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Ο κυτταρικός κύκλος στα λιποκύτταρα σε φυσιολογικές και παθολογικές καταστάσεις

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Summary and foreword

Even though obesity is one of the major health care challenges of the 21st century, there is still a huge gap in our knowledge regarding the fundamental biology of mature adipocytes. The fact that mature adipocytes are categorized as post-mitotic cells is a deep-rooted notion in the scientific literature that has never been challenged directly but a handful of times. Thus, the aim of this study was to investigate the cell cycle in human mature adipocytes and the physiological and pathological effects it has in the biology of the tissue.

In the first part of the study we investigated whether mature human adipocytes can re-enter cell cycle *in vivo* and *in vitro*. Using immunocytochemistry and immunohistochemistry techniques, it was shown that human primary adipocytes were able to express several cell cycle markers spanning from G1 to G2 phase. These results were confirmed *in vitro* in a modified culture environment of isolated human mature adipocytes. In this model adipocytes were found to re-enter cell cycle, as suggested by the elevation of cyclin D1 nuclei levels. Moreover, the EdU nucleotide incorporation into cells' DNA indicated that the adipocytes were able to pass through S-phase. These phenomena were augmented when insulin was added to culture medium which is proof for insulins mitogenic effects in mature adipocytes.

Despite cell cycle re-entry, however, there is no indication that adipocytes can go through cytokinesis. This might suggest that what is really happening is an alternative to cell cycle, called endoreplication which is responsible for development of polyploid cells. In order to investigate the possibility of adipocyte polyploidy we developed and improved qualitative techniques for staining of adipocytes' nuclei and quantitative techniques, like the staining of Barr body, as a second part of the study. In addition to this, a new flow cytometry analysis method was proposed, that would allow for the simultaneous investigation of adipocyte hypertrophy (hallmark of polyploid cells) and other cell markers. If validated, these techniques can be applied in future studies of adipocyte polyploidy and hypertrophy.

In addition to possible polyploidy, the fact that mature adipocytes are able to re-enter cell cycle without finishing it has the implication that mature primary adipocytes might develop senescence. Senescence could potentially act as a unifying theory to explain the hypertrophic, pro-inflammatory and insulin resistant characteristics of "unhealthy adipocytes". In the third part of the study we investigated the development and the factors leading to adipocyte *senescence in vivo* and in culture, as well as the role of cell cycle re-entry as a condition for senescence. When isolated human primary adipocytes were stained for senescence associated β-Galactosidase (SA-β-Gal), some of the adipocytes were found positive. Parallel to SA-β-Gal staining, two more known markers of senescence were used, the increased size of SA-β-Gal positive cells and their correlation with HMGB-1 nuclear absence, which established the presence of a senescent

phenotype in the adipocytes. More importantly, the percentage of SA-β-Gal positive adipocytes was found to have a true physiological effect, as it was shown to correlate with the metabolic profile of adipose tissue donors in a cohort of 51 patients. In addition, *in vitro* experiments revealed that the presence of insulin, which previously led to cell cycle re-entry, increased senescence. The use of palbociclib that arrests cell cycle progression, also, augmented senescence, while metformin, which restricts cell cycle entry and inhibits the mTOR mitogenic signals, reduced senescence.

Finally, in order to investigate the heterogeneity between adipocytes, which might be responsible for leading some cells into senescence while protecting others from it, the insulin receptor expression was studied in human mature adipocytes, since insulin had such a pivotal role in cell cycle and senescence. Using flow cytometry the presence of an insulin receptor negative adipocyte population and its correlations to the metabolic profile of 14 patients was investigated, yielding however inconclusive results for the time being.

In conclusion, mature human adipocytes were found to be able to re-enter cell cycle which is an important condition for the development of senescence. Parallel to those, this study provides new methods for the investigation of adipocyte ploidy and hypertrophy. Finally, a new adipocyte population of insulin receptor negative cells was discovered and its correlation to patients' metabolic profile was investigated. Future studies can address how these new biological processes affect adipose tissue and human health.

Περίληψη της πτυχιακής εργασίας

Παρότι η παχυσαρκία αποτελεί μια από τις κυριότερες προκλήσεις για την υγεία στον 21ο αιώνα, υπάρχουν ακόμα μεγάλα κενά στις γνώσεις μας όσον αφορά τη θεμελιώδη βιολογία του λιποκυττάρου. Ένα μεγάλο μέρος της επιστημονικής κοινότητας θεωρεί τα ώριμα λιποκύτταρα post-mitotic κύτταρα που δεν έχουν τη δυνατότητα να επανεισέλθουν στο κυτταρικό κύκλο. Η μελέτη αυτή έχει ως στόχο τη διερεύνηση της ύπαρξης κυτταρικού κύκλου στα ώριμα ανθρώπινα λιποκύτταρα και τον προσδιορισμό των επιπτώσεων που το φαινόμενο αυτό επιφέρει στο λιπώδη ιστό.

Στο πρώτο τμήμα της μελέτης διερευνήθηκε η ικανότητα των ανθρώπινων ώριμων λιποκυττάρων να επανεισέρχονται στον κυτταρικό κύκλο *in vivo* και *in vitro*. Χρησιμοποιώντας τεχνικές ανοσοκυτταροχημείας και ανοσοϊστοχημείας, βρέθηκε ότι τα ανθρώπινα ώριμα λιποκύτταρα είναι ικανά να εκφράσουν πολλαπλούς δείκτες του κυτταρικού κύκλου, από τις φάσεις G1 έως και G2. Τα αποτελέσματα επιβεβαιώθηκαν *in vitro,* με την χρήση ενός τροποποιημένου μοντέλου κυτταροκαλλιέργειας ανθρώπινων λιποκυττάρων. Ορισμένα από τα λιποκύτταρα του μοντέλου εισήλθαν στον κυτταρικό κύκλο, όπως αποδείχθηκε από τα αυξημένα επίπεδα κυκλίνης D1 στον πυρήνα. Επιπλέον παρατηρήθηκε ενσωμάτωση νουκλεοτιδίων EdU στο κυτταρικό DNA, φαινόμενο ενδεικτικό της δυνατότητας των λιποκυττάρων να αντιγράφουν το DNA τους και να περνούν από την φάση S. Και τα δύο αυτά φαινόμενα ενισχυθήκαν με τη προσθήκη ινσουλίνης στο καλλιεργητικό μέσο, πράγμα ενδεικτικό της μιτογόνου δράσης της ινσουλίνης στα ώριμα λιποκύτταρα.

Παρόλο που τα λιποκύτταρα μπορούν να εισέλθουν στον κυτταρικό κύκλο, δεν βρέθηκαν δεδομένα που να στοιχειοθετούν την έξοδο από τον κυτταρικό κύκλο με κυτταροκίνηση. Το γεγονός αυτό πιθανόν υποδηλώνει την ύπαρξη ενός εναλλακτικού κυτταρικού κύκλου, ονόματι endoreplication, που έχει ως αποτέλεσμα τη δημιουργία πολυπλοειδών κυττάρων. Στο δεύτερο τμήμα της μελέτης και για τη περαιτέρω διερεύνηση του φαινομένου αυτού αναπτύχθηκαν ποιοτικές και ποσοτικές τεχνικές όπως η βελτίωση της χρώσης του πυρηνικού DNA και η σήμανση του σωματίου Barr. Παράλληλα, αναπτύχθηκε μια μέθοδος ανάλυσης δεδομένων κυτταρομετρίας ροής στα λιποκύτταρα, η οποία και επιτρέπει την ταυτόχρονη ανάλυση της υπερτροφίας (χαρακτηριστικό των πολυπλοειδών κυττάρων) με άλλους κυτταρικούς δείκτες. Οι τεχνικές αυτές θα μπορούσαν να εφαρμοστούν στο μέλλον για την μελέτη της πολυπλοειδίας και της υπερτροφίας των λιποκυττάρων.

Πέραν της πολυπλοειδίας, το γεγονός ότι τα ώριμα λιποκύτταρα δύνανται να επανεισέλθουν στον κυτταρικό κύκλο χωρίς να τον τελειώσουν μπορεί να έχει και άλλες προεκτάσεις, όπως την ανάπτυξη senescence. Η ανάπτυξης senescence στα ώριμα λιποκύτταρα θα μπορούσε θεωρητικά να δράσει σαν μια ενοποιός θεωρεία για τον φαινότυπο των παθολογικών λιποκυττάρων, που χαρακτηρίζονται από υπερτροφία, προ-φλεγμονώδη κατάσταση και αντίσταση στην ινσουλίνη. Στο τρίτο τμήμα της μελέτης διερευνήθηκε κατά πόσον τα ώριμα

λιποκύτταρα μπορούν να αναπτύσσουν senescence *in vivo* και σε κυτταροκαλλιέργειες, καθώς και ο ρόλος του κυτταρικού κύκλου στο φαινόμενο αυτό. Ορισμένα ώριμα ανθρώπινα λιποκύτταρα ήταν θετικά για την χρώση με senescence associated β-Galactosidase (SA-β-Gal). Παράλληλα, ο φαινότυπος επιβεβαιώθηκε με την χρήση δύο ακόμα δεικτών για το senescence, καθώς τα θετικά λιποκύτταρα μετρήθηκαν να έχουν μεγαλύτερο μέγεθος από τα αρνητικά, αλλά και συσχετίστηκε το ποσοστό τους με την απουσία του πυρηνικού HMGB-1. Στη συνέχεια πραγματοποιήθηκε αναδρομική ανάλυση δειγμάτων λιπώδους ιστού 51 ατόμων όπου αναδείχθηκε η συσχέτιση της παρουσίας των SA-β-Gal θετικών λιποκυττάρων με το μεταβολικό προφίλ των ασθενών. Όσον αφορά στα πειράματα in vitro βρέθηκε ότι η προσθήκη ινσουλίνης, που προηγουμένως είχε διευκολύνει την επανέναρξη του κύκλου, προωθεί και την ανάπτυξη του senescence. Το ίδιο αποτέλεσμα επιτευχθεί με τη χρήση παλμποσικλίμπης, που δρα αναστέλλοντας την πορεία του κυτταρικού κύκλου. Αντίθετα η χρήση μετφορμίνης, ενός αντιδιαβητικού φαρμάκου με πολλαπλές δράσεις, όπως αποτροπή της εισόδου στον κυτταρικό κύκλο και της αναστολής του μιτογόνου παράγοντα mTOR, μείωσε τα επίπεδα του senescence.

Στο τελευταίο κομμάτι της μελέτης, για την διερεύνηση της ετερογένειας μεταξύ των λιποκυττάρων, η οποία προάγει κάποια από τα κύτταρα στο senescence ενώ προστατεύει άλλα, μελετήθηκε η έκφραση του υποδοχέα ινσουλίνης. Αυτός επιλέχθηκε, καθώς η ινσουλίνη είχε καθοριστικό ρόλο στην σύνθεση DNA και την παρουσία senescence στα προηγούμενα πειράματα. Με χρήση κυτταρομετρίας ροής αναδείχθηκε ένας υποπληθυσμός λιποκυττάρων τα οποία ήταν αρνητικά για τον υποδοχέα, ενώ διερευνήθηκε η συσχέτιση αυτού του πληθυσμού με το μεταβολικό προφίλ 14 ασθενών, χωρίς όμως να βγουν συμπεράσματα για την ώρα.

Εν κατακλείδι, φάνηκε ότι τα ανθρώπινα ώριμα λιποκύτταρα μπορούν να εισέλθουν ξανά στον κυτταρικό κύκλο, γεγονός σημαντικό για την ανάπτυξη και του senescence. Παράλληλα, αναπτύχθηκαν νέοι μέθοδοι για την διερεύνηση της πολυπλοειδίας και της υπερτροφίας των λιποκυττάρων. Τέλος, ανακαλύφθηκε ένας πληθυσμός λιποκυττάρων που δεν έχουν υποδοχέα ινσουλίνης στην μεμβράνη τους και ελέγχθηκε η συσχέτισή τους με το μεταβολικό προφίλ παχύσαρκων ασθενών. Μελλοντικές μελέτες είναι απαραίτητες για να αναδείξουν πως οι νέες αυτές καταστάσεις επηρεάζουν τον λιπώδη ιστό και την υγεία.

Introduction

The mature white adipocytes and their role in metabolism: a brief overview

The mature white adipocytes are considered post-mitotic differentiated cells that mainly reside in the adipose tissue, as the dominant cell both in number and volume. Their distinct morphology characterized by one massive unilocular lipid droplet, which displaces the cytoplasm and nucleus, had made many researchers dismiss them as nothing more than nutrient storage. However, by the second half of the 20th century this attitude started shifting as more of the adipocytes' functions, other than temperature insulation and mechanical protection, were investigated. Nowadays, mature adipocytes and the white adipose tissue in general are recognized for their roles as endocrine organ and master regulator of energy and nutritional homeostasis. The last one became even more relevant with the tremendous surge of obesity and its related comorbidities. (1,2)

According to the world trends in BMI, it is estimated that around 18% of all men and 21% of all women worldwide will be classified as obese by the year 2025 (3). This trend has exposed the world population in an exceedingly high risk for the development of obesity-associated diseases, with type 2 diabetes (T2D) prevalence estimated at 422 million people worldwide in the year 2014 (4). The association of obesity to the development of T2D as a risk factor is generally accepted (5,6), with the majority of T2D patients being categorized as overweight or obese (7). However the presence of "metabolically healthy obese" (8) and "metabolically obese normal-weight" (9) individuals indicates that there is no strict cause and effect relationship. Rather, the relationship between obesity and T2D is more complex, where specific characteristics of adipose tissue, like distribution and morphology (10) correlate to the development of insulin resistance, which will eventually lead to metabolic syndrome and T2D. More specifically, the increased visceral adiposity that characterizes central body fat distribution is one of the strongest predictors for the development of insulin resistance (11), as is the presence of hypertrophic adipocytes in white adipose tissue (12–14). In general, adipose tissue can respond in two major ways to the caloric excess for its expansion, either with hyperplasia or with hypertrophy. A healthy adipose expansion is believed to occur with hyperplasia (15), as the mobilization of pre-adipocytes for differentiation increases the number of small and healthy adipocytes in the tissue. Contrary, in hypertrophy adipocytes respond with an increase in size. These hypertrophic cells are not only found to be insulin resistant and pro-inflammatory, but it is suggested that their inability to expand further leads to the development of lipotoxicity and subsequently the ectopic accumulation of lipids in other organs like liver, skeletal muscles and heart, thus directly contributing to the development of T2D (6,16).

Cell cycle

The phases of Cell Cycle

Cell cycle, as the procedure that leads to the faithful replication of the cell, is one of the fundamental cellular processes that life is based on. Specifically for most of the eukaryotic somatic cells, cell cycle consists of four tightly regulated and coordinated phases called G1 (Gap 1), S (Synthesis), G2 (Gap 2) and M (mitosis). The phases G1, S and G2 are called collectively interphase and are responsible for the faithful replication of the cells' genome and the increase in cell size and organelles number, while ensuring that the genetic information will be inherited intact to the daughter cells, thanks to the implementation of the G1/S and G2/M checkpoints. The actual DNA duplication happens in S phase as the name suggests. Finally, the last part of cell cycle is M phase and it consists of two parts, mitosis and cytokinesis. While mitosis involves all the steps that lead to the creation of two separate nuclei with the correct amount of genetic information in each, cytokinesis completes the cell cycle through the division of cytoplasm for the creation of the two daughter cells. (17,18) (Figure 1)

Apart from these 4 phases, all the cells that are not actively into cell cycle are considered to be in G0 or quiescent state. Cells enter into quiescence when they don't receive sufficient mitogenic signals, growth factors and nutrients or when they are fully differentiated. However it is possible for quiescent cells to reenter into cell cycle even after years of dormancy. This is one of the main differences with senescent cells that are permanently arrested from cell cycle entry (19).

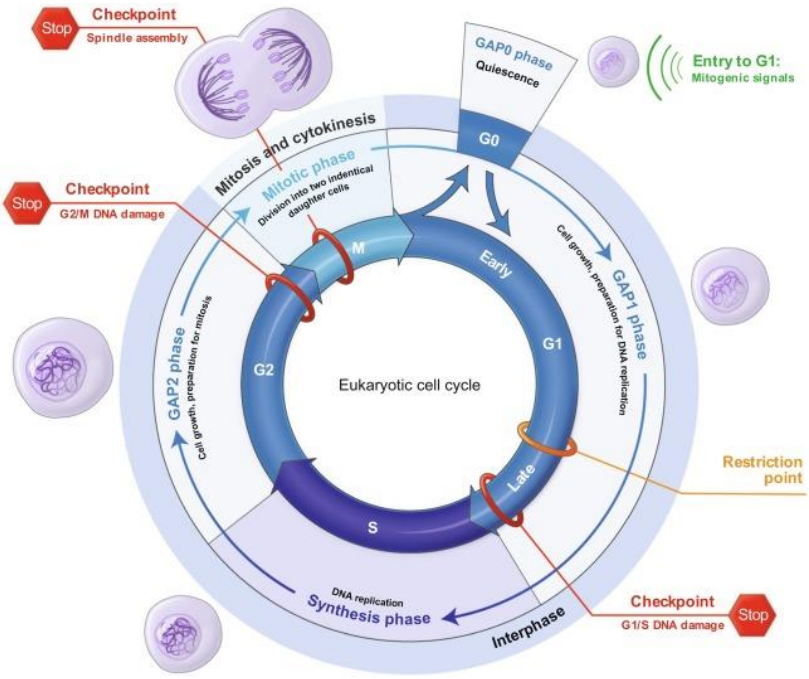


Figure 1:
A diagram of the cell cycle
Source: Ferry I, Kuzan-Fischer CM, Ernoult E, Rutka JT. Targeting Cell Cycle Proteins in Brain Cancer. In: Handbook of Brain Tumor Chemotherapy, Molecular Therapeutics, and Immunotherapy. Elsevier; 2018. p. 271–90.

Markers of cell cycle phases

A useful tool for the identification of cell cycle's entry and phases is the utilization of cell cycle markers that usually appear in the cells' nuclei. Below is a list of the cell cycle markers used in this study and their specific expression pattern in cell cycle according to the literature. (Figure 2)

- **Cyclin D1**: Important regulator of cell cycle entry in G1 early phase. Cyclin D1 levels are high during G1 and it is also present in G2, while it gets degraded during S phase. (20)
- **Cyclin E1**: It is expressed during late G1 and S phase. (21)
- **Cyclin A2**: The expression pattern spans from the very beginning of S phase until the end of G2. (22,23)
- **Cyclin B1**: It is located in the cytoplasm in minimal levels in G1 with steady increase in S and peak activation (x25 increase from G1) during G2 and M (24). The translocation from the cytoplasm to the nucleus signals the pass through G2/M checkpoint and the commitment to exit G2. (25)
- **PCNA**: The "proliferating cell nuclear antigen" protein aids DNA transcription via the facilitation of polymerase binding to DNA. Its nuclear expression is uniform during G1 and G2, in S it has a dot pattern and during M it is present almost everywhere in the nucleus except from parts of condensed chromatin. (26)
- **Ki67**: It's a nuclear marker of cell proliferation. It's levels start to increase in late G1, being a transcriptional target of E2F, and it peaks during G2 and M phase (27)
- **Anillin**: A scaffold protein of cytoskeleton that resides in the nucleus from late G1 until the end of G2, while it is later found in cell cortex, the contractile ring and midbody, sequentially during the many phases of mitosis and cytokinesis. (28)
- **pHH3**: The phosphorylation of histone H3 at Ser10, is a marker of DNA condensation. During G2 it appears in a dot pattern in the nucleus, while its main use is as a marker of mitosis, due to the distinct bright staining. (29)

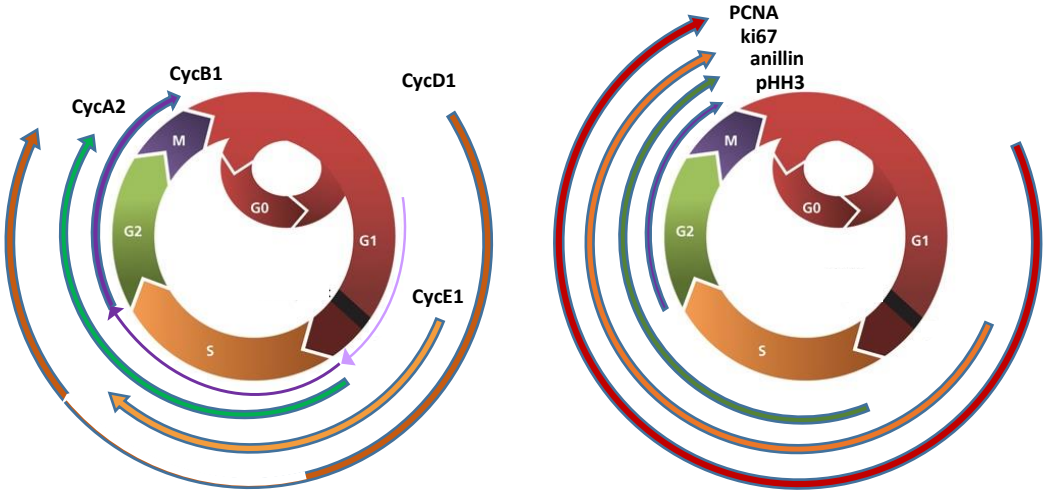


Figure 2: A diagram of the cell cycle markers used in this study and their specific time of expression

The cyclin-CDK complexes

The main players that orchestrate the smooth and regulated transition between the cell cycle phases consist of two groups of proteins the cyclins and the cyclin dependent kinases (CDK) (30). The cyclins, as their name suggests, have variable expression levels that peak only once per cell cycle and they act as co-activators and regulators in the heterodimer complex they create with the catalytically active and stably expressed CDKs. There are 4 main cyclin-CDK complexes classes, the G1 cyclin-CDK complex that regulate cell cycle entry and the G1/S, the S and the M cyclin-CDK complexes that dictate the cell cycle events.

The proteins involved in the G1 complex are the cyclins D (D1, D2, D3) (31), with D1 being the most relevant, and the cyclin dependent kinases CDK4 and CDK6. In an environment where the cell receives enough growth factors, the mitogenic signals of Ras/Raf (32–34) and MAPK/Mek/Erk (35) promote the transcription of Cyclin D1, while the Ras/PI3K/Akt pathway (36) protect the short-lived (37) cyclin from ubiquitination and premature degradation. Thus, the levels of cyclin D1 rise during the early G1 phase (and are kept in lower levels at G2 as well) (20) and the Cyclin D1-CDK4/6 complex puts in motion the first part of cell cycle commitment by phosphorylating the retinoblastoma (Rb) protein (38). In its hypophosphorilted state, Rb stays in a complex with the E2F transcriptional factor (39) and inhibits cell cycle progression by directly impeding E2F activation (40) and by activating histone deacetylaces, in order to silence E2F target genes (41). However, after Rb's phosphorylation that leads to its disassociation from E2F, the transcription factor is free to promote cell cycle progression into S phase (42), with one of its main targets being cyclin E (43).

Next comes the G1/S complex constituted of cyclin E (E1 and E2) and CDK2 (31). A crucial moment for independent cell cycle progression comes by the end of G1, when the cell is past the Restriction point (R point), after which the cell commits irreversibly to cell cycle, regardless of the presence of mitogenic signals (44). The R point coincides with the complete phosphorylation of Rb (45) and the inhibition of Cdh1, a substrate-recognition subunit of the anaphase-promoting complex (APC/C) (46), both targets of cyclin E-CDK2 complex. In addition to cell cycle entry, the G1/S complex has pleotropic actions on DNA synthesis and the preparation of S phase in general (47). However, cyclin E gets quickly degraded in the beginning of S phase, giving up its place for the rise of S complexes.

The S complex is created through the dimerization of Cyclin A2 and the CDK2 that coordinate the events of DNA synthesis (48) and the exit from S phase (49). The presence of Cyclin A2 is also important for the uninterrupted continuation through G2 phase (50), while it can be part of complexes with CDK1 as well (51). Finally the M complex proteins Cyclin B1 and B2 as well as CDK1 orchestrate the events of mitosis like centrosome separation (52), chromosomal condensation (53), nuclear envelope degradation (with the help of cyclin A2) (54,55), Golgi apparatus disassembly (56) and others. Interestingly enough cyclin B1 is located in the cytoplasm

during G2 phase and it is cyclin A2 that promotes its nuclear translocation in the beginning of M phase (55).

Cell Cycle Checkpoints

The end point of cell cycle, with the creation of two identical daughter cells, cannot be achieved without the meticulous and scrutinizing processes that take place in the cell cycle checkpoints. Their main goal is to ensure the integrity of genomic information with the G1/S, intra-S and G2/M checkpoints and the proper alignment of chromosomes in metaphase plate with the M checkpoint or spindle assembly checkpoint (SAC) (57).

Integral parts to the checkpoint control are the CDK inhibitors (CKIs), which include the INK4 proteins and the Cip/Kip proteins family. The INK4 are direct inhibitors of CDK4 and CDK6, that are part of the G1/S complex and they include $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$. On the other hand the Cip/Kip work by binding to multiple cyclin-CDK complexes, thus being involved in different checkpoints, with the $p21^{Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$ being the best recognized proteins of the family. (58)

The G1/S checkpoint at the end of G1 phase coincides with the restriction point and it ensures that the cell has a favorable environment to continue the cell cycle. In G1/S checkpoint both INK4 and some of the Cip/Kip are employed while in the presence of DNA damage it is the ATM and ATR kinases that activate CHK1, CHK2 and tumor suppressor p53 that arrest the cell cycle. The intra-S checkpoint cannot stop the cell cycle, but it delays passing through S-phase when the cell experiences genotoxic stress. The G2/M checkpoint regulates the entry into mitosis and it ensures that the cells' DNA has not sustained any damage or it is incompletely replicated. Finally, SAC acts as an "anaphase wait" signal that is there to ensure the correct segregation of the chromatids. (17)

Cell cycle in adipocytes

The topic of cell cycle in adipocytes has always been divisive in the research community. From early on there have been studies that have established the idea that mature adipocytes are post-mitotic, based on the absence of mitosis, as described from numerous histological preparations of the tissue (59,60). According to Simon "the moment when lipids appear, the cell loses the capacity to divide." (60).

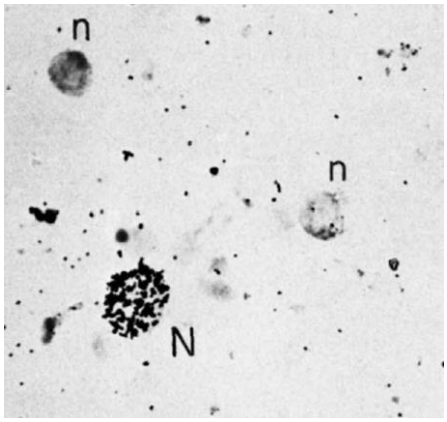
However, in the 60s and 70s there was a series of papers dedicated in the investigation of cellular and DNA turnover of cells in the adipose tissue utilizing ^{3}H thymidine incorporation and they painted a more complex picture of the issue. Hollenberg and Vost showed in their 1968 study

that after 12 hours of ^{3}H thymidine injection in rats they could detect 1% of the total radioactivity of DNA in the adipocytes' fraction of cells (61). Following a similar research approach Greenwood and Hirsch found ^{3}H thymidine incorporation in the adipocyte fraction as well (62). Even though both groups dismissed it as a possible contamination, a more thorough study was conducted in 1979 by Klyde and Hirsch in order to address these possible concerns (63). According to them, it was shown that ^{3}H thymidine can be incorporated *in vivo* and *in vitro* into nuclei of cells from the adipocyte fraction of the adipose tissue (Figure 3 a), however this time they took various precautions in order to ensure that this was not a false positive resulting from contamination and it is part of DNA synthesis. The same group has also shown that rats fed with high fat diet (HFD) for 7 days have a 14-fold increase in ^{3}H thymidine incorporation into the DNA of cells of the adipocyte fraction, when compared to chow fed rats. This effect was noticed after 15-21 hours of ^{3}H thymidine injection (64). The effect of ^{14}C Thymidine incorporation in cells from the adipocyte fraction of adipose tissue has even been reported to occur in 30 minutes after the injection in 5-days old rats (65).

