UNIVERSITÀ DEGLI STUDI DI MILANO

Effects of Lipid mixture and a selective PPARγ modulator on the differentiation capabilities of human derived mesenchymal stem cells (hADSCs) derived from healthy and breast cancer patients.

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2019-2020 – Cycle XXXIII

"We must believe that we are gifted for something and that this thing, at whatever cost, must be attained."

"Marie Curie"

Dedicated to whom

he believed in me

and in my possibilities

Abstract

Metabolic syndrome is associated with many complications especially leading to life threatening disorders such as obesity and cancer. To be able to identify solutions and natural treatments, we need to investigate the underlying causes of this syndrome. Nutrition is one important factor to consider in the prevention and treatment of the metabolic syndrome. Nutrition effects almost all metabolism mechanisms in the human body. One provident effect of nutrition is adiposity. Over the recent years, an interest was noted to studying adipogenesis in relation to obesity. Different factors affect adipogenesis including natural dietary compounds to help decrease adiposity, therefore the risk of developing obesity and later on obesity related diseases such as breast cancer. To be able to study this correlation *in-vitro*, a wide choice of cell models can be used. Human adipose derived mesenchymal cells (hADSCs) are one of the top choices used to study adipogenesis overcoming the limitations that other cell models have in their applicability to humans regarding the prevailing difference in their metabolism and physiology. In this study, the aim was to study adipogenesis using hADSCs in presence of dietary compounds such as lipids and GMG-43AC, a natural selective peroxisome proliferator-activated receptor γ (*PPAR γ*) modulator, that seems to have a positive effect on inhibiting adipogenesis in murine 3T3-L1 cells. We wanted to investigate further on its application on human cell models and try to understand its mechanism in inhibiting this phenomenon. The protocols were set up using the THP-1 cell line, which we noticed upon using a Lipid mixture cocktail (Composition: Non-animal fatty acids; 2 μg/ml arachidonic; 10 μg/ml linoleic acid; 10 μg/ml linolenic acid: 10 μg/ml myristic acid; 10 μg/ml oleic acid; 10 μg/ml palmitic acid; 10 μg/ml stearic acid; 0.22 mg/ml cholesterol from New Zealand sheep′s wool; 2.2 mg/ml Tween-80; 70 μg/ml tocopherol acetate), a decrease in pro-inflammatory cytokines *IL-6* and *IL-1β*. We also noticed a dose-

dependent increase of *FABP-4*. Our findings regarding hADSCs, that *PPARγ* expression and lipid accumulation was restored upon the presence of lipid mixture in breast cancer hADSCs that were derived from breast tissue. Secondly, GMG-43AC in both concentrations (0.5mM and 2mM) inhibited lipid accumulation and showed a significant decrease in the expression of adipocyte-specific genes, such as *PPARγ* and *FABP-4* even after the full differentiation of hADSCs that were derived from lipoaspirates. This suggests that dietary compounds are important factors in adipose differentiation and diet has a big influence in the progression and prevention in many metabolic diseases, such as obesity and cancer.

Riassunto

La sindrome metabolica è associata a molte complicanze che portano in particolare a malattie potenzialmente letali come l'obesità e il cancro. Per essere in grado di identificare soluzioni e trattamenti efficaci, dobbiamo indagare le cause alla base di questa sindrome. La nutrizione è un fattore importante da considerare nella prevenzione e nel trattamento della sindrome metabolica. La nutrizione ha effetto su quasi tutti i meccanismi del metabolismo del corpo umano, anche sull'adipogenesi Negli ultimi anni, è stato posto un enorme interesse sulo studio dell'adipogenesi in relazione soprattutto all'obesità. Diversi fattori influenzano l'adipogenesi primi fra tutti i nutrienti presenti nella dieta. Tra i nutrienti con effetto di contrasto all'obesità, i composti dietetici naturali godono di particolare interesse per aiutare a diminuire l'adiposità, quindi il rischio di sviluppare l'obesità e successivamente malattie correlate all'obesità come le malattie cardiovascolari e il diabete ma anche il cancro al seno. Per poter studiare questa correlazione in vitro, è possibile utilizzare un'ampia scelta di modelli cellulari. Le cellule mesenchimali isolate dal tessuto adiposo (hADSCs) sono una dei modelli sperimentali in vitro più usate per studiare l'adipogenesi superando i limiti che altri modelli cellulari hanno nella loro traslabilità all'uomo. In questo studio, lo scopo è stato di studiare l'adipogenesi utilizzando hADSCs anche in presenza di composti dietetici come lipidi e GMG-43AC un modulatore del recettore γ (*PPAR γ*) recettore gamma attivato dai proliferatori dei perossisomi. che ha mostrato un effetto positivo sull'inibizione dell'adipogenesi in cellule murine 3T3-L1. Inoltre, abbiamo indagato ulteriormente la sua applicazione su modelli di cellule umane per capire il suo meccanismo d'azione specifico che porta all' inibizione di questo fenomeno. La parte sperimentale è stata impostata utilizzando la linea cellulare THP-1 differenziate a macrofagi in co-cultura con le hADSCs. Abbiamo notato che trattando le hADSCs con un cocktail di miscela lipidica,

si verifica la diminuzione dell'espressione delle citochine pro-infiammatorie *IL-6* e *IL-1β*, valutata mediante real-time RT_PCR. Abbiamo anche notato un aumento dose-dipendente dell'espressione di *FABP-4*. Inoltre, abbiamo anche dimostrato che le capacità differenziative di hADSCs isolate da tessuto adiposo peri-tumorale in casi di tumore della mammella, sono alterate. In questi casi le hADSCs hanno scarse capacità differenziative, valutate mediante saggi istologici ed espressione dell'mRNA di *PPARγ* e *FABP-4*. Al contrario, la presenza nel terreno di coltura delle hADSCs di una miscela lipidica (Composizione: acidi grassi non animali; 2 μg / ml arachidonico; 10 μg / ml di acido linoleico; 10 μg / ml di acido linolenico; 10 μg / ml di acido miristico;10 μg / ml di acido oleico; 10 μg / ml di acido palmitico; 10 μg / ml di acido stearico; 0,22 mg / ml di colesterolo dalla lana di pecora della Nuova Zelanda; 2,2 mg / ml di Tween-80; 70 μg / ml di tocoferolo acetato) ripristina l'espressione di *PPARγ* e l'accumulo di lipidi. In secondo luogo, GMG-43AC in entrambe le concentrazioni (0,5 mM e 2 mM) ha inibito l'accumulo di lipidi e ha mostrato una significativa diminuzione nell'espressione di geni specifici degli adipociti, come *PPARγ, FABP-4* anche dopo la completa differenziazione di hADSC derivati da lipoaspirati . Ciò suggerisce che i composti dietetici sono fattori importanti nel differenziamento adipocitario e la dieta ha una grande influenza nella progressione e nella prevenzione di molte malattie metaboliche, tra cui l'obesità e il cancro.

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Chapter 1

Introduction

1.1 Introduction to Metabolic disorders

The metabolic syndrome has long been defined that some cardiovascular and risk factors related to metabolic functions come together and cause complications (1). Most authors relate their definition of metabolic syndrome to Reaven's 1988 of syndrome X or the insulin resistance syndrome. However, the base of their information was deeper and broader than what is covered in the description. The origin of the definition of "metabolic syndrome" comes from the observation that certain metabolic and biological characteristics, associated with a high risk of diseases occur together, such as diabetes and cardiovascular diseases (2-4).

The metabolic syndrome is very common among various adult populations (5). The primary cause of the metabolic syndrome is the western lifestyle, and genetic disposition, which results in obesity. Obesity leads to insulin resistance and hyperinsulinemia, glucose intolerance, high levels of serum triglyceride (dyslipidemia), low high-density cholesterol lipoprotein (HDL), and arterial hypertension. These are all risk factors for several diseases that can lead to life-threatening conditions (6). In addition, it was seen that there is a geographical variation in the metabolic syndrome. This is important and useful as a preventive solution and a predictor of adjoining disease solution in metabolic syndrome. Gurke et al. has studied the geographical variation in the USA and have found that the geographical variation is a predictor of cardiovascular disease in the studied population. They identified different areas where a huge part of the adult population tends to be at risk. Therefore, noting that some geographic areas

tend to need more efforts of prevalence in the metabolic syndrome (5). Another study was conducted with the same purpose to determine the prevalence of the metabolic syndrome among the Chinese population. It was seen that women living in the rural areas and older persons, tend to have an increased risk factors to developing metabolic syndrome (7). Changing lifestyle should be the course of prevention and treatment, by reducing the calorie intake from saturated fats and sugars and increasing physical activity. However, when such diseases are established, pharmacological treatments are necessary to avoid the consequences of the disease (8, 9). In this study, we are focusing on how nutrition affects obesity from an *in-vitro* view using different nutritive compounds that could halt the underlying cause of obesity as well as its linkage to breast cancer.

1.2 Obesity

Obesity has become a primary health problem worldwide, contributing to morbidity and mortality that is causing increased risk for many chronic diseases such as breast cancer (10). Obesity is a metabolic disease, that is classified as a body mass index $\geq 30kg/m^2$. Its risk contributes to type 2 diabetes, coronary artery disease, dyslipidemia, stroke, and cancer. Obesity as a metabolic syndrome is caused by various factors including high calories intake, lack of physical activity, and genetic predisposition(11). Obesity prevalence has nearly tripled worldwide since that 1970s (12). In 2016, over 340 million children and adolescents aged between 5 to 19 years old were overweight, 650 million adults were obese. In 2019, around 38.2 million children under the age of five were overweight or obese(12). This suggest that the role of lifestyle habits may strongly influence the development of obesity(13).

1.3 Etiology

Obesity contributes to many health complications. Therefore, there is a keen interest to identify the main factors that contribute to its development. The identification of the *Ob gene*, which codes for leptin, a peptide secreted protein by adipocytes, which was seen to be correlated with

the amount of body fat(14). The chronic excess of energy intake over the energy requirements which defines obesity as an energy balance disorder (15). Therefore, positive energy balance is a decisive etiology factor of obesity that promotes body fat accumulation (16, 17). Several studies have represented that sedentary and nutritional habits seemed to be a risk factors for the etiology of obesity (12, 18).

1.4 Genetics

The role of genetics in obesity and body fat regulation is abundantly investigated since the discovery of *Ob gene* in 1994 by Friedman et al.(14, 19). However, it is seen that the rising incidence of obesity is not caused by recent change in the patient's genetic background. The tendency of occurrence mostly results from environmental factors and lifestyle, such as the increased availability of high calorie food combined with low physical activity. The occurrence of obesity can be caused by a single gene mutation (monogenic) or it can be a symptom of genetics syndrome referred to as syndromic(20, 21). The main cases seemed to be caused by the interaction of several gene variants and the environment known as polygenic. The genome wide association studies have showed many genetic variations in the human genome related to obesity(22-24). Putting into concentration their individual effects, these allelic morphisms have a minor effect on their body weights. Regardless the sum of their effects, these variations define an individual's predisposition to gain weight and display polygenic Obesity. Therefore, the identification of these genetic variants predisposing to obesity may be a beginning to understand body weight changes in a given population (25-28). The predisposing genes code for the physiological systems that regulate energy balance. Examples of the genes are leptin (LEP), leptin receptors (LEPR), ghrelin (GHRL), Ghrelin receptors (GHSR), and brain derived neurotrophic factor (BNDF). Leptin is secreted majorly in adipose tissue, but it is also secreted in the stomach, placenta, heart, and skeletal muscle.

Leptin reduces hunger. On the other hand, Ghrelin is secreted in the lining of the stomach and increases hunger. Both hormones activate the hypothalamus, and in obesity, both hormone signals are at a disequilibrium. Leptin is secreted and reaches the hypothalamus signaling a saturation in adipose tissue therefore suppressing hunger(29). Ghrelin is secreted in the stomach and travels in the circulatory system to reach the hypothalamus and signals the state of hunger(30)(Fig. 1).
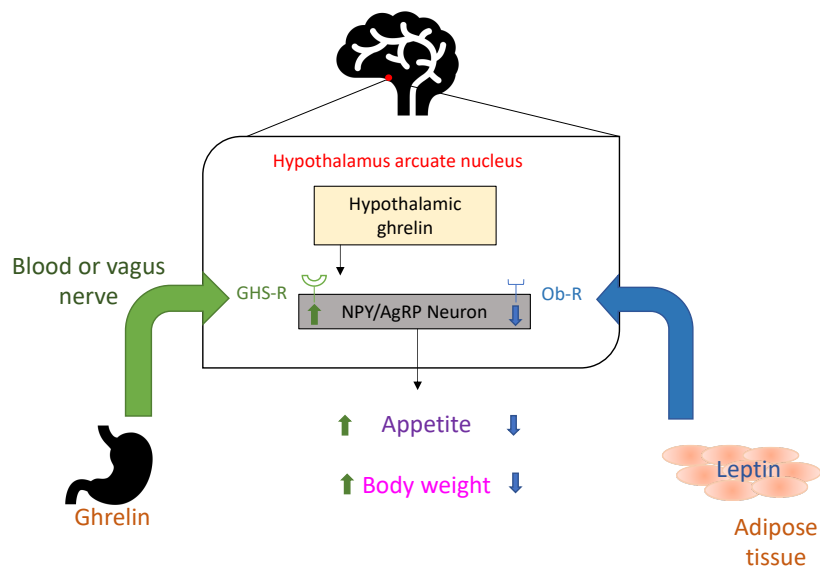
Figure 1. Leptin and Ghrelin act on the brain by the hypothalamus.

These genes show to have variants strongly related to the pathophysiology of obesity and its complications(25). In this framework, the differences in single nucleotide polymorphisms (SNPs) that are present in these genes contribute with a higher risk of obesity (25). Several mechanisms are involved to avoid the occurrence of obesity. The first mechanism is the feedback signaling system considering the amount of adipose energy stores (31), which was by cloning the *Ob gene* and the identification of leptin (19). The second mechanism is cell signaling at regulatory sites in the central nervous system mostly the hypothalamus, which is known to be responsible for regulation of metabolism and energy expenditure. Therefore, many physical or chemical injuries in this area can result in severe effects such as hypo/hyperphagia

along with dramatic metabolic disturbances (32). Thus, the leptin receptor and its expression in the hypothalamus is a key association between peripheral signals and the major pathways involved in the regulation of energy balance (33).

Chapter 2

Introduction to Adipogenesis

In a multicellular organism, several cell types are known to be pluripotent and develop into other types of cells. These are known as stem cells. The process to which these cells can originate new lineages of cells is known as cellular differentiation. Cellular differentiation is activated by signals at the organism level that are translated into specific processes such as activation, inactivation of transcription factors, differential expression of genes, and cellular signaling proteins. Examples of these cells are adipose derived mesenchymal cells. Adipose tissue was known as a dormant tissue and its only function is the constitution of the energy reservoir in an organism, by the accumulation of triglycerides. But after it has been extensively studied, it is considered as a very active tissue with various functions such as hormonal, immunological, and regulatory of the energy homeostasis(34, 35). Over the past years, an increased interest was seen to study obesity in relation to adipogenesis. Animal cell models such as murine preadipocytes were commonly used to study adipogenesis(36). Examples of used cell lines are; 3T3 murine cell lines(37); 3T3-F442A mouse cell line, which are derived from the murine swiss 3T3 cells (38); OP9 mouse cell line, a bone marrow derived stromal cell line derived from the calvaria of newborn mice that are genetically deficient in functional macrophage-colony stimulating factor (39); C3H/10T1/2 mouse cell line that is derived from 14-17 day old C3H mouse embryonic stem cell precursors(40); MEFs, which are a primary Mouse Embryonic Fibroblasts that are derived from totipotent cells in early mouse mammalian embryos(41); Porcine primary adipocytes (42); Feline primary adipocytes(43), and lastly human cell models that are derived from adipose tissue (44). Nevertheless, 3T3 murine models

are the most commonly used *in-vitro* (36). However, these models have limitations regarding their applicability to humans with regards to existing differences in their metabolism and physiology(45). This helped in developing a keen interest in using human cell models such as the adipose derived stem cells (ADSCs), which is one of the most important cell types used to study adipogenesis due to its easy accessibility and minimal invasiveness(46).

