

**SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)**

**ROLE OF AMPK IN THE REGULATION OF BREAST  
CANCER METABOLISM AND BEIGE ADIPOCYTE  
DIFFERENTIATION**

by Tamás Fodor

Supervisor: Péter Bay, PhD, DSc



**UNIVERSITY OF DEBRECEN  
DOCTORAL SCHOOL OF MOLECULAR MEDICINE  
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# **Role of AMPK in the regulation of breast cancer metabolism and beige adipocytes differentiation**

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The Examination takes place at the Library of Department of Biochemistry and Molecular Biology and Department of Medical Chemistry (LSB 3.009-3.010), Faculty of Medicine, University of Debrecen,  
22<sup>th</sup> of May, 2018 at 11:00 am

Head of the **Defense Committee**: Zoltán Papp, MD, PhD, DSc

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, 22<sup>th</sup> of May, 2018 at 13:00 pm

## THEORETICAL BACKGROUND

Living organisms must accommodate to changes in the environment, such as the availability of nutrients (i.e. nutrient availability or nutrient scarcity) or changes to the external temperature. Therefore, cells possess an energy and nutrient sensor system that fine tunes metabolism to meet the needs of the organism. The pathological function of this sensory system leads to pathological metabolic adaptation that may in fact contribute carcinogenesis and cancer progression. My dissertation presents two studies that assess the regulation of both physiological and pathological metabolic adaptation.

### Structure of AMPK

Cells continuously need an optimized energy state that is necessary to maintain the cellular energy homeostasis which is essential for cell survival. Therefore, ATP utilization and production needs to be balanced. The major energy sensor that enables sensing of the AMP to ATP ratio is a heterotrimeric enzyme 5' adenosine monophosphate-activated protein kinase (AMPK). The most notable feature of AMPK is its sensitivity to cellular energy state. Decreases in the ATP/AMP ratio can activate AMPK through interdependent allosteric and post translational modifications. AMPK is a heterotrimeric enzyme complex made up of the catalytic  $\alpha$  and the regulatory  $\beta$  and  $\gamma$  subunits all of which are necessary for AMPK activity. There are different isoforms of the  $\alpha$ ,  $\beta$  and  $\gamma$  subunits, namely,  $\alpha 1$ ,  $\alpha 2$ ,  $\beta 1$ ,  $\beta 2$  and  $\gamma 1$ ,  $\gamma 2$  and  $\gamma 3$ , respectively. Nevertheless, due to alternative splicing other isoforms exist, too. In mammals, these isoforms are coded by seven genes. The  $\alpha$  and  $\beta$  subunits are encoded by two genes namely PRKAA1, PRKAA2 and PRKAB1, PRKAB2, while the  $\gamma$  subunit is encoded by an additional three genes PRKAG1, PRKAG2 and PRKAG3. The  $\alpha 1$ ,  $\beta 1$ , and  $\gamma 1$  (the common isoforms) are expressed in most mammals cells. The  $\alpha 2$ ,  $\beta 2$ ,  $\gamma 2$ , and  $\gamma 3$  isoforms are abundant in cardiac and skeletal muscle. The  $\gamma$ -subunit has the major role in direct sensing and binding with cytoplasmic AMP/ADP in order to stimulate the enzymatic activity of AMPK. The  $\gamma$  subunit has four cystathionine beta synthase (CBS) domains which

provide AMPK with the capability to sensitively detect the alterations in the AMP/ATP ratio. AMPK is activated when the metabolic balance is perturbed by a deficit of nutrient energy status. Consequently AMPK turns on the ATP-generating processes (stimulation of hepatic fatty acid oxidation, ketogenesis, stimulation of skeletal muscle fatty acid oxidation and glucose uptake), meanwhile it switches off the ATP consuming processes (inhibition of cholesterol synthesis, lipogenesis, and triglyceride synthesis, inhibition of adipocyte lipolysis and lipogenesis, and inhibition of insulin secretion by pancreatic beta-cells).

### **Regulation of AMPK by the AMP/ATP ratio**

In an energetically quiescent cell, the ATP/ADP ratio is 10:1 and the ATP/AMP ratio is 100:1. Energetically unfavorable reactions require high ATP/ADP ratio. Elevated levels ATP consumption which are not covered by sufficient ATP production will lead to an increment of ADP. ADPs will be quickly converted to ATP and AMP by adenylate kinases in the following reaction:



This reaction dramatically increases AMP levels and consequently AMP boosts the activity of AMPK by 5-fold. The activation of AMPK takes place at the  $\gamma$ -subunit, where AMP can bind. Increased AMP concentration will lead to the conformational alterations on the  $\gamma$ -subunit of AMPK in a way that two AMP can connect to the four Cystathione beta synthase (CBS) domains which also referred to as the Bateman domains. This conformational change leads to a moderate increase in AMPK activity 2 to 10-fold. This conformational change also enhances the phosphorylation of Thr172 of the  $\alpha$ -subunit, which further activates AMPK. The phosphorylation of AMPK results in activation by at least 100-fold. Interestingly, a recent study pointed out that AMPK regulated by ADP and not only by AMP and ATP.

### **AMPK in health and disease**

Every organism continuously face with energy demands during their lifetime. In response to these initiator stress factors cells evolved complex metabolic processes to be able to stock fuel substrates when food source is ample and to be able

to reduce energy consumption and mobilize stores when food is scarce. These energy balancing processes are essential for survival in both short term and prolonged nutrient deprivation.

