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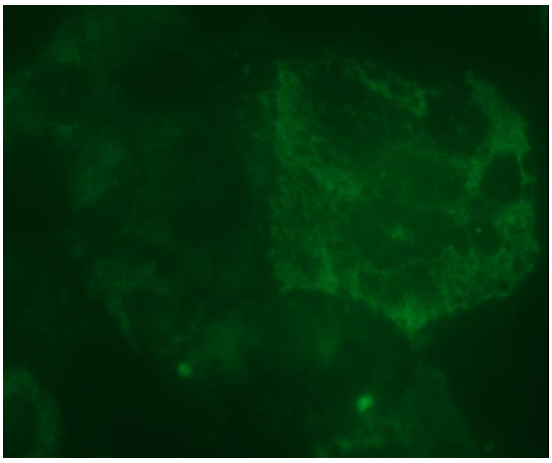
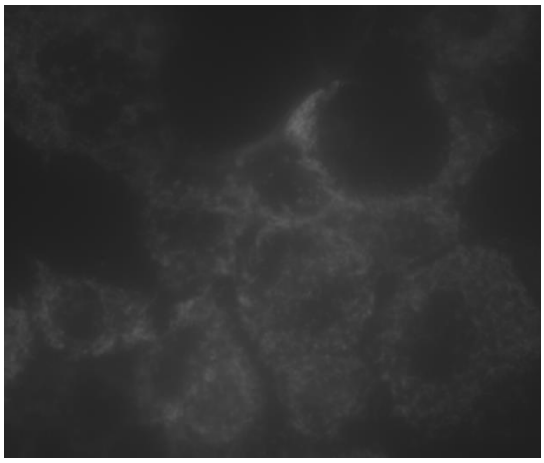
Effect of energy substrate switching on mitochondrial respiration of Caco-2 cells.

Master Thesis





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HAP-80424


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Abstract

Caco2 cells are often used as an *in vitro* model to obtain a deeper knowledge about the biological reactions of the intestinal barrier. During the differentiation process, Caco2 cells develop many similarities with small intestinal epithelial cells. However, the metabolism of Caco2 cells still differs from human healthy cells; in the first case is principally glycolytic while in the second case is mainly aerobic. In some experiments, these metabolic differences could affect outcomes and Caco2 cells may not represent a reliable *in vitro* model. One reason of these differences may be due to the high glucose availability of the medium in which Caco2 cells are routinely cultured, which favours the anaerobic metabolism. Switching the energy substrates of the medium, from glucose to galactose, may improve the aerobic metabolism. Undifferentiated and differentiate Caco2 cells were seeded in glucose (control group) or galactose(treatment group) medium and eventually differences in the expression of oxidative phosphorylation (OXPHOS) complexes were analysed through Western Blot. Moreover, differentiated Caco2 cells were stimulated with CI OXPHOS inhibitors, rotenone or piericidin A, and there were measured the levels of phosphorylated AMPK (pAMPK) and phosphorylated ACC (pACC)after the stimulation. Differentiated Caco2 cells, grown in glucose or galactose, were treated with the CI inhibitor rotenone or piericidin A at 0nM (control group) or 200nM (treatment group) and eventually differences in pAMPK and pACC ratios were detected through Western Blot. Differentiated Caco2 cells cultured in galactose medium had higher expression of CI, CIII, CIV and the sum of all OXPHOS complexes. For the undifferentiated Caco2 cells, there was find a significant increase of CIV expression only. The ratio of pAMPK/AMPK was found to be significantly higher for Caco2 cells cultured in galactose only when treated with 200nM of both CI inhibitors while for pACC/ACC significant differences were not found in any of the treatment groups. The result suggest that the switching of energy substrate in the culture medium, from glucose to galactose, caused an increase of aerobic metabolism, which could lead to have a more representative *in vitro* model of the intestinal barrier

Abbreviations

Acetyl-CoA carboxylase	ACC
Adenosine triphosphate	ATP
AMP-activated protein kinase	AMPK
Colorectal cancer cell line	Caco2
Flavin adenine dinucleotide	$FADH_2$
Indomethacin	INDO
Nicotinamide adenine dinucleotide reduced	NADH
oxygen consumption rate	OCR
Oxidative Phosphorylation	OXPHOS
Phosphorylated Acetyl-CoA carboxylase	pACC
Phosphorylated AMP-activated protein kinase	pAMPK
polyphenols	PPs
Tricarboxylic acid cycle	TCA cycle

1. Introduction

1.1 Intestinal barrier

The small intestine is the site for the organism responsible of macro and micro nutrient absorption in the bloodstream. The intestinal wall is divided in four layers: mucosa, submucosa, muscularis and serosa. The mucosa is composed by a monolayer of epithelial cells, called enterocytes, which are organized in villi and microvilli. The presence of villi and microvilli allows a larger exchange surface with nutrients, which increaseS the capacity of nutrient absorption(1). At the same time, the enterocytes layer blocks the passage of potential pathogen agents (such as bacteria or virus) and toxic molecules (such as mycotoxins)(2). For this reason intestinal barrier is considered as a semipermeable barrier.

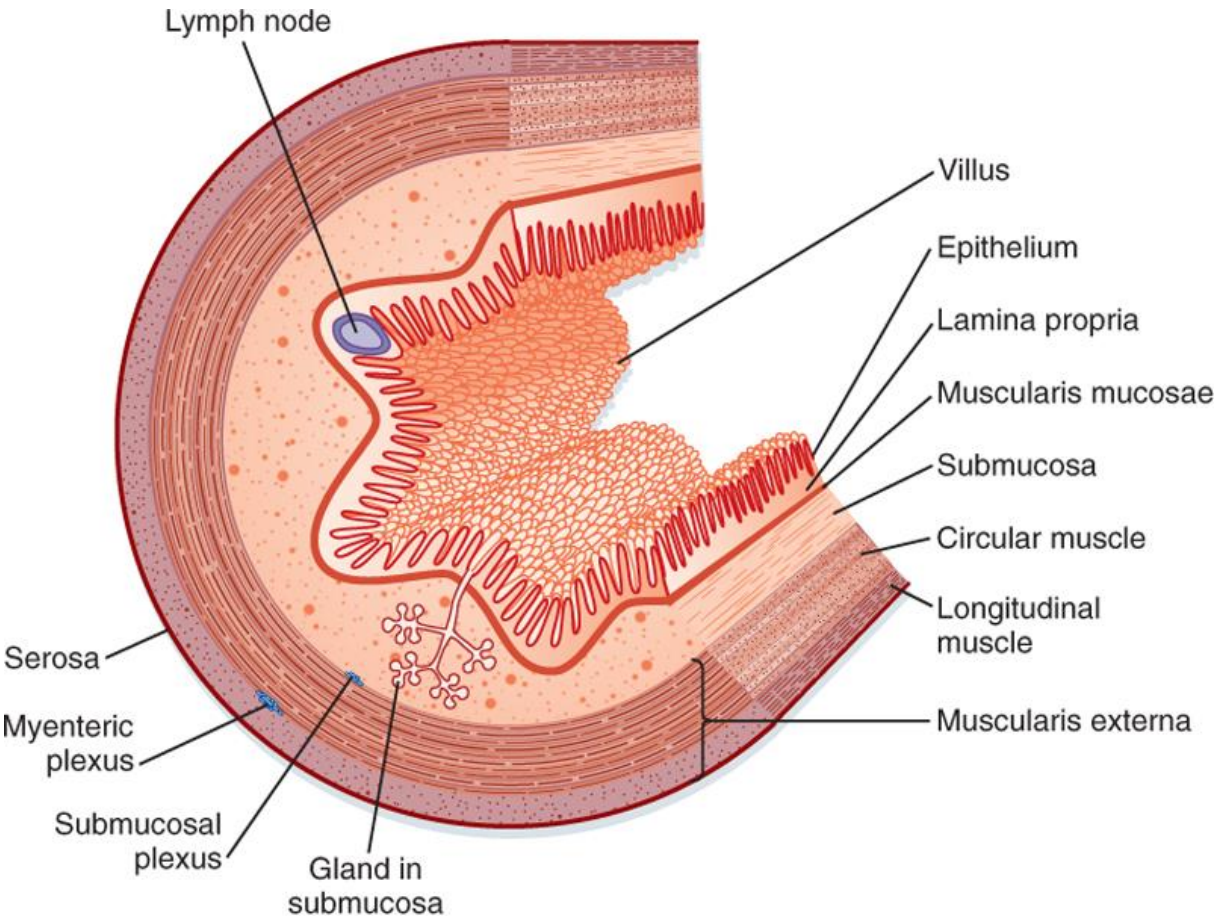

Figure 1 Gross anatomy of intestinal (users.atw.hu). *Layers of intestinal barrier: mucosa, submucosa, muscularis and serosa. Higher absorption of nutrients is made possible by the presence of villi and microvilli, which increases the exchange surface.*

Stress situations may compromise the blocking activity of enterocytes thereby increasing intestinal permeability (3), which may lead to abdominal cramps, nausea and diarrhoea (4)

and higher infection risk (5). To study this mechanism *in vitro* cell models, which use animal or human cells. For example,cancer cells are often used as *in vitro* model and one of the most studied are Colorectal cancer cell line(Caco2).

1.2 Caco2 Cells

Caco2 are human epithelial colorectal adenocarcinoma cells, which after differentiation and formation of a monolayer develops characteristics of enterocytes (6). After differentiation, Caco2 cells have a cylindrical morphology with microvilli, tight junctions between cells and the presence of some hydrolase as sucrase-isomaltase, lactase and aminopeptidase (6). Therefore, Caco2 cells are useful to recreate an *in vitro* intestinal model suitable for many experiments. For example Caco2 cells are used for toxicological research, for example to study the possible toxic effect and the mechanism of chemicals compounds in the intestine (7; 8). Other examples of cells used are HT-29, HCT-EB and HCT-GEO, however, Caco2 cells seem to have higher differentiation activities compared with these cancer cell lines (9).

Caco2 cells, after differentiation, develop many characteristics of human intestinal cells, but other traits still differ from healthy human cells. For instance, energy metabolism of Caco2 cells has significant differences with enterocytes. Tumour cells rely principally on anaerobic metabolism while primary human cells (and tissues) use mainly their aerobic metabolism (9). In some situations, intestinal permeability may be influenced by the energy metabolism the cell is (9) For this reason, the use of Caco2 cells as *in vitro* model could be more complicate or not be a representative model. If Caco2 cells develop an aerobic metabolism, they may represent a more reliable model of intestinal barrier.

1.3 Energy Metabolism

1.3.1 Glycolysis

In aerobic situations the glycolysis process a molecule of glucose is converts a molecule of glucose in two molecules of pyruvate. During this process two molecules of Nicotinamide adenine dinucleotide reduced (NADH) and two molecules of Adenosine triphosphate (ATP) are released (10). In healthy cells, pyruvate is converted in acetyl CoA, catalysed by pyruvate dehydrogenase, which enters the Tricarboxylic acid cycle (TCA) cycle located in the mitochondria. NADH and Flavin adenine dinucleotide ($FADH_{2)}$, produced by glycolysis and TCA cycle, are transformed in ATP through Oxidative Phosphorylation (OXPHOS), which completes the aerobic process. The conversion of NADH and $FADH_2$ yields respectively three and two molecules of ATP (10). In anaerobic situations or in conditions of high glucose availability, pyruvate is converted in lactate, through lactate dehydrogenase, which consumes a molecule NADH completing the fermentation process. The net of ATP yields, at the end of fermentation, is two molecules of ATP (10).

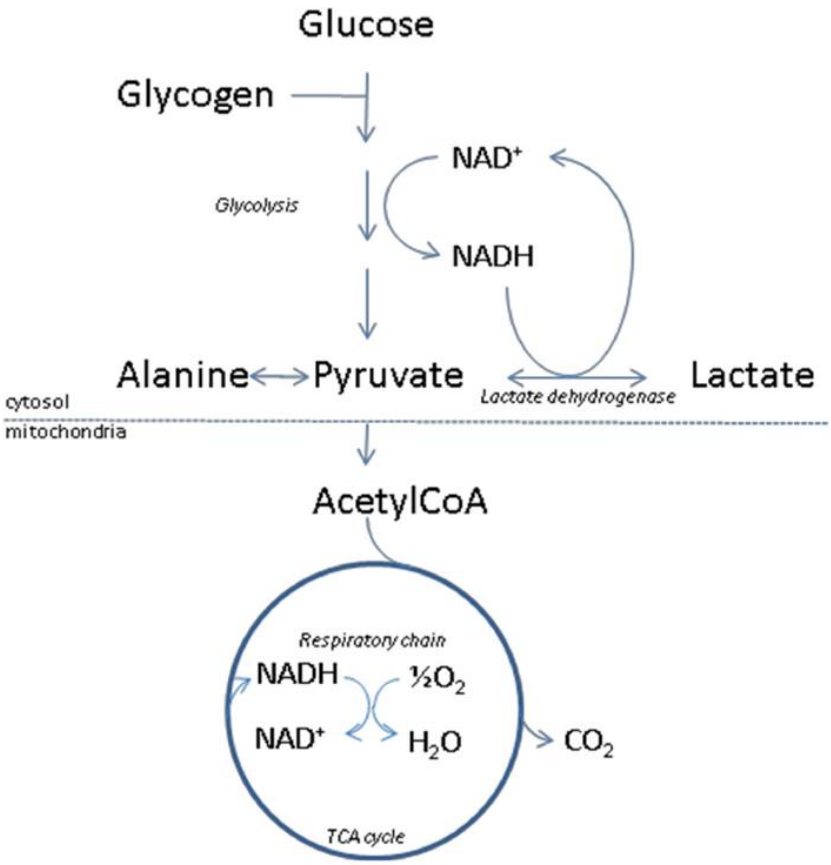

Figure 2 Glycolysis pathways (Kruse,2011). *One molecule of glucose is catabolized in two molecules of pyruvate releasing two molecules of NADH and two of ATP. In anaerobic or high glucoseavailability situations pyruvate is converted in lactate through lactate dehydrogenase and the oxidation of NADH. In aerobic situations pyruvate moves into mitochondria where it is transformed into Acetyl-CoA, which enters the TCA cycle.*

1.3.2 Mitochondria

Mitochondria are the organelles where TCA aerobic metabolism is located and therefore known to have an important role in ATP production. Mitochondria are composed by two membranes: mitochondrial outer membrane, which separates mitochondria from cytosol of the cell, and mitochondrial inner membrane between the mitochondrial matrix and the inter-membrane space (Fig 5). The TCA cycle is situated in the mitochondrial matrix, while the OXPHOS in the mitochondrial inner membrane (11).

