AMERICAN UNIVERSITY OF BEIRUT

# TRANSCRIPTOMIC ANALYSIS OF COLON CANCER CELLS TREATED WITH AN ANTI-DIABETIC DRUG

by
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submitted in partial fulfillment of the requirements
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of the Faculty of Medicine
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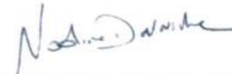

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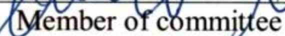

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# AN ABSTRACT OF THE THESIS OF

Jamila Mostafa Hijazi for Master of Science
Major: Biochemistry

Title: Transcriptomics Analysis of Colon Cancer cells Treated with an Anti-Diabetic Drug

**Background:** Metformin, the anti-diabetic drug which is widely used as a hypoglycemic agent in treating type-2 diabetes is also known for its anti-cancer effects. Data obtained from in-vitro and in-vivo studies showed that metformin influences cancer progression since it affects cell cycle arrest and apoptosis. Most studies refer this anti-proliferation mechanism to the inhibition of mTor by metformin; however the genetic pathway analysis underlying the downstream targets is still rarely reported.

**Aims:** In this study, we aim to unravel the genetic pathways using in-vitro cell model, and to examine the effect of metformin on cell cycle and on cancer proliferation in colon cancer cells.

**Methods:** Four different metformin concentrations were used (1mM, 2mM, 5mM and 10 mM) on the HCT116 colon cancer cell line and its genetic modified forms HCT-116 p53 null and p21 null. To assess the percentage of cell viability, a colorimetric assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide for assay: MTT assay) was applied at 24 h and 48 h of treatment. Cell cycle and apoptosis rate were measured using the flow cytometer propidium iodide based assay.

Finally, RNA-seq was performed on HCT116 after 24hr of metformin treatment and the results were analyzed using Galaxy. Pathway analysis was undertaken using the Pathway studio software, and significantly differentially expressed genes were confirmed by qPCR.

**Results** showed a significant decrease in the percentage of cell viability between the control and the treated cancer cells in a dose and time response manner. Analysis of cell cycle has shown an increased in the subG0 phase (apoptosis phase). Transcriptomic analysis revealed more than 3,000 significantly down-regulated genes and 1,200 up-regulated genes ($p<0.05$).

**Conclusion** metformin shows high potential in inhibiting colorectal cancer proliferation and in inducing apoptosis through increasing cell death and cell fragmentation at the level of SubG0 especially in the absence of *p21* gene as *p53* could be the main player in stimulating apoptosis in colorectal cancer cells but it’s not alone. Metformin affected the

molecular pathways implicated in colorectal cancer, but still we need more next generating sequencing to be implicated at different levels of the disease.

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# ABBREVIATION

| | |
|---|---|
| 4EBP1 | 4E-binding protein 1 |
| ACC | Acetyl-CoA carboxylase |
| AMPK | Adenosine monophosphate activated protein kinase |
| AMPK | AMP-activated protein kinas |
| APC gene | Adenomatous polyposis coli |
| BAX | BCL2 associated X |
| BMP | Bone morphogenetic proteins |
| CCND1 | Cyclin D1 |
| CDC4 | Cell division control protein 4 |
| CDK | Cyclin-dependent kinases |
| CIN | Chromosomal instability |
| CSC | Cancer stem cell |
| CRC | Colorectal Cancer |
| DDIT4 | DNA damage inducible transcript |
| EMT | Epithelial-to-mesenchymal transition |
| FAP | Family adenomatous Polyposis |
| FBS | Fetal Bovine Serum |

| | |
|---|---|
| FBXW7 | F box and WD40 domain protein |
| FOXO3 | Fork head box O3 |
| GPDH | Gglycerol-3-phosphate dehydrogenase |
| HIF-1 alpha | Hypoxia-inducible factor 1-alpha |
| HNPCC | Hereditary non polyposis colorectal cancer |
| IGF2R | Insulin-like growth factor 2 receptor |
| IPMK | Inositol polyphosphate multi kinase |
| IR | Insulin receptor |
| LKB1 | Liver kinase B1 |
| MT | Metformin |
| miRNA | Mmicro RNA |
| MMR | Mismatch repair |
| MSI | Microsatellite instability |
| mTOR | Mammalian target of rapamycin |
| mtt | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| PARP | Poly (ADP-Ribose) Polymerase |
| PI | Propidium Iodide |
| PI3K | Phosphoinositide 3-kinase |
| PTEN | Phosphatase and tensin homolog |

| | |
|---|---|
| SREBF1 | Sterol regulatory element binding transcription factor 1 |
| STAT | Signal Transducer and Activator of Transcription |
| TCA | Tricarboxylic acid |
| TGF-B | Transforming growth factor beta |
| TGFBR2 | Transforming growth factor beta Receptor 2 |
| TP53 | Tumor protein |
| VEGF | Vascular Endothelial Growth Factor |
| ΔΨM | Mitochondrial membrane potential |

# CHAPTER I

# INTRODUCTION

## A. Colorectal Cancer

### *1. Overview*

Colorectal Cancer usually emerges from the glandular, epithelial cells of the large intestine. The cancer arises when certain cells of the epithelium acquire a series of genetic or epigenetic modifications that confer on them a selective advantage (Rawla, Sunkara, & Barsouk, 2019). With abnormally heightened replication and survival, these hyper-proliferative cells give rise to a benign adenoma, which may then evolve into carcinoma and metastasize over decades (Ewing, Hurley, Josephides, & Millar, 2014).

The primary function of the colon is the reabsorption of water and remaining minerals and nutrients in the chyme. The large intestine contains diverse microflora that can break down remaining starches and proteins. In order to facilitate absorption, the gastrointestinal epithelium is organized as an axis of crypts and villi. Colon stem cells and progenitor cells are located in the bottom of the crypt. These pluripotent cells function in self-renewal (Peifer, 2002). As the progenitor cells differentiate into specialized epithelium cells, they migrate out of the crypt and up the villus. Differentiated epithelial cells include Paneth, goblet, and enteroendocrine cells as well as enterocytes. Once these cells arrive at the top of the villus after about 14 days, they undergo apoptosis, i.e. programmed cell death, and are shed and eliminated with the feces (Kosinski et al., 2007) This process is highly regulated by a gradient of signaling proteins, among which the most common are Wnt, BMP, and TGF-B (Medema & Vermeulen, 2011) . However,

in colorectal cancer these orchestrated pathways are deregulated and the underlying mechanisms are still unclear.

The colorectal cancers actually comprise a very heterogeneous group of diseases driven by a vast array of mutations and mutagens. And because not all colorectal cancers share similar driving mutations, it has been difficult to design a "catchall" molecular therapy (Ogino & Goel, 2008).Thus, a strong understanding of the pattern of colorectal cancer development, the environmental and genetic risk factors, and the molecular evolution of the disease can empower researchers and physicians to prevent and treat this deadly neoplasm.

## 2. *CRC Initiation and Stages*

CRC progresses gradually through three precisely-connected stages: Initiation – a process that alters the molecular message of the normal cell, promotion - aberrant signal transduction cascades and progression - phenotypically-altered, transformed cells.
CRC can be divided into five stages, stage 0 to IV (Figure 1). Disease severity and the corresponding therapeutic options depend on the stage (Hatano et al., 2013).

Stage 0 can be characterized by a tumor at the region of the mucosa or inner lining of the colon. CRC stage I is when cancer cells grow in the mucosa, yet their invasive capacity is restricted to the muscular region and not present in the neighboring tissues of the colon (Robinson, Newcomb, Hardikar, Cohen, & Phipps, 2017). Stage II can be subcategorized into three types based on invasive growth into: the walls of the colon, the muscular layer of abdomen lining, and nearby tissues. Depending upon the growth of the cancer, stage III can be further divided into three types. During this stage, the cancer grows into the inner lining of the colonic muscular layer and forms lymph

nodules in surrounding tissues. Based on the number of nodule formations, this stage can be named IIIA, IIIB or IIIC. Stage IV describes the worst stage of the disease where the cancer has spread to distant parts of the body, such as the liver and lungs (Rawla et al. 2018)

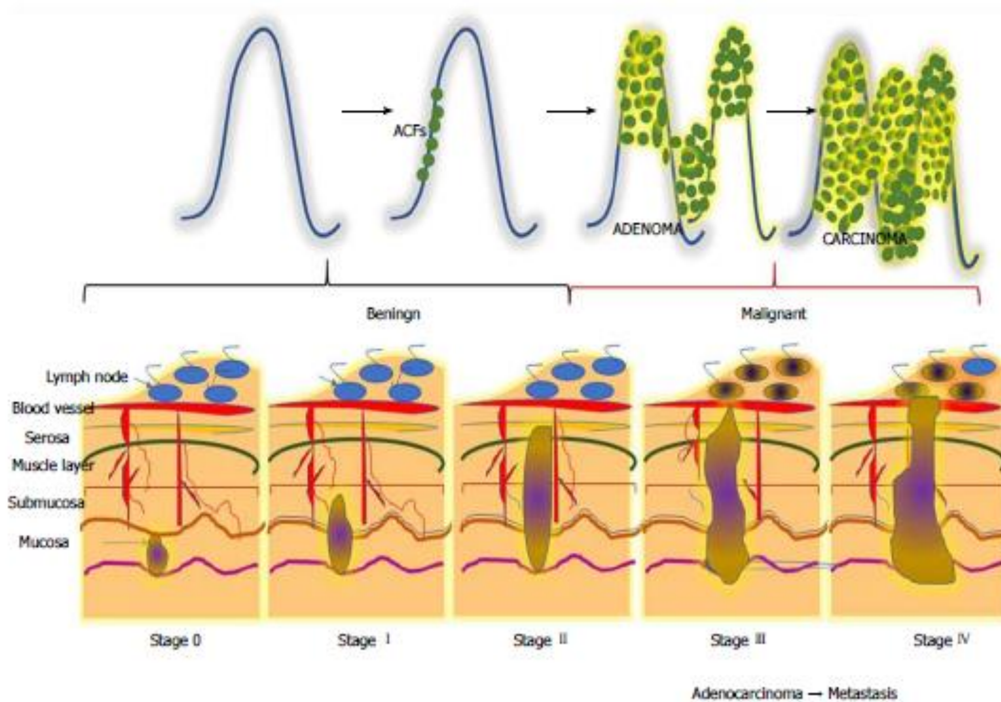



Fig.1 Different stages during the progression of colorectal carcinogenesis (adapted from Rawla et al. Gastroenterology Review, 2018)

The majority of CRCs (>90%) are adenocarcinoma, a malignant neoplasm that develops from glandular epithelial cells of the colon and rectum; other rare types include squamous cell carcinoma, adenosquamous carcinoma, spindle cell carcinoma and carcinoma (Keum & Giovannucci, 2019).Approximately 60–65% of CRC cases arise sporadically (that is, occur in individuals without a family history of CRC or inherited

genetic mutations that increase CRC risk) (Fig. 2) through acquired somatic genetic and epigenetic aberrations largely attributable to potentially modifiable risk factors

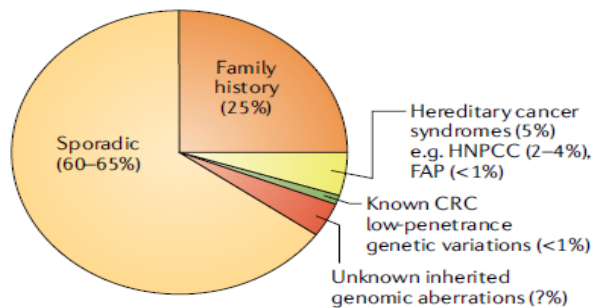



Fig. 2. Proportion of colorectal cancer cases associated with sporadic and hereditary factors (adopted from Keum and Giovannucci, Nature Reviews Gastro, 2019)

(Jasperson, Tuohy, Neklason, & Burt, 2010). Approximately 25% of CRC cases have a family history of CRC without any obvious genetic cancer syndrome. Only 5% are attributed to hereditary cancer syndromes such as hereditary non polyposis colorectal cancer (HNPCC, also known as Lynch syndrome) or familial adenomatous polyposis (FAP), caused by inherited germ line mutations in rare but high penetrance susceptibility genes (for example, *MLH1* and *APC*, respectively)(Jiao et al., 2014)

### 3. *Subtypes and Genetic Mutations in CRC*

A clinicopathological changes with genetic abnormalities is observed in the progression of chromosomally unstable colorectal cancer (Walther et al., 2009) .The initial step in tumorigenesis is that of adenoma formation, associated with loss of adenomatous polyposis coli (*APC*). Larger adenomas and early carcinomas acquire mutations in the small *GTPase KRAS*, followed by loss of chromosome 18q with *SMAD4*, which is downstream of transforming growth factor-β (TGFβ), and mutations in *TP53* be consistnet throughout either the gene is *TP53* or *p53* in frank carcinoma.

Microsatellite instability (MSI+ ) CRCs, characterized by a deficiency of the mismatch repair system that leads to slippage in microsatellites (the caretaker pathway involving genes that maintain genomic stability)(Grady & Carethers, 2008), only carry the above changes infrequently; therefore, development of CRC must involve different, but analogous, genetic changes to those described in chromosomal instability (CIN)+ CRC (Fig. 3).