5'-adenosine monophosphate AMP activated protein kinase called AMPK is a well-studied heterotrimeric enzyme complex, a Ser/Thr kinase with very prominent role in the maintenance of the cellular energy equilibrium in mammalian cells. It is sensitive for changes in energy charge reflected by changes in the ATP/AMP ratio. When AMPK activated by decrease in ATP/AMP ratio, AMPK switches on catabolic mechanisms and in parallel switch off the anabolic processes to rebalance the energy state (Hardie 2007). Besides these protective effects AMPK has diverse role in detecting and regulating not only the metabolism of peripheral tissues (e.g adipose tissue, liver, pancreatic  $\beta$  cells, skeletal muscle) but also energy sensing in the central nervous system, more closely, the hypothalamus and hence fine-tune whole body energy homeostasis. Moreover, AMPK is down regulated in most common chronic diseases like cancer, diabetes, inflammation, and obesity. Due to these facts, AMPK became a possible therapeutic target in the above mentioned diseases.

### **AMPK Activators**

AMPK acts as an energy sensor by sensing the ATP/AMP ratio. Therefore, AMPK activation under physiological conditions aims to restore energy balance in metabolic or energetic crisis where ATP content is compromised like in hypoxia, ischemia, low nutrient status or when ATP consumption elevated. As consequence, AMPK activation initiate catabolic processes to generate ATP and suppresses ATP consuming processes that are not necessary for the imminent survival of the cell.

Plenty of natural drugs, plant compounds, several hormones and artificial drugs are able to activate AMPK. We can distinguish four classes based on their mechanism of action. The first group of activators act indirectly via the inhibition of the mitochondrial ATP synthesis either through inhibiting Complex I (e.g., metformin or phenformin), Complex III (e.g., antimycin A) or the mitochondrial F1 ATP synthase (e.g., oligomycin) in the respiratory chain. All of these compounds also increase

cellular ADP/ATP and ATP/AMP ratios, although correlations between such ratios and changes in AMPK activity do not prove that AMPK activation by AMP or ADP is the only mechanism of activation.

The second group of activators are the pro-drugs that are converted inside the cells to AMP analogs, such as 5-aminoimidazole-4-carboxiamide riboside (AICAR). In our studies we applied AICAR to induce AMPK activation. AICAR is taken up by adenosine transporters and it is consequently phosphorylated by intracellular adenosine kinase to 5-aminoimidazole-4-carboxiamide-1-D-ribofuranosyl-5'-monophosphate (ZMP). ZMP is an AMP mimetic that binds to AMPK exactly the same sites as AMP and has the same effect on the activation of AMPK as AMP does. A study pointed out that antimetabolite methotrexate, we used combined with AICAR in our study, dramatically sensitizes cells to the activating effects of AICAR and showed a remarkable additive effect.

### **AMPK in Warburg metabolism**

Otto Warburg revealed that cancer cells have different metabolism as compared to normal, non-transformed cells. He hypothesized that reprogrammed metabolism is the essential cause of cancer.

Warburg effect can be identify in most cancer cells, among others, in breast cancer, which preferably produce energy via enhanced glycolysis in aerobic environment and produce lactate in thy cytosol. Furthermore, these cells are characterized with down regulated mitochondrial oxidation and elevated flux of glycolysis and pentose phosphate shunt in order to support rapid cell proliferation. Cancer cells have outstanding rate of glucose and glutamine consumption from external source and glycogen from internal source as substrates, of which, glucose breakdown via glycolysis is the main source of ATP. Moreover, elevated glycolytic rate can also boost the pentose phosphate pathway that enhance the de novo nucleotide synthesis. In tumor cells glycolysis and the TCA cycle are uncoupled. In the cytosol glycolysis provide intermediates for the pentose phosphate shunt to support nucleotide

production. Meanwhile, in the mitochondria, TCA cycle via alternative enzymes use glutamine for citrate production further supporting uncontrolled cell proliferation.

In cancer cells metabolic reprogramming increases the rate of aerobic glycolysis, furthermore, glycolysis and the TCA cycle uncouples. Glycolysis supports the pentose phosphate shunt with intermediers for nucleotide synthesis. TCA cycle is charged from alternative sources, such as glutamine, to produce citrate for fatty acid synthesis. In our experiments we inhibited nucleotide synthesis by the folate analog methotrexate (MTX) and used AICAR to stimulate AMPK.

With regard to rapid cell division, it is important to emphasize that the G1/S checkpoint is under metabolic control. Any interruption to the metabolic blocks the cell cycle at G1 to S transition. AMPK has intricate connections with other metabolic and energy sensing pathways like Akt, mTOR, Sirt1, PARPs and coordinate them. Importantly, upon AMPK activation mitochondrial oxidation and consequently mitochondrial biogenesis enhances that had been related to an anti-Warburg and antiproliferative effect on lymphomas.

To assess the possible role of AMPK in reverting Warburg metabolism and to investigate its antiproliferative effect we applied the pharmacological activator of AMPK 5-aminoimidazole-4-carboxiamide 1- $\beta$ -ribofuranoside (AICAR) and a folate-dependent 1-carbon metabolism analog methotrexate (MTX).

In our study, as an in vitro model, we used MCF7 breast cancer cells. Breast cancer is the most common cancer in women worldwide with close to 1.7 million new cases diagnosed in 2012. This represents about 12% of all new cancer cases and 25% of all cancers in women. Despite of the extensive prevention programs, it is still the fifth most common cause of death from cancer in women.