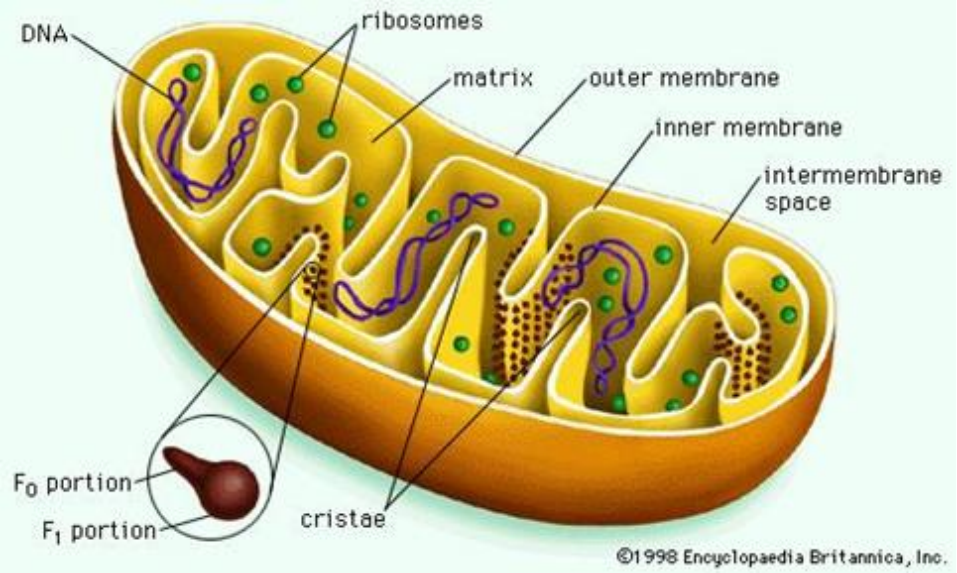

Figure 5: Mitochondria structure (www.britannica.com). *Mitochondria are composed by two membranes: mitochondrial outer membrane and mitochondrial inner membrane .The first one separates mitochondria from the rest of the cell while the second one separates the mitochondrial matrix from the inter-membrane space.*

1.3.3 Tricarboxylic acid cycle

Pyruvate, released by glycolysis, is transported from the cytosol to the mitochondrial matrix and converted into acetyl CoA by pyruvate dehydrogenase. Acetyl CoA transits in the TCA cycle and binds with oxaloacetate forming a molecule of citrate. Citrate is oxidized by a sequence of reactions (fig.3) which yield three NADH, one ATP, and one $FADH_2$ molecules and one of oxaloacetate allowing repetition of the cycle (12) .

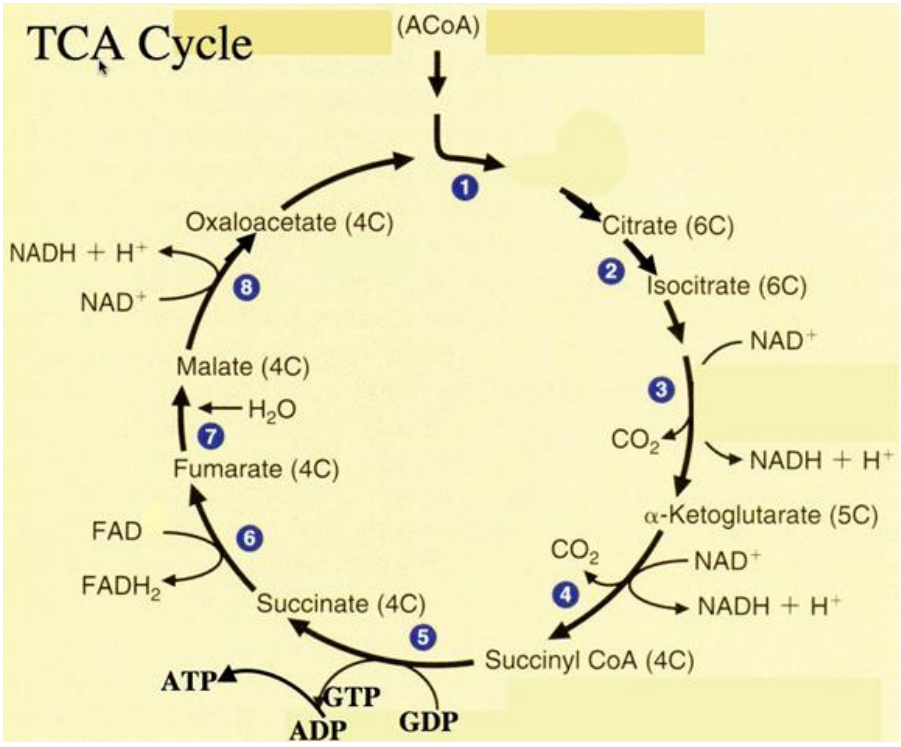

Figure 3 Tricarboxylic acid cycle **(www.unm.edu).** *Acetyl CoA binds with Oxaloacetate and starts a sequence of reactions which end with the formation of Oxaloacetate. For each molecule of Acetyl CoA that enters the TCA cycle, three molecules of NADH, one FADH2 and one ATP are released.*

1.3.4 Oxidative Phosphorylation

OXPHOS is the process where $NADH_2$ and FADH molecules, released by glycolysis and TCA cycle, are converted into ATP (13). The OXPHOS system is located in the mitochondrial inner membrane and it is composed by five complexes (CI, CII,CIII,CIV and CV). The first four complexes form the electron transport chain where the electrons, released by the oxidation of NADH or $FADH_2$, are transported to (O_2), which acts as the final acceptor, producing H_2O. The oxidation of NADH2 and $FADH_2$ starts respectively at CI and CII. A special carrier, called coenzyme Q_{10}, transports the electrons (released by the oxidation of NADH and $FADH_2$) to CIII. Cytochrome-c is the electron carrier from CIII to CIV where the electron transport chain ends with the donation of the electrons to O_2. During this process protons (H^+) are expelled out of the matrix into the inner membrane space. This creates an electrochemical proton-motive force between the mitochondrial matrix and the inter-membrane space. This force drives protons to re-enter in the mitochondrial matrix through CV (ATP synthase protein) and actives the synthesis of ATP from a molecule of ADP and inorganic phosphate (13) (fig4).

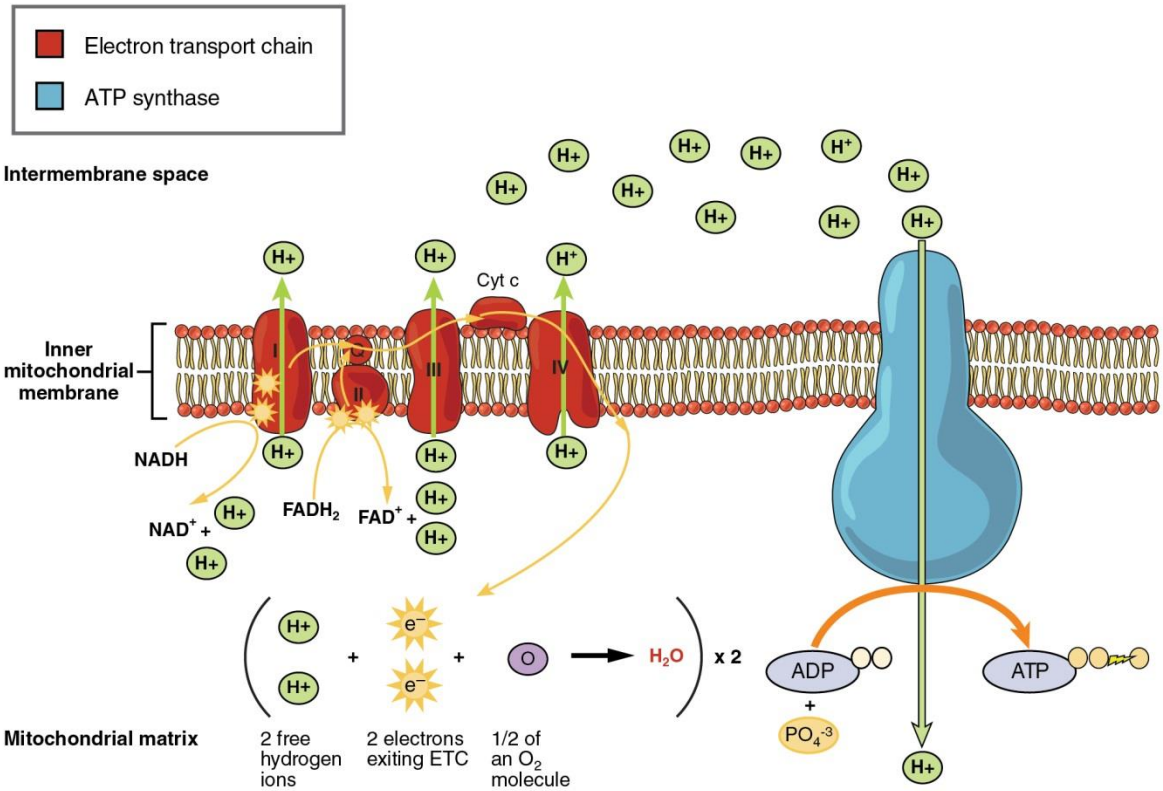

Figure 4 Oxidative phosphorylation (www.slideshare.net). *NADH and $FADH_2$ are oxidized by CI and CII, respectively. The electrons, released by the oxidations, transit to CIII and subsequently to CIV where the Electron transport chain ends with the donation of two the electrons to O_2.The electrons migration to CIV pumps out protons, from the Inner mitochondrial membrane, creating an electrochemical proton-motive force. Protons renters into mitochondrial matrix through ATP synthase (CV) which generates ATP.*

1.4 Metabolism of Caco2 cells and human healthy cells

In vivo situations healthy human cells, in presence of oxygen, produce ATP through glycolysis, TCA cycle and OXPHOS (16; 17). However *in vitro* situations, tumour cells mainly use glycolysis instead of TCA and OXPHOS, even in presence of O_2. This process is called "aerobic glycolysis" or "Crabtree effect "(14;15). The mechanism, which explain this process, is not totally clear, but high glucose availability, of the medium used during cells cultivation, seems to be one of the main factors that explain the inhibition of OXPHOS (16;17). Also environments of low O_2 availability favour glycolysis instead of mitochondrial respiration ("Warburg effect") (14). The combination of glycolysis, TCA cycle, and OXPHOS yields 36 molecules of ATP from one molecule of glucose, while glycolysis process yields two molecule of ATP. To reach adequate energy levels, tumour cells develop higher glycolytic activity compared to healthy cells (14).

1.5 Galactose metabolism

Substrate switching already improved the OXPHOS activity of some cancer cells (16;18). One example of substrate switching is the change of the medium energy substrate from glucose to galactose (16;18). Galactose is converted to pyruvate trough Glycolysis, as for glucose. Before entering in the glycolysis pathway galactose is catabolized into galactose-1-phosphate and subsequently in glucose-1-phosphate by two different enzymes: Galactokinase and Uridyl transferase respectively (19). Glucose-1-phosphate enter in the glycolytic pathway through his conversion in Glucose-6-phosphate, catabolized by phospho-glucomutase (fig6).

