Examination of the Inhibitory Protein P21 in Omento Fat as an Indication of Adipocyte Proliferation

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Abstract

Determining whether adipocytes are post-mitotic as opposed to proliferating may be of significance in developing efficient pharmaceuticals for treating symptoms of obesity. Recent studies have observed indications of proliferate cell activity in mature adipocytes. This study therefore aims to distinguish if there are any trends between obesity in terms of a higher Body Mass Index (BMI) and cyklin-dependant kinase inhibitor protein P21 as an indication of senescence and an attempt of proliferating. Four samples of omento adipose tissue from four separate patients were indirectly immunofluorescently stained and analysed in a confocal microscope. The results indicated a correlation between elevated senescence and a higher BMI. Obesity induced factors such as increased leptin and adiponectin levels also correlated well with adipocyte attempts to proliferate. Subsequently, the study was successful as it further substances the questioning of whether adipocytes are fully mitotic and the findings urge for further studies on the matter.

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Contents

1 Introduction

The recent surge of interest in adipocytes and their functions in the human body is often linked to the worldwide obesity epidemic. Preassembled data clearly indicates that obesity or overweight causes noticeable damage to both the physical and psychological state of health, resulting in for instance abnormal cholesterol levels, insulin resistance, adipokine secretion as well as type 2 diabetes [1]. By studying adipocyte proliferation, it is possible to further understand part of the complex issues of obesity. This field of interest attempts to pinpoint the specific factors involved in the increase of fat mass and their specific negative impact so as to inhibit them. Studies concerning proliferation in adipose tissue could consequently be vital for developing adequate and precise pharmaceuticals with the purpose of treating pathogenesis of obesity.

1.1 Cell Proliferation

The cell-cycle is divided into different stages, initiated by the G_1 state of interphase. During this phase, the cell grows and increases its amount of organelles in addition to its supply of proteins. Thereafter, the cell proceeds to the S phase which consists of DNA replication followed by the G_2 phase, which is yet another rapid growth phase with the addition of microtubule-restructuring. G_2 marks the completion of interphase and thus the cell is ready to go through mitosis. The entire course of the cell-cycle is illustrated in Figure 1.

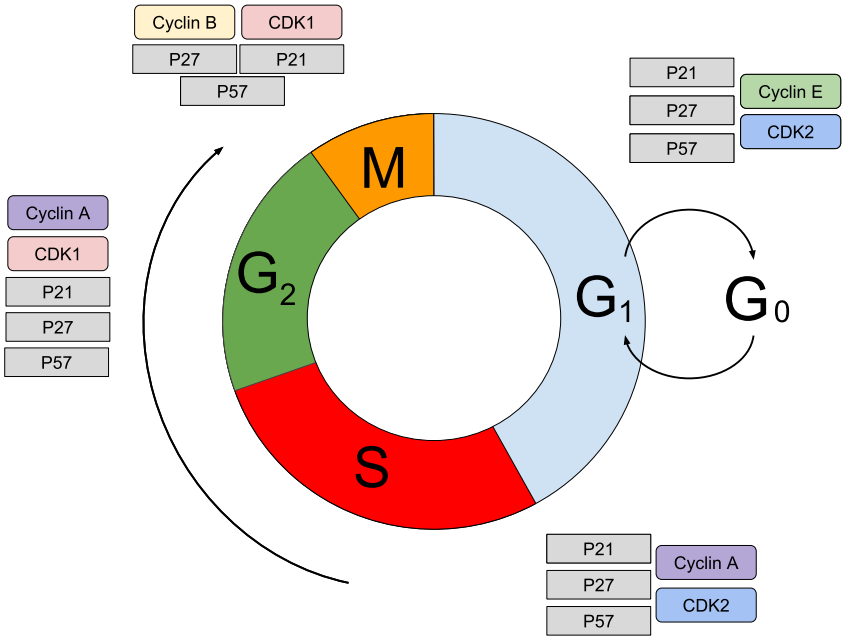


Figure 1: The cell-cycle with corresponding CDKs and CDKIs for each phase. G_1 is the initial growth phase, followed by the DNA synthesising S phase, growth G_2 phase and mitotis period, M. Cells which have completed or abrubted the cell cycle are referred to the quiescent G_0 phase. P21 is an important cell-cycle inhibitor throughout the cycle.

