CHARACTERIZATION OF ADIPOSE MESENCHYMAL STROMAL CELLS (ASC) IN AGING AND OBESITY

Margarida Maria Terrón Puig

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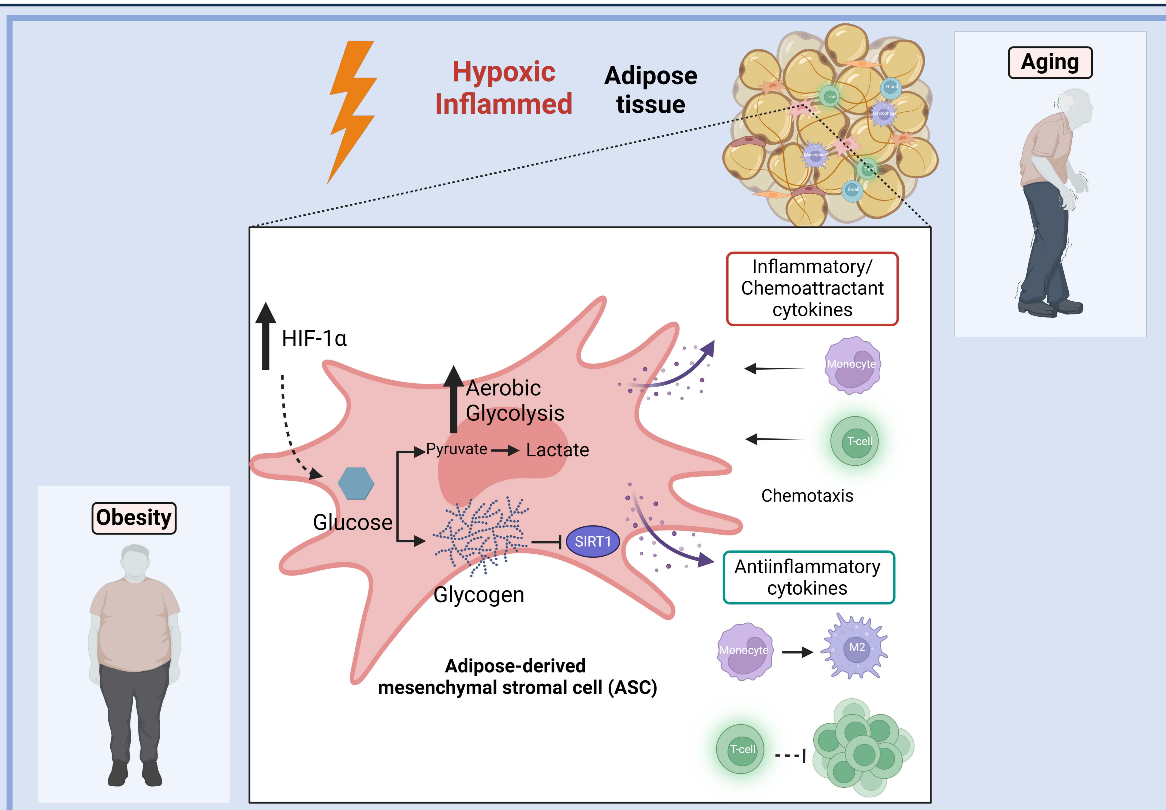
Characterization of Adipose Mesenchymal Stromal Cells in Aging and Obesity



MARGARIDA MARIA TERRÓN PUIG

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2024

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Characterization of Adipose Mesenchymal Stromal Cells in Aging and Obesity

DOCTORAL THESIS

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STATE THAT

The study entitled "**Characterization of Adipose Mesenchymal Stromal Cells in Aging and Obesity**" has been developed by Margarida Maria Terrón Puig under our supervision and fulfils the requirements to be eligible for the award of the degree of Doctor with international mention.

As evidence thereof to all appropriate effects, we sign the present document in Tarragona on December 14, 2023

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Com bé diu el poeta:

Nulla ethica sine æsthetica, nulla scientia sine lætitia

No hi ha ètica sense estètica, no hi ha ciència sense alegria

There is no ethics without aesthetics, there is no science without joy

Quan un científic mira una pedra
no veu tan sols un objecte contundent,
hi veu tot un entramat de molècules,
l'estructura tridimensional dels silicats,
l'acumulació ofegada de foraminífers.
Quan mira un arbre coneix el perquè dels vius colors,
la distribució espacial dels àtoms de clorofil·la,
les cadenes de carbonis asimètrics que li han donat vida.
Quan era un infant i es demanava el perquè
de la duresa de les roques,
el canvi del vi en vinagre,
per què la sobrassada torna blanca,
no sospitava la bellesa dels símbols,
el bell alenar del coneixement,
i que la mirada seria un acte de creació.
De la natura de les coses
cal extreure'n el plaer de viure.

Àngel Terrón Homar
Iniciació a la química (1977)
Tafal

When scientists look at a stone
they don't just see a blunt object,
they see a whole network of molecules,
the three-dimensional structure of silicates,
the drowned accumulation of foraminifers.
When they look at a tree, they know the origin of the bright colours it has,
the spatial distribution of chlorophyll atoms,
the asymmetric carbon chains that have given it life.
As children, they wondered why
rocks were hard,
why wine turns into vinegar,
why sobrassada131turns chalky white,
they did not suspect yet the beauty of symbols,
the sweet breath of knowledge,
and that gazing at things could be an act of creation.
from the nature of all things.
the pleasure of living must be gleaned

Rafael Peñas Cruz
The Poetry of science (2021)
Goat Star Books

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ABBREVIATIONS

ALP: Alkaline phosphatase
AMPK: 5'AMP-activated protein kinase
ANG: Angiopoietin
APC: Antigen-presenting cells
ASC: adipose-derived mesenchymal stromal cells
AT: Adipose tissue
BAT: Brown adipose tissue
B cell: B lymphocyte
BDNF: Brain-derived neurotrophic factor
BER: Base excision repair
bFGF: Basic fibroblast growth factor
BMAT: Bone Marrow Adipose Tissue
BMI: Body Mass Index
CCL2: Chemokine (C-C motif) ligand 2/ **MCP-1**: Monocyte chemoattractant protein 1
CCL3: Chemokine (C-C motif) ligand 3/ **MIP-1α**: Macrophage inflammatory protein 1-alpha
CCL4: Chemokine (C-C motif) ligand 4/ **MIP-1β**: Macrophage inflammatory protein 1-beta
CCL5: Chemokine (C-C motif) ligand 5/ **RANTES**: Regulated on activation, normal T cell expressed and secreted
CCL7: Chemokine (C-C motif) ligand 7/ **MCP-3**: Monocyte-chemotactic protein 3
CCL8: Chemokine (C-C motif) ligand 8
CCL11: Chemokine (C-C motif) ligand 11/ **Eotaxin-1**
CCL17: Chemokine (C-C motif) ligand 17/ **TARC:** Thymus-and activation-regulated chemokine
CCL20: Chemokine (C-C motif) ligand 20/ **MIP-3α**: Macrophage Inflammatory Protein-3
CCL26: Chemokine (C-C motif) ligand 26/**Eotaxin-3**
CD40: Cluster of differentiation 40
CD74: Cluster of differentiation 74
CD80: Cluster of differentiation 80
CD86: Cluster of differentiation 86
CDKN2A: Cyclin Dependent Kinase Inhibitor 2A
Ck β 8-1: Ck β 8-1 protein
CM: Conditioned media
COL1A1: Collagen type I alpha 1 chain
COMP: Cartilage Oligomeric Matrix Protein
CTSB: Cathepsin B
CTSS: Cathepsin S
CXCL1: C-X-C motif chemokine 1/ **GROα**
CXCL2: C-X-C motif chemokine 2/ **GROβ**
CXCL5: C-X-C motif chemokine 5/ **ENA-78**: Epithelial-derived neutrophil-activating protein 78
CXCL9: C-X-C motif chemokine 9/ **MIG**: monokine induced by gamma interferon
CXCL10: C-X-C motif chemokine 10/ **IP10**: Interferon gamma-induced protein 10
CXCL11: C-X-C motif chemokine 11/ i-TAC: Interferon-inducible T-cell alpha chemoattractant
CXCL12: C-X-C motif chemokine 12/ **SDF-1**: Stromal cell-derived factor 1
FABP4: Fatty Acid Binding Protein 4
FASN: Fatty Acid Synthase