More recently, in a study from 2011 Rigamonti et al made some similar observations. While focusing on adipose tissue turnover, via the investigation of BdU incorporation and ki67 expression, Rigamonti et al suggested that these markers are not present just in pre-adipocytes, but in mature adipocytes as well. According to their data, sections from human adipose tissue stained with ki67 (marker of proliferation), C/EBPa (marker of pre-adipocytes and adipocytes), perilipin (marker of adipocytes) and DAPI (DNA stain) included cells that stained positively for all four markers, thus indicating the presence of mature adipocytes being into the cell cycle (Figure 3 b). Moreover, in mice that were treated with BrdU, a nucleotide analogue that gets incorporated into DNA during replication, incorporation could be seen in single lipid-filed cells just mere 6 hours after the infusion (Figure 3 c). (66)

The above literature is mainly focused on *in vivo* and *in vitro* models of primary adipocytes. However there are publications with cell cycle in focus that are based on cell lines or specific culturing techniques that are out of the scope of this study. Briefly, differentiated 3T3-L1 cells have been recorded to have multiple nuclei and even divide, even though they have already accumulated lipids (67). Moreover, an adipocyte culture technique called "ceiling culture" allows the mature adipocytes to become multilocular or lose their lipid content and thus de-differentiate and retrieve their ability to proliferate (68–71).

a



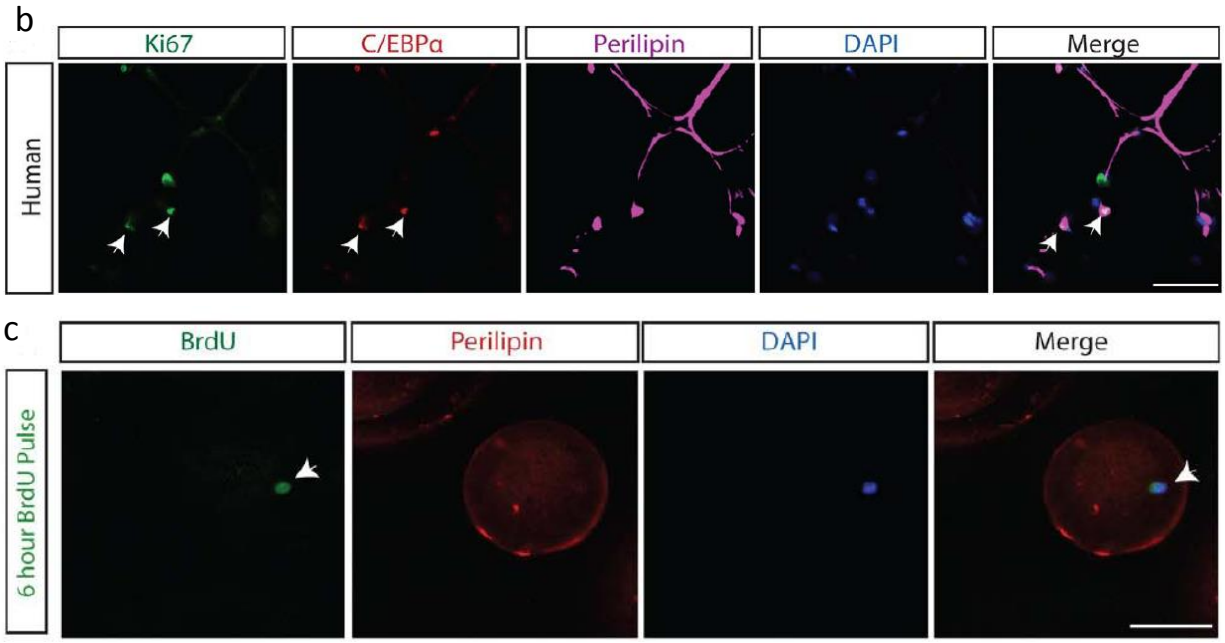


Figure 3:
(a) Isolated adipocyte nuclei from rat treated with 3H thymidine 12 hours before their sacrifice. In the radioautography the nucleus marked with "N" is positive for ^{3}H thymidine, indicating active DNA synthesis, while the "n" nuclei are negative.
Sourse: Klyde BJ, Hirsch J. Isotopic labeling of DNA in rat adipose tissue: evidence for proliferating cells associated with mature adipocytes. J Lipid Res. 1979;20(6):691–704.
(b) Immunofluorescence image of human adipose tissue section stained for Ki67 (green), C/EBPa (red), DAPI (blue) and perilipin (purple). White arrow indicates triple positive cell. Scale bars 50 mm.
Source: Rigamonti A, Brennand K, Lau F, Cowan CA. Rapid cellular turnover in adipose tissue. PLoS One. 2011;6(3).
(c) Immunocytochemistry image of isolated single lipid-filed cells from adipose tissue of mouse treated with BrdU pulse 6 hours before sacrifice. The cell is stained with BrdU (green), DAPI (blue) and perilipin (red). White arrow indicates triple positive cell. Scale bars 100 mm.
Source: Rigamonti A, Brennand K, Lau F, Cowan CA. Rapid cellular turnover in adipose tissue. PLoS One. 2011;6(3).

Endoreplication and Polyploidy

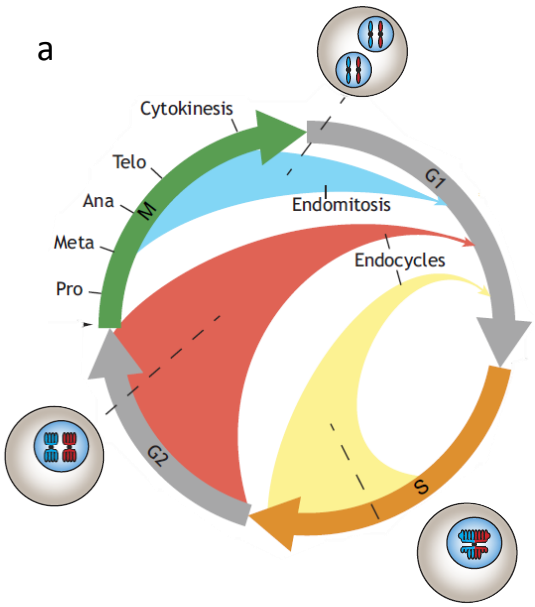
An alternative to cell cycle

Even though a cell cycle consisting of "G1-S-G2-M" sequence is the most well-known and prevalent type in nature, this does not negate the presence of alternative variations to the cell cycle default. One such example is the process of endoreplication, a cell cycle variation that leads to the increase of cell's DNA content, without following up with the subsequent cell division (72). Consequently, this phenomenon leads to the rise of polyploid cells, which hold more than the diploid amount of genome in their nucleus or multiple nuclei (73–75).

Under the general term "endoreplication" or "endoreduplication" many different subtypes are included, depending on the point of cell cycle that is being skipped (Figure 4 a). The classic endocycle is characterized by the complete absence of mitosis, where only alternating G and S phases occur; leading to the creation of mono-nucleus cells of increased ploidy (Figure 4 b). However, when there is initiation of mitosis that is abruptly abrogated, the phenomenon is called either "endomitosis" or "acytokinetic mitosis". The former term refers to a cell with one big lobular polyploid nucleus, while the later depends on the ability of the cell to achieve nuclear division and thus produce multiple nuclei respectively. Outside of cell cycle-dependent creation of polyploid cells, there is also a process that is cell cycle-independent, via cell fusion (75).

The presence of polyploid cells is best described in plants, where either the whole organism or specific tissues are known to consist of polyploid cells, in a process called autopolyploidy (happens in germline cells and characterizes the whole resulting organism) and endopolyploidy (happens in somatic cells) respectively (75). However the presence of polyploid cells is not exclusive to the plant kingdom, as polyploid cells' importance in development, function and stress response of tissues is recognized in animals as well, from invertebrates to human. Some of the best characterized human cells that are polyploid include the hepatocytes (76,77), megakaryocytes (78,79), placenta trophoblast giant cells (80), endometrial stroma cells (81), cardiac myocytes (82,83), vascular smooth muscle cells (84,85), uterine smooth muscle cells (86), keratinocytes (87), retinal pigment epithelium cells (88) and mammary gland cells (89) among others. The sheer magnitude of tissues incorporating these cells indicates that polyploidy is not the exception in humans, but rather an overlooked phenomenon.

a



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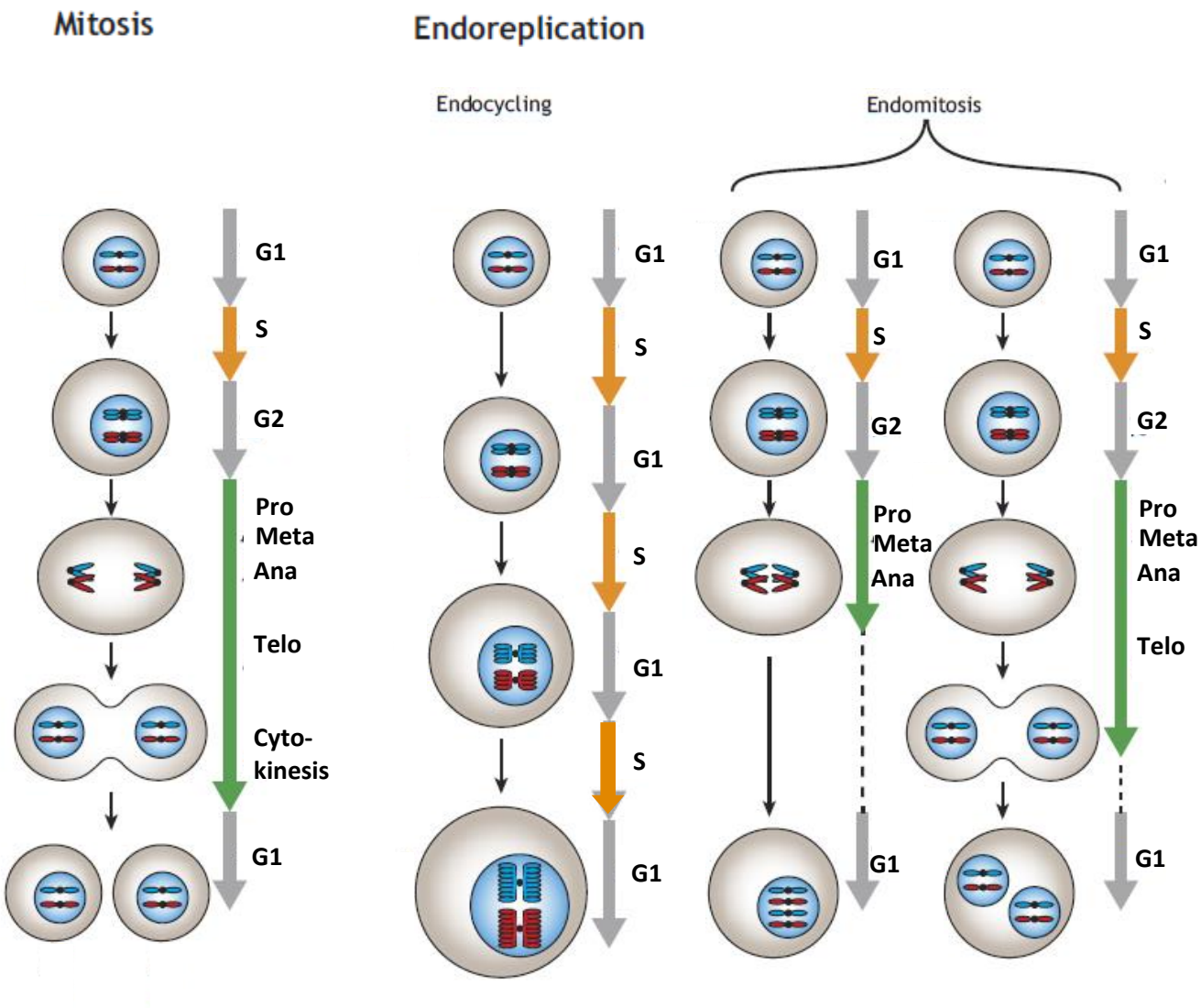


Figure 4:

(a) Diagram of the different phases of cell cycle that can be truncated during endoreplication. In cases where endoreplication starts before the end of S phase (yellow arrow), like Drosophila's saliva gland cells, then the resulting cell is polytene, with multiple DNA strands stuck to one another and areas of undereplication. The red arrow represents endocycle where G2 continues directly to G1, leading to the creation of mononucleated polyploid cells, while the blue arrow shows an endomitosis cycle leading to multinuclear cells.

(b) Diagram of the different variations of endoreplication. From left to right there is the normal mitosis, the endocycling, the endomitosis and the acytokinetic mitosis.

Source: Øvrebø JI, Edgar BA. Polyploidy in tissue homeostasis and regeneration. Dev. 2018;145(14).

Biological significance of endoreplication

The endoreplication program exerts a multitude of benefits for the different tissues, mainly through polyploid cells' ability to increase in size (90,91). Polyploidization is, thus, essential for the support of cellular hypertrophy, a mechanism crucial for the expansion of tissues constituted of post-mitotic cells. Early on, it had been proposed that there is a minimum ratio of nuclear to cell size that is necessary for sustaining increased cellular volumes with enhanced RNA and protein production and although this hypothesis is not confirmed to its entirety, there are plenty of evidence to support that there is a positive correlation between cell volume and DNA content. This holds true across cells from different species (Figure 5 a) (92), different cells from the same species (Figure 5 b) (93) and even same tissue cells with different ploidy levels (Figure 5 c).

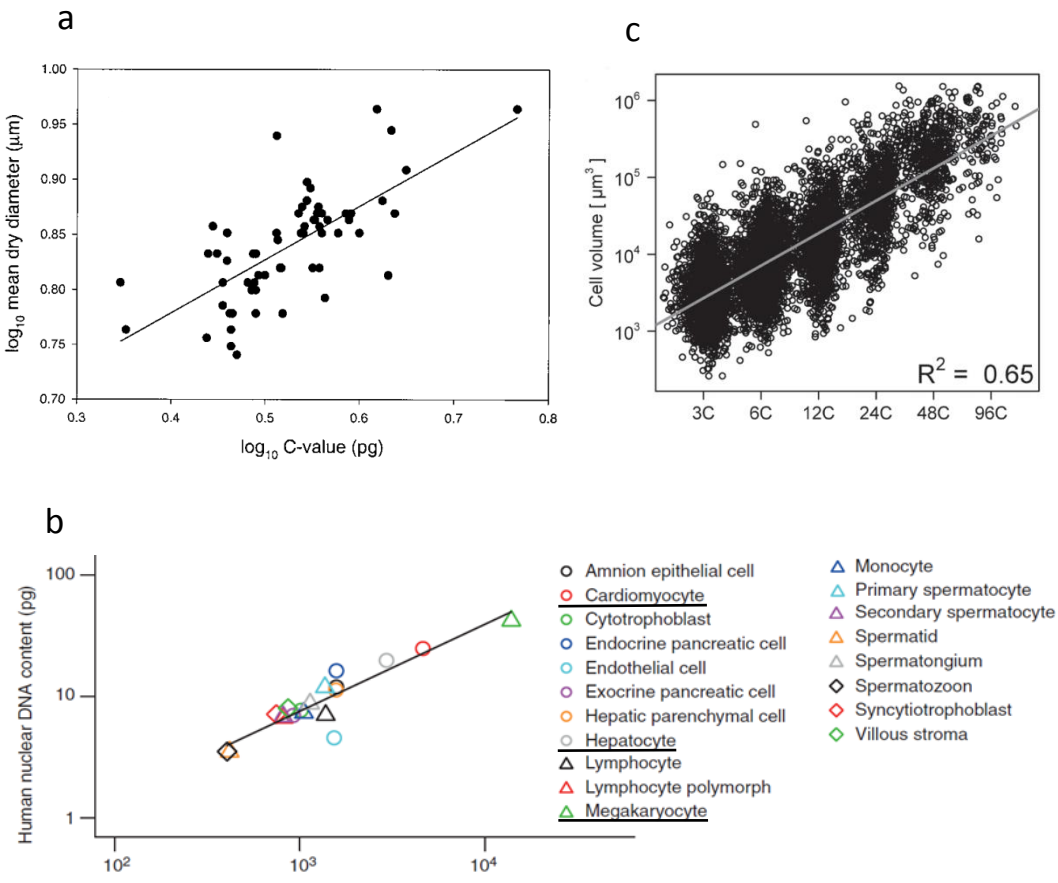


Figure 5:
(a) Diagram of the different mammalian erythrocyte diameter versus their DNA content; measured as C-value aka the total chromatin content of the haploid cell.
Source: Gregory TR. The Bigger the C-Value, the Larger the Cell: Genome Size and Red Blood Cell Size in Vertebrates. Blood Cells, Mol Dis. 2001 Sep;27(5):830–43.

(b) Plot of mean cell volume versus mean nuclear DNA content of 19 different human cells gathered from a meta-analysis. Cells that are recognized as polyploid in literature, like megakaryocytes, cardio myocytes and hepatocytes are depicted with greater DNA content and cell size when compared to diploid cells, while spermatids and spermatozoa are smaller, as they are haploid cells.
Source: Gillooly JF, Hein A, Damiani R. Nuclear DNA content varies with cell size across human cell types. Cold Spring Harb Perspect Biol. 2015;7(7):1–27.
(c) Plot of cell size versus different ploidy level of endosperm of Sorghum bicolor. The greater the ploidy the bigger the cell size.
Source: Kladnik, Ales. (2015). Relationship of nuclear genome size, cell volume and nuclei volume in endosperm of Sorghum bicolor. Acta Biologica Slovenica. 58. 3-11.

Where cells undergo hypertrophy without mitosis, during endoreplication, there is no actual need for cytoskeleton rearrangement and detachment from neighboring cells. Thus, polyploidy can be advantageous in retaining the tissue architecture, that would have otherwise been compromised with mitosis. This function is characteristic for tissues that partake in barrier functions like trophoblast giant cells of the placenta (94) and keratinocytes of the skin (91). On the same note, cardiomyocytes allow heart development after adolescence (95) and respond to loss of tissue from cardiac injury through endocycling (83), as mitosis could potentially disturb the sarcomere's actin-myosin structure and hearts contractility (96).

Additionally, the presence of polyploidy can be vital for the normal development and proper function of cells. This is the case for both megakaryocytes and mammary gland epithelium. For the former, it has been shown that the level of megakaryocyte polyploidy can have a direct effect on the number of created platelets (97). For the later, it was recently showcased that the presence of binucleated cells or rather the DNA replication are indispensable in the lactation process (89,98).

The role of polyploidy is central in wound healing biology as well. The surgical partial hepatectomy is known for decades to be reversible thanks to liver regeneration. And even though the details on how the liver can sense the reduction of its size are unclear, it is well established that the process of regeneration is achieved through endoreplication either totally or partially, depending on the extend of hepatectomy (99). It is even estimated that more than 40% of human hepatocytes are polyploid in the adult human and this number only increases with age (100,101), indicating that endoreplication has a significant role in liver development in general; even though polyploidization itself is dispensable in the tissue (102,103). Hepatocytes are quite plastic when it comes to endoreplication variations, as both mononucleated and polynucleated cells are found, while these cells have the additional ability to continue mitosis later on, which can lead to aneuploidy and genetic variation (104,105). It is also interesting to note that the driver for hepatocyte polyploidy is the activation of the insulin/PI3K/Akt pathway (101,106).

Other important advantages of polyploidy include the increase of cell's metabolic activity (91), even though there are limitations to it (107). Additionally, the presence of polyploid cells is hypothesized to give an adaptation advantage to cells that are endowed with genetic variation

in their polyploid genome (108,109). Lastly, one the more interesting benefits is DNA damage resistance and lengthened cell lifespan. Since polyploid cells have by definition multiple DNA copies, there is a lower chance for them to be susceptible to the ominous effects of DNA damage; thus they can continue tissue expansion despite the possible mutations. Endoreplication as an alternative to cell cycle can also lower the chances for the creation of cancer-prone daughter cells. The hypothesis is being suggested for keratinocytes and hepatocytes that are subjected to carcinogenic UV radiation and toxins on a daily basis. Polyploidy in these cases leads to both a more stable differentiated cell state and lengthens cells' lifespan (72), through apoptosis repression (110).

Unfortunately, there is no reference in the literature about *in vivo* polyploidy in mammalian mature adipocytes. However this is not the case for differentiated 3T3-L1 cells. In a paper by Xu and colleagues they present differentiated 3T3-L1 adipocytes with multiple nuclei (67) (Figure 6). Moreover, while polyploidy has not been proven for mammalian adipocytes, this is not the case for all animals. *Drosophila*'s fat body, that is analogous in function to adipose tissue and liver, is known to consist of adipocyte polyploid cells (111–113).

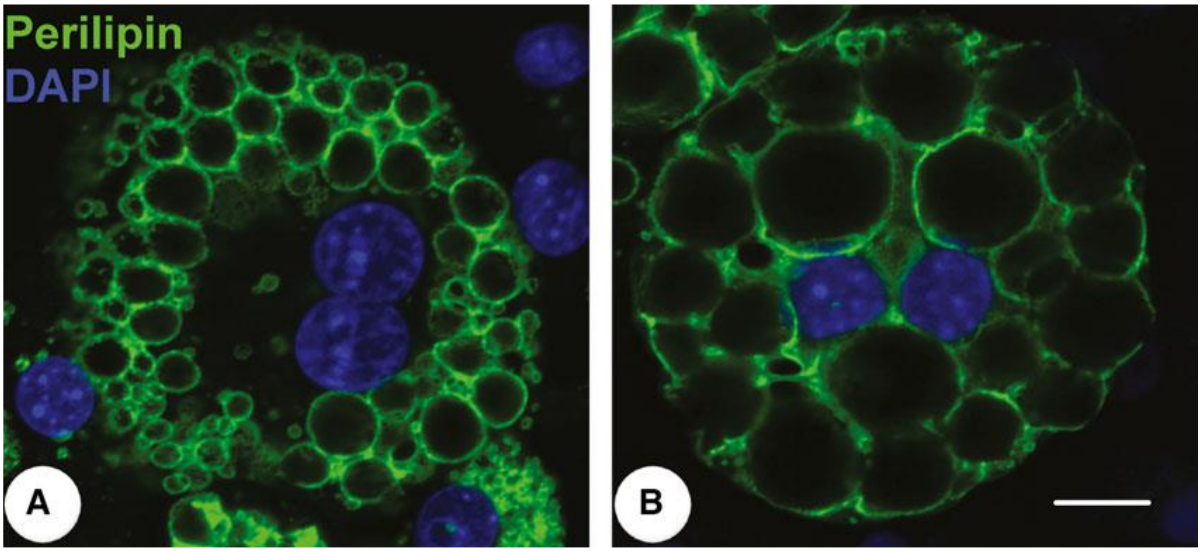


Figure 6: Induced differentiated binucleated 3T3-L1 cells. The cells are stained with blue for Dapi and green for perilipin (a protein surounding lipid droplets in the cell). Cells at the (A) 10th and the (B) 20th day of differentiation.

Source: Xu et al. Mature adipocytes observed to undergo reproliferation and polyploidy FEBS Open Bio. 2017;7(5):652–8.

DNA condensation

Around 90 years ago, Emil Heitz was the first to recognize that DNA is organized into highly condensed heterochromatin and less condensed euchromatin, according to its stainability by DNA dyes (114). From that point on scientists have tried to unravel the mechanisms behind DNA condensation that allows the DNA macromolecules to fit into a space with a diameter of less than 10 μm. It is now recognized that DNA is organized into the euchromatin that represents the areas of high accessibility where the majority of active genes reside, and the heterochromatin that is condensed and essentially inaccessible to the transcription machinery. The later, though, is not static, but it has a rather dynamic role helping the cell regulate its gene expression and by extension many functions pertaining to differentiation (115,116), cell division (117), evasion of genetic instability and DNA repair (118). Heterochromatin is further categorized as constitutive and facultative. Found in centromeres, telomeres and transposons, constitutive heterochromatin is stable throughout cell cycle and similarly located in many cells of the same organism. On the other hand, facultative heterochromatin is found in specific genomic loci pertaining to cell's type and it is significant for cell differentiation and morphology (119,120).

In order to initiate, maintain and regulate DNA condensation, the cells employ a series of different mechanisms. The main proteins involved are DNA- and histone-modifying proteins, and ATP-dependent chromatin remodeling complexes (CRCs) (120). DNA modifications include cytosine methylation that leads to transcriptional silencing (121) and demethylation through TET proteins which has the opposite effect (122). Histone modifications are more complex than that of DNA, including more than 200 post-translational modifications of the core proteins of the nucleosome, ranging from methylation / demethylation, acetylation / deacetylation, phosphorylation / dephosphorylation, citrullination, SUMOylation, ADP ribosylation, deamination and crotonylation among others (123,124). The best characterized post translational modifications of heterochromatin include mono-, di- and tri- methylation of histone H3 lysine 9 (H3K9me) or lysine 27 (H3K27me) (119,125,126). Finally CRCs utilize ATP hydrolysis in order to reposition DNA, making it more available for transcription (127).

Another mechanism of DNA condensation involves the use of non-coding RNAs (ncRNA) (119,128). From small RNAi in yeast to long-non coding RNA that can even inactivate whole mammalian chromosomes like XIST, which is involved in X chromosome's silencing (129), RNA has been established as integral components of DNA condensation. These ncRNA are transcribed from heterochromatin itself and work as recruiters of silencing factors sometimes via binding to them directly.

Furthermore, a new idea that is recently getting momentum in the scientific community suggests that the DNA condensation in heterochromatin could be attributed to the phenomenon of phase separation (130,131). During phase separation, a previously soluble compound becomes demixed from its solvent leading to either liquid droplet-like formations or gel-like formations

that exist in different phases than the solvent. Characteristically, the protein HP1, which in combination with H3K9me3 marker of histone characterizes a major type of heterochromatin, was found able to form phase separation *in vitro* (125). This phenomenon is electrostatic in nature, meaning that it can be manipulated through changes in the pH and salt concentrations, as showcased by the suppression of phase separation when salt concentration was increased (131). Interestingly enough, the effects of salts and pH were already recognized in DNA condensation, before the idea of phase separation. In principle, neutralization of DNA's negatively charge can lead to its condensation, either with the aid of cations or acidic environment. On the one hand, salts and cations with different valences were found to do so, with higher valences (≥3) in general leading to more robust condensation, the monovalent Na+ inducing condensation in lower concentration than K+ (132) and different combinations of divalent and trivalent cations reversing the effect (133). On the other hand, thanks to this neutralization effects, lower pH values lead to DNA condensation while higher pH values lead to de-condensation in general (134,135).

Unpublished data from our lab suggest that the adipocytes' nuclei might be more condensed than nuclei originating from other cell types. This phenomenon could potentially have an effect on the accuracy of the measurement of DNA content in the adipocytes' nuclei. According to Hardie et. al., without proper percussions , extremely condensed nuclei, like the one in human sperm, can be found to have falsely lower integrated optical density (IOD) and consequently less measured DNA content than what they truly carry (Figure 7) (136).