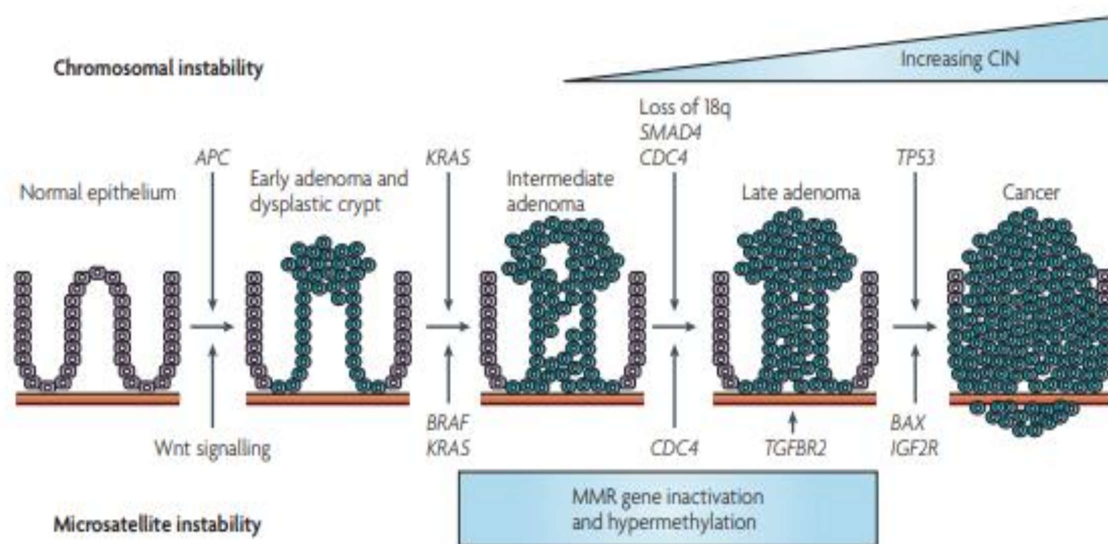



Fig. 3. Adenoma–carcinoma sequence model for chromosomal instability in colorectal cancer (adapted from Walther et al. Nature Reviews, 2009)

MSI is uncommon in adenomata, and the initial step is thought to involve alteration in Wnt signaling (Segditsas & Tomlinson, 2006), possibly involving axin. Mutations in *BRAF*, common in MSI+ CRC, are likely to occur in the place of *KRAS* mutations (Rajagopalan et al., 2002), although the latter do occur in a minority of cases. Mismatch repair (MMR) deficiency in sporadic CRC occurs predominantly by down-regulation of *MLH1* through promoter methylation(Mao et al., 2004) and MSI status is

increased by positive selection of tumor cells with mutated microsatellites in *MSH3* and *MSH6*(Cunningham et al., 2004).

Further positive selection occurs for mutations affecting microsatellites in TGFβ receptor 2 (*TGFBR2*), insulin-like growth factor 2 receptor (*IGF2R*) and *BAX*, which in turn provides a *TP53*-independent mechanism of progression to carcinoma (Samowitz et al., 2005).FBXW7 (F box and WD40 domain protein) inactivation may precede TP53 mutation, leading to increasing CIN144, although it is not always associated with CIN and may also have a role in the MSI pathway (Cheng et al., 2008)

## B. Targeting cell death in colorectal cancer: implications for therapy

An interesting finding about cancer is that several genes that are responsible for cancer development are very much active during embryogenesis and fetal development, particularly regulating embryonic growth and organ formation (Costanzo, Bardelli, Siena, & Abrignani, 2018). These genetic programs remain silent throughout the rest of the life of an organism; however, they are turned on in cells during cancer formation (Wong et al., 2008)

Apoptosis is a tightly regulated process essential to maintain tissue homeostasis. Acquisition of a resistance to apoptosis plays a pivotal role in tumorigenesis by disrupting the balance between cell proliferation and cell destruction and by allowing cancer cells to resist radiation and chemotherapy (Elmore, 2007). Identification of mechanisms that can antagonize apoptosis is essential to devise therapeutic strategies aimed at enhancing the efficiency of cancer treatment (Mohammad et al., 2015).

Recent technological advances have allowed the successful targeting of individual genes (ex. *p53* and *p21*genes) in human somatic cells, thus permitting studies of

activation or inactivation of a particular gene on the response of cancer cells to therapeutic agents.(Abreu Velez & Howard, 2015)

During these sequential events from benign polyp formation to adenomas and finally carcinomas, cell death plays an essential role (Testa, Pelosi, & Castelli, 2018).

A low rate of apoptosis in the base of the crypt, where stem cells are expected to reside, is fundamental to the function of the normal intestine (Bach, Renehan, & Potten, 2000). It is interesting to note that epithelial cells residing in the villi of the small intestine and colon are resistant to apoptosis (Marshman, Ottewell, Potten, & Watson, 2001)

Changes in the expression patterns of several apoptotic proteins during the transformation of adenomas into carcinomas reveal the importance of apoptosis during colon cancer progression (Ashokkumar et al., 2018).

Since 70% of reported CRCs are associated with mutations in the tumor suppressor *p53* gene, the transition from adenomas to carcinomas in the colorectal region is considered to involve a mechanism whereby apoptosis machinery fails (Li, Zhou, Chen, & Chng, 2015)

Apart from apoptosis, several signaling cascades are known to regulate apoptosis. Among these, PI3K/Akt/mammalian target of rapamycin (mTOR) is an important signaling pathway that acts as a checkpoint in apoptosis and promotes cancer progression. Interestingly, PI3K/Akt hyper activation, *PIK3CA* mutations, and both *PTEN* mutations and deletions have been reported in the incidence of CRC (Danielsen et al., 2015)

## C. Tumor Suppressor Gene: *p53*

Most colorectal cancers develop from the sequential inactivation of tumor suppressor genes including *APC, TP53*, and *SMAD4*, as well as activation of oncogenes (e.g., *KRAS*) (Zhao, Mishra, & Deng, 2018). Recent studies have defined the "driver" mutations involved in colorectal cancer. In one study, the introduction of five key mutations, *APC*, *TP53, SMAD4, KRAS, and PIK3CA*, in normal human intestinal organoid induces transformation and tumorigenesis (Matano et al., 2015).

*P53* is well known gene for its tumor suppressor role and is one of the most mutated genes in all forms of human cancer. Activation of the *p53* DNA damage stress response induces DNA repair and regulates the cell cycle to prevent oncogenic mutation (Hsieh et al., 2005). Alteration of p53 signaling in colon cancer, which results in the loss of apoptosis and cellular checkpoints while altering genetic integrity, ultimately leads to malignancy.

In addition to the tumor-suppressing role of p53, it has been shown that some missense-type mutations of the *p53* gene at the DNA binding domain induce acquisition of oncogenic functions (Nakayama & Oshima, 2019).

Therefore we need to consider the role of *p53* mutation in colorectal cancer development from two separate aspects: the loss of wild-type p53 function and the possible gain of oncogenic function of missense-type mutant *p53*.

Genetic alterations in these genes(Driver genes)are thought to cause acquisition of stemness, increased cell proliferation, suppression of differentiation, and impaired genome DNA maintenance, which may contribute to ‘hallmarks of cancer’ (Hanahan & Weinberg, 2011) as major components of the cancer cell signaling pathways (Vogelstein et al., 2013). Furthermore, the modification of these pathways by the same genetic

alterations is widely found in many types of cancers, suggesting that these are key driver pathways for oncogenesis, irrespective of tissue type (Sanchez-Vega et al., 2018)

**D. P53 and other driver genes in tumorigenesis**

The nuclear accumulation appears to be required for the oncogenic function of mutant p53. Although loss of wild type p53 may cause stabilization of mutant p53, it has also been shown that even after the loss of wild-type p53, mutant p53 stabilization and nuclear localization are still regulated by exogenous signaling (Nakayama & Oshima, 2019). Accordingly, further studies will be required to understand the mechanism underlying mutant p53 stabilization and subcellular localization by microenvironment derived signaling (Nakayama & Oshima, 2019). However, such signaling will be an ideal target for therapies to prevent colorectal cancer progression.

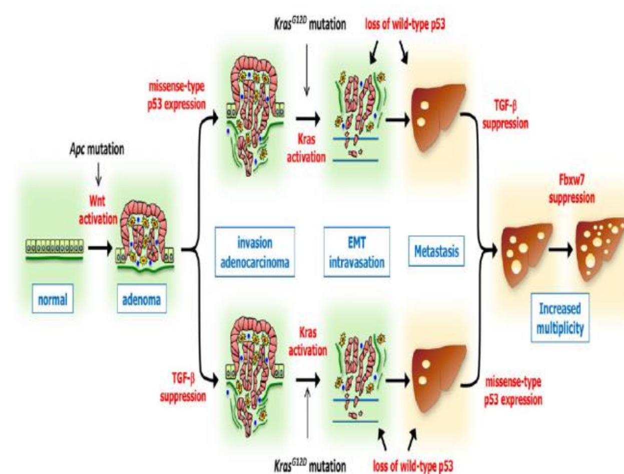



**Fig. 4. Multistep tumorigenesis of colorectal cancer development and malignant progression based on the results of multi-crossing mouse genetic experiments ( adopted from Sakai et al. ,Cancer Res.,2018)**

## E. Role of *P21* in inducing apoptosis and cell arrest

### *1. P21 as a Tumor Suppressor Gene*

P21 mediates various biological activities by sensing and responding to multiple stimuli, via p53-dependent and independent pathways (Al & Gali, 2019). *P21* is known to act as a tumor suppressor mainly by inhibiting cell cycle progression and allowing DNA repair. Significant advances have been made in elucidating the potential role of *P21* in promoting tumorigenesis (Kreis, Louwen, & Yuan, 2019)

The Cdk inhibitor *p21* is often responsible for stress-induced p53-dependent and p53-independent cell cycle arrest (Eekhout & De Veylder, 2019). Cell cycle arrest permits cells to pause and to repair damage and then to continue cell division. On one hand, the function of p21 to inhibit cell proliferation may contribute to its ability to act as tumor suppressor (Pasz-Walczak, Kordek, & Faflik, 2001).

### *2. p21 as Oncogene*

On the other hand, the capacity of p21 to induce cell cycle arrest after stress can protect cells from stress-induced apoptosis. Anti-apoptotic activity of p21 may contribute to its potential to act as an oncogene (Shamloo & Usluer, 2019). Similar to the growth-promoting oncoproteins *Myc* and *E2F1* that have an "antagonistic duality" because they possess the ability to promote apoptosis (anticancer) to counter to their proliferative activity (Kreis et al., 2019),the growth-arresting protein p21 has an "antagonistic duality" in that it often inhibits apoptosis (procancer) to counter to its anti-proliferative effects (anticancer)(Morgan, 1995).Now its role in human cancer has become more controversial because it may also possess Anti-apoptotic (procancer) capabilities(Shibata, Yoshidome, Shibata, Jorcyk, & Green, 2001).

## F. The P53-P21 Axis

*p53* protein is stabilized and mediates apoptosis and cell cycle arrest. Whereas the mechanisms of p53-dependent apoptosis are not well understood, p53-dependent cycle arrest is primarily mediated by the CDK inhibitor *p21* (Pietsch, Sykes, McMahon, & Murphy, 2008). There is a mounting evidence that p21 is a major inhibitor of p53-dependent apoptosis (Fig. 5). It is not entirely clear how a cell chooses between apoptosis and p21-dependent cell cycle arrest after DNA damage and stabilization of p53, but often high levels of p21 expression mediate cell cycle arrest and protect from p53-dependent apoptosis (Javelaud & Besançon, 2002).

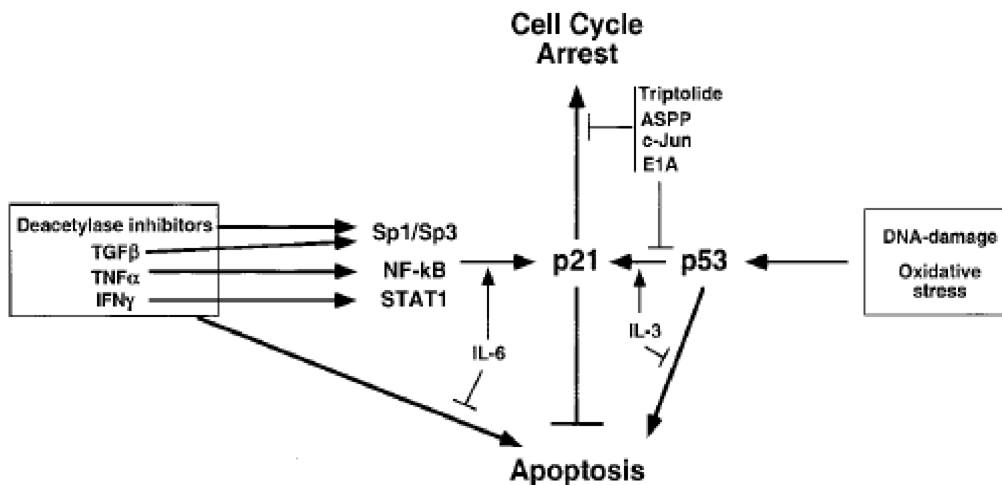



Fig. 5. p21 is a major inhibitor of p53-dependent and p53-independent apoptosis. (Adopted from Andrei et al., Molecular Cancer Therapeutics, 2002)

Anticancer drugs kill cancer cells by inducing p53-dependent and p53-independent apoptosis, and p21 protects cells from anticancer drug-induced apoptosis (Hientz, Mohr, Bhakta-Guha, & Efferth, 2017). Because loss of *p21* usually increases

sensitivity of tumor cells to apoptosis induced by different chemotherapeutic agents, small molecules that eliminate p21 expression may improve the action of anticancer drugs (Lane, Cheok, & Lain, 2010). Therefore, functional p21 may suppress tumor growth in the organism, but at the same time elimination of p21 may be beneficial during cancer treatment.