FFA: Free fatty acids
FLT-3 LG: Fms-related tyrosine kinase 3 ligand
GBE1: Glycogen branching enzyme
GCP-2: Granulocyte chemotactic protein 2
G-CSF: Granulocyte colony-stimulating factor
GDNF: Glial cell line-derived neurotrophic factor
GLB1: Galactosidase Beta 1
GM-CSF: Granulocyte-macrophage colony-stimulating factor
GS: Glycogen synthase
GSK3: Glycogen synthase kinase 3
GYS: Glycogen synthase
hASC: human adipose-derived mesenchymal stromal cells
HFD: High-fat diet
HGF: Hepatocyte Growth Factor
HIF-1α: Hypoxia inducible factor 1α
HK2: Hexokinase 2
HLA: Human Leucocyte Antigen
HLA-D: Human Leucocyte Antigen D
HLA-DM: Human Leucocyte Antigen DM
HLA-DR: Human Leucocyte Antigen DR
HR: Homologous recombination
IDO: Indolamine-2,3-dioxygenase
IFN-γ: Interferon gamma
IGF: Insulin-like growth factor
IGFBP-2: Insulin-like growth factor-binding protein-2
IGFBP-3: Insulin-like growth factor-binding protein-3
IGFBP-4: Insulin-like growth factor-binding protein-4
IL-1α: Interleukin-1-alpha
IL-1β/IL1B: Interleukin-1-beta
IL-2: Interleukin-2
IL-5: Interleukin-5
IL-6/IL6: Interleukin-6
IL-7: Interleukin-7
IL-8: Interleukin 8/ **CXCL8**: C-X-C motif chemokine 8
IL-10/IL10: Interleukin-10
IL-12 p70: Interleukin-12 p70
IL-13: Interleukin-13
IL-15: Interleukin-15
iNOS: Inducible nitric oxide synthase
IRS: Insulin receptor substrate
KLF4: Krüppel-like factor 4
LA: Lean adult
LDHb: Lactate dehydrogenase b
LE: Lean elderly
LIF: Leukemia inhibitory factor
LIGHT: Tumor necrosis factor superfamily member 14
LPL: Lipoprotein Lipase
MCP-4: Monocyte chemoattractant protein 4
M-CSF: Macrophage colony-stimulating factor
MDSC: Myeloid-derived suppressor cells

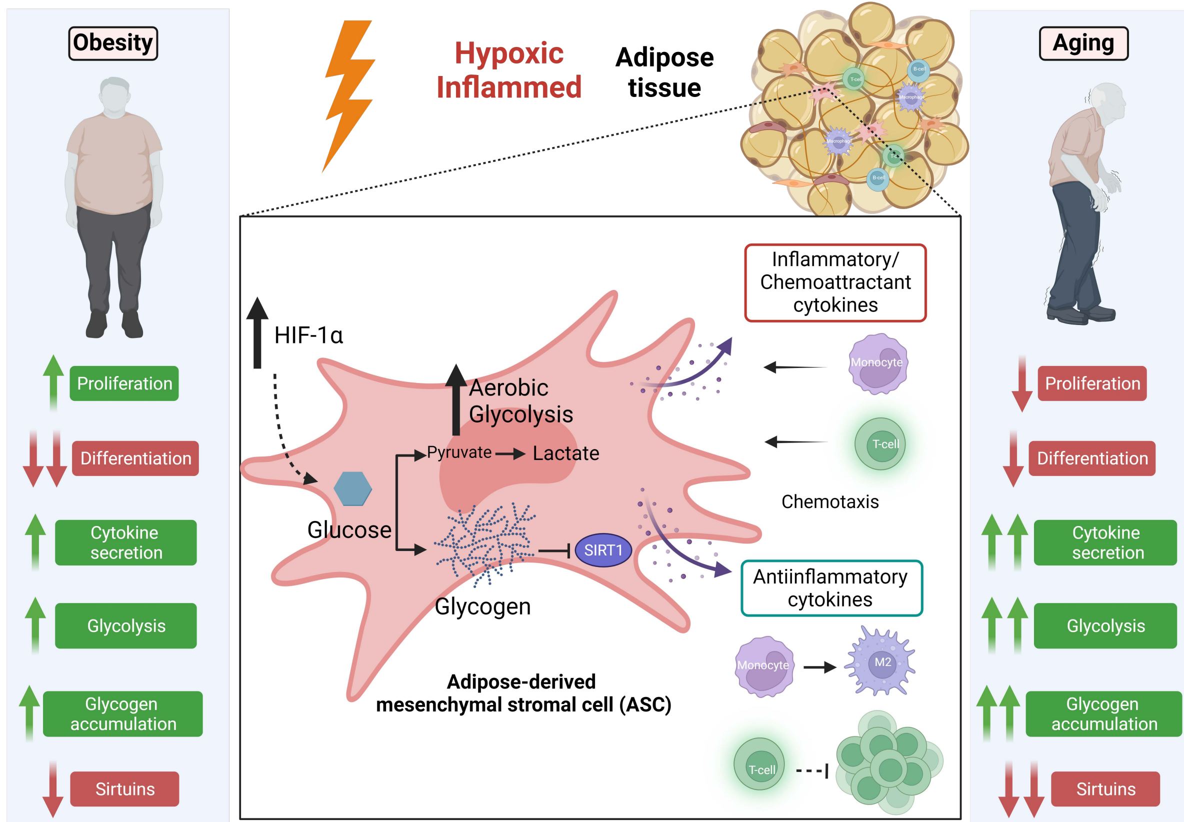
MHC-1: Major Histocompatibility Complex, Class I
MHC-2: Major Histocompatibility Complex, Class II
MIF: Macrophage migration inhibitory factor
MRC1: Mannose receptor c-type 1
MSC: Mesenchymal stromal cells
Myf5: Myogenic factor 5
NAFLD: Non-alcoholic fatty liver disease
NER: Nucleotide-excision repair
NF-kB: Nuclear factor-kB
NHEJ: Non-homologous end joining
NK: Natural killer cells
NO: Nitric oxide
NT-3: Neurotrophin-3
NT-4: Neurotrophin-4
OA: Obese adult
OE: Obese elderly
OGDH: Alpha-ketoglutarate dehydrogenase/ 2-oxoglutarate dehydrogenase
OPG: Osteoprotegerin
OPN: Osteopontin
OSM: Oncostatin M
OVA: Ovoalbumin
OXPHOS: Oxidative phosphorylation
p: phosphorylated
PARC: Pulmonary and activation-regulated chemokine
Pax7: Paired box 7
PBMC: Peripheral blood mononuclear cells
PBS: Phosphate-buffered saline
PDGF-BB: Platelet-derived growth factor
PDK4: Pyruvate dehydrogenase kinase, isozyme 4
PD-L1: Programmed death-ligand 1
PFKM: Phosphofructokinase M
PGE2: Prostaglandin E2
PGM-1: Phosphoglucomutase 1
PLIN1: Perilipin 1
PPARG: Peroxisome proliferator-activated receptor
PPP: Pentose phosphate pathway
PPP1R3C: Protein Phosphatase 1 Regulatory Subunit 3/ **PTG**: Protein targeting to glycogen
PYGL: Liver-type Glycogen phosphorylase
ROS: Reactive oxygen species
RUNX2: RUNX Family Transcription Factor 2
SASP: Senescence-Associated Secretory Phenotype
SAT: Subcutaneous adipose tissue
SCF: Stem cell factor
SDHb: Succinate dehydrogenase b
Sir2: Silencing information regulator 2
SIRT1: Sirtuin1
SIRT6: Sirtuin6
SLC2A3: Solute carrier family 2 member 3 **GLUT3**: Glucose transporter type 3
SLC2A1: Solute carrier family 2 member 1 **GLUT1**: Glucose transporter type 1
SPP1: Secreted Phosphoprotein 1

SVF: Stromal vascular fraction
T2D: Type-2 Diabetes
TCA: Tricarboxylic acid cycle
T cell: T lymphocyte
TGF-β: Transforming growth factor β
TGF-β1: Transforming growth factor β1
TGF-β2: Transforming growth factor β2
Th1: T-helper type 1
Th2: T-helper type 2
THPO: Thrombopoietin
TIMP-1: Tissue inhibitor of metalloproteinases-1
TIMP-2: Tissue inhibitor of metalloproteinases-2
TNF-α/TNFA: Tumor necrosis factor-alpha
TNF-β: Tumor necrosis factor-beta
TP53: Tumor Protein P53
Tregs: Regulatory T cells
UV: ultra-violet
VAT: Visceral adipose tissue
VEGF: Vascular endothelial growth factor
WAT: White adipose tissue
WHO: World Health Organization
WT1: Wilms tumor 1