Breast cancer cells are described by Warburg rearrangements and importantly, endeavors to revert Warburg metabolism support chemotherapy. MTX can inhibit *de novo* nucleotide synthesis by hampering the folate-dependent 1 carbon metabolism. Methotrexate acts specifically during DNA and RNA synthesis, and thus it is cytotoxic during the S-phase of the cell cycle. In breast cancer chemotherapy

treatment MTX is used in combination with cyclophosphamide and 5-fluoro-uracil that is called CMF protocol.

The obvious relevance of Warburg metabolism in breast cancer suggested that the anti-Warburg metabolic rearrangements upon AMPK induction may have antiproliferative effect and possibly act synergistically with methotrexate.

### **The role of AMPK in beige adipocyte physiology**

The energy homeostasis of living organisms is determined by energy input and energy consumption. Any acute or long term disturbance to the energy balance leads to chronic metabolic diseases. Energy dissipation depends on the energy generated by the active biochemical processes (e.g. phosphorylation-dephosphorylation), physical exercise and mitochondrial oxidative phosphorylation (e.g. cardiac and skeletal muscle or brown adipose tissue).

Adipose tissue has fundamental role in maintaining organismal energy balance. There are three types of adipose tissue: white adipose tissue (WAT), brown adipose tissue (BAT) and the beige adipose tissue (merged of brown and white). WAT is the main adipose tissue in mammals with a function of storing excess energy. BAT and beige adipocytes have similar functions in rodents and mammals. Both of them have thermogenic capacity to break down glucose and fat and produce heat via the activation of uncoupling protein 1 (UCP1) which uncouple cellular respiration and mitochondrial ATP synthesis.

Upon adrenergic activation beige adipocytes enhance lipolysis, mitochondrial oxidation, mitochondrial biogenesis and creatin phosphate cycle. Several factors are able to induce beige differentiation and browning such as environmental stress, hormones, and inflammation. Functionally active and transplantable beige cells were found in humans and it is plausible the beige adipocytes have similar proportions in heat generation as skeletal muscle. Hormones such as irisin, BMP4, FGF<sub>21</sub>, GLP-1, NRG4 with the signal of AgRP neurons and serotonergic system can stimulate beige differentiation and browning.

It is important to note that fibrates or thiazolidinedione can also induce browning. Activation of beige adipocytes induce sirtuin 1 (Sirt1) that in turn deacetylates PPAR $\gamma$  that boosts peroxisome proliferator activated receptor cofactor-1 $\alpha$  (PGC1 $\alpha$ ) which results in elevated level of mitochondrial oxidation and mitochondrial biogenesis.

AMPK is not only an upstream regulator of Sirt1 and a master regulator of energy balance, but is also known to play role in brown adipose tissue differentiation through inducing mitochondrial oxidation and mitochondrial biogenesis.

It is therefore very likely AMPK can also affect beige adipose tissue differentiation.

## **AIM OF THE STUDY**

AMPK is a central actor in adaptation to energetic stress. We wanted to enlarge the current envelope of knowledge by studying the role of AMPK in reverting Warburg metabolism and better understanding its role in beige adipocyte differentiation.

We planned answering the following questions.

- What are the metabolic effect of the joint application of AICAR and methotrexate (MTX)?
- What are the molecular actors in the metabolic rearrangements caused by the joint application of AICAR and MTX?
- Does the joint application of AICAR and methotrexate change cellular proliferation?
- What is the role of AMPK in the differentiation of beige adipocytes from human adipose–derived mesenchyme stem cells (hADMSCs)?

## MATERIALS AND METHODS

### Cell cultures

Mouse mammary gland/breast tumor cells (4T1) were maintained in MEM (Sigma-Aldrich) supplemented with 10 % heat-inactivated FBS (Sigma-Aldrich) and 2mM L-glutamine (Invitrogene), and 1 % Penicillin/Streptomycin (Invitrogene).

Human breast adenocarcinoma (MCF-7) cells were maintained in MEM completed with 2 mM L-glutamine, 1% Penicillin/Streptomycin (Invitrogene) and 10 % heat-inactivated FBS.

Human adipose-derived mesenchymal stem cells (hADMSCs) were prepared from WAT of patients undergoing cardiac surgery (planned heart surgery, coronary bypass surgery, valve surgery, or Batista operation). Tissue samples were from the Institute of Cardiology, University of Debrecen, Debrecen, Hungary. Cells were isolated from the pericardial adipose tissue. hADMSC cells were cultured in DMEM-F12 medium enriched with 10 % heat-inactivated FBS (Gibco).

Human mammary gland/breast cancer cells (SKBR-3) were maintained in MEM (Sigma-Aldrich) supplemented with 10 % heat-inactivated FBS (Sigma-Aldrich) and 2mM L-glutamine (Invitrogene), 1 % Penicillin/Streptomycin (Invitrogene). All four human cell lines were subcultured in 37 °C incubator with humidified atmosphere of 5 % CO<sub>2</sub> between 60-90% confluency, and were passed after 2-3 days by trypsin-EDTA solution.

### AICAR and MTX treatment schemes

Cells were treated with the pharmacological activator of AMPK, 5'-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) and the antimetabolite methotrexate (MTX). 10uM MTX and 100um AICAR and their combination 10uM MTX+100uM AICAR were applied in the subsequent experiments. In control samples we use PBS as vehicle. Cells were treated with the previously described drugs for 1 or 6 days.