A variety of types of cellular stress lead to induction of p21 expression by both p53 dependent and -independent mechanisms (Wierød et al., 2008). However, a number of reports also suggest that p21 possesses pro apoptotic functions under certain conditions in specific systems (Al & Gali, 2019).

## G. Metformin

### *1. Overview*

Metformin (chemically designated as 1,1-Dimethylbiguanide hydrochloride) is a drug of first choice for the treatment of type II diabetes as it functions and primary inhibits hepatic gluconeogenesis, but a clear mechanistic understanding of its effects has remained elusive (Castillo-Quan & Blackwell, 2016; Hundal et al., 2000; Kirpichnikov, McFarlane, & Sowers, 2002)

Metformin has also been shown to have anticancer effects against a variety of malignancies, including colorectal cancer. Although inhibition of the mTOR pathway is known to be the most important mechanism for the antitumor effects of metformin, other mechanisms remain unclear (Kang et al., 2018).

Several reports suggested that metformin slows cancer cell growth and protects against multiple human cancers (Bruno et al., 2015; Pryor & Cabreiro, 2015; Rodríguez-Lirio et al., 2015; Wu et al., 2016), although the majority of available clinical data on the

anti-cancer potential of metformin are based on observational studies (Gadducci, Biglia, Tana, Cosio, & Gallo, 2016).

### 2. *Metformin way of action*

Metformin absorbed from the gut, acts via systemic and intracellular molecular pathways. Systemic actions of metformin via metformin uptake through OCT in the liver, AMPK inhibition of gluconeogenesis, and consequent reduction in insulin reduces insulin receptor (IR) stimulation and downstream intracellular effects (including via PI3K, AKt, and HIF-1a) and leads to increased glucose uptake in tissues such as skeletal muscle(Rena, Hardie, & Pearson, 2017). Hepatic functions are adequate with the potential to avoid lactic acidosis (Klubo-Gwiezdzinska et al., 2013) and potentially enhance cancer cell kill. (Fig.6)

The master sensor of cellular energy, the Ser/Thr protein kinase complex AMP-activated protein kinase (AMPK), remains central to studies of metformin(Hardie, 2011). AMPK activation stimulates TSC2, hence indirectly as well as AMPK directly inhibits mTOR and consequently by down-regulating S6 kinase leads to reduced protein synthesis, reduced cellular growth, and lowers proliferation (Thompson, 2014). A complex containing Liver kinase B1 (LKB1), classed as a tumor suppressor gene, activates heterodimers of AMPK subunits through phosphorylation of AMPKa2Thr-172, established as a link between LKB1, AMPK, and cancer (Hawley, Gadalla, Olsen, & Hardie, 2002).

Metformin affects key energy and metabolic processes such as the mitochondrial respiration (complex I), TCA cycle, fatty acid β-oxidation, gluconeogenesis, and

glycolysis (Schulten, 2018). Metformin affects cellular fate processes such as cell cycle, cell growth, EMT, autophagy, and apoptosis. AMPK, the cellular key energy sensor, is phosphorylated in response to an increased AMP/ATP ratio and implicated in exerting several pleiotropic MTF effects. AMPK-dependent mechanisms include, e.g., inhibition of 4EBP1 via the Akt/PI3K/mTOR pathway. AMPK-independent mechanisms include, e.g., inhibition of mTORC1 by DDIT4. The pleiotropic effects of Metformin on gene regulation include, e.g., downregulation of differentiation markers and modulation of epigenetic and mRNA features. Direct activation or inhibition processes are thoroughly known only for a limited number of molecules, such as LKB1 activation of AMPK (Fig.6)

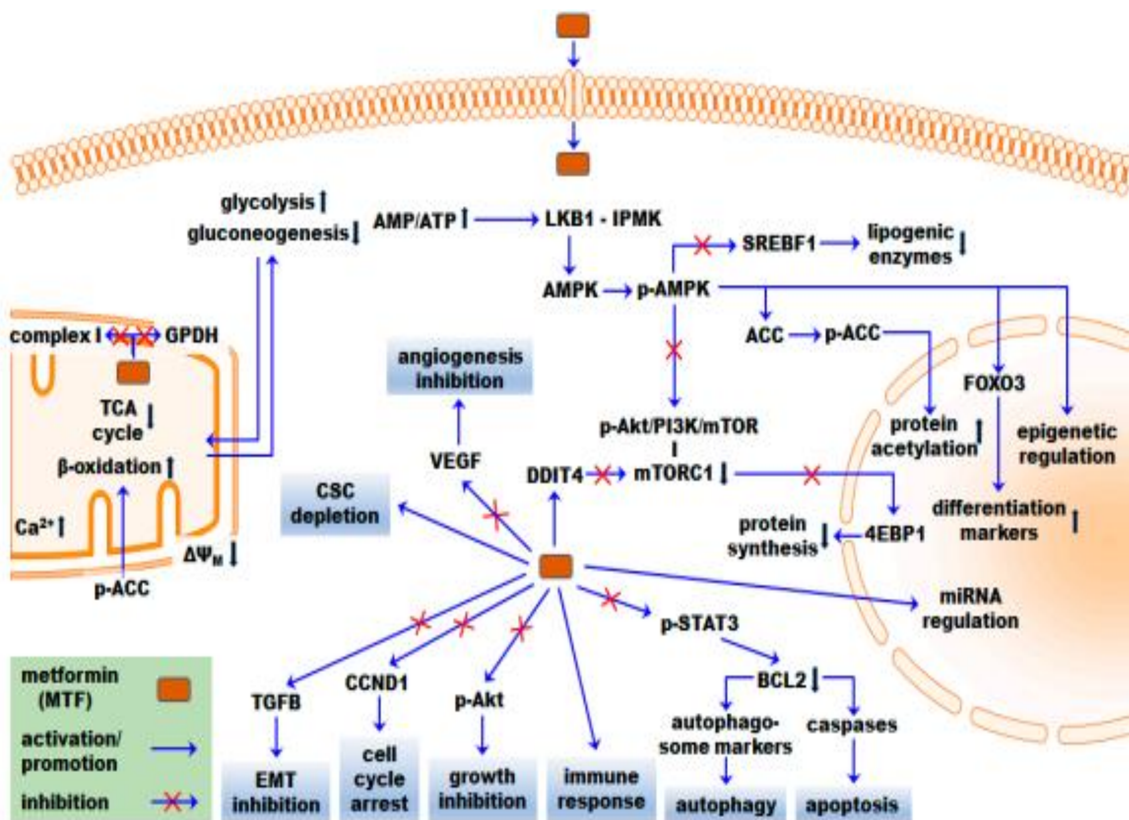



**Fig. 6. Putative anticancer effects of metformin exemplified by molecular and cellular key events (adapted from Schulten, International Journal of Molecular Sciences, 2018)**

Metformin regulates mitochondrial-nuclear communication and modulates the epigenetic landscape in pre-cancerous cells, and this might guide the development of new metabolic-epigenetic strategies for cancer prevention and therapy (Cuyàs, Fernández-Arroyo, Joven, & Menendez, 2016; Jin et al., 2017). However, the molecular mechanisms underlying the anticancer properties of metformin remain elusive (Zhong et al., 2017)

# Aim of the Study

Colorectal cancer is hard to be detected earlier in early stages when the treatment is well sufficient to remove polyps and cure the patient. However, most cases were detected at stage 3 or 4 when there is a little chance for the surgery or for the chemotherapy to stop cancer metastasis and recover colon function and hemostasis. This illustrates challenges and potential opportunities for primary needs for prevention or early detection of colorectal cancer. As for Metformin, it was shown to have anti-cancerous effect which meets with cancer prevention in the first place. However, the mechanism of action of metformin in targeting cancer is still unknown.

In this study we will shed the light over these prospective as follows:

- ❖ Validate the potential effect of metformin in reducing colorectal cancer proliferation and in inducing apoptosis
- ❖ Dissect the effect of Metformin on the cell cycle and its players P21 and P53.
- ❖ Use the unbiased RNA-seq approach to unveil the molecular mechanisms induced by Metformin.
- ❖ Use bioinformatics tools to comprehend the RNA-seq data by building a gene network map that could help targeting colorectal cancer.

# CHAPTER II

# MATERIALS AND METHODS

## A. Cell culture

The human colorectal cancer cell lines, HCT-116 (purchased from American Type Culture Collection ATCC) and its derivates HCT-116 $p21^{-/-}$ and HCT-116 $p53^{-/-}$ were supported from Dr. Nadine Darwiche’s lab (Kindly provided from Dr. Carlos Maria Galmarini, Pharma Mar, Madrid, Spain) .Cells were maintained and grown in Dulbecco's Modified Eagle Medium-F12 (SIGMA), supplemented with 10 % fetal bovine serum, 1% sodium pyruvate and 1 % penicillin/streptomycin. Cells were cultured in a humidified incubator at 37 °C in 5 % carbon dioxide (CO2).

## B. Cell Viability Assay

Cells were seeded in 96-well plates at a density of ~5000 cells/well, after 24 h of incubation, cell were treated with different concentrations of Metformin (1, 2, 5 and 10mM ). After 24 and 48 h, Metformin- treated cells were inducted with MTT for 3h in the cell culture incubator. To stop the reaction MTT stop solution (100ul) was added to each well, and kept in incubator for 1h. The absorbance at 570 nm of the mixture was determined by Elisa. Cell viability was calculated based on the following formula: cell viability (%) = mean experimental absorbance/mean control absorbance x 100%.

### C. Flow Cytometry to determine cell cycle and apoptosis

The cell apoptosis and cell cycle of CRC cells were determined by flow cytometry experiments. Briefly, cells were seeded in 12 well plate, treated with metformin (1, 2, 5 and 10mM) for 24 and 48 h, and then cells were collected and washed with phosphate buffer saline (PBS) twice. For the cell cycle evaluation, cells were fixed overnight with cold 80% ethanol and followed by incubation with propidium iodide and RNase for 1 h at $4^0$C. For the cell apoptosis evaluation, cells were treated with PI. Cell apoptosis was analyzed by a FACS flow cytometer (BD Biosciences).

### D. RNA-sequencing Analysis

Using the NextSeq 550 Illumina we performed RNA-Seq of HCT-116 cell line treated with 10mM metformin after 24 h interval as well as for the control sample (untreated). The experiment was run in triplicates.

Briefly, after fragmentation of total RNAs for short read sequencing, RNA samples were reverse-transcribed into cDNA. Adaptors were then ligated onto both ends of the cDNA fragments followed by amplification of the fragments. Sequenced raw reads were first subjected to quality control (QC) using FastQC. (fig7)

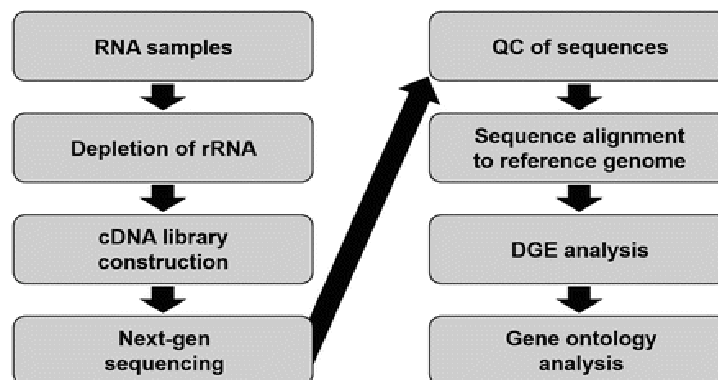



**Fig. 7. A generalized workflow for an RNA-seq protocol examining differential gene expression (adapted from Lang et al., Methods in Molecular Biology, 2018)**

Reads were then mapped to the reference human genome using Bowtie2 mapping. Transcripts were then assembled from aligned reads using Feature-Counts. Using DESeq2, we assessed variation patterns within replicates and between conditions via Principal Component Analysis (PCA) on counts. To view the association between variables, correlation matrix plots were conducted. Also, significantly differentially expressed features between treated and untreated samples from count tables were identified. A p-value/p-adjusted value of less than 0.05 was used in order to account for statistical significance. Finally, fold-change threshold of 1 was further applied. With the use of the Transcript Per Million (TPM) method, expression levels within and across samples were compared once raw counts were normalized by scaling gene counts within a sample to a total of 1 million transcripts while also accounting for gene length.

### E. Pathway Analysis

Differentially expressed gene features were then functionally analyzed and topologically organized into gene-gene interaction networks using the commercially available software Pathway Studio. This program allows us to examine functional associations among the genes and generate functional (with predicted activated or inhibited states) gene-gene interaction networks based on the presence of inter-connected genes, based on the amount of publications in the software's knowledgebase supporting the connections. The pathways, gene network and gene set analyses permit selection of genes predicted to have key roles in phenotypes and diseases based on their expression and number of functional associations within significant interaction networks.

## F. Two-step Reverse Transcription Quantitative Polymerase Chain Reaction (RTqPCR)

In order to validate the differential expression of genes (up-regulated versus down-regulated in CRC cells), we conducted RT-qPCR.