ABSTRACT

Dysfunctional adipocyte precursors are increasingly recognized as significant contributors to the inflammation associated with aging and obesity, yet the underlying mechanisms remain unclear. Here, we explored the dysfunctional adipose tissue of elderly subjects and individuals with obesity, concentrating on the metabolic alterations in human adipose-derived mesenchymal stromal cells (hASC). These alterations may modify hASC's differentiation potential and immunomodulatory properties. We particularly examined the role of sirtuins as potential mediators bridging metabolism and inflammation. Our findings revealed that while both obesity and aging impaired the differentiation potential of hASC, their effects on proliferative capacity differed. We observed upregulated glycolysis-related genes in hASC from elderly individuals (≥65 years), a phenotype accentuated by obesity. This upregulation corresponded with increased lactate secretion and glycogen storage. Through multiplex protein profiling, we associated the metabolic switch towards glycolysis and glycogenesis with a transformation in the secretome pattern, which implied an elevated secretion of inflammatory-chemotactic and immunosuppressive cytokines. Furthermore, hASC isolated from obese and elderly settings, characterized by increased glycolytic flux and glycogen deposition, demonstrated an increased capacity to suppress T-cell proliferation and promote lymphocyte and monocyte migration. Additionally, these cells could polarize macrophages towards a mixed-M2 phenotype. Enforced glycogen deposition, through the overexpression of protein targeting to glycogen (PTG), resulted in the decline of the adipocyte differentiation potential and in the downregulation of SIRT1/6 protein levels. On the other hand, a decrease in the protein expression of SIRT1 and SIRT6 was evident in hASC isolated from obese and aged environments. Knockdown of SIRT1 in hASC from lean adults via siRNA increased the expression of proinflammatory and glycolysis-related markers and induced macrophage polarization towards a mixed-M2 phenotype, mirroring the behavior of hASC from elderly subjects and individuals with obesity. In conclusion, our data point to a glycogen-SIRT1/6 signaling axis as a driver of age-obesity inflammation and as a modulator of hASC functions. The metabolic reprogramming observed in hASC may be instrumental in modifying their secretome, thereby altering their immunomodulatory capacities. Like the effects of inflammatory priming or hypoxic environments, metabolic preconditioning could serve as an additional and potentially superior method for enhancing the immunosuppressive properties of hASC.

GRAPHICAL ABSTRACT



INTRODUCTION

1. INTRODUCTION

1.1. ADIPOSE TISSUE

1.1.1. PHYSIOLOGY AND COMPOSITION

The adipose tissue (AT) consists of adipocytes, cells within the stromal vascular fraction (SVF), connective tissue matrix, and nerve tissue[1]. Adipocytes account for 80-90% of the tissue volume, highlighting the central role of these within AT, and are characterized by a single large lipid droplet. However, most cells are found within the SVF despite its comparatively small volume. The SVF encompasses adipose-derived mesenchymal stromal cells (ASC) (which are the Mesenchymal stromal cells (MSC) found in the AT), preadipocytes, endothelial cells, fibroblasts, and immune cells[2] (Figure 1.1). ASC account for 15-50% of AT cells, signifying a substantial proportion of the cellular components in this tissue[3].

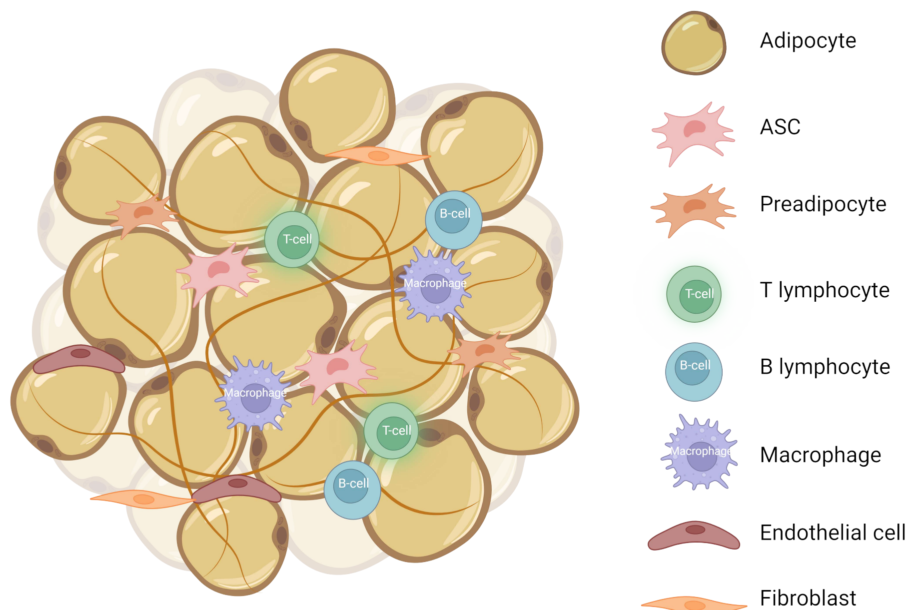


Figure 1.1 Adipose tissue composition.
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When excess energy is available, adipocyte hypertrophy (an increase in adipocyte volume) occurs, providing more space for storing triglycerides. Once adipocytes reach a specific size, hyperplasia (an increase in adipocyte numbers) is triggered, as hypertrophy alone cannot accommodate the additional triglycerides. These changes are the basis of the critical

fat cell size hypothesis, and both adipocyte volume and number dictate fat mass[4]. It is proposed that the number of adipocytes is determined mainly in early adulthood[4]. Nonetheless, an annual adipocyte turnover of approximately 10% is observed, allowing the replacement of dead adipocytes. New adipocytes arise from the differentiation of adipocyte precursors into mature adipocytes[4]. Hypertrophic expansion of adipocytes is considered the primary mechanism for lipid storage in adults[5], with adipocyte number remaining constant when body weight is stable[4]. Fat reduction is typically attributed to a decrease in adipocyte cell size rather than a decrease in adipocyte number[6]. It has been proposed that once the number of adipocytes increases, it never reverts to the original level, meaning that adipocyte numbers can only expand[6].

1.1.2. FUNCTIONAL ROLES OF ADIPOSE TISSUE

AT is as a major energy storage hub, primarily for triglycerides, making it a crucial long-term energy reserve in mammals[7,8]. The tissue adjusts its activity based on substrate availability, storing or releasing triglycerides as needed. AT also provides physical protection, insulating and cushioning internal organs[9]. Brown AT contributes to thermoregulation by burning fatty acids to generate heat in response to cold conditions—a process known as thermogenesis. Moreover, subcutaneous AT creates an insulating barrier to prevent heat loss[10].

Significantly, AT is a major endocrine organ, capable of receiving signals from the central nervous system and hormones and producing hormones such as leptin and adiponectin that trigger systemic effects[1,11]. The mediators generated by adipocytes and non-adipocyte fractions, known as adipokines, respond to energy status changes[9]. It has been described that adipocytes account for 10% of adipokine secretion, the remaining 90% being secreted by non-adipocyte cells[12]. These AT-secreted factors can modulate metabolism[8], insulin resistance, and inflammatory signaling[13], influencing other organs such as muscles, liver, bone, and brain. For example, leptin, a hormone AT produces, regulates food intake and energy expenditure[14]. Its levels increase with AT mass and decrease with caloric restriction[12]. Adiponectin has anti-inflammatory properties, promotes insulin sensitivity, and aids in resolving inflammation[8]. Other hormones, such as plasminogen activator inhibitor-1 (PAI-1)[15], resistin, adipsin, and proteins of the renin-angiotensin system, are also produced by AT[1].

Furthermore, AT houses enzymes in the metabolism of sex steroid hormones and glucocorticoids, like 11βHSD1, which converts cortisone into cortisol. Although the gonads and adrenal glands are the main contributors to sex steroid hormones in the bloodstream, AT can also serve as a source[1].

Remarkably, emerging research illustrates the role of AT in immune modulation, wound healing, and tissue regeneration. Extended literature has described the fat body in invertebrates as fundamental for innate immunity[16]. Specifically, the fat body in Drosophila flies produces antimicrobial peptides and increases the production of cytokines when in contact with bacteria or fungi[17]. Moreover, the production of antimicrobial peptides by adipocytes after an acute infection has been described in mice[18]. The omentum (a VAT) is recognized in humans for its immune and regenerative properties, particularly in peritonitis or abdominal wounds. The omentum was called the "abdominal policeman" for these immunological and angiogenic features[19]. These properties are due to lymphoid clusters, known as milky spots in the omentum, that remind of secondary lymphoid tissues. The presence of leukocytes in the AT has to be taken as an indicator of its behavior as an immune organ[20]. Indeed, the AT can be considered a reservoir for memory T cells, therefore contributing to secondary immunity responses[21]. Harboring memory T cells is not the only mechanism by which the AT contributes to host defense, the synthesis and secretion of cytokines and chemokines that induce the migration and activate lymphocytes and macrophages and the secretion of antimicrobial peptides by adipocytes and leucocytes in the tissue also play a decisive role[18].