2.1 Human adipose derived mesenchymal cells (hADSCs)

Stem cells are of interest for researchers especially in regenerative medicine. This interest comes from the fact that these cells are known to be undifferentiated cells that have the capability to undergo differentiation(47). These cells are characterized to be multipotent or pluripotent, which can be induced to differentiate into different somatic cells for this reason, it has advantages therapeutic effects for treating injured tissues or organs(48). Specifically, this technique of differentiation has gained attention as a source for transplantation. Some examples of such cells are tissue progenitor cells, hemopoietic cells, and mesenchymal cells(49). Research was interested to access stable, safe and easily accessible stem cell source that can be utilized in regenerative medicine. The mouse bone marrow isolated cells showed plastic adherence properties upon culture. They formed spindle shaped colonies known as fibroblasts(50). These cells have the ability to differentiate into various cellular lineages that are developed from the mesoderm. Therefore, they were named multipotent mesenchymal cell. Mesenchymal cells can be obtained from various adult and fetal tissues from both murine and humans. Tissue examples are bone marrow and adipose tissue (51).

Mesenchymal stem cells (MSCs) Derived from adipose tissue are known as Adipose-derived mesenchymal stem cells (ADSCs). They are easily accessible in great amount and require minimal invasiveness as they are mostly obtained from subcutaneous adipose tissue by liposuction. Human adipose derived stem cells are found in the stromal-vascular region and can be obtained *ex vivo* using two steps; Washing and enzymatic digestion (48). In literature it

was seen that human Adipose Derived Stem Cells are characterized for growth features, phenotype, and the potential multipotent differentiation. Therefore, upon isolation they are defined as mesenchymal stem cells for the above features but specifically for their positivity to specific mesenchymal markers (Fig. 3) that determine their stemness. ADSCs can be isolated from likewise traits from liposuction adipose tissue, periumbilical adipose tissue, or breast adipose tissue(52). Many studies have differentiated hADSCs in too many cell lineages. Such as in the endothelial like cells, dopaminergic neurons, chondrocyte, and osteoblasts (53, 54).

2.2 The role of dexamethasone, 3-isobutyl-methylxanthine, and insulin in ADSCs differentiation

The process of adipogenesis results in functional mature adipocytes, which contain the machinery necessary for lipid metabolism and secrete adipokines (55). This mature adipocyte loses its proliferation potential and the insulin sensitivity increases (41, 55). Inducing *in-vitro* cell adipocyte differentiation can happen by culturing them at the confluence and exposing them to different stimuli known as a classic cocktail composed of a glucocorticoid (dexamathesone), a phosphor-diesterase inhibitor (3-isobutyl-1-methylxanthine (IBMX)), a cyclooxygenase inhibitor (indomethacin), and insulin. Each constituent activates a different signaling pathway involved in the differentiation process (41).

2.3 Key molecular factors in adipose differentiation process

Due to the complexity of the classical cocktail differentiation solution used, the molecular events involved with them MSC differentiation into the adipocytes have been difficult to interpret (56, 57). Upon the exposure of the classical cocktail, these adipocyte-like cells are identified by two methods; 1) according to their morphology (Fig. 2A) (accumulation of oil droplets), and their transition from spindle shaped to spherical (Fig. 2B); 2) The expression of terminal gene and protein markers of adipogenesis (Fig. 3) (56-59).
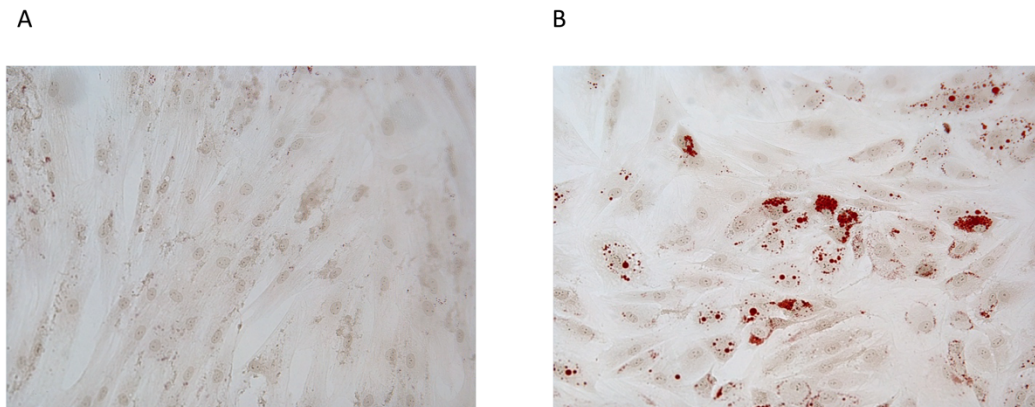
Figure 2. hADSCs differentiation. A) hADSCs before the exposure to the classical differentiation cocktail (87% DMEM 1 g/l glucose medium (Microtech); 10% FBS (Microtech); 1% Penicillin G potassic salt (100 U/ml) (Euro Clone);1% Streptomycin Sulfate (100 μg/ml) (Euro Clone); 1% Glutamine (Euro Clone) 1% Amphoteracin. **B) Lipid droplet accumulation stained with Oil Red O (lysochrome (fat-soluble dye) diazo dye that binds to triglycerides in hADSCs after the exposure to the classical differentiation medium (**87% DMEM 4.5g/l glucose (Microtech); 10% FBS (Microtech);1% Penicillin G potassic salt (100 U/ml) (Euro Clone);1% Streptomycin Sulfate (100 μg/ml) (Euro Clone);1% Glutamine (Euro Clone);1% Amphoteracin 10 μg/ml insulin (Humalog®);1 μM DEXA (SIGMA);0.5 mM IBMX (SIGMA))**.**

The biomarkers expressed in adipocyte tissue correspond to their adipogenicity character. These markers are *peroxisome proliferator-activated receptor γ (PPAR γ),* and *fatty acid binding protein 4 (FABP4).* The main regulators of adipogenesis in mammalian cells are *PPAR γ* and *CCAAT/enhancer binding protein α (C/EBP)*. *C/EBPα* is a mid-marker of adipogenesis (Fig. 3) (60).
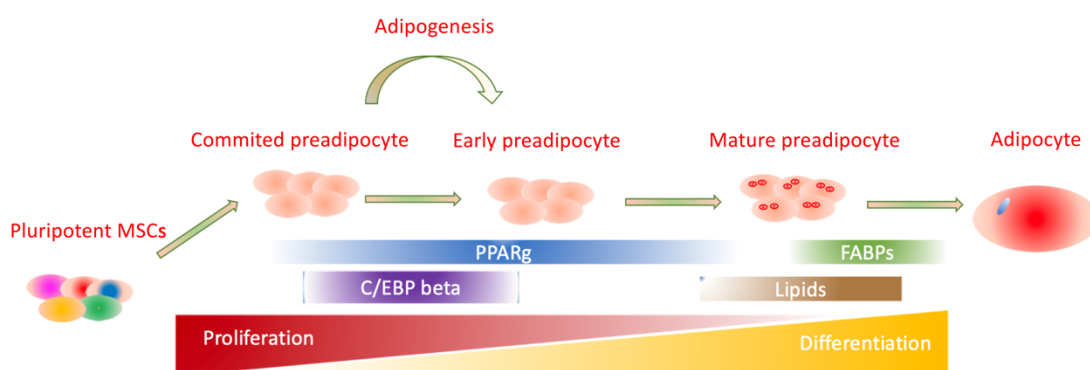
Figure 3. Adipogenic differentiation marker expression.

The induction of *PPAR γ* is essential during the differentiation of preadipocyte to adipocytes(61). *PPAR γ* and *C/EBPα* functions and seen to overlap over their transcriptional targets. *PPAR γ can* promote adipogenesis in that cells that are *C/EBPα* deficient but the

opposite is not possible. This shows that *PPAR γ* is a fundamental regulator of adipogenesis(62). On the other hand, *C/EBPα* deficient cells seem to differentiate into adipocytes but the process is not complete because these cells do not express *PPAR γ* and their lipid accumulation is minimal. Therefore, this shows that that cross-regulation between these two proteins is a very crucial to maintain the differentiated state of the cells(63).

The differentiation pathway happens chronologically by the expression of numerous genes that at the end yields the adipocyte phenotype. The changes involve the appearance, the early/intermediate and late gene markers (RNA/protein), and lipids accumulation. The four stages of adipogenesis in mesenchymal lineages are; 1) growth arrest; 2) mitotic clonal expansion; 3) early differentiation; 4) terminal differentiation(64).

Chapter 3

Introduction to Cancer

3.1 Types and effects of Cancer

Cancer is defined as an uncontrollable growth of abnormal cells overcoming the nature of normal cell division(65, 66). Usually cells are exposed to signals that drive them to divide normally, differentiate or die. On the other hand, cancer cells overcome these signals, which results in an uncontrolled growth and proliferation of the abnormal cells. This growth and perforation if spreads can be fatal. In fact, cancer related deaths are due to their outspread, which is commonly referred to as metastasis. Cancer research in the past decades have reached new insights as to how cancer develops. For this matter, cancer now is defined as a disease that involves deoxyribonucleic acid (DNA) changes that produce proteins that that retired cellular division and quiescence of the cells, which results in the uncontrollable division of cells to form cancer in different parts of the body. Mutations that occur in the genetic material of the cell cause the uncontrollable division in these cells (66). Any cell type in the body can undergo mutations to form tumors. This process is called carcinogenesis or tumorigenesis. If a tumor can metastasize, it is referred to as a malignant tumor. DNA damage in a region where the cell is not driven to its quiescence can cause a mode of survival for these cancer cells. Gene-environment replication can cause DNA damage and cause susceptibility inheritance in the cell from their parental genes. For example, the inheritance of the BRCA1 gene can cause a more susceptibility to developing breast cancer in women. For a mutation to happen in the cells, DNA damage control factors are disabled. These are regulated by two factors the intrinsic and extrinsic risk modulators. Pharmacogenics are known as an intrinsic risk modulator, are traits that are inherited that do not directly cause DNA damage but causes environment modulation

when exposed to cells(66). Examples of this is how enzyme metabolism functions in drug modulation and activity of hormones(67). Chemo-prevention agents such as antioxidants, are known as the extrinsic risk modulator. Examples are dietary components such as vitamin C, that was seen to remove free radicals from the cells(68). Our body is composed of many cells that are categorized where they reside according to their function. Examples are connective tissue cells, blood cells, epithelial cells, and lymphatic cells. Carcinogenesis in almost all kinds of cells present a common pattern of growth. The process has three steps: 1) Initiation; 2) promotion; 3) progression (66) (Fig. 4).
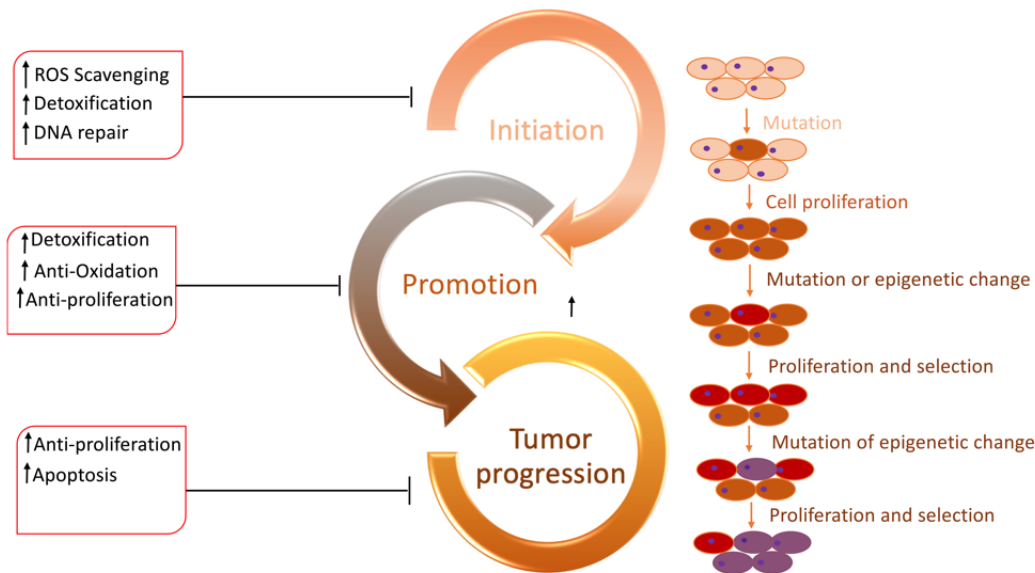
Figure 4. The development of Cancer

3.2 Breast Cancer

Breast cancer is a disease that affects the cells in the breast. That type of cancer depends on what type of cells turn into cancer. The cancer can start in various parts of the breast. The main parts that the breast is made of are: 1) glandular tissues (lobules and ducts) that produce and carry milk to the nipple; stromal tissues (connective tissue), that are mostly fatty tissue. Breast

cancer has a tendency to spread to other parts of the body through lymph vessels and blood this is commonly referred to as metastasis (67).

Types of breast cancers include non-invasive which refers to being localized to the ducts and do not spread to the nearby fatty tissue of the breast. Examples of non-invasive breast cancer are ductal carcinoma in situ (DCIS), and lobular carcinoma in situ (LCIS). Every invasive breast cancer refers to the tissue cells that evade their confinement of glandular tissue and invade connective and fatty tissue. The most common types of breast cancers are LCIS and DCIS. There are also various types of breast cancer which occur less frequently (Table 1)(66).

Name	Description
Medullary carcinoma	Invasive tumor that forms a distinct boundary between the tumor tissue and normal tissue.
Mutinous carcinoma	Rare occurring breast cancer formed by mucus-producing cancer cells. Also known also as *colloid carcinoma*.
Tubular carcinoma	An infiltrating invasive breast carcinoma.
Inflammatory breast cancer	Appearance of inflamed breasts with dimples caused by the cancer cells blocking the lymph vessels in the skin over the breast.
Paget's disease of the nipple	A rare form of breast cancer which develops in the milk ducts and spreads to the skin of the nipple and areola.
Phylloides Tumor	Tumor develops in connective tissues of the breast. Can be benign or malignant.

Table 1. List of rarely occurring breast cancers

Breast cancer is the most common in women, and includes 18% of all the cancer affecting females(69).

3.3 Etiology

That exposure to estrogen was seen to be a risk factor for breast cancer. It was found that catechol estrogen quinones, which is an oxidative metabolite of estrogen, have the capability to react to DNA and become chemical carcinogenesis endogenously (70, 71). This catechol estrogens were not removed by the production of a protective enzymes; they covalently bond to DNA causing mutations. These mutations can initiate abnormal cells perforation and cancer

(72). The excessive production of catechol estrogen 3,4 quinones that are referred to as oxidative metabolites of estrogens can react with DNA and have the possibility of becoming chemically endogenous carcinogens and produce mutations, therefore initiating cancer (70, 73).

3.4 Genetics

Around 5 to 10% of breast cancer are due to genetic predisposition(74). In general, susceptibility of breast cancer is inherited as an autosomal dominant with limited penetrance. This can be passed down in families and can be transmitted by members that don't develop cancers themselves (75). BRCA1 and the BRCA 2 are some gene variants that are common examples high penetrance predisposition genes(76). These genes in humans produce tumor suppressor proteins and they usually help repair the DNA if damaged. If a mutation occurs in one or both genes, the DNA cannot be repaired properly and upon that, cells begin to develop genetic alterations that could initiate cancer (77).

Chapter 4

Nutrition

Nutrition is a key factor in many diseases that are causes of death. But still, it is not stressed enough in our everyday lifestyle and following the recommendations of nutrition and physical activity in our routinely manner could help prevent and halt the progression of disease. Therefore, ameliorating the nutrition delivery or quality of food to all the population also in hospitals and private clinics can help improve the overall population health especially now since a variety of chronic diseases related to obesity, aging, and the way of living has a great impact to public health (78).