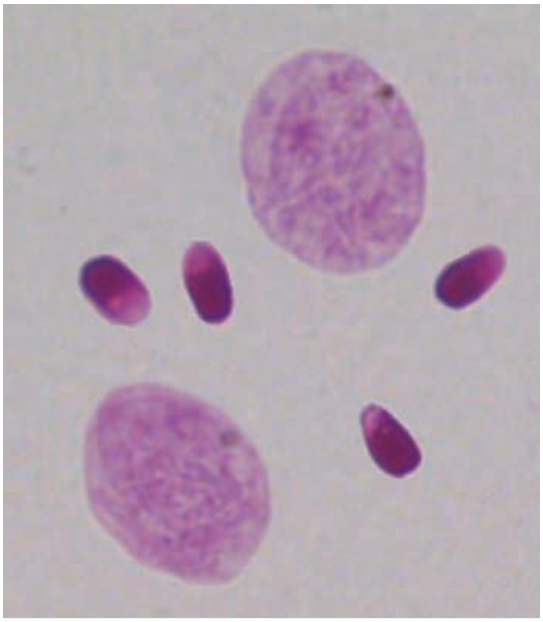


Figure 7:

The IOD measure-ment of human sperm (1C 3.5 pg, IOD500) versus buccal epithelium (2 C 7.0 pg, IOD 1370) shows a ratio of 1:2,7 instead of 1:2, thus sperm appears to have less DNA

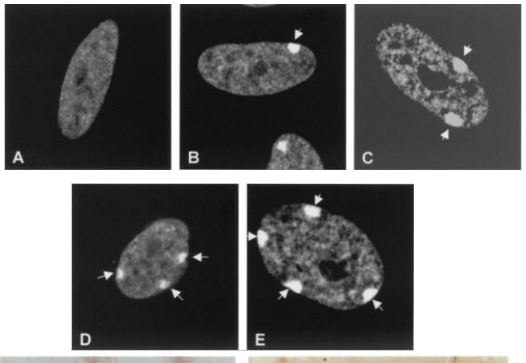
Source: Hardie DC, Gregory TR, Hebert PDN. From pixels to picograms: A beginners' guide to genome quantification by Feulgen image analysis densitometry. J Histochem Cytochem. 2002;50(6):735–49.

The Barr body

The Barr body or sex chromatin was first recognized by Barr and Bertram in 1949, as a distinct intranuclear structure of the interphase nuclei of placental mammals that was found mainly in nuclei of female origin, while it was completely absent in males (137). This distinction was not based on the subjects' sex, but rather on the presence of X chromosomes, as female Turner patients (45 XO) did not have nuclei with Barr body, while the opposite was true for male Klinefelter patients (47 XXY) (138,139). It was later discovered that this Barr body actually represented the condensed form of X chromosome that is normally silenced/inactivated in female cells during embryogenesis. This phenomenon is called "lyonization", and occures in cells with more than 1 X chromosome, that ought to stochastically silence all the excess X chromosomes, in order to do dosage compensation and achieve equal expression of X-linked genes between the two sexes (140).

While the main application for Barr body usually revolves around the determination of biological-sex between subjects, it is worth noting that it can be used in more creative ways as well. For example, the Barr body is bound to appear every time there are more than one X chromosomes, which holds true both for female origin diploid cells (46 XX) and for most polyploid cells, regardless of sex (141). While all the female polyploid cells appear with one enlarged or multiple Barr bodies, only half of the male confirmed polyploid cells have Barr bodies. However, as stated by Klinger and Schwarzacher «It remains clear that female nuclei with abnormally large or multiple sex chromatin bodies and male nuclei with large distinct heterochromatic bodies are always polyploid» (141). This means that the Barr body could at least be used as a crude indicator and proof of concept of cell's polyploidy in cases where it is otherwise unclear or modern techniques fail to show a definitive result. (Figure 8 a and b)

a



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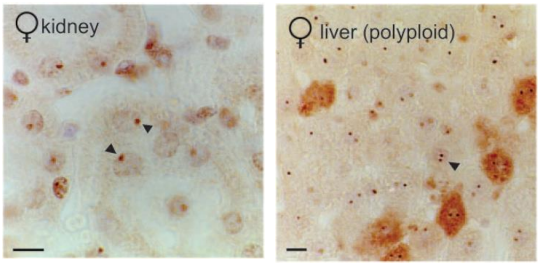


Figure 8: The Barr body
(a) A series of 5 confocal microscopy nuclei images showing the Barr body stained with specific autoimmune antibodies. From left to right there is a male nuclei (46,XY), a female nuclei (46,XX) nuclei from cell lines with (47,XXX), (48,XXXX) and (49,XXXXX). (142)
Source: Hong B, et al Identification of an autoimmune serum containing antibodies against the Barr body. Proc Natl Acad Sci U S A. 2001;98(15):8703–8.
(b) Immunohistochemistry of kidney (diploid) and liver (polyploid) tissue with ubi-H2A, a marker of Barr body (143). Source: Baarends et al. Silencing of Unpaired Chromatin and Histone H2A Ubiquitination in Mammalian Meiosis. Mol Cell Biol. 2005;25(3):1041–53.

Senescence

Senescence and its types

Senescence is a cellular program leading to permanent cell cycle arrest as a regulated response to stress (144–147). From this definition it is insinuated that senescence can practically occur only in proliferating cells that have an active cell cycle program. Moreover, there is a distinction between senescence and the other types of cell cycle arrest, namely quiescence and terminal differentiation. When it comes to the quiescent state the difference is clear, as quiescence is a reversible cell cycle arrest arising from mitogen deprivation (19). On the other hand, terminal cell differentiation is a developmental process leading to the permanent withdrawal from cell cycle. It is interesting, though, that there are some examples in literature suggesting the presence of senescence in differentiated post mitotic cells as well (148).

Aging is the best recognized condition linked to cellular senescence, with the two concepts often intertwined (149). However, there is also a great variation of stressors, pertinent or not to aging, which initiate the different senescence programs. The first senescence type to have ever been described was the replicative senescence that develops in cell cultures after multiple cell divisions (150) and it is attributed to telomere shortening (151). Another well recognized senescence category is stress-induced premature senescence (SIPS) (152) that includes DNA damage-induced senescence (153), oncogene-induced senescence (OIS) (154), oxidative stress-induced senescence (155), mitochondrial dysfunction-associated senescence (MiDAS) (156), epigenetically induced senescence (157) and many others. The pharmacological induction of senescence with the use of drugs like chemotherapy or cell cycle inhibitors (e.g. palbociclib) is called drug-induced senescence (chemical-induced SIPS) and it is currently utilized against cancer (158). Even the presence of senescent cells in a tissue can trigger the development of senescence in neighboring cells via paracrine senescence (159). Finally, physiological senescence partakes in embryogenesis, tissue homeostasis, wound healing and other physiological processes (160).

Hallmarks of senescence

The senescence program is heterogeneous not only in the ways it is activated but also in the expression of its phenotypes, depending on cell type, cause and stage of senescence. Thus, there is a plethora of senescence markers that are usually presented in senescence, even though not one senescence marker is unanimous across all these cells. (Figure 9)

The first distinction between normal and senescent cells comes from the cells' morphology. Senescent cells are typically larger and flatter, with irregularities in their shape and enlarged

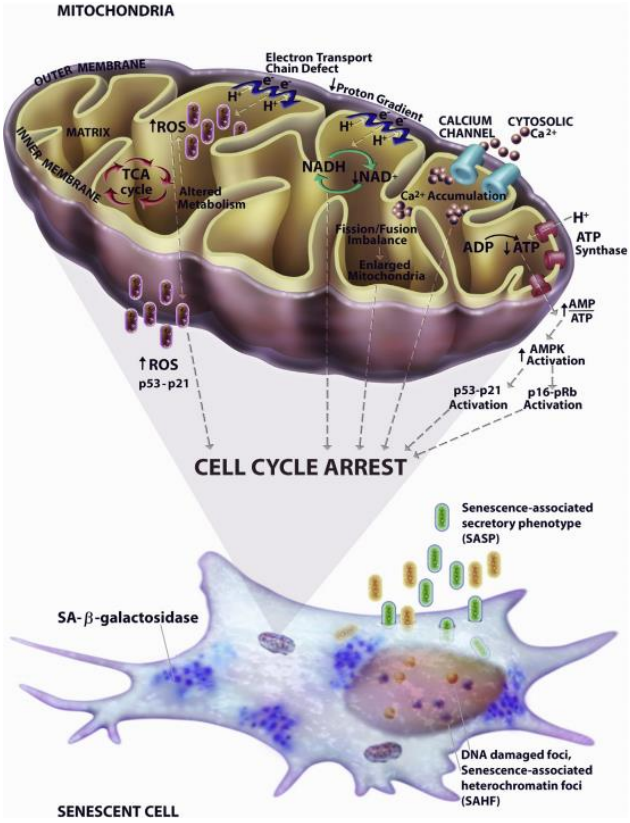
nuclei (161). The cells have also enlarged Golgi apparatus, modified ER and most importantly lysosomes that are greater in size and are highly active (147). One of the best characterized markers of senescence is the robust enzymatic activity of senescence associated β-galactosidase (SA- β-gal) at pH 6 that is being used in different assays in order to distinguish between senescent and non-senescent cells (162). Other lysosomal enzymes that have been reported to be active in senescence are α-l-fucosidase (α-Fuc), β-glucuronidase, acid phosphatase, β-hexosaminidase, α-mannosidase and N-acetyl-β-glucosaminidase (163). Apart from the enzymes, lipofuscin, a non-degradable, auto-fluorescent aggregate of lipids, proteins and other oxidized macromolecules has been described to accumulate in senescent cells' lysosomes (164).

Another important hallmark of senescence is cell cycle arrest. This is manifested through the overexpression of tumor suppression proteins that belong to the cell cycle inhibitors (CDKI) and especially $p16^{Ink4a}$ and p21 (145). These proteins are crucial components of the two main and intertwining pathways employed during senescence, the p16/pRB and the p53/p21, which will be briefly discussed later on (146). Moreover, even the absence of proliferation markers like ki67 and the lower BrdU incorporation have been proposed as potential biomarkers for senescence (147).

The transcriptional program of senescence, called senescence associated secretory phenotype or SASP is one of the most important, yet very heterogeneous (165), hallmarks of senescence (166). The SASP usually consist of secreted cytokines, chemokines, proteases and growth factors that can influence neighboring cells and extracellular matrix of senescent cells. The SASP response could vary from fairly minimal (156) to robust pro-inflammatory (167) or promoting tissue remodeling (168), based on cell type, cause of senescence (169) and activated molecular mechanism (170).

Some changes in the nucleus and chromatin compaction are also noticed in senescent cells. The loss of nuclear structural component LaminB1 has been linked to the development of specific chromatin structures termed senescence- associated heterochromatin foci (SAHF) (171). Thus, both LaminB1 and SAHF have been used as negative or positive markers of senescence respectively, even though they are not present unanimously among senescent cells (172). As the integrity of the nuclear membrane is getting compromised with LaminB1 loss, cytoplasmic chromatin fragments (CCFs) appear as well (173), which could be one of the reasons for the lower DNA content in deep-senescent cells (174). Finally, the HMGB1, a DNA chaperon that is normally found in the nucleus, gets secreted to the cytoplasm during senescence (175).

Other distinct characteristics of senescent cells is their resistance to apoptosis (176). It has been shown that senescent cells are overexpressing anti-apoptotic proteins from the BCL-2 family, like BCL-XL and BCL-W (177). Even senescent cells' metabolism is altered but still quite active, with increases in the ratio of AMP/ATP that lead to the activation of AMPK (178).



Then there are all the markers that are more relevant to the cause a senescence. For instance, in the cases where senescence was initiated by DNA damage response (DDR), there is an increase in the levels of p53, the phosphorylation of ataxia–telangiectasia mutated (ATM) and the presence of γH2AX histone nucleic foci (179). Whereas, in replicative senescence, cells' telomeres length has been cited as a potential biomarker (180).

Figure 9: Hallmarks of senescence
Source: Sultana Z et al. Is there a role for placental senescence in the genesis of obstetric complications and fetal growth restriction? Am J Obstet Gynecol. 2018 Feb;218(2):S762–73.

Pathways involved in senescence

The best characterized pathways involved in senescence are tied with the establishment of permanent cell cycle arrest. These include the p53/p21^{CIP1} and the p16^{INK4a}/phosphorylated Rb pathways that act parallel and complimentary with one another (146). (Figure 10)

In cases of DNA damage; one of the most common causes of cellular senescence; but also independently of DDR (181,182), there is an increase in the expression of p53 tumor suppressor protein. And while a transient expression favors DNA repair in a quiescent cell state, the prolonged presence of p53 leads to the gene expression of p21^{CIP1}, which aids the initiation and establishment of senescence. p21 is a CDK inhibitor having an effect on both CDK2 and CDK4 (183), thus promoting cell cycle arrest on a broader scale.

The other molecular axis depends on the transcriptional and epigenetic regulation of INK4/ARF locus. The p16^{INK4a} and p15^{INK4b} (human gene), transcribed from this locus, are CDK inhibitors directly acting on CDK4 and CDK6 (184), which inhibit the Rb dephosphorylation and thus they preserve the E2F inactivation (185). Parallel to this, ARF or human p14^{ARF} is a suppressor for the MDM2, which normally targets p53 for proteolytic degradation(186), hence promoting p53 stabilization and activation of its pathway.

However, there are some researchers who propose that permanent cell cycle arrest is not sufficient for the induction of senescence on its own. Instead, it is suggested that there is a second step needed for senescence; that is the continuous and inappropriate mitotic signaling on top of already cell cycle arrested cells. This step is otherwise called geroconversion and it describes the development of senescence on quiescent or cell cycle-arrested cells via the action of mTOR or other mitogens; thus explaining the hypertrophic and hyperactive profile of senescence (187,188).

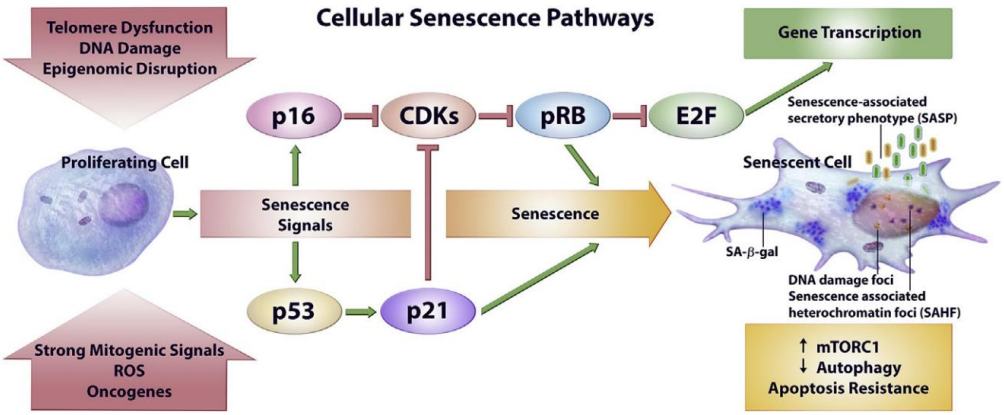


Figure 10: Schematic of cellular senescence's pathways
Source: Sultana Z et al. Is there a role for placental senescence in the genesis of obstetric complications and fetal growth restriction? Am J Obstet Gynecol. 2018 Feb;218(2):S762–73.

Senescence in the adipose tissue

There are many reasons why the adipose tissue poses as a compelling target for the development of senescence. The microenvironment of adipose tissue in obesity closely resembles the conditions that lead to senescence. Increases in ROS (189), mitochondrial dysfunction (190), short telomeres (191), DNA damage (192) and pro-inflammatory cytokines (193) are all involved in the pathophysiology of obesity and metabolic syndrome, while they are also crucial instigators of senescence. Thus it comes as no surprise that the senescent phenotype has been described in almost all cells of the tissue *in vivo* and *in vitro*.

Focusing on adipose tissue as a whole, aging of the tissue has been implicated in the worsening of carbohydrate metabolism and the development of insulin resistance in rats, independent of adipocyte diameter (194). Deterioration of insulin resistance has also been linked with the expression of p53 in adipose tissue of mice and human. p53, one of the best characterized players of senescence and DNA damage, was found increased in the adipose tissue of genetically diabetic mice (Ay mice) and diabetic patients, leading to the expression of senescence-like phenotype; while the adipocyte specific haplo-insufficiency of p53 ameliorated insulin resistance and

senescence phenotype (195). Furthermore, in the XP-V mouse model (xeroderma pigmentosum), where polymerase-η (pol-η) is knocked out, leading to increased DNA damage, the mice developed obesity and insulin resistance that was attributed to adipose tissue senescence. XP-V mice had increased levels of DNA damage in adipose tissue accompanied by augmented expression of senescence markers like p53, $p16^{INK4a}$, p21, SA-β-Gal, IL-6 and TNF-α; while p53 inhibitor was able to alleviate part of the symptoms (196). The other axis of senescence via $p16^{INK4a}$ has also been shown to play an important role in adipose tissue senescence. Pivotal in this line of research was the development of transgene INK-ATTAC mouse model, which allows for the clearance of $p16^{INK4a}$ overexpressing (senescent) cells via cell death induced after drug administration. In a progeroid background, mice treated for the elimination of senescent cells had a healthier adipose tissue and even late-onset treatment could attenuate age-related pathologies in adipose tissue (197). These results were recapitulated in naturally-aged mice after the elimination of $p16^{INK4a}$ overexpressing cells (198), while it was also shown that the clearance contributed to mice longevity in general (199). Finally, in a study that tracked senescent cells with the use of $p16^{INK4a}$-EGFP transgenic mice it was shown that senescent cells increase in the visceral adipose tissue during high fat diet, but the effect is reversible thanks to exercise (200).

The best described cell type of the adipose tissue that undergoes senescence is by far the pre-adipocyte, otherwise called stromal vascular fraction (SVF) cells or mesenchymal precursor cells. It has been shown by multiple studies that the development of senescence in these cells reduces their ability to differentiate into mature adipocytes, while they simultaneously promote senescence at neighboring cells. This reduction in adipogenesis can have detrimental effects in a person's metabolic profile by promoting adipocyte hypertrophy over hyperplasia and leading to insulin resistance (6). First and foremost, the differentiation capacity of pre-adipocytes is known to diminish with age progression both in animal models (201,202) and humans (203,204). This effect of aging on senescence can be recapitulated with the induction of replicative senescence in cultures of pre-adipocytes, presenting with multiple markers of senescence and reduced differentiation (205). The presence of senescent pre-adipocytes has been uncovered in human adipose tissue *in vivo,* as well. Consistent with all the previous results, pre-adipocytes expressed multiple markers of senescence and had reduced differentiation, while their presence correlated with patients' age, BMI, adipocyte cell size, risk of developing T2D and established T2D (206). The effect of these senescent pre-adipocytes is not confined in adipose tissue, but it can have pleotropic effects on the organism level, extending to physical strength and longevity. Mice that were inoculated with small numbers of senescent pre-adipocytes had greater burden of induced senescent cells both in adipose tissue and in muscle, a tissue that was not infiltrated by the xenographic pre-adipocytes; thus indicating the spread of senescence through SASP. The effect was even enhanced with aging and high fat diet, but was ameliorated with the use of senolytic drugs, which act by inducing apoptosis in senescent cells (207).

Even the endothelial cells (EC) of adipose tissue have been involved in obesity-related pathologies, via the development of senescence. *In vitro* experiments show that EC can express senescence markers and that the effect is more robust when they are cultured with omental

adipocytes' condition media than when treated with subcutaneous (208). The effect of senescence in EC has be shown to alter lipid handling and promote inflammation in the tissue, as well (209).

Finally, there are some models from the 3T3-L1 cell line or differentiated pre-adipocyte cultures that describe a possible mechanism of cellular senescence in mature adipocytes. Adipocytes derived from 3T3-L1 differentiated cells showed expression of SA-β-Gal, p53, $p16^{INK4a}$, pro-inflammation markers and reduced glucose uptake when exposed to oxidative stress (210). DNA damage in differentiated adipocytes leads to the activation of p53 pathway and promotes changes in cell metabolism pertinent to insulin resistance and inflammation (192). In a 3T3-L1 *in vitro* model that simulates the conditions of adipocyte aging, the differentiated cells were found to express both SA-β-Gal and other senescence markers in hypoxic and oxidative conditions as the time of culture progressed (211). Moreover, mice that were adipose-specific MDM2 knock out developed a phenotype of lipodystrophy, with the adipose tissue cells involved in either apoptosis or senescence due to vast expression of p53 in adipocytes. Adipocytes differentiated from SVF of this mouse model were expressing different senescence markers like p21, TNF-a, IL-6, and MCP-1 (212). Finally in the XP-V mouse model, that was discussed earlier, it was shown that mature adipocytes derived from pre-adipocyte differentiation cultures could express SA-β-Gal and other markers of senescence, after the accumulation of lipids (196).

The insulin receptor

The insulin receptor

The adipocyte metabolism as we know it is deeply connected to the effects of insulin on the cells. Insulin can message its anabolic signals by binding to its receptor, rightfully called insulin receptor (IR), while it is also possible to bind to the insulin like growth factor receptor 1 (IGF-IR) with a lower affinity. Besides insulin, IR can bind with a lower affinity to insulin like growth factor 1 (IGF1) and 2 (IGF2) as well. The insulin receptor protein consists of two subunits, the α that is extracellular and acts as a binding site and the β that is intracellular and has the tyrosine kinase activity for signal transduction. Moreover, IR has two different isoforms, depending on the splicing of exon 11, which encodes a 12 amino acid sequence located in the α subunits' C-terminus. The isoform IR-A, which lacks the sequence, is the main isoform expressed during embryogenesis and in cancer, exerting mitotic actions. On the contrary, IR-B is found mainly in post-natal tissues of predominant insulin targets, like liver, muscle, kidney and adipose tissue, where it transduces metabolic signals. In order for the insulin receptor to properly function, dimerization needs to occur either between IR-A or IR-B homodimers and heterodimers, or between one of IR isoforms and IGF-IR, with the last one called hybrid receptor (HRs). The

different combinations are binding with different affinity to their ligands, while changes in this combinations have been found in many pathological conditions including metabolic syndrome and diabetes. (213–215)

Insulin receptor in adipose tissue

One of the best ways to evaluate the action of INSR is by utilizing genetically engineered mouse models. Through the years many research groups have created their own mice where the efficiency of KO method and the phenotype was determined by the gene promoter used for Cre and the cell type specificity of the KO (215,216). It is really interesting to note that the phenotype of all these mice showed consistently reduction of adipose tissue and varying morphological changes in the tissue, but the metabolic profile of the mice had a wide variability. The first mouse model called FIRKO (217), was created with aP2-Cre and besides the low fat mass, the morphologic heterogeneity of the tissue and the adipocyte unresponsiveness to insulin stimulation, FIRKO was protected from obesity and generalized insulin resistance, it had paradoxically high leptin for its amount of adipose tissue and it was even found to display a 18% increase in longevity (218). In a combined IR and IGF1R KO mouse model (FIGIRKO) using again the aP2-Cre, the mice were once again protected from age and HFD induced obesity and insulin resistance, but were faced with challenges regarding their thermogenesis (219). The main problem with this model was that it was later revealed that aP2 is expressed in many off target tissues besides the adipose (216). Moreover, the metabolic health phenotype changed dramatically when GLUT4-Cre and Adiponectin-Cre mouse models were investigated. The GLUT4-Cre mouse model called GIRKO had adipose tissue heterogeneity in its morphology, comparable to that of FIRKO, but this time it developed severe diabetes that was attributed to the effect of IR KO on both skeletal muscle and adipose tissue (220). Finally, the Adiponectin-Cre mouse models were introduced and they are considered the most specific and efficient in knocking out the insulin receptor. The AIRKO mouse model developed severe lipodystrophy, with overt diabetes, dyslipidemia, extremely low levels of leptin, hyperphagia, β-cell hyperplasia and ectopic lipid accumulation (221,222) that led to non-alcoholic fatty liver disease (223). While the white adipose tissue (WAT) was reduced in AIRKO to less than 90%, the brown adipose tissue (BAT) was paradoxically increased more than 50%, with the phenomenon attributed to increased lipid accumulation, resembling WAT. However, treatment with leptin was enough to almost completely reverse the severe hyperglycemia in 2 weeks (221). A year later, a study on tamoxifen induced AIRKO mouse model was published, in order to differentiate the effects of IR between development and maintenance of adipose tissue. According to this research after 3 days of tamoxifen treatment the majority of adipocytes were no longer present in the adipose tissue in their normal form, a phenomenon attributed by the researchers to apoptosis, even though there was no apparent inflammation. This conditional AIRKO developed metabolic syndrome, resembling a milder version of AIRKO, which was reversed in 30 days due to adipose tissue

regeneration from newly differentiated pre-adipocytes. The adipose tissue reduction with tamoxifen could be repeated multiple times in the same mouse and interestingly enough leptin treatment once again prevented any metabolic disturbance other than adipose tissue reduction (224). It is still worth noting that the reduction of IR in the adipose tissue, even in the cases of lipodystrophy, didn't have a strong metabolic effect when the leptin levels are normal. Moreover, it seems that even in extreme lipodystrophy there were some adipocytes that survived the phenotype, although we cannot be certain that they indeed participated in cre recombination. (Figure 11)

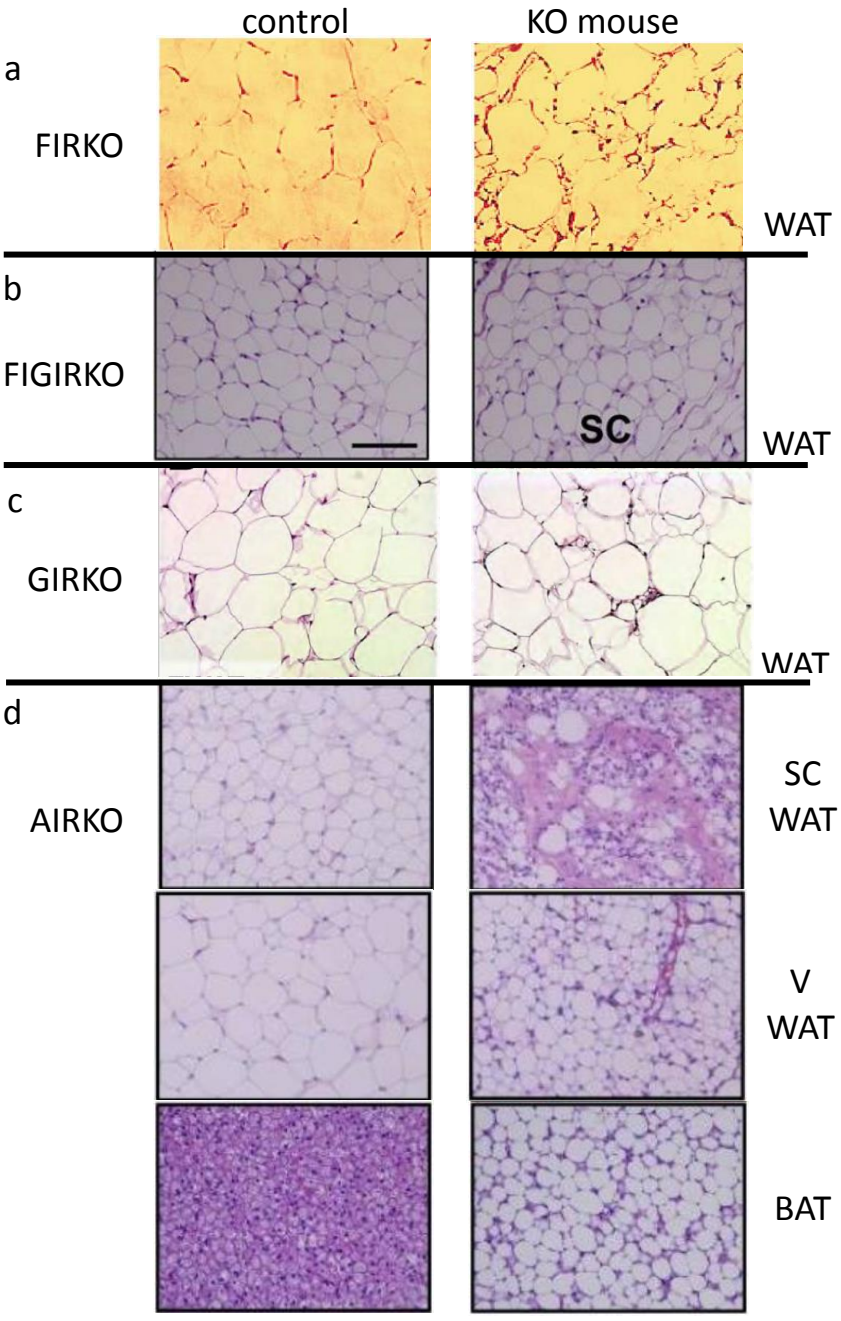


Figure 11:
Adipose tissue morphology of control mice and the different mice models.
(a) FIRKO Source: Blüher M et al. Adipose tissue selective insulin receptor knockout protects against obesity and obesity-related glucose intolerance. Dev Cell. 2002;3(1):25–38.
(b) FIGIRKO Source: Boucher Jet al. Impaired thermogenesis and adipose tissue development in mice with fat-specific disruption of insulin and IGF-1 signalling. Nat Commun. 2012 Jan 12;3(1):902
(c) GIRKO Source: Lin H V et al. Diabetes in Mice With Selective Impairment of Insulin Action in Glut4-Expressing Tissues. Diabetes. 2011 Mar;60(3):700–9.
(d) AIRKO Source: Boucher J et al. Differential Roles of Insulin and IGF-1 Receptors in Adipose Tissue Development and Function. Diabetes. 2016 Aug;65(8):2201–13.
WAT (white adipose tissue), SC (subcutaneous), OM (omental), V (visceral), BAT (brown adipose tissue)

While the animal studies can be very informative about the systemic effects of IR knock out in adipose tissue, there are always many variables that can distract us from the true effect. In a much simpler *in vitro* model, differentiated 3T3-L1 adipocytes had their IR acutely knocked down with tamoxifen (225). In this experiment, the elimination of IR had little effect in lipid accumulation the first days after the knockdown, however, after 14 days of culture from their differentiation, the adipocytes had a remarkable delipidation phenotype, while they continued to express adiponectin.