The remarkable plasticity o AT allows it to adapt and perform diverse functions in response to physiological and pathological changes, such as nutrient availability, temperature, beta-adrenergic tone, and tissue damage. This adaptation implies structural changes in the tissue, altering the proportions of different AT tissues. Cold exposure[22] or the practice of physical activity[23], for example, increases the number of brown adipocytes. However, this plasticity can sometimes have adverse effects[24]. Obesity exemplifies how sustained AT changes initially intended to store excess fat can eventually lead to tissue dysfunction.

1.1.3. TYPES OF ADIPOSE TISSUE

AT is a complex and heterogeneous organ encompassing the white adipose tissue (WAT) and the brown adipose tissue (BAT), with distinct composition and function. AT can be categorized by its location and origin, which influences its characteristics and functions. White and beige adipocytes originate from a paired box 7 (Pax7)-/ myogenic factor 5 (Myf5)- MSC, while brown adipocytes come from Pax7+/ Myf5+ MSC[25]. WAT is an energy reservoir of triglycerides with endocrine and immune functions, while the primary role attributed to BAT is as a thermoregulator, because of its capacity to dissipate the energy produced as heat in a process known as thermogenesis[26]. Adipocytes from WAT contain a single droplet, while adipocytes from BAT have multiple smaller droplets[27]. Beige AT emerges when white adipocytes turn into brown-like adipocytes after stimulus like exposure to cold[28]. Besides WAT, BAT, and beige AT, another type of AT within the bone marrow has been reported. The Bone Marrow Adipose Tissue (BMAT) accounts for 10% of the total AT volume. BMAT makes up for 70% of the bone marrow volume, and although it shares more similarities with WAT, many of its functions are still unclear[29].

The two main WAT depots -subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT)- differ in adipocyte size and number, vascularization, adipokine secretion, metabolic function, inflammatory cell infiltration, susceptibility to cell senescence and apoptosis[3,26], response to hormones like insulin and fatty-acid release[3]. The differences between SAT and VAT are due to differences in location and in ASC origin, as they appear to come from different mesenchymal lineages. Adipocytes from VAT (but not from SAT) come from ASC that expressed the Wilms tumor 1 (WT1), a mesothelial cell marker[30]. Apart from SAT and VAT, which constitute the two most abundant AT depots, there are other depots in the heart, in the kidneys, in the bone marrow, in the lungs and in the adventia of major blood vessels[8].

1.1.3.1.SAT

SAT represents 80% of all fat[4] and primarily functions as an energy storage and endocrine organ[26]. A larger quantity of ASC is present in SAT compared to VAT, leading to a predominance of hyperplasia in this depot[24]. ASC from SAT demonstrated enhanced adipogenic differentiation potential compared to VAT ASC[31], reducing the need for hypertrophy. Lower levels of hypertrophy in SAT result in diminished tissue inflammation[32]. SAT has lower inflammatory cytokine secretion and higher adiponectin levels than VAT[26] and is more insulin-sensitive, which reduces tissue lipolysis[26]. Accordingly, the SAT is recognized for its protective role against cardiovascular diseases[32].

1.1.3.2.VAT

VAT accounts for 10-20% of total fat mass, and one of its prominent roles is to protect the internal organs from trauma. The capacity of ASC present in VAT to differentiate into adipocytes is lower than that of SAT[31]. Adipocyte hypertrophy alters adipokine secretion patterns, resulting in low adiponectin and high inflammatory factors, contributing to low-grade systemic inflammation[33].

VAT accumulation is linked to metabolic disorders more than SAT[34], contributing to insulin resistance, diabetes mellitus, dyslipidemia, hypertension, atherosclerosis, hepatic steatosis

1.2. MESENCHYMAL STROMAL CELLS. ADIPOSE-DERIVED MESENCHYMAL STROMAL CELLS

1.2.1. DEFINITION AND FEATURES

1.2.1.1. Definition

MSC are multipotent precursor cells with fibroblast-like characteristics originating from mesoderma. Their differentiation potential is restricted to a limited number of mesenchymal lineage cell types, including adipocytes, osteocytes, chondrocytes, and others[35]. Despite the restriction of differentiation due to lineage boundaries, some MSC cases have been reported to differentiate into ectodermal origin neural cells[36]. They were first described in 1966 by *Friedenstein and al.,* who found adherent, colony-forming, fibroblast-like, non-hematopoietic cells in the bone marrow that could undergo osteogenic differentiation[37]. Although MSC can be expanded in vitro, they are not classified as "stem" cells due to their limited self-renewal capacity and inability to regenerate the entire tissue in vivo[38]. Typically, these non-differentiated cells are located perivascularly, adjacent to both arterial and venous vessels[32].

MSC can be easily obtained from different tissues, including adipose depots, bone marrow, and umbilical cord[35]. They were first isolated from bone marrow, but they have been found in a wide range of tissues like periosteum, connective tissue[39], muscle, AT, perichondrium[40], umbilical cord blood, fetal tissues[41], amniotic fluid[42] and placenta[43,44].

Among all of them, the AT provides a very accessible source of MSC. The MSC isolated from AT are called ASC[45]. The adipose depot from which ASC are isolated can influence their properties, potentially explaining differences between SAT and VAT.

1.2.1.2. Minimal criteria for identification

The “Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy” proposed a minimum criterion to identify MSC. This criterion stipulates that MSC must be plastic-adherent when cultured; they must express CD90, CD73, and CD105 on their surface and lack the expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and Human Leucocyte Antigen DR (HLA-DR); and they must show multipotent differentiation to adipocytes, chondrocytes, and osteocytes in vitro[46] (Figure 1.2). ASC differ from bone-marrow-derived MSC by expressing CD36 and lacking CD106 expression[47]. Thus, to verify MSC identity, it is essential to perform immunophenotyping

using flow cytometry and a selected panel of positive and negative surface markers that recognize a specific MSC population, as established by the International Society of Cell Therapy and the International Federation for Adipose Therapeutics and Science). Note, some markers can change their expression in vitro, like the CD34, which is positive in ASC in vivo or in SVF but becomes negative in ASC in the late stages of culture[47].

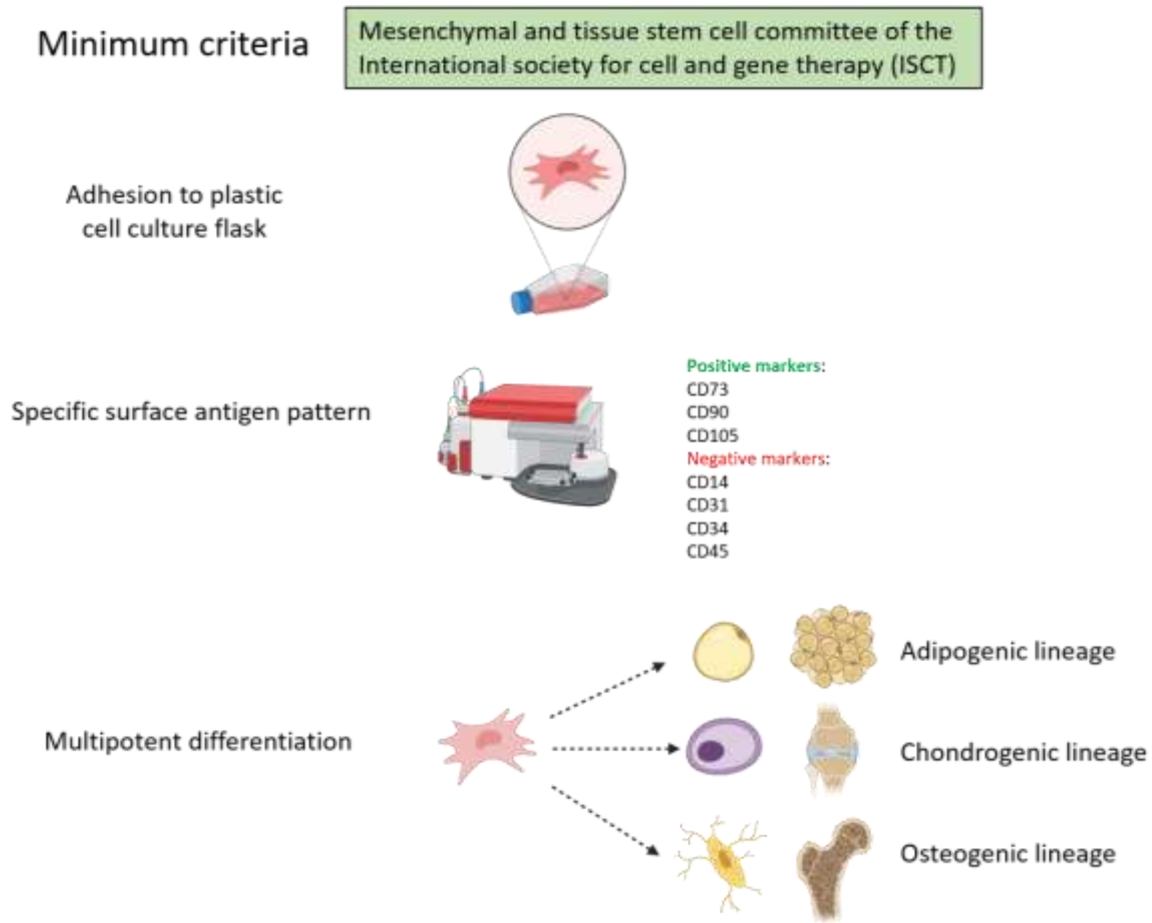

Figure 1.2. Minimal criteria that MSC must fulfill in vitro to be identified as MSC. Created with *BioRender.com*

Hypo-immunogenicity

One of the significant advantages of MSC is their hypo-immunogenic nature, meaning they generally do not provoke an immune response when administered. This diminished immunogenicity arises from several mechanisms. This immune-privileged status is primarily due to their non-or very low expression of Major Histocompatibility Complex, Class II (MHC-II)/Human Leucocyte Antigen D (HLA-D) and the absence of CD80/86 and CD40 (costimulatory molecules needed for antigen presentation). Major Histocompatibility Complex, Class I (MHC-I) is expressed in their surface but at very low levels.

