarcinoma cells promoted tumor invasion. Our findings were similar to these results and showed that miR-21 was expressed at a significantly high level in pancreatic CAFs. The co-culture of CAFs that are highly expressing miR-21 and pancreatic cancer cells could significantly accelerate tumor formation.

The study of the mechanism suggested that abnormal expression of miR-21 can inhibit the expression of phosphatase and tensin homolog (PTEN), a critical negative regulatory target, by activating the extracellular-signal-regulated kinase (ERK) pathway, thus mediating tumor progression[31]. One of the biological manifestations is the regulation of the cell metabolic program via miR-21 to change and affect tumor progression[32]. Thus, metabolic enzymes, oncogenes, and tumor suppressor genes are directly or indirectly regulated through multiple pathways to affect tumor metabolism, including glucose and lipid metabolism and amino acid biosynthesis[32]. The knockdown of miR-21 has an impact on aerobic glycolysis in T24 bladder cancer cells[33]. Glucose uptake and lactate production were reduced, indicating a decrease in the rate of aerobic glycolysis[33]. At the same time, the enzymatic activity of hexokinase, a well-known rate-limiting enzyme of glycolysis, decreased, suggesting that the knockdown of miR-21 reduces the level of aerobic glycolysis in T24 cells[33]. Moreover, Bartman et al. demonstrated that increased plasma miR-21 levels are associated with increased activity of phosphofructokinase, a well-known, rate-limiting enzyme in glycolysis[34]. Jiao et al. demonstrated that miR-21 protected the kidney against renal ischemic preconditioning via HIF-1α by inhibiting its target renal ischemic preconditioning PHD2[35]. MiR-21 was significantly upregulated by hypoxia/reoxygenation in HK-2 cells, while PHD2 levels decreased significantly. Hypoxia inducible transcription factor prolyl-hydroxylase 2 (HIF-PHD2) is a key regulator of the hypoxia response via hydroxylation of specific HIF-α proline residues, which may lead to the degradation of HIF-α[36]. Thus, upregulation of miR-21 and subsequent downregulation of the HIF-α suppressor PHD2 stimulates HIF-1α expression and HIF-1α-promoted glycolysis. Jiang et al. also demonstrated that miR-21 upregulated HIF-1α, resulting in the promotion of the key enzymes of glycolysis and radioresistance in...
non-small cell lung cancer cells[37]. Additionally, exosome-derived miR-21 should also be considered part of the CAF-cancer cell interaction. A miRNA signature in circulating exosomes including miR-21 has been demonstrated to be superior to exosomal glypcan-1 levels for diagnosing pancreatic cancer[38]. Exosome-delivered miR-21 plays a key role in the pathogenesis of glioblastoma[39] and malignant melanoma[40]. There are also nutritional sources of exosomal miR-21 provided by dairy milk consumption[41], which should be considered in future research.

In this study, we found that inhibiting the expression of miR-21 in pancreatic CAFs suppressed the level of glycolysis in CAFs and indirectly suppressed tumor progression. This is a key and novel finding that has not been previously reported. Previous studies have shown that the increased level of glycolysis mediated by high expression of miR-320 in stromal cells reflects a similar phenomenon[18]. This study suggested that miR-21 was involved in the regulation of the Warburg effect in stromal cells, thus participating in the regulation of pancreatic cancer cell development. The key molecules involved in the regulation of the Warburg effect by miR-21 might be therapeutic targets for the treatment of pancreatic cancer. However, the specific regulatory mechanism remains to be studied further. With progress in the study of miRNAs, especially in the in-depth understanding of tumor cell regulatory mechanisms and networks, more miR-21 functions and targets of action may be found.

In conclusion, miR-21 was involved in the metabolic alteration of CAFs and affected the development of cancer cells. This metabolic alteration may be an important mechanism by which the microenvironment promotes tumor progression in a nonvascular manner. Further studies should focus on how CAF reshapes the metabolic transition.

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Competing Interests
The authors have declared that no competing interest exists.

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