Finally, it is worth checking the phenotype of patients born with INSR gene mutations and how their adipose tissue presents. There are 3 main syndromes associated with this mutation: leprechaunism or Donohue syndrome, Rabson-Mendenhall syndrome, and Type A syndrome. Donohue syndrome is the most severe, leading to infant mortality by the age of 2. These patients present with embryonic growth retardation and infantile failure to thrive, severe insulin resistance with hyperinsulinemia and hyperglycemia, facial malformations, hyperandrogenemia (presenting as hirsutism and polycystic ovaries in females), organomegaly (heart, kidneys, liver etc) and acanthosis nigricans. These patients are also born with nearly absent subcutaneous adipose tissue. The Rabson-Mendenhall syndrome shares many similarities in the phenotype with Donohue syndrome, but it is less sever, thus the patients can survive into their 20s'. Finally, the Type A insulin resistance syndrome patients get diagnosed as teens, when they have developed overt diabetes. The insulin resistance and diabetes developed in these patients requires massive amounts of insulin units per day for its treatment. In contrast to all the other syndromes that present with severe insulin resistance, however, these 3 are usually exempt from the obesity phenotype and the patients of the most severe syndromes even have scarce fat tissue (226,227), even though INSR mutations are not regarded as causes of lipodystrophy (228). Moreover, it was found that these patients have paradoxically high adiponectin despite their severe insulin resistance (229). While it is impossible to recognize which of those patients' tissues contributed the most to the development of diabetes, adipose tissue was found affected in a similar way to the knock out models. This comes to show that insulin signaling is important for the development of the tissue, which is otherwise less developed and resistant to expansion.

Biological significance and Objectives

The aim of this study is to investigate the presence of cell cycle in human mature adipocytes and the different biological phenomena that arise as a consequence. Even though mature adipocytes have been portrayed as post mitotic in the vast majority of the literature, this observation is challenged in multiple occasions. The problem with the previous studies was that they were scrutinized over the lack of strong imaging techniques and the shortage for multiple cell cycle markers. In this study, however, the focus was shifted on accomplishing a comprehensive analysis of multiple cell cycle markers in human primary adipocytes in *in vivo* conditions, while employing multiple imaging techniques. Moreover, with the use of suspension cultures we tried to recreate the physiological aspect leading to the expression of cell cycle markers and visualize the entry into cell cycle and the DNA replication that took place *in vitro*.

Another possibility that can be investigated is the presence of polyploidy in human primary adipocytes. If this is established, it could act as a supporting evidence of polyploidization being a vital component in adipocytes' hypertrophy. In order to do so, this study lays the foundation for future experiments that will investigate polyploidization and hypertrophy. Better staining technics were developed so in the future one could achieve an accurate DNA measurement of adipocytes DNA content. Barr body staining was used as an alternative to FISH, in order to recognize the chromosome count carried by cells. Lastly, a new FCM analysis strategy is proposed for white adipocyte FACS that has the potential to distinguish between hypertrophic cells, which could be sorted and analyzed further in the future.

The development of senescence in the adipose tissue has been implicated in the pathology of insulin resistance and obesity-related diseases, with the systemic implications of this phenomenon reaching as far as compromising the organism's longevity. However, while both adipose tissue as a whole and many different cell types of the adipose tissue specifically have been shown to develop senescence, this is not the case for human primary adipocytes, the most prominent and metabolically relevant of all cell types of the tissue. The main obstacle for such a claim was the post-mitotic status of mature adipocytes that is in direct contrast to the very essence of senescence definition. However, since there are data to indicate that mature adipocytes can in fact enter into cell cycle, that could be aberrant in nature, it is vital to investigate further this possibility. Thus, human primary adipocytes were isolated for the investigation of senescence and the possible factors that contribute to its development *in vivo*. Parallel to these, *in vitro* experiments were conducted using the suspension culture, so as to manipulate the cell cycle and explore the mechanisms involved in the development of senescence.

The insulin receptor has a significant effect in the development of adipose tissue and its regulation has been heavily implicated in the pathology of metabolic syndrome. Moreover, throughout this study the effects of insulin have been found central in the advancement of other

biological processes in mature adipocytes. Thus it was important to develop a method for the quick and accurate distinction of human primary adipocytes expressing varying amounts of the IR. With the use of flow cytometry, this study begins the investigation into the presence of an IR negative adipocyte population and checks its presence in different fat depots and its distribution among obese patients of different metabolic statuses.

Materials and methods

Human subjects

All the patient samples were obtained from patients undergoing bariatric, gallbladder or hernia surgery at Ersta Hospital in Stockholm, Sweden. The samples used were obtained from surgical biopsies of subcutaneous adipose tissue and only when it is specifically indicated the adipocytes origin is that of omental adipose tissue. For the different adipocyte cultures 51 patients were included (BMI 36.8 ± 6 kg/m^2, 16 male and 35 female, age 46.1 ± 15 years), but due to scarcity of adipocytes, it was impossible to try all culture conditions using cells from the same patient. The table below describes the 51 patients, whose adipocytes were also used for the untreated adipocytes SA-β-Gal staining:

Table 1

	Non Obese (7)	Obese Healthy (11)	Obese Unhealthy (27)	Type 2 Diabetes (6)	NO vs OH	NO vs OU	NO vs T2D	OH vs OU	OH vs T2D	OU vs T2D
	Mean (SD)				p value					
age	69.57 (9.36)	43.82 (11.33)	39.63 (10.62)	52.17 (15.83)	0.0183	0.0003	0.6481	>0.9999	>0.9999	0.3787
BMI	24.21 (2.45)	37.20 (2.75)	39.31 (3.81)	39.58 (2.74)	0.0457	<0.0001	0.0032	0.7415	>0.9999	>0.9999
C-peptide	0.59 (0.14)	0.729 (0.16)	1.174 (0.32)	1.09 (0.38)	>0.9999	0.0006	0.0926	0.0052	0.402	>0.9999
Insulin	6.30 (1.86)	7.90 (4.34)	17.66 (8.07)	15.76 (9.33)	>0.9999	0.0016	0.1887	0.0037	0.4345	>0.9999

Table 1:
Patient categorization into “non obese” (BMI<30), “obese healthy” (BMI>30, c-peptide <1 nmol/L and / or insulin <20mU/L), “obese unhealthy” (BMI>30, c-peptide >1 nmol/L and/ or insulin >20mU/L) and diagnosed diabetics. The number of subjects for each group is given in the title in brackets, while the mean and standard deviation are provided.
In order to analyses the groups, first normality was investigated using D'Agostino & Pearson test. Since some of the groups were smaller than what was needed to show significance in normal distribution, non-parametric Kruskal-Wallis test were used (instead of one way ANOVA) and the post-hoc analysis was done with Dunn's multiple comparisons test, that gave the p-value. For p-value ≤ 0.05 statistical significance between the groups was recognized (white box), while if the p-value was higher, then no statistical significance was recognized between groups (grey box).

For the flow cytometry experiments 15 patients were included (BMI 36.3 ± 4.5 kg/m^2, 3 males and 12 females, age 41.2 ± 10.3 years).

All patients were fasted before surgery and blood was drawn for the analysis of serum glucose, triglycerides, cholesterol, HDL, LDL, Apo A1, Apo B, HdA1c, C-peptide, insulin, IGF-1, leptin and

adiponectin. Moreover, somatometric characteristics and percentage of body fat were noted as well. All the patients provided written informed consent prior to their surgery.

Isolation of human mature adipocytes

Intact human adipose tissue was collected during surgeries and it was the main source of human mature primary adipocytes used in our lab experiments. The tissue was firstly treated mechanically with scissors' mincing and then digested enzymatically with 25ml of warm "KRB buffer" (127 mM NaCl, 12.3 mM $NaPO_4$, 1.36 mM $CaCl_2$, 5.07 mM KCl, 1.27 $MgSO_4$, pH 7.4) plus 400µl of "Digestion mix" (0.05% collagenase I from *Clostridium histolyticum* (#C0130, Sigma), 5 mM D-glucose, 50 µg/ml gentamicin and 4% albumin) for every 10-15 gr of fat. After an incubation for 30-120min at 37^{o}C in water bath the reaction came to an end with the addition of 500µl of 0,1M EDTA (Sigma) pH=8 to the mix. The suspension was then filtered through 250µm mesh and it was rinsed with pre-warmed "Isolation washing buffer" (1% BSA, 400nM Adenosine, 5mM D-glucose, 50µg/ml gentamicin in PBS) into a 50ml falcon. After a waiting period of 5-10 min all the adipocytes would flow to the top of the suspension, while the stroma vascular fraction (SVFs) and the rest of cells residing in the tissue would sediment in the liquid below. The liquid was then collected for different experiments and the adipocytes were washed with 50ml of washing buffer, repeating the procedure for at least 4-5 times, in order to receive adipocytes free from contaminations.

According to the experiment the adipocytes could be used either fresh, froze at -80^{o}C or they could be fixed. For fixation 300-500µl of packed adipocytes were incubated for 30 min in 1ml "Fixative solution" (2% PFA, 1% sucrose in PBS) and then washed twice with "Isolation washing buffer".

Cell culture of mature adipocytes in suspension

For the culture of mature adipocytes 500µl-1µl of cells were plated into 5ml of culture media in 6-well plates (Costar) and they were left to float freely in the well. In this culture method it was not required to change the media during the 4 or 7 days period of culture.

The culture media that was mainly used was Low glucose media (50% DMEM (gibco Thermo Fisher), 50% DMEM/F12+GlutaMAX-I (gibco Thermo Fisher) and 1% Pen Strep (gibco Thermo Fisher)). The culture conditions for the experiments, according to the abbreviations, were:

- Basal: Low glucose media, 10% FBS (gibco Thermo Fisher) that was not heat deactivated
- Basal + Ins: Low glucose media, 10% FBS, 100nM Insulin (Sigma)
- Basal + Ins+ Met: Low glucose media, 10% FBS, 100nM Insulin, 5mM metformin (Sigma)

- Basal + Ins + Palb: Low glucose media, 10% FBS, 100nM Insulin, 0.5μM palbociclib-isethionate (Sigma)

In order to measure EdU incorporation during the culture conditions of adipocytes, EdU ("Click-iT™ Plus EdU Cell Proliferation Kit for Imaging, Alexa Fluor™ 488 dye" (Thermo Fisher)) was supplemented in the culture medium in 50μM concentration.

Immunohistochemistry of adipose tissue paraffin sections

Immunohistochemistry (IHC) staining of adipose tissue was performed using the "Cell and Tissue Staining Kit HRP-DAB system" for rabbit and mouse (R&D System). Through IHC b-galactosidase, cyclin B1, ki67, pHH3, PCNA protein content and topology were validated. The paraffin sections were first de-parafinazed through heating for 60 min at 60^{o}C and then through incubation to xylene and consecutively decreasing concentrations of ethanol. Antigen retrieval was performed by microwaving slides within a Citrate buffer (10mM Citric acid, 0,05% Tween-20 in MQ water, pH 6.0) bath. When the temperature decreased, the slides were adjusted to a specific contraption that hold a coverslip tightly close to the slide in order to minimize the amount of reagents and antibody needed for the IHC. The slides were incubated consecutively with peroxidase blocking reagent, serum blocking reagent, avidin blocking reagent, biotin blocking reagent (provided by the kit) and primary antibody overnight. The next day the slides were incubated with the biotinylated secondary antibody, HSS-HRP and DAB+chromogen solution. For contrast the tissues were stained with hematoxylin (HistoLab Mayers HTX) and finally they were dehydrated and mounted with Eukitt® Quick-hardening mounting medium (Sigma-Aldrich).

For positive control, lymph node sections were used for every different staining. For negative control sections were stained without first antibody for the anti-mouse and anti-rabbit biotinylated antibodies. Perilipin staining was also used to show that tissues from different patients are stained equally and there is no internal bias between patients.

The quantification was done using ZEISS Axio Lab.A1 light microscope.

Immunocytochemistry of isolated adipocytes

Immunocytochemistry (ICC) was performed in fixed mature adipocyte samples either for quantification of protein expression (Cyclin D1, HMGB1), or for the evaluation of protein expression in general (Anillin, cyclin A2, Cyclin D1, cyclin E1, HMGB1, ki67, CDKN2A / p16INK4a, p21, PCNA, Histone H3 (Ser10) Phospho).

Due to the difficulty of staining adipocytes' nuclei with standard protocols, two other techniques were previously developed in the lab in order to facilitate the permeabilization step; one being trypsinazation and the other methanol incubation. Refining for the best technique was done individually for each of the proteins stained.

The fixed cells (200µl-800µl) were kept in 2ml round bottom tubes in suspension. After removing the liquid with a clean syringe that is equipped with a large diameter blunt needle, the cells were incubated with 0,5% Triton X-100 (Sigma) in PBS for 30 minutes, on the shaker. Next came the permeabilization step that was either 10 minutes of incubation in Trypsin solution (1:10000 trypsin (25µg/ml) in PBS) at +37oC followed by the addition of trypsin inhibitor or it was 7-10 minutes' incubation in 100% ice cold methanol at -20^{o}C followed by a 30 seconds spin at 100G, removal of methanol and three washes with PBS. Subsequently the cells were blocked for 30 - 120 minutes in ICC blocking buffer (2% fatty acid free BSA (SIGMA) in PBS (filtered)) and then they were left to incubate over night with the right primary antibody in ICC blocking buffer on the shaker. The next day the cells were washed three times with ICC washing buffer (0,1% BSA, 0,05% Tween (Sigma) in PBS) and incubated with the correct conjugated secondary antibody for 2 hours in the dark. Finally, after washing them again three times with ICC washing buffer, the cells were left to incubate with 1:500 Hoechst 33342 (Molecular Probes) and 1:500 green lectin (Fluorescein labeled Lens Culinaris Agglutinin (LCA) VECTOR) in PBS. The slides were prepared by suspending the cells in 80% glycerol (Sigma-Aldrich) in PBS solution and placing them on a glass slides (VWR) that were covered with coverslips (22x40mm 1,5) (Mrnzel-Gläser) and secured.

Images and quantification were done using ZEN software (Carl Zeiss, Germany) and images were taken with an inverted confocal Zeiss LSM 700 or LSM 880 confocal laser scanning. The laser power was set according to the highest possible settings that did not produced overexposed images and the settings were kept comparable for the quantifications. The quantifications were done by taking the photo of at least 100 nuclei for each sample.

Adipocyte Nuclei preparation

For the experiments that require isolated nuclei, frozen adipocytes or PBMCs were utilized.

The adipocytes were firstly thawed in lukewarm running water and they were transferred to 15ml falcons with 10ml "Adipocyte lysis buffer" (1% BSA, protease inhibitor (one tablet (cOmplete EDTA-free protease inhibitor cocktail tablets (Roche))), 5mM MgCl2, 0,6% NP40 (Igepal (Sigma)) in isotonic Hepes buffer or KHCO3 buffer) for a 10min incubation in the shaker. The cells were then dounced 25 times using pre-rinsed douncer and subsequently filtered through 100µm mesh to pre-rinsed 50ml falcons. After a 10 min centrifugation at 800G and 4^{o}C a pellet is formed. The supernatant is carefully removed and the nuclei in the pellet are re-suspended to 20µl of "Isotonic Hepes buffer" (10 mM Hepes, 5 mM NaCl, 140 mM KCl, 0,3 mM MgCl2 in MQ water) or

"KHCO3 buffer" (25 mM KHCO3, 2 mM NaCl, 0,3 mM MgCl2 in MQ water), according to the experimental conditions.

Regarding PBMCs nuclei isolation, the thawed cells are transferred to 15ml falcons with 10ml "PBMCs lysis buffer" (1% BSA, protease inhibitor (one tablet (cOmplete EDTA-free protease inhibitor cocktail tablets (Roche))), 5mM MgCl2, 0,1% NP40 (Igepal) in isotonic Hepes buffer) and after a quick vortex they are let on ice for 5 min. The nuclei pellet is collected after a centrifugation of 7 min, 400G, at 4^{o}C. The rest is done similarly to the adipocytes.

DNA de-condensation procedures

For the DNA de-condensation of adipocytes' nuclei a multitude of different methods were used in order to decide on the optimal conditions.

The isolated nuclei were prepared for either flow cytometry analysis or for confocal microscopy. For the flow cytometry analysis, the different conditions were tested on nuclei in suspension. For the confocal microscopy the nuclei were placed as drops on Silane-Prep slides (Sigma) and they were left to dry overnight, so the nuclei could be prepared and stained appropriately.

Different buffers used for the nuclei preparation and final suspension including "Isotonic Hepes buffer" or "KHCO3 buffer"

For nuclei fixation either by adding PFA fixation buffer or by adding ethanol and leaving the cells at -20oC overnight

The pH alkalization was achieved with the addition of NaOH on different concentrations, depending on the desired output.

The RNase treatment was prepared with the addition of 1:10 RNase (Invitrogen)

Barr body staining

The staining was based on the protocol provided by (230). Fixed isolated nuclei from a female patient attached to X slides were used. The slides were immerged to hematoxylin (HistoLab Mayers HTX) for 12 minutes and then they were submerged to Acid alcohol (1% HCl in 96% ethanol) for 90 seconds. After that the slides were rinsed with tap water for 5 minutes and mounted using 80% glycerol (Sigma). The slides were observed using a ZEISS Axio Lab.A1 light microscope and images were taken using Canon DS126311 EOS Rebel T3i Digital Camera at x40 magnification.

Flow cytometry of mature adipocytes

For each staining condition, 300-500μl of freshly isolated adipocytes were placed in pre-rinsed 2ml round bottom tubes that contained 1ml FCM blocking buffer (10% FBS in PBS). The cells were left to incubate for 30 minutes in the shaker and then they were kept on the bench standing overnight. The next day the adipocytes were incubated for 2 hours with the conjugated anti-insulin receptor antibody or the isotype control in the dark. The cells were then washed twice with PBS and they were placed in special flow cytometry tubes (Falcon® Round-Bottom Polypropylene Tubes, 5 mL) that contained 200μl PBS.

The flow cytometry was conducted using a MoFlo XDP cell sorter together with the Summit 5.4 software (Beckman Coulter) and applying the settings as described in Hagberg et al (231).

The analysis of the results was done with FlowJoTM 10 software. Figure 12 includes the steps for the analysis as used in the majority of the experiments. The population of enucleated adipocytes was refine using the information from Hagberg et al (231) and confirming it with nuclei Hoechst staining (Figure 12 a) and a new singlets gate (Figure 12 b). The 2 populations arising from the singlets gate are not present in all samples, but they appear under stochastic conditions that were yet to be defined, but pertain to the samples or MoFlo machine handling.

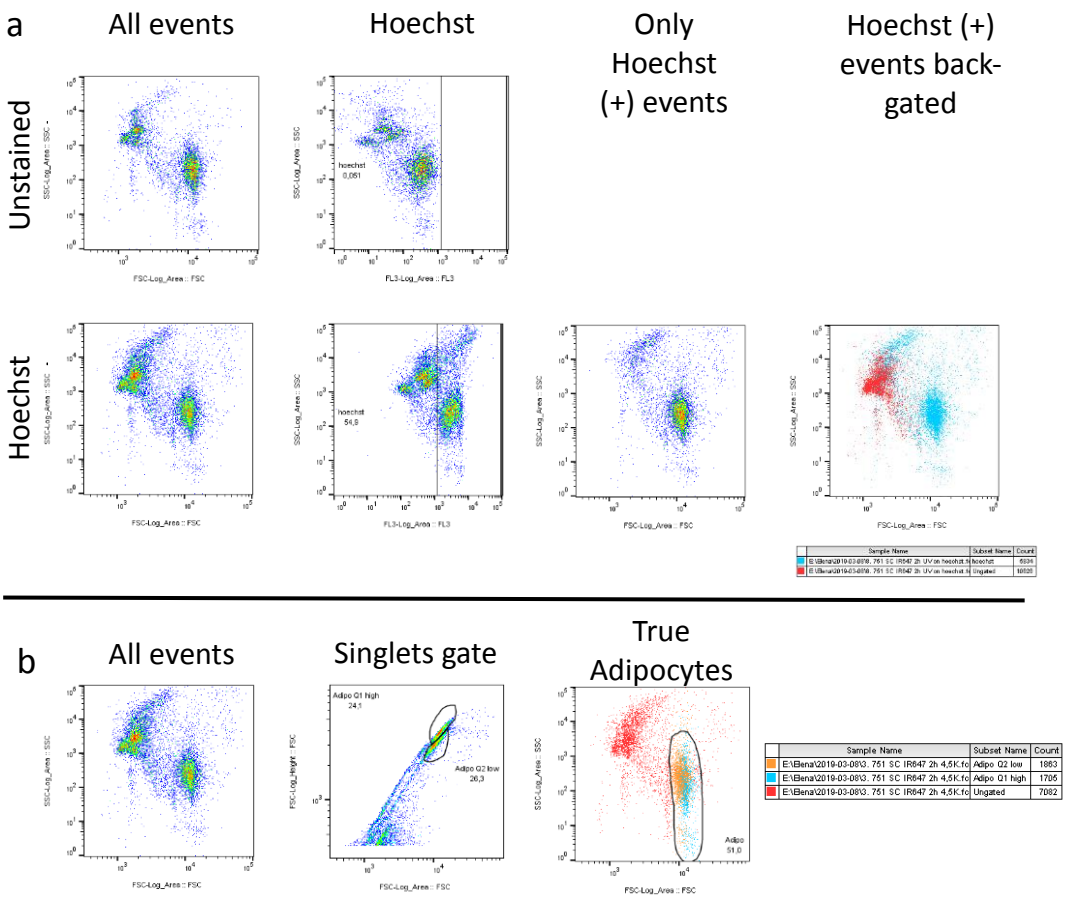


Figure 12:
(a) Flow cytometry analysis of subcutaneous adipocytes that were either unstained or stained with Hoechst DNA dye. From left to right there is the standard SSC-A vs FSC-A gate that depicts all events, the SSC-A vs Hoechst showing the positive and negative adipocytes, the standard gate with only the Hoechst positive events and the back-gate showing the area of Hoechst positive events (blue) in the standard gate.
(b) Flow cytometry analysis of the same sample as above showing the true adipocytes. From left to right is first the standard gate with all the events, then the singlet gate where only the events that belong in the diagonal line are counted and finally the standard gate were the singlets are back-gated (blue and orange).
Combining the information for the Hoechst positive events and the singlet events we can define the area where the true adipocytes appear in flow cytometry, into the above gate. These results are in agreement with Hagberg et al. (231)

EdU staining of cultured adipocytes

The "Click-iT™ Plus EdU Cell Proliferation Kit for Imaging, Alexa Fluor™ 488 dye" (Thermo Fisher) was used in order to stain all the adipocytes that incorporated EdU as they progressed through S-phase during their culture. The collected cells were fixed as it is described above and then they were washed with EdU blocking buffer (3% fatty acid free BSA in PBS (filtered)). Permeabilization was achieved through a 15 minutes' incubation in 0,5% Triton X-100 in PBS, followed by a second wash with EdU blocking buffer. The cells were then incubated for 30 minutes in the dark with the reaction mix, prepared according to the kit's instructions. One last wash was done using EdU blocking buffer and the adipocytes were left to incubate in 1:500 Hoechst 33342 (Molecular Probes) and 1:500 Rho-conjugated lectin (Rhodamine labeled Lens Culinaris Agglutinin (LCA) VECTOR) in PBS. Slides were prepared after the cells were suspended in 80% glycerol.

The quantification was performed using the ZEN software (Carl Zeiss, Germany) and images were taken with an inverted confocal Zeiss LSM 700 or LSM 880 confocal laser scanning. At least 500 cells were counted in order to determine the percentage of EdU positive cells.

B-Galactosidase staining of adipocytes

In order to depict the enzymatic activity of senescence associated beta galactosidase (SA-β-gal) the "Senescence β-Galactosidase staining kit" from "Cell signaling technology" was utilized. Fixed mature adipocytes were prepared according to the kit's instructions and they were left to

incubate in the pH=6 mix, at +37^{o}C, in tubes covered with parafilm, for 48 hours. Slides were then prepared with 80% glycerol and the percentage of SA-β-gal positive cells was determined after counting at least 200 cells per sample using x40 magnification in ZEISS Axio Lab.A1 light microscope. For the determination of cell size between SA-β-Gal positive and negative adipocytes, images were taken using Canon DS126311 EOS Rebel T3i Digital Camera at x10 magnification and the size was determined by ImageJ software.