4.1 Nutrition and Obesity

Nowadays, it is widely known that the consumption of foods high in fats and sugars and lack of physical activity is on the rise in the developing world (79). The shift in the dietary practices and physical activity or inactivity, body composition, to tackle the widespread cases of obesity is known as the "nutrition transition", along with other diet related diseases (80).

One of the main nutrition related disorders is obesity (13). As explained before, the etiology of obesity is dependent on many factors including the interactions between dietary factors and various exposures such as genomic, epigenomic, metabolic factors, and lifestyle (81). It has been noted that some genetic variants may interact with dietary factors, especially in obesity and weight change (82, 83). Favoring energy dense and fatty foods that contain high caloric values, was a human evolution factor to sustain living, which caused humans to be more prone to modern obesogenic lifestyle, therefore raising the risk of obesity(84). Examples are fast foods and takeaway food, which can cause a great imbalance in energy input and output. It was shown that this type of diet has an crucial role in obese individuals in the strategy of a

successful long-term weight loss and prevention of weight gain(85, 86). Many studies have been done to prove the association between fast food and obesity risk (87, 88). Nutrition is a very important part of research to study the roles of food or diet and nutrients in the prevention of or treatment of disease to secure a healthy lifestyle. At the present time, nutritional recommendations are standard for all. However, many studies have collected evidence from genomics and other studies that suggest that the "one size fits all strategy" is likely misleading (89). Therefore, the personalized nutrition concept was introduced where patients are treated based on their personal characteristics. This concept addresses the nutrition related health problems such as obesity with combining the genomics with the nutritional research to help understand the interactions between the nutritional factors and the features of the human body. This approach produces a personalized nutritional recommendation for a better health outcome (90). In addition, it is known that obesity is related to breast cancer progression and some nutraceutical products are favored as treatments because of their effectiveness and the minor side effects they leave in patients with comorbid situations such as obesity and cancer(91).

4.2 Nutrition and Tumor microenvironment (TME)

A tumor as explained previously, is characterized by uncontrolled growth and disorganized morphology of cells. Along with cancer cells, other types of cells are involved such as stromal cells, mesenchymal cells, fibroblasts, adipocytes and immune cells (Table 2) (92). These cells initiate the tumor microenvironment with an unusual structure and different characteristics (93). The interaction between the tumor parenchyma is formed by the cancer interaction with stromal cells, which is directed by many factors such as metabolites and cell to cell interaction. Therefore, to establish adaptation, these cells alter the metabolic functions to support their demands of uncontrolled growth and progression of disease. These metabolic alterations are the result of oncogenic signaling that reach out to different metabolic pathways that cause nutrient based competition between these cells(94). These cells need a lot of macromolecules

such as lipids, amino acids, and nucleic acids to make up for their energy consuming activities in promoting tumor growth and progression as well as keeping up their cellular redox balance (95).

Cell type	Roles within tumor
Endothelial cells	Generation of blood vessels that serve for sustains nutrients and oxygen.
Fibroblasts	Remodels the tumor extracellular matrix
Macrophages	Utilizes cancer cell migration
Neutrophils	Supports stem cells and can play as a pro/anti-tumorigenic
Dendritic cells	Collects antigens for T cells
Cytotoxic cells	Serves for killing tumor cells and the expression of neo-antigens

Table 2. A list of the major cells present along with tumor cells in the tumor microenvironment.(92)

Recent studies have stated that nutrients found in the tumor microenvironment are important molecules that affect the strange metabolic a phenotypic plasticity present in tumor cells, which allow them to adapt to these strange features of the TME they approach, such as nutritional stress. Tumors and tumor associated stromal cells communicate with one another through metabolic connections creating a metabolic symbiosis supplying each other with different metabolites coming altering the anabolic and catabolic reactions(96). Nutrients are exchanged in the TME in this mode. Examples of some of these nutrients are lactate, glutamine, citrate, and lipids (96). It was seen that adipose tissue is a very important metabolic organ that has several functions including heat production and hormonal functions. Adipose tissue is composed of several cell types such as stromal vascular cells. Adipose stem cells (ADSC) are one of these cells, which has antiapoptotic, anti-inflammatory , and immunomodulatory purposes by the secretion of cytokines, chemokines, and growth factors (97). ADSC's are

associated with the promotion of tumor progression (98). Recently it was seen that these cells show that the cellular functions that are associated with invasion and spreading of the tumor are not produced by carcinoma cells , instead they are transient response signals that the tumors receives from the stromal microenvironment (99, 100). Mi et al. have seen that breast cancer cells mixed with bone marrow derived MSCs increase the metastasis suggesting an invasion and spreading of cancer. In addition, they observed that the interaction of cancer cells and MSCs favored the transformation of these MSCs into cancer associated fibroblasts (CAFs) (101). Once CAFs are activated, they no longer contain a normal phenotype or submit to apoptosis like other normal fibroblasts(102). The role of how diet plays a role in breast cancer is taking a high interest in research, especially low and high fat diets (103-105). Various studies have noted that a low-fat diet has improved the survival of patients (94, 103, 106). Cellular responses were investigated, lipid mixtures were used to mimic a high fat diet in vitro (107). Ecker and colleagues showed that obesity is associated with a higher with persistence survival of tumor cells in murine models (108). In addition, it was seen that dietary fat was a modulator of hemostasis of the adipose tissue that activates autophagy and apoptosis (109). Evidently it is seen that obesity and cancer was strongly related (10, 108). In a recent study, post-menopausal women with a high BMI were seen to have a high risk of developing breast cancer. Moreover, even women with normal BMI but high fat composition also had a higher risk of developing breast cancer. In addition, it was seen that the chemotherapy used for treatment of breast cancer in obese patients rendered an increased risk of metastasis compared with patients with lean composite body composition. Also, a worse disease free survival (DFS) and overall survival (OS) was reported in these patients (110).

Chapter 5

PPARγ modulation

Peroxisome proliferator-activated receptors (PPARs) as explained previously are a nuclear receptor superfamily that are characterized as a regulator of fatty acid synthesis, and glucose metabolism. The PPARs family is composed of 3 different isoforms: *PPARα, PPARβ*, and *PPARγ*. *PPARγ* is a principle promoting factor of adipogenesis (111, 112). From the clinical pharmacology point of view *PPAR γ* is considered a potential interesting druggable target and serves as such in the treatment of some metabolic diseases (113). In addition, *PPAR γ* has been seen to have a role in controlling the differentiation of monocytes to macrophages, therefore having a crucial role in the immune system (114). Furthermore, selective *PPAR γ* agonists such as thiazolidinediones(115), have anti-inflammatory properties, which can influence the inflammatory response in the immune system especially when regulating the major pathways in the abdominal fat (63, 116). Moreover, *PPARγ* activation can lead to different effects and responses depending on the nature of the modulating factor (117-120). For example, several studies showed that *PPAR γ* has a molecular role in cancer chemoprevention (121, 122). Some natural products or nutraceuticals, that have anti-inflammatory effects can activated *PPARγ*. Therefore, making it a promising source of therapeutics and emerging of new drugs (123). An example is dietary lipids is linoleic acid (124). It was reported that Conjugated Linoleic acid (CLA) provides a strong ligand for *PPARγ*, which was seen to have an effective anti-carcinogenic outcome(125, 126). Moreover, various known nutraceuticals are now considered as supportive treatments in various conditions such as obesity and related disorders, and inflammatory diseases. This is due to their capability to modulate the expression of

PPARγ. In this manner, overcoming many side effects that results from synthetic pharmacological drugs (127).

To study the aims of this project, we have published a recent study, where *PPARγ* modulation was noted in breast cancer adipose derived mesenchymal cells treated with lipid mixture. The study investigated that ADSC's derived from these patients were not able to be differentiated in comparison with healthy patients derived cells suggesting altered capabilities of differentiation(128). In another published study, a selective *PPARγ* modulator derived from propanoic acid, was seen to halt adipogenesis in adipose derived mesenchymal cells, suggesting that *PPARγ* modulation is an important factor in the future to develop new strategies to prevent adipogenesis and therefore open an insight on preventing and treating obesity(129).

Chapter 6

Aims

Obesity as stated before, is a metabolic syndrome caused by various factors including high calories intake, lack of physical activity, and genetic predisposition(12). Studying adipogenesis in relation to obesity using nutritive compounds to reduce adiposity or fat content, could serve as a potential to be able to involve the diet a much as possible to decrease the risk of obesity and related disorders. Adipose tissue has important metabolic functions not only serving as an energy storage but it also has a function as a major endocrine organ secreting adipokines, cytokines, and chemokines(129, 130). These regulate different metabolic processes especially in adipose tissue. Therefore, the outcome of the dysregulation of these processes in obese individuals can cause a pro-inflammatory microenvironment rendering a higher risk of developing cancers(130).

The principal goal of this inter-related project is to test the effects of different nutritive compounds on the differentiation and inflammatory response in THP-1, a monocyte human cell line and hADSC capability of adipocyte differentiation using different schedules.

The initial aim was to try and differentiate THP-1 cells:

1. To see the effect of nutritive compounds on the differentiation and the inflammatory response.

2. Setting up a startup protocol to optimize for the analysis of this phenomenon. Afterwards, the protocol was applied on hADSCs.

The active concentrations and the toxicity of each used compound were determined and assessed. The compounds used are Lipid Mixture and GMG-43AC (a selective $PPAR\gamma$ modulator). (details in Materials and Methods).

1. The first specific objective is to investigate whether ADSCs isolated from the adipose tissue of breast cancer bearing patients have specific cellular and functional characteristics that may be influenced, in a bidirectional manner, by the breast cancer microenvironment and by the tumour itself. Indeed, there could be differences in qualitative and quantitative characteristics of these ADSC populations, in terms of their adaptation to extended culture and multipotency, or their response to specific stimuli such as lipid mixture. (**DOI: <u>10.1155/2019/1480314</u>)**

2. The second objective is to study the effects of GMG-43AC on hADSC and verify if adipogenesis is reversed or halted using two different concentrations of the drug. This will help evaluate if GMG-43AC has a potential in halting adipogenesis and therefore could be furthermore studied to be a potential drug in obesity whether in a preventive or treatment manner. (**DOI: 10.3390/ijms21124415)**

To achieve the objectives of the study, the effects of the compounds on adipogenesis were evaluated through cell count and triglyceride determination after staining with Oil Red O. The mechanism of action of the compounds used was studied with RT-PCR. RT-PCR is to verify the gene expression that is crucial for adipocyte differentiation.

Chapter 7

Materials and Methods

7.1 Materials and stock solutions

Human recombinant Insulin (Humalog®, Eli Lilly) was used at the final concentration of 10 mg/ml. Dexamethasone (DEXA, Sigma-Aldrich) was dissolved in DMSO at the concentration of 1 mM. 3-isobutil-1-metylxantine (IBMX, Sigma-Aldrich) was dissolved in DMSO at the concentration of 0.5 M. Troglitazone (Sigma-Aldrich) was dissolved in DMSO at the concentration of 10 μM. GMG-43AC, was kindly given by Giuliani Sp.A. (Milano, Italy) and dissolved in DMSO at the concentration of 200mM. For all the compounds the final concentration of DMSO added to the growth medium was less than 0.001%.

7.2 Solutions

PBS 0.01 M

137 mM NaCl (SIGMA)

2.7 mM KCl (BDH)

1.47 mM KH_2PO_4 (BDH)

8.1 mM Na_2HPO_4 (BDH)

in dH_2O.

THP-1 Growth Medium

92% RPMI 1640

1% Penicillin G potassic salt (100 U/ml) (Euro Clone)

1% Streptomycin Sulfate (100 μg/ml) (Euro Clone)

5% FBS (Microtech)

1% Amphoteracin

THP1 Differentiation and activation

To the above reported medium add:

- 200nM Phorbol 12-myristate 13-acetate (PMA)
- 5ng/ml Lipopolysaccharide

hADSCs Maintenance medium

87% DMEM 1 g/l glucose medium (Microtech)

10% FBS (Microtech)

1% Penicillin G potassic salt (100 U/ml) (Euro Clone)

1% Streptomycin Sulfate (100 μg/ml) (Euro Clone)

1% Glutamine (Euro Clone)

1% Amphoteracin

hADSCs adipose differentiation Medium 1

87% DMEM 4.5g/l glucose (Microtech)

10% FBS (Microtech)

1% Penicillin G potassic salt (100 U/ml) (Euro Clone)

1% Streptomycin Sulfate (100 μg/ml) (Euro Clone)

1% Glutamine (Euro Clone)

1% Amphoteracin

10 μg/ml insulin (Humalog®)

1 μM DEXA (SIGMA)

0.5 mM IBMX (SIGMA)

hADSCs adipose differentiation Medium 2

87% DMEM 4,5 g/l D-glucose (Microtech)

10% FBS (Euro Clone)

10 µg/ml insulin (Humalog®)

1% Penicillin G potassic salt (100 U/ml) (Euro Clone)

1% Streptomycin Sulfate (100 µg/ml) (Euro Clone)

1% Glutamine (Euro Clone)

Oil Red O stock

0.5g Oil Red O (SIGMA)

in 100% isopropanol

MTT Assay

MTT assay kit reagent

3   mM HCL

 0,1% NP40

100% Isopropanol

Lipid Mixture (LM) (Sigma)

Composition: Non-animal fatty acids

- 2 µg/ml arachidonic
- 10 µg/ml linoleic acid
- 10 µg/ml linolenic acid

- 10 μg/ml myristic acid

- 10 μg/ml oleic acid

- 10 μg/ml palmitic acid

- 10 μg/ml stearic acid

- 0.22 mg/ml cholesterol from New Zealand sheep′s wool

- 2.2 mg/ml Tween-80

- 70 μg/ml tocopherol acetate

- 100 mg/ml Pluronic F-68 solubilized in cell culture water

GMG-43AC (Giuliani SpA Milan, Italy)

Composition:

- White powder with molecular weight 237.22 gr/l.
- Soluble in DMSO (dimethyl sulfoxide) (up to 0.1-1 gr/ml) and also in water (up to 1-10 gr/l).

Calculated physic-chemical proprieties:

Conditions:

- Temperature 25^0C

- Pressure 760 mmHg

- Vapor pressure -12.56 log(atm)

- Boiling point 350.7^0C

- Water diffusion 6.06×10^{-6} cm^2/sec

- Density 1.19 g/cm^3

- Refractive index 1.53

- Electron affinity -0.24 eV

- Heat of vaporization 34,97 Kcal/mol

- Air diffusion 0.044 cm^2/sec

- Volume 199.3 cm^3/mol

- Polarizability 24.44 $\mathring{A}^3$

- logP (octanol/water) 1,79

Solvent	Henry constant log(atm/(mol/l))	Activity coefficient log	Solubility log(mole fraction)	Solubility log(mole/l)
Water	-9.58	3.69	-4.72	-2.98
DMSO	-14.32	1.65	-1.03	0.12
n-Octanol	-10.96	1.36	-2.39	-1.59
Tetrahydrofuran	-11.24	1.37	-2.40	-1.32
Ethyl ether	-10.92	1.60	-2.63	-1.64
Acetone	-12.20	0.47	-1.33	-0.18
N,N-Dimethylformamide	-12.56	0.05	-1.03	-0.06
Chloroform	-9.89	2.74	-3.77	-2.67
Methanol	-10.94	1.97	-3.00	-1.62
Ethanol	-11.04	1.72	-2.75	-1.51
Cyclohexane	-7.37	5.12	-6.15	-5.19
1,2-Dichloroethane	-9.95	2.67	-3.71	-2.61
1,2-Dichlorobenzene	-8.73	3.73	-4.77	-3.82
Toluene	-9.46	3.04	-4.07	-3.10
Acetonitrile	-13.32	0.52	-1.03	0.24
Dioxane	-11.18	1.41	-2.44	-1.38

Figure 5. Physio-chemical proprieties of GMG-43AC.