## **Caspase assay**

Caspase-3-like activity was measured by the cleavage of the fluorogenic tetrapeptide-amino-4-methylcoumarine conjugate (DEVD-AMC). After AICAR, MTX, and AICAR+ MTX treatment cells were pooled and resuspended in lysis buffer (10 mM HEPES, 0.1% w/v CHAPS, 5 mM DTT, 2 mM EDTA, 10 µg/ml aprotinin, 20 µg/ml leupeptin, 10 µg/ml pepstatin A, and 1 mM PMSF, pH 7.25). Cell lysates and substrates (50 µM) were combined in triplicates in caspase reaction buffer (100 mM HEPES, 10% sucrose, 5 mM DTT, 0.1% CHAPS, pH 7.25) at 37°C. Fluorescence of released AMC has been measured by a microplate fluorimeter (Thermo Labsystems Multiskan MS) at excitation wavelength of 380 nm and emission wavelength of 460 nm.

## **Cell cycle analysis**

MCF7 cells were seeded in 6-well plate (10 000 cells/well). Cells were treated with 100 µM 5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) and 10 µM Methotrexate (MTX) and its' combination at one and six days. Cell cycle analysis of AICAR and Methotrexate treated cells was carried out by the following fixation in cold 70 % EtOH and staining with 50 µg/ml propidium iodide by fluorescence-activated cell sorting (FACS) using a FACS Calibur instrument (Beckton Dickinson, Mountain View, CA,) at excitation wavelength of 535 nm and emission wavelength of 617 nm and the acquired data were processed with the BD CellQuest™ Pro. Software (Beckton Dickinson, Mountain View, CA).

## **Constructs, transfections**

For silencing assay the pSuper RNAi system was used. To create a small hairpin RNA (shRNA)-expressing construct, double stranded DNA oligonucleotides were cloned into the pSuper vector. The oligonucleotides (containing the siRNA sequence) were annealed in annealing buffer (150 mM NaCl, 1 mM EDTA, 50 mM Hepes, pH 8.0). The resulting duplexes carried BglIII and HindIII sites and were cloned into pSuper using these sites (siPGC1α1, siPGC1α2, siPGC1β, FOXO1, AMPKα1), resulting a ready to transfect mammalian expression vector that directs

intracellular synthesis of shRNA-like transcripts. Transfections were performed each day throughout the six days treatment using polyethylenimine (PEI) as a transfection reagent.

### **Isolation, culture and differentiation of human adipose derived mesenchymal stem cells (hADMSCs)**

Tissue samples were collected and processed on the day of the heart surgery. The adipose tissue were disentangled from fibrous parts and blood vessels, minced into small pieces and remaining tissue parts were digested in PBS with 120 U/ml collagenase for 1 hour at 37 °C with gentle agitation. The separated tissue was filtered through at a 100 µM pore size sieve to remove the unnecessary tissue parts. The cell suspension was centrifuged for 10 min at 1300 rpm and the pellet of stromal cells (hADMSCs) were resuspended in DMEM-F12 medium containing 10% FBS (Gibco) and seeded to the appropriate plate. After the cells reached confluency, differentiation was started. The protocol of Fischer–Posovszky and coworkers (Fischer-Posovszky et al. 2008) were used for white adipose cells differentiation, the protocol of Elabd and co-workers (Elabd et al. 2009) was used for brown adipose cell differentiation. 100 µM AICAR was applied to white adipocytes differentiation medium to activate AMPK. During the differentiation FBS free medium was used. Cells were differentiated for 14 days.

### **Sulforhodamine B assay**

Cells were seeded in 96-well plate (3000–5000 cells/well). Cells were grown in the presence of vehicle and treated with AICAR, MTX, AICAR+MTX at one and six days. At the end of the treatment, after fixation in situ by 50% trichloroacetic acid (TCA) cell were stained with sulforhodamine B (SRB) solution (0.4% in 1% acetic acid). Unbound dye was removed by washing with 1% acetic acid. Bound stain was solubilized with 10 mM TRIS base. Absorbance was read on an automated plate reader (Thermo Labsystems Multiskan MS) at 540 nm.

### **Measurement of mitochondrial membrane potential**

Mitochondrial membrane potential was determined by DioC6 (3,3-dihexyloxacarbocyanine iodide) staining. Cells were seeded in 96-well plate (25 000 cells/well). After one and six days treatment, cells were harvested by adding trypsin/EDTA, and the detached cells were stained with 40 nM DioC6 for 30 min then washed with phosphate-buffered saline (PBS). Cells were subjected to flow cytometric analysis (FACSCalibur, BD Biosciences) with 20,000 events collected for each sample; each measurement point was repeated in 3 parallel replicates. Control cells were treated with 10  $\mu$ M Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) to dissipate mitochondrial membrane potential. The value measured in the FCCP-treated cells were subtracted from all groups. The FCCP-corrected values were displayed and were used for statistical analysis.

### **Measurement of superoxide production**

Superoxide was measured using hydroethidine (HE) staining. Cells were prepared similarly to mitochondrial membrane potential measurement and were stained by 2 mM HE for 30min. Fluorescence was analyzed by flow cytometry (FACSCalibur, BD Biosciences). Superoxide production was indicated as a mean of HE fluorescence in each sample. Cells were subjected to flow cytometry analysis with 20,000 events collected for each sample, each measurement point was repeated in 3 parallel replicates.