Statistical analysis

The statistical analysis was conducted using GraphPad Prism 6 software. All the parameters were checked for normal distribution using D'Agostino-Pearson normality test. If the parameters had a Gaussian distribution then the tests chosen for the analysis was paired or unpaired t-test, depending if the data were paired or not. For non-gaussian distribution or if the parameter did not have enough data for the D'Agostino-Pearson normality test to determine the distribution, then the test of choice was either Wilcoxon matched pairs test for paired nonparametric data or Mann-Whitney test for unpaired nonparametric data. The statistical significance was reached when $p<0,05$. (ns for $P > 0.05$, * for $P \leq 0.05$, ** for $P \leq 0.01$, *** for $P \leq 0.001$ and **** for $P \leq 0.0001$)

Table of Antibodies

Antibody	Technique		ICC method	Concentration	Antibody type	Company	number
Anillin	-	ICC	none	1:250	Goat Polyclonal IgG	abcam	ab5910
b-Gal	IHC	-	-	1:150	Mouse monoclonal IgG2a	Santa cruz	sc-377257
cyclin A2	-	ICC	trypsin	1:250	Rabbit monoclonal	abcam	ab32498
Cyclin B1	ICH	-	-	1:200	Rabbit monoclonal	abcam	ab227844
Cyclin D1	-	ICC	methanol	1:250	Rabbit monoclonal	abcam	ab190563
cyclin E1	-	ICC	methanol	1:250	Rabbit monoclonal	abcam	ab33911
HMGB1	-	ICC	trypsin	1:250	Rabbit monoclonal	abcam	ab79823

Insulin receptor alpha	FCM		-	1:50	Mouse monoclonal IgG1	abcam	ab36550
ki67	IHC	ICC	methanol	1:5000 (IHC) 1:250 (ICC)	Rabbit polyclonal IgG	abcam	ab15580
PCNA	IHC	ICC	Trypsin	1:500 (IHC) 1:250 (ICC)	Mouse monoclonal IgG2a	Invitrogen	133900
Perilipin	IHC	-	-	1:250	Rabbit monoclonal IgG	Cell signaling	9349
Histone H3 (Ser10) Phospho	IHC	ICC	Trypsin	1:250 (IHC) 1:250 (ICC)	Rabbit polyclonal	Millipore	06-570
Anti-rabbit	-	ICC	-	1:1000	Donkey polyclonal IgG	ThermoFischer / Molecular Probes	A-31572
Anti-mouse	-	ICC	-	1:1000	Donkey	Life/Molecular Probes	A-31570
Anti-goat	-	ICC	-	1:1000	Donkey	Invitrogen Life	A-21432
Mouse IgG1 isotype control	FCM		-	1:50	Mouse monoclonal IgG1 K	BD	557714

Results

Investigation of cell cycle in mature adipocytes *in vivo* and *in vitro*

Immunocytochemistry imaging of primary human adipocytes for cell cycle markers

One of the crucial challenges of the project was the convincing depiction of mature adipocytes' ability to express cell cycle markers that span throughout most of cell cycle phases. In order to achieve this, freshly isolated and fixed human mature adipocytes were stained with representative cell cycle nuclear markers as well as membrane and nuclei counterstaining. As a measure to rule out exogenous nuclei contamination, the images were recreated using confocal microscopy z stacking, thus providing evidence that the cell membranes are intact and the nuclei are located inside the adipocytes. The selected images depict at least one positively and one negatively stained adipocyte simultaneously (**Images are not shown because they will be used in an upcoming publication**).

For the investigation of cell cycle markers' presence in the mature human adipocytes, seven well established proteins were chosen, which are found in different phases of the cell cycle. PCNA, ki67 and Cyclin D1 (Figure 13 a, b, c) are general markers of cell cycle, whose presence is constant in almost all the interphase (20,232,233) . Cyclin E1 (Figure 13 d) is detectable during G1 and early S phase while Cyclin A2 (Figure 13 e) follows the preceding E1 rising during S and G2 phase (21,22). Anillin and phosphorylated histone H3 (pHH3) (Figure 13 f, g) are markers rising during S and G2 phase respectively and continue being present well into mitosis. Both of these last markes have a distinct pattern of expression during G2 phase drastically altering when the cell transitions to mitosis (28,29). Anillin relocates from the nucleus to the cytoplasm in early M-phase, and later in cytokinesis to the contractile ring, while pHH3 expression spikes during mitosis and thus becomes immensely brighter in ICC imaging. None of the above patterns, pertinent to mitosis, were recognized in cells stained for anillin or pHH3 and additionally throughout all the confocal microscopy sessions there was an almost complete lack of cells with a mitotic phenotype and universal absence of cytokinesis. According to the images of adipocytes taken using confocal microscopy, isolated human mature adipocytes are able to express a plethora of different cell cycle markers found during G1, S and G2 phase, while there is absence of markers' patterns recognized during mitosis or cytokinesis.

Immunohistochemistry imaging for multiple cell cycle markers

Though highly convincing, the images of ICC depicting the cell cycle markers in isolated human adipocytes cannot fully represent adipocytes' biology as is in vivo. In order to receive a more faithful to reality depiction, adipocytes residing in their natural environment of adipose tissue were stained using ready-made sections of fixed and paraffin embedded adipose tissue. Immunohistochemistry (IHC) techniques were employed to reaffirm the presence of some of the cell cycle markers, like ki67, PCNA and pHH3 (Figure 13 a, b, c) that were found in ICC. Another advantage of IHC is the greater depiction of cytoplasmic proteins that are more difficult to observe with ICC techniques. In this case, Cyclin B1, a marker of G2 and M phase that characteristically translocates from the cytoplasm to the nucleus when the cell enters mitosis (25) was found in the cytoplasm of many adipocytes but not in their nuclei (Figure 13 d). These findings are in line with our ICC results that adipocytes can express cell cycle marker pertinent to G1, S and G2 phase.

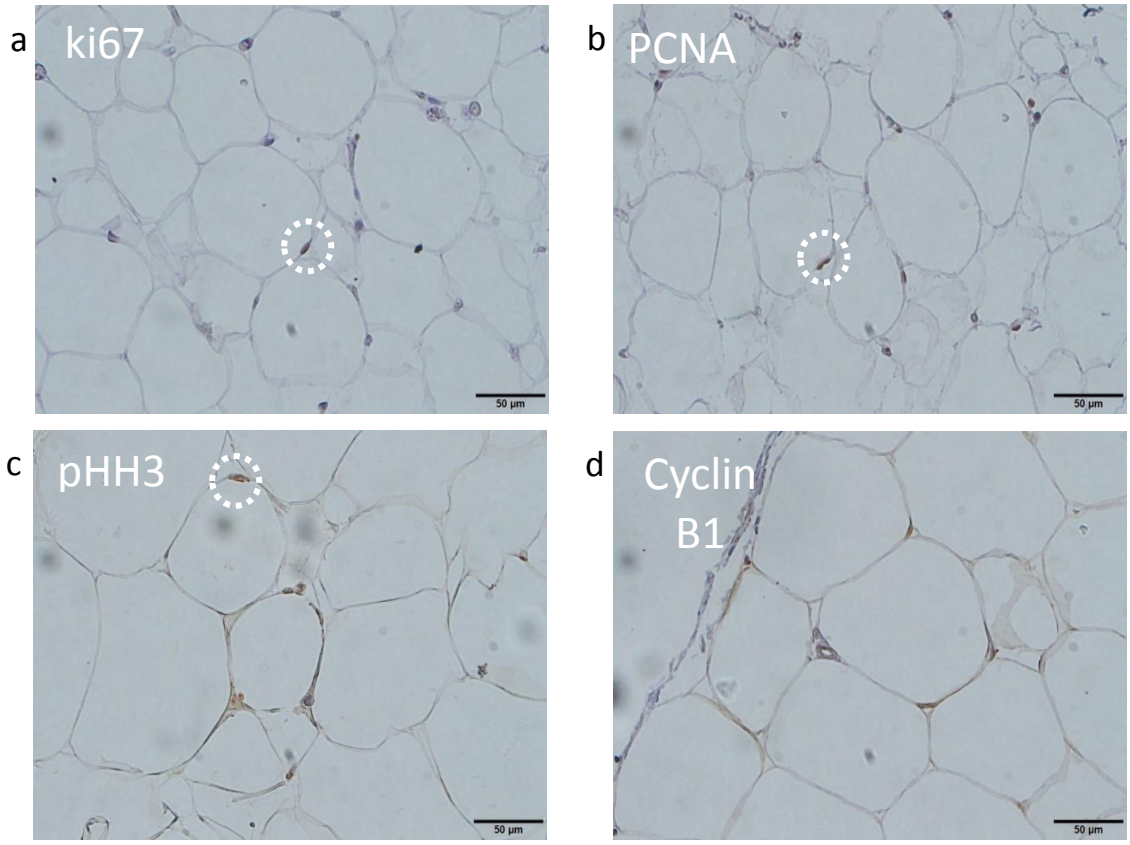
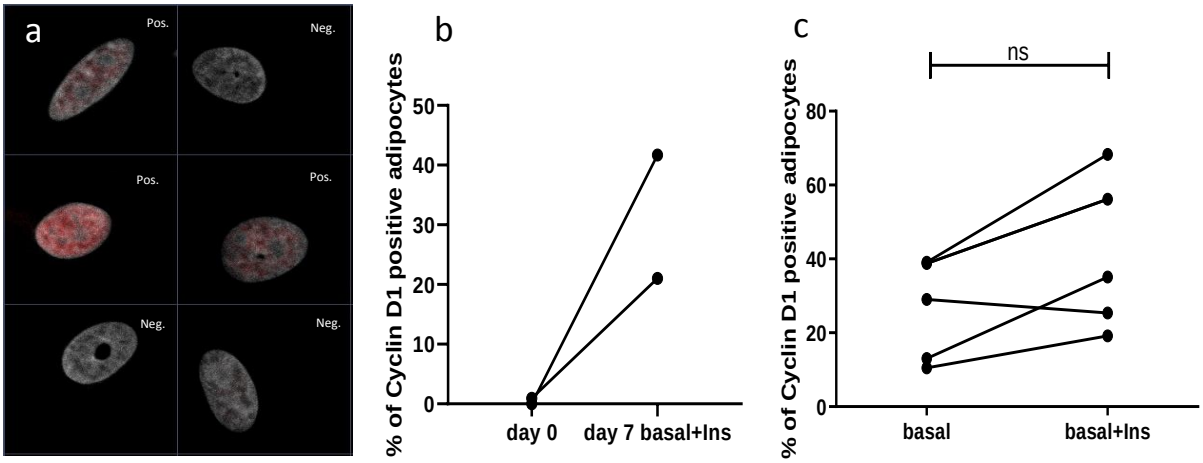


Figure 14: Immunohistochemistry images of adipose tissue stained for ki67 (a), PCNA (b), pHH3 (c) and Cyclin B1 (d). The white circles point to selected positive nuclei

Cell cycle entry and S-phase passing of adipocytes *in vitro*

The above findings serve as an indication that adipocytes are not circumstantially accumulating different cell cycle markers with non-canonical functions, but rather cell cycle actions are normally involved in the adipocyte biology. In order to investigate this possibility of active entry and passage through cell cycle, isolated adipocytes' suspension cultures was employed. Besides the basal culture media, some comparative experiments were conducted with the supplementation of insulin, which is a known growth enhancer promoting cell cycle (234,235) and a vital hormone in adipocytes' biology. After 7 days of culture the cells were retrieved, fixed and stained for Cyclin D1 (Figure 14 a), one of the cell cycle markers that are present throughout most of the interphase. The cells that were found positive using confocal microscopy are actively out of G0 phase and partaking in cell cycle. Matched samples of adipocytes that were fixed right after isolation were compared to adipocytes from the same sample that were cultured for 7 days in basal media plus insulin and they show a dramatic increase in the levels of Cyclin D1 (Figure 14 b). In addition adipocytes that were cultured for 7 days with in either basal media or with the addition of insulin showed a clear tendency towards cell cycle entry when insulin was added (Figure 14 c).

Another key advantages of cell culture is the added ability to detect the passage through S phase. This was made possible by visualizing the incorporation of a special nucleotide analogs, called EdU, in the replicating DNA of the cell. Adipose tissue pieces as well as isolated adipocytes were cultured for four days in suspension cultures with basal media supplemented with insulin and EdU. After fixing and staining for EdU, Hoechst and red lectin, it is possible to detect adipocytes that have readily incorporated EdU as they passed through S-phase during their culture period. (**Images are not shown because they will be used in an upcoming publication**) As with Cyclin D1, the EdU incorporation was also increased in a time manner and in response to insulin. Edu incorporation was greater after 7 days of culture compared to pulled 3 or 4 day of culture in isolated adipocytes (Figure 14 d) and the presence of insulin in the media had a statistically significant effect as well, compared to basal (Figure 14 e).



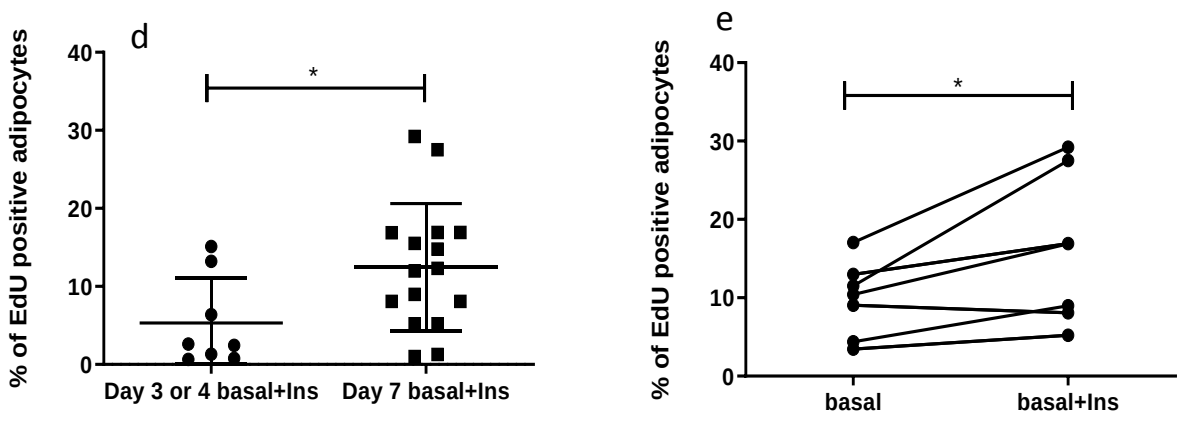


Figure 14:
(a) Zoomed in images of adipocytes' nuclei stained for Cyclin D1 (red) and Hoechst (white) showing the variability in the staining.
(b) The percentage of Cyclin D1 positive cells for paired samples from day 0 and after 7 days of culture with basal media and insulin.
(c) The percentage of Cyclin D1 positive adipocytes after 7 days of culture in basal media or with the addition of insulin. Analysis with Wilcoxon matched-pairs signed rank test.
(d) Diagram showing the percentage of EdU incorporation after 3-4 days of culture and after 7 days of culture in basal plus insulin (Unpaired t test).
(e) The percentage of EdU incorporation after 7 days of culture in basal media or media supplemented with insulin (Paired t test). Ns for $p>0.05$, * for $p<0.05$

Investigation of polyploidy in mature human adipocytes

The idea that mature adipocytes are not post-mitotic, as ones thought, but rather they are able to enter into cell cycle, surely broadens our perspectives on further biological functions they might hold. Combining the two concepts of “mature adipocytes’ cell cycle entry” and “lack of cytokinesis”, indicates that they might actually be able to enroll into endoreplication and thus become polyploidy cells. In order to investigate further this possibility, mature human adipocytes’ nuclei were evaluated qualitatively and quantitively so as to measure the amount of DNA they carry. Moreover, some flow cytometry experiments were conducted in order to find a way of efficient analysis of hypertrophic adipocytes in high quantities during the simultaneous investigation of other markers in flow cytometry.

Optimization of DNA de-condensation protocols

In order to detect adipocyte ploidy, the primary idea was to stain the isolated nuclei with DNA dye and measure the emitted fluorescence either with flow cytometry or colorimetric techniques. If the estimations were correct, there would have been distinct 4n, 6n, 8n etc. populations of adipocytes that would appear separate from the 2n population. Primary experiments in flow cytometry indicated that there was indeed an increase in the DNA content of the adipocyte nuclei when compared with control 2n nuclei. However, this increase was not detected on the area where 4n nuclei were calculated to be found but rather in between 2n and 4n (Figure 15 a). However, when “Comparative Genomic Hybridization (CGH) array” experiment was conducted previously (data not shown), it overruled the possibility that adipocytes are aneuploidy. So, since this discrepancy in fluorescence can’t be attributed to aneuploidy, another possible explanation could be the increased DNA condensation, that is shown to lead to falsely lower DNA content measurements in some cases (136).

With this in mind adipocytes’ nuclei were prepared using different conditions that would allow the DNA to de-condense. Isolated nuclei from clean populations of “peripheral blood mononuclear cells” (PBMCs-control 2n nuclei) or adipocytes were treated with: pH adjustments (Figure 15 d, e, p, q), RNase enzymes (Figure 15 d, e, f, g, h, i, n, o, p, q), surfactant agents like NP40 (Figure 15 f, g) and triton X100 (Figure 15 h, i), dithiothreitol (DTT) (Figure 15 h, i), FBS media (Figure 15 f, g), different fixation methods like PFA (Figure 15 j,k) and ethanol (Figure 15 l-q), and different DNA dyes like PI (Figure 15 b-e) and Dapi (Figure 15 f-q) among others. While interventions like RNAse treatment, higher pH in the solution, use of potassium salts and intercalating DNA dyes (propidium iodide, draq5 etc.) increased substantially the nuclei stainability, others were not as effective. Less impressive were the results from the use of

proteases, ATP, DTT, fixatives, surfactants or DNA dyes of minor groove binders (Hoechst, dapi etc.). From the different combinations of methods it was shown that the greatest de-condensation and staining of DNA was consistently achieved when the nuclei were treated with RNase, pH higher than 12 and PI DNA dye.

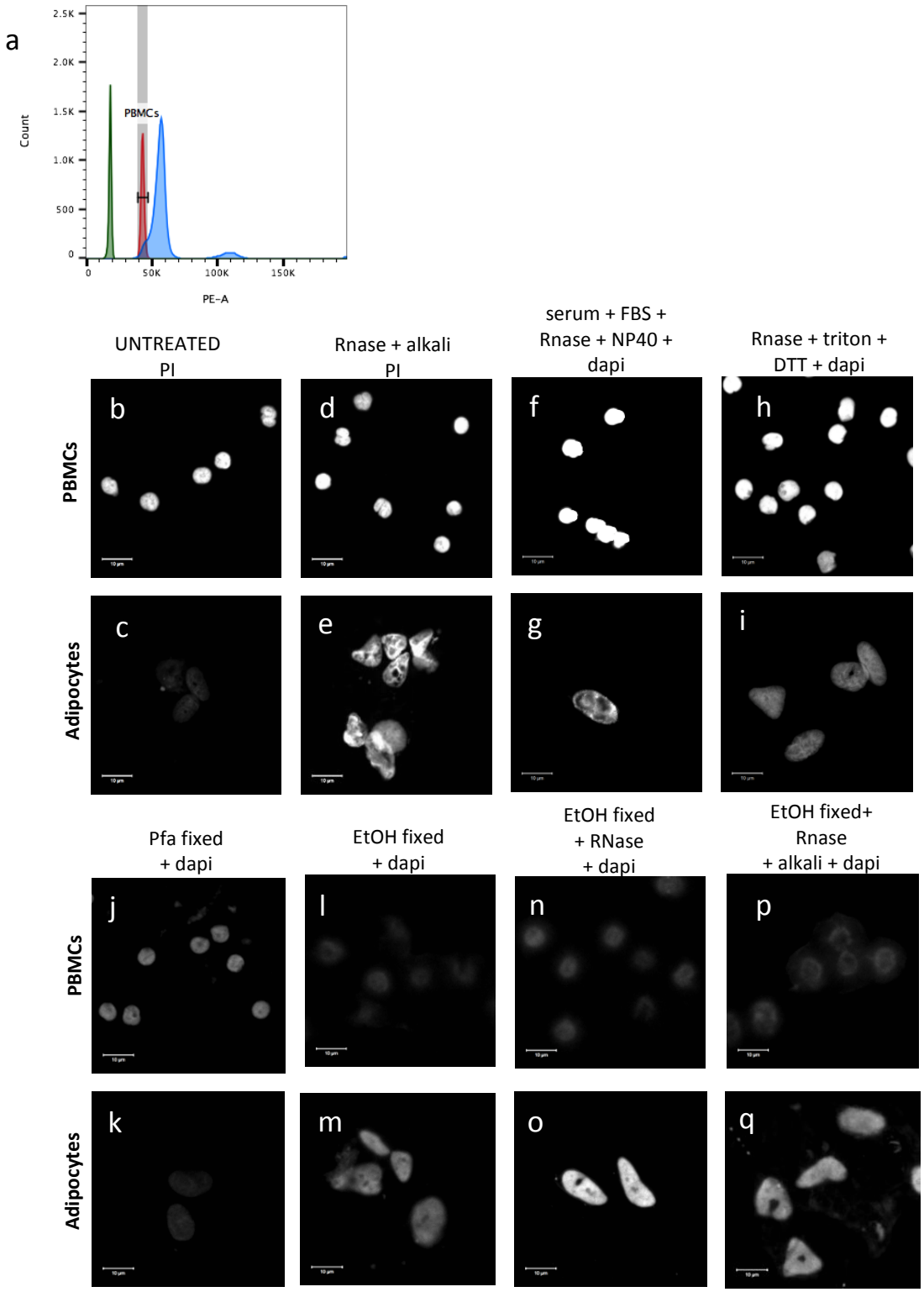


Figure 15:
(a) Flow cytometry of adipocytes nuclei showing the DNA content of adipocytes (blue), PBMCs (red) and chicken erythrocyte nuclei (green).
(b-q) Confocal microscopy images of PBMCs and adipocyte isolated nuclei which are untreated (b, c), with RNase, high pH, and PI (d, e), with serum, FBS, RNase, NP40 and dapi (f, g), with RNase, triton-X100, DTT and dapi (h, i), with PFA fixation and dapi (j, k), with ethanol fixation and dapi (l, m), with ethanol fixation, RNase and dapi (n, o) and with ethanol fixation, RNase, high pH and dapi (p, q)

Utilizing Barr body staining for quantitative measurement of chromosomal content

A different approach that could reveal the presence of ploidy comes from the immediate quantification of chromosomal content. Previously, RNA FISH was used with this purpose in mind (data not shown). The idea was that the adipocytes, would show that they are polyploid through the expression of multiple sites for FISH at a time. However, the condensed nature of DNA did not always allow the clear distinction of FISH signaling, since the dots were located close to one another.

Keeping this plan in mind a simpler approach was employed. Instead of focusing on smaller targets and fluorescence through FISH, a whole chromosome would be utilized instead. Naturally, every cell containing more than two X chromosomes condenses the excess X chromosomes into structures called Barr bodies, so that the cell will only have genetic information from one X chromosome (236). In that case the Barr body can be found in all 2n female cells as well as all the polyploid cells that happen to possess more than two X chromosomes regardless of sex (141). Since the Barr body is a clearly defined structure in the nucleus (237), the idea was to stain adipocyte nuclei for the Barr body and determine the polyploid cells as either female-originated with more than 2 Barr bodies or male-originated with at least 1 Barr body. A modified protocol using hematoxylin staining was enough to reveal the presence of Barr body (Figure 16). However, the presence of other intranuclear structures, like the nucleolus or SAHF, which can be stained similarly to Barr body's pattern, rendered this technique less reliable.

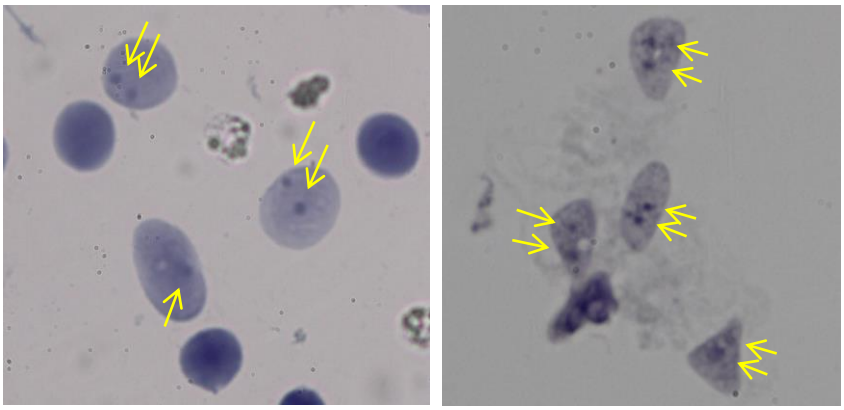


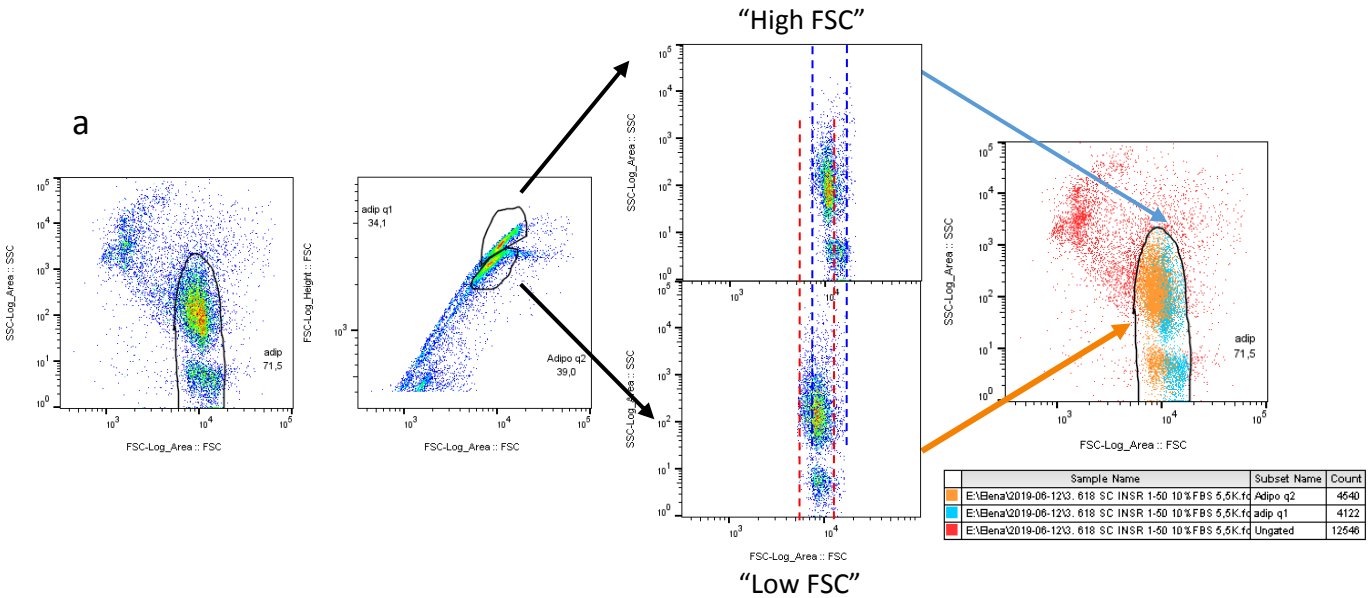
Figure 16: Isolated nuclei of human mature adipocytes from a female (46 XX) that were stained with modified hematoxylin protocol. The yellow arrows indicate the areas of possible Barr bodies in the nuclei.

Recognizing a possible hypertrophic adipocyte population through flow cytometry

The concept of adipocyte hypertrophy is a well-established one in the literature as a mechanism of adipose tissue expansion (2). However, there are only a few techniques that could efficiently distinguish between hypertrophic and normal adipocytes while simultaneously evaluating other characteristics of these populations in a single-cell basis. Previous work in this lab has established flow cytometry (FCM) protocols for the accurate analysis of adipocytes (231). However, this old analysis was based on mouse adipocytes and it didn't show distinct hypertrophic populations on the plots, despite finding areas with bigger or smaller cells. However, a new analysis strategy that was developed has the potential to now pinpoint the hypertrophic population (Figure 12 b, page 40, materials and methods & Figure 17 a).

In this new analysis, in the singlets gate created with "FSC log height" and "FSC log area" two distinct populations the "FSC high" and "FSC low" can arise in the established area of adipocytes. For unknown reasons, these populations were not present in all the experiments that were conducted. When these two populations of "FSC high" and "FSC low" are back-gated to the initial gating panel of FSC-SSC, it is finally revealed that they presented as partially overlapping populations and thus it was impossible to distinguish them before (Figure 17 a).

An interesting twist was the discovery that these two populations have a different profile, at least when it comes to the expression of insulin receptor (IR) (Figure 17 b). In a small cohort of 8 patients, which showed the distinct populations in their analysis, it was found that the "low FSC" population has significantly lower expression of the extracellular cell membrane IR, compared to "high FSC" population that almost unanimously expressed it (Figure 17c).