1.1.1 Senescent Cells

The G_0 phase is when cell division has stopped, either due to completion or abortion, and cells enter a mitotically passive phase. Quiescent cells are cells that have successfully completed the cell cycle while senescent cells have undergone proliferate arrest as a consequence to cellular distress. In contrast to quiescent cells, senescent cells are completely unresponsive to mitabolic stimuli. However, they remain metabolically active and are able to influence adjacent cells. Senescent cells are also resistant to apoptosis and considered very resilient. Cell proliferation is abrubted by a shortened mitotis period. Alternatively, senescence may be initiated during G_2 before the mitosis or, most commonly, between the G_1 and the S phase. Depending on when the cell-cycle is interrupted, the senescent cell can be larger, unaffected or contain excessive genetic information.

Senescent cells are characterised by a specific phenotype referred to as the senescence-associated secretory phenotype (SASP). The SASP often impacts surrounding tissue by

promoting cell proliferation, altering the pre-established pattern of cell differentiation, changing the immune response and furthering cell motility [2]. Conversely, the homoeostasis and secretory activity within a certain tissue become imbalanced. In the case of adipose tissue, senescence is believed to result in nutrient signalling dysfunction, chronic inflammation, stem cell exhaustion and proteostatic dysfunction [3]. Additionally, formations of oxidised unsaturated fatty acids, protein and metals, known as lipofuscins, gather in aged, senescent tissue [4]. It has been determined that an excess of lipofuscins is implicated in severe morbidity and mortality.

1.1.2 P21

P21 is a cyklin-dependent kinase inhibitor protein (CDKI). When the DNA in a cell is exposed to cellular stress, P21 is activated through P53 dependent and independent regulation. In obese patients, adipocytes tend to be most influenced by oxidative damage. The protein is activated by phosphorylation and ubiquitylation wherafter it will inhibit one of the cyklin-dependant kinase proteins (CDK) regulating cell-cycle progression. This often results in cell senescence. [5]

1.1.3 Leptin and Adiponectin

Leptin is a hormone responsible for the sensation of feeling full after a meal. In patients with excessive energy intake, leptin levels will be higher due to leptin resistance. As of yet, there is no definite explanation for leptin resistance, however, some studies indicate that there could be something wrong with the pathway to the hypothalamus [7, 8]. Consequently, the signal is interrupted and the body requires more leptin to respond appropriately. Similarly, adiponectin is a protein hormone with the function of regulating glucose levels and the breakdown of fatty acids. Adiponectin levels are also shifted during energy imbalance, however the causes are still unclear [9].

1.2 Previous Research

Progress has been rather slow concerning studies on the metabolic activity of adipocytes. A study by Naaz A, Holsberger DR et al. [10] compared different groups of mice and concluded that mice who had had their CDKIs removed, became obese with a distinct increase of fatmass and volume compared to wild mice. The study specifically examined the effect of the CDKI named P21. It inhibits the activity of Cdk1/cyclin B1 which, in its phosphorylated complex, is responsible for cell cycle progression. In response, P21 consequently sends the adipocyte into the G_0 phase. The results from the experiment indicated that lack of proliferate inhibitors may lead to obesity. Absence of CDKIs was therefore concluded to increase differentiation by expanded hypertrophy and hyperplasia. Subsequently, the metabolic change was directly linked to the differentiation of mesenchymal preadipocytes into adipocytes through adipogenesis. Moreover, the possibility that not only preadipocytes, but also mature adipocytes, could have proliferated was not considered. Similarly, two other studies reached the same final conclusion through separate experiments on adipocytes [11, 12]. The validity of the notion "all adipocytes are terminally differentiated" was therefore not questioned.