### **Oxygen consumption**

Oxygen consumption was measured using an XF96 oximeter (Seahorse Biosciences, North Billerica, MA, USA). Cells were seeded in 96-well XF 96 assay plates (~2000 cell/well) and were treated as described before. Oxygen consumption rate (OCR, reflecting mitochondrial oxidation) and changes in pH, extracellular acidification rate (ECAR, reflecting glycolysis) were recorded every 30 min to follow transfection effect. Cells were treated with etomoxir (50  $\mu$ M), then oligomycin (10  $\mu$ M) and finally, antimycin (10  $\mu$ M). Data were normalized to protein content and normalized readings were used for calculations. OCR values after antimycin

treatment were subtracted from all other values. We named the readings for the untreated cells baseline OCR. OCR after etomoxir treatment represents the oxygen consumption related to glucose and amino acid oxidation (*Glc+AA*), while the difference between baseline and *Glc+AA* OCR represents fatty acid oxidation (*FAO*). The oligomycin-resistant respiration gives information on the leakage through the inner membrane of the mitochondria (*proton leak*). Data were normalized to protein content and normalized readings were displayed.

In the case of adipocytes cells were seeded and also differentiated in 96-well XF96 well assay plates. After differentiation the baseline oxygen consumption was rerecorded, than the cells were given a bolus dose of dibutryl-cAMP (500  $\mu$ M final concentration) pretending the adrenergic stimulation. Then the oxygen consumption was recorded every 30 minutes and the last reading took place at 7 hours post-treatment. Finally, a single bolus dose of antimycin A (10  $\mu$ M) were given to the cells for baseline correction. In both case data were normalized to protein content and normalized readings were displayed.

### **RNA isolation, reverse transcription and QPCR**

Total RNA was prepared using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Two micrograms of RNA were used for reverse transcription (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Foster City, CA, USA). Diluted cDNA was used for reverse transcription-coupled quantitative PCR (RT-qPCR). The qPCR reactions were performed with the qPCRBIO SyGreen Lo-ROX Supermix (PCR Biosystems) except for *TBX1* where the primer and probes were designed and supplied by Applied Biosystems (Taqman Hs00271949\_m1, Applied Biosystem) using a Light-Cycler 480 system (Roche Applied Science) for detection. Gene expression was normalized to the geometric mean of human *36B4*, *18S*, and *cyclophyllin* and *G6PD* values. Gene expression values were calculated based on the  $\Delta\Delta$ Ct method, where white adipose sample were designated as calibrator.

## **SDS-PAGE and Western Blotting**

Cells were lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 10% SDS, 1% Nonidet P-40, 1 mM Na<sub>3</sub>VO<sub>3</sub>, 1 mM NaF, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture, pH 8.0). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 8% acrylamide gels and blotted onto nitrocellulose membranes. After blocking in 5% (w/v) non-fat dry milk, the membranes were washed with 1x TW-TBS. Membranes were probed with primary antibodies. Polyclonal phospho-acetyl-CoA-carboxylase antibody (pACC, 1:500) (Cell Signaling, MA, USA), Cell Signaling anti rabbit antibody 1:1000) and polyclonal AMPK $\alpha$  (Sigma Aldrich, anti-rabbit antibody, 1:1000) were applied overnight at 4C° as a downstream sign of AMPK activity and monoclonal Anti- $\beta$ -Actin –Peroxidase antibody (1:20000) for 1 hour at room temperature.

## **Database screening**

AMPK $\alpha$ 1, FOXO1 and PGC1 $\alpha$  expression on breast cancer survival was assessed through the Kaplan-Meier plotter database (<http://kmplot.com/analysis/>) including those patients, where ER status was derived from gene expression data. Overall survival was analyzed.

## **Statistical analysis**

Significance was analyzed by Student's t test, for multiple comparisons ANOVA test was applied. Error bars represent  $\pm$  S.D., unless noted otherwise.

## RESULTS

### AMPK in Warburg

#### **Combined treatment of AICAR and MTX inhibits MCF-7 proliferation, lowering proliferation rate**

As the first step of the study, we determined the sensitivity of MCF-7 cells (a cellular model of invasive ductal breast carcinoma) to AICAR and MTX. Cells were treated with MTX (3–300  $\mu$ M) or AICAR treatment (100–1000  $\mu$ M) and in combination.

Cell proliferation was estimated through an invert microscope and on the sixth day of the assay total protein-that corresponds to cell number-was assessed using SRB assay. When cells were treated with MTX or AICAR individually we did not detect decreases in total protein content, however, the combination of AICAR and MTX resulted in a marked decrease of total protein. On day six, 10  $\mu$ M MTX + 100  $\mu$ M AICAR combination (abbreviated as AICAR+MTX) resulted 50% reduction in cell numbers; these AICAR and MTX concentrations were used in the subsequent experiments.

As next step we performed a time course experiment. The efficiency of AICAR+MTX combination became evident on the third day of treatment and turned statistically significant on day six. To measure the dynamics of biochemical changes we carried out all measurements at an early time point (1 day post treatment, short treatment) and late time point (6 days post treatment, prolonged treatment). An obvious explanation for reduced total protein upon AICAR+MTX treatment could be cell death. Importantly, we did not detect major increase in PI positive cells neither on day 1, nor on day 6 or increases in caspase activity (data not shown), suggesting that increased cell death is not the cause of reduced cell numbers, it is due to rather proliferation slow down.