### *1. Reverse Transcription of RNA to cDNA*

Total RNA samples were reverse transcribed to cDNA. RNA samples, as well as the kit reagents were thawed manually, and then gently mixed by flicking the tubes. Before being kept on ice, all tubes were briefly centrifuged in order to collect any residual liquid from the sides of tubes. DNase/RNase free water was added to each RNA sample. Then, the reverse-transcription reaction was performed by preparing a master mix. The master mix was then transferred into each tube of template RNA, reaching a total volume of 20µl, then mixed gently. The tubes were then briefly centrifuged and placed in the BioRad T100 Thermal Cycler.

### *2. Quantitative Polymerase Chain Reaction (qPCR)*

All the following RT-qPCR experiments were conducted in biological duplicates under sterile conditions, using filtered tips and molecular grade nuclease-free water. DNA samples and primers were left to thaw on ice, mixed thoroughly and centrifuged briefly to collect solutions at the bottom of the tubes. For each transcript reaction, a master mix was prepared on ice (containing SyberGreen supermix (Sigma), forward and reverse primers, and DNase/RNase free water)

In order to ensure homogeneity and be able to dispense equal aliquots into all the wells of the PCR plate, the assay master mixes were mixed carefully and spun down.

After loading 19µL of the master mix into each reaction well of a 96-well PCR plate (BioRad), a 1µl of cDNA was distributed in each well except for the control well (instead of cDNA, 1µl of DNase/RNase free water was added)

Lastly, the PCR plate was sealed using an adhesive sealer, centrifuged at 1,000 rpm for 1 min and loaded into the Real-Time PCR BioRad CFX machine.18S gene was used as a reference gene. (Table.1)

**Table 1. Primer sequences for selected genes**

| **Gene Name** | **Primers Sequence (5'-3')** |
|---|---|
| ***C-JUN*** | F: CCC CAA GAT CCT GAA ACA GA<br>R: CCG TTG CTG GAC TGG ATT AT |
| ***MAPK4*** | F: AAG CTG CTC CCT GAA GTG AA<br>R: GGA CAG CTG GCT CTG GTT AG |
| ***SH2D3C*** | F: ATG GGG AGA GGC TAA AGG AA<br>R: GGG TCT GCA TCT CCT TGG TA |
| ***KDM4D*** | F: TTT CCC TAT GGC TAC CAT GC<br>R: TCA TAG CGT TCA GGT TGC AG |
| ***WNT9A*** | F: GCA AGC ATC TGA AGC ACA AG<br>R: TGC TCT CGC AGT TCT TCT CA |
| ***PFKFB4*** | F: GGT GGG CTA GGT TTC CAA CA<br>R: ACT GGC CAA CAT TGA ACT CC |
| ***18S*** | F: GTA ACC CGT TGA ACC CCA TT<br>R: CCA TCC AAT CGG TAG TAG CG |

### G. Statistical analysis

Data are represented as mean ± SEM and all statistical tests were performed using GraphPad Prism 8 software. To determine whether there are any statistically significant differences between two experimental data sets, a non-paired student's t-test was performed. In analyzing significance, all *P*-values less than 0.05 were considered significant.

# CHAPTER III

# RESULTS

## A. Metformin inhibits proliferation of HCT cell lines

The three cell lines, HCT-116, HCT-116 $p21^{-/-}$ and HCT-116 $p53^{-/-}$ were seeded to be treated with metformin doses (1, 2, 5 and 10 mM) at 24 h and 48 h time interval. We have noticed under the microscope a decrease in cell proliferation as metformin concentrations and time increase. (fig.8)

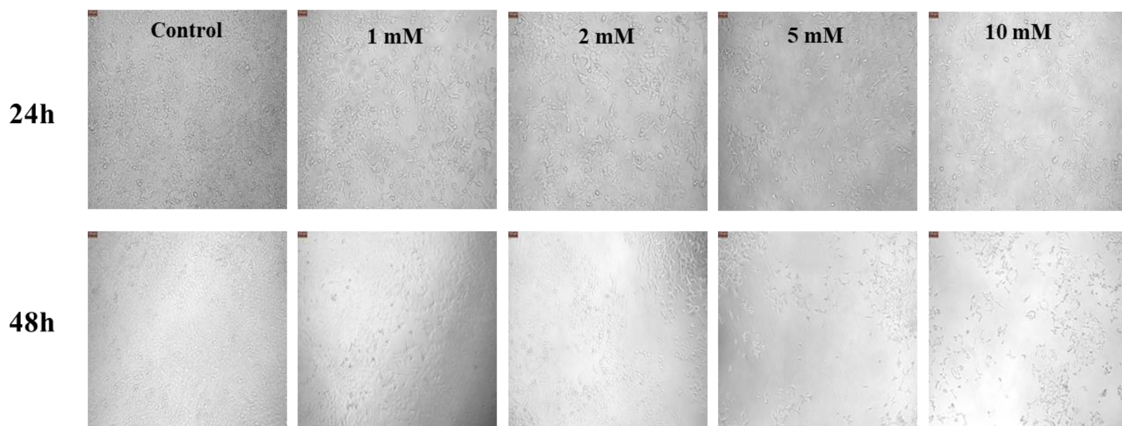



**Fig. 8. HCT116 cells after treatment with metformin (1, 2, 5 and 10 mM) at 24 and 48 h. (Objective lens 10x)**

**B. Effect of metformin on cell viability**

To investigate the effects of metformin on cell viability of CRC cells, MTT assay were performed to evaluate cell growth in vitro. The proliferation of HCT116 cells was all significantly diminished by metformin in a time- and dose-dependent manner (Fig. 9)

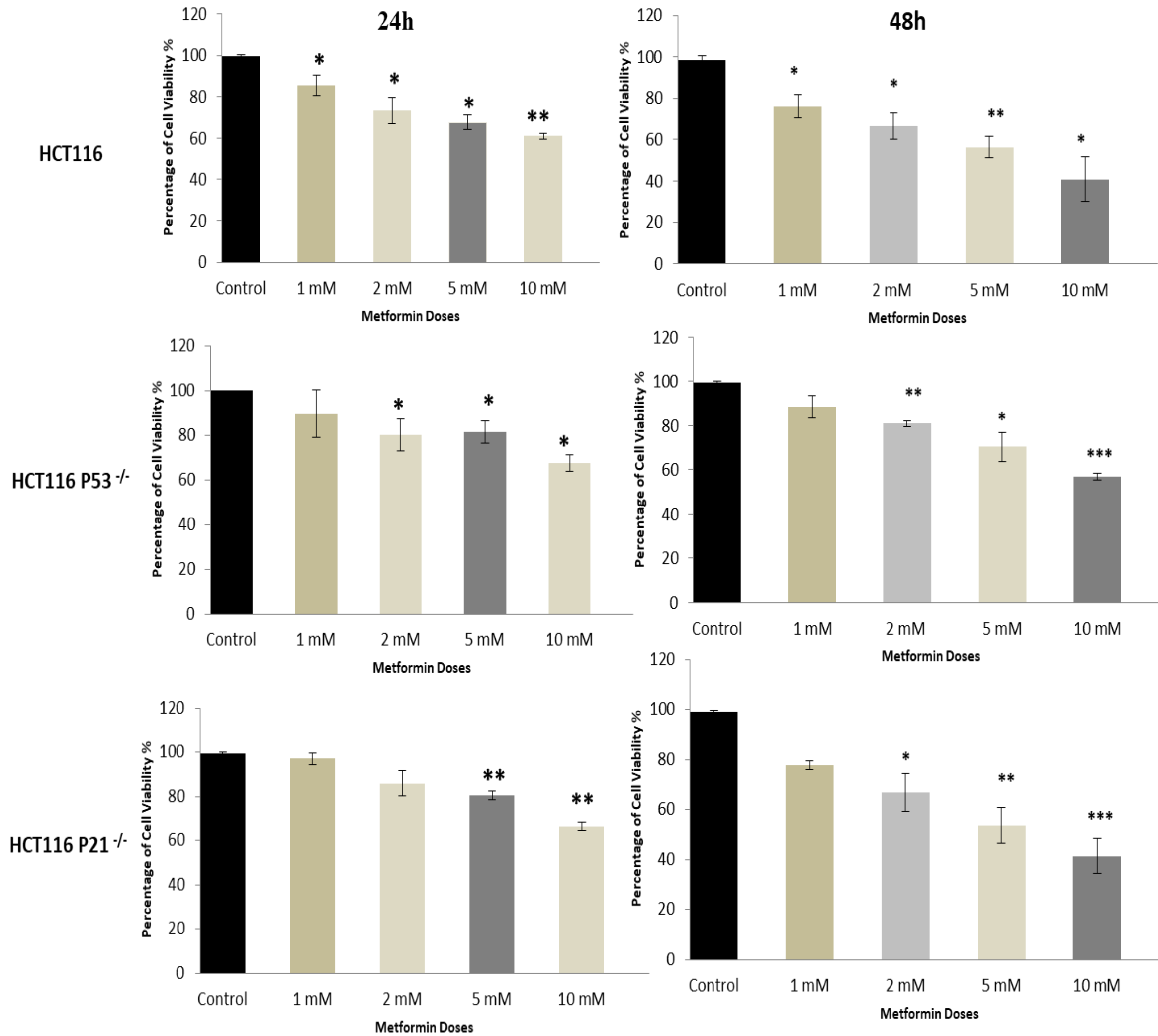



**Fig. 9. Cell viability was affected after metformin treatment.** Data are represented as mean ± SEM (n=3), *P < 0.05;**P < 0.01; ***P < 0.001 compared to the untreated control (Student's t-test).

### C. Does replenishing metformin affect cell Viability?

Since metformin is an amphoteric compound, we thought replenishing the cell lines again with metformin after 48 h could increase cell death; however, results of the MTT assay after replenishing were not significant as controlled with the one without replenishing. (fig.10)

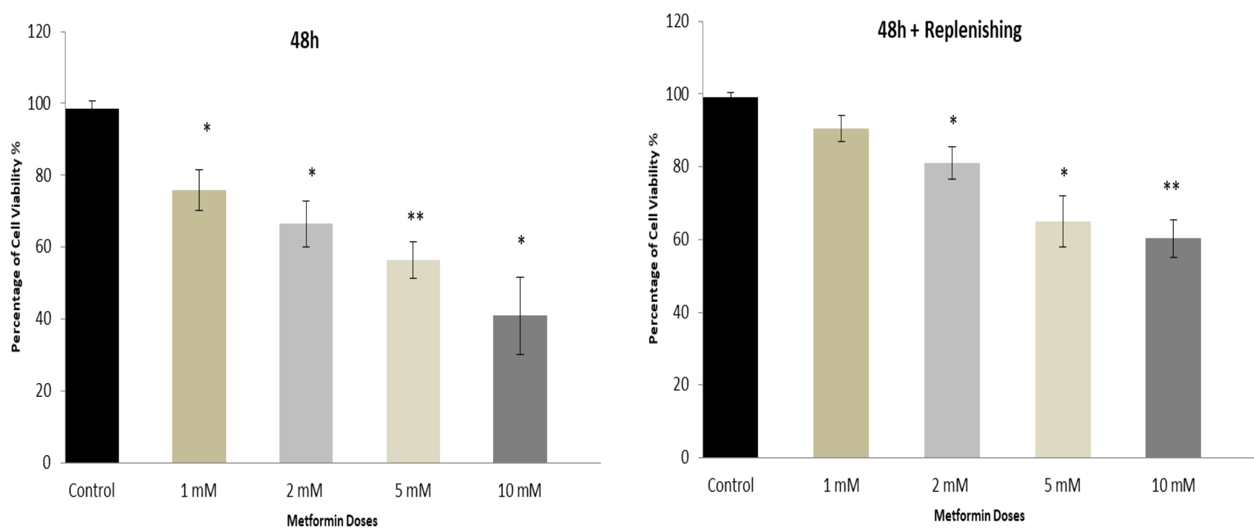
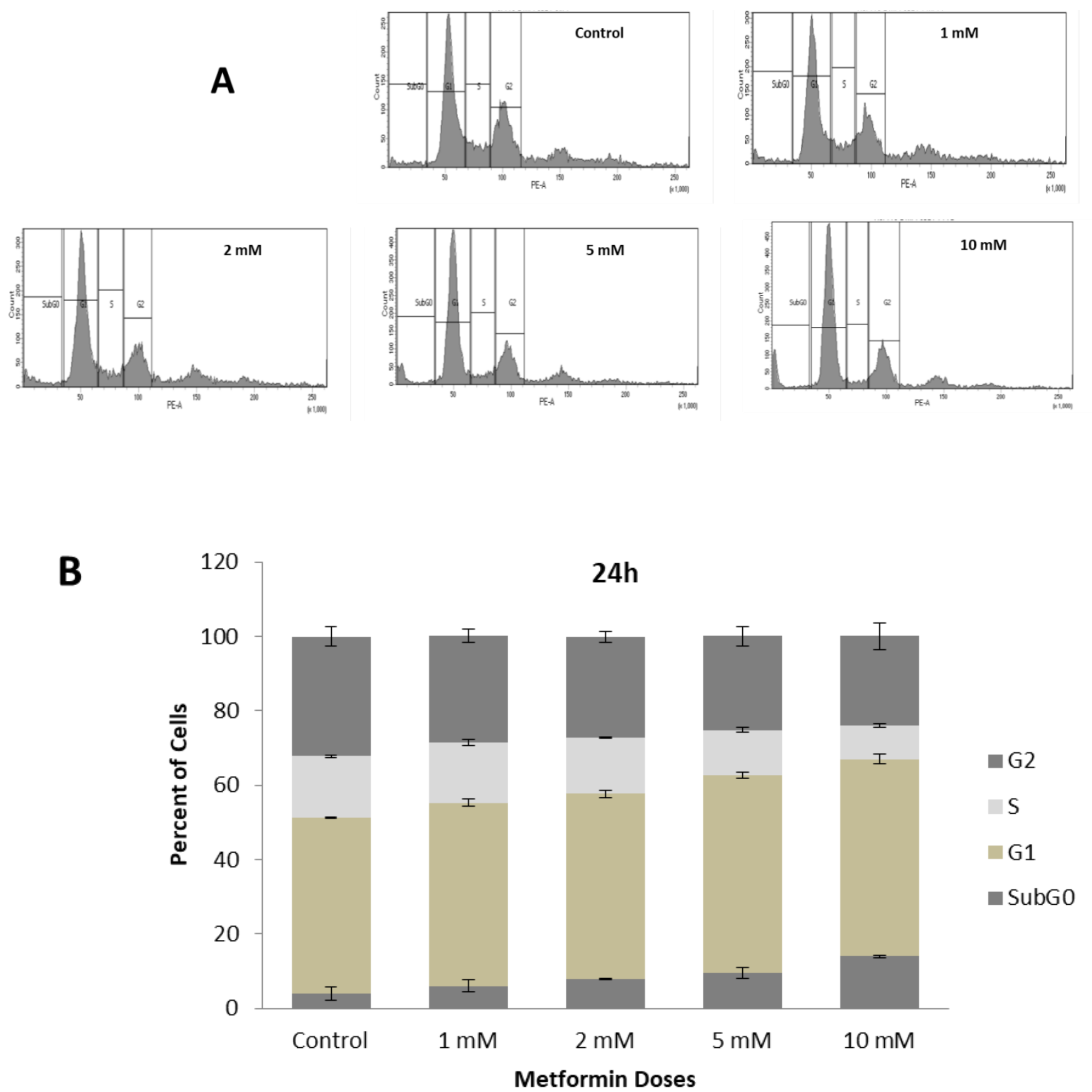