In contrast to previous findings however, observations of mature adipocytes in obese patients with two nuclei or double sets of DNA per cell have been reported at The Integrated Cardio Metabolic Centre at Karolinska Institutet. This would suggest that there is, in fact, ongoing proliferate activity in mature adipocytes. If so, it would also contradict previous beliefs that mature adipocytes are post-mitotic and incapable of further proliferating [13]. These observations suggest that adipocytes most likely do not complete the cell-cycle but are sent back to the G_0 phase in a senescent state as a cause of hyperglycemia, hyperlipidemia, increased muscle activity as well as mitochondrial dysfunction etc. [14].

The theory can be further studied by examining the P21 levels in mature adipocytes from adipose tissue in obese patients considering that high levels could indicate proliferate cell activity [5]. As the body becomes exposed to energy imbalance, the demand for

energy storage in the form of adipocytes increases. This may be achieved by preadipocytes differentiating or existing adipocytes expanding in size and proliferating. However, since obesity also implies high levels of free fatty acids in the body, adipocyte DNA is subjected to oxidative damage and therefore become senescent due to involvement of first P53 and thereafter P21. [6]

1.3 Aim of the Study

The aim of the study is to examine the concentration of P21 in adipocytes of obese patients compared to lean patients. This would then constitute an indication of whether adipocytes are fully differentiated or if they may attempt another cycle of mitosis in response to energy over-consumption.

2 Method

In this section, the method of distinguishing P21 abundance is depicted. Adipocyte samples were stained, photographed in a confocal microscope and later analysed in accordance to their positive or negative expression of P21.

2.1 Study Participants

Surgical biopsies from omWAT and scWAT were obtained from one lean and five obese patients during bariatric surgery at Ersta hospital in Stockholm, Sweden. Patients had a BMI of 36 ± 10.3 kg m^{-2}, an age of 44 ± 10 years and were exclusively female. None of the patients were influenced by medicine targeting adrenergic signalling. Blood glucose, serum insulin, triglycerides and high density lipoprotein cholesterol (HDL-C) levels were measured the morning of the surgery after a fasting protocol had been implemented overnight. Results were then analysed at Karolinska universitetslaboratoriet. Body fat percentage was also measured prior to the procedure. Omento adipose tissue was dissected from the great omentum while subcutaneous fat was disected from the epigastric region

of the abdomen. Glucose and insulin were used to calculate insulin sensitivity index. The study was approved by the Regional ethics committee in Stockholm (2014/1115-31/2) and written informed consent for participation in the study was obtained from all participants. Fat was purposefully obtained from patients with varying degrees of obesity in order to be able to compare the results as well as conclude a correlation between P21 and degree of obesity. Two sample exclusions were made due to lack of substantial amount of stained fat left to examine.

2.2 Fixation of Adipocytes

In order to stain the adipocytes, they first had to be permeabilised. To prevent the cell structure from falling apart prior to further examination, the adipocytes had to be fixed before staining. 1 mL of fixing solution (2% PFA, 1% Sucrose, PBS, water) was added to 300–500 µL of freshly isolated adipocytes and was thereafter placed on shaker for 30 minutes at room temperature. Adipocytes were let to float, PFA was removed and 1 mL washing buffer was added. Samples were kept at room temperature until immunostaining.