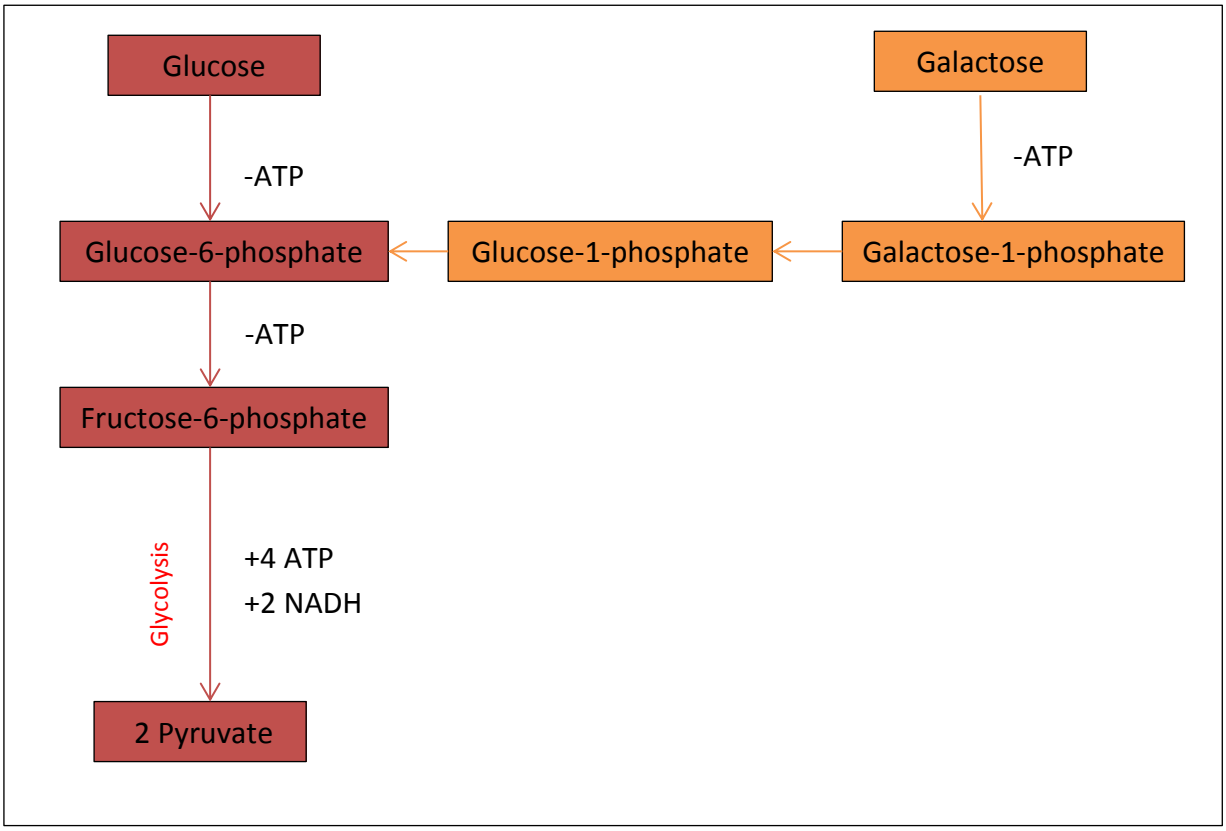

Figure 6 Metabolism of glucose and galactose. *Galactose is converted in Galactose -1-phosphate before enter in the glycolytic process and subsequently in Glucose-1-phosphate respectively by Galactokinase and Uridyl transferase. Glucose-1-phosphate is transformed in Glucose-6-phosphate and entering the glycolysis. No differences in ATP yields are present between the two substrates.*

The pathway mechanism of the improvement of aerobic metabolism due to galactose switching is not totally clear. One possibility could be related the initial steps of galactose metabolism before enter in the glycolytic pathway, which may slow the glycolysis process and consequently improving aerobic metabolism (20).

1.6 Inhibitors of OXPHOS

1.6.1 Chemical inhibitors

OXPHOS can be inhibited by chemical agents, which can impair the complex activity of the electron transport chain. For example, CI can be inhibited by piericidin A and rotenone limiting the oxidation of NADH (21). Other examples of chemical agents that may impair protein complexes of OXPHOS are antimycin A and oligomycins that inhibit CIII and CV, respectively.

1.6.2 Natural Inhibitors

Situations of high glucose availability, such as the regular Caco2 cells medium, may inhibit OXPHOS even in the presence of O_2 (18). This process, previously described, is called "Crabtree effect" which is more common in yeasts, but it can also be found in cancer cells and healthy cells (15). Another potential inhibitor of OXPHOS is hypoxia ("Warburg effect") due to a lack of the final acceptor of electrons (O_2) which is indispensable for the OXPHOS (22).

1.6.3 Energy status after OXPHOS complex inhibitors

As explained previously, in healthy cells most ATP is released by the combination of TCA and OXPHOS, while in tumour cells the main source of energy is the glycolytic process"(14). The administration of OXPHOS inhibitors, rotenone and piericidin A, to human and cancer cells should have different effects on the energy status. There would be a significant decrease of ATP levels for human cells which are more dependent on OXPHOS for their energy, while in the case of cancer cells there would be less effects on energy status because most ATP is produced by glycolysis (23).

1.7 Aim

Caco2 cell line is a representative *in vitro* model of the intestinal barrier, but the metabolism of these tumour cells differs from human cells. The aim of the research was to have a deeper knowledge about the effects of the energy substrate changing on OXPHOS protein density. Studies already demonstrated that energy substrate switching, from glucose to galactose increases OXPHOS protein density in many cancer cells, such as HeLa, HT-29 and HepG2 (16;18). Based on past studies, we expect the same increase in OXPHOS protein density in Caco2 cells upon the substrate switch from galactose to glucose. This should eventually improve the aerobic metabolism in Caco2 cells. The second aim was to study the effect of CI inhibitors, rotenone and piericidin A, on Caco2 cells energy status cultured in galactose and glucose. For the Caco2 cells seeded in galactose medium the contribution of aerobic metabolism should be higher compared to the glucose medium conditions. For this reason, the administration of OXPHOS inhibitors may cause higher energy stress for Caco2 cells incubated in galactose medium.

2. Material and Methods

2.1 Caco2 cells culture

Dulbecco's modified eagle media(DMEM) (Gibco), at concentration 25mM, was used to maintain the cell layer, with the supplementation of PenStrep (100U/L penicillin and 100ug/L streptomycin), 1mM Na-pyruvate, 2mM glutamax and 10% foetal bovine serum. For the galactose sample also DMEM was used with the same supplementation of glucose medium, without glucose and L-glutamine but with the addition of 25 mM galactose and 25nM HEPES. Caco2 cell lines, were incubated at 37˚C in humidified atmosphere with 5% of CO_2; the medium of the samples was refreshed every 3-4 days (Appendix I).

2.1.1 Undifferentiated Cells

Caco2 cells were grown in 75cm^2 cell culture flasks (Corning®) for an incubation period of ten days. Caco2 cells cultured in galactose medium grow slower than Caco2 cells grown in glucose medium. Therefore, Caco-2 cells were sub-cultured with a different starting cell number to achieve the similar confluency (70%-80%) at the end of the incubation period (table1). Cells were counted with the Ceilometer™ auto T4 (Nexcelom Bioscience).

2.1.2 Differentiated cells

Caco2 cells were seeded, at density of 1*10^5 cells/cm^2, in Thincert cell culture insert for 24 well plates with 0,4 µm pore diameter (Greiner Bio-One International) for an incubation period of 14 days, during which the cells are able to differentiate.

TABLE 1: SUBSTRATE USED, OPTIMAL SEEDING AMOUNT, INCUBATION PERIOD, CONFLUENCE REACHED AT THE END OF INCUBATION PERIOD.

Substrate	Status	sample	Optimal seeding density	Incubation	Confluence
Glucose	undifferentiated	4	0.03-08*10^6	10 days	80%
Galactose	undifferentiated	4	1.2-1.7*10^6	10 days	80%
Glucose	differentiated	3	1*10^5	14 days	100%
Galactose	differentiated	3	1*10^5	14 days	100%

2.2 Harvest and preparation of the samples

Caco2 cells, after the incubation period, were harvested adding of 50mM Tris, 1% triton lysate buffer. Samples were stored at -20˚C until western blot preparation. The Branson® Sonicator was used to break the cells (Appendix II) and the protein concentration was determined with the Bio-Rad DC protein assay (Appendix III). Before gel electrophoresis , cell solution concentrations were corrected for the lowest found concentration with lysate buffer. Before loading in the gel electrophoresis 1M Dithiothreitol and NuPAGE® LDS Sample Buffer (Novex®).

2.3 Western blot

OXPHOS complex protein expression and the energy status, after CI inhibitors administration, of Caco2 cells were studied. OXPHOS protein expression was analysed for undifferentiated and differentiated cells. The AMP/ATP levels was estimated through the detection of phosphorylated Acetyl-CoA carboxylase (pACC) and phosphorylated AMP-activated protein kinase (pAMPK) expression. Rotenone or piericidin A at 200nM concentration, were used as CI inhibitor only for differentiated cells.

2.3.1 OXPHOS complex protein expression

Samples were divided in two groups: the control, where Caco2 cells were seeded in glucose medium, while the treatment group was performed with galactose medium. Undifferentiated and the differentiated cells had the same, western blot protocol (appendix IV). Four glucose samples and four galactose samples were used for the analysis of undifferentiated cells. Three glucose samples and three glucose samples were used for the differentiated cells analysis. For the undifferentiated and differentiated cells 40µg and 50µg of proteins were loaded respectively in the gel electrophoresis.

Samples were heated for five minutes at 50 degrees Celsius and loaded in 12% acrylamide gels (appendix V) at 70V for 30 minutes and subsequently switched to 150V for one hour. The proteins separated, by gel electrophoresis, were blotted to the membrane (Immobilon® transfer membranes, Millipore) for one hour at 100V. The membrane was blocked by TBST-5% BSA, for one hour at room temperature. The membrane was incubated overnight at 4 degrees Celsius while gently rocking with (label) with Total OXPHOS Human WB Antibody Cocktail (ab110411) (1:1000,Abcam®), overnight at 4 degrees Celsius while gently rocking with with mixer Polymax 2040 (Heidolph). Goat ant-mouse IRDye® 800CW Secondary Antibodies and(1:10000, Odyssey). Anti-beta Actin antibody (ab8227)(1:1000, Abcam) , was chosen as loading control.

2.3.2 Rotenone and piericidin A

For the ATP levels, 12 Caco2 cells cultures were treated with rotenone and other 12 were treated with piericidin A. After 14 days of differentiation, cells were stimulated with CI inhibitors, rotenone or piercidin A. Caco2 cells were treated with CI inhibitors ,rotenone or piericidin A, at the end of the incubation period and 24 hours after the stimulation cells were harvest. In both treatments two equal groups were formed; six samples were seeded in glucose medium and six samples in galactose medium. Three cell cultures, of each groups, were treated with 0nM of CI inhibitor and the other three with 200nM of CI inhibitor (Fig7).

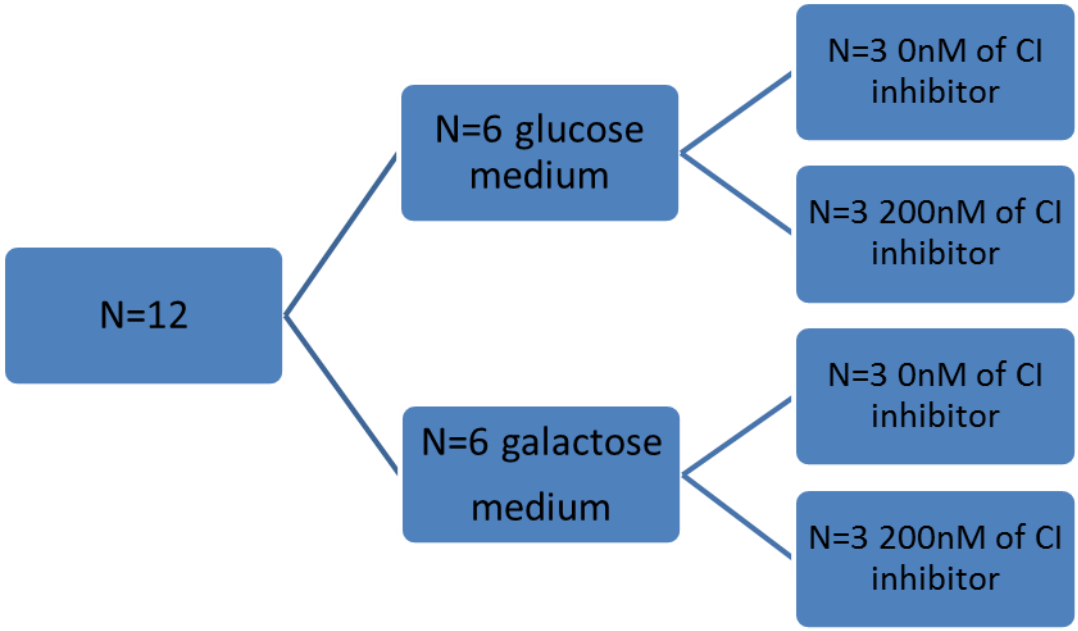

Figure 7: Sample size of ATP/AMP analysis after CI inhibitors: *For each CI inhibitor 12 samples were used and two equal groups were formed. Six samples were seeded in glucose and six samples were seeded in galactose. Each group was divided in two equal sub-groups, which were treated with 0nM or 200nM of CI inhibitor.*

The protocol, previously used for the OXPHOS complex protein density, was adjusted for the cells treated with rotenone and piercidin A(Appendix VI). Samples were heated at 70 degrees Celsius before been loaded in duplicate at the same time in two different NuPAGE™ 4-12% Bis-Tris Protein Gels (Novex™1.0 mm, 15-well). One membrane was incubated with the antibody cocktail composed by Phospho-Acetyl-CoA Carboxylase (Ser79) Antibody and Phospho-AMPKα (Thr172) (40H9) Rabbit mAb (1:1000, Cell Signaling Technology). The other membrane was incubated with Acetyl-CoA Carboxylase Antibody and AMPKα (23A3) Rabbit mAb antibody(1:1000, Cell Signaling Technology). The incubation period was overnight at 4 degrees Celsius while gently rocking with mixer Polymax 2040 (Heidolph). Donkey ant-rabit IRDye® 800CW Secondary Antibodies and(1:10000, Odyssey) and Anti-beta Actin antibody (ab8227)(1:1000,Abcam) were chosen as secondary antibody and loading control respectably.