**Fig. 10. Comparison of cell viability in HCT116 cells after 48h with and without metformin replenishment.** Data are represented as mean ± SEM (n=3), *P < 0.05;**P < 0.01; ***P < 0.001 compared to the untreated control (Student's t-test).

### D. Potential of metformin in inducing apoptosis

Then, we estimated the effect of metformin on cell cycles distribution by treating HCT116 cells with different metformin doses. Figure 11 show that metformin has increased the cell populations in SubG0 phase. Furthermore, the apoptotic cell populations were upregulated by metformin in a time and dose-dependent manner



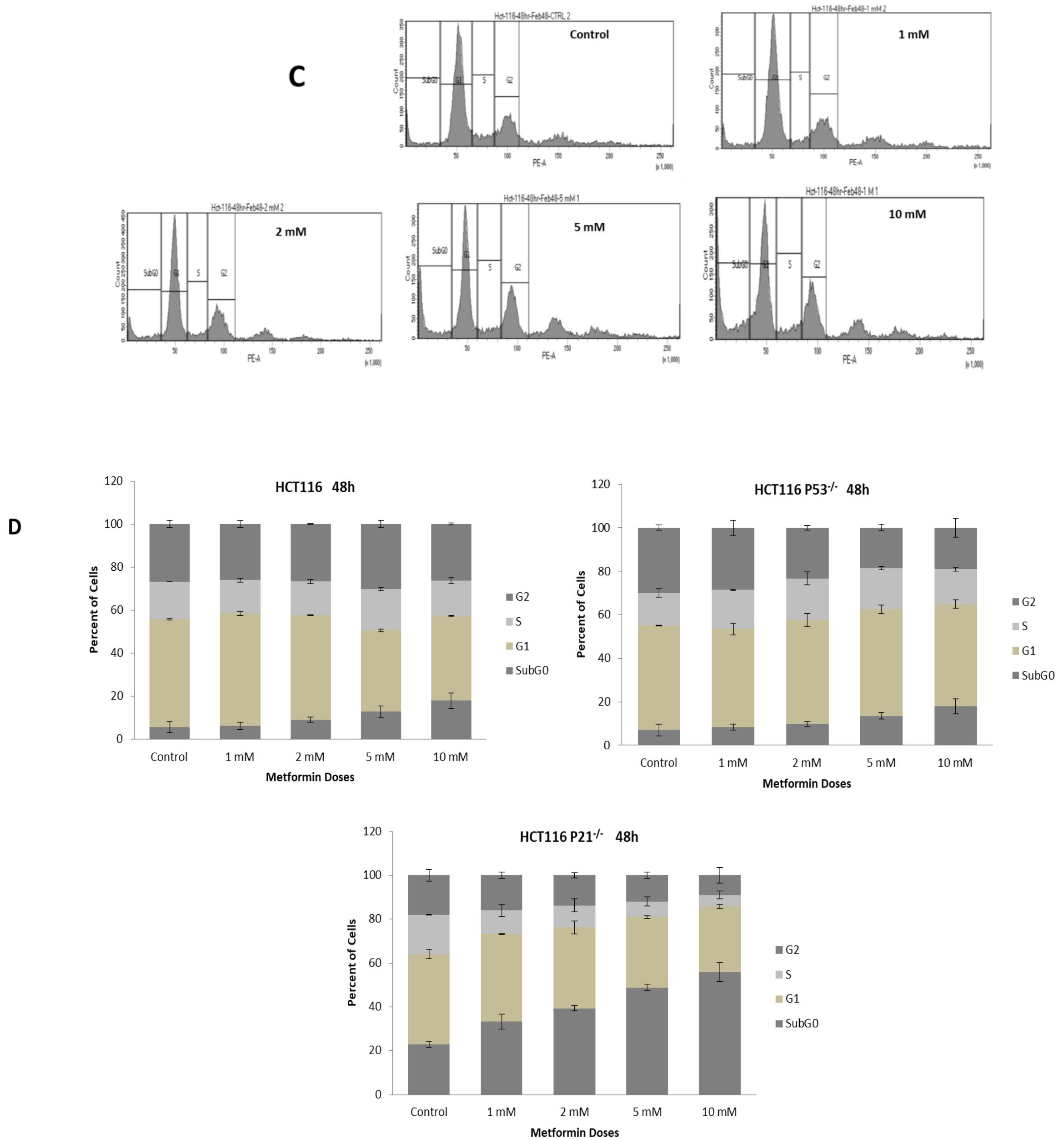



**Fig. 11. Metformin increases SubG0 counts in HCT116 cell line and its derivatives HCT116p21 and p53 null**. (A) Represents initial plots (counts) for the cell cycle taken from flow cytometry on HCT116 after 24 h (A) and after 48 h(C).Percent of cell counts reflecting effect of metformin on cell cycle and in inducing apoptosis after 24 h(B) and after 48 h (D). (D) Represents HCT116, HCT p53 null and HCT116 p21 null.

## E. Whole transcriptome sequencing analysis

Duplicates of each sample were sent for RNA-sequencing which was conducted in the Pillar Genomics Institute of Precision Medicine at AUBMC. After sequencing was carried out, data was processed using the DESeq2 tool using bam files of individual samples in the Galaxy platform. Correlograms were conducted in order to assess the magnitude of variability between the different experimental groups. The dendrogram on Fig. 12 demonstrates the magnitude of similarity between samples. Knowing that the shorter the dendrogram arm is, the more similar samples are, our duplicates demonstrate high levels of association. Another way to determine sample-to-sample distances, based on their overall gene expression, is Principal Component Analysis (PCA). Data points (samples) are projected onto the 2D plane such that they spread out in the two directions that elucidate most of the variances (Fig. 13). The samples are separated according to two principal components, PC1 and PC2, whereby they represent factors lying behind the variances observed. The treated samples were located far from the controls in the gene expression PCA plot, hence suggesting that the treated samples are distinct to the untreated ones. Furthermore, in terms of gene expression profiles, the closer the duplicates emerge on the PCA plot, the more alike they are as seen in Fig. 13. So as to look at the pattern of gene expression, MA plots were generated (Fig. 14). The plot demonstrates the variances between measurements after transforming the raw data onto M (log ratio) and A (mean average) scales. The scatter plot shows the Log2 fold change on the y-axis versus the mean expression of normalized counts for a given sample on the x-axis. Genes with an adjusted p-value less than 0.05 are represented in red.

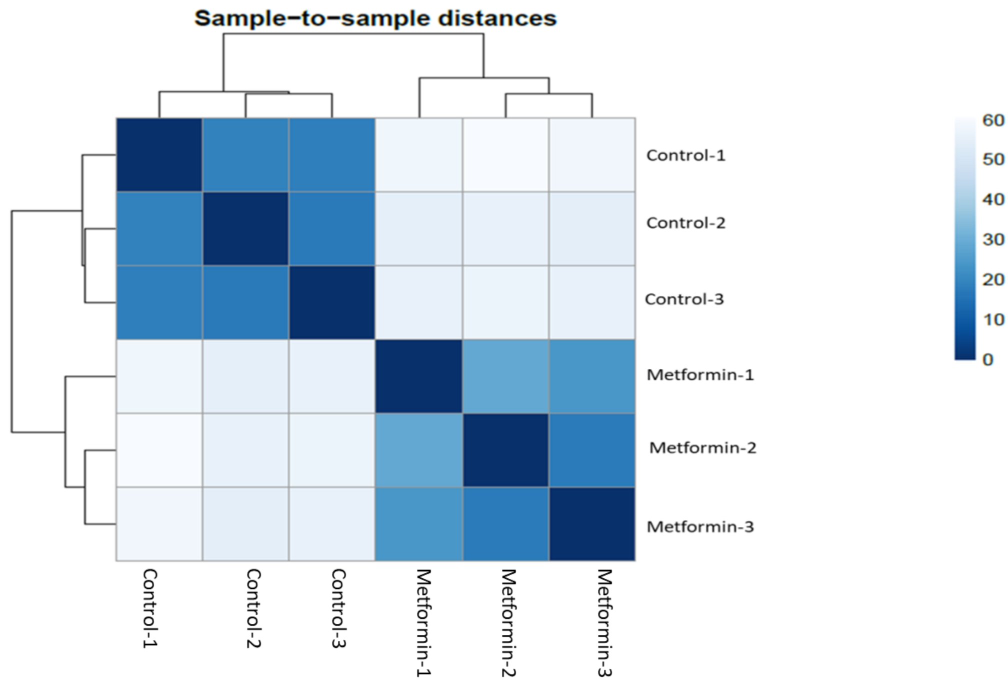



**Fig. 12. Correlation Matrix on Aligned Reads.** Spearman correlation on aligned reads visualizing the correlation distance between samples. Correlation matrix was generated using the DESeq2 tool.

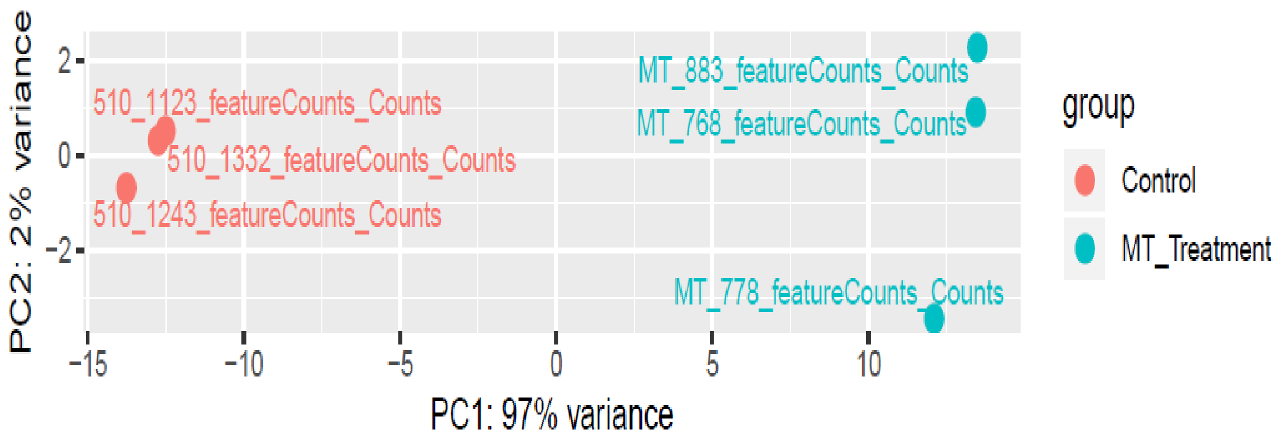



**Fig13. Principal Component Analysis Plot.** PCA plots of RNA-seq data assessing inter and intragroup variability displaying HCT 116 treated metformin RNA samples as opposed to controls. Each dot indicates a sample where treatments are given their own color