Consequently, T lymphocytes do not proliferate in response to MSC, as their antigens are not recognized due to negligible or low expression of MHC-II and MHC-I[48], but primarily due to the lack of costimulatory molecules[49]. The presence of Interferon-gamma (IFN-γ) induces the expression of MHC-II and CD40 in ASC[50]; but it still does not trigger an alloreactive lymphocyte response[51]. On the other hand, MSC are resistant to the lysis induced by cytotoxic CD8+ T cells[52], suggesting a mechanism by which MSC may evade the immune system. Natural killer cells (NK) generally do not target MSC; however, upon activation, NK cells may exhibit alloreactivity[53].

This hypo-immunogenic characteristic of MSC is critical, implying that non-autologous/allogenic MSC could be used broadly without safety concerns. There will not be a requirement for Human Leucocyte Antigen (HLA) matching, simplifying the process of developing an allogenic product that could be used for all patients, optimizing the production process, and providing logistic advantages.

1.2.2. FUNCTIONS

1.2.2.1.Precursors of adipocytes

They can differentiate into adipocytes, osteocytes, chondrocytes, and other connective tissue cells to generate fat, bone, cartilage and tendon. ASC, in particular, can differentiate into adipocytes, osteocytes, chondrocytes, muscle myoblasts[54], cardiomyocytes[55], endothelial cells[56], hepatocytes[57], pancreatic cells[58] and hematopoietic-supporting cells[59]. Specifically, the differentiation into adipocytes and osteocytes is a process that starts with lineage commitment and continues with the maturation of committed progenitors into mature cells[60]. The duration of the process is approximately 20 days. During adipocyte differentiation, Peroxisome proliferator-activated receptor (PPARG), Fatty Acid Binding Protein 4 (FABP4), Lipoprotein Lipase (LPL), Perilipin 1 (PLIN1) and Fatty Acid Synthase (FASN) expression is enhanced. For osteocyte differentiation, the expression of RUNX Family Transcription Factor 2 (RUNX2), Alkaline phosphatase (ALP), Collagen type I alpha 1 chain (COL1A1) and Secreted Phosphoprotein 1 (SPP1) is required, while for chondrocyte differentiation, Cartilage Oligomeric Matrix Protein (COMP) and COL1A1 transcription is triggered.

1.2.2.2.Regenerators of tissue

Their capacity to home to the damaged tissues and regenerate them has sparked huge interest. MSC have the capacity to migrate or "home" to sites of injury where their restorative functions are required, leaving their perivascular natural location[61,62]. They can sense damaged or inflamed blood vessels via the action of various chemoattractants. The expression of different receptors on their membrane allows chemokines, growth factors, and extracellular matrix to impact them and direct their migration to the injury site[63]. Additionally, MSC expressed receptors for molecules presented on the surface of activated endothelial cells: The Integrin a4/b1 serves as a receptor for vascular cell adhesion molecule 1 (V-CAM-1), and CD44 operates as a receptor for E-selectin. V-CAM and E-selectin bind to their receptors on the circulating MSC, attaching them to the damaged endothelium[64]. This critical feature assures that exogenously administered MSC will home to the targeted injury site in patients, as proved in animal models[65].

The current knowledge establishes two mechanisms through which MSC induce regeneration: immunomodulation and trophic activities[66,67]. The regenerative properties that MSC are endowed with have been traditionally attributed to their capacity to target the injured area, their engrafting and their differentiation into the mature cells of the tissue. But this theory is being challenged by the day. The fact that MSC can help repair non-mesodermal tissues, although they are unable to differentiate into the cells of the tissue, as they are not pluripotent cells (only multipotent), emphasizes the crucial roles of these cells besides their function as progenitors[38,62,68]. It is not an overstatement to say that MSC have an indirect action rather than a direct one, as their potential to stimulate the endogenous physiological repair mechanisms seems to be more important than their capacity to engraft in the inflamed area for a long time or even to differentiate into mature cells to replace the damaged tissue[62,63]. It has been proposed that their main action is to create a regenerative environment at the site of injury by secreting bioactive molecules involved in trophic, anti-scarring, chemoattractant, and immunomodulatory activities[63,66,67].

Evidence suggests that, in a clinical setting, the mechanism behind the anti-inflammatory and immunomodulatory actions of the MSC administered to a patient is their paracrine action on the patient's own MSC. The MSC engraftment seems to be temporary, and the chemotactic cytokines they secrete induce the migration of the patients' MSC to the site of injury. The soluble factors they secrete seem to be key factors in blunting excessive

inflammation and promoting the survival of the damaged cells. This has also been demonstrated by 3D decellularized structures replenished with MSC. The success of these structures relies on the fact that they provide an extracellular matrix, thus, a microarchitecture that serves as a scaffold to be colonialized by the donor's MSC first and by the patient's MSC second. Cells of interest (like MSC) can recellularize the structure before inserting it into the patient. These structures and the MSC first introduced in the scaffold are ultimately reabsorbed, meaning after some time, they are eliminated. The scaffold and the MSC are just a way to activate the endogenous reparation systems for tissue growth and regeneration.

Therefore, the MSC's secretome is essential to carry out their actions. Their secretome comprises cytokines, chemokines, growth factors, matrix proteins, miRNAs, and extracellular vesicles (EV)[45] that help them accomplish their function as tissue repairers and mitigators of excessive inflammation.

Specifically talking about AT, studies have revealed that ASC-conditioned media (CM) helps regenerate the damaged area with similar efficiency as ASC[69], corroborating this hypothesis. That's why increasing interest has been set on the soluble factors secreted by ASC rather than in the cells per se.

The contribution of ASC to the AT secretome has been proven to be essential. Modification of their secretome as a response to different situations (like obesity) changes the profile of the whole AT secretome[70,71]. The plasticity of the AT is partly attributable to the plasticity of ASC, namely their capacity to adapt their responses according to environmental factors. Various receptors on their surface, such as growth factors receptors, allow them to respond to diverse stimuli. For instance, hypoxic conditions stimulate the secretion of angiogenic factors by ASC, helping the survival of the tissue in ischemic conditions[72]. Nevertheless, this adaptability suggests that pathological conditions altering their niche can significantly affect their functionality and characteristics. This is evident in states such as obesity[73] and diabetes[74,75]. This must be taken into account when using these cells in regenerative medicine.

1.2.2.2.1. Trophic activities

The trophic effects include inhibition of apoptosis, promotion of angiogenesis by secretion of Vascular endothelial growth factor (VEGF), stabilization of new vessels by adopting the pericyte phenotype[76], and enhancement of proliferation and differentiation of tissue progenitors by secretion of mitogens[62].

The inhibition of apoptosis by the molecules secreted by MSC limits the extension of cell death. Intracarotid administration of MSC in murine models protected against ischemic acute renal failure by lowering apoptosis and renal injury. Moreover, these effects were mediated by paracrine mechanisms through VEGF, Insulin-like growth factor (IGF-1) and Hepatocyte Growth Factor (HGF), cytokines with antiapoptotic properties[77,78]. ASC express VEGF, HGF, and also Transforming growth factor β (TGF-β), basic fibroblast growth factor (bFGF), and Granulocyte-macrophage colony-stimulating factor (GM-CSF). The secretion of all of these cytokines (particularly of VEGF) by MSC is enhanced in hypoxic conditions, known to be a part of the first stages of tissue damage. Therefore, the ASC secretome helps conserve the tissue surrounding the damaged areas by limiting apoptosis and upregulating angiogenesis, which will compensate for the lack of oxygen in the tissue[78]. VEGF, Interleukin-6 (IL-6), and Monocyte chemoattractant protein 1 (MCP-1) act at the same time as anti-apoptotic, preventing the necrosis of the cells, and as pro-angiogenic, re-establishing blood supply[79]. In cases of cerebral ischemia, MSC can prevent programmed cell death in neurons through the secretion of neurotrophins and also promote the formation of vessels by the production of angiogenic factors like VEGF and Angiopoietin-1 (ANG)[80].