2.4 Image quantification and data analysis

The membrane was scanned with Image Studio™ Software for the Odyssey® (LI-COR) and the intensity of the protein bands scan was quantified by Image Studio Lite Ver 5.2 (LI-COR).All the values found were adjusted for the loading control (β-actin). The statistical analysis was carried out with Graphpad prism software using one-tailed t test for the OXPHOS complex protein density analysis while for the pACC/ACC and pAMPK/AMPK test two-Way ANOVA and Bonferroni multiple comparisons were used. Significant differences were considered when p-value < 0.05.

3.Results

3.1 Optimizing the detection of the OXPHOS complexes

To optimize the detection of all five OXPHOS complexes, different dilutions (1:500-1:2000) of Rodent OXPHOS antibody cocktail were tested first (fig 8A). At all these antibody dilutions CIII, CIV, and CV were detected successfully and based on these results the antibody dilution of 1:1000 will be used. However, CI and CII was difficult to detect and therefore Rodent OXPHOS antibody cocktail was tested with mouse muscle tissue to check an eventual defect of the antibody cocktail (fig 8B). All five complexes were visible when using mouse muscle tissue and therefore the difficult detection of CI and CII was not attributable to defective Rodent OXPHOS antibody cocktail. Increasing the loading of protein (fig 8C; only galactose samples) and decreasing the heating temperature during the sample preparation to 50 degrees Celsius (fig 8D) did not improve the detection of CI and CII. When using the Human OXPHOS antibody cocktail (1:1000) all five OXPHOS complexes could be identified in protein samples (with 50µg of protein loaded) derived from Caco2 cells cultured in glucose and galactose (fig 8E).

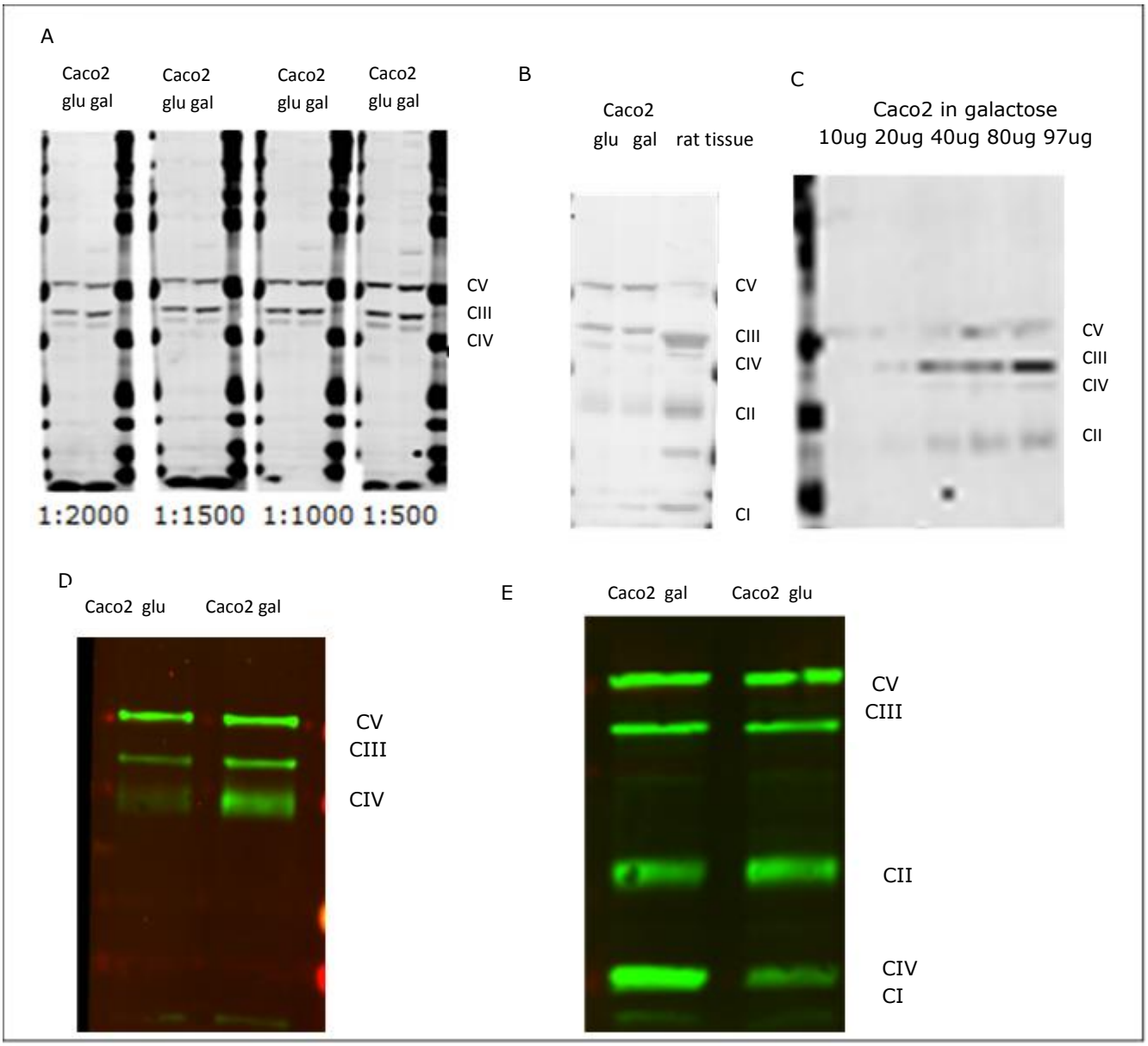

Figure 8: Western Blot Optimisation of Rodent OXPHOS antibody and Human OXPHOS antibody cocktail*. A) Comparative analysis of Rodent OXPHOS antibody cocktail at dilutions 1:2000, 1:1500, 1:1000 and 1:500. B) Rodent OXPHOS antibody tested with Caco2 cells grown in glucose or galactose and rat muscle tissues. C) Analysis of Caco2 grown in galactose with 10, 20, 40, 80, 97µg of protein loaded Rodent OXPHOS antibody cocktail. D) Rodent OXPHOS antibody cocktail with samples (derived from Caco2 cells seeded in glucose or galactose)heated at 50 degrees Celsius. E)Caco2 seeded in Glucose and galactose were tested with Human OXPHOS antibody cocktail at dilution 1:1000 and all five complexes were detected.*

3.2 OXPHOS complex protein density

Undifferentiated and differentiated Caco2 cells were grown in two different medium conditions: in glucose and galactose medium where the first one correspond to the control group while the second one correspond to the treatment group.

3.2.1 Increased expression of CIV in undifferentiated Caco2 cells when cultured with galactose

Differences between the protein expression levels of the different OXPHOS complexes were first studied in undifferentiated Caco2 cells grown in glucose and galactose medium for 10 days (figure 9A. The protein expression, of OXPHOS complexes grown in galactose, was found higher in CIII, CIV and CV respectively with an increase of 16%, 113% and 15% compared to the cells seeded in glucose medium. CIV showed a significant difference (P=0.012) (figure 9B). Also the sum of the expression all OXPHOS complexes protein density was calculated, however, no significant differences were found between glucose and galactose sample (figure 9C).

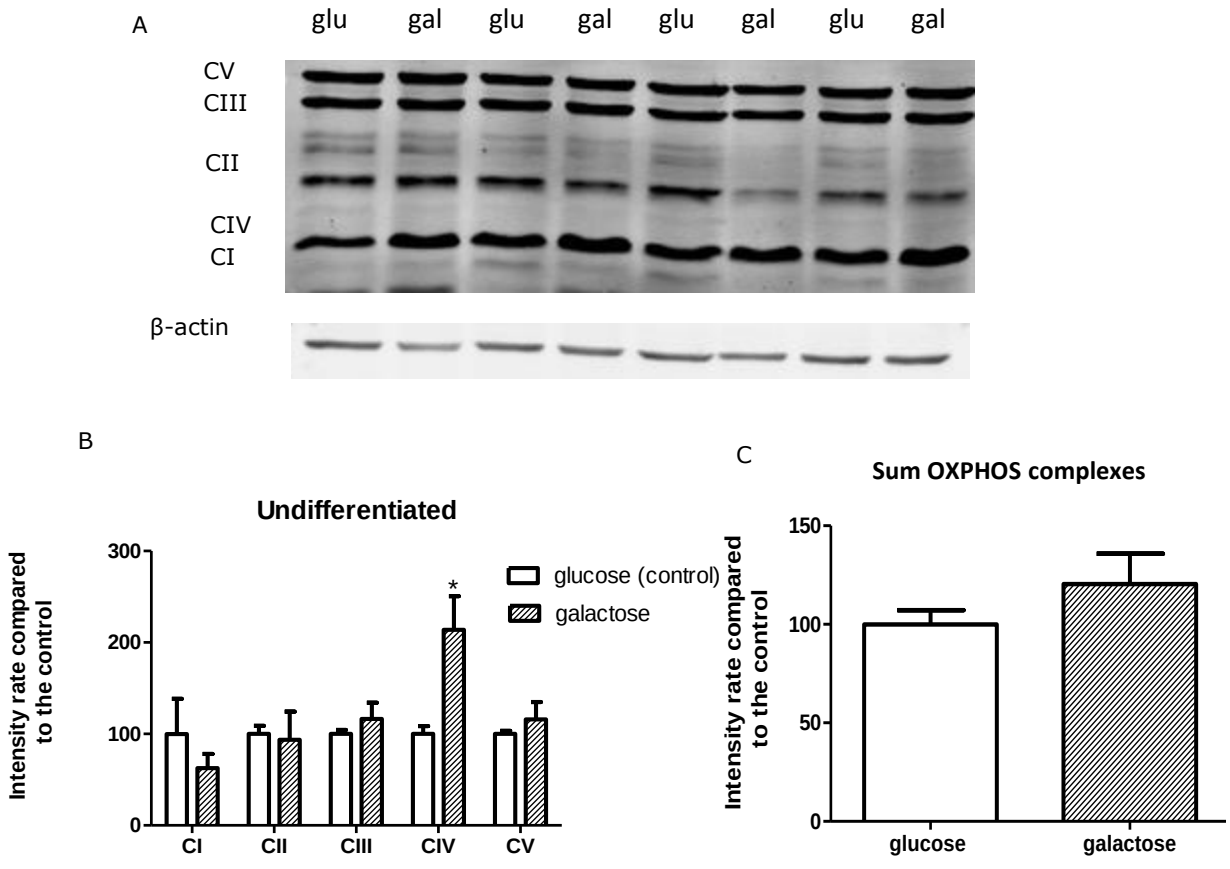

Figure 9:Western Blot of OXPHOS complexes: *A) Scan of the membrane with all five OXPHOS complexes loading control (β-actin). B)test confirm not C) Complex protein density of Caco2 cells grown in galactose medium had significant differences for CIV(P =0.012). D) No significant differences for the sum OXPHOS complexes expression.*

3.2.2 Increased OXPHOS expression in differentiated Caco2 cells when cultured with galactose

The OXPHOS complex protein expression of differentiated Caco2, grown for 14 days in glucose or galactose, was analysed through Western Blot (figure 10A). Caco2 cells cultured in galactose had higher protein expression for all the OXPHOS complexes. Significant differences were found for CI (P value 0.0024), CIII (P =0.0073), CIV (P =0.005), with an increase of 327%, 55% and 147% respectively (figure 10B). Moreover the of the OXPHOS complex expression in the galactose samples was two times higher ($P < 0.001$) than the glucose medium conditions (figure 10C). The β-actin (the loading control) bands had higher expression (figure 10A) in the glucose samples. at the beginning, we were thinking β-actin could be influenced by the treatment. But after Protein stain(Thermo Scientific™)it was shown the glucose samples had effectively higher amount of protein loaded(appendix VII).