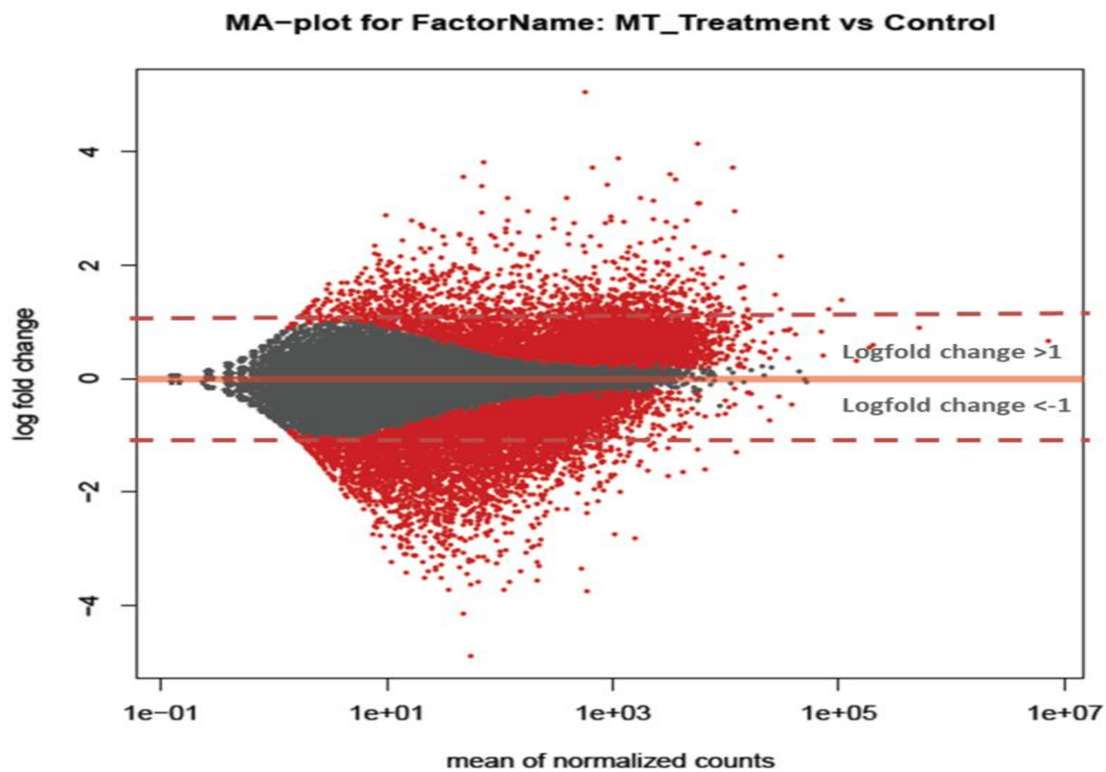



**Fig14. MA plot for differential expression analysis in HCT116 treated metformin RNA samples**. The circles represent different genes, whereby the red color indicates the most significant differently expressed observation. The y-axis indicates the Log2 fold change and the x-axis indicates the normalized mean. The upper zone represents up-regulated gene compromising a log2fold change less than 1,whereas the lower zone represent the down-regulated genes compromising the log2fold change less than -1.

### F. Investigation of the Transcriptomic Data

RNA-sequencing analysis showed around 34,798 regulated genes. However, this number was decreased significantly when applying a Log2 fold change of ± 1 and a P-adjusted < 0.05. The pattern of up-regulated and down-regulated genes in the experimental groups is depicted in Table 1 below:

**Table2. The variation in the regulated gene numbers in the treated HCT116 cells**

| | Gene numbers |
|---|---|
| **Significant Genes P<0.05** | 13,067 |
| **Up-regulated genes** | 1,242 |
| **Down-regulated genes** | 3,061 |

Differential gene expression obtained after Deseq2 analysis shows a bundle of interesting genes that can be studied for later investigation that help to understand early transformation and alteration in colorectal cancer genetic. The most Up-regulated and down-regulated genes are represented in table 2 and table 3, respectively.

**Table 3. Comparative list of up-regulated genes in HCT116 after metformin treatment for 24 hours compared to control**.

| Gene_ID | log2_FC | P-value | P-adj |
|---|---|---|---|
| ***HSPE1-MOB4*** | 5.040515374 | 2.26E-141 | 6.10E-138 |
| ***CHORDC1*** | 4.152824248 | 3.60E-298 | 9.73E-294 |
| ***ZFAND2A*** | 3.880736558 | 1.49E-138 | 3.36E-135 |
| ***HSPH1*** | 3.719781293 | 6.09E-221 | 8.21E-217 |
| ***DUSP2*** | 3.714896718 | 3.06E-111 | 3.59E-108 |
| ***FOS*** | 3.600144754 | 3.16E-163 | 1.70E-159 |
| ***HSPA1B*** | 3.52240083 | 1.03E-115 | 1.46E-112 |
| ***CCN1*** | 3.412962041 | 1.08E-163 | 7.31E-160 |
| ***CCDC190*** | 3.405601069 | 1.33E-27 | 8.47E-26 |
| ***DUSP8*** | 3.196324192 | 2.10E-129 | 3.55E-126 |
| ***CNMD*** | 3.182646756 | 5.25E-36 | 5.83E-34 |
| ***FOSB*** | 3.181313077 | 1.20E-92 | 8.72E-90 |
| ***TECR*** | 3.13293725 | 6.42E-153 | 2.48E-149 |
| ***DDIT3*** | 3.099851523 | 6.89E-176 | 6.20E-172 |

| | | | |
|---|---|---|---|
| ***PPP1R15A*** | 3.082702056 | 1.07E-144 | 3.22E-141 |
| ***DNAJB1*** | 2.958522399 | 4.02E-157 | 1.81E-153 |
| ***NR4A1*** | 2.807422746 | 1.12E-152 | 3.79E-149 |
| ***DNAJB1P1*** | 2.802896207 | 1.95E-47 | 4.00E-45 |
| ***BCAS2*** | 2.783042001 | 1.74E-109 | 1.96E-106 |
| ***JUN*** | 2.782690489 | 4.07E-98 | 4.06E-95 |
| ***DRC1*** | 2.77863248 | 1.24E-25 | 6.84E-24 |
| ***TENT5B*** | 2.759911433 | 1.29E-112 | 1.66E-109 |
| ***GADD45B*** | 2.754113998 | 9.30E-123 | 1.48E-119 |
| ***UBALD1*** | 2.73864795 | 5.95E-100 | 6.42E-97 |
| ***HSPA1A*** | 2.674260989 | 1.20E-10 | 1.23E-09 |
| ***TRIB3*** | 2.666507171 | 1.02E-138 | 2.50E-135 |
| ***SEPT7P9*** | 2.65714636 | 3.78E-32 | 3.23E-30 |
| ***MAP7D2*** | 2.572589324 | 7.27E-34 | 6.86E-32 |
| ***RFTN2*** | 2.570576793 | 2.70E-22 | 1.07E-20 |
| ***MBD6*** | 2.56527676 | 7.06E-73 | 3.53E-70 |
| ***RP9P*** | 2.516757129 | 1.08E-85 | 6.76E-83 |
| ***CCN2*** | 2.508280864 | 1.69E-46 | 3.21E-44 |
| ***DNAJA1*** | 2.488042746 | 1.33E-99 | 1.38E-96 |
| ***PLAC9*** | 2.480437129 | 6.48E-21 | 2.21E-19 |
| ***EDN1*** | 2.471282324 | 2.00E-17 | 4.70E-16 |
| ***NOCT*** | 2.455674966 | 2.86E-132 | 5.15E-129 |
| ***MAFF*** | 2.414435304 | 6.75E-115 | 9.11E-112 |
| ***CWF19L2*** | 2.393008781 | 6.64E-97 | 5.97E-94 |
| ***PDE1C*** | 2.366739459 | 3.37E-25 | 1.78E-23 |
| ***NIM1K*** | 2.365265114 | 2.03E-11 | 2.31E-10 |
| ***PMEPA1*** | 2.361652547 | 1.70E-50 | 3.96E-48 |
| ***RPL39P5*** | 2.345654499 | 1.92E-24 | 9.34E-23 |
| ***MSMO1*** | 2.321819061 | 6.35E-79 | 3.73E-76 |
| ***SORBS1*** | 2.318369796 | 9.45E-54 | 2.52E-51 |
| ***AK3P5*** | 2.294009475 | 1.68E-16 | 3.53E-15 |
| ***SERPINH1*** | 2.285811305 | 2.58E-89 | 1.83E-86 |
| ***DUSP1*** | 2.232986434 | 7.27E-58 | 2.42E-55 |
| ***RAB39A*** | 2.230192648 | 2.00E-08 | 1.52E-07 |
| ***TNFSF18*** | 2.229469174 | 9.62E-15 | 1.64E-13 |
| ***DUSP10*** | 2.224096955 | 1.86E-58 | 6.28E-56 |
| ***NAALAD2*** | 2.219270183 | 5.81E-29 | 4.12E-27 |
| ***MARS*** | 2.218714879 | 1.50E-136 | 3.11E-133 |
| ***RNU6-927P*** | 2.212658641 | 8.98E-06 | 4.50E-05 |
| ***FDFT1*** | 2.212299686 | 3.36E-74 | 1.82E-71 |
| ***RAB30*** | 2.180876826 | 2.87E-28 | 1.92E-26 |
| ***SRF*** | 2.174232233 | 8.43E-97 | 7.11E-94 |

| | | | |
|---|---|---|---|
| *GDF15* | 2.163022214 | 3.30E-69 | 1.46E-66 |
| *TRIM26* | 2.160106387 | 4.21E-94 | 3.34E-91 |
| *HSPA8* | 2.158590569 | 7.24E-93 | 5.42E-90 |
| *NR4A3* | 2.150994883 | 3.21E-36 | 3.60E-34 |
| *MXD1* | 2.134850921 | 5.08E-77 | 2.91E-74 |
| *MATN3* | 2.119847503 | 4.08E-07 | 2.55E-06 |
| *PSMA2P2* | 2.117143671 | 3.08E-08 | 2.28E-07 |
| *ATF3* | 2.110173785 | 2.84E-88 | 1.87E-85 |
| *ZFP64P1* | 2.093835454 | 1.67E-05 | 7.96E-05 |
| *DNAJA4* | 2.07305862 | 4.22E-97 | 3.93E-94 |
| *KLHL25* | 2.071600654 | 2.25E-57 | 7.04E-55 |
| *MAG* | 2.068939275 | 1.06E-05 | 5.27E-05 |
| *PDS5B* | 2.062219712 | 3.54E-44 | 5.79E-42 |
| *TUFT1* | 2.058682419 | 2.78E-57 | 8.64E-55 |
| *RPL7P32* | 2.041442957 | 1.16E-05 | 5.69E-05 |
| *CKS2* | 2.033227942 | 2.11E-88 | 1.42E-85 |
| *MAP2K3* | 2.032721908 | 2.54E-95 | 2.08E-92 |
| *UBE2B* | 2.031096387 | 6.23E-54 | 1.70E-51 |
| *DUSP5* | 2.023608358 | 3.93E-86 | 2.52E-83 |
| *AGR2* | 2.02274284 | 7.73E-10 | 7.20E-09 |
| *B3GALT2* | 2.001080946 | 1.10E-18 | 2.97E-17 |

**Table 4. Comparative list of down-regulated genes in HCT116 after metformin treatment for 24 hours compared to control.**

| Gene_ID | log2_FC | P-value | P-adj |
|---|---|---|---|
| *PFKFB1* | -2.000543374 | 7.16888E-10 | 6.70138E-09 |
| *GDPD2* | -2.001956525 | 0.000102939 | 0.000420961 |
| *ZRANB2-AS2* | -2.002062536 | 8.63452E-25 | 4.37039E-23 |
| *NUTM1* | -2.008694878 | 8.56446E-05 | 0.000355958 |
| *ZNF613* | -2.008829293 | 1.71797E-12 | 2.23361E-11 |
| *CA9* | -2.01025414 | 1.12483E-06 | 6.58257E-06 |
| *MSH4* | -2.010992905 | 1.13614E-23 | 5.11698E-22 |
| *TTLL6* | -2.018950097 | 1.61778E-09 | 1.43285E-08 |
| *MASP2* | -2.021378883 | 3.21264E-07 | 2.03883E-06 |
| *PRSS37* | -2.02562464 | 2.29039E-05 | 0.000106315 |
| *NREP* | -2.06324576 | 8.36069E-28 | 5.39605E-26 |
| *THAP2* | -2.063522993 | 1.56607E-16 | 3.29817E-15 |
| *CARMN* | -2.071255289 | 3.15609E-08 | 2.33466E-07 |
| *KIF19* | -2.073163391 | 1.26393E-05 | 6.15938E-05 |