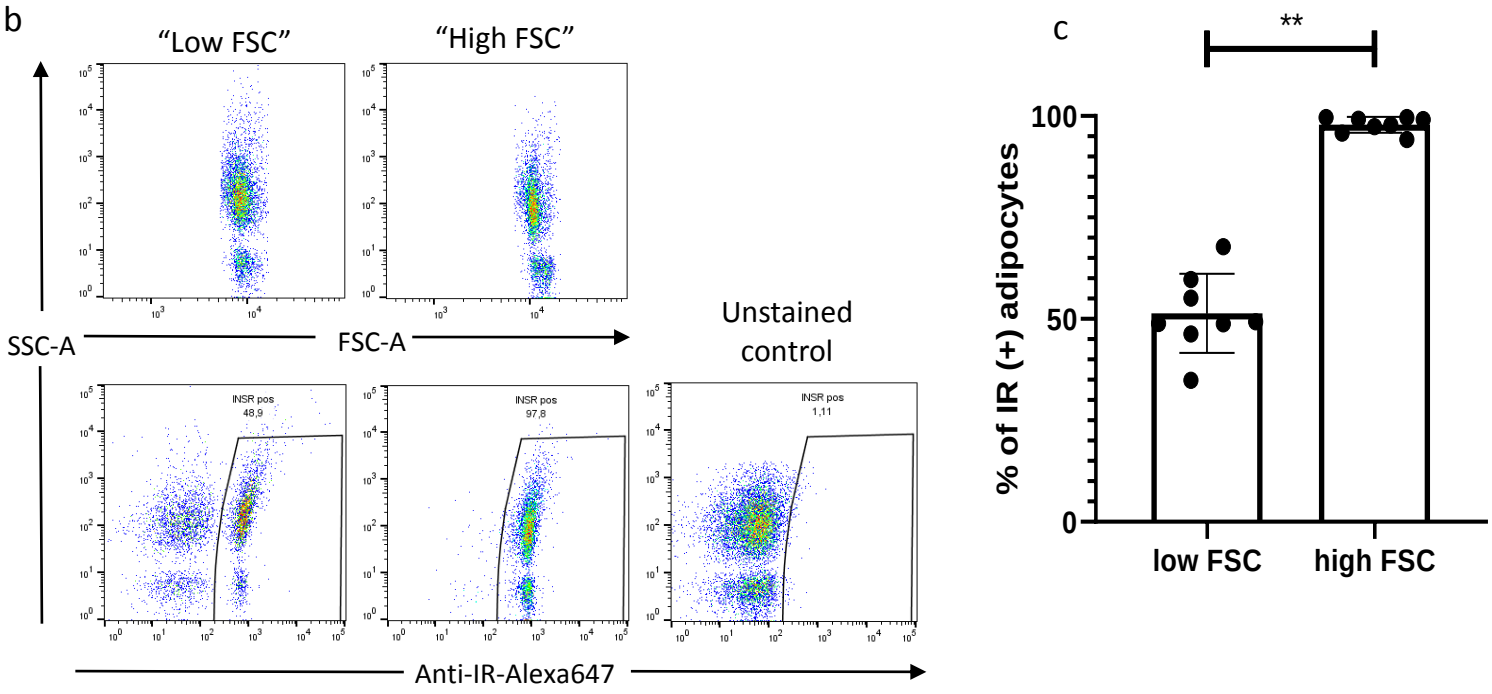


Figure 17:
(a) Flow cytometry analysis of subcutaneous adipocytes and the presence of the two populations. From left to right is the standard SSC-A vs FSC-A gate, the FSC-A vs FSC-H singlets gate (of all events: adipocytes, debris and free lipids) that revealed the two populations, the SSC-A vs FSC-A gate for "high FSC" and "low FSC" populations and finally the two populations back-gated to the original gate.
(b) Flow cytometry analysis of a patients "low FSC" and "high FSC" in the SSC-A vs FSC-A gate (top) and in the SSC-A vs IR-Alexa647 (bottom). The sample was stained with anti-IR antibody conjugated with Alexa 647 and it shows the positive and negative populations when comparing with the unstained control.
(c) Analysis of the expression levels of IR (+) adipocytes from the "low FSC" and "high FSC" populations. The analysis was done with Wilcoxon matched-pairs signed rank test and was statistically significant with $P \leq 0.01$.

Senescence of primary human adipocytes

Investigation of the *in vivo* state of senescence in primary adipocytes

One of the major biological functions that are tightly correlated with cell cycle and specifically cell cycle inhibition is senescence. The presence of senescence is well characterized in the adipose tissue (196), the pre-adipocytes (206) and the other cells of the tissue (208). However, there is still skepticism if the phenomenon is extended to the most abundant cell of the adipose tissue, the mature adipocyte; mainly due to the notion that they are considered post-mitotic

cells. However, in the light of the above findings it is imperative to investigate this hypothesis, given that adipose tissue senescence has been really tightly correlated to the development of insulin resistance and metabolic syndrome (207,238,239).

In order to verify this hypothesis, isolated mature human adipocytes were first stained with Senescence Associated β Galactosidase (SA-β-Gal) (**Images are not shown because they will be used in an upcoming publication**). SA-β-Gal at the pH of 6 is considered the gold standard in the depiction of senescent cells (240). In our case the adipocytes were stained blue with SA-β-Gal in the area close to the nuclei, where the majority of cytoplasm resides in the adipocytes. The topology of the SA-β-Gal staining was then confirmed with IHC of b-Gal (Figure 18 a), showing it right next to the adipocytes' nuclei. These SA-β-Gal positive adipocytes were further investigated and they were associated to additional markers of senescence; namely cell size and absence of nuclear HMGB1. It is known that cells going through senescence have increased size compared to their non-senescent counterparts (161) and this was depicted with statistical significance ($p \leq 0.0001$) in three separate adipocyte samples stained for SA-β-Gal (Figure 18 b). Additionally, HMGB1 has been described to translocate outside of the nucleus when cells enter into senescence (175).This was true in our case as well, where in a small cohort of 9 patients the percentage of SA-β-Gal positive adipocytes was significantly correlated with the absence of nuclear HMGB1 (Figure 18 c).

The most intriguing part of our study, however, is how the percentage of mature adipocytes in senescence correlates with the metabolic status of the patient, whose adipose tissue samples were investigated. In a cohort of 51 surgery patients, the subjects were stratified as non-obese (BMI <30), obese healthy (BMI>30, c-peptide <1 nmol/L and / or insulin <20mU/L), obese unhealthy (BMI>30, c-peptide >1 nmol/L and/ or insulin >20mU/L) and diagnosed diabetics (Figure 18 d). Subcutaneous adipose tissue was collected during surgery and the isolated mature adipocytes were stained with SA-β-Gal. The non-obese patients had the lowest percentages of SA-β-Gal positive adipocytes (7 patients, range 0%-0.5%, mean=0.142%, std. dev.=0.24) but were not found significantly different from the obese healthy subjects (11 patients, range 0%-3%, mean=0.87%, std. dev.=1.01). On the contrary, both groups were found to be different with statistical significance, when compared to the obese unhealthy group (27 patients, range: 0%-35%, mean=9.890%, std. dev.=10.00) and the diabetic group (6 patients, range: 1%-7%, mean=3.921%, std. dev.=2.00). The unhealthy obese and the diabetic group bared no statistically significant difference between them, even though the distribution of obese unhealthy patients was much wider. Upon further investigation, it seems that the c-peptide levels of the patients are the true drivers of this wide distribution in this obese unhealthy group. Out of a variety of metabolic parameters including BMI, waist to hip ratio, lipid profile, blood pressure, adiponectin, and lectin among others; it was shown that only c-peptide correlated positively with the percentage of SA-β-Gal positive adipocytes, when we were exclusively investigating the unhealthy obese group (Figure 18 e).

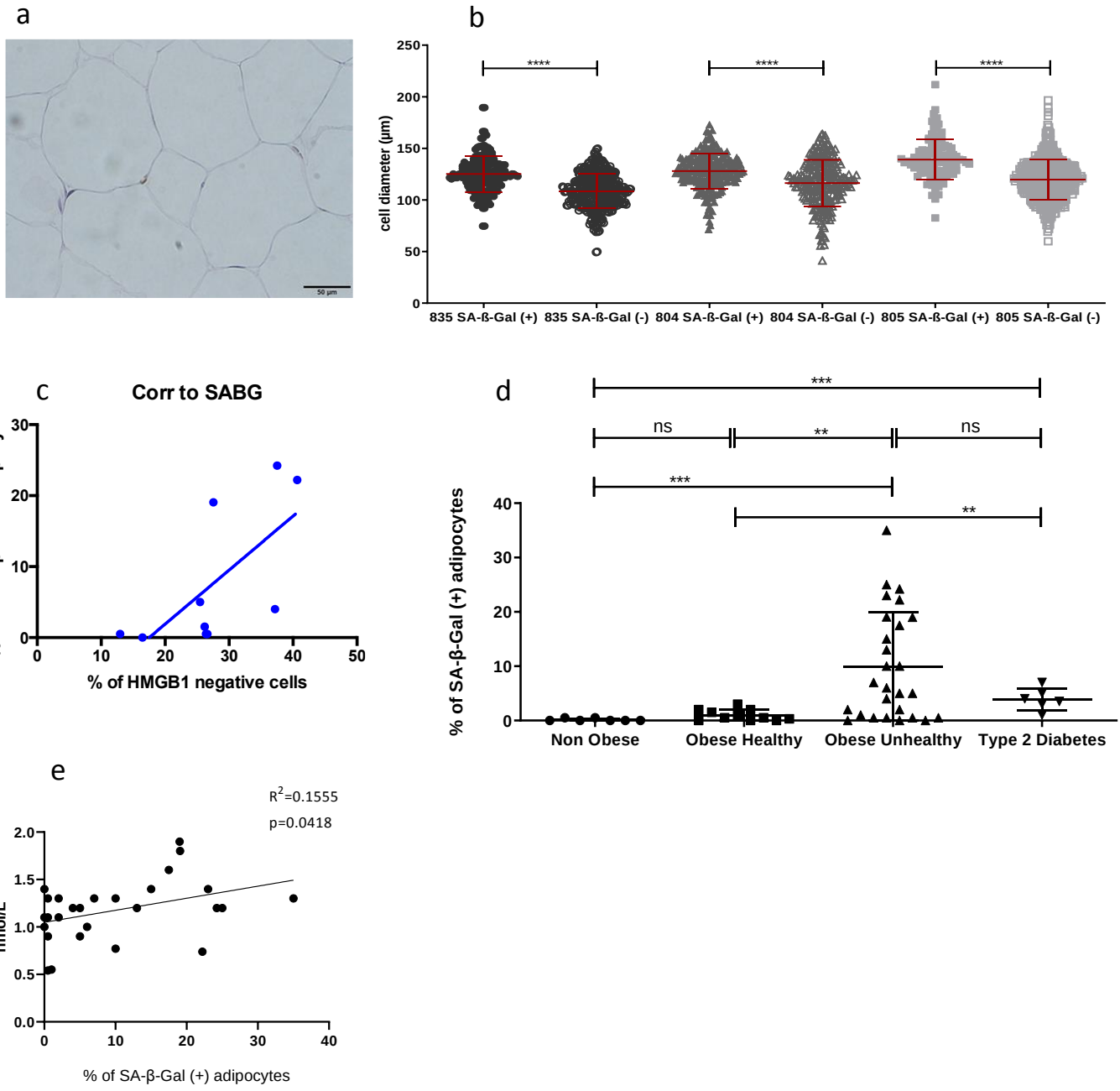

a
b
cell diameter (µm)
****
****
****
835 SA-ß-Gal (+)
835 SA-ß-Gal (-)
804 SA-ß-Gal (+)
804 SA-ß-Gal (-)
805 SA-ß-Gal (+)
805 SA-ß-Gal (-)
c
Corr to SABG
% of SABG positive adipocytes
% of HMGB1 negative cells
d
% of SA-β-Gal (+) adipocytes
***
ns
**
ns
***
**
Non Obese
Obese Healthy
Obese Unhealthy
Type 2 Diabetes
e
R2=0.1555
p=0.0418
C-peptide nmol/L
% of SA-β-Gal (+) adipocytes

Figure 18:
(a) Immunohistochemistry imaging of adipose tissue stained for SA-β-Gal. The white circles indicate the area near the adipocytes' nuclei positively stained.
(b) Diagram of three adipocyte samples (835, 804, 805), where at least 100 SA-β-Gal positive and 100 SA-β-Gal negative adipocytes were measured and their diameter was displayed. Mann Whitney test was performed for each paired group showing that adipocytes' diameters between positive and negative were statistically significant different, with $p < 0.0001$ in all three cases.
(c) Diagram prepared from a cohort of 9 patients, showing the positive correlation between the percentages of HMGB1 negative nuclei and the percentages of SA-β-Gal positive adipocytes, when paired samples were prepared from each patient.
(d) Diagram prepared from a cohort of 51 patients. The patients were stratified into four groups (non-obese, obese healthy, obese unhealthy and type 2 diabetics), according to their metabolic state, and their percentages of SA-β-Gal positive adipocytes were measured. The statistical analysis was conducted using Mann Whitney test or unpaired t test (only for obese healthy vs obese unhealthy).
(e) Diagram depicting the correlation between the percentages of SA-β-Gal positive adipocytes and the c-peptide levels, for the 27 patients of the obese unhealthy group.

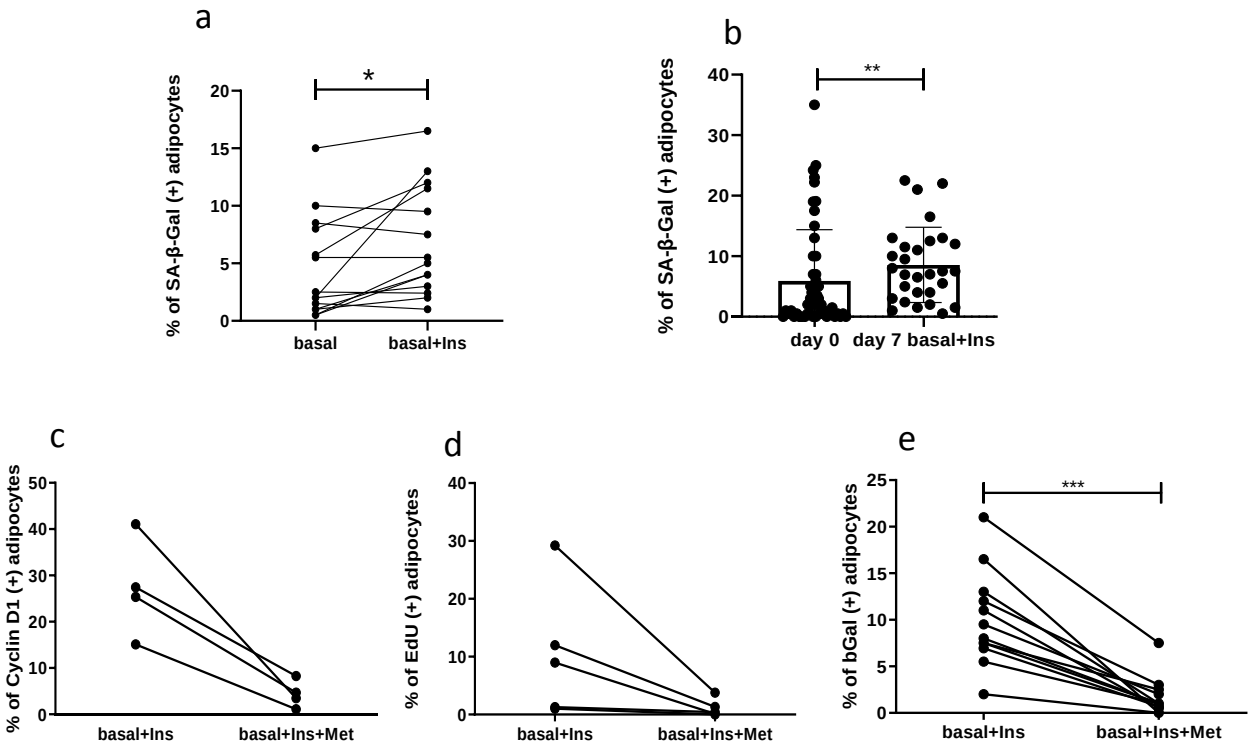
In vitro investigation of senescence's driving factors in primary adipocytes

In order to further investigate potential driver for senescence and the different effects they elicit, from manipulating cell cycle entry, adipocytes were once again cultured in suspension, under different experimental conditions. The presence of extra insulin in the culture media, that was previously found pivotal in the entrance to cell cycle and DNA synthesis, had once again a significant effect, leading to the increase of SA-β-Gal positive human primary adipocytes after 7 days of culture (Figure 19 a). Moreover, the effect of culture time was taken into consideration. When all samples from day 0 (51 subjects) and day 7 (29 subjects) were pulled for the analysis, there was a statistically significant increase of SA-β-Gal positive adipocytes after culture (Mann-Whitney test $p= 0.0025$) (Figure 19 b). However, the percentages of SA-β-Gal after the culture with insulin did not correlate significantly with any of the parameters of the patient (data not shown).

Moving forward, the effects of cell cycle inhibitors were put into test, in order to investigate how cell cycle manipulation affected mature adipocyte senescence. The two cell cycle inhibitors chosen for this task, metformin and palbociclib, target different parts of cell cycle and are also widely available drugs used in the everyday medical practice. Metformin, among others, has a cell cycle inhibitory effect, as it leads to Cyclin D1 degradation, hence effectively preventing cell cycle entry (241,242), while palbociclib is a CDK4/6 inhibitor, thus allowing cell cycle entry but preventing cell cycle progression past G1/S checkpoint (243). Mature human adipocytes cultured in basal media with insulin and metformin for 7 days show a decrease in cyclin D1 expression (Figure 19 c) and EdU incorporation (Figure 19 d). Additionally these samples contained, with

statistical significance, lower levels of SA-β-Gal positive adipocytes after the 7 days of culture (Figure 19 e). On the other hand, the adipocytes cultured with palbociclib had lower levels of Cyclin D1 expression and EdU incorporation, but in this instance the percentage of SA-β-Gal positive adipocytes was much higher when compared to the control (Figure 19 f, g, h).

Another important aspect of the above experiments was to identify how the patients' metabolic profile could determine adipocytes response to the two drugs. The analysis indicated that SA-β-Gal positive adipocytes' final levels, both for metformin and palbociclib treatment, were correlating with patients' initial levels of serum insulin (Figure 19 i, k). In addition, when the SA-β-Gal positive percentage of "basal+Ins+Met" was subtracted from the respective percentage for "basal+Ins" treatment, to highlight the effect of metformin on its own, it was shown that the difference correlates significantly with patients' triglycerides (Figure 19 j) as well as systolic blood pressure, cholesterol, LDL, Apo B, visceral adiposity index, insulin and C-peptide levels (data not shown). Using a similar approach, it was found that the net effect of palbociclib to the cultured adipocytes was a positive correlation between the SA-β-Gal positive percentage and triglycerides (Figure 19 l), cholesterol, LDL, Apo B, visceral adiposity index and insulin levels (data not shown).



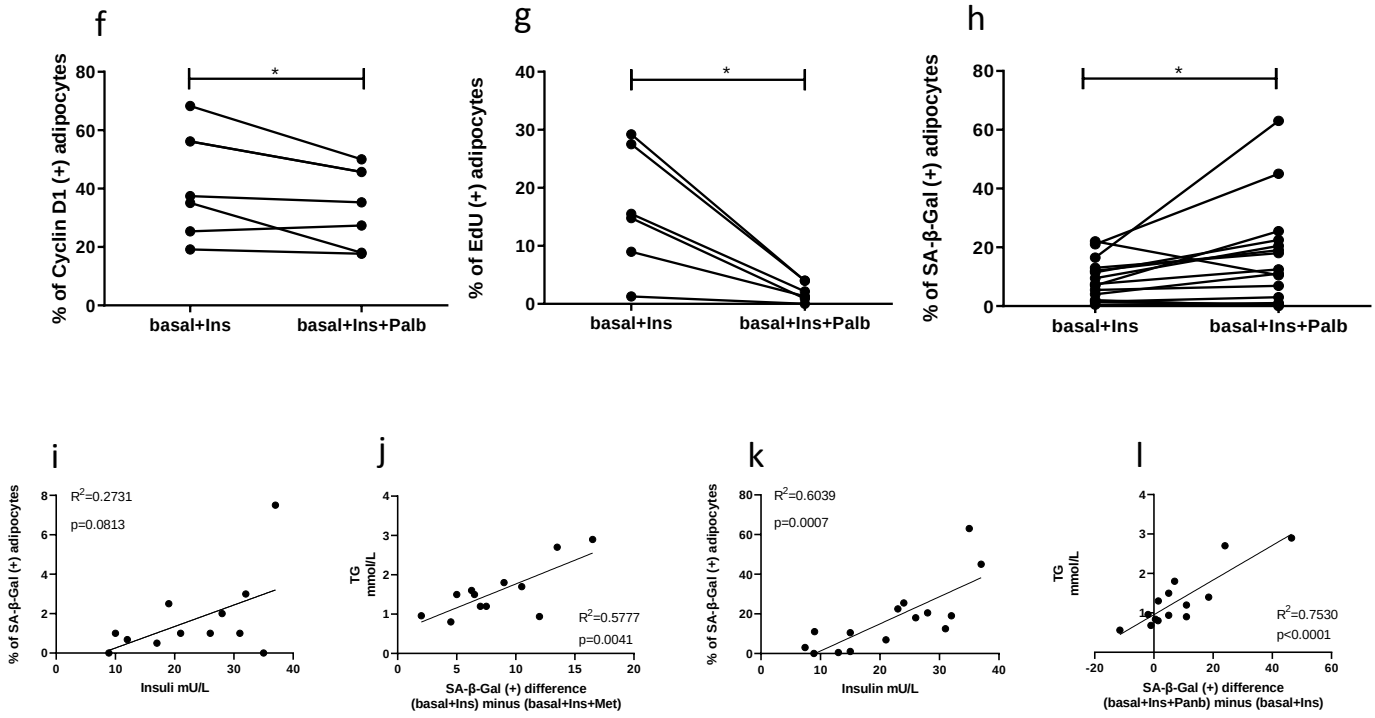


Figure 19:

(a) Diagram of SA-β-Gal positive mature adipocyte percentage of paired samples cultured for 7 days in basal media or basal plus insulin. Analysis with paired t-test showed significant difference between the two groups.
(b) Diagram of SA-β-Gal positive mature adipocyte percentage of all the samples that were either untreated (day 0) or treated with insulin supplementation for 7 days of culture. Mann-Whitney test revealed p=0.0420 which is statistically significant
(c) The effect of metformin in the expression of Cyclin D1 in adipocytes. Paired samples were cultured for 7 days with either basal media plus insulin or basal media plus insulin and metformin. Analysis with Wilcoxon matched-pairs signed rank test showed no significance due to the low number of samples.
(d) The effect of metformin in the incorporation of EdU in adipocytes. Paired samples were cultured for 7 days with either basal media plus insulin or basal media plus insulin and metformin. Analysis with Wilcoxon matched-pairs signed rank test showed no significance due to the low number of samples.
(e) The effect of metformin in the induction of SA-β-Gal staining in adipocytes. Paired samples were cultured for 7 days with either basal media plus insulin or basal media plus insulin and metformin. Analysis with Wilcoxon matched-pairs signed rank test showed statistical significance.
(f) The effect of palbociclib in the expression of Cyclin D1 in adipocytes. Paired samples were cultured for 7 days with either basal media plus insulin or basal media plus insulin and palbociclib. Analysis with Wilcoxon matched-pairs signed rank test showed statistical significance, though the effect is not as drastic as to plunge the expression close to zero.
(g) The effect of palbociclib in the incorporation of EdU in adipocytes. Paired samples were cultured for 7 days with either basal media plus insulin or basal media plus insulin and palbociclib. Analysis with Wilcoxon matched-pairs signed rank test showed statistical significance.

(h) The effect of palbociclib in the induction of SA-β-Gal staining in adipocytes. Paired samples were cultured for 7 days with either basal media plus insulin or basal media plus insulin and palbociclib. Analysis with Wilcoxon matched-pairs signed rank test showed statistical significance.
(i) Diagram of the corelation between patients' intial insulin levels and the SA-β-Gal levels after 7 days of culture with basal media plus insulin and metformin.
(j) Diagram of the corelation between patients' intial triglyceride levels and the net effect of SA-β-Gal levels reduction due to the effect of metformin.
(k) Diagram of the corelation between patients' intial insulin levels and the SA-β-Gal levels after 7 days of culture with basal media plus insulin and palbociclib.
(l) Diagram of the corelation between patients' intial triglyceride levels and the net effect of SA-β-Gal levels increase due to the effect of palbociclib.

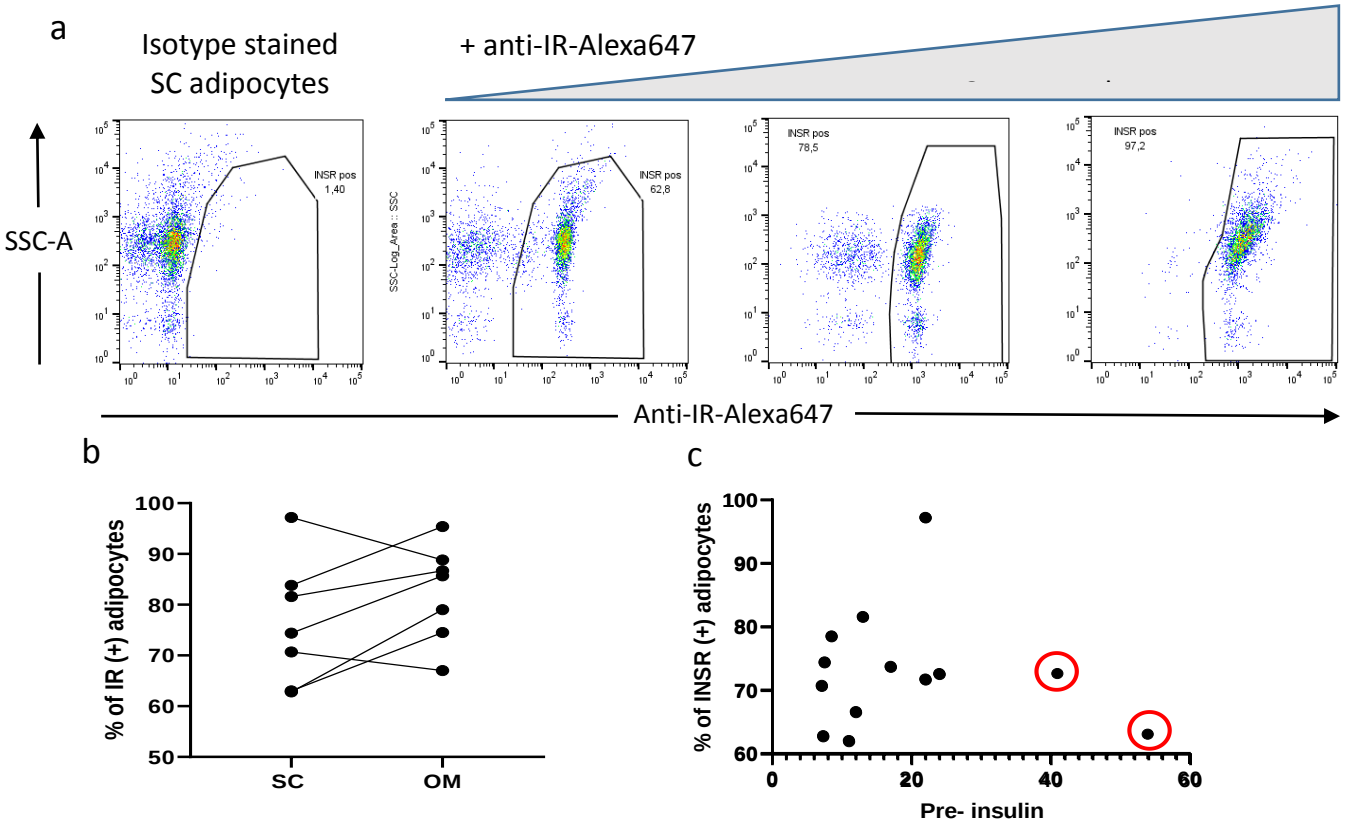
Investigation of insulin receptor expression in mature human adipocytes

Expression levels of insulin receptor in regards to patients' metabolic profile

During the course of this study it became abundantly clear that one of the major players in cell cycle and senescence processes is the insulin pathway. The addition of insulin into adipocyte cultures had major effects, even though many of the donor patients were morbidly obese with established metabolic syndrome. This begs the question if all adipocytes have a unanimous response to their insulin pathway or if there is heterogeneity in the tissue. Unpublished data from our lab have shown, through single cell RNA sequencing of subcutaneous mature human adipocytes from obese patients, that there is an established heterogeneity in adipocytes, which cluster into 3 subgroups. While it was somewhat expected that the "unhealthy" cluster will have lower levels of expression of insulin receptor (IR) compared to others, what is novel is that there are mature adipocytes completely lacking INSR mRNA and these cells were gathered mainly in the "healthy" cluster. (Data not shown)

In order to validate these mRNA findings and indicate if they hold true for the protein levels, adipocytes from obese patients were stained with conjugated antibodies against IR and the samples were analyzed with flow cytometry (FCM). As it was shown previously from the single cell data, there is a fraction of mature adipocytes that lack the IR in the surface of the adipocytes. In line with the single-cell data it seems that the percentage of these IR (-) adipocytes is higher in patients with better metabolic profile, while it lowers as the patient becomes more insulin resistant (Figure 20 a). A cohort of 7 patients where both subcutaneous (SC) and omental (OM) adipocytes were analyzed shows that there might be a higher expression of IR in the adipocytes from omental origin, however this increase was not proven statistically significant (Figure 20 b). A diagram from a bigger cohort of 14 obese patients was prepared showing the percentage of IR

(+) adipocytes from SC versus the patients' early insulin levels (Figure 20 e). Analyzing the graph, it seems that the percentages increase parallel with the increase of insulin levels. Only two patients with disproportionally high insulin levels sick out as potential outliers. When all 14 patients were used for the analysis there was no significant correlation between patients' IR (+) primary adipocytes and any of the metabolic factors, like C-peptide and insulin levels as well as HOMA-IR (Figure 20 d, e, f). However, when these two patients were excluded the rest of the obese patients' IR (+) levels correlate significantly with patients' c-peptide and insulin levels, as well as HOMA-IR index (Figure 20 g, h, i). On the other hand the adipocytes originating from the OM adipose tissue did not show any significant correlations (data not shown).