Another important trophic feature of MSC is their ability to support the differentiation of tissue progenitors. MSC can be found in bone marrow, where they regulate hematopoiesis by modulating the microenvironment of the bone marrow and becoming the supportive BM stroma[81,82]. ASC support hematopoiesis as MSC from BM do[83]. Studies have demonstrated that MSC support hematopoiesis in culture; in other words, MSC support the differentiation of hematopoietic progenitor cells[82]. Among the cytokines secreted by MSC responsible of this action there's Stem cell factor (SCF), IL-6, Macrophage colony-stimulating factor (M-CSF), Granulocyte colony-stimulating factor (G-CSF), GM-CSF, C-X-C motif chemokine 12 (CXCL12)/ Stromal cell-derived factor 1 (SDF-1)[63]. They are essential for hematopoietic cell transplantation, as they promote engraftment of autografts[81]. Studies showed that MSC's CM promoted the differentiation of lung progenitor cells into distal lung epithelial

cells[84]. In addition, MSC are thought to be responsible for the recruitment and support of neuronal precursors after ischemic brain injury. Again, MSC undertake their function by secretion of soluble factors like SDF-1, ANG, and Brain-derived neurotrophic factor (BDNF)[80,85,86]. These neurotrophins not only promote the survival of neurons; they also induce differentiation of neural progenitor cells to form neurons at the site of injury[80].

1.2.2.2.2. Antiscaring

MSC have been shown to display anti-scarring activities, preventing the establishment of fibrosis. bFGF and HGF seem to mediate this effect in ischemic tissues[87]. MSC injections reduced cardiac fibrosis in a rat model of heart failure. In this case, another molecule secreted by MSC, adrenomedullin, was found to be responsible for the anti-scaring effects[88].

1.2.2.2.3. Chemoattractants

MSC promote the migration of cells to the site of injury by the secretion of a wide range of chemoattractant molecules: Chemokine (C-C motif) ligand 2 (CCL2)/ MCP-1, Chemokine (C-C motif) ligand 3 (CCL3)/ Macrophage inflammatory protein 1-alpha (MIP-1α), Chemokine (C-C motif) ligand 4 (CCL4)/ Macrophage inflammatory protein 1-beta (MIP-1β), Chemokine (C-C motif) ligand 5 (CCL5)/ Regulated on activation, normal T cell expressed and secreted (RANTES), Chemokine (C-C motif) ligand 7 (CCL7)/ Monocyte-chemotactic protein 3 (MCP-3), Chemokine (C-C motif) ligand 20 (CCL20)/ Macrophage Inflammatory Protein-3 (MIP-3α), Chemokine (C-C motif) ligand 26 (CCL26)/ Eotaxin-3, C-X-C motif chemokine 1 (CXCL1)/ GROα, C-X-C motif chemokine 2 (CXCL2)/ GROβ, C-X-C motif chemokine 5 (CXCL5)/ Epithelial-derived neutrophil-activating protein 78 (ENA-78), C-X-C motif chemokine 8 (CXCL8)/ Interleukin 8 (IL-8), C-X-C motif chemokine 10 (CXCL10)/ Interferon gamma-induced protein 10 (IP10), C-X-C motif chemokine 11 (CXCL-11)/ Interferon-inducible T-cell alpha chemoattractant (i-TAC) and CXCL12/SDF-1[89]. These molecules trigger the migration of monocytes, neutrophils, eosinophils, basophils, T cells, B cells, NK cells, dendritic cells, and hematopoietic and endothelial progenitors.

1.2.2.2.4. Immunomodulators

Their actions as immunomodulators, regulators of the immune response, provide them the capacity to regenerate damaged or aged tissue and explain how they escape the immune system. The modulation of the local immune response occurs through the secretion of

molecules and by cell-to-cell contact mechanisms. T lymphocytes (T cells) and B lymphocytes (B cells), regulatory T cells (Tregs), dendritic cells, and NK cells are involved in the regeneration of the tissue[90–92]. Although MSC are known for their role in the resolution of inflammation, they exert inflammatory actions in the early-stages of inflammation[93]. MSC secrete chemotactic cytokines to recruit neutrophils to the site of injury. The anti-inflammatory MSC actions are triggered when a certain level of TNF-α and IFN-γ in the environment is reached[94,95]. MSC primed by this inflammatory milieu can now display their immunosuppressive properties by the secretion of soluble factors. Indolamine-2,3-dioxygenase (IDO), Prostaglandin E2 (PGE2), and TGF-β are the three main molecules accountable for these properties[93].

Several reports have long demonstrated MSC's immunosuppressive properties, mainly by the induction of an anti-inflammatory and tolerant environment[49]. MSC are known to suppress in vitro the proliferation of T-cell and B-cell previously stimulated with mitogens like Phytohematoaglutinin (PHA) or in a mixed lymphocyte reaction (MLR)[48,96]. They also limit the secretion of IFN-γ by T-helper type 1 (Th1) lymphocytes and stimulate the secretion the secretion of IL-4 by T-helper type 2 (Th2) lymphocytes[49]. MSC-secreted PGE2 is responsible for T-cell suppression because when levels of PGE2 secretion are reduced, T-lymphocytes suppression is blunted[49]. Moreover, MSC increase the secretion of IDO to reduce T-lymphocyte proliferation[93].

Moreover, MSC are responsible for the induction of CD4+CD25+FoxP3+ Tregs[97]. On the one hand, they stimulate the proliferation of Tregs by secreting Transforming growth factor β1 (TGFβ1), IDO and IL-6. On the other hand, they stimulate an increase in the number of Tregs in an indirect manner, when MSC-induced M2 macrophages secrete CCL18[93].

They can also suppress NK proliferation and activation against mismatched HLA-expressing cells or against HLA-negative expressing cells (cells infected by viruses or cancer cells). They inhibit both the cytotoxicity and the cytokine secretion of NK cells[53]; specifically, they reduce the secretion of IFN-γ[49]. Cell-to-cell contact might be imperative for some of these effects, while the CM of MSC is enough for others. TGF-β and PGE2 are some soluble factors released by MSC that trigger the inhibition of NK cells[53].

MSC inhibit dendritic cells differentiation and maturation, impairing their action as antigen-presenting cells (APC), thus reducing their capacity to stimulate allogenic T-cell proliferation[98]. Moreover, they change the secretion pattern DC secretion, reducing the secretion of TNF-α by DCs type I and increasing the secretion of Interleukin-10 (IL-10) by DCs type II, thus modulating tolerogenic dendritic cells[49,97].

Furthermore, MSC modify the secretion pattern of macrophages, reprogramming them to secrete anti-inflammatory cytokines like IL-10[99]. PGE2, a cytokine involved in M2 polarization[93], is the soluble factor secreted by MSC that mediates the performance of this function[99]. ASC can switch the macrophage phenotype from an M1-proinflammatory phenotype towards an M2-anti-inflammatory one[100]. Studies show that subcutaneous ASC inhibit Interleukin-1-beta (IL-1β) expression in activated macrophages[101].

1.2.3. THERAPEUTIC APPLICATIONS

MSC gained attention in recent years because of their anti-inflammatory and regenerative properties, becoming a promising tool in the treatment of many diseases. MSC are classified as Advanced therapy medicinal products (ATMP), specifically under the subcategory of somatic cell therapy medicinal products (sCTMP). sCTMP encompasses viable cells or tissues that have undergone substantial manipulation, such as in vitro expansion and culture, leading to altered biological characteristics, physiological functions, or structural properties relevant for the intended clinical application or whose essential biological function is not the same in donor and recipient (non-homologous use)[102]. The feasibility of using allogenic MSC is crucial for industrial production, allowing for the ready availability of large quantities of "off-the-shelf" MSC suitable for any patient
The tolerance induced by MSC might help prevent and treat graft versus host disease (GVHD) following bone marrow transplantation, particularly in acute cases[49]. In addition, MSC can facilitate the engraftment of bone marrow transplants and prevent the rejection of MHC-mismatched skin grafts by inhibiting the allogenic response[103]. Allogenic MSC, such as ATMP based on bone marrow-derived MSC *Prochymal*, have gained conditional approval for treating pediatric steroid-refractory GVHD in New Zeland and Canada, and in Japan[81,102].