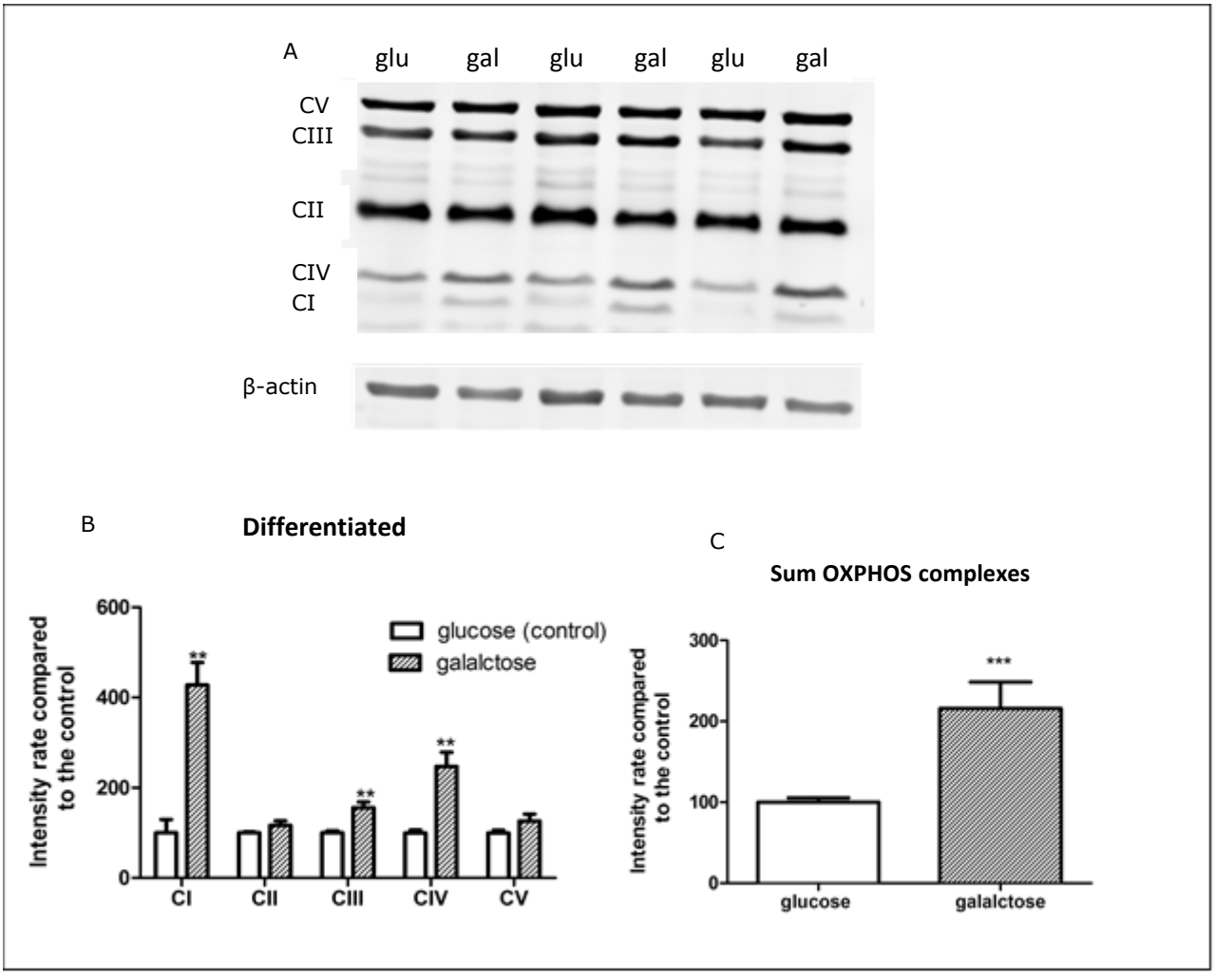

Figure 10: Western blot of OXPHOS complexes for differentiated cells: *A)Scan of the membrane with OXPHOS complex and loading control (β-actin. B) Significant differences, in complex protein density, found for CI (P =0.0024), CIII (P =0.0073), CIV (P =0.005).C)Total OXPHOS protein complex density of Caco2 cells grown in galactose was significant high compared to glucose samples(P < 0.001).*

3.3 AMP/ATP levels after administration of CI inhibitors

Differentiated Caco2 cells were treated with CI inhibitors, with Rotenone and piericidin A, at the end of the incubation period and harvest after 24 hours. The concentration used of CI inhibitors was 0nM (the control) and 200nM (the treatment);both medium condition, glucose or galactose, were tested. The effect of CI inhibitors on AMP/ATP levels in Caco2 cells was analysed.

3.3.1 Rotenone increased pAMPK levels in differentiated Caco2 cells cultured with galactose

To determine the AMP/ATP ratio of Caco2 cells the levels of phosphorylated ACC (pACC) and phosphorylated (pAMPK) were measured (figure 11A&B). No significant differences were found in pACC/ACC ratio in both medium conditions (figure 11D). PAMPK/AMPK levels in Caco2 cells, grown in galactose medium and treated with 200nM rotenone, were statistically higher ($P < 0.01$) compared to the 0nM rotenone concentration (figure 11C). While for Caco2 cells cultured in glucose no significant differences were found. In both membrane β-actin (loading control) showed there were not an homogeneus protein loadding (figure11 A&B). PACC/ACC and pAMPK/AMPK ratios were adjusted for β-actin, thus the validity of the result were not impired by the different amount of protein loaded.

3.3.2 Piercidin A administration increased pAMPK levels in differentiated Caco2 cells cultured with galactose

The ratio of pAMPK/AMPK was used to determinate the energy status of Caco2 Cells after the stimulation with Piercidin A. The cells grown in galactose medium and treated with 200nM Piericidin A had significant higher levels of pAMPK/AMPK ($P < 0.001$) compared to the control (Fig12C). As for rotenone, piercidine A did not have significant effect on Caco2 seeded in glucose(Fig12C).The presence of air bubbles in one membrane made impossible the quantification and consequently the calculation of pACC/ACC ratio. The β-actin showed the same amount of proteins were loaded (figure 12 A&B).

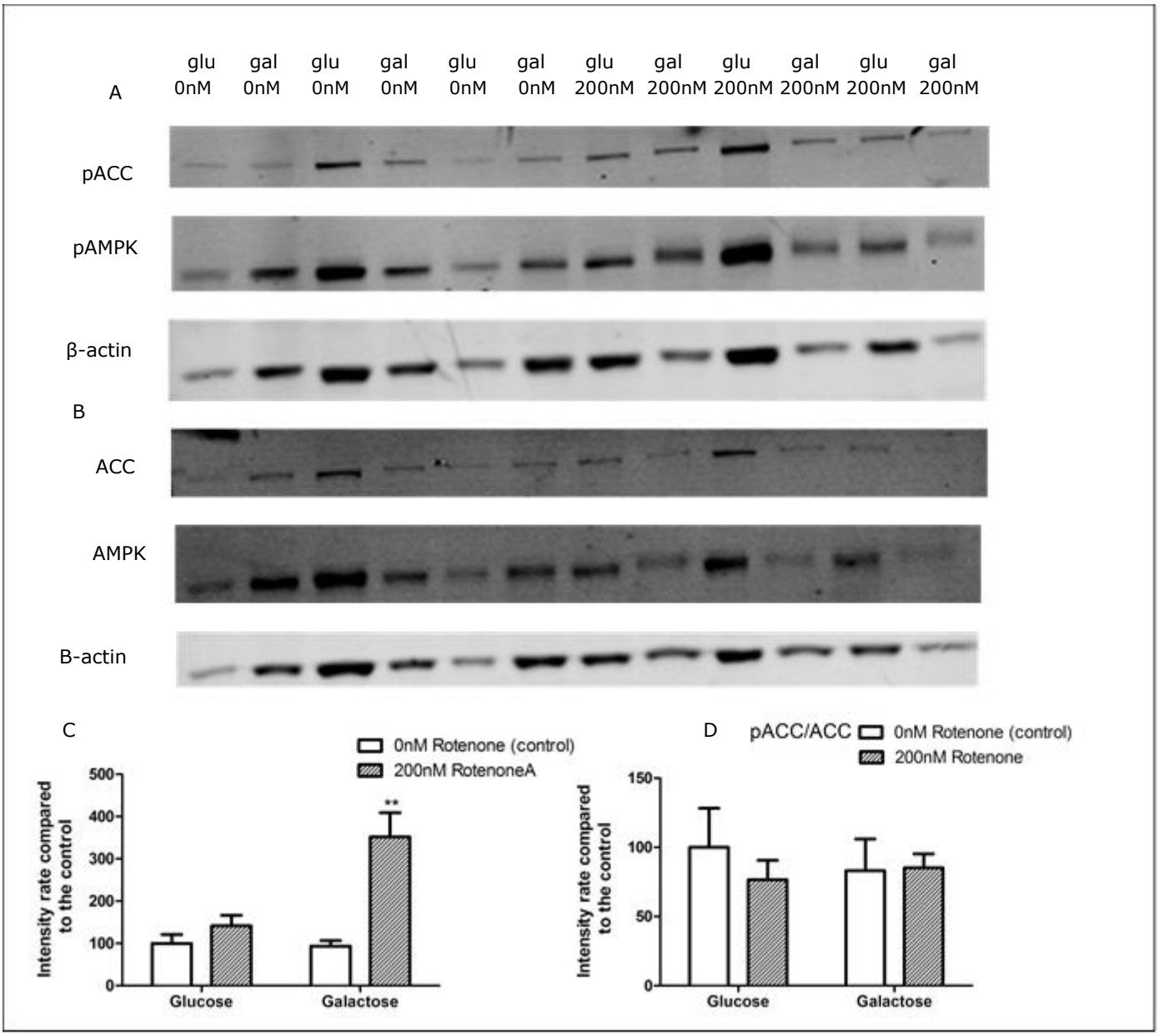

Figure 11:Western Blot of Caco2 cells treated with Rotenone: *A)Membrane scan of pACC, pAMPK and loading control of pACC and pAMPK membrane. B) Membrane scan of ACC and AMPK and loading control. C)Caco2 cells grown in galactose and treated with 200nm of Rotenone had significant differences for pAMPK/AMPK ratio respect to the control (0nM Rotenone) ($P < 0.01$) F)no significant differences found for pACC/ACC.*

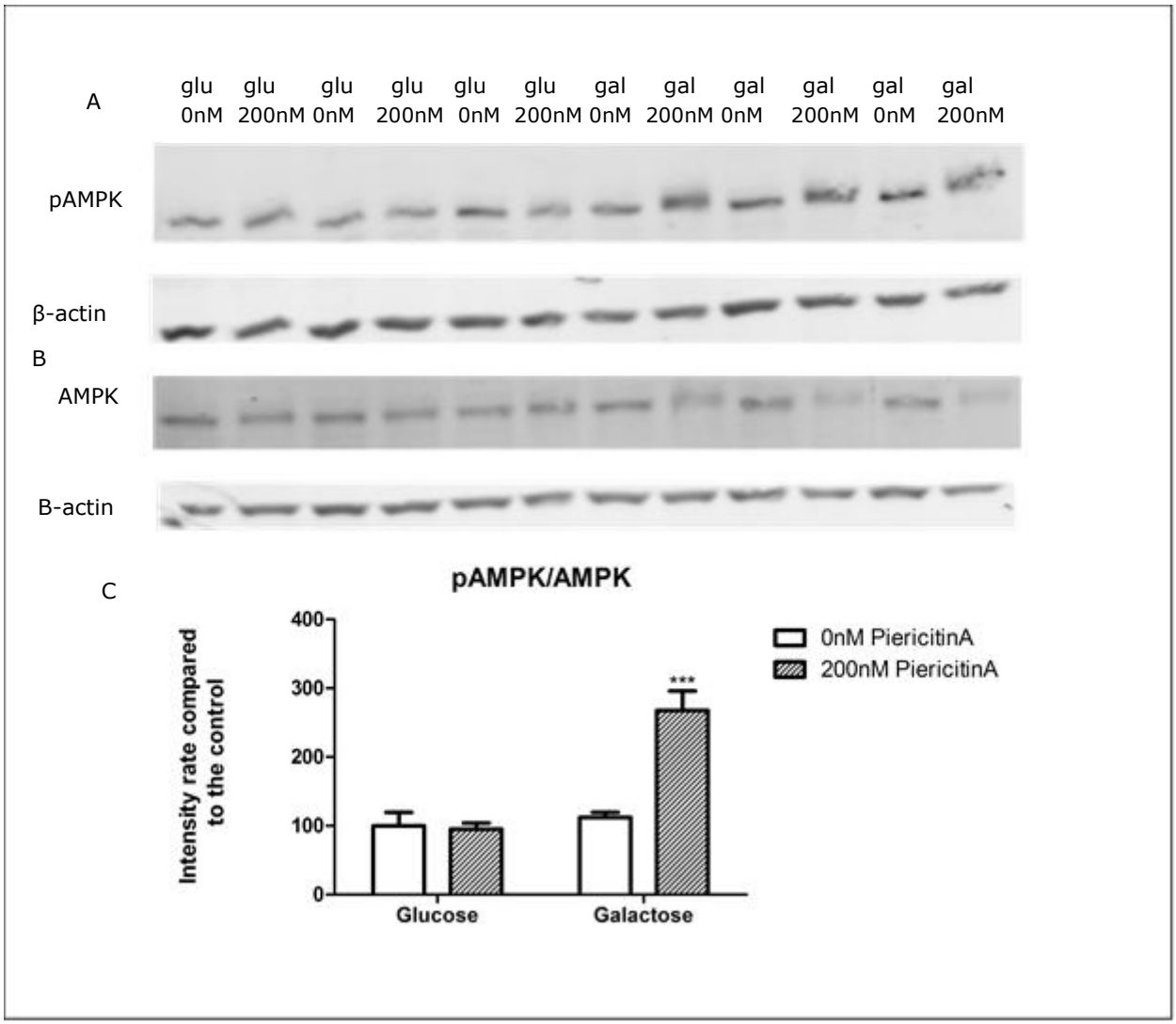

Figure 12: Western Blot of Caco2 cells treated with Piericidin A : *A) Membrane scan of pAMPK and loading control. B) Membrane scan of AMPK and loading control of AMPK. C) Caco2 cells grown in galactose and treated with 200nm of Piercitin A had significant differences for pAMPK/AMPK ratio ($P < 0.001$) respect to the control (0nM Piericitin A).*

4.Discussion

Caco2 cells, as intestinal model, are already used in several studies (7; 8). However, a major drawback is the metabolic differences between cancer and human cells may cause compliances for some experiments or impairs some results. One of the most accredited reason of these metabolic differences is due to the high glucose medium conditions, in which Caco2 cells use to grow . The change of energy substrate from glucose to galactose may reduce these differences increasing the aerobic metabolism.