2.3 Indirect Immunofluorescent Staining

The washing buffer (0.1% BSA in 0.05% PBS-Tween) was removed and 1 mL PBS 0.5% Triton-x100 was added before placing the adipocytes on the shaker for 30 minutes at room temperature. Triton was removed, MetOH was added for 7 minutes at -20°C and the samples were thereafter centrifuged for 30 seconds at 100 g and 4°C. The MetOH was removed and 1 mL blocking buffer (2% BSA) was added. Tubes were placed on the shaker for 90 minutes. Permeabilization was completed and samples were exposed to primary antibody (Pure Rabbit Monoclonal for P21 waf1/cip1) prepared in 500 µL blocking solution (1:200). Adipocytes were left on the shaker overnight at room temperature. Primary antibody was rinsed with washing buffer and tubes placed on the shaker for 3×10 minutes. Secondary antibody (Alexa 555 Conjugated Anti-Rabbit Donkey Polyclonal lgG,

1:1000) in addition to membrane dye (Fluoroscein, 1:500) and nuclei stain (Hoechst 33342, 1:500), were prepared in 1000 µL washing buffer. A staining solution was introduced and the tubes placed on the shaker for 2 hours at room temperature. Samples were washed with PBS and placed on the shaker for 15 minutes. 400 µL was prepared with 100 µL of cells in a new tube. Cells were mounted on cover-slides for further observations.

2.4 Visual Analysis

Visuals were captured using a confocal microscope and during immunostaining, the excitation levels of the three separate dyes were accounted for in the microscope controls. All images were processed and interpreted with Figi (ImageJ) software. A MACRO for finding P21 in the nuclei, and provide numbers of dots and their intensities, was used. The threshold for each patient was in turn determined by interpreting the patients' respective intensity values and microscope images which then allowed for defining of a positive or negative P21 expression for each patient. There was therefore no absolute intensity value. The thresholds were established based on the correlation between the various staining results of the nuclei.

3 Results

The results indicate some correlation between obesity and cell senescence. Figure 2 displays the percentage of cells expressing high levels of P21 in relation to the severity of obesity in regards to the BMI of the patients. The results of the study indicate that the percent of senescent adipocytes in omento adipose tissue increases as patients become progressively obese.

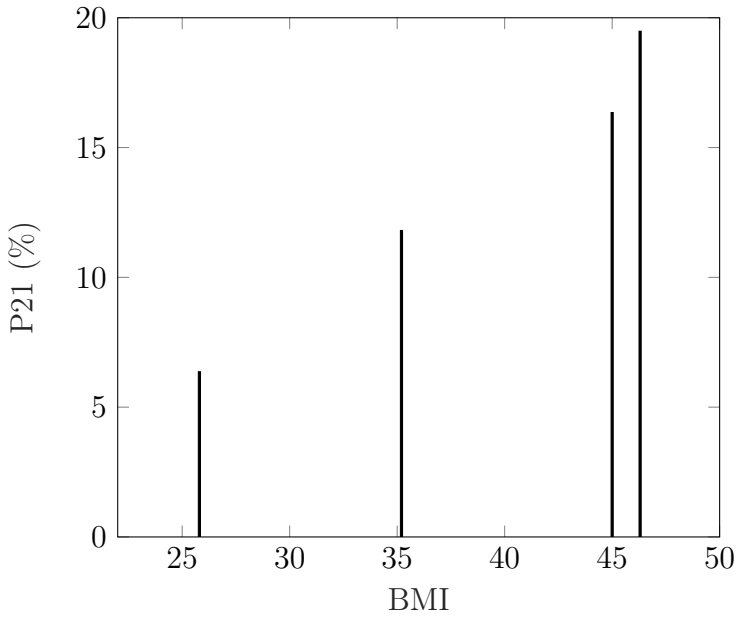
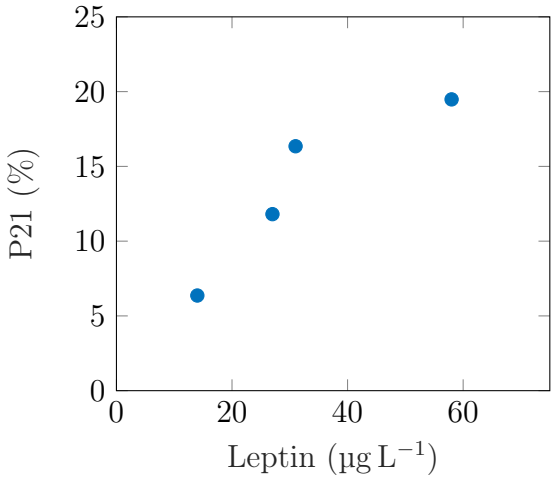
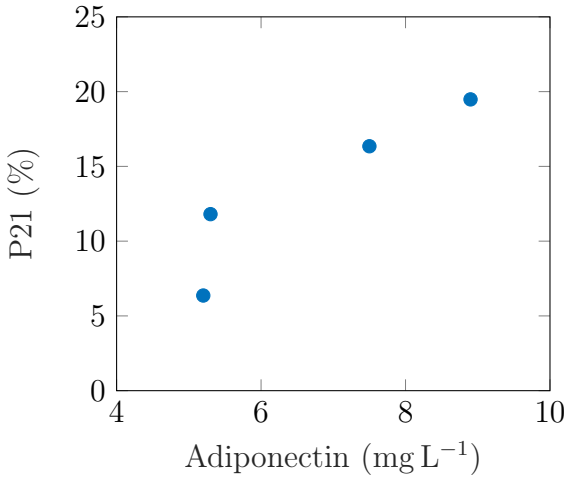