Due to their anti-inflammatory properties, MSC are also useful for treating inflammatory conditions. For instance, *Cartistem*, an ATMP based on umbilical cord-derived MSC, treats

knee cartilage defects caused by traumatic and degenerative osteoarthritis. They have also effectively treated Crohn's disease-related perianal fistulas and ulcerative colitis[104]. *Darvadstrocel (Alofisel)* is another therapy based on allogenic ASC expanded ex vivo, exhibiting anti-inflammatory and immunomodulatory properties, especially at the site of injurie or inflammation. Alofisel is an ATMP approved by the EMA, indicated for treating perianal complex fistulas in patients with luminal inactive or mild Crohn's disease who have not responded well to conventional or biological drugs[105]. The marketing authorization holder is Takeda Pharma A/S. It has been designated as an orphan drug and is reimbursed in Spain. MSC have also been tested in the treatment of autoimmune diseases like multiple sclerosis and systemic lupus erythematosus[106]. While the therapeutic potential of MSC is evident, considerable work remains in optimizing treatments and gaining a deeper understanding of the mechanisms behind their action. This could improve the efficacy of MSC currently used in clinical settings and foster the development of new strategies.

1.3. DISRUPTION OF THE ADIPOSE TISSUE BY INFLAMMATORY STATES AND METABOLIC DISORDERS

Many parallels between Obesity and Aging have been drawn regarding AT[107,108]. Changes in AT metabolism due to either expansion from obesity or senescence associated with aging can initially lead to localized inflammation, which ultimately evolves into a chronic systemic effect[109,110]. Certain stimuli in aged individuals and subjects with obesity can shift the secretory pattern of AT towards a more proinflammatory phenotype[111,112].

ASC play a pivotal role in these alterations in the AT secretion profile. Specifically, in aging and obesity, the cytokines and chemokines secreted by ASC can trigger inflammation in adjacent adipocytes[111,112], impairing lipid handling and comprising their functional integrity. Conversely, the secretion of some anti-inflammatory cytokines can also be amplified during aging and obesity[113]. This underlines the complexity of classifying ASC as inflammatory or anti-inflammatory concerning aging or obesity, as some of their characteristics may be compromised in an inflammatory milieu. In contrast, others may be stimulated by the same conditions.

1.3.1. PHYSIOLOGICAL SITUATION: AGING

1.3.1.1. Definition and features

With the global population aging, age-related illnesses such as cardiovascular diseases, cancer, or dementia are becoming increasingly prevalent[114], posing significant challenges to healthcare systems and associated economic concerns for governments.

The aging process is characterized by the accumulation of DNA damages[115], epigenetic alterations, diminished autophagy, disrupted cell-to-cell communication, alteration in protein homeostasis, Reactive oxygen species (ROS) accumulation, and exhaustion of stem cells[116,117]. A progressive decline in mitochondrial function is also acknowledged as a key aspect of the aging process[118].

1.3.1.2. **Pathophysiology of aging**

Aging is a gradual process involving ongoing deterioration of the body's biological functions. A low-grade inflammation is established due to cellular senescence and lack or exhaustion of progenitor cells[114].

The AT has a notable influence on longevity, as it is widely acknowledged that caloric restriction and other therapeutic interventions targeting the AT can extend lifespan[114]. Moreover, obesity, as the paradigm of AT dysfunction, might exemplify a case of accelerated aging. Similarities between aging and obesity (see next point for detailed information) include visceral adiposity, metabolic dysfunction, and systemic effects like insulin resistance and inflammation[114,119]. A dysfunctional AT characterizes both processes, though the origin of the dysfunction differs. While nutrient excess is the cause of obesity, the consequent deterioration of the biological systems due to the passage of time is the cause of aging. Aging triggers changes in the AT in abundance, localization, cellular composition, and endocrine signaling[120]. Age-related increase in body mass and body fat is associated with reduced insulin sensitivity in older individuals[121]. Simultaneously, there is a decrease in lean mass and bone mineral density[122]. Furthermore, fat redistribution occurs: SAT decreases while VAT increases, a pattern reminiscent of lipodystrophy syndromes. A physiological change accompanying aging is the increase in the number of white adipocytes, thus reducing the BAT proportion of AT[122]. Moreover, aging, as obesity, is characterized by ectopic lipid deposition. Due to the reduction in ASC's adipocyte differentiation potential, the AT cannot originate sufficient adipocytes to store free fatty acids (FFA) as triglycerides. This results in an overflow of FFA accumulating in other organs (liver, muscle, kidneys, pancreas, and heart), causing damage [123]. This lipotoxicity from excessive FFA can lead to conditions such as fatty liver disease, apoptosis of beta cells in the pancreas, and sarcopenia. The regenerative potential of the body is also affected by age. Many factors are involved in body aging and loss of regeneration potential, including the depletion or exhaustion of ASC. This reduction in ASC number and functionality relates to tissue aging and loss of homeostasis[124].

Like other tissue cells, ASC can undergo cellular senescence, characterized by irreversible cell cycle arrest in response to stress stimuli and resistance to apoptosis[117]. Senescent cells exhibit enlargement and test positive for beta-galactosidase. In addition, they secrete a specific mix of soluble factors known as the Senescence-Associated Secretory Phenotype

(SASP)[113]. As previously explained, unlike stem cells, ASC have a limited number of cell divisions due to the lack of telomerase, a reverse transcriptase that maintains the telomeres length in stem cells[117]. Therefore, their telomeres shorten with each division. DNA replication becomes impossible when the telomeres become excessively short, leading to ASC senescence and proliferation arrest. Other factors that can induce ASC senescence include DNA damage, metabolic stress, or oncogene activation[125]. Precisely, in the course of cellular aging, there's a progressive accumulation of DNA damage. Senescence is a defense mechanism that induces cell-cycle arrest to limit the proliferation of tumoral cells or cells that accumulate DNA damage[126]. Some tumor-suppressor proteins like p16, p21, and p53 are inducers of senescence, functioning as cycle-inhibitory proteins[117]. The AT is one of the organs with a higher accumulation of senescent cells[120]. Strategies like the blockage of p16, p21, and p53[126] or that of factors included in the SASP[127] have been put into place to counteract the effects of senescence, for instance, aging-related insulin resistance. Also, removing senescent cells from the AT can enhance adipogenesis from progenitors, allowing proper storage of nutrient excess and reducing inflammation and insulin resistance[127].

This dysfunctional AT abundant in senescent cells is accompanied by a low-grade chronic inflammation, termed "Inflammaging"[107]. Primarily, the SASP secreted by senescent cells induces inflammation in surrounding cells, and inflammation spreads through the tissue, ultimately resulting in a systemic effect. Indeed, one of the main contributors to the systemic inflammation that appears in aging is the AT[120]. Senescent cells of the AT (like adipocytes and ASC), macrophages (M1-phenotype), and other immune cells infiltrated in the tissue are responsible for secreting inflammatory mediators[114] .

Loss of function is associated with ASC senescence, as their intrinsic capacity for self-renewal is lost[117]. However, the proliferation arrest is not the most important effect of ASC cell senescence on tissue damage. Changes in their secretome have a greater impact on the tissue, as one senescent ASC can spread senescence to others in a paracrine mode[128]. The particular cytokines, growth factors, chemokines, and metalloproteinases that are secreted by senescent cells are responsible for the detrimental effects attributed to senescence[113]. The SASP results from activating transcription factors like nuclear factor-kB (NF-kB), mTOR, C/EBPβ, and GATA4 among others[129]. SASP comprises cytokines like IL-6, IL-8, IL-1, and CCL2. For instance, the SASP factors IL-6 and MCP-1 secreted by senescent ASC

recruit immune cells to the AT. As senescence spreads through the cells in the tissue and more cells acquire this phenotype, the microenvironment surrounding them all becomes inflamed. Sustained low-level inflammation is established in the affected tissue.

Aging predisposes the body to hormone resistance, insulin resistance being a prominent example. Indeed, the prevalence of type 2 diabetes is increased in the elderly population due to a combination of beta-cell dysfunction plays and insulin resistance[130]. Elevated systemic levels of proinflammatory cytokines lead to the imbalance of insulin signaling, particularly the Insulin receptor substrate (IRS)1/PI3K/AKT pathway, resulting in impaired glucose uptake in muscle and AT, diminished glycogen synthesis and increased gluconeogenesis in the liver, along with impaired lipid uptake and storage[130]. Increased leptin plasma levels are also observed in the aging population, leading to leptin resistance[131] as a counteracting effect, just like for insulin levels. On the contrary, adiponectin levels decrease in old age, which agrees with the decrease in insulin sensitivity[132].

All the features mentioned above are shown in Figure 1.3.