7.3 Methods

7.3.1 Activation of THP-1 cells, maintenance in culture, and stimulation to macrophage differentiation

THP-1 are a monocytic cell line that has minimal receptor expression before activation. They can be differentiated by PMA (phorbol myristate acetate) or Vitamin D3, a classic activator for THPs, which will adhere and up-regulate phagocytic receptors to yield an overall macrophage-like state. They are able to induce inflammatory responses, such as the production of *IL-1* and *TNF-α* (131). Exposure of THP-1 cells to bacteria such as Lipopolysaccharide (LPS) leads to activation of inflammation, cell proliferation, differentiation, migration, and cell survival. This is because can the NF-κB transcription factor is activated. This are mediated by the release of chemokines and cytokines(132).

It was seen that MSCs have immunomodulatory effects especially through changes in monocytes and macrophages(133). It was interesting to see how THP-1 effects alone with the presence of lipids in this concept. The THP-1 cells were purchased at ATCC ((ATCC® TIB-202™)

The cells were expanded in RPMI 1640 complete medium and cultured at 5% CO_2 at 37°C in a Biological Safety Cabinets (HERA Safe, HERAEUS, USA). The cells were split after 3 days to keep the cells not exceeding $1,0 \times 10^6$ cells/ml. For the experimental procedure, the cells were collected in an 50ml Falcon tube and centrifuged at 1300 rpm for 10 mins. The supernatant was discarded, and the pellet resuspended in 5 ml of medium. The number of live cells was determined by means of trypan blue exclusion method by using a Neubauer chamber (mix 10µl of the sample and 10µl of trypan blue). Then the volume of the medium was increased to obtain the proper cell density for the experiment and for the PMA, LPS needed to differentiate the cells and the lipid mixture concentration. The cells were than seeded in 3 6-multiwell plates for RT-PCR, and 96-multiwell plates for MTT assay as described below (Fig. 6).

THP-1 cells treated with Lipid mixture (LM)

Three groups:
Activated with 200nM PMA and 5 ng/ml LPS

- **Pre-incubation**: 24 hr incubation with LM then 18 hrs with LPS.
- **Co-incubation**: 24 hr incubation with LM and LPS.
- **Post-incubation**: 18 hr incubation with LPS then 24 hr incubation with LM.

	1	2	3
A	Positive control	Negative control	Treatment 1 50µl LM
B	Treatment 2 100µl LM	Treatment 3 150µl LM	Treatment 4 200µl LM
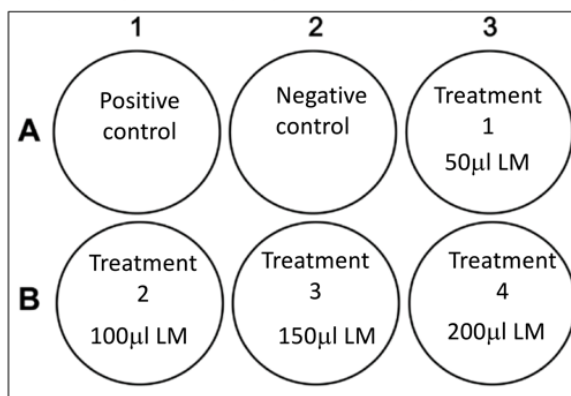
Figure.6 Experimental plan of THP-1 treatment with Lipid mixture**.**

7.3.2 Cell cultures and induction of differentiation in hADSCs.

Primary cell cultures from human adipose tissue samples were obtained from voluntary patients undergoing elective liposuction procedures under local anesthesia or undergoing breast biopsies suspected to have breast tumors. Patients gave their informed consent according to the Declaration of Helsinki. Lipoaspirates samples were obtained f under local anesthesia (Lidocaine; AstraZeneca, London, UK. The protocol involved an infiltration step in which a solution of saline and the vasoconstrictor epinephrine (2 µg/ml; Key Customer Solutions S.A.S, Basiglio, Milan, Italy) was infused into the adipose compartment to minimize blood loss and contamination of the tissue by peripheral blood cells(48). The breast adipose tissue was obtained from patients undergoing breast biopsy. The tissue was obtained 5 cm away from the tumor, therefore from the microenvironment. The adipose tissue is enclosed in a sterile container and transported to the laboratory under sterile conditions. The tissue is washed with a solution of saline and cut into small pieces (diameter < 1cm) and placed in a petri dish with 1 ml Dulbecco's modified Eagle's medium (DMEM) (Euro Clone) and incubated for 10 days.

The cells were isolated and expanded in a Biological Safety Cabinets (HERA Safe, HERAEUS, USA) and cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) (Euro Clone) containing 1g/l D-glucose 10% heat-inactivated fetal bovine serum (FBS) supplemented with antibiotics at 37°C in a humidified, 5% CO_2 incubator (HERAcell 150- Thermo electron, USA) (134) (Fig. 7). For adipogenic differentiation, two days after confluence (defined as *day 0*), cells were exposed to the adipocyte differentiation medium (DMEM) containing 4,5 g/l D-glucose, 10% FBS, antibiotics and supplemented with dexamethasone (DEX; 1 µM), 3-isobutil-1-metylxantine (IBMX; 0.5 mM) and insulin (10 µg/ml) (Fig. 8).
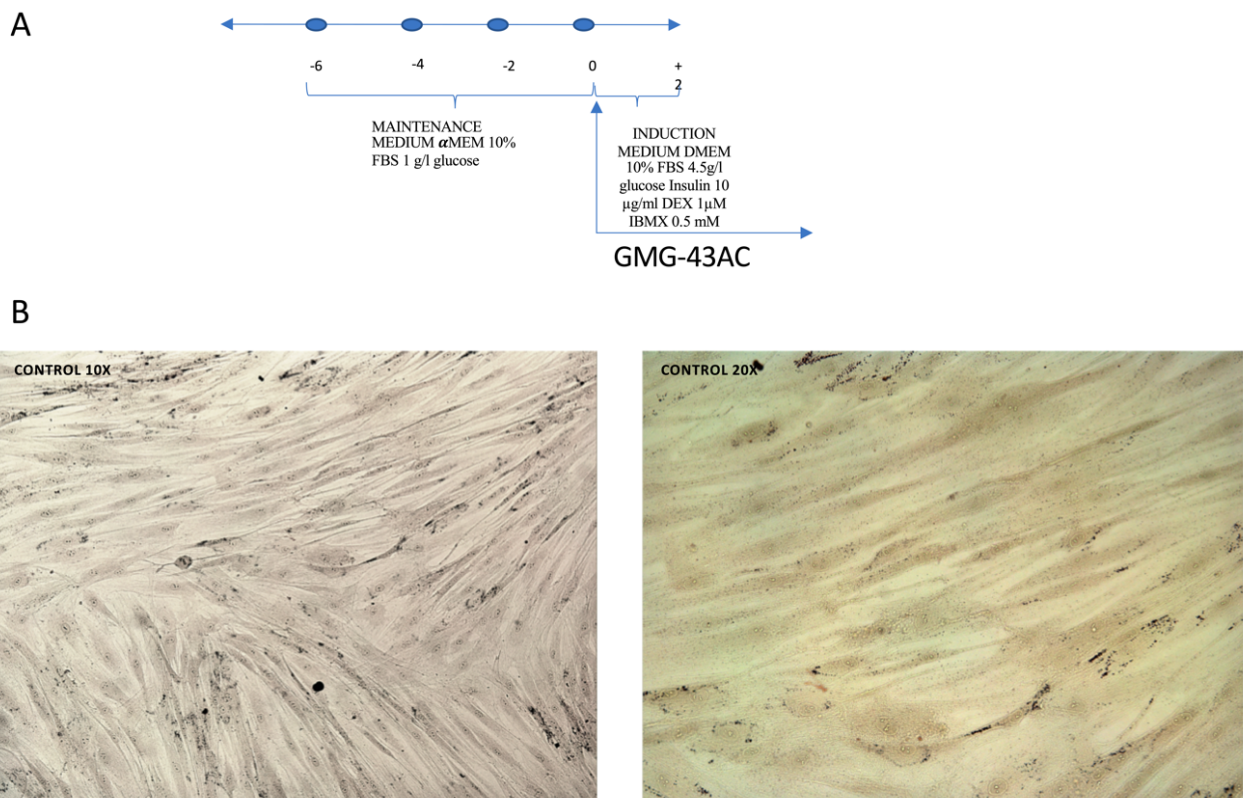
Figure 7. hADSCs maintenance. (A) Schematic timeline of maintenance of hADSCs. (B) Images of hADSCs in maintenance medium.

A
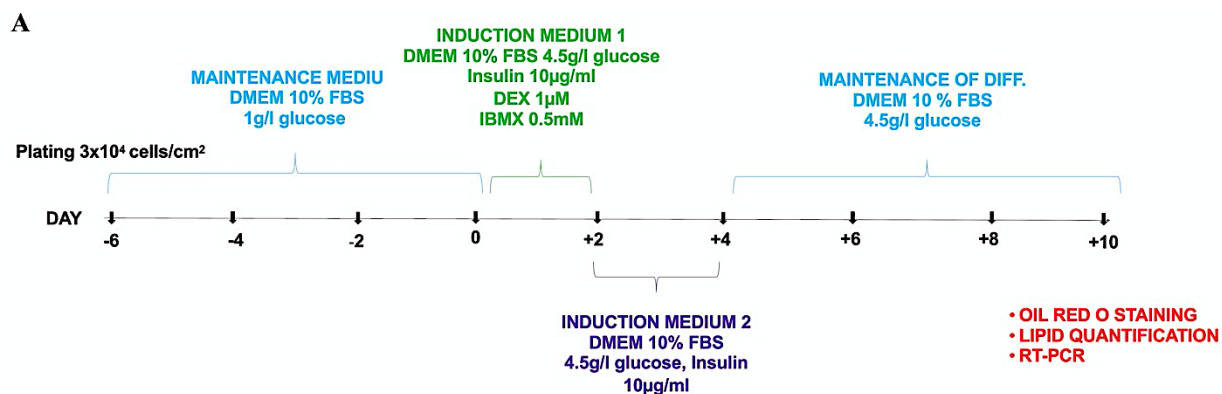
Figure 8. Schematic timeline of hADSCs differentiation

7.3.3 Evaluation of the GMG-43AC effects on in-vitro induction of adipogenesis.

GMG-43AC was diluted in DMSO and were added to the culture medium of hADSCs to reach a final concentration of 0.5 µM and 2 µM. Cells were incubated with the drug in according to the protocol described below:

I.   GMG-43AC was added to the medium at *day 0* of the differentiation process and maintained thereafter for the following *10 days* (Fig.9).

A
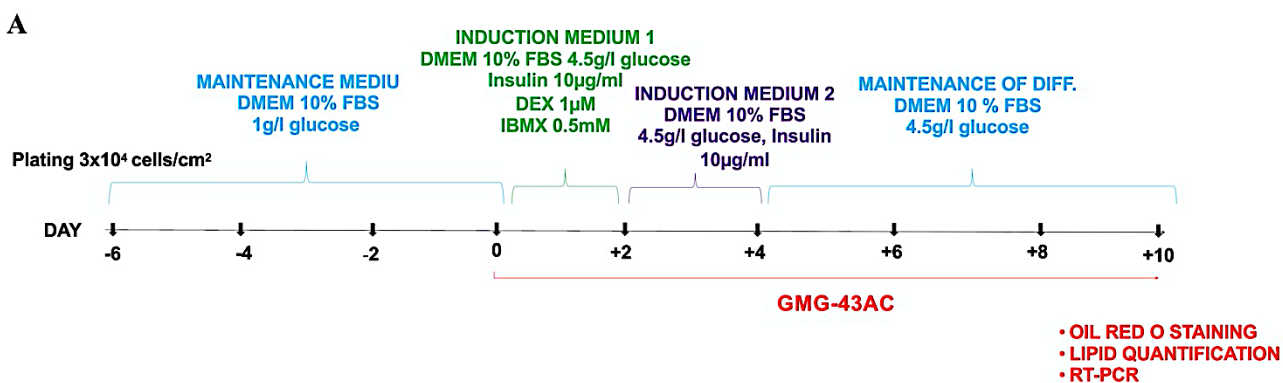
Figure 9. Schematic timeline of hADSC differentiation with GMG-43AC

7.3.4 Adipogenesis reversion process by GMG-43AC

hADSCs were differentiated for 7 days as described previously. The ability of GMG-43AC to revert adipogenesis in these cells was evaluated on day 14 of differentiation. GMG-

43AC was added at day 7 of differentiation and kept for 14 days. The dosages used were 0.5 µM and 2 µM (Fig 10).
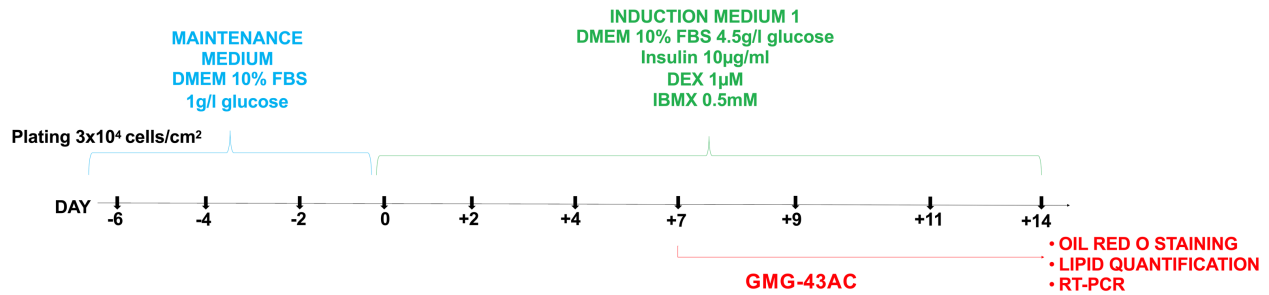
Figure 10. Schematic timeline of hADSCs reversion of differentiation with GMG-43AC.

7.3.5 Oil Red-O staining and quantification of lipid accumulation in adipocytes

Cells were cultured and differentiated in 6-multiwell and 48-multiwell plates. Eight to ten days after the induction of differentiation, cells were rinsed twice with phosphate buffered saline (PBS), fixed with 4% paraformaldehyde in PBS for 1 hour and washed with PBS (10 min, RT). After that were stained with Oil Red O. Oil Red O staining is the standard technique utilized to visualize droplets of neutral lipid accumulated in cytoplasm (135). Briefly, this dye specifically interacts with triacylglycerol and stains droplets in red. Oil Red O preparation procedure and adipocyte staining procedure used were following:

o Prepare Oil Red O stock. Dissolve 0.5 g Oil Red O in 100 ml of isopropanol, stir overnight at room temperature and then filter with 0.45 µm and store at $+4^0$C.

o Prepare Oil Red O working solution. Dilute 6 parts of the stock stain with 4 parts of distilled water, allow to stand for 20 min, and filter with 0.20 µm. The working solution should be made up fresh from the stock solution each time.

o Wash wells once with 60% isopropanol (10 min, RT).

o   Let the wells dry completely.

o   Add Oil Red O working solution min (without touching the walls of the wells) and incubate for 15.

o   Remove all Oil Red O and immediately add dH_2O, wash with dH_2O twice and then wash under running tap water.

o   Take pictures if desired.

o   Remove all water and let dry.

o   Elute Oil Red O by adding 100% isopropanol, incubate for about 20 minutes in agitation.

o   Pipet the isopropanol with Oil Red O up and down several times to be sure that all Oil Red O is collected.

o   Transfer to a 24-multiwell plate or 1.5 Eppendorfs.

o   Measure OD at 500 nm, 0.5 sec reading.

o   As blank use 100% isopropanol. As control use isopropanol from empty well stained as previously described.