4.1 OXPHOS protein expression

The slower metabolism of galactose may cause a decrease of glycolysis in favour of TCA cycle and OXPHOS. Studies suggest that the increase of aerobic metabolism could be related to higher amounts of OXPHOS protein units (24). OXPHOS protein expression of Caco2 cells, were studied in glucose and galactose medium conditions to detect eventually differences. The experiment was carried out with differentiated and undifferentiated cells.

Differentiated cells grown in galactose medium had a higher OXPHOS protein expression compared with the samples grown in glucose medium, in particular, for CI, CIII and CIV. CI and CIV could be two indicators of a possible increase of aerobic metabolism (13). CI is the site where NADH is oxidized, which is released mainly through TCA cycle (part of aerobic metabolism). Thus, high CI density may be related to higher TCA cycle activity, which means an improvement of aerobic metabolism. Also high amount of CIV may indicate higher aerobic activity. CIV is the complex where the electron transport chain ends with the donations of two electrons to the acceptor (O_2). For these reasons higher amounts of CIV present in the mitochondria mean higher O_2 consumption (13).

For the undifferentiated cells only significant difference were found for CIV which means that energy substrate switching had a lower effected compared to the differentiated cells. Literature about metabolic differences between differentiated and undifferentiated cells is not available. However, studies done with stem cells, demonstrated differentiation process already increase mitochondrial biogenesis and induces oxidative metabolism (25; 26).Caco2 cells metabolism differs from the human healthy cells, thus in the case of undifferentiated Caco2 cells these differences could be even larger to impair the effect of the galactose switching.

4.2 Effect of OXPHOS inhibitors

Energy status of Caco2 cells, and other cells, is indicated by AMP/ATP ratio (27) . In normal situations, AMP/ATP is low and tends to increase with energy stress situations (27). High levels of AMP turns the inactive form of protein kinase AMPK to its active form pAMPK. The activation of AMPK favors the phosphorylation of ACC to its inactive form (pACC) which increases fatty acid and glucose oxidations (27). For this reason, pAMPK/AMPK and pACC/ACC are used as protein signal for the energy status. High levels of pAMPK/AMPK and pACC/ACC mean high levels of AMP/ATP. In the organisms with high aerobic metabolism,

ATP is mainly produced by OXPHOS and TCA cycle instead glycolysis . Thus the administration on OXPHOS inhibitors, as rotenone and piericidin A, may have higher effects on ATP production for cells with aerobic metabolism compared to cells with more glycolytic metabolism. If Caco2 cells developed higher aerobic in galactose the treatment, with rotenone and piericidin A, would increase the AMP/ATP levels and consequently raise pAMPK and pACC levels.

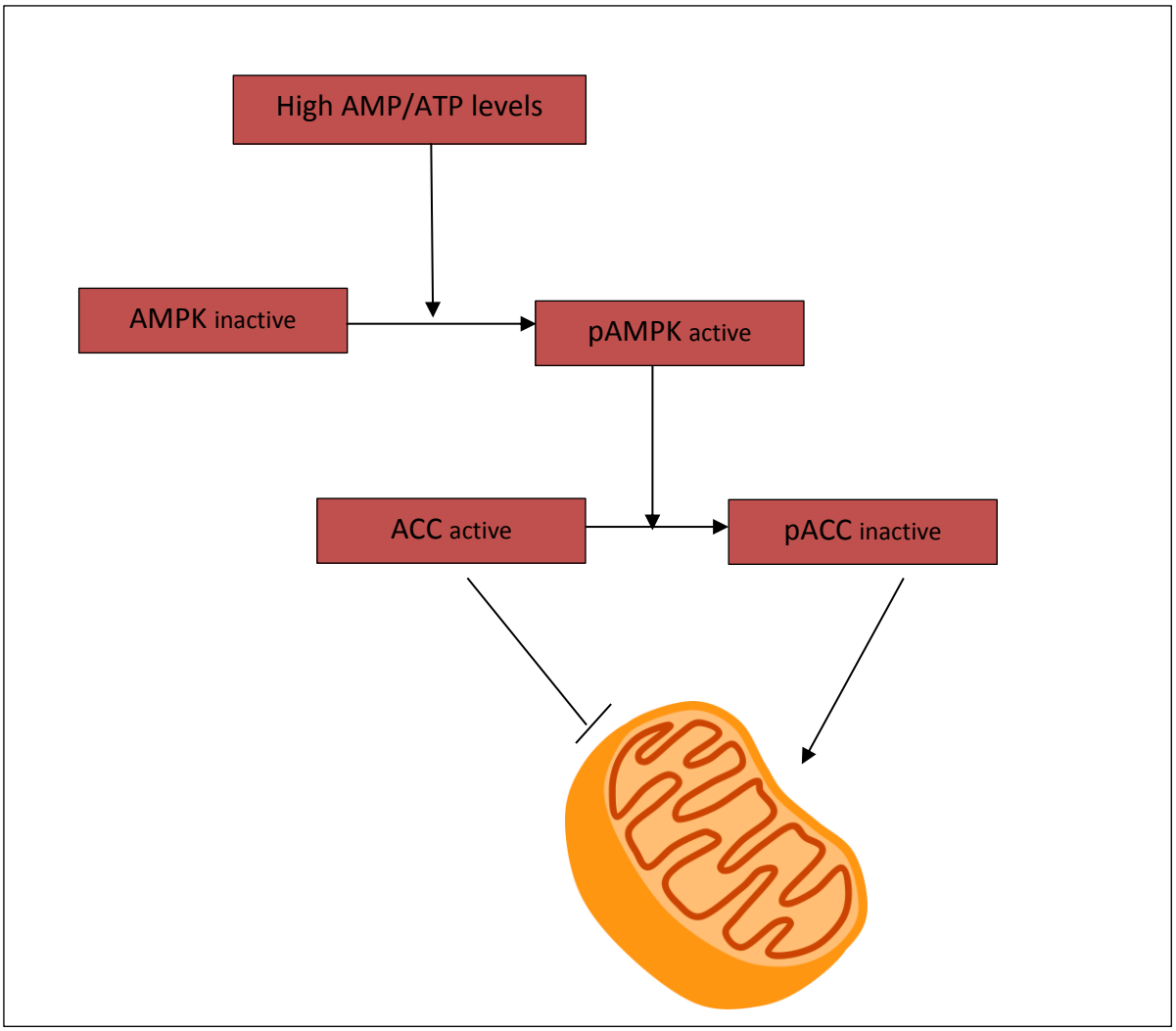

Figure 12: pAMPK and pACC proteins signal of energy status: *High levels of AMP/ATP activate AMPK in its phosphorylated form. The release of pAMPK inactivates ACC into pACC which improves the oxidation of glucose and fatty acids. High levels of pAMPK and pACC mean energy stress situation.*

The samples grown in galactose the treatment with CI inhibitor raised the levels of pAMPK/AMPK, which means there were a reduction of ATP levels. This result suggest that Caco2 cells grown in galactose may have developed an aerobic metabolism, which contributes, in higher rate, in the ATP productions compared to the glucose growing conditions. On the other hand, for the Caco2 grown in glucose, the administration of both CI

inhibitors did not have significant effect on the ATP/AMP energy status. This result means, for the cells cultured in glucose medium the aerobic metabolism contributes in a lower rate, compared to the galactose medium, in favour of a more glycolytic metabolism.

In all samples treated with Rotenone, no significant differences were found for pACC/ACC ratios. The presence of air bubbles on the membrane site of pACC protein bands, did not allow the quantification of pACC /ACC of the samples treated with piericidin A. A previous study, where AMP/ATP levels were altered, by other OXPHOS inhibitors, the increase of pAMPK/AMPK was parallel to higher pACC/ACC levels (27). In this studies, rat neurite tissues were used. One possible explanation could be due to experimental conditions; for example the use of dated ACC antibodies were available. More over another explanation could be due to the blotting time (of one hour) which could be too short for a protein of ACC (over 200 KDa.)

4.3 Caco2 cells as *in vitro* model

The Caco2 cell line represents an *in vitro* model of intestinal barrier with similarities of *in vivo* situations. However, the high glucose availability of the medium, in which the Caco2 cells are routinely cultured, impairs the aerobic metabolism, which is the main ATP source of intestinal cells as enterocytes. Due to the metabolic differences, between Caco2 cells and human cells, the use of this *in vitro* model could impair the results of some experiments, as studies about mitochondrial dysfunctions.

One example is the study of Carrasco-Pozo (28). Caco2 cells were treated with another CI inhibitor Indomethacin (INDO) and eventually beneficial effects of polyphenols (PPs) against this stress situation were studied. Significant effects were found for quercetin but not for other compounds. During this study before the PPs administration different concentrations of INDO were tested to evaluate the lowest concentration to use, whose causes a significant decrease of ATP levels. INDO at concentration 250 nM was chosen for all the experiments. In Carrasco-Pozo study, Caco2 were seeded in glucose medium and probably the contribution of the ATP production from OXPHOS was low. For this reason, to have significant decrease of ATP by INDO, higher concentrations of that compound were needed. How suggested in my research, the OXPHOS inhibitors should have higher negative effects on ATP levels ,for cells cultured in galactose. Thus, in the Carrasco-Pozo study, lower INDO concentrations would be needed if galactose medium was used for the Caco2 cells culturing. An eventual lowering of INDO concentration may increase the effectiveness of the treatment with other PPs compounds.

4.4 Conclusion

As explained previously, the switching of energy substrate of Caco2 cells, from glucose to galactose, could increase the metabolic similarities with human cells passing from anaerobic to aerobic metabolism. The results of my research suggest there was an improvement of

aerobic metabolism, through energy substrate changing. In particular refers to OXPHOS expression, differentiated Caco2 cells seeded in galactose had a significant higher protein density for CI, CIII, CIV and the sum of all complexes. This raise in the protein density could leads to an increase of OXPHOS activity and consequently contribute in higher rate to ATP production. The lowering of ATP levels caused, by CI inhibitors, in Caco2 cells grown galactose indicated that the cells rely more on mitochondrial ATP production. New studies, for example the measurement of oxygen consumption of Caco2 cells in the different medium conditions, may confirm the hypothesis the energy substrate shifting to galactose improves the aerobic metabolism . The lack of studies about this topic makes it difficult to quantify the similarities of Caco2 cells cultured in galactose with intestinal cells. However, with the developing of new researches, a more representative *in vitro* model would be possible

5. Suggestion for future researches

5.1 Short term researches

As mentioned previously, the measurement of oxygen consumption may help to have a deeper knowledge about the effect of galactose medium on Caco-2metabolism. OROBOROS analysis allows the analysis of oxygen consumption rate (OCR) ,of Caco2 cells in the medium. OCR reflects the OXPHOS activity of cell cultures, as CIV consumes O_2. The samples are incubated and OROBOROS monitors the oxygen consumption rate (OCR) over time under administration of inhibitors (such as oligomycin and antimycin A) and uncouple agent phenylhydrazone. Basal Respiration, Leak respiration, Maximal respiration and Non-mitochondrial Respiration are detected and Caco2 cells grown in galactose should have higher Basal Respiration and Maximal respiration due to the developing of aerobic metabolism.

Another suggestion could be the analysis of gene expression of AMPK and ACC through real time quantitative polymerase chain reaction (qPCR). Energy deficits, caused by external agents, may increase the gene expression of AMPK and ACC (27). If OXPHOS inhibitors cause an increase in gene expression, of AMPK and ACC for Caco2 for cells grown in galactose, the results found on AMPK expression would be confirmed.

5.2 Polyphenols

As already discussed, the switching of energy substrate from glucose to galactose increased the aerobic metabolism of Caco2 cells lines. This improvement allows having an *in vitro* model with metabolic similarities of intestinal barrier. However, these similarities could be refined through other treatments. One suggestion may be the use of polyphenols (PPs) as a promoter of OXPHOS.

PPs are aromatic compounds present principally in fruits, vegetables and herbs (ref). In the last decades, the study of this compound became more popular due to its potential beneficial effect on human health (ref). Studies already demonstrate some beneficial effects of PPs. Some example refers to their potential beneficial effects against oxidative stress, improvement of fatty acid oxidation and reduction of glucose uptake (29;30).The mentioned PPS activities suggest aerobic metabolism of Caco2 could be improved through administration of PPs through two possible mechanisms.

One mechanism could be attributable to the inhibition of intestinal glucose carriers (30).The study of Johnson, performed already on Caco-2, cells suggests that some classes of polyphenols, as catechins and aglycones could inhibit SGLT1 and GLUT2 transporters. In particular, quercetin seems to have competitive inhibitor effects on SGLT1 carrier (31). As previously mentioned, Caco2 cells medium conditions use to have high glucose availability

which leads to a decrease of OXPHOS activity ("Crabtree effect") (18). The eventually reduction of glucose uptake, due to PPs administration, may decrease the glycolysis in favour of OXPHOS.

Another possible mechanism refers to the potential effects of PPs on mitochondrial biogenesis and in the regulation of both carbohydrate and lipid metabolism. Studies with mice, with high PPs diet, had significant increase in phosphorylation and gene expression of AMPK and ACC (32). PPs were administered through high soy diet (which is rich of PPs) and subsequently liver, muscle and in white adipose tissue of mice were analysed. Thus, the direct administration of PPs on Caco2 cells could have the same effects.