| | | | |
|---|---|---|---|
| ***APOLD1*** | -2.074468378 | 1.8611E-38 | 2.36834E-36 |
| ***FALEC*** | -2.075095883 | 3.52423E-05 | 0.000157882 |
| ***BCL11B*** | -2.090065927 | 5.42537E-15 | 9.54143E-14 |
| ***ATP4A*** | -2.098133394 | 1.14232E-05 | 5.61544E-05 |
| ***TIGD5*** | -2.09915109 | 4.95365E-19 | 1.37773E-17 |
| ***TEX45*** | -2.10760215 | 1.07072E-06 | 6.29183E-06 |
| ***NAT1*** | -2.114959267 | 6.71945E-11 | 7.13971E-10 |
| ***NRG4*** | -2.126185475 | 4.36619E-18 | 1.10188E-16 |
| ***PALMD*** | -2.141026788 | 1.4799E-06 | 8.49827E-06 |
| ***ZNF629*** | -2.142160265 | 4.44534E-38 | 5.55215E-36 |
| ***BNIP3P1*** | -2.1443469 | 1.96407E-56 | 5.95356E-54 |
| ***JRKL*** | -2.145653927 | 1.67867E-23 | 7.41195E-22 |
| ***PROZ*** | -2.202493421 | 5.62879E-06 | 2.92419E-05 |
| ***FAM47E*** | -2.209110841 | 6.23397E-43 | 9.72138E-41 |
| ***APOBEC2*** | -2.209854652 | 2.79223E-11 | 3.12827E-10 |
| ***NDNF*** | -2.219026597 | 8.14611E-06 | 4.10777E-05 |
| ***ZNF19*** | -2.223288946 | 1.11712E-08 | 8.82251E-08 |
| ***UPK1A*** | -2.225298405 | 2.28472E-20 | 7.34651E-19 |
| ***HLA-V*** | -2.230137042 | 3.08868E-14 | 4.91892E-13 |
| ***RHD*** | -2.230476119 | 3.26593E-08 | 2.40798E-07 |
| ***CHRNA10*** | -2.231872179 | 6.93944E-07 | 4.19194E-06 |
| ***PARS2*** | -2.257112837 | 2.8734E-23 | 1.24229E-21 |
| ***RNVU1-6*** | -2.265297691 | 1.48193E-07 | 9.8788E-07 |
| ***ZFP30*** | -2.269727825 | 6.30409E-25 | 3.2333E-23 |
| ***NLRC3*** | -2.273417963 | 5.68405E-09 | 4.67941E-08 |
| ***ZNF416*** | -2.27635798 | 3.27837E-17 | 7.48255E-16 |
| ***TRIM17*** | -2.277766232 | 1.76606E-11 | 2.03437E-10 |
| ***CRB1*** | -2.280657653 | 3.99084E-07 | 2.505E-06 |
| ***CXCR4*** | -2.285500637 | 1.04341E-10 | 1.08017E-09 |
| ***TMC3*** | -2.289110824 | 1.34452E-10 | 1.37605E-09 |
| ***HIST1H3PS1*** | -2.3150168 | 1.86736E-08 | 1.43037E-07 |
| ***AKAP3*** | -2.318558567 | 1.92736E-16 | 4.0276E-15 |
| ***TMC2*** | -2.323620278 | 3.74704E-06 | 2.0065E-05 |
| ***CXCL8*** | -2.329463698 | 1.53421E-45 | 2.67032E-43 |
| ***MYO15A*** | -2.333711261 | 2.16855E-33 | 1.9899E-31 |
| ***SMC1B*** | -2.334162824 | 4.62019E-07 | 2.86899E-06 |
| ***TTC34*** | -2.334585426 | 2.92549E-08 | 2.17601E-07 |
| ***PTPRN*** | -2.369015687 | 3.55077E-08 | 2.60519E-07 |
| ***BCL11A*** | -2.410333537 | 1.41255E-11 | 1.64755E-10 |
| ***PECAM1*** | -2.415642102 | 3.72581E-13 | 5.26807E-12 |
| ***ADAM12*** | -2.544793076 | 1.56804E-11 | 1.81791E-10 |
| ***MFAP4*** | -2.599506074 | 4.43205E-08 | 3.19359E-07 |

| ***CCL20*** | -2.600189627 | 2.57636E-07 | 1.65883E-06 |
|---|---|---|---|
| ***FANK1*** | -2.622167205 | 8.86906E-51 | 2.09886E-48 |
| ***ATCAY*** | -2.670456513 | 1.22335E-09 | 1.10899E-08 |
| ***AKAP5*** | -2.681366865 | 2.70259E-14 | 4.34249E-13 |
| ***GNB4*** | -2.791860875 | 1.90648E-15 | 3.54956E-14 |
| ***CD4*** | -2.794492437 | 1.5388E-12 | 2.01621E-11 |
| ***WNT9A*** | -2.959965721 | 1.31834E-57 | 4.23408E-55 |
| ***RORA-AS1*** | -3.018578803 | 2.09806E-39 | 2.76105E-37 |
| ***RORA*** | -3.038557497 | 7.71814E-36 | 8.23004E-34 |
| ***NPAS3*** | -3.143445205 | 1.56107E-27 | 9.86289E-26 |
| ***HOXB-AS4*** | -3.192570927 | 1.44659E-24 | 7.16074E-23 |
| ***ZBED8*** | -3.199261794 | 5.1316E-31 | 4.12025E-29 |
| ***DIRC3*** | -3.22663672 | 4.04846E-66 | 1.70655E-63 |
| ***RAB30-AS1*** | -3.286103061 | 2.74135E-67 | 1.17391E-64 |
| ***CLDN14*** | -3.32116687 | 3.46733E-16 | 7.02264E-15 |
| ***CHRNG*** | -4.874121945 | 7.38782E-33 | 6.59963E-31 |

### G. Validation of the Transcriptomic Data via RT-qPCR

We next assessed the gene expression of differential expressed genes that we have got from Deseq2. Fig. 15 illustrates that *CJUN* expression was up-regulated after the treatment, whereas *KDM4D, SH2D3C, WNT9A, PFKFB4 and MAPK4* genes were down-regulated after metformin treatment which is compatible with the list of differential genes that we have from the RNA sequencing.

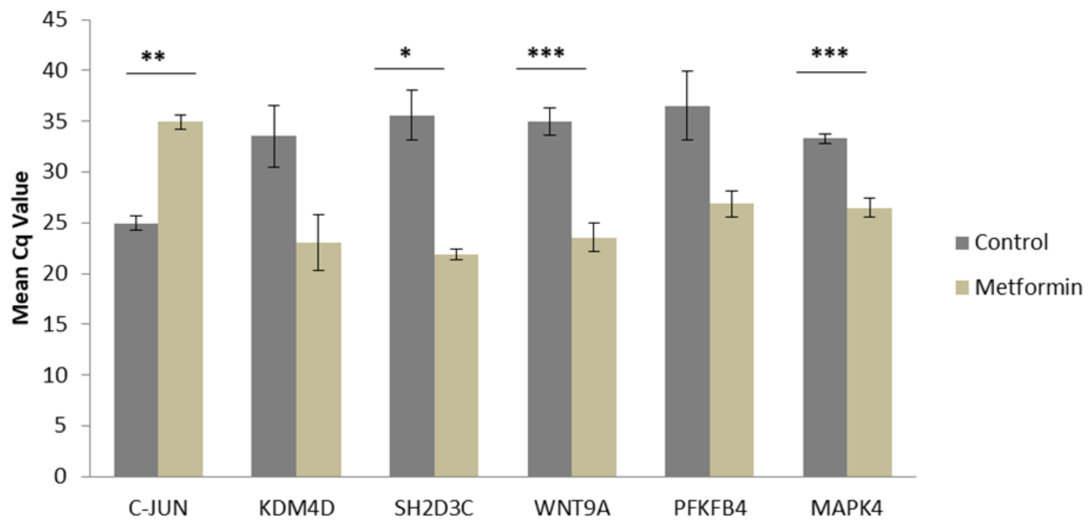



**Fig. 15. Representative graph of gene expression for HCT116 cells after treatment with 10 mM metformin (24 h)**. Data are represented as mean ± SEM (n=3), *P < 0.05;**P < 0.01; ***P < 0.001 compared to the untreated control (Student's t-test).

### H. Network Pathway Analysis of Transcriptomic Data

Following RNA-sequencing analysis, the transcriptomic data was complemented with a stringent bioinformatics analysis using Elsevier's Pathway Studio software. This was performed in order to assess for pathway and protein interaction enrichment analysis, linking expressed proteins to cell processes and diseases. The program builds interactions within proteins and cellular states, with symbols on the arrows representing the type of interaction, according to the number of publications in the software's knowledge-based supporting this link. Fig.16 and 17 demonstrate the constructed pathways for the up-regulated and down-regulated genes, respectively, after metformin treatment.

**A**

DEFB106A GDF15 MATN3 CCN2 AGR2 EDN1 CCN1 CNMD TNFSF18 TUFT1 SERPINH1

DNAJA4 AMOTL2 MAG

DNAJB1 PDE1C DNAJA1 UBE2B MAP2K3 DUSP2 DUSP8 DUSP12 DUSP10 DUSP1 PPP3CA SLA2 DUSP5

HSPA1A CRNN CCNA2 HSPH1 HSPA1B apoptosis CKS2 PPP1R15A

MXD1 MAFF SORBS1 DDIT3 ATF3 HSPA8 FOSB BCAS2 MARS NR4A1 CCNL1 PDS5B TRIM26 GADD45B TRIB3 SRF NR4A3 FOS JUN

PMEPA1 FDFT1

Cell Process

Protein

Protein (Ligand)

Protein (Protein kinase)

Protein (Protein phosphatase)

Protein (Receptor)

Protein (Transcription factor)

Regulation

**B**

Cell Process

Protein

Protein (Ligand)

Protein (Protein kinase)

Protein (Protein phosphatase)

Protein (Transcription factor)

Regulation

DUSP2 DUSP1 DNAJB1 BCAS2 TUFT1 NR4A3 PDS5B SERPINH1 NR4A1 DUSP5 DRC1 CCNA2 ATF3 PPP3CA PLAC9 DDIT3 HSPA8 CRNN PMEPA1 DNAJA1 PPP1R15A AGR2 TRIB3 cell cycle MARS MAP2K3 CNMD GDF15 EDN1 HSPA 1A CKS2 CCNL1 UBE2B SRF GADD45B FOSB FOS CCN1 MXD1 CCN2 JUN

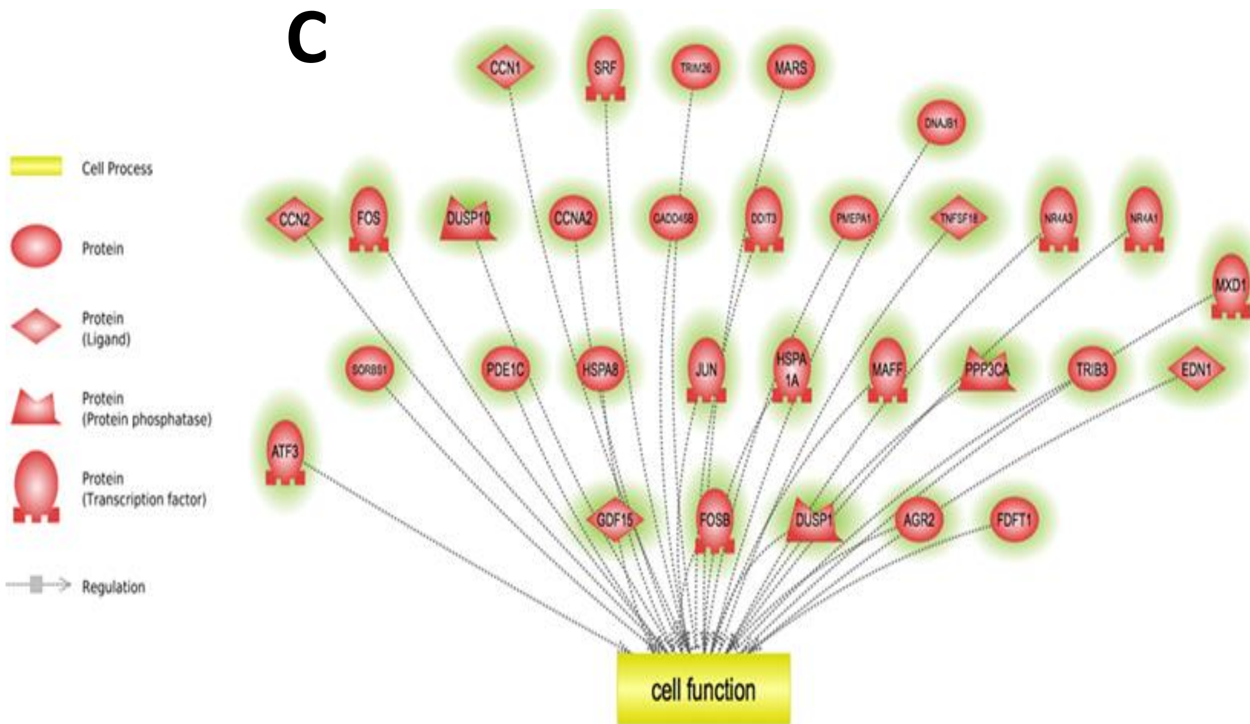
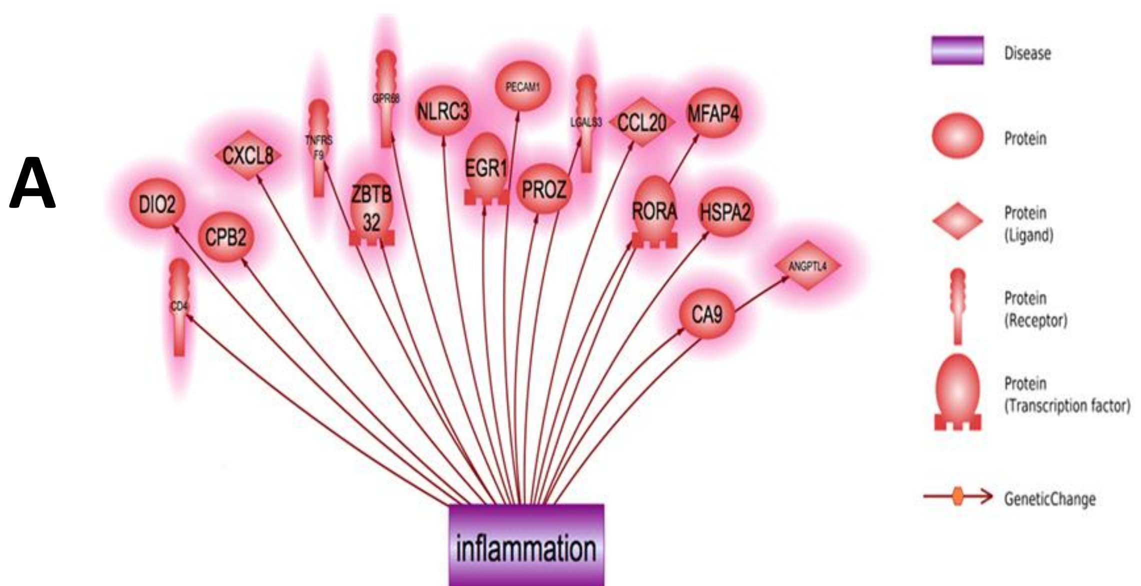
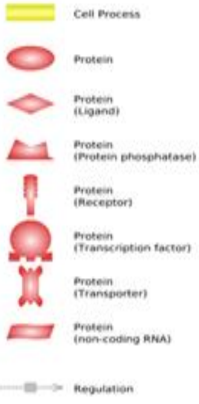
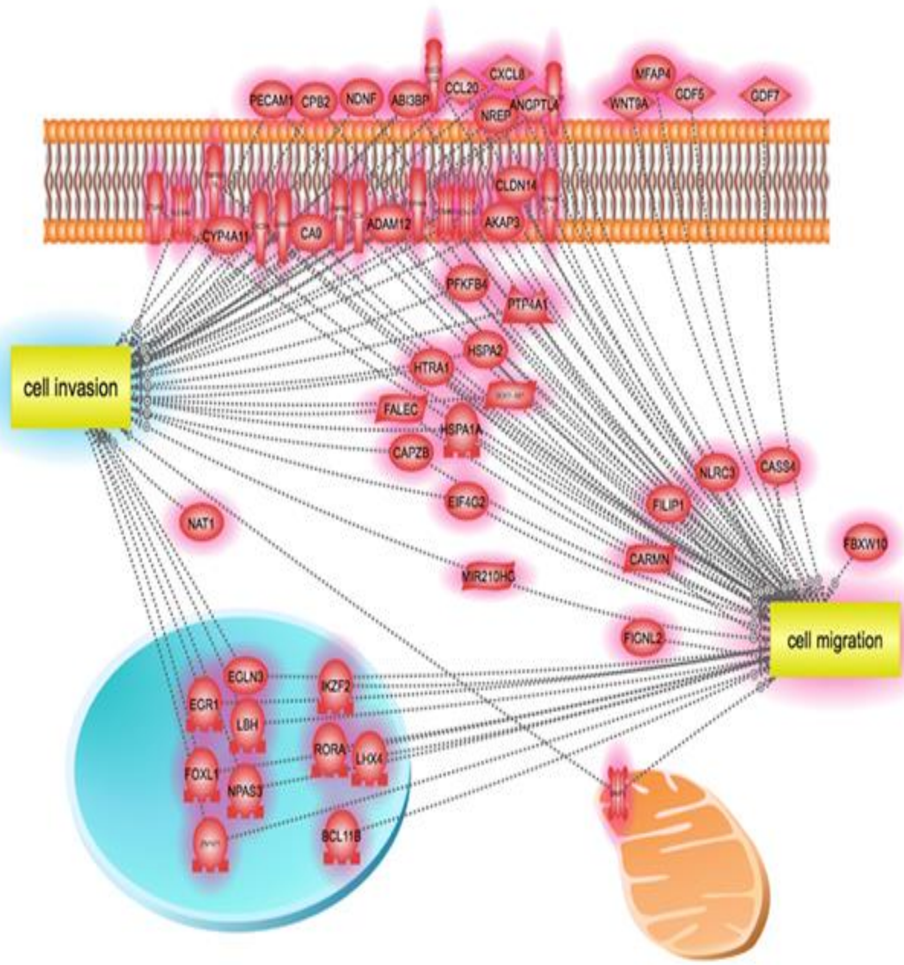