Figure 2: Percentage of cell-cycle inhibitor P21 expression in nuclei of omento adipose tissue in one lean patient and three varying degrees of obese patients.

The P21 percentage was also compared to leptin and adiponectin levels of the patients. Some correlation was found, depicted in Figure 3. It would appear that an increase in leptin and/or adiponectin, correlates to increased P21 activity in adipocyte nuclei.



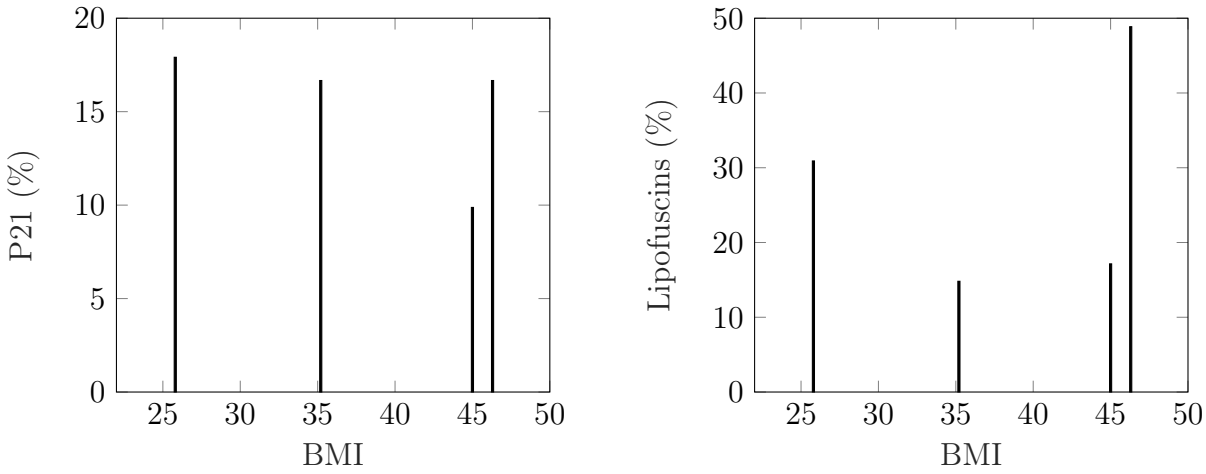
(a) Concentration of leptin in correlation to P21 percentage.



(b) Concentration of adiponectin in correlation to P21 percentage.

Figure 3: The correlation between the cell-cycle inhibitor, P21, expression in omento fat nuclei and the abundance of protein hormones leptin and adiponectin in patients of varying severity of obesity.