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Appendix I: General tissue culturing procedures

Precautions

- Wash your hand and arms
- Roll up your lab coat sleeves (use coat with green sleeves)
- Change coats regularly
- Work sterile and clean the working surfaces with 70% ethanol.
- Spray everything you bring into the laminar flow cabinet thoroughly with ethanol
- Use (filter-) sterile solutions
- Write the date of first opening of general solutions
- Use filtertips
- Do not wear gloves, unless you have small injuries.
- Mark and use your own filter tips and eppy jars
- Do not cross with your arm over plates, open vials, open eppy boxes etc.
- Work on the back side of the laminar flow cabinet
- Preferably aliquot most of the solutions.

Preparation of buffers, solutions, Media

HBSS buffer (Hanks' balanced Salt Solution)

- Ready to use, (4 °C)

DMEM (Dulbecco's Modified Eagle's Medium, low glucose)

- Ready to use, (4 °C)

RPMI 1640 medium Sigma R6504

- Dissolve all powder from one bottle RPMI 1640 and 2 g $NaHCO_3$ in 0.8 liter MQ
- Stir until dissolved
- Adjust pH when necessary (see data sheet)
- Add additional water to bring the solution to final volume (= 1 liter)
- Sterilize immediately by filtration using a membrane (0.22 microns)
- Store at 4 °C

Media additives

Before use check the vendor of the cell line (mostly the ATCC) what additives need to be added to the media.

In general we culture our cells in medium with 1 g/l glucose which is a more physiological concentration than 4,5 g/l (which is mostly used), 10% FBS and 1x Pen/Strep.

Other commonly used additives (final concentration in medium):
Check concentrations and expiration dates for the used media and additives. Stocks may

vary per brand in concentration, this protocol is based on described concentrations,

5-10% Foetal Bovine Serum (Heat-inactivated), (-20 °C)
in our lab we have different lot numbers, check the used lot number for your study on the list on the freezer and use that lot number always. After thawing store the serum in the fridge.
Fetal Bovine serum is the most common supplement in animal cell culture media. It is used as a relatively low-cost supplement to provide an optimal culture medium for cultivating animal cells. These supplements provide carriers or chelators for labile or water-insoluble nutrients, provide hormones and growth factors, protease inhibitors, and bind and neutralize toxic moieties.

Penicillin/Streptomycin (-20 °C)
Antibiotics are often used to control the growth of bacterial and fungal contaminants
Stock is 100x concentrated, so use 1 ml/100 ml. If you open a new bottle, please aliquot it over 10-15 tubes.

Non-essential amino acids, (4 °C)
Gibco® MEM Non-Essential Amino Acids are used as a supplement for cell culture medium, to increase cell growth and viability. Stock is 100x concentrated, so use 1 ml/100 ml.
If you open a new bottle, please aliquot it over 10-15 tubes.

Glucose (-20 °C)
Some cell lines need a higher level of glucose, therefor add a filter sterile solution of D-Glucose to a final concentration of 4.5g/l (or another concentration if needed).

Glutamax (-20 °C)
Gibco® GlutaMAX™ supplement provides an alternative to L-Glutamine with improved stability and cell health.
Stock is 100x concentrated, so use 1 ml/100 ml. If you open a new bottle, please aliquot it over 10-15 tubes.

Sodium Pyruvate (4 °C)
Sodium Pyruvate is commonly added to cell culture medium as an energy source in addition to glucose. Since the cells make sodium pyruvate as an intermediate metabolite in the glycolysis pathway, it is not necessary to supplement all cultures. However, if the cells have been grown in medium supplemented with sodium pyruvate, we recommend the supplementation is continued as the cells become accustomed to this addition and their growth will lag without it.
Stock is 100x concentrated, so use 1 ml/100 ml. If you open a new bottle, please aliquot it over 10-15 tubes.

Hepes 1M (4 °C)
Gibco® HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid) is a zwitterionic organic chemical buffering agent commonly used in cell culture media. The addition of 10–25 mM HEPES provides extra buffering capacity when cell culture requires extended periods of

manipulation outside of a CO_2 incubator. HEPES is a good buffering choice for many cell culture systems because it is membrane impermeable, has limited effect on biochemical reactions, is chemically and enzymatically stable, and has very low visible and UV light absorbance. If you open a new bottle, please aliquot it over 10-15 tubes.

Other needed solutions

Trypsin

Trypsin is used to detach adherent cells for sub culturing. It is stored as 1x 0.5% in the middle drawer. If finished thaw 10x trypsin 5%, dilute the 10x concentrated trypsin in HBSS to obtain 1x trypsin 0.5%; Gibco cat. 15400; bottom drawer freezer cell culture lab)
For cells which are difficult to detach, 25% 10x stock is available, and 2.5% 1x working solution.
If you open a new bottle, please aliquot it over 10-15 tubes.

Tryphan blue

0.2% Tryphan blue in HBSS is used for live/dead analysis when counting cells. Damaged cells have a permeable membrane and therefore after staining turn out to become blue. Healthy cells remain white. Tryphan blue solution is not a sterile solution.

Sterilisation and handling of solutions

Most of the used solutions in the tissue culture lab are sterile.
Home-made solutions need to be sterilised or by autoclaving, or by filtration through a 0.22 uM filter. Not every compound is stable enough for autoclaving so filtration is the most used method. In the cabinet bottle top filters are present which can be placed on top of a sterile red-capped bottle (yellow dot means that the rad-capped bottle is baked in the oven for at least 4 hours and suitable for culturing media). When the aspirator tubing is connected and the vacuum system is switched on, the filtered solution is sterile.
For smaller solutions, take a sterile tube, fill a syringe with the solution to be sterilized, place a mini filter disk (molecular lab, white cabinet) on the syringe and filter the solution to the sterile tube.

When you use sterile solutions (media, washing salt solution, additives) for culturing, dry the bottles if pre-warmed in the waterbath with a tissue and thoroughly spray it with 70% ethanol for outside sterilisation. Then place the sterile bottle in the cabinet.

When using solutions, try to open bottles and/or culturing flasks as short as possible. Do not touch the rim with your hands, place caps up-side down on the working surface, but preferable hold them in your fingers, with the opening downwards (this needs some practicing...)

Mark sterile solutions with a yellow dot/label.

Write on sterile bottles the date of preparing and the date of opening (labels are available on the CO2 incubator or in the main lab). Writing should be performed with a pencil, because of the readability after rinsing with 70% Ethanol.

Method

Tissue Culturing- Basic procedures

All protocols below are for culturing in T75 flasks. Other volumes are described at the end of this protocol.

Remarks
Do not move hands or objects above open flasks, plates or bottles.
Do not pipette liquids on the cells (except trypsin).
Keep the cross flow as empty as possible (objects will disturb the sterile flow).
If necessary, wear gloves (Lentiviral work, wounds on hands).
Work organized and accurate.

Flow cabinets and CO2 incubators
Two flow cabinets are present in the lab, the left one is equipped for right-handed persons, and the right one for left-handed persons ☺.
There are 3 incubators in the lab, one for routine culturing, one for experimental culturing, and one for lentiviral production/incubation. This is indicated on the door of the incubators.

Procedure before culturing
- Wash your hands extensively before culturing, wear a lab coat
- Reserve a cross flow (Left or Right) for the time you need it in the agenda in the tissue culture lab.
- Turn the cross flow on 30 min before use
- Clean the cross flow and the red or white cabinet next to the flow with 70% ethanol
- Put all your media and/or HBSS buffer in the 37 °C water bath
- Take your peronal trypsine/EDTA (TRP) out of the fridge and let it acclimatise at room temperature. Do not put it at 37 °C (repeated heating and cooling decreases the activity of trypsin.

Medium refreshment
- Medium refreshment is mostly performed on Monday, Wednesday and Friday.
- Clean all flasks on the outside with 70% ethanol before entering the cross flow.
- Switch on the aspiration system.
- Remove the cloth from a 2 ml pipet using forceps and attach it to the aspirator.
- Open the culture flask and aspirate the medium. Do not touch the cells with the pipet.
- In case of many death cells, wash cells with 5 ml of HBSS (do not pipet directly onto the cells), add HBSS, gently let the HBSS cover the cells and let it gently run over a few times.
- Aspirate the HBSS. Do not touch the cells with the pipet.
- Add 10 ml of new medium (swirl bottle first to mix the ingredients well), (do not pipet directly onto the cells), and gently let the medium cover the cells.
- Inspect the cells with the microscope and place them back as quick as possible in the incubator at 37^{o}C and 5% CO_2.

Subculturing

- Subculturing is mostly needed when the cells reach a confluency of 80-90% or when cells tend to grow too fast to for example keep healthy during the weekend. Overgrowing of cells could end up in differentiated cells, or unhealthy cells and should always be prevented.
- Remove medium using the aspirator.
- Wash gently with 5 ml of HBSS, remove HBSS using the aspirator.
- Add 1.0 ml 0.5% trypsin, let the trypsin cover the cell layer and incubate 3-5 minutes at 37^{o}C and 5% CO_2.
- Tick mildly against the flask to loosen the cells. To hard ticking may result in clumps of cells.
- Inspect using the microscope, to long incubation of cells in trypsin may be harmful for the cells
- Resuspend the cells in the appropriate volume of culture medium (mostly 10 ml).
- Add the appropriate volume of new medium to the new flask(s) and dilute the cells to the appropriate dilution (in case of a 1:10 dilution, add 10 ml to the trypsinised cells, add 9 ml new medium to the new cells, add 1 ml cells to the new flask)
- Write down on the flask: Name user, Cell line, passage number, date
- Inspect the cells with the microscope and place them back as quick as possible in the incubator at 37^{o}C and 5% CO_2

Numbers for most commonly used cell culture volumes

Type	dimension	Appr.surface mm^2	Cells at confluency	Medium ml	Trypsine ml	HBSS ml
Wells plate	6 wells	950	$9.5 * 10^5$	3-5	0.5	2
Wells plate	12 wells	380	$3.8 * 10^5$	1-2	0.5	1
Wells plate	24 wells	190	$1.9 * 10^5$	0.5-1	0.2	1
Wells plate	96 wells	32	$3.2 * 10^4$	0.1-0.2	0.05	0.2
disk	10 mm	6010	$6.8 * 10^6$	10	1	5
flask	T75	7500	$8.5 * 10^6$	10	1	5
flask	T25	2500	$2.8 * 10^6$	3-4	0.5	2
flask	T225	22500	$1.75 * 10^7$	30	3	10

The number of cells on a confluent plate, disk or flask will vary with cell type, this table is based on HeLa cell cultures.

<u>Counting cells:</u>

Using the cellometer T4 cell counter

- Trypsinize the cells.

- Resuspend the cells in 10 ml of medium (75 cm² flask).
- Add 20ul celsuspension to 20 ul 0.2% tryphan blue in an eppendorf tube .
- Fill a chamber slide twice with 20 ul of cell suspension.
- Count cells according to manual provided.

Remarks: perform a cell size analysis, indicating that you count the whole cell population. Check the dilution factor in the software (must be 2).

Using the counting chamber

- Trypsinize the cells.
- Resuspend the cells in 10 ml of medium (75 cm² flask).
- Pipet 50 µl of celsuspension in an eppendorf tube.
- Add 50 µl of 0.2% trypan blue.
- Fill counting chamber with celsuspension/ trypanblue.
- Count cells in xxx squares and calculate the average.

To be continued....

Procedure after culturing

- Clean the cross flow with 70% ethanol.
- Rinse the tube of the pump with 70% ethanol, switch off.
- Collect all culture trash in folded bags in the RVS waste buckets.
- Collect paper and plastics trash in normal waste buckets.
- Turn the cross flow, microscope and waterbath off.
- Organize the lab
- Wash your hands

Appendix II: Sonicator manual

Principle
Sonication is the use of ultrasonic frequencies (> 20 kHz), which can be used to break open cells and cell structures in the making of homogenates.

Precautions
-

Preparations/required materials
Ice box (preferably glass) + ice
Homogenized samples (e.g. use HAP 127; Protein extraction for this)
Ethanol (70%) and tissues for cleaning

Apparatus
Sonicator in the HAP lab (SLPE-150)

Method

1. Switch ON the power, 'RDY' will display on the screen

2. Press 'mode' button and 'set' button until time (min/sec) and amplitude LED is on
3. Set the pulser by press the set button
   a. Set 'on' and 'off' time (in seconds) of the pulse by 'UP/DOWN' button
   b. Time on = 00:01, Time off = 00:03
      Set the 'amplitude' by 'UP/DOWN' button
   c. Amplitude = 40
      Set the experiment time (in minute) by 'UP/DOWN' button
   d. Experimental time = 00:03
4. Press 'enter' to save the setting
5. Clean the probe with EtOH
6. Put the sample in a box with ice, preferably a glass box to be able to see through
7. Immerse the probe halfway into the sample
8. Press 'START/STOP' to start the pulse
9. Press 'START/STOP' after required pulses achieved
   Required pulses = 10 pulses
10. Clean the probe with EtOH

Appendix III:Protein concentration determination

Principle
Protein determination is needed prior to several assays, as western blotting, triglyceride measurements etc. Two methods are described, with a different detection range.

Precautions
See kit instructions and chemicals used

Preparations/required materials

Kit:
RC DC protein assay (range 0.2-2 ug/ul).
Bio-Rad, # 500-0113 Protein Assay Reagent A
500-0114 Protein Assay Reagent B
500-0115 Protein Assay Reagent S

Or #500-0116, complete package

Micro BCA Protein assay kit (range 0.002-0.04 ug/ul)
Pierce: # 23235

Matrix
serum, tissue, human, mice

Apparatus
Biotec plate reader

Method
Protocol Bio-Rad: RC DC protein assay (range 0.2-2 ug/ul).