## **The joint application of AICAR and MTX induces AMPK that reverts Warburg metabolism**

We tested whether AICAR indeed activates AMPK by monitoring the phosphorylation of acetyl-CoA carboxylase (pACC). On day 1, similarly to the results on proliferation, AICAR did not induce AMPK individually, only when applied in combination with MTX. At prolonged treatment, on day 6, MTX, AICAR and MTX+AICAR robustly induced AMPK. AMPK activation was translated into mitochondrial activation indicated by higher DioC6 fluorescence indicating increases in mitochondrial membrane potential. We continued our research with the measurements of the major metabolic regulator and effector genes. At day 1 we observed the induction of peroxisome proliferator activated receptor gamma coactivator (PGC)-1 $\alpha$  and forkhead transcription factor-1 (FOXO1) upon AICAR+MTX treatment. In line with these, the expression of ATP5g1, a subunit of ATP synthase and isocitrate dehydrogenase-2 (IDH2), a marker of tricarboxylic acid (TCA) cycle were induced slightly, suggesting that higher expression of PGC1 $\alpha$  and FOXO1 was translated into gene expression programs supporting mitochondrial activity. The expression of PGC1 $\alpha$  and FOXO1 further enhanced by day 6, furthermore, PGC1 $\beta$ , another key mitotropic regulator boosted upon AICAR+MTX treatment. Consequently, at day 6 enhanced expression of ATP5g1 and IDH2 in the AICAR+MTX treated cells was exacerbated, furthermore, other TCA cycle enzymes expression level, fumarase and aconitase-2 (ACO2) were also elevated. As it was showed that anti-Warburg rearrangement of metabolism leads to the suppression of glycolysis, we tried to compare the rate of glycolysis and mitochondrial oxygen consumption by using the Seahorse XF96 instrument. Surprisingly, on day 1 in AICAR+MTX treated cells glycolysis was induced, evidenced by decreases in the ratio of oxygen consumption rate (OCR) / extracellular acidification rate (ECAR). In contrast to that, on day 6 OCR/ECAR ratio increased in AICAR+MTX pointing out increased mitochondrial activity and lower dependence of cells on glycolysis. Taken together treating MCF-7 cells with AICAR+MTX at early time point (i.e. day 1) moderately induces mitochondrial metabolism, but supports glycolysis-paradoxically

bringing about rather pro-Warburg changes. However, prolonged (i.e. day 6) AICAR+MTX treatment largely induces mitochondrial oxidation and suppresses glycolysis setting off true anti-Warburg changes.

### **AICAR+MTX treatment leads to G1/S and G2/M blockade**

Our preliminary data suggested an anti-Warburg rearrangement of cellular metabolism upon AICAR+MTX treatment, therefore we set out to analyze changes in cell cycle. Short term AICAR+MTX treatment (day 1) reduced the number of cells in S and slightly elevated the number of cells in G2. When we checked the ratios between different cell cycle phases, it was apparent that G1/S and G2/S ratios increased, while G1/G2 ratio remained constant suggesting G2/M and probably G1/S block. Prolonged AICAR+MTX treatment (day 6) showed similar changes, elevated proportion of G2 cells with decreased number of cells in S phase and increases in the proportions of cells in G1 and G2 as compared to the number of cells in S-signs of simultaneous G1/S and G2/M block in the cell cycle.

### **Combined treatment of SKBR-3 and 4T1 cells with AICAR and MTX reduces cell**

We verified our findings on two other breast cancer cell lines. The human SKBR-3 and the murine 4T1. SKBR-3 cells, similarly to MCF-7, were not sensitive to MTX and AICAR when administered alone. Meanwhile the AICAR+MTX combination slowed down cellular proliferation that coincided with increases in the expression of several markers of mitochondrial oxidation (FOXO1, PGC1 $\alpha$ , PGC1 $\beta$ , fumarase, IDH2 and ACO2) that are the same as in the case of MCF-7. 4T1 cells were very sensitive to MTX, roughly thousand fold, less MTX was already antiproliferative in 4T1 cells as compared to MCF-7 or SKBR-3. Despite the changes in sensitivity we did observe a slightly enhanced antiproliferative effect of the 300  $\mu$ M AICAR + 7.8 nM MTX or 300  $\mu$ M AICAR + 15.6 nM MTX combination as compared to the individual components alone. Similarly to MCF-7 and SKBR-3 300  $\mu$ M AICAR + 7.8 nM MTX treatment induced slightly the expression of FOXO1, ATP5g1, fumarase and IDH2. Taken together, the additive effect of AICAR+MTX

treatment is not specific for MCF-7 but it acts similarly in other breast cancer cell lines as well. In order to determine the specificity of AICAR+MTX treatment we tested two other cell lines, WM35 that is a model for melanoma and SAOS that is a model for osteosarcoma; none of them was susceptible to the MTX+AICAR combination (data not shown).