The dye retained in the cells was evaluated with 2 different approaches:

1.   By elution with 1,5 ml isopropanol for at least 10 min. Specific optical density was measured at 500 nm in spectrophotometer Bio UV/VIS® Parkin Helmer (UK).

2. By the counting of positive stained cells compared to all the cells indicated by haematoxylin (SIGMA) staining (5 min). Slides were photographed by using optical microscope Leica and digital camera Leica (German). Data was plotted reporting the percentage of positive cells as the mean of the quantification of at least 5 fields for each condition.

7.3.6 Real Time RT-PCR

Real time Reverse Transcription-Polymerase chain reaction (RT-PCR), is a very sensitive and specific quantitative method that is useful for the detection of expression profiles of a selected genes that are available in low quantities (136). Real-time PCR is quantifies genes based on the fluorochrome and the melting curve analysis of the amplified products(137). The components needed to perform a RT-PCR are: 1) DNA template (cDNA), which can be single or double stranded; 2) Two oligonucleotide primers that bind the DNA sequence to amplify the product; 3) dNTPS (4 nucleotides triphosphates); 4) a heat stable polymerase (enzyme); 5) magnesium ions. The amplification reaction is performed based on temperature cycling. The high temperature is applied to separate the strands of the double helix DNA, and the low temperature to let the primers anneal to the template, and the last temperature is set depending on the polymerase used. Which should be optimum for the extension of the primers. The three steps are referred to denaturation, annealing, and elongation(138). (Fig. 11).
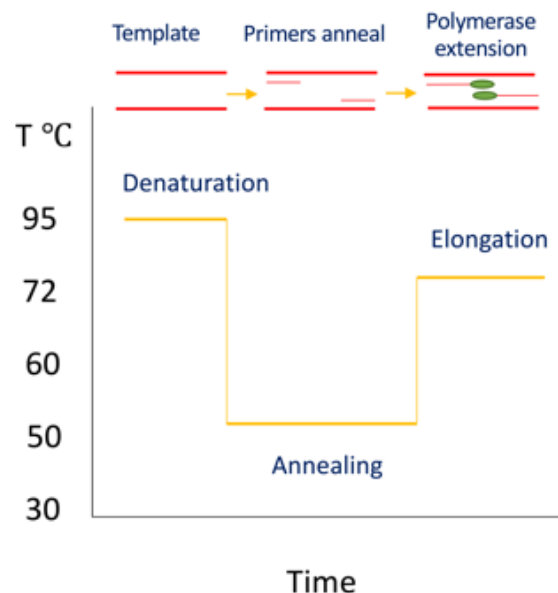
Figure 11. The PCR Temperature cycle

Currently four different polymerases are available for RT-PCR, TaqMan®, Molecular Beacons, Scorpions® and SYBR® Green (BIO-RAD). All of these enzymes allow detection

of PCR products via the generation of a fluorescent signal. TaqMan probes, Molecular Beacons and Scorpions depend on Förster Resonance Energy Transfer (FRET) to generate the fluorescence signal by the coupling of a fluorogenic dye molecule and a quencher moiety to the same or different oligonucleotide substrates. SYBR Green is a fluorogenic dye that exhibits little fluorescence when in solution but emits a strong fluorescent signal upon binding to double-stranded DNA. The threshold cycle or C_t value is the point associated signal increases and is detected by the system. This starts with the exponential growth of PCR product during the log-linear phase. This phase very useful to inform us about the reaction and the slope of the log-linear phase is a reflection of the amplification efficiency. The efficiency (Eff) of the reaction is calculated by the formula: $Eff=10^{(-1/slope)} - 1$. The efficiency of the PCR should be 90 - 100%. The efficiency of PCR is affected by various issues such as the length of the amplicon, the secondary structure and primer quality. The C_t is an important factor for quantitation. The higher the initial amount of genomic DNA, the earlier the accumulated product is detected in the PCR process, and vice versa. The threshold should be placed above any baseline activity and within the exponential increase phase. Two strategies are commonly

employed to quantify the real-time RT-PCR data: the standard curve method known as absolute quantification, and the comparative Ct method known as relative quantification (Fig. 12).
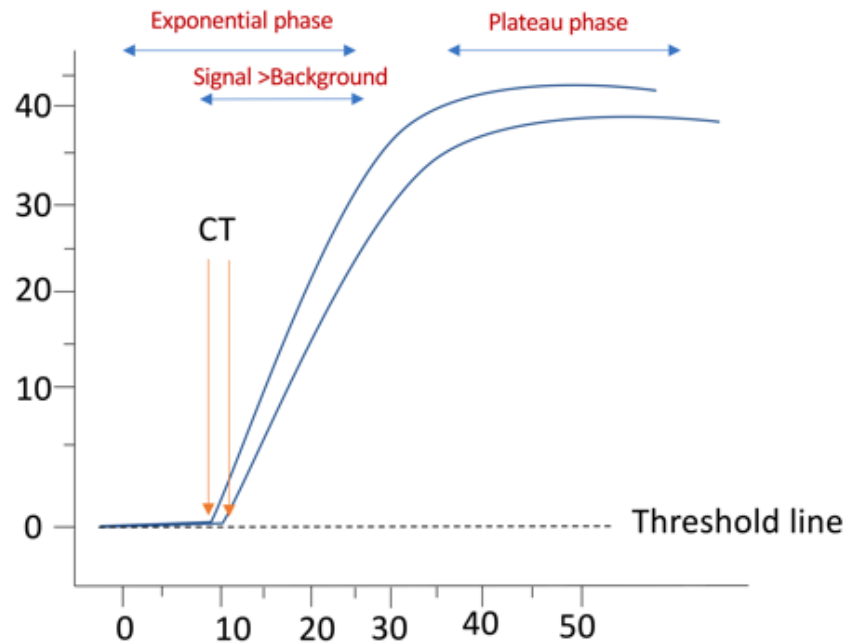
Figure 12. The RT-PCR Reaction. Relative Fluorescence vs. cycle number

In the standard curve method, a standard curve is first constructed from an RNA of known concentration. This curve is then used to construct the standard curve for any cDNA sample expressing the target gene. Spectrophotometric measurements at 260 nm can be used to assess the concentration of these DNAs, which can then be converted to a copy number value based on the molecular weight of the sample used. The comparative C_t method involves comparing the C_t values of the samples of interest with a control or control such as a non-treated sample or RNA from normal tissue. The C_t values of both the control and the samples of interest are normalized to an appropriate endogenous housekeeping gene. The comparative C_t method is also known as the $2^{-\Delta\Delta C_t}$ method (139), where $\Delta\Delta C_t = \Delta C_{t,sample} - \Delta C_{t,reference}$. $\Delta C_{t,sample}$ is the C_t value for any sample normalized to the endogenous housekeeping gene and $\Delta C_{t, reference}$ is the C_t value for the control also normalized to the endogenous housekeeping gene. For the $\Delta\Delta C_t$

calculation to be valid, the amplification efficiencies of the target and the endogenous reference must be approximately equal. This can be established by looking at how ΔC_t varies with template dilution. If the plot of cDNA dilution versus delta C_t is close to zero, it implies that the efficiencies of the target and housekeeping genes are very similar. Relative gene expression comparisons work best when the expression of the chosen internal control is abundant and remains constant among the samples. By using an invariant endogenous control as an active reference, quantitation of an mRNA target can be normalised for differences in the amount of total RNA added to each reaction. For this purpose, the most common choices are 18S RNA, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and β-actin.

Real-time PCR requires an instrumentation consists of a thermal cycler, a computer, optics for fluorescence excitation and emission collection, data acquisition, and analysis software. These machines differ in 1) sample capacity, some are 96-well standard format, others process fewer samples or require specialized glass capillary tubes; 2) method of excitation, some use lasers, others broad spectrum light sources with tuneable filters; 3) sensitivity.

In the experimental conditions, Real-time PCR was performed in a StepOne system (Applied biosystems) using iQ™ SYBR Green Supermix (BIO-RAD) following the manufacturer's instructions. GAPDH was used as reference housekeeping gene for normalization. An analysis using the ΔΔCt was performed, this procedure can be used since the replication efficiencies (slopes of the calibration or standard curves) for the genes of interest and housekeeping gene were previously determined. All the amplification reactions were performed in triplicates. Primers were designed using the NCBI's Primer-BLAST.

The primers were:

Gene name	Forward primer	Reverse primer
PPAR γ	5'CAAGAGTACCAAAGTGCAATCA AAGTGGAG-3'	5'-GTTCTCCGGAAGAAACCCTTGCATCCTTCA-3'
FABP-4	5'-CTGGGCCAGGAATTTGACGA-3'	5'-ACCAGGACACCCCATCTAA-3'
GAPDH	5'-CTTTTGCGTCGCCAG-3'	5'-TTGATGGCAACAATATCCAC-3'

Table 2. Primer sequences for THP-1 and human Adipose Derived Mesenchymal Cells

Gene name	Forward primer	Reverse primer
IL-1 β	5'-ACAGATGAAGTGCTCCTTCCA-3'	5'-GTCGGAGATTCGTAGCTGGAT-3'
IL-6	5'-GGTACATCCTCGACGGCATCT-3'	5'-GT GCCTCTTTGCTGCTTTCAC-3'
GAPDH	5'-CTTTTGCGTCGCCAG-3'	5'-TTGATGGCAACAATATCCAC-3'

Table 3. Primer sequences for THP-1 inflammatory markers

Real-time RT-PCR analysis

For these experiments, cells were grown and differentiated in 6-Multiwell dishes and each well was 25cm^2. Total RNA was isolated by using TRI Reagent® (SIGMA) in accordance with the manufacturer's instructions. In briefly, the procedure used was the following:

- o Add 500 ml of TRI Reagent directly on the culture dish. After addition of the reagent, the cell lysate should be passed several times through a pipette to form a homogenous lysate.

- o Phase Separation: To ensure complete dissociation of nucleoprotein complexes, allow samples to stand for 5 minutes at room temperature. Add 0.1 ml of chloroform per ml of TRI Reagent used. Cover the sample tightly, shake vigorously for 15 seconds, and allow to stand for 15 min at room temperature. Centrifuge the resulting mixture at 12,000 x g for 15 minutes at 4°C.

- Centrifugation separates the mixture into 3 phases: a red organic phase (containing protein), an interphase (containing DNA), and a colourless upper aqueous phase (containing RNA).

- Transfer the aqueous phase to a fresh tube and add 0.5 ml of isopropanol per ml of TRI Reagent used in Sample Preparation and mix. Allow the sample to stand for 10 min at room temperature. Centrifuge at 12,000 x g for 10 min at 4°C. The RNA precipitate will form a pellet on the side and bottom of the tube.

- Remove the supernatant and wash the RNA pellet by adding 500 μl of 75% ethanol per 500 μl of TRI Reagent used in Sample Preparation. Vortex the sample and then centrifuge at 7,500 x g for 5 min at 4°C.

- Briefly dry the RNA pellet for 5–10 minutes by air-drying. Do not let the RNA pellet dry completely, as this will greatly decrease its solubility. Add an appropriate volume of 0.5% SDS water, or to the RNA pellet. To facilitate dissolution, mix by repeated pipetting with a micropipette at 55–60 °C for 10 min.

The RNA quality and the amount of RNA in the sample was quantified spectrophotometrically. The ratio between A260/A280 was calculated to verify RNA purity. 500 ng/sample was used for cDNA synthesis.

Reverse transcription-PCR (RT-PCR)

The synthesis of single-strand cDNA was carried out on 1 μg of RNA template, using iScript™ Reverse Transcription Supermix for RT-qPCR (BIO-RAD) following the manufacturer's instructions.

Component	Volume per reaction
5X iScript reverse transcription Supermix	4 μl
RNA template (1 μg total RNA)	11 μl
Nuclease-free water	5 μl
Total volume	20 μl

Reaction protocol:

Priming	5 min at 25^0C
Reverse transcription	20 min at 46^0C
RT inactivation	1 min at 95^0C

7.3.7 MTT assay

Cell viability was measured by a quantitative colorimetric MTT assay, sensitive of the metabolic statuses of cells, particularly the mitochondrial status, thus reflecting early redox changes. Briefly, THP-1 and hADSCs were seeded in a 96-well plate at a density of 2×10^3 cells/well. After the cellular treatments were over, 10 µl of MTT assay kit reagent (Sigma-Aldrich) was added to each well, and the cells were incubated for an additional three hours. MTT was eluted with a solution containing HCl 4 mM, 0,1% NP40, Isopropanol for 30 min. The absorbance of each reaction product was measured with EnSight™ multimode plate reader (PerkinElmer) at a wavelength of 560 nm. The results are expressed as a percentage of the MTT absorbance of the control cells, which was set to 100%.

Statistical analysis

All data were expressed as mean ± SEM. Multiple comparisons were analyzed by one-way analysis of variance (ANOVA) followed by Turkey post hoc test. Statistical significance was accepted at a level $P<0.05$.

Chapter 8

Results

8.1 THP-1 Differentiation with lipid mixture

The rationale behind this experiment is to test the effects of high lipid diet on macrophages, since it was stated that this family of cells tend to interact with of immune cells and induce inflammation affecting different tissues(140, 141). THP-1 are maintained in THP-1 growth medium (see Materials section), they were seeded at a density of 1 x 10^6 cells/ml. THP-1 cells were differentiated by 200nM PMA for 24 hours and activated as described in the methods section (7.3.1), therefore they will stop dividing and adhere to the plate and take on an closer appearance to macrophages. LPS is added to induce inflammation. The experiment was conducted in three groups: 1) Pre-incubation (24-hr incubation with lipid mixture followed by 18-hr LPS); 2)Co-incubation (18-hr incubation with LPS followed by 24-hr incubation with lipid mixture); 3) Post-incubation (18-hr incubation with b0th LPS and lipid mixture). THP-1 showed that they accumulated lipids in all three experimental groups, which is important to know, meaning that this can activate and induce an inflammatory phenotype (47) (Fig. 13).