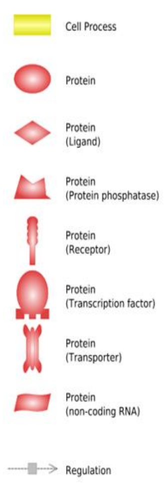

**Fig. 16. Pathway studio pathways relating the up-regulated expressed proteins with cell process and diseases**. The up-regulated proteins play a role in inducing apoptosis (A), regulating cell cycle (B) and in maintaining cell function (C).



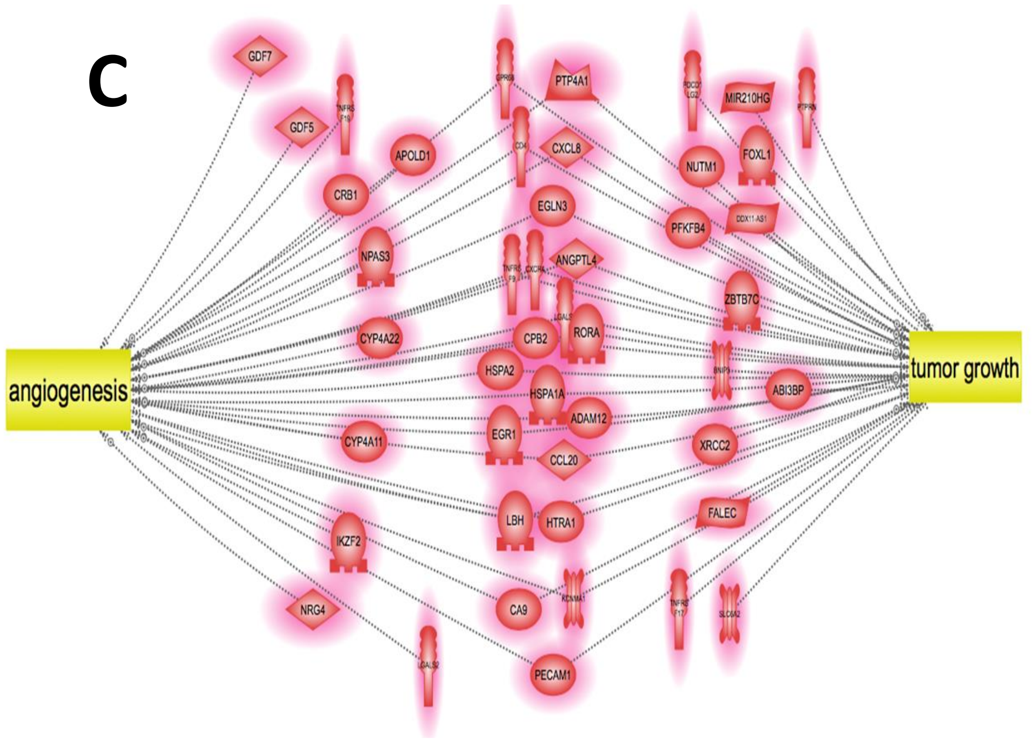



**B**




**C**



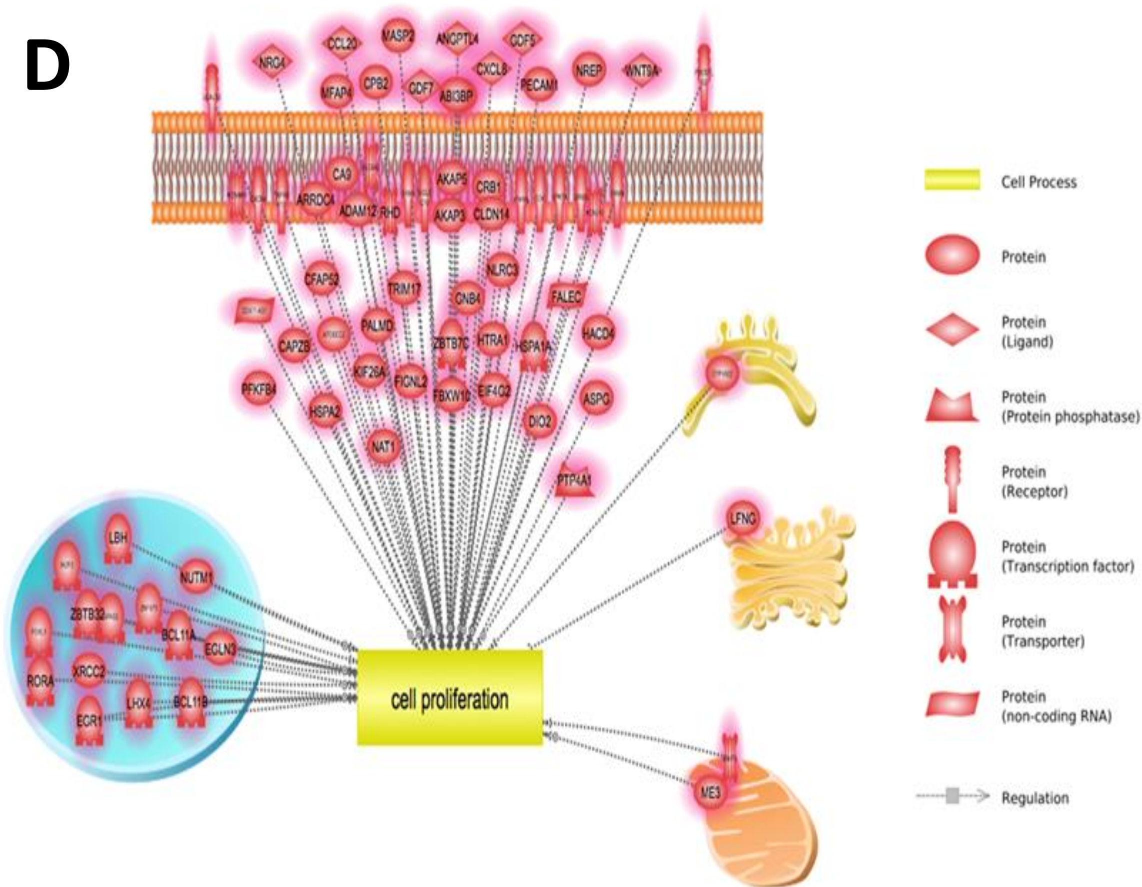



**Fig. 17. Pathway studio pathways relating expressed proteins with cell process and diseases**. The down-regulated proteins play a role in decreasing inflammation in cancer (A), reducing cell invasion and migration (B), inactivating angiogenesis and inhibiting tumor growth (C) and in reducing cell proliferation (D)

# CHAPTER IV
# DISCUSSION

Metformin has shown anti-carcinogenic activities in colon cancer; however, its molecular mechanisms of action are still unknown. In this study, we showed that metformin affects cell cycle regulation depending on the *p21* status of the cells. Metformin inhibited cell growth (high subG0 counts) in HCT 116 and its isogenic HCT116 ($p53^{-/-}$ and $p21^{-/-}$) human colon cancer cell lines with significant induction of apoptosis in HCT116 $p21^{-/-}$ cells only. According to a study done by Javelaud & Besançon,it was shown that p53 activity was 3 times higher in HCT116 $p21^{-/-}$ as compared to HCT116 $p21^{+/+}$ this leads us to hypothesize thatpP53 may stimulate apoptosis in a p21-independent manner. The absence of *p21* gene in HCT116 $p21^{-/-}$ cells correlates with apoptosis induction, This why we thought that p53 could be a main player in inducing apoptosis in the absence of *p21*gene ,but the fact that HCT116 and HCT116 p53 $^{-/-}$ cells also arrested at SubG0 phase implies that p53 is not the only inducer of this arrest.

The differential gene analysis in HCT116 cells has shown a high expression for *C-JUN*, as it was reported in a review by Al and Gali, induces p53-dependent apoptosis partly by negatively regulating the association of p53 with p21 promoters. Our results from RNA-sequencing validate this observation as it highlights the importance of studying the *C-JUN* role in inducing early apoptosis in colorectal cancer. Perhaps a knockout of *C-JUN* could be done to validate this point.

Moreover, It was shown from the literature that the overexpression of *ADAM1*, *CCL2* and *PECAM1* genes play a role in strengthening and directing cancer cell signaling

towards metastasis and invasion (Bhattacharyya, Feferman, & Tobacman, 2014; Itatani et al., 2016; Przemyslaw, Boguslaw, Elzbieta, & Malgorzata, 2013), in addition to *BCL11B* that showed once overexpressed apoptosis resistance (Huang, Du, & Li, 2012).In our study, these genes were highly down-regulated after metformin treatment. This highlights again the importance of metformin as an early therapy in colorectal cancer that can prevent cancer from progression and from invading other body organs.

In parallel, *SRF* the central regulator of genes involved in apoptosis (Ro, 2016), *ATF3* the tumor suppressor in colon cancer and anti-metastatic factor in HCT116 colon cancer (Hackl et al., 2010), all were up-regulated after treatment; this is why metformin is believed to stimulate cancer cells for early apoptosis and cell death.

Now what is the mechanism of action behind these differentially expressed genes in altering colorectal cancer function, more studies and investigation should be done to assess the underlying molecular network and cell signaling involved in colorectal tumorigenesis.

One of the limitations of our study, it lacks a normal colorectal cell line model, hence if it was found it will allow us to understand the earlier shift in gene expression before and after treatment, in normal and cancer colon cell lines, this will widen our understanding of the molecular transition from normal to cancer state.

Second, we have done RNA-seq only in HCT116 cell lines and after 24 h, however apoptosis was highly reached after 48h and more specifically in HCT116 $p21^{-/-}$. If genome sequencing was applied also to p21 null cell line, the expression of p53 with respect to other genes could be checked out.

It is recommended to implement more of the next-generation sequencing in colorectal cancer to understand the genetic architecture of tumors, along with acquired

mechanisms of drug resistance, which will guide the development of tumor-specific inhibitors and combination therapies in the future.

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