### **The inhibitory properties of the AICAR+MTX treatment can be reverted by the silencing of mitotrophic transcription factors**

AICAR+MTX treatment brought about anti-Warburg alterations in metabolism and led to G1/S and G2/M block in cell cycle. Enhanced expression of PGC1 $\alpha$ , PGC1 $\beta$  and FOXO1 correlates with enhanced mitochondrial activity and the slowdown of proliferation suggesting central role for these proteins in the antiproliferative effect of AICAR+MTX treatment. We prepared shRNA expressing constructs (siPGC1 $\alpha$ 1, siPGC1 $\alpha$ 2, siPGC1 $\beta$ , FOXO1, AMPK $\alpha$ 1) targeting these proteins in order to assess their possible role. Both short (until day 1) and long term (until day 6) silencing (transfection each day) efficiently reduced the expression of target mRNA. When these constructs were transfected into MCF-7 cells the proliferation of the cells doubled. Furthermore, reduced cell proliferation upon AICAR+MTX treatment was abolished by the end of the 6 day treatment.

### **Expression of AMPK $\alpha$ 1 and FOXO1 positively correlate with survival in breast cancer**

The previously performed experiments nominated AMPK and other mitochondrial transcription factors (PGC's, FOXO1) as possible targets in human breast cancer. To validate this possibility we screened a publicly available cancer gene expression database, (Kaplan-Meier plotter, [kmplot.com](http://kmplot.com)). When comparing the lowest and the highest expression quartile, higher expression of AMPK $\alpha$ 1 and FOXO1 conferred significantly longer survival to the high expression quartile as compared to the lowest expression quartile. Higher expression of PGC1 $\alpha$  did not confer longer survival (data not shown).

## **The role of AMPK in beige adipocyte differentiation**

### **AICAR-induced AMPK activation leads to beige-like morphological changes in hADMSCs-derived white adipocytes**

In the study we used hADMSC cells to model white and beige adipocytes. Each hADMSCs cell line (= each individual) was differentiated in three directions, namely towards beige adipocytes, white adipocytes and AICAR-treated white adipocytes. First, we measured the AMPK activity in the three groups at the end of the differentiation. AMPK activity was higher in beige than in white adipocytes. Importantly, the treatment of white adipocytes with 100  $\mu$ M AICAR enhanced AMPK activity almost to the same extent as in beige adipocytes that suggested a role for AMPK in beige adipocyte differentiation and function. AICAR treatment of hADMSCs-derived white adipocytes does not yield functional beige adipocytes. We continued our investigation by assessing the biological functions of the beige adipocytes. Beige cells rely on the mitochondrial oxidation and mitochondrial biogenesis. As next step, we investigated mitochondrial oxygen consumption rate. A study highlighted that beige adipocytes showed higher basal and cAMP-simulated oxygen consumption rate than white adipocytes. AMPK activation by AICAR did not increase the oxygen consumption rate of the white adipocytes. Although, the lack of the induction of OCR upon treatment with AICAR was surprising, we continued our experiments by measuring the mRNA levels of some beige differentiation marker genes, Uncoupling protein-1 (UCP1), cell death-inducing DFFA-like effector A (CIDEA), PR domain containing 16 (PRDM16), transmembrane protein 26 (TMEM26) and T-box protein 1 (TBX-1). The above-mentioned markers were expressed in higher levels in beige cells compared to white adipose cells, but we were not able to measure any increment upon AICAR treatment comparing to white adipose cells.

## SUMMARY

The AMP-activated protein kinase (AMPK) is one of the most important energy sensor in the human body. AMPK is activated by decreases in the ATP/AMP ratio. AMPK activation has several organ or tissue specific physiological or pathological effects that all finally result in enhanced glucose and fatty acid oxidation and mitochondrial biogenesis to restore cellular ATP levels. Cancer cells often undergo metabolic alterations that can be characterized by depressed mitochondrial oxidation and enhanced glycolysis in order to support the uncontrolled and rapid cell proliferation that is called Warburg metabolism. AMPK can exert an anti-Warburg effect through enhancing of mitochondrial biogenesis. We provided evidence a combination of AICAR, a pharmacology activator of AMP-activated protein kinase, together with the folate-analog methotrexate (MTX) can slow down breast cancer cell proliferation. The AICAR+MTX combination enhanced mitochondrial oxidation and reduced glycolytic rate. These metabolic alterations went together with a slowdown of the G1/S and G2/M transition that slowed down the cell cycle. The high level of expression of transcription factors, PGC1 $\alpha$ , PGC1 $\beta$ , and FOXO1 were responsible for enhanced mitochondrial oxidation. The slowdown of cell proliferation was abolished when the mitochondrial transcription factors, PGC1 $\alpha$ , PGC1 $\beta$ , and FOXO1 were silenced.

We also assessed the possible role of AMPK in beige cell differentiation using human adipose-derived mesenchymal stem cells (hADMSCs) originated from pericardial adipose tissue as model. In the differentiation process of hADMSCs, white adipocytes were induced by 100  $\mu$ M AICAR. AICAR was able to boost AMPK activity in white adipocytes to a similar extent as in beige adipocytes. AICAR treatment did bring AMPK activation of white adipocytes similar to the one in beige adipocytes. However, we were unable to detect functional changes in the AICAR-treated white adipocytes, neither mitochondrial oxidation, nor the expression of marker genes (TBX1, UCP1, CIDEA, PRDM16 and TMEM26) was comparable to beige adipocytes.

Our experiments provided evidence that AICAR-induced AMPK activation has an exploitable potential in treating metabolic and neoplastic diseases.