-ve CTRL + 200 nM PMA

+ve CTRL + 200 nM PMA+ 5ng/ml LPS

Treatment 1 50 µl Lipid Mixture

Treatment 2 100 µl Lipid Mixture

Treatment 3 150 µl Lipid Mixture

Treatment 4 200 µl Lipid Mixture

Pre-incubation 200nM PMA + 50 µl LM (no LPS)

Pre-incubation 200nM PMA + 100 µl LM (no LPS)

Pre-incubation 150nM PMA + 100 µl LM (no LPS)

Pre-incubation 200nM PMA + 200 µl LM (no LPS)

Co-incubation 200nM PMA + 5ng/ml LPS+50 µl LM

Co-incubation 200nM PMA + 5ng/ml LPS+100 µl LM

Co-incubation 200nM PMA + 5ng/ml LPS+150 µl LM

Co-incubation 200nM PMA + 5ng/ml LPS+200 µl LM

Post-incubation 200nM PMA+ 5ng/ml LPS (no LM)

Post-incubation 200nM PMA+ 5ng/ml LPS (no LM)

Post-incubation 200nM PMA+ 5ng/ml LPS (no LM)

Post-incubation 200nM PMA+ 5ng/ml LPS (no LM)

Figure 13. Images of THP-1 differentiated with PMA and activated with LPS and LM at different time

8.2 THP-1 Relative gene expression studies by RT-PCR

The expression of *PPAR γ* and *FABP-4* was investigated to confirm lipid accumulation in these cells along with the association to pro-inflammatory features (*IL- 6* and *IL-1β*). The expression of *PPAR γ* seems to be upregulated in all the pre-, co- and post-incubation with LM especially with the higher concentrations (150μl and 200 μl) (Fig 14 A-C). Suggesting a dose-dependent manner of lipid mixture. These findings indicate that *PPAR γ* has an important role in macrophage development even in the absence of LPS. *PPAR γ* is seen to increase therefore following a differentiating manner. Whereby, *FABP-4* seemed to be minimally present in all the three groups but was significantly expressed mostly in the higher concentrations of LM. These could suggest that if the incubation periods with LM were longer, *FABP-4* could be accelerating the lipid accumulation in the THP-1 cells. (Fig. 14 D-F)
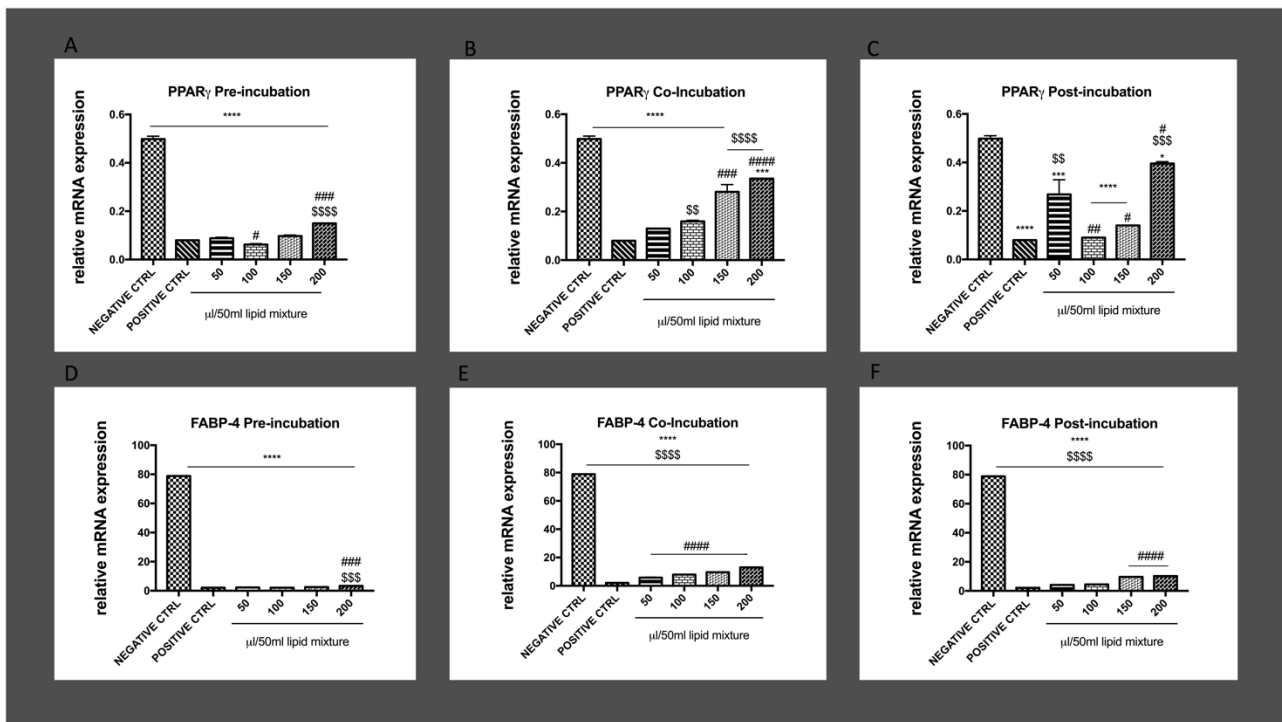
Figure 14. Expression of *PPARγ* and *FABP-4* in differentiated THP-1 cells with lipid mixture. (A*) PPARγ* Pre-incubation (24-hr incubation with lipid mixture followed by 18-hr LPS); **(B) *PPARγ* Co-incubation** (18-hr incubation with LPS followed by 24-hr incubation with lipid mixture); **(C) *PPARγ* Post-incubation** (18-hr incubation with b0th LPS and lipid mixture). **(A) *FABP-4* Pre-incubation** (24-hr incubation with lipid mixture followed by 18-hr LPS); **(B) *FABP-4* Co-incubation** (18-hr incubation with LPS followed by 24-hr incubation with lipid mixture); **(C) *FABP-4* Post-incubation** (18-hr incubation with b0th LPS and lipid mixture).

The inflammatory markers that were expressed upon the exposure of different dosages of LM at different times were *IL-6* and *IL-1β*. *IL-6* was seen to be significantly expressed in the positive control, which is expected. Whereby it was significantly reduced as the concentrations of LM increased in the Pre-incubation group (Fig. 15 A). In the Co-incubation and post incubation it was significantly decreased (Fig.15 B, C). This is interesting to observe, which means that LM has potent in decreasing the pro-inflammatory factors. *IL-1β* expression was seen to be significantly decreased in comparison to the negative control (Fig. 15 D). But also, there was a significance between the experimental groups and the positive control, which was more prominent in the co and post-incubation groups (Fig. 15 E, F). This confirms that LM downregulates the expression of proinflammatory markers in macrophages, suggesting that the components present in the LM could have an anti-inflammatory effect.
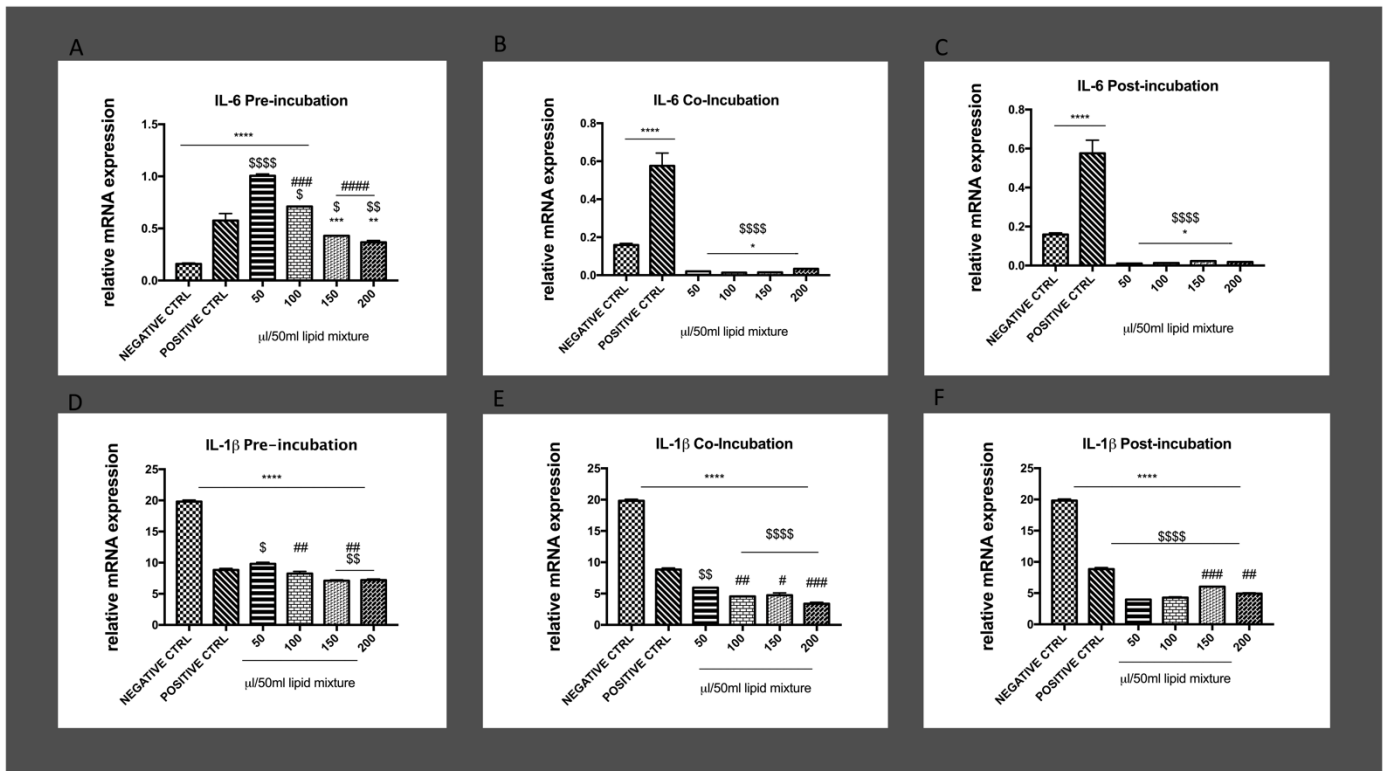
Figure 15. Expression of *IL-6* and *IL-1β* in differentiated THP-1 cells with Lipid mixture. **(A*)* *IL-6* Pre-incubation** (24-hr incubation with lipid mixture followed by 18-hr LPS); **(B)** *IL-6* Co-incubation** (18-hr incubation with LPS followed by 24-hr incubation with lipid mixture); **(C)** *IL-6* Post-incubation** (18-hr incubation with b0th LPS and lipid mixture). **(A) IL-1β Pre-incubation** (24-hr incubation with lipid mixture followed by 18-hr LPS); **(B)** *IL-1β* Co-incubation** (18-hr incubation with LPS followed by 24-hr incubation with lipid mixture); **(C)** *IL-1β* Post-incubation** (18-hr incubation with b0th LPS and lipid mixture).

The adipose tissues were derived from breast tissue from patients undergoing surgery in the mammary gland for the tumor removal. The biopsy was taken 5 cm away from the tumor location therefore from the microenvironment of the tumor. The observation that BRCA ADSCs seem to present a different and reduced differentiation ability compared to healthy ADSCs. Therefore, this experiment was conducted to investigate whether the process can be stimulated by exogenous factors such as situations when the adipogenic differentiation was reduced. To be able to study this, the differentiation with a lipid mixture of free fatty acids (10% v/v; Lipid Mixture), used to mimic a high-fat diet (107). The reasoning is that there could be an altered *PPARγ* modulation, as it was mentioned in literature reports indicating an alteration in *PPARγ* expression in breast cancer cells (142) (Fig. 16).
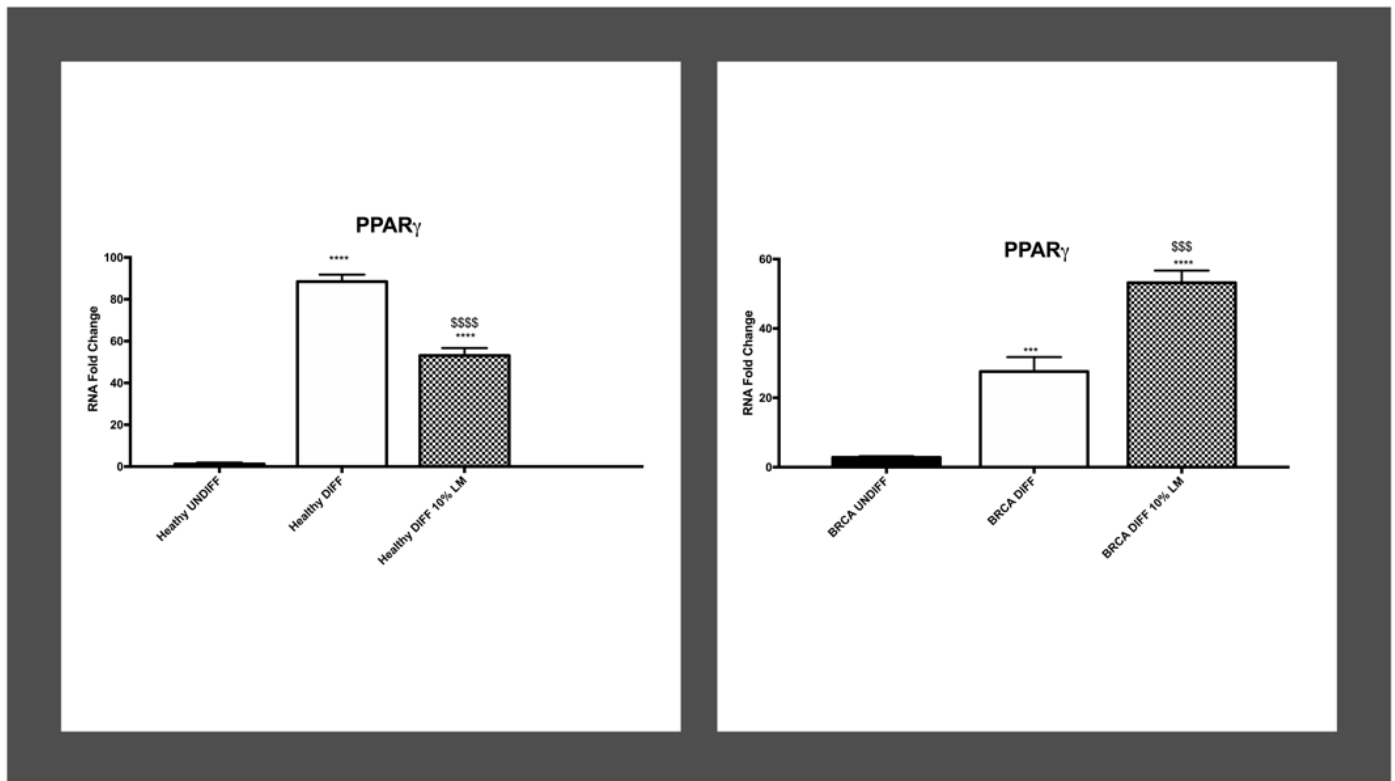
Figure 16. *PPARγ expression* in differentiated Healthy hADSC **(left),** and BRCA hADSC **(right).** Student's t-test; ****$p < 0.0001$, ***$p < 0.001$ **$p < 0.01$ vs. UNDIFF ADSC. $$$$ $p < 0.0001$ vs DIFF 10% LM.

Furthermore, an identification of a potential mechanism for the obesity correlation with breast cancer (10, 104, 105, 108). The accumulation of lipid droplets, investigated by Oil Red O staining, was increased in both conditions, and this is significantly relevant when the lipid mixture was added (Fig. 17).
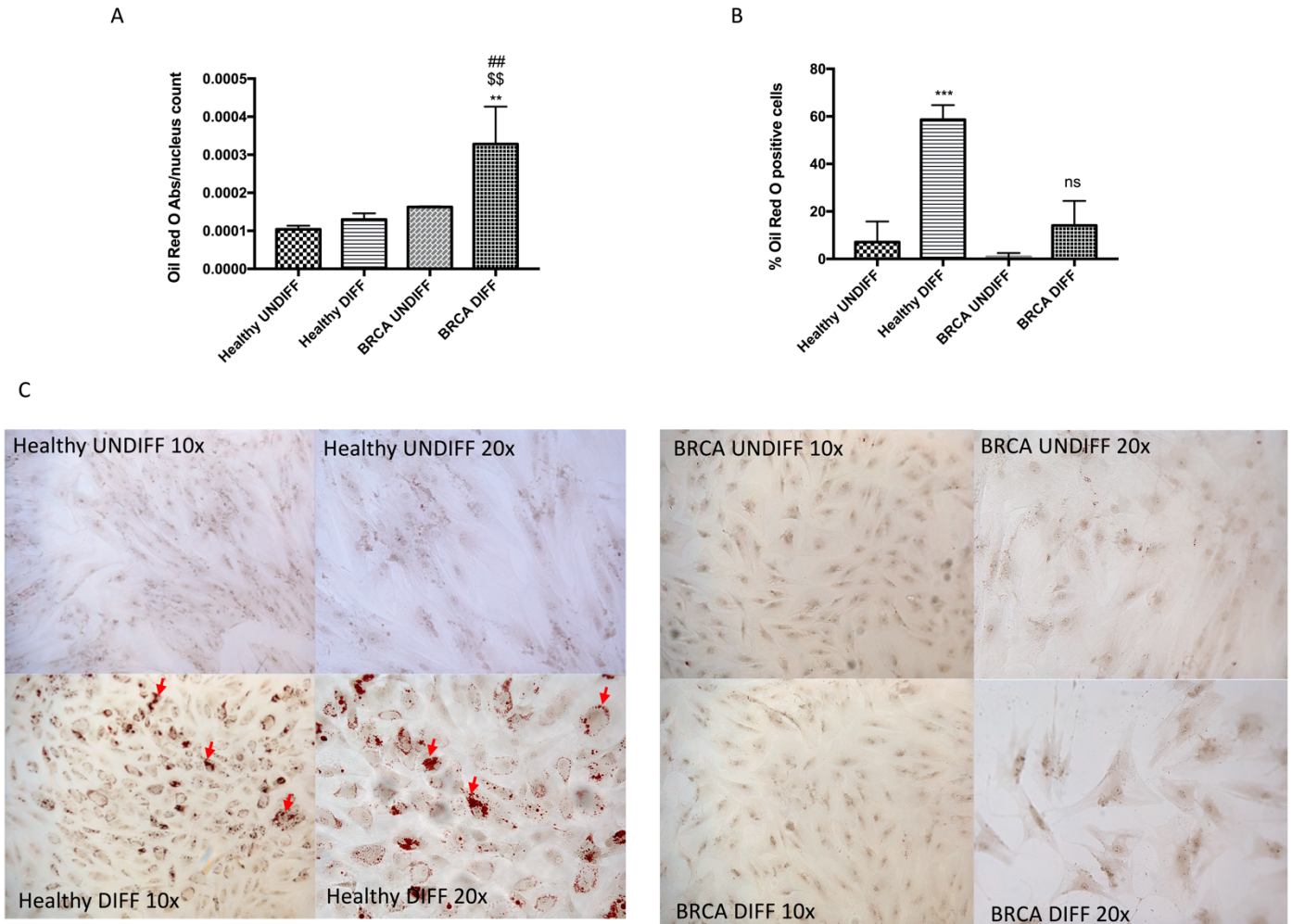
Figure 17. Adipogenesis was revealed by Oil Red O staining for lipid droplet accumulation. (A) Percentage of cells positive to Oil Red O staining. **(B) Quantification of Oil Red O extracted from lipid droplets measured at 520 nm normalized over nucleus counts. (C) Representative images of healthy ADSCs and BRCA ADSCs in control conditions and after adipogenic differentiation for 7 days (differentiated ADSCs).** Scale bars: 200 μm. Data are representatives of five different isolates. Magnifications of images of Healthy ADSCs and BC ADSCs in control conditions and after adipogenic differentiation for 7 days (differentiated ADSCs). Accumulation of lipid droplets, stained with Oil Red O, is highlighted with red arrows. Scale bars: 100 μm. Data are representatives of five different isolates Data are reported as the mean ± SD of 5 different isolates. The statistical significance was determined by Student's t-test **$p < 0.01$; ***$p<0.001$ vs. UNDIFF Healthy ADSC. $$ $p <0.01$ vs DIFF Healthy ADSC, ## $p<0.01$ vs BRCA DIFF