Upon the introduction of previous experiments on subcutaneous samples, the difference in P21 expression between the two different tissues of fat is noticeable. Figure 4a illustrates the percentage of P21 in subcutaneous, as opposed to omento, fat. In subcutaneous fat, the percentage of nuclei with abundant P21 is rather high but does not seem to be affected much by patients BMI as long as they are obese. In contrast to Figure 2, there is no clear correlation between increase of P21 in nuclei and a higher BMI in Figure 4a.



(a) P21 % in subcutaneous adipocytes.

(b) Lipofuscin % in omento adipose tissue.

Figure 4: Figure 4a illustrates the percentage of the CDKI, P21, expressed in nuclei of subcutaneous tissue from patients with varying BMI and Figure 4b displays the percentage of nuclei with surrounding lipofuscins out of all visually captured nuclei in correlation to the BMI of the patients.

Furthermore, another indication of senescence was measured. Figure 4b displays the calculated percentage of lipofuscins surrounding the nuclei. There may be an interrelation between obesity and increased lipofuscins which would indicate a concordance with adipocyte senescence. There is no clear correlation since there is an increase in lipofuscins the more obese the patients are, however, the lean patient expresses a relatively high percentage of lipofuscins compared to the obese samples.

4 Discussion

In this study, we examined the percentage of nuclei expressing levels of P21 high enough to indicate senescence in four different patients and compared the data. The results imply a possible correlation between higher BMI and an increased percentage of nuclei expressing P21 which translates to an escalated amount of adipocytes becoming senescent in omento tissue. The fact that adipocytes become senescent in obese patients insinuates that adipocytes are not post-mitotic, but will attempt proliferating before becoming senescent.

Adipocytes likely re-enter the cell-cycle because of insulin resistance which follows once patients become obese [1], however, during this experiment it was impossible to examine any possible correlation between P21 and insulin or glucose levels since fat was dissected from patients who had not fasted properly prior to surgery. Leptin and adiponectin levels could nonetheless be compared and evaluated. Due to leptin resistance in obese patients, their leptin levels were elevated. So were the adiponectin levels but instead because of insulin resistance. The results suggest a correlation between the hormones and P21. As leptin and adiponectin levels increase, so does the P21 percentage in the adipose tissue. Despite this, it is difficult to determine if these factors are the main actuators, if one is of more importance or if there is something else of bigger impact triggering cell proliferation.

Lipofuscins were also used as an indication of senescence, although they are not entirely conclusive in terms of the results. This is due to the fact that the abundance of lipofuscins is partly determined by the age of the patient. Old tissue tends to gather more lipofuscins than young tissue [15]. Therefore, the results are somewhat effected by the varying ages of the patients, the oldest patient being the lean one. Nevertheless, all obese patients express higher values of lipofuscins than would be expected for their age. This then suggests that there is ongoing senescence in these adipocytes. Conversely, the adipocytes in obese omento adipose tissue do show some signs of proliferating in regards to the amount of lipofuscins surrounding the nuclei.

In contrast to omento fat, subcutaneous fat does not seem to be affected by how high BMI the patient has. This could possibly subsequent to the fact that subcutaneous fat is the most metabolically active tissue of fat. In energy imbalance, the subcutaneous fat will be the first to be effected since the omento fat is implemented mostly when the subcutaneous fat has already met its potential. The results are not to insinuate that adipocytes in subcutaneous fat are completely unaffected by obesity and lack the proliferate ability as mature adipocytes. Rather, this indicates that perhaps the subcutaneous adipocytes are affected before the omento fat but once patients reach a certain BMI, an upper limit is achieved and adipocytes will, or can, no longer proliferate or be sent to the senescent state. The high levels of P21 expression in the subcutaneous tissue of all examined obese patients further supports this claim.

Since no other study has been conducted on the examination of P21 percentage in adipocyte nuclei as an indication of senescence, the collected data so far is not sufficient for a complete analysis. However, the results do indicate a trend which verifies our hypothesis that adipocytes may not be post-mitotic and therefore function as incentive to initiate further studies on the topic.

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