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### List of publications related to the dissertation

1. Abdul-Rahman, O., Kristóf, E., Doan-Xuan, Q. M., Vida, A., Nagy, L., Horváth, A., Simon, J., Maros, T. M., Szentkirályi, I., Palotás, L., Debreceni, T., Csizmadia, P., Szerafin, T., **Fodor, T.**, Szántó, M., Tóth, A., Kiss, B. K., Bacsó, Z., Bai, P.: AMP-Activated Kinase (AMPK) Activation by AICAR in Human White Adipocytes Derived from Pericardial White Adipose Tissue Stem Cells Induces a Partial Beige-Like Phenotype.  
*PLoS One*. 11 (6), e0157644, 2016.  
DOI: <http://dx.doi.org/10.1371/journal.pone.0157644>  
IF: 2.806
2. **Fodor, T.**, Szántó, M., Abdul-Rahman, O., Nagy, L., Dér, Á., Kiss, B. K., Bai, P.: Combined Treatment of MCF-7 Cells with AICAR and Methotrexate, Arrests Cell Cycle and Reverses Warburg Metabolism through AMP-Activated Protein Kinase (AMPK) and FOXO1.  
*PLoS One*. 11 (2), 1-16, 2016.  
DOI: <http://dx.doi.org/10.1371/journal.pone.0150232>  
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### List of other publications

3. Mikó, E., Kovács, T., **Fodor, T.**, Bai, P.: Methods to assess to role of poly(ADP-ribose) polymerases in regulating mitochondrial oxidation.  
*Methods Mol. Biol.* 1608, 185-200, 2017.
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*Arch. Dermatol. Res.* 306 (10), 885-902, 2014.  
DOI: <http://dx.doi.org/10.1007/s00403-014-1511-5>  
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## ORAL AND POSTER PRESENTATIONS

### Presentations

**Tamás Fodor**, Magdolna Szántó , Judit Márton , Lilla Nagy , Péter Bai The role of AMPK in reversing Warburg metabolism Galyatető (2016)

Marton J, Nagy L, **Fodor T**, Bhattoa Pal H, Bai P *PARP2 interferes with skeletal muscle cholesterol homeostasis and steroid metabolism*. PARP Family and Friends, Cold Spring Harbor, NY, USA (2016)

**Tamás Fodor** , Magdolna Szántó , Omar Abdul-Rahman, Judit Márton , Lilla Nagy , Péter Bai Combined treatment of MCF7 cell with AICAR and methotrexate, arrests cell cycle and reverses Warburg metabolism through AMP-activated protein kinase (AMPK) and FOXO1, Debrecen (2015)

**Fodor T**, Mikó E, Vida A, Kovács T, Abdul-Rahman O, Nagy L, Trencsényi Gy, Bai P *New ways to revert Warburg-type metabolism*. FEBS3+ meeting, Protoroz, Slovenia (2015)

**Fodor T** The analysis of AMPK activation in MCF7 cell modell, UK, Cambridge (2014)

**Fodor T** The analysis of AMPK activation in different cell models. Signaling pathways in cancer biology, Mátraháza (2014)

Szántó M, **Fodor T**, Nagy L, Márton J, Csumita M, Kiss B, Gergely P, Virág L, Bai P Poly (ADP-ribose) polymerase enzymes in metabolic regulation. MÉT Vándorgyűlés, Budapest (2013)

**Fodor T** (2013) The role of AMPK activation in the changes of mitochondrial activity in various cell models. Signaling pathways in cancer biology, Egerszalók (2013)

Szántó M, Brunyánszki A, Kiss B, Cantó C, Tóth A, **Fodor T**, Bai P

The role of PARP-2 in the regulation of SIRT1 expression and mitochondrial respiration – metabolism and oxidative stress. PARP2013, Quebec City, Canada (2013)

### **Poster presentations**

Nagy L, Docsa T, Tóth A, Somsák L, **Fodor T**, Gönczi M, Gergely P, Bai P (2015) Glycogen phosphorylase inhibition has positive effect on metabolism. FEBS3+ Portoroz, Slovenia

Szántó M, Brunyánszki A, Márton J, Nagy L, **Fodor T**, Kiss B, Bai P (2014) The role of poly(ADP-ribose) polymerase enzymes in the regulation of cholesterol homeostasis *Frontiers in Metabolism: From Molecular Physiology to Systems Medicine* EMBL Heidelberg 2014

Márton J, Szántó M, Nagy L, **Fodor T**, Kiss B, Bai P (2014) The role of poly(ADP-ribose) polymerase-2 in the cholesterol homeostasis. *MBKE Debrecen* 2014

**Tamás Fodor**, Magdolna Szántó, Judit Márton, Lilla Nagy, Péter Bai

Study of anti-Warburg effect of AMPK activators and antimetabolite drugs on MCF7 cell model *Conference of the Hungarian Society for Biochemistry, Debrecen, Hungary* (2014) Poster award winner 2nd prize

**Tamás Fodor**, Magdolna Szántó, Judit Márton, Lilla Nagy, Péter Bai

Study of anti-Warburg effect of AMPK activators and antimetabolite drugs on MCF7 cell model *FEBS, Paris, France* (2014)

**Fodor T**, Szántó M, Nagy L, Brunyánszki A, Gergely P, Virág L, Bai P

The role of PARP10 enzyme in mitochondrial metabolism. *Hungarian Molecular Life Sciences Siófok, Hungary* (2013)

**Fodor T**, Szántó M, Nagy L, Brunyánszki A, Kiss B, Gergely P, Virág L, Bay P  
The role of PARP10 in metabolic regulation. Conference of the Hungarian Society  
for Physiology, Budapest, Hungary (2013)

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