Figure 18. Adipogenesis was revealed by Oil Red O staining for lipid droplet accumulation. Representative images of healthy ADSCs *(left),* and BRCA ADSCs (*right)* in control conditions and after adipogenic differentiation in the presence of 10% LM for 7 days**.**

8.4 Pharmacological Selective PPAR γ modulator inhibits Triglycerides Accumulation and an effect the expression of adipocyte-specific genes in Human Adipose Derived Stem Cells (hADSCs) (DOI: 10.3390/ijms21124415)

GMG-43AC is an experimental drug presenting with a structure derived from propionic acid (PA)where PA is fermented in the colon by microbiota. It can reach the blood and adipose tissue where it can decrease fatty acids in plasma by the inhibition of lipolysis and induce lipogenesis and suppress fatty acid production in the liver (129).

The adipose tissue used was derived from Lipoaspirates. The accumulation of triglycerides by means of Oil Red-O staining for 10 days (Fig. 9) and 14 days (Fig.10) were evaluated. Briefly, hADSCs were differentiated in the presence of 0.5 mM or 2 mM GMG-43AC for 10 days (Fig. 19A). We observed a significant inhibitory effect with both 0.5 mM and 2 mM GMG-43AC concentrations on intracellular lipid accumulation. The quantification of lipid accumulation

obtained by measuring the absorbance at 500 nm after the extraction of the triglycerides stained with Oil Red-O further consolidated this finding (Fig. 19 B).

Figure 19. Morphology and Oil Red O staining and cell counts for 10 days. (A) hADSCs were differentiated and treated with 0.5- and 2-mM GMG-43AC for 10 days; **(B)** Percentage of Oil Red O positive cells and levels of accumulated triglycerides (labelled with Oil Red O in hADSCs undifferentiated and differentiated treated with GMG-43AC as evidenced by quantitative absorbance 500 nm wavelength ($n = 3$). (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. UNDIFF; $$$ $p < 0.001$, $$$$ $p < 0.0001$ vs. ADIPOCYTES; ## $p < 0.01$, ### $p < 0.001$, vs. experimental groups).
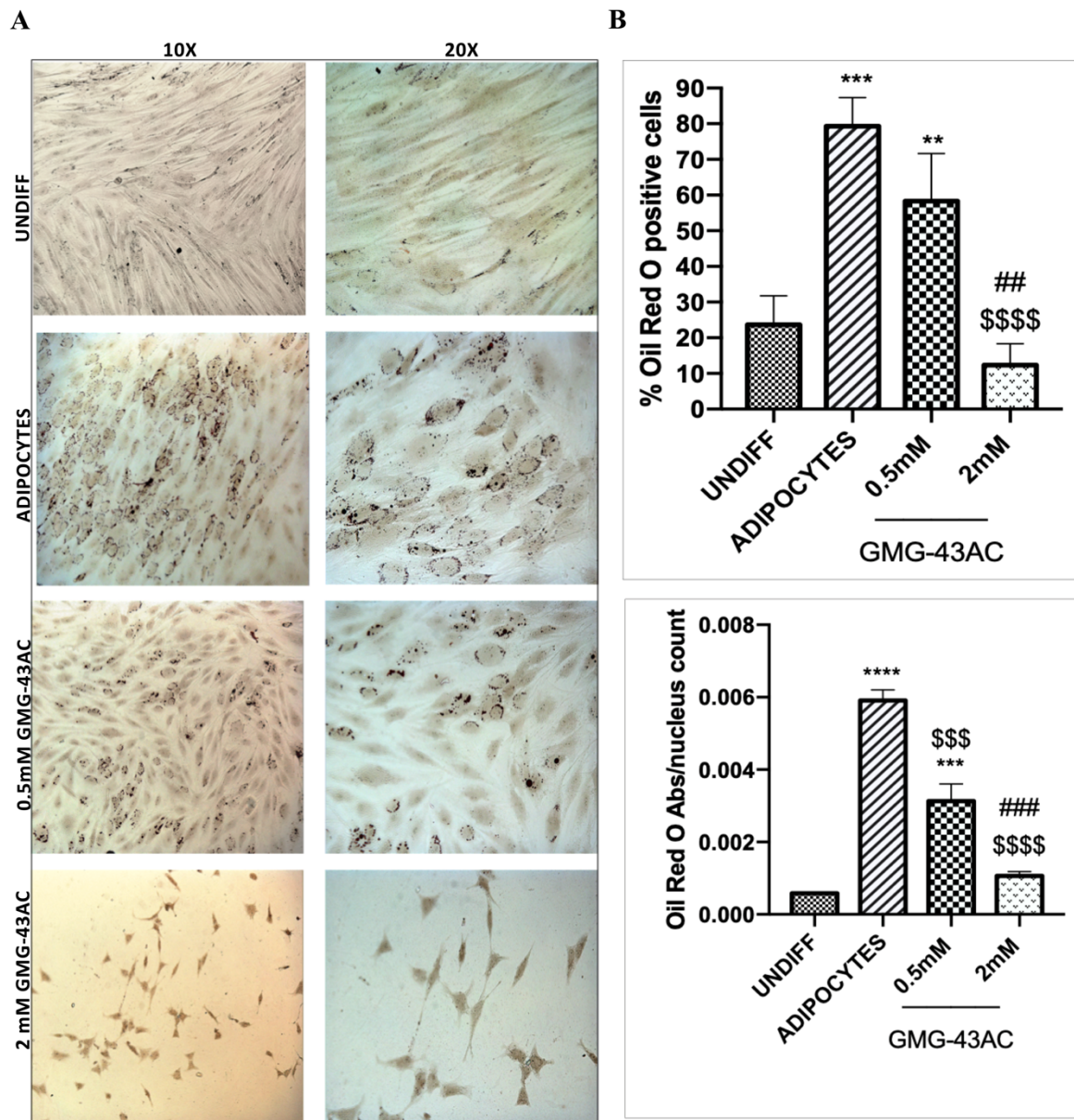
We evaluated *PPARγ* and *FABP-4* in hADSCs and we observed that in 10-day differentiation, both *PPARγ* (Fig. 20 A), and *FABP-4* (Fig. 20 B) were highly expressed in the 0.5 mM GMG-43AC treated cells whereby extremely downregulated in the 2 mM GMG-43AC. These results indicate that in the 10-day differentiation, the concentration of 2 mM GMG-43AC has a preventive effect of differentiation in hADSCs (Fig.20).

A

B
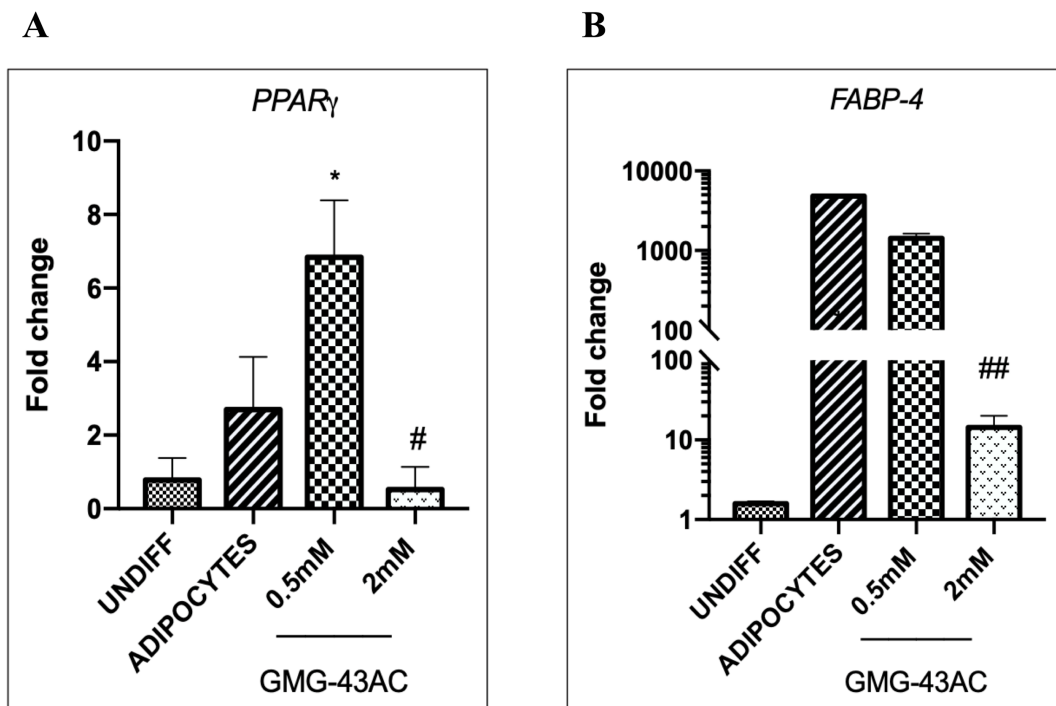
Figure 20. Expression of *PPARγ* and *FABP-4* during human Adipose Derived Stem Cells (hADSCs) differentiation for 10 days. **(A)** *PPARγ*; **(B)** *FABP-4*; Reported values (mean ± SEM) are the result of 3 independent experiments, and for each experiment at least three independent fields were considered for every condition (* $p < 0.05$ vs. UNDIFF; # $p < 0.05$, ##, $p < 0.01$, vs. experimental groups).

Another group of hADSCs were differentiated for 7 days without the presence of the drug, then 7 days with the presence of 0.5 mM or 2 mM GMG-43AC for a total of 14 days (Fig.10). Similar to the 10-day differentiation with GMG-43AC, both concentrations of GMG-43AC also had a significant inhibitory effect in the 14-day differentiation with reduced number of cells positive to Oil Red-O staining (Fig. 21 A, B) The quantification of lipid accumulation obtained by measuring the absorbance at 500 nm after the extraction of the triglycerides stained with Oil Red-O further consolidated this finding (Fig. 21 B).
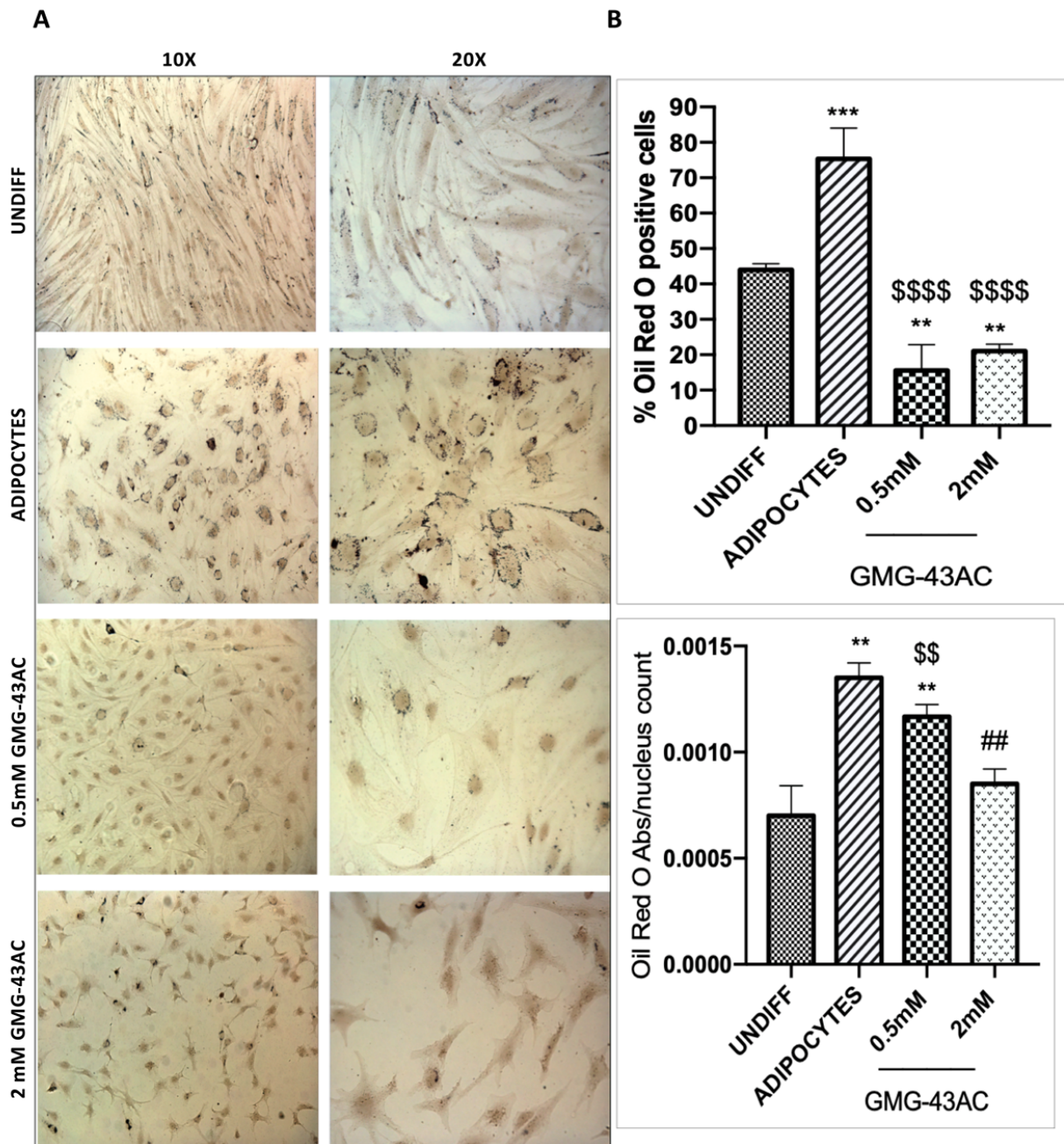
Figure 21. Morphology and Oil Red O staining and cell counts for 14 days. **(A)** hADSCs were differentiated and treated with 0.5- and 2-mM GMG-43AC for 14 days; **(B)** Percentage of Oil Red O positive cells and levels of accumulated triglycerides (labelled with Oil Red O in hADSCs undifferentiated and differentiated treated with GMG-43AC as evidenced by quantitative absorbance 500 nm wavelength ($n = 3$). Reported values (mean ± SEM) are the result of three independent experiments, and for each experiment at least three independent fields were considered for every condition (** $p < 0.01$, *** $p < 0.001$ vs. UNDIFF; $$ $p < 0.01$, $$$$ $p < 0.0001$ vs. ADIPOCYTES; ## $p < 0.01$ vs. experimental groups).