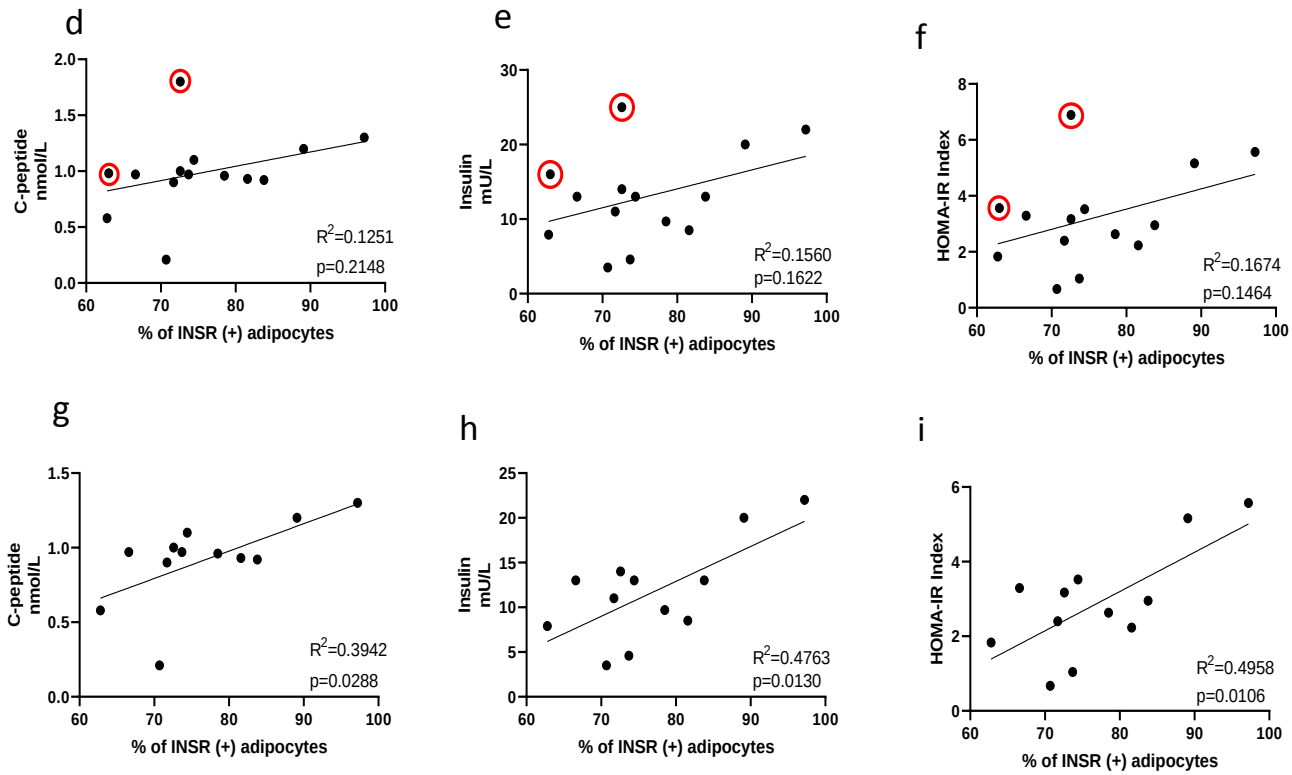


Figure 20:
(a) Flow cytometry analysis of subcutaneous adipocytes stained with anti-IR antibody conjugated with Alexa 647 and the unstained control. The stained samples are arranged based on patients HOMA-IR index.
(b) Analysis of the percentages of IR (+) cells from paired SC and OM samples. Wilcoxon matched-pairs signed rank test showed no significant difference between the two groups.
(c) Correlation analysis between patients' IR (+) % of SC adipocytes and their insulin levels. In red circle are the two samples that present as possible outliers.
(d) Correlation analysis between patients' IR (+) % of SC adipocytes and their c-peptide levels. In red circle are the two samples that present as possible outliers.
(e) Correlation analysis between patients' IR (+) % of SC adipocytes and their insulin levels. In red circle are the two samples that present as possible outliers.
(f) Correlation analysis between patients' IR (+) % of SC adipocytes and their HOMA-IR index. In red circle are the two samples that present as possible outliers.
(g, h, i) Same diagrams as (d, e, f), where the potential outliers are excluded.

Discussion

Part 1: Cell cycle of Human Primary Adipocytes

Proving that human mature adipocytes are able to enter into cell cycle is a challenge to the longstanding consensus that they are post-mitotic cells, permanently arrested in G0 phase. Even though previous studies have hinted that this is not always the case (63,65,66,244), most of their claims were dismissed. Their main problems were lack of reliable imaging or prof that the cells depicted were true adipocytes. In some cases it was unlikely to exclude contamination from other proliferating cell types. Moreover, even though there were reports that ^{14}C Thymidine or BrdU can be incorporated into adipocyte fraction cells' DNA extremely fast, only 30 minutes (65) and 6 hours (66) respectively, critics pointed out that it is impossible to exclude that the incorporation didn't happen in pre-adipocytes that were directly differentiated or that the incorporation was part of DNA repair.

In this study, however, there was a more meticulous approach into directly imaging multiple cell cycle markers in adipocytes by using immunocytochemistry techniques combined with confocal microscopy or confirmatory and complimentary immunohistochemistry. With this approach we were able to depict human primary adipocytes expressing cell cycle markers, including Cyclin D1, Cyclin E1, Cyclin A2, Cyclin B1 (cytoplasmatic), PCNA, ki67, anillin and pHH3 *in vivo*. Seven out of these eight markers were found in nuclei that were inside intact mature adipocytes imaged from top to bottom, so the possibility of foreign origin nuclei contaminating the samples can me dismissed in the imaging data. Finally, the 8th marker, Cyclin B1, was localized in adipocytes' cytoplasm in varying intensities, but not in the nuclei. Combined with the presence of anillin only in the nucleus and the dotty pattern of pHH3, it seems that adipocytes cannot progress normally into M phase. It is also really interesting to note that a very recent publication on the single cell mRNA analysis of mature mouse adipocytes, has shown that there is a cluster (type 11 adipocytes) expressing cell cycle markers (245). The presence of cell cycle in adipocytes would have been more certain if we had managed to stain the adipocyte for multiple cell cycle markers at once, however this was proven difficult to achieve. Nonetheless, it was shown that human adipocytes can be found *in vivo* expressing some of the most strategic proteins of cell cycle's interphase, thus providing a strong case for the presence of cell cycle.

The fact that mature adipocytes express cell cycle markers, however, is not a clear case for the entrance into cell cycle. Many of these markers are reported to have non-canonical actions as well, some pertinent to metabolism among others (246). For instance, the actions of CDK4 and CDK5 have been linked to the translocation of glut4 transporter, glucose transport and lipolysis (247). Moreover Cyclin D3, CDK4 and E2F have been linked with the activation of PPARγ, while Cyclin D1 with its repression, as reviewed in (248).

Some really interesting insights come from knock out (KO) and transgenic (TG) mouse models of cell cycle genes as well. The genetic ablation of cyclin D3 (249), has been linked to mouse phenotypes of resistance to high fat diet, decreased adipocyte mass, smaller adipocytes and better metabolic profile. Mice that are CDK4 KO have lower weight and atrophy of their adipocytes, while the complete opposite was true for the mice with constitutively active CDK4. This transgenic mice model, despite having its β-cells spared from the mutations and its insulin levels normal, presented with adipocyte hypertrophy and increased body weight (250). Finally, E2F1 KO mice are resistant to high fat diet induced obesity while E2F1 TG had the opposite response on this diet, even though the effect was solely attributed to adipogenesis (251) and β-cell function (252) by the authors. On the contrary, ablation of the cell cycle inhibitors p21 and p27 led to increased adipose tissue mass (253), with other studies giving the exact opposite results (254). While the whole body KO is not the best model to draw conclusions about mature adipocytes, the fact that the KO models have an effect on adipocyte size and hypertrophy acts as an indication for a possible action. With the data of this study in mind, however, we can suggest that these cell cycle proteins are not acting only through non-canonical actions in adipocytes, but in fact they regulate cell cycle as well. This would explain how inhibition of cell cycle progression (KO cyclin D3, CDK4 and E2F1) leads to smaller adipocytes, while overexpression (TG CDK4, E2F1) leads to cell cycle progression and adipocyte hypertrophy. There is a possibility, however, that the effects of cell cycle progression in mature adipocytes might be biologically significant only in the context of obesity and metabolic syndrome models. This would explain why in chow fed mice, the cell cycle arrest of mature adipocytes, with conditional p27 overexpression, did not elicit any significant effect on white adipose tissue (255).

In order to further our understanding on the nature of adipocyte's cell cycle, the isolated primary adipocytes of humans were put into suspension cell cultures. There, the cyclin D1 was chosen as a marker of cell cycle entry, since it is the first cyclin to spike during G1 phase and it can be found during G2 as well, with its expression spanning across most of the interphase (20). The data suggest that that human primary adipocytes can enter into cell cycle while they are kept for 7 days in the culture conditions. Moreover, the cell cycle entry is seems to increase with the supplementation of insulin. Parallel to these results, EdU incorporation into adipocytes' DNA was inspected *in vitro*, with this phenomenon acting as a direct proof of DNA replication and of passage through the S phase. In this study, human primary adipocytes were found to incorporate EdU into their nuclei, as it had been previously reported with the incorporation of ^{3}H or ^{14}C thymidine (63,65) and BrdU (66). The experiment was positive both for isolated cells and for whole tissue pieces, demonstrating that this is a phenomenon occurring regardless of the isolation protocol and the effects of collagenases. Similar to cyclin D1, EdU incorporation increased as the days of culture progressed and it was further enhanced with the addition of insulin into the culture media. Furthermore, the fact that CDK4/6 inhibitor, palbociclib, reduced the incorporation of EdU indicates that this phenomenon is part of the DNA replication. If the EdU incorporation was part of DNA repair, then palbociclib, a G1-arrest-inducing drug, would not have exercised such a robust inhibition. We can also dismiss that cells used in the cultures are

something other than mature adipocytes, since all cells counted were visualized with microscope and also the 7-days suspension culture would impede the survival of other cell types. For once, only pre-adipocytes can evolve to look like adipocytes, but the culture media lacked any of the drugs (glucocorticoids, 1-methyl-3-isobutylxanthine (IBMX), thiazolidinediones (TZD)) used for their differentiation into mature cells (256). Thus our data suggest that human primary adipocytes are able to enter cell cycle and pass through S phase in vitro, with the results being enhanced by the duration of the suspension culture and by the supplementation of insulin.

The fact that insulin acts as a mitogen and promotes DNA synthesis in different cell types is well established in literature, both in cultured cells (257) and during hyperglycemia in patients (258). Patients with established endogenous (259) or iatrogenic hyperinsulinemia (260) have increased BMI, mainly attributed to an increase in their fat mass (261). Moreover, serum insulin levels have a positive correlation with adipocyte size in patients (262) and this relationship becomes apparent in the case of lipohypertrophy (263), a skin nodular lesion characterized by a subcutaneous adipocyte hypertrophy located in the site of recurrent insulin injections (Figure 21 a, b). It could be then hypothesized that hyperinsulinemia that usually accompanies high fat diet (264) could be one of the contributors for the augmented ^{3}H thymidine incorporation in the DNA of adipocyte fraction cells found in rats after 7 days of high fat diet (HFD). The label incorporation (^{3}H thymidine) was allowed for 15-21 hours before rats' sacrifice and showed a 14-fold increase in for the epididymal fat pad and 8-fold increase for the retroperitoneal fat pad between HFD and chow fed rats (64) (Figure 21 c).

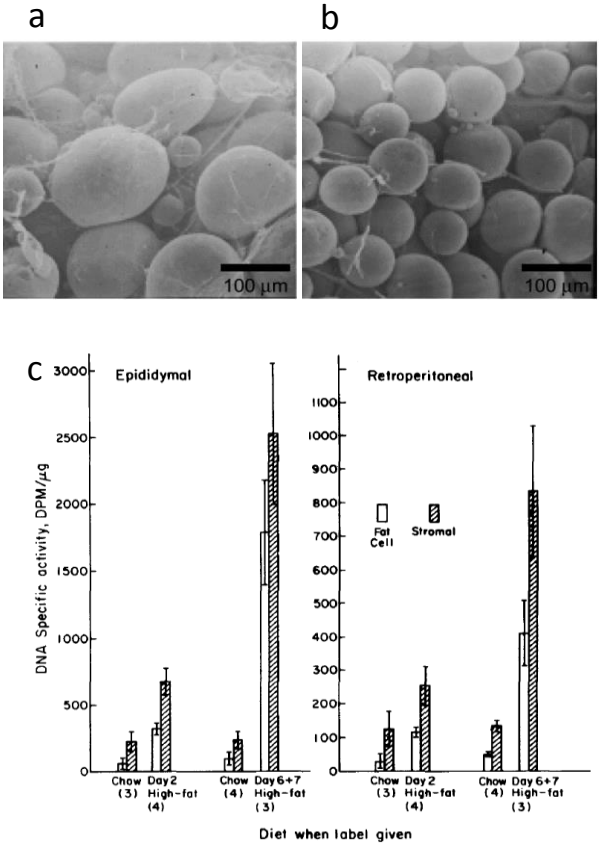


Figure 21:
Electron microscopy image of adipocytes from the (a) lypohypertrophy area and (b) the healthy adjacent adipose tissue, showing the great discrepancy in size.
Source: FUJIKURA J et al. Insulin-induced Lipohypertrophy: Report of a Case with Histopathology. Endocr J. 2005;52(5):623–8.
(c) Diagram of ^{3}H thymidine incorporation in the DNA of adipocyte and stroma vascular fraction (SVF) cells from rats on chow or HFD after a short pulse (15-21 hours). The rats were kept in 2 days or 7 days diet prior to the experiment.
Source: Klyde BJ, Hirsch J. Increased cellular proliferation in adipose tissue of adult rats fed a high-fat diet. J Lipid Res. 1979 Aug;20(6):705–15.

Even though it seems contradictory that adipocytes as fully mature post-mitotic cells can enter into cell cycle, this is not the first cell type to be reported to do so. Both neurons and muscle cells are known to enter into cell cycle during pathological conditions, even though they don't seem to complete the process with mitosis (265). In these cells, cell cycle re-entry has been linked to the pathophysiology of many neuromuscular disorders, like Alzheimer's, Parkinson's, inclusion body myositis and polymyositis, epilepsy, ALS, traumatic brain injury (TBI) and cerebral hypoxia-ischemia. Some of the drivers for cell cycle re-entry include oxidative stress (266) and hypoxia (267) that are established players in the pathology related to obesity as well (268). With these in mind it seems that further research in the adipocyte cell cycle entry could be of significant importance in order to determine if this is part of an adaptive mechanism or a cause for the obesity related diseases.

Part 2: Polyploidy of Human Primary Adipocytes

Having established that entry into cell cycle and passing through S phase is not excluded from the human primary adipocytes' biology, it was critical to investigate the potentials that this phenomenon would entail. Since we could not find any strong evidence that mitosis and cytokinesis take place, it is possible that adipocytes follow an endoreplication program instead of a classical cell cycle. Parallel to this, polyploidy arising from endoreplication comes hand in hand with cellular hypertrophy that is ingrained in adipocyte physiology and pathology. In order to investigate this hypothesis, this study laid the ground by developing and refining procedures that will aid future research on these fields.

More specifically, the focus of the first part was to refine the DNA staining method of adipocytes' nuclei. This refining was of utmost importance, as adipocytes' nuclei were not properly stained with classical methods. We can better view this in Fig 16 a page 50, where in a FACS experiment the adipocytes' nuclei appear to have DNA content between 2n and 4n. However, aneuploidy had already been excluded as a potential reason for staining disparities, thanks to "Comparative Genomic Hybridization (CGH) array". This technique has a relatively high resolution and can detect imbalances that are found in as low as 8% of all cells in the sample (269). So, we next hypothesized that there is a distinctive quality of adipocytes' nuclei that might be preventing the correct penetration and binding of the dye to the DNA, leading to these inaccurate results. This idea is reinforced from the fact that with small manipulations, like a 30 minute RNAse treatment, the measured DNA content of adipocytes but not that of PBMCs increased. Of course, it is not possible that the actual DNA increased in those isolated and sometimes fixed nuclei, but rather the phenomenon is explained by the fact that more of the DNA became available for staining.

It was found that a treatment of RNAse, pH>12, potassium salts in the mixture and propidium iodine DNA dye gave the best results out of all combinations. These results resonate with the working hypothesis that it is the extreme DNA condensation that leads to low stainability. RNA

acts as a vital mechanism in heterochromatin formation (128), so its lysis with RNAse treatment could potentially lead to DNA de-condensation. However, even if RNAse treatment has undoubtedly increased stainability consistently in our experimental setting, there is some literature which argues that RNAse treatment increases DNA condensation (270). Nonetheless, RNAse treatment is important when using dyes with affinity for both DNA and RNA (like PI and Draq5), so as to obtain accurate results (271). Alkalization of nuclear environment is also key for DNA de-condensation (134); however substantially high values are known to lead to DNA denaturation (272) and DNA loss, which was found to be partially rescued in the presence of ethanol (273). Another variable that should be taken into account is that with alkali denaturation single strand DNA (ssDNA) are produced and when they are stained (274) they could account for some confusing results. This can be somewhat balanced with the use of a good control (PBMCs). Finally potassium is recognized as the cation with the weakest ability to condense DNA when compared to other biologically significant cations (132), so it makes sense that its use would lead to better results.

Nevertheless, there are some ideas that are not quite consistent with the model of DNA condensation acting as the masking factor for adipocytes' nuclei. First of all, heterochromatin is the part of the DNA that gets strongly stained compared to euchromatin and not the other way around, while there are many experimental settings where condensed DNA is robustly stained, like in apoptosis (275). Additionally, DNA dye binding to DNA is stoichiometric, meaning that lower binding could be the result of DNA conformation variables. For instance, the intercalated ruthenium-compounds produce stronger luminescence when bound to triplex-DNA, Z-form DNA and B-form DNA, while they are the least intense when bound to A-form DNA (276). Moreover, there are publications from the early 80's, which demonstrate that terminally differentiated cells have lower stainability compared to their undifferentiated counterparts and this staining discrimination is more prominent when using intercalating dyes, than with minor groove dyes (277). According to Darzynkiewicz, the DNA of these differentiated cells was less accessible to the DNA dyes due to the interference of nuclear proteins and they managed to show that by extracting the proteins with 0.1M HCl they could not only increase nuclei stainability from +45% to 13 fold while testing 7 different dyes, but also achieve almost equal staining between the differentiated and undifferentiated cells (277) (Figure 22). Since adipocytes are fully differentiated cells they could fall into this category; while it is also important to note that other researchers have demonstrated the huge changes in stainability they can achieve, just by meddling with the environment of the nuclei. Lastly, what sets adipocytes used in these experiments apart from all the other adipocytes used in this study is the long refrigeration period at -80^{o}C. Adipocyte freezing and thawing is notoriously unsuccessful in keeping adipocytes viable, especially when cryopreservatives are not used (278), which might contribute to lower quality nuclei. Freezing and thawing has been implicated in higher-order chromatin structure defects as well (279).

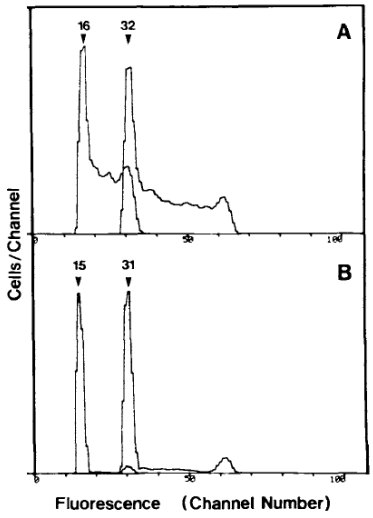


Figure 22:
Frequency distribution histograms representing fluorescence of FL cells stained with Propidium iodine. A) Exponentially growing cells before (peak value = 16) and after treatment with 0.1N HCl (peak value = 32). B) Differentiated cells before (peak = 15) and after extraction with 0.1N HC1 (peak = 31). (277)
Source: Darzynkiewicz Z, Traganos F, Kapuscinski J, Staiano-Coico L, Melamed MR. Accessibility of DNA in situ to various fluorochromes: Relationship to chromatin changes during erythroid differentiation of friend leukemia cells. Cytometry. 1984 Jul;5(4):355–63.

The second part on polyploidy was focused on recognizing a quick method to quantify chromosomes inside the nuclei. While our lab has already some data using RNA FISH, the results from them are not clear cut for every case. In order to obtain a different perspective on the quantitative measurement of chromosomes, the Barr body was employed as marker for the X chromosome. The Barr body is recognized as the distinct intra-nuclear structure arising from X chromosome's inactivation and it is usually present in all the cells with more than one X chromosome. While it is not a very accurate technique, it can be a quite easy and cost effective method to investigate polyploidy and it could be effortlessly adopted into a clinical setting, if future studies reveal any prognostic value in determining adipocytes' polyploidy. Most importantly in this particular study the presence of more than 2 Barr bodies in a female origin nuclei or the presence of at least one Barr body in a male one are enough evidence of polyploidy in adipocytes, just as a proof of concept.

The main problem that was encountered was the difficulty of faithfully distinction between the Barr body and other structures of the nucleus, namely the nucleolus. With this in mind, future experiments could focus on more specific staining methods, like the one described by Klinger (280), where RNA specific dyes can be used in order to distinguish between Barr body and nucleolus. Moreover, extra care should be given in order to distinguish Barr body from the senescent associated heterochromatin foci (SAHF) as well (281).

Finally, the third part of the study was devoted in finding a way of efficiently analyzing hypertrophic adipocytes on a single-cell level. For this purpose, freshly isolated adipocytes were analyzed using a MoFlow flow cytometer where a new strategy used in post analysis was able to pull out two populations that differ in the FSC scale. Interestingly enough, these populations are not only different in FSC, but they were found to include different percentages of IR positive and negative cells. The "low FSC" population was found to contain more or less equal amount of positive and negative cells, while the "high FSC" was constituted mainly of IR positive cells. More on the nature of these two IR populations will be discussed later on in the final part of "Discussion".

Since the use of FSC scale is the standard when analyzing relative cell size with flow cytometry (282), it is not baseless to assume that adipocyte populations with hypertrophy will pop up in the analysis as “high FSC”. However, in a recent paper from our lab, Hagberg et al describes the SSC scale as the one distinguishing the size between adipocyte in FACS analysis (231), even though this was conducted using mouse adipocytes. Nonetheless, it is important to contact FACS sorting of these populations in order to define the mean diameter for the cells and prove that “high FSC” represents hypertrophy.

It is also worth noting that the “low FSC” and “high FSC” populations were not distinguishable in all the adipocyte samples analyzed in the course of this study, however there are strong indications that the populations arise from specific machine operation methods and they are not related to the tissue quality. Although there is still a chance that these populations are an artifact, the clear and strong statistics for the distribution of the IR-positive and negative populations indicate that there is a basis for biological differences in adipocytes landing on different ends of FSC scale. At the very least, this analysis provides a perspective that IR-negative adipocytes are generally smaller than the positive ones, which should be further investigated as well.

Even though data from this study suggest that adipocytes can pass through S phase and thus double their genome size, there are still different theories that could explain this phenomenon of polyploidy other than endoreplication in differentiated adipocytes. Cell fusion, a cell-cycle independent pathway to polyploidy has been implicated in the adipocyte’s biology. The key points from this hypothesis stems from animal transplantation models, showing donor’s genome into the receiver’s adipocyte pool. However it is still debatable if bone-marrow derived cells from the donor differentiate into adipocytes, or if they fuse with them leading to polyploidy (283). Furthermore, we cannot exclude the possibility that endoreplication happens in pre-adipocytes and thus we end up with polyploid mature adipocytes that have not gone through endoreplication themselves. The 3T3-L1 murine cell line is central in the research of adipocyte differentiation, because with the right differentiation protocol the cells can transform from fibroblast-like phenotype to lipid filled cells (284). 3T3-L1 don’t only require mitotic clonal expansion in order to differentiate (285), but they were also found to become binucleated and polyploid during their differentiation and after the accumulation of lipids (67). Thus, further research is required in order to determine if the point of endoreplication is during pre-adipocytes’ differentiation, during mature adipocytes’ lifespan or if it can happen in both cases.

As closing remarks, it is important to note that the model of endoreplication complements really nicely the biological processes known to happen in adipocytes. First and foremost, polyploidy could be a fitting model to explain the mechanism behind adipocyte hypertrophy. According to our data on cell cycle, insulin is a major contributor in cell cycle entry and potentially endoreplication, which fits with literature that places the insulin pathway as central activator not only to the polyploidization of other cells, like hepatocytes (286,287), but also as a contributor to adipocytes’ hypertrophy (288). Moreover, polyploidization is found to develop in models of NAFLD as a response to extreme oxidative stress (289); with oxidative stress of adipocytes being

a well-established player in the pathophysiology of obesity and metabolic syndrome (290). It has even been proposed that polyploid cells have reduced metabolic activity and fitness, due to the biophysical constrains that a larger cell encounters due to intracellular distances and volume to surface ratio (107). All the above paint the image of endoreplication as being a part of adipocytes' response mechanism during the development of metabolic syndrome. The increase mitogenic signaling from insulin and overnutrition and the oxidative stress in the tissue correlate with the development of polyploidy and hypertrophy that could render the cells less metabolically active.