Preferably perform the assay in triplicate.

Make a calibration curve of BSA in the appropriate homogenization solution (same matrix as the samples) ranging 0-2 ug/ul (5 ul per well is needed, so 15 ul in total): Weigh a few ug of BSA. Add half the amount of ul homogenization buffer to the BSA to dissolve, → 2 ug/ul

nr	Conc ug/ul	ul BSA	ul buffer
1	2	undiluted	
2	1.75	87.50	12.50
3	1.5	85.71	14.29
4	1.25	83.33	16.67
5	1	80.00	20.00
6	0.75	75.00	25.00
7	0.5	66.67	33.33
8	0.25	50.00	50.00

Dilute the sample 1:2, 1:3, 1:5 and 1:10 (if needed) in the appropriate solution:

dilution	ul extract	ul buffer
1:2	10	10
1:3	10	20
1:5	10	40
1:10	10	90

Add 5 ul of sample or standard to each well.
Add 25 ul of reagent A (standard S must be added to reagent A before, if a detergent is used in the homogenization, see original manual).
Add 200 ul of reagent B, mix well by pipetting and leave on a mixer for 15 minutes.
Read absorbance after 15 minutes at 750 nm on the plate reader (650-750 nm).
The signal is stable for at least 1 hour.

Calculate the protein concentration of the undiluted homogenate.

Protocol Pierce: Micro BCA Protein assay kit (range 0.002-0.04 ug/ul)

Needs to be written by the first user.

Appendix IV: Western blot analysis OXPHOS complexes.

Harvesting cells for energy status WB:

1.Wash with ice-cold HBSS.

2.Add 0.5 mL ice-cold lysis buffer (50mM TRIS pH 7.4, 1% Triton).

3.Incubate shortly and harvest cells (pool transwells per treatment).

4.Sonicate samples (10 pulses per sample).

5.Measure protein content with BioRad DC protein assay.

6.Make 140uL cell protein lysate solution and correct for the lowest found

7.Add 50uL Sample loading buffer and 10uL DTT.

8.Heat samples 5min at 50°C.

9.Cool down samples on ice.

10.Freeze samples at -20°C for electrophoresis and western blotting next day.

Gel electrophoresis

1.Make 1x running buffer: 40mL 20x MOPS running buffer+ 760mL MQ.

2.Put gels in the system (wells on the inside).

3.Fill up middle compartment with running buffer.

4.Add 200uL of NuPAGE antioxidant (AOX).

5.Pipet 10uL sample(total protein loaded 80µg) and 2uL of marker.

6.Start run at 100V.(Total estimated time: 100-120min).

Western blotting

1	Make 1x transfer/blot buffer: Make TBT-T (0.1% Tween 20):	Make 1x transfer buffer without MeO:
	30mL 20x transfer buffer 100mL 10x TBS	2.5mL 20x transfer buffer
	60mL MeOH	47.5mL MQ
	510mL MQ	200uL 10% SDS
	600uL NuPAGE AOX	

2.Prepare PVDF blotting membrane: 8.2*8.2cm, eventually mark one corner. Activate membrane 15 sec in MeOH and store in blot buffer for at least 5min and mix.

3.Prepare gel and cut stacking gel from the gel, keep in blot buffer until use. Put the gel in transfer buffer without MeO for 5 minutes.

4.Make blotting sandwich in all prewet layers: Black plastic transfer cassette- fibre pad- filter paper- gel- membrane- filter- fibre path. Prevent and remove any air bubbles.

5.Fill tank with blot buffer and place transfer cassette (black to black).

6.Insert cold pack from -20 and stirring magnet, place tank on stirring plate.

7.Use small power supply. Run at 100V (should be approximately 300mA, otherwise switch to lower voltage), for 45 minutes.

8.Wash membrane 5 min in 5mL TBS-T with gentle rocking.

9.Add some ponceau S stain to see whether transfer of protein was successful.

10.Make a picture.

11.Rinse the membrane with demi water until pink colour disappears. Cut membrane in pieces (Cut in half above 75kDa mark).

12Wash membrane twice for 5min in 5mL TBS-T while gently rocking.

13.Block membrane in TBS-T 5% BSA 1hr at RT.

14.Incubate covered membrane in 1:1000 diluted Rodent WB Antibody Cocktail antibody or OXPHOS CI (x-ndufa9) in dilution 1:1000 (in TBS-T 5% BSA) overnight at 4°C while gently rocking.(Rabbit antibodies against: AMPK/pAMPK/ACC/pACC).

15.Rinse membrane 3 times for 5min with TBS-T.

16.Incubate covered membrane donkey anti-mouse IRDye 680 antibody dilution 1:10000 (in TBS-T 5% BSA) for 1h at RT, while gently rocking.

17.Rinse membrane 5 times for 5min with TBS-T.

18.Scan membrane or store membrane at 4°C for up to 1 month.

Imaging the western blot

1.Clean glass plate with 70% EtOH.

2.Put membrane with proteins down on the glass plate with some TBS-T and remove air bubbles.

3.Start Odyssey program.

4.Parameters to be completed:

Preset: membrane.

Resolution: 84 and medium for more clear pictures.

Channels: 700 (marker) and 800nm (donkey anti-mouse IRDye 680 antibody).

Intensity: start with 5, switch later to higher value when bands are too light.

Area: select the area of the blot.

5.Start scan.

6.Save image in the project.

7Adjust images and quantify band intensity with Image Studio Lite .

Appendix V: Making an SDS-PAGE gel.

1. Clean the spacers, combs and glass plates with detergent and subsequently with alcohol.
2. Assemble the gel casting apparatus, making sure that the sandwich of glass plates and spacers will make a good seal.

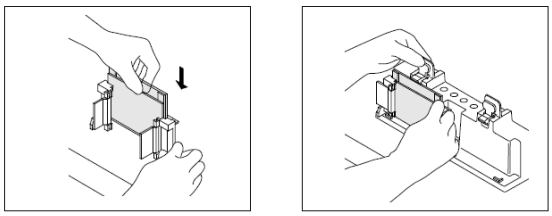

Fig. 2. Assembling the Mini-PROTEAN Tetra cell casting frame and casting stand.

3. Prepare the Separating Gel solution according to the acrylamide concentration needed. (A gel with low density will resolve the larger polypeptides while cutting off the lighter ones, and one of higher density will reveal the smaller polypeptides, while compressing and possibly distorting the larger ones.) Mix well. 10 ml is sufficient for 1x 1.5 mm mini gel or 2x 0.75 mm mini gels.

Separating gel

kDa:	**>100**	**60-100**	**30-80**	**20-60**	**<20**
Final acrylamide conc	**7.5%**	**10%**	**12%**	**15%**	**20%**
MilliQ	5.525 ml	4.9 ml	**4.4 ml**	3.65 ml	2.2 ml
40% acrylamide	1.875 ml	2.5 ml	**3.0 ml**	3.75 ml	5.0 ml
1.5 M Tris pH 8.8	2.5 ml	2.5 ml	**2.5 ml**	2.5 ml	2.5 ml
10% SDS	100 µl	100 µl	**100 µl**	100 µl	100 µl
10% APS	100 µl	100 µl	**100 µl**	100 µl	100 µl

TEMED	10 µl	10 µl	**10 µl**	10 µl	10 µl
Total	**10 ml**	**10 ml**	**10 ml**	**10 ml**	**10 ml**

4. Load the apparatus with the separating gel solution.

5. Immediately top with ethanol or MilliQ to equalize the surface and to isolate the polymerization from oxygen.
6. After polymerization (~30 minutes), pour off the ethanol/milliQ, and remove any droplets from the inside of the casting apparatus with a paper towel.
7. Prepare the stacking gel solution. Mix well.

Stacking gel (4% acrylamide)

MilliQ	3.2 ml
40% acrylamide	488 µl
0.5 M Tris pH 6.8	1.25 ml
10% SDS	50 µl
10% ammonium persulfate (APS)	75 µl
TEMED	5 µl
Total	**5 ml**

8. Immediately load the stacking gel solution, and insert the comb for the stacking gel, take care not to introduce air bubbles around the comb.
9. Allow the stacking gel to polymerize completely (~45 minutes) before removing the comb.

Appendix VI: Western blot analysis pAMPK/AMPK and pACC/ACC.

Harvesting cells for energy status WB:

1.Wash with ice-cold HBSS.

2.Add 0.5 mL ice-cold lysis buffer (50mM TRIS pH 7.4, 1% Triton).

3.Incubate shortly and harvest cells (pool transwells per treatment).

4.Sonicate samples (10 pulses per sample).

5.Measure protein content with BioRad DC protein assay.

6.Make 140uL cell protein lysate solution and correct for the lowest found

7.Add 50uL Sample loading buffer and 10uL DTT.

8.Heat samples 5min at 70°C.

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Gel electrophoresis

1.Make 1x running buffer: 40mL 20x MOPS running buffer+ 760mL MQ.

2.Put gels in the system (wells on the inside).

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4.Add 200uL of NuPAGE antioxidant (AOX).

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Western blotting

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	510mL MQ	200uL 10% SDS
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3.Prepare gel and cut stacking gel from the gel, keep in blot buffer until use. Put the gel in transfer buffer without MeO for 5 minutes.

4.Make blotting sandwich in all prewet layers: Black plastic transfer cassette- fibre pad- filter paper- gel- membrane- filter- fibre path. Prevent and remove any air bubbles.

5.Fill tank with blot buffer and place transfer cassette (black to black).

6.Insert cold pack from -20 and stirring magnet, place tank on stirring plate.

7.Use small power supply. Run at 100V (should be approximately 300mA, otherwise switch to lower voltage), for 1 hour.

8.Wash membrane 5 min in 5mL TBS-T with gentle rocking.

9.Add some ponceau S stain to see whether transfer of protein was successful.

10.Make a picture.

11.Rinse the membrane with demi water until pink colour disappears. Cut membrane in pieces (Cut in half above 75kDa mark).

12Wash membrane twice for 5min in 5mL TBS-T while gently rocking.

13.Block membrane in TBS-T 5% BSA 1hr at RT.

14.Incubate covered membrane in 1:1000 diluted Rodent WB Antibody Cocktail antibody or OXPHOS CI (x-ndufa9) in dilution 1:1000 (in TBS-T 5% BSA) overnight at 4°C while gently rocking.(Rabbit antibodies against: AMPK/pAMPK/ACC/pACC).

15.Rinse membrane 3 times for 5min with TBS-T.

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17.Rinse membrane 5 times for 5min with TBS-T.

18.Scan membrane or store membrane at 4°C for up to 1 month.

Imaging the western blot

1.Clean glass plate with 70% EtOH.

2.Put membrane with proteins down on the glass plate with some TBS-T and remove air bubbles.

3.Start Odyssey program (New scan: username and password: Odyssey).

4.Parameters to be completed:

Preset: membrane.

Resolution: 84 and medium for more clear pictures.

Channels: 700 (marker) and 800nm (donkey anti-mouse IRDye 680 antibody).

Intensity: start with 5, switch later to higher value when bands are too light.

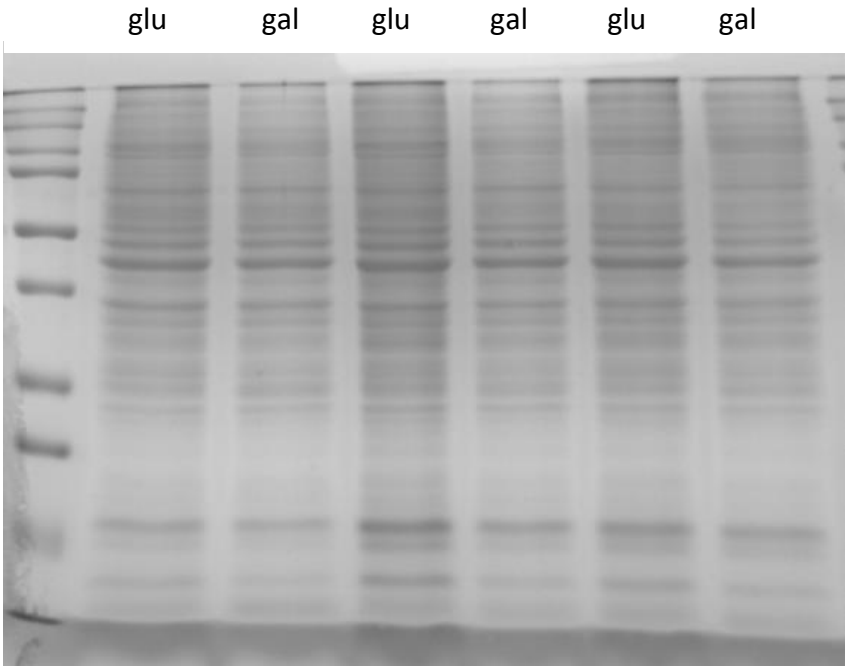
Area: select the area of the blot.

5.Start scan.

6.Save image in the project.

7Adjust images and quantify band intensity with Image Studio Lite .

Appendix VII: Picture of protein stain.



The protein stain showed higher amounts of protein in the glucose samples as reported by β-actin. Probably, during the protein assay there were an underestimation of glucose sample concentrations which cause an over load of protein in the gel electrophoresis. We can deduce β-actin were not influenced by the tratment.