Interestingly, *PPARγ* (Fig. 22 A) and *FABP-4* (Fig. 22 B) were subjected to a different regulation to what was observed when cells were differentiated directly for 14-days. Both were significantly downregulated in both concentrations of GMG-43AC. These results suggest that GMG-43AC inhibited lipid accumulation in hADSCs in a dose-dependent manner and showed a potential prevention and treatment capability in these cells (Fig. 22).

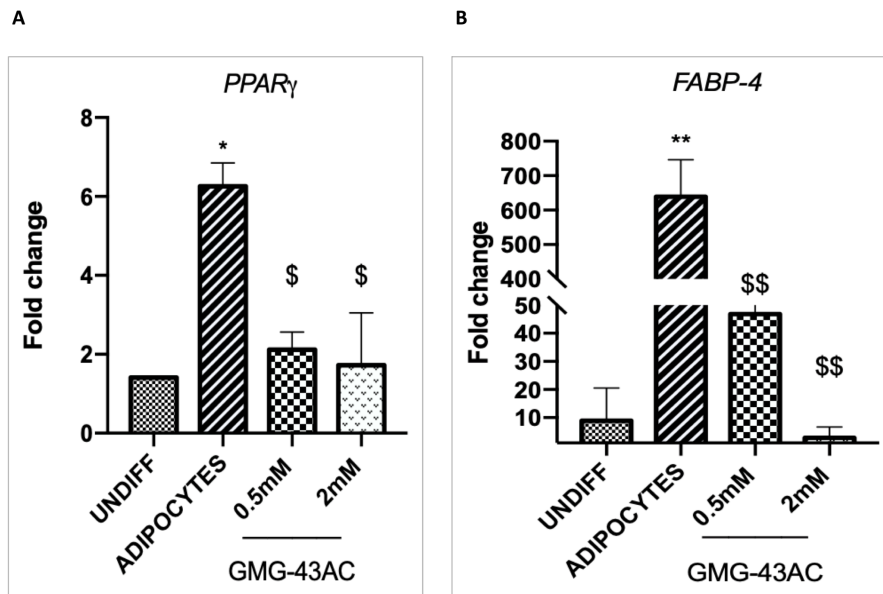**Figure 22. Expression of *PPARγ* and *FABP-4* during human Adipose Derived Stem Cells (hADSCs) differentiation for 14 days. (A) *PPARγ*; (B) *FABP-4*;** Reported values (mean ± SEM) are the result of three independent experiments, and for each experiment at least three independent fields were considered for every condition (* $p < 0.05$, ** $p < 0.01$ vs. UNDIFF $ $p < 0.05$, $$ $p < 0.01$ vs. ADIPOCYTES).

Moreover, cell viability was studied by means of MTT assay and the results show that in comparison to control cells, demonstrating the lack of toxicity induced by the drug at 0.5 mM. However, we noticed toxicity in the 2 mM dosage of GMG-43AC (Fig. 23).
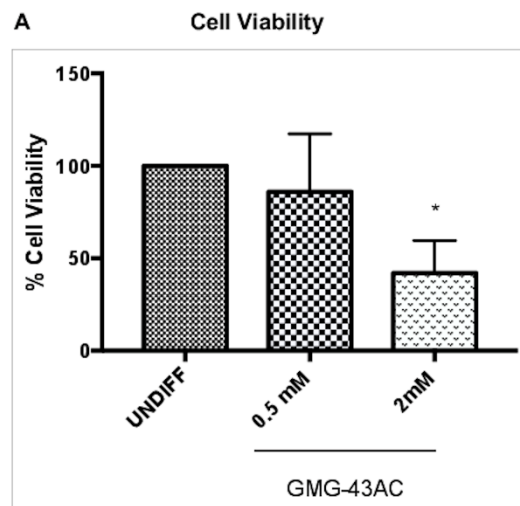
Figure 23. GMG-43AC halts cells proliferation in high concentration. **(A)** Effect of GMG-43AC on cell proliferation of hADSCs was determined by MTT assay. Values are expressed as a percentage of the MTT absorbance of the control cells, which was set to 100%. Data are expressed as the mean ± SEM of three independent experiments (4 wells/experiment, $n = 12$; * $p < 0.05$ vs. UNDIFF).

Chapter 9

Discussion

Metabolic syndrome is a global condition that affect a variety of the population with elevated risk of developing chronic inflammation. Obesity, CVD, and Diabetes are examples of Metabolic syndrome (143). Adiposity in relation to obesity are associated to a higher risk of the development of breast cancer (144). It was seen that obesity has a detrimental effect on breast cancer outcome in women patients (145). Breast cancer is the most common fatal malignancy among women worldwide (146). Many studies have established the fact that the development of breast cancer is dependent on the tumor microenvironment, such as adipocytes playing a role in its initiation, growth, and spread of the tumor. Therefore, not only defining adipose tissue as an energy storing organ but an important endocrine organ that has an influence on many systematic pathways related to energy expenditure, immune functions, and reproduction (147).

In this study, we evaluated many factors related to nutritive dietary compounds that we believe have a positive effect on adipocytes and adipogenesis. To be able to reach this goal, we started with using the THP-1 cell line to test the efficacy of these compounds on macrophages, which initiate inflammation. Therefore, testing how these dietary compounds effect inflammation by measuring the released cytokines *IL-6* and *IL-1β*. We noticed upon using a Lipid mixture cocktail a decrease in pro-inflammatory cytokines *IL-6* and *IL-1β* as a preventive and treatment in these cells especially with increasing concentrations suggesting a dose-dependent downregulation of pro-inflammatory marker. In addition, we suggest that *PPARγ* has an important role in macrophage development even in the absence of LPS. To assess the lipid accumulation in these cells, we noticed a dose-dependent increase of *FABP-4*.

To apply this knowledge to the differentiation of hADSCs, we differentiated hADSCs isolated from breast tissue that were derived from both healthy and breast cancer affected patients using a well-known and defined differentiation protocol and noticed that cells derived from breast cancer patients had an altered *PPARγ* modulation, in comparison with the cells from healthy patients, which underwent a normal adipogenesis. This was seen by RT-PCR where *PPARγ* expression was decreased in hADSCs from breast cancer patients. Furthermore, to validate this result qualitatively, we noticed that the lipid accumulation in these cells were much lower than the cells derived from healthy patients. This suggesting that *PPARγ* expression was impaired in hADSCs from breast cancer patients. While with the addition of lipid mixture, adipocyte differentiation was restored as well as the *PPARγ* expression in the hADSCs from breast cancer patients. Moreover, we noticed that the lipid accumulation in these cells treated with lipid mixture were more or less the same as the cells derived from healthy patients suggesting an important role of diet in the adipogenesis of these cells rendering a positive effect in these patients that have breast tumors. These results demonstrate that hADSCs from breast cancer patients are incapable to differentiate when a standard differentiation protocol is used, but this can be partially restored by the supplemention of lipids in the differentiation medium. The idea of the experiment was derived from a number of evidences , which suggests that there is a a role for breast cancer ADSCs during tumour progression course and relapse actions. It was noted that such interactions between tumours and their local microenvironment can regulate different characteristics such as the growth, spreading, and angiogenesis(100, 101). This experiment can show some preliminary, yet interesting aspects correlating the nutritional role on the tumor microenvironment, but more studies should be done to furthermore understand the mechanisms of how to positively affect the tumor microenvironment.

In the second part of the project, we treated hADSCs that were derived from lipoaspirates from healthy patients with a natural selective *PPARγ* modulator GMG-43AC to also assess the

differentiation capabilities. Were noticed that in both doses of GMG-43AC (0.5mM and 2mM) has a preventive effect as well as being effective even after the cells fully differentiated. This was seen from the inhibitory effect of *PPARγ* and *FABP-4* in the 14-day differentiation period. On the contrary, in the 10-day differentiation, *PPARγ* was significantly reduced in the 2mM concentration of GMG-43AC. On the other hand, it was significantly induced in the 0.5 mM concentration. This difference in expression can be explained from literature where it was stated that different possible transcriptional activities of *PPARγ* could be induced by specific modifications in the 3D conformation of the *PPARγ* receptor. Therefore, rendering a distinct gene expression. This has been investigated with many selective PPARγ modulators, such as in sebocytes, where *PPARγ* was shown to inhibit the accumulation of lipids, which a factor-dependent mechanism (148-150). We consider that hADSCs are sensitive to the 0.5mM concentration of GMG-43AC, which was evident from the low number of apoptotic cells. Therefore, suggesting a dose sensitive pharmacological approach to reduce adiposity and fat contents by adipogenesis- inducing agents. This is a promising outcome in relation to obesity treatment, which has a strong risk to breast cancer.

Chapter 10

Conclusions

In conclusion, during this study with all the different yet related concepts we were able to see that there is noticeable difference between the hADSCs cells that are derived from mammary glands of healthy patients and breast cancer patients. This can be seen especially when observing the adipogenic differentiation markers and the adipogenic differentiation capability upon the addition of dietary compounds in a dose dependent manner. Nonetheless, many studies should continue to investigate these theories with different methods to furthermore analyze the mechanisms behind this discrepancy. Furthermore, this does not negate the fact that the diet has a crucial role in the differentiation of hADSCs, which can help developing more preventive pharmacological approaches to fight life threatening metabolic diseases such as obesity, which was seen to have a strong relation to breast cancer.

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Publications

Papers

- Chemical Characterization and Nematicidal Activity of the Essential Oil of Nepeta nuda L. ssp. pubescens and Nepeta curviflora Boiss. from Lebanon / L. Musso, B. Scaglia, **G. AL HAJ,** Nelly Apostolides Arnold, F. Adani, G. Scari', S. Dallavalle, M. Iriti. - In: JOURNAL OF ESSENTIAL OIL-BEARING PLANTS. - ISSN 0972-060X.20:6(2017), pp. 1424-1433.

- Adipose-Derived Stem Cells from Fat Tissue of Breast Cancer Microenvironment Present Altered Adipogenic Differentiation Capabilities / F. Rey, E. Lesma, D. Massihnia, E. Ciusani, S. Nava, C. Vasco, G. **AL HAJ, G**. Ghilardi, E. Opocher, A. Gorio, S. Carelli, A.M. Di Giulio. - In: STEM CELLS INTERNATIONAL. - ISSN 1687-966X. - 2019(2019 Aug 14), pp. 1480314.1-1480314.15.

- A New Selective PPARγ Modulator Inhibits Triglycerides Accumulation during Murine Adipocytes' and Human Adipose-Derived Mesenchymal Stem Cells Differentiation / **G. AL HAJ**, F. Rey, T. Giallongo, M. Colli, B. Marzani, G. Giuliani, Gorio, G.V. Zuccotti, A.M. Di Giulio, S. Carelli. - In: INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES. - ISSN 1422-0067. - 21:12(2020 Jun 21).

Abstracts

- Embryonic Cell Extracts Ameliorate Wound Healing in Diabetic Mice / C. loretelli, M.B. nasr, **G.A. HAJ**, F. rocchio, S. faravelli, A.M. malvandi, F. d'addio, A. maestroni, T. letizia, E. assi, V. usuelli, E.C. leon, R. meronI, G. zuccotti, P. fiorina. - In: DIABETES. - ISSN 0012-1797. - 67: Supplement 1(2018 May), pp. 1231-P. A330-1231-P. A330. ((Intervento presentato al convegno Scientific Sessions of the American-Diabetes-Association: June 22nd - 26th tenutosi a Orlando (FL, USA) nel 2018.

- The Role of Nutrients in the Tumor Microenvironment / **G. AL HAJ**, F. Rey, E. Lesma,D. Massihnia, T. Giallongo, S. Nava, C. Vasco, G. Ghilardi, E. Opocher, S.

Carelli, A. M. Di Giulio. ((Intervento presentato al convegno Congresso DISS 5 edizione tenutosa Milan nel 2019.

- The GMG-43AC drug seems to inhibit adipocytes' differentiation / **G. AL HAJ**, F. Rey, T. Giallongo, D.M. Hebda, G. Ghilardi, S. Carelli, A.M. Di Giulio. ((Intervento presentato al convegno Congresso DISS 5 edizione tenutosi a Milan nel 2019.

- Therapeutic effect of neural progenitor cells expanded in the 3D nano-engineered Nichoid substrate in a Parkinson's disease preclinical model / B. Barzaghini, T. Giallongo, F. Rey, **G. AL HAJ**, T. Zandrini, G. Cerullo, R. Osellame, A.M. Di Giulio, M.T. Raimondi, S. Carelli. Intervento presentato al 28. convegno SINS tenutosi a Perugia nel 2019.

- Therapeutic effect of neural progenitor cells expanded in the 3D nano-engineered Nichoid substrate in a Parkinson's disease preclinical model / S. Carelli, T. Giallongo, F. Rey, B. Barzaghini, **G. AL HAJ**, T. Zandrini, G. Cerullo, R. Osellame, A.M. Di Giulio, M.T. Raimondi. Intervento presentato al convegno ESB-ITA Meeting 2019 tenutosi a Bologna nel 2019.

Acknowledgements

I wish to thank all the people whose assistance was a milestone in the completion of this Doctorate. All my colleagues that I met and worked with. Tonia, Federica, Bianca, Andrea, Riccardo, Alice, Letizia, Francesca, Alessandra, and finally, Stephana. Stephana you gave me faith in this field, and you taught me that it is never about how much time you spend in the laboratory that matters, but how you use it and what comes out of it. You were so helpful and so patient and very loving and kind. You taught me in two years what I need to spend long years in learning. You made everything smooth and I wish for any student that God could grant and guide them to a mentor like you. You are fair and I never felt that you sided with anyone against the other. You taught equally and that is the hardest thing to do whilst balancing teaching and other academic work and a family. I thank you from the bottom of my heart and as I always tell you I gained a friend not just a mentor. Professor Anna Maria Di Giulio you also were an excellent mentor. You have also given me a lot of strength to continue this journey when I was really thinking to quit but here I am now at the end because you gave me a chance to keep going. Dr. Alassandra Barassi, thank you for raking over and reaching the end with me. Also, I would like to thank all those who made it difficult for me at the beginning. You made it impossible at the beginning, but I guess what doesn't kill you makes you stronger. It was a very rough road for me especially that I tried to put all my strength to push through till the last second. Nobody warned me before that pursuing a PhD was really hectic and full of emotional ups and downs. The fear of failure, not having enough time to finish your due points, not keeping up with your everyday life, lack of funding, and most importantly if you have a family you tend to neglect them at times and feel guilty of doing so. But with perseverance I pushed through even though I have two children that depend on me just as much as depending on their father. Their father, my husband, my other half, Toufic. He helped me severely, it was as if he was also on this road rather than me. I left him to last to thank him because he was always and

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still is there for me at every beginning and never gave up on me till I reached the very end. Just like my family did for me when I was a little child. This is where I have to give them credit to driving me to where I am today. My mother, father, and my three brothers Tarek, Ziad, and Nabil, and my nephews and nieces. You all gave me strength, you got me where I am, you pushed me off many cliffs to learn how to spread my wings and fly and here I am probably at the last stop of academic life. I feel on top of the world with this achievement I am glad I did it!