Part 3: Senescence of Human Primary Adipocytes

The fact that human mature adipocytes are susceptible to senescence is an important step in furthering our understanding of the pathologies related to obesity and metabolic syndrome. While adipose tissue as a whole has been involved in the development of senescence (195,196,200), there were no concrete evidence in the literature to suggest that mature adipocytes share this fate, due to their post-mitotic status. Thus, by proposing that mature adipocytes are able to partake into cell cycle, this study lays the foundation to investigate our hypothesis that human mature adipocytes can become senescent *in vivo* and *in vitro* and that this cell cycle entry is essential for the development of senescence.

Data from this study suggest that senescent adipocytes can be detected with the use of SA-β-Gal staining (162) that is found near the nucleus. By using isolated primary adipocytes the staining localization becomes clear and can't be attributed to contamination from other cells. More importantly the validation that these cells are indeed senescent comes from the use of two other markers of senescence. More specifically, by utilizing the fact that senescent cells are larger in size (161), it was shown that indeed SA-β-Gal positive adipocytes have greater diameter than the negative ones, on a single-cell level, with statistical significance reaching $p<0.0001$. Finally the levels of SA-β-Gal positive adipocytes are correlating with the absence of nuclear HMGB1 from cells of the same sample, another characterized marker of senescence (175). The fact that multiple markers of senescence were used strengthens our claim for the senescent condition of mature adipocytes.

Even though not a single marker is unanimously expressed in all senescent cell types, many agree that SA-β-Gal is one of the most valuable, citing it as the "gold standard" (291). It is also worth noting that even though this study is one of the first to describe the SA-β-Gal staining in mature adipocytes, it is not the first to detect it. In the literature there are papers discussing adipose tissue senescence, like Xu et al (198), Schafer et al (200), Ogrodnik et al (292) and Hickson et al (293), where the SA-β-Gal staining of the tissue shows mature adipocytes stained, among the other senescent cells of the tissue; even though they don't comment further on that (Figure 23). The fact that mature adipocytes from four different and independently published studies have a consistent pattern of SA-β-Gal staining, similar to the isolated mature adipocytes from this

investigation, strengthens the argument that this is a true senescence staining. Moreover, the combination of SA-β-Gal and cell size that was used in this study for the investigation of senescence in a single-cell level and *in vivo* setting has been proposed to be a reliable indicator of senescence (294). It is finally important to note that mature adipocytes with markers of senescence are larger in size than the negative cells, with regard to adipocytes' hypertrophy. This idea comes hand in hand with the literature that connects insulin resistance with adipocyte hypertrophy (295), as both conditions would fit the concept of senescence in adipose tissue. From this study it is not clear if hypertrophic adipocytes are prone to senescence, or if senescent cells are becoming hypertrophic, but it is certainly a valid question to investigate further upon.

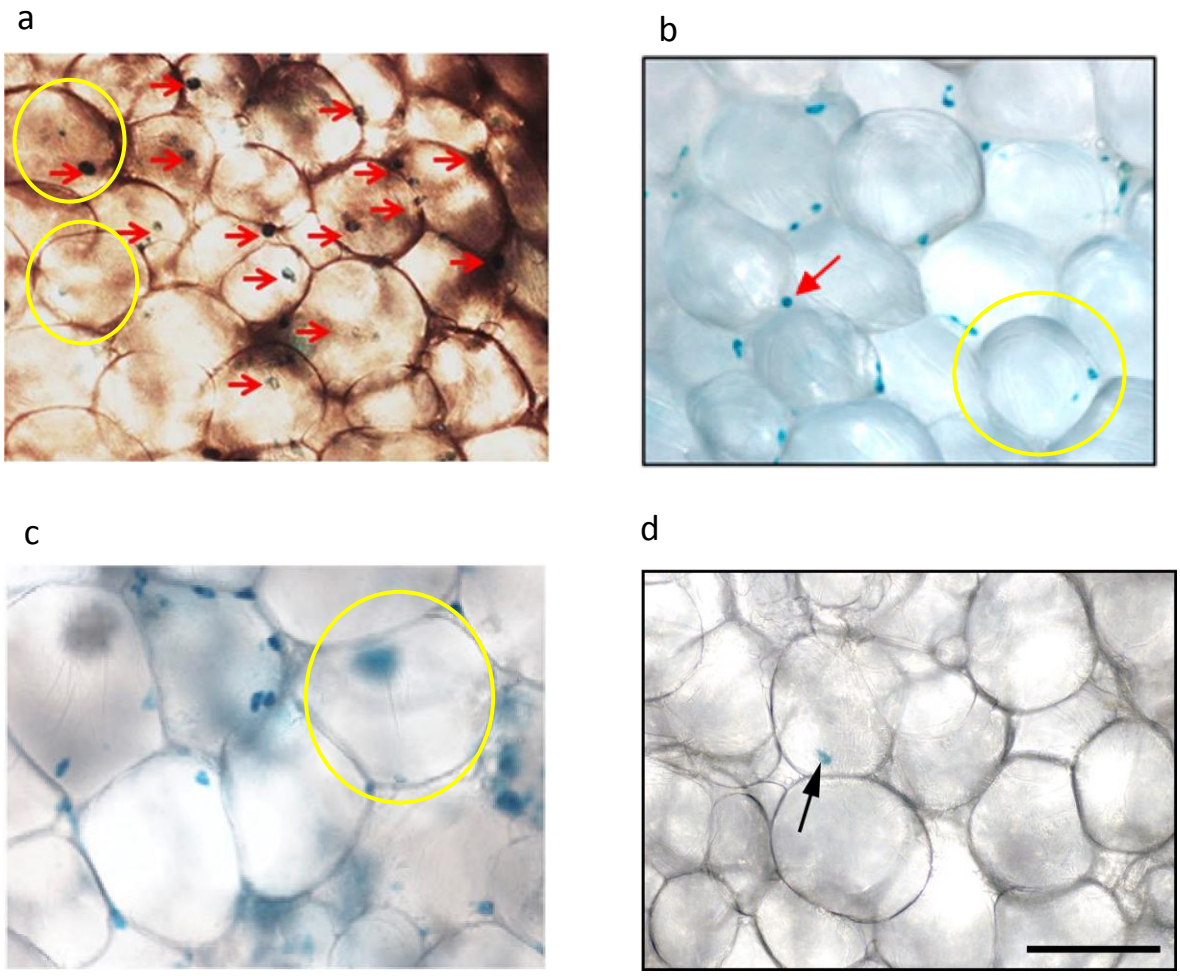


Figure 23:
Adipose tissue stained for SA-β-Gal. Some of the senescent mature adipocytes are marked in yellow circle.
(a) Source: (Supplementary material) Xu M, et al. Targeting senescent cells enhances adipogenesis and metabolic function in old age. Elife. 2015;4(DECEMBER2015):1–19.
(b) Source: Schafer MJ et al. Exercise prevents diet-induced cellular senescence in adipose tissue. Diabetes. 2016;65(6):1606–15.

(c) Source: Ogrodnik M et al. Obesity-Induced Cellular Senescence Drives Anxiety and Impairs Neurogenesis. Cell Metab. 2019 May;29(5):1061-1077.e8.
(d) Hickson LJ et al. Senolytics decrease senescent cells in humans: Preliminary report from a clinical trial of Dasatinib plus Quercetin in individuals with diabetic kidney disease. EBioMedicine. 2019 Sep;47:446–56.

Next, a cohort of 51 patients was prepared, in order to investigate the possible factors that contributed to the development of senescence in mature adipocytes *in vivo*. Coming as no surprise, it was the insulin sensitivity state of the patients that determined the level of senescence. Insulin resistance, obesity and diabetes have all been linked to the increased senescence burden in organisms (296). Moreover, the severity of insulin resistance, as described by the levels of c-peptide in the "obese unhealthy" patients, was the sole driving factor that described the heterogeneity of senescent adipocytes' percentage in this group.

It is also really noteworthy that "obese healthy" patients were spared from the increased senescent burden that the "obese unhealthy" and diabetic patients had. These results complement the point that "obese healthy" patients generally have hyperplastic obesity, while the "obese unhealthy" have adipocyte hypertrophy. From the literature one would suggest that the healthy adipose tissue is free to expand normally with adipogenesis (15). On the contrary a senescent adipose tissue would have been challenged with the low differentiation rate of senescent pre-adipocytes (206) and possible increased insulin resistance and hypertrophy from senescent adipocytes. Yet again, we can't be sure if senescence contributed to worsened metabolic profile, or the other way around, however, research on the elimination of senescent cells has uncovered that targeting these cells could result in improved metabolic profile and amelioration of symptoms from diabetes associated diseases (239).

Even though it should have been expected that the most affected patients were the diabetic ones, the results from this cohort put them in the same level as the "unhealthy obese". One should keep in mind that these patients are all treated for their condition with anti-diabetic drugs, like metformin, thus their results don't mirror the true effect of diabetes.

Lastly, age as a factor did not yield the expected correlations to senescence (297). This is probably the case, because the obese patients/donors undergoing bariatric surgery were significantly younger than the lean patients/donors that were enrolled from laparoscopic surgeries (gallbladder removal, hernia repair). The correlation with age could be possibly uncovered with the design of a larger cohort with metabolically healthy subjects and a wide spectrum of ages, so as to remove the confounding strong effect of insulin resistance.

Another important aspect of this study was how the senescence phenotype of mature human adipocytes could be manipulated by the entrance and progression to cell cycle. Insulin that was previously described as an important factor in inducing cell cycle entry and DNA repliction in primary human adipocytes is now shown to do the same thing with senescence. Interestingly enough insulin on its own has been implicated in the development of senescence in endothelial

cells in an Akt-dependent manner that promoted the p53/p21 pathway (298). Mirroring this study's results, a new publication highlights hyperinsulinemia as an instigator of neuronal senescence (299). According to this study, the age-related hyperinsulinemia in type 2 diabetes promotes insulin resistance in neurons. The pathways leading to this impaired glycolysis are also responsible for the reduced degradation of β-catenin, which translocates to neurons nuclei. This promotes neuronal cell cycle entry *in vitro*, with the elevation of cyclin D1; as a direct target of β-catenin; and continues with the induction of senescence a day later, as shown by increased SA-β-Gal, $p19^{ARF}$, $p16^{INK4a}$, PML and p21 among others. This paper is really relevant to this thesis, since it doesn't only show senescence as a consequence of hyperinsulinemia and failed cell cycle entry, but it does so in neuron cells that have been deemed as irreversibly post-mitotic as well.

Finally, this study delves into the effects of two readily available drugs, palbociclib and metformin, that can manipulate cell cycle at different points and investigates how human primary adipocytes' cell cycle entry, DNA synthesis and senescence get affected. With the use of metformin, mature primary adipocytes in the culture have an astonishing reduction of their Cyclin D1 levels, their EdU incorporation and the expression of SA-β-Gal. Moreover, patients with better metabolic profile as described by lower triglycerides, systolic blood pressure, cholesterol, LDL, Apo B, visceral adiposity index, insulin and C-peptide levels had a better response of their adipocytes to metformin treatment.

Metformin is one of the most prescribed anti-diabetic drugs worldwide (300) and a treatment option for conditions related to metabolic syndrome, like polycystic ovarian syndrome (301). Even though it is widely available and used for many decades, metformin's mechanism of action is quite complicated (302), with new potential therapeutic effects discovered to this day. For instance, several mouse models (303), clinical studies (304,305) and meta-analysis (306) have emerged showing that metformin has a potential anti-aging effect, that is not related to its glycemic control actions per se. The most probable mechanism for this effects is the inhibition of mTOR signaling pathway (307), that has been linked to longevity thanks to the actions of rapamycin (308). In regards to senescence, according to the geroconversion theory (188), mTOR and other mitogenic signals need to be present in cycle arrested cells in order for senescence mechanism to unfold. Thus, by inhibiting mTOR signaling, metformin rescues cells from senescence. Another possible explanation comes from the literature on the anti-cancer effects of metformin. It is shown that metformin can induce G0/G1 arrest by reducing Cyclin D1 levels on multiple cancer types (241,242,309–312). This ties up with this study's results, where cultured adipocytes are not able to enter cell cycle, as manifested by the really low Cyclin D1 levels and thus they can't enroll in a senescence program.

The fact that mature adipocytes have lower levels of senescence after the metformin treatment should probably not be interpreted as senescence reversal. It is more likely that senescent cells could not survive in the cell culture, while the healthy cells were protected from senescence with a different mechanism. The metformin concentration used in the 7 days culture was 5mM and it is generally acceptable in the literature. It has been also shown that pre-adipocyte derived

adipocytes can be viable in a 24 hours culture with metformin concentrations from 0.1 to 10mM without statistical differences in apoptosis between the groups (313). However, there is no real consensus in the literature about the therapeutic concentration for metformin (314) that could have acted as an indicator for the best concentration in the culture media. For example, in the mouse longevity experiment, metformin serum levels of 0.45 ± 0.09 mM promoted longevity, while the 5.03 ± 0.87 mM serum levels had the opposite effect, reducing the lifespan, probably due to toxicity (303). Moreover, concentrations of metformin as low as 5-10 mM have been linked to enhancement of apoptosis in cancer cell cultures (315–317). Without further research we can't be sure that mature adipocytes are not entering senescence because of the protective mechanisms of metformin via mTOR inhibition or G0/G1 arrest, or because they respond to cell cuclture stress and metformin concentrations with a different cell programs, like apoptosis. Nevertheles, our results are on par with the anti-diabetic effects of the drug, since the net effect was the actual reduction of senescence.

On the other hand, human primary adipocytes treated with palbocyclib had a diffetent response compared to the metformin treated. This time, while cyclin D1 levels decreased and there was almost no new DNA synthesis, the percentages of SA-β-Gal positive adipocytes were found much higher in the treated sample than in the paired control. Furthermore, this percentage in senescent cells was correlating with patients insulin levels, and the net effect of palbociclib was associated with patients' triglycerides, cholesterol, LDL, Apo B, visceral adiposity index and insulin levels. This makes sense, as patients with worse metabolic profile will probably have poorer results and will be affected more.

In the case of palbociclib it is much easier to pinpoint the biological mechanism behind its cell cycle and senescence effects. Palbociclib is a specific cell cycle inhibitor, targeting selectively the CDK4/6 that bind with cyclin D1 and are involved in the progression of G1/S checkpoint (243). Thus, this would explain the low cyclin D1 levels and the absence of DNA synthesis directly associated with a G1/S cell cycle arrest. Moreover, the mechanism leading to senescence is much easier to assumed, since palbociclib is a known promoter of drug-induced senescence and it has been already approved for breast cancer treatment for this reason (318). The systemic metabolic effects of chronic palbociclib exposure have been mainly attributed to its adverse actions in pancreatic β-cells homeostasis (319). However, there is also literature on the non-canonical action of CDK4 in adipocytes and how it is an important regulator of the insulin pathway, thus palbociclib might exert multiple effects in the adipose tissue, promoting insulin resistance (250). Lastly, it is worth noting that insulin media-supplementation leading to increased adipocyte senescence did not abolish cell cycle entry or DNA synthesis as palbociclib did. Thus, it would be safer to assume that *in vivo* the adipocyte senescence is linked to S phase or G2/M arrest in at least some cases of SA-β-Gal positive adipocytes. This comes to show that results of adipocyte senescence due to the use of palbociclib might differ from the *in vivo* mechanism.

As a final commentary on this section I would like to bring up the relationship between cellular senescence and polyploidy. It has been described in the literature that senescence and polyploidy

often coincide both in cancer and in normal tissues. Focusing on the non-cancerous cells, the effects of senescence on polyploid cells have been recognized in giant trophoblast cells, megakaryocytes, hepatocytes (101) and vascular smooth muscle cells (320), with the activation of the senescence program sometimes being actually vital for the proper function of these cells. For example, during the normal placenta development the trophoblasts that invade the endometrium undergo endoreplication and the development of senescence in these cells acts as a polyploidization-break. Disturbances in the senescence pathway, however, have been connected with the development of complete hydatidiform moles (80). Senescence has also been found to have a similar cell cycle-arrest effect in normal megakaryocytic differentiation and maturation, with the senescence program being absent in certain myeloproliferative neoplasms, like myelofibrosis (321).

In conclusion, human primary adipocytes are susceptible to the development of a senescence-like phenotype that correlates to a patient's metabolic status. This study makes a strong case about how the inappropriate cell cycle entry of adipocytes can be one of the drivers for the development of senescence. Whether, it is cell cycle, the paracrine effect of neighboring senescent cells, the shorter telomeres, the ROS or the DNA damage that contribute to senescence, it is important to explore it further in the future.

Part 4: The Insulin Receptor Expression in Human Primary Adipocytes

All throughout this study, insulin has been one of the most important drivers both for cell cycle re-entry and for the development of senescence. However, not all mature adipocytes responded to the same extend to insulin signaling. This begs the question if there is an intrinsic ability of these cells to resist to the effects of insulin. Unpublished data from our lab have confirmed the presence of heterogeneity between mature adipocytes and one of the most bizarre results was the occurrence of some INSR mRNA negative cells that were otherwise similar to the INSR positive cells. Thus it is tempting to theorize that this might be a true population that would give us further insights into the adipose tissue biology.

For the recognition of this population it was important to employ a single-cell based technique that distinguishes cells according to their protein level of IR expression. Thus, flow cytometry of mature adipocytes was employed, as described by Hagberg et al (231) and it indeed revealed the presence of an IR negative population. For the further investigation of IR negative population two cohorts were employed, one that checked the difference between matched subcutaneous (SC) and omental (OM) adipose tissue samples and one that evaluated its presence in the subcutaneous adipose tissue of obese patients with different metabolic profiles. The first cohort showed a tendency towards more IR positive adipocytes in the OM than the SC, but probably due

to the small number of patients there was no statistical correlation. The second cohort had some interesting results. The majority of the patients (12 out of 14) had IR (+) percentages that could correlate with their metabolic parameters like c-peptide, insulin levels and HOMA-IR index. The worse the patient's metabolic profile was, the lower the percentage of the negative IR cells. However, there was this small group of 2 people that despite their initially really high insulin levels they also seemed to have high percentages of IR (-) adipocytes.

This disparity in the results of the second cohort could possibly have two explanations. It could either be the result of the variability found in the human population, or it could be an indication of the limits of flow cytometry technique. In the first case, the difference will disappear with an increased sample size of the cohort, or it will be revealed that there is no true correlation. However, the second case might be the result of low sensitivity of flow cytometry with the antibody used. From the single cell data, two populations with extremely low INSR mRNA levels have emerged; the adipocytes that had close to no expression and were predominately found as a small population in the "healthy sub-cluster" and the adipocytes that had very low levels of expression and characterized the majority of cells in the "unhealthy sub-cluster". It wouldn't be a stretch to assume that adipocytes with unhealthy characteristics would be insulin resistant, which might lead to reduced IR levels as it has been shown in gestational diabetes (322). Thus, if the flow cytometry cannot distinguish between "absence" and "very low" levels of IR, it could lead to some conflicting results.

Regarding the results between OM and SC adipose tissue, the literature is split as well. Even though there is no published paper to my knowledge that specifically looks into the expression of IR of subcutaneous versus omental adipocytes in humans, it is possible to extrapolate some results from when the total amount of IR is used as a control for the phosphorylated state of the receptor. In general, there are studies showing an increase of total mRNA (323) or protein levels of IR (324) in the omental tissue, and others that could not identify any difference between them (325–327). Of course, the bulk amount of IR protein could not possibly distinguish between even distribution of IR among the adipocytes of the tissue or the presence of adipocytes with really high or low levels, however it could act as an indicator of how abundant this signaling pathway is. A difference in IR levels could also explain why visceral adipose tissue of humans is more responsive to insulin than the subcutaneous tissue (324).

Moreover, adipocytes with no insulin receptor protein expression have been found in the literature. Even in the most extreme case of IR knock out mice, the AIRKO, these mice still had some remaining adipocytes that were smaller than the control, like in the visceral fat (221) or the bone marrow adipocytes (222). It is not yet clear though if these cells will undergo fast delipidation, like the differentiated 3T3-L1 (225), thus representing a transient phase of some adipocytes biology, or if these are specific cells that can operate without the need of IR.

From the patients' cohort it seems that the percentage of these IR (-) cells correlates with patients insulin sensitivity markers and not with any of the somatometric measurements like BMI or total percentage of body fat. Thus one could assume that IR (-) cells could hold some metabolically

beneficial characteristics. In all the mouse models that the leptin levels were normal to begin with (217) or the levels were normalized after treatment (221,224), the adipocyte specific knock down of IR had no negative effects on metabolism for the mice and in one case it even led to longevity (218). Moreover, in the subcutaneous tissue of the tamoxifen induced AIRKO mice, while the expression of all the other genes related to insulin signaling were low, Ucp-1 mRNA levels were elevated both in the IR knock out and the IR/IGF1R double knock out mice (224). In addition, the patients suffering from less severe IR mutations are usually lean and have increased adiponectin levels (229). Even our own results from the analysis of this population reveal that there is a good chance that the IR (-) adipocytes belong mainly in the small-sized adipocytes and not in the hypertrophic population. Thus one could suggest that a small population of adipocytes with these characteristics might actually be beneficial. In the end, more research is needed in order to confirm this adipocyte population, once and for all, and investigate its unique identity and role in the adipose tissue.

Future plans

Cell cycle

This study provides some compelling evidence about the cell cycle re-entry of human primary adipocytes and the factors that promote it. However, the absence of multiple cell cycle marker stainings, due to the difficulties arising from working with adipocytes, and the absence of DNA synthesis / S phase passing evidence from *in vivo* experiments, keep us from a definite confirmation of the phenomenon. Since, for obvious reasons, humans cannot be used in experiments involving EdU incorporation or live *in vivo* microscopy, lineage tracing mouse models could be utilized in order to give a final proof. For instance, the AdipoChaser mouse (328) can be genetically engineered to permanently label all of its mature adipocytes created before a specific point in time and thus distinguish between newly differentiated and old adipocytes. Using this mouse model we can potentially conduct experiments, in an *in vivo* setting, like EdU incorporation and investigate the effects of insulin, high fat diet, overnutrition, growth factors and other drugs, without having a doubt that the cells stained are mature adipocytes and not newly differentiated cells. Moreover, the human primary adipocyte single cell data that have already been produced in the lab can be analyzed in a way that would arrange the cells in subpopulations, according to their cell cycle phase and their size, as described in (329).

Ploidy and Hypertrophy

Since the main focus of this study was to lay the ground for the investigation of polyploidy and its connection to adipocyte hypertrophy there are more to be done in this field in the future. More specifically attention will be given in three fronds: definite confirmation of polyploidy, accurate measurement of polyploidy and the role of ploidy in hypertrophy.

For the confirmation of polyploidy the first that needs to be done is a better Barr body staining that will differentiate it from nucleolus, using the technique described by Klinger (280). Even though site-specific RNA FISH did not give clear results, there are another FISC techniques that could be used in interphase nuclei (330), while even a hybrid FACS-FISH technique can be utilazed, where it’s the amout of flourescence and not the chromosome localization that determine the ploidy (331). Finally, as with cell cycle phases above, the single cell data from human mature adipocytes previously prepared in the lab could be put to use, in order to recognise cells with specific signature, as described for cells undergoing endoreplication (332).

Aside from confirmation of polyploidy, the accurate measurement of polyploidy in multiple cell could give us a chance to prepare a cohort of lean and obese subjects and better understand the correlation of the percentage and degree of polyploidy to patients' metabolic profile and obesity status. With this in mind we will first try to see if the de-masking protocol we prepared is enough for a colorimetric analysis of ploidy. This effort could be potentially improved by altering the nuclei isolation protocol, so as fresh adipocytes will be used instead of frozen ones. If all else fails, we can use the nuclei protein extraction protocol proposed by Darzynkiewicz et al (277).

Finally we can try to decipher what is the relationship between polyploidy and hypertrophy in mature adipocytes. In this case the two FSC differentiated populations of adipocytes will be sorted using MoFlow in order to find the mean diameter for each population and determine if we can do parallel analysis of hypertrophic adipocytes using FACS. If this goes as planned, then we can check the polyploidy of the shorted adipocytes and find any potential correlations. Lastly, from that point on we could further our analysis on the different factors that drive hypertrophy and assess them with FACS analysis (just like IR), or sort the cells for rtPCR and Western blot.

Senescence

The fact that mature human adipocytes can become senescent has great potential implications and it is worth investigating further. First of all, it would have been interesting to investigate all the other instigators of senescence and how they act on mature adipocytes, like ROS, mitochondria damage, DNA damage, short telomeres, cytokines etc. Moreover, it would have been really interesting to investigate the crosstalk between mature adipocytes and macrophages of different polarization or pre-adipocytes. The investigation of mature adipocyte SASP could act as a potential indicator for the senescent adipocytes' actions and communication with its environment.

Furthermore, many new questions arise regarding the effects of senescence on adipocytes' metabolism and insulin resistance. Through our current model, SA-β-Gal activity is found on fixed cells, however there are new fluorescent agents acting on live cells that might also come in handy for different experimental settings. Besides potential insulin resistance effects, the possible correlation with adipocyte hypertrophy should also be investigated further. For a really crude method of isolating adipocytes according to their cell size, cells can be isolated depending on the time they need to float on top of a liquid column, as described in (194,333,334). Thus we could very quickly check if the percentage of SA-β-Gal positive cells change according to their relative size.

Moreover there are still some experiments that would answer some questions directly coming from the data produced from this study. It would be really interesting to investigate the possible effect of metformin on adipocyte apoptosis, the effects of different metformin concentrations on senescence and what are the possible targets of metformin in adipocytes. Moreover, we can investigate the geroconversion theory. If the senescence comes as the combined effect of cell

cycle entry push and cell cycle arrest, by culturing cells with palbociclib (cell cycle arrest) in a charcoal-treated culture media (no mitogenic signal), there should theoretically be minimal senescence. In addition, it would have been important to research the characteristics of cells that enter senescence and the ones that don't; are there any factors protecting the adipocytes or is it a stochastic effect. For this line of work the single cell data could be analyzed further on this direction. Finally, the relationship between adipocyte senescence and the potential polyploidy should be further characterised.

IR positive and negative populations

The discovery of a new population of adipocytes that completely lack the insulin receptor and have potential metabolic benefits is a totally new concept. Thus it is really important to ensure that what the flow cytometry analysis suggests is true. One way to investigate that is to sort the IR positive and negative adipocytes so as to perform rtPCR and Western blot for the insulin receptor expression. Moreover, a second anti-IR antibody can be used that might be more sensitive in detecting possible differences between "very low levels of IR" and "absent IR" adipocytes.

After ensuring the quality of the methods used, it would be really interesting to expand the cohort to more patients and to paired samples for SC and OM adipose tissue, in order to delve deeper into the expression patterns and the adipose tissue heterogeneity. It is also important to check if this population is present in lean and diabetic subjects and thus if there is any benefit in manipulating this adipocyte population for therapeutic purposes.

Moreover, one must check the integrity of the insulin pathway in these IR (-) adipocytes and if it is still responsive to evaluate the other possible receptors that transduce the signal. One way to do this is through the analysis of the single cell data that could reveal if the IR (-) cells still have the newly discovered transcriptional effects of insulin receptor (335). Another way is to detect in flow cytometry the direct effects of insulin treatment in the adipocytes, for example by measuring the glucose uptake of fluorescent probes like 2-NBD-Glucose and how this differs between IR (+) and IR (-) adipocytes.

Finally it would be interesting to characterize this population and see if it indeed exerts any beneficial effects, or if it is a transient phase in adipocyte biology. The analysis of the single cell data can give a direction for the identity of these cells that can be later confirmed with flow cytometry analysis or rtPCR and Western after sorting them. In the literature there is already a microarray experiment conducted with the adipocytes of FIRKO mice that could give some guidance in regards to their identity as well (336). Lastly, one should check if these IR (-) adipocytes are prone to enter cell cycle of express markers of senescence, since the insulin signaling has been linked to the development of those conditions in the adipocytes.

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