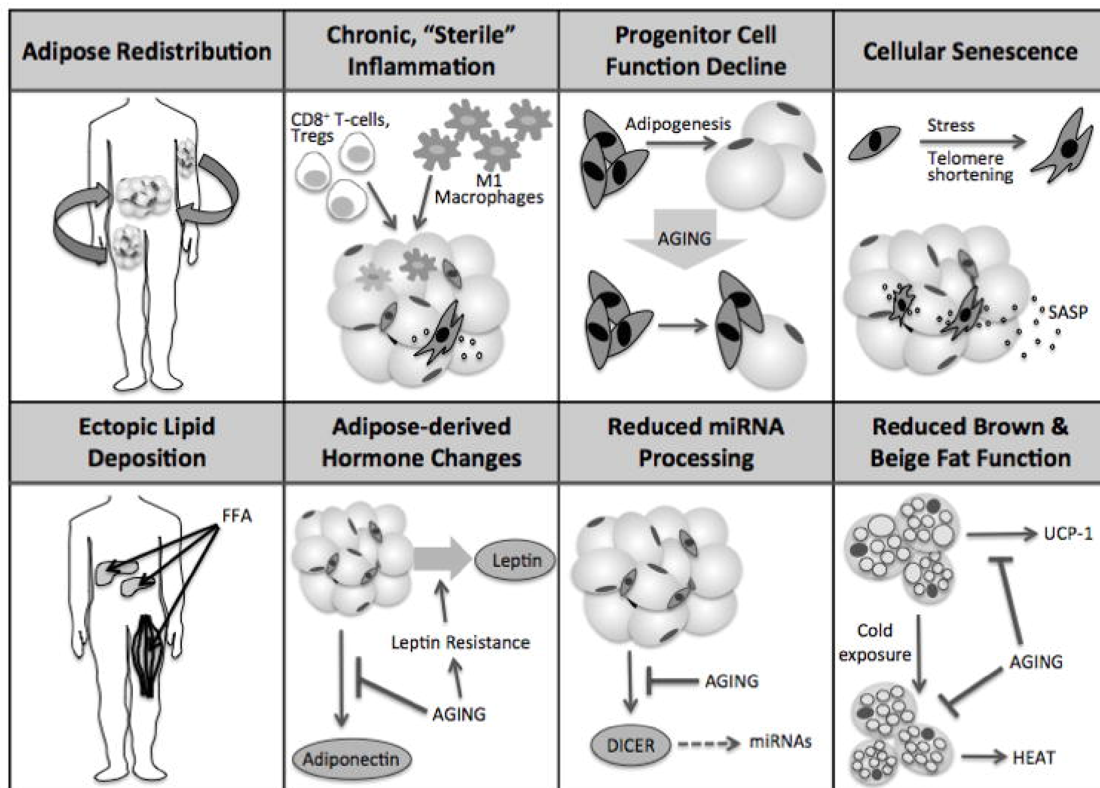

Figure 1.3. The aging effects on Adipose tissue (Palmer et al., 2016. *Experimental Gerontology*)

1.3.1.3. Influence of immune cells on inflammation and metabolism in Adipose tissue

Aging and obesity share biological hallmarks, like the presence of a dysfunctional AT. Hence, it is not unexpected that obesity and aging both show similar alterations in the immune cell population within AT, although some disparities also occur. Thus, there is an observed increase in the count of T lymphocytes in the AT of obese subjects. However, while obesity is associated with a decrease in the number of Tregs, aging is characterized by an increase in the AT Tregs[133].

The number of macrophages rises in the SAT depot of elderly subjects[134,135] and correlates with age and adiposity[136]. However, the total number of macrophages is unaltered in VAT of elderly subjects, contrary to what happens in obesity, where the number of VAT macrophages is increased.

What is expected in obesity and aging, for both depots, is the increased proportion of M1-phenotype macrophages (proinflammatory macrophages) compared to M2-phenotype macrophages (anti-inflammatory macrophages). Aging increases the M1/M2 ratio in both SAT and VAT, which accounts for the reduction of the M2 macrophages[133]. The macrophages isolated from elderly individuals increase the secretion of TNF-α, CCL2, and IL-6 (markers of M1-phenotype) and show a decreased expression of PPARG (marker of M2 phenotype), corroborating the decrease in the M2-macrophages number[133].

1.3.1.4. Contribution of SAT and VAT to systemic inflammation

Despite the increased body fat with age, the redistribution of AT (increase of visceral depot at the expense of subcutaneous depot) appears to be the key factor affecting the inflammation status[137].

The AT dysfunction that comes with age originates in the SAT depot. The telomere length shortens quicker in the cells in the SVF of SAT, than in VAT[138]. As previously mentioned, the shortened of telomeres is a trigger for senescence[125]. Accordingly, a higher senescence burden is found in SAT, a stimulus that induces the shift in the AT secretome towards a more proinflammatory phenotype[113,114]. As indicated, in the context of aging, macrophages accumulate in SAT (but not in VAT)[134,135], making this depot the first in which inflammation is triggered in the course of aging, acting like a sensor for the systemic changes that will follow.

1.3.2. PATHOLOGICAL CONDITION: OBESITY

1.3.2.1.Definition and features

Obesity is an excessive or abnormal accumulation of fat with multifactorial origin (biological, nutritional, genetic, environmental, and social) that represents a risk to health[139]. Metabolic-associated diseases comprise a heterogenous group of conditions that can appear to accompany obesity: atherosclerosis, hypertension, ischemic cardiovascular disease, dyslipidemia, non-alcoholic fatty liver disease (NAFLD), and type 2 diabetes (T2D), among others[140]. These diseases have overlapping mechanisms related to the chronic inflammation that obesity produces.

Obesity is a significant global health concern, as demonstrated by current epidemiology data (2016 statistics). It is estimated that nearly two billion people worldwide are overweight, and 650 million are obese. Over four decades, obesity prevalence has doubled, leading to a worrying situation where one in three individuals globally is classified as either overweight or obese. Although obesity affects all genders and age groups, it is slightly higher in women and the elderly. Particular attention must be paid to childhood obesity: 340 million children aged 5-19 are overweight or obese, and 39 million children under the age of 5 are overweight or obese. Moreover, the actual prevalence of obesity might be underestimated as it is typically assessed by the Body Mass Index (BMI). This measure does not account for other parameters such as fat distribution or the ratio of fat to lean mass[139,141].

1.3.2.2.Pathophysiology of obesity

Obesity is a systemic metabolic disease that appears due to an energy imbalance when caloric intake is superior to energy expenditure, characterized by excess adiposity and a dysfunctional AT.

One adaptation of the AT to excess fat is the hypertrophy of the adipocytes. Obesity predominantly leads to VAT depot enlargement through adipocyte hypertrophy[142]. This enlargement of the adipocytes allows them to store higher amounts of triglycerides. Another mechanism to store excess energy is hyperplasia, a rise in the number of adipocytes in the tissue. The number of adipocytes can increase thanks to the differentiation of ASC into new adipocytes[143]. The remodeling of the extracellular matrix helps accommodate the newly-formed adipocytes[144]. When the hypertrophy reaches its maximum, adipocytes cannot store

more FFA and triglycerides. As a result, fat accumulates in ectopic depots like the liver, kidneys, skeletal muscle, and heart, causing lipotoxicity[123].

Moreover, as the tissue expands, many adipocytes are not vascularized enough and are put in a hypoxic atmosphere that ultimately leads to necrosis and death of the adipocytes[145]. The excess of triglycerides, the pseudohypoxic state, and the products derived from adipocyte necrosis change the secretome of the adipocytes towards an inflammatory one, especially by the secretion of TNF-α and MCP-1, and these signals stimulate macrophage migration[9]. Necrotic adipocytes and macrophages create crown-like structures, significantly contributing to tissue inflammation[146]. The local inflammation is due to changes in the cell composition of the AT (structural, immune and vascular cells). Consequently, changes in the AT secretory pattern lead to a systemic chronic inflammation[147]. This systemic inflammation is exemplified by elevated IL-6 and C-reactive Protein (CRP) levels in the serum of subjects with obesity, which correlate with the development of type 2 diabetes[148] and other metabolic diseases.

Nutrient excess induces a type of inflammation different from the classical inflammatory response. Inflammation as a response to injury, based on swelling, redness, pain, and fever, is beneficial and necessary to trigger tissue repair. This type of inflammation is resolved in the short term. Metabolic diseases, such as obesity, lead to long-term inflammation that doesn’t show the features of classic inflammation and is commonly known as chronic or low-grade inflammation. Hotamisligil[149] referred to this inflammation triggered by metabolic surplus as “metainflammation” for the first time. The response to pathogens and different status of nutrient availability is done through common pathways. For instance, Toll-like receptors (TLR), a type of pattern recognition receptors (PRR), which are designed to detect pathogens or tissue damage, are also activated by nutrients, triggering the inflammation that initially was presumed to act against the pathogens[150,151]. The inflammatory outcome is the same independently of whether pathogens or nutrients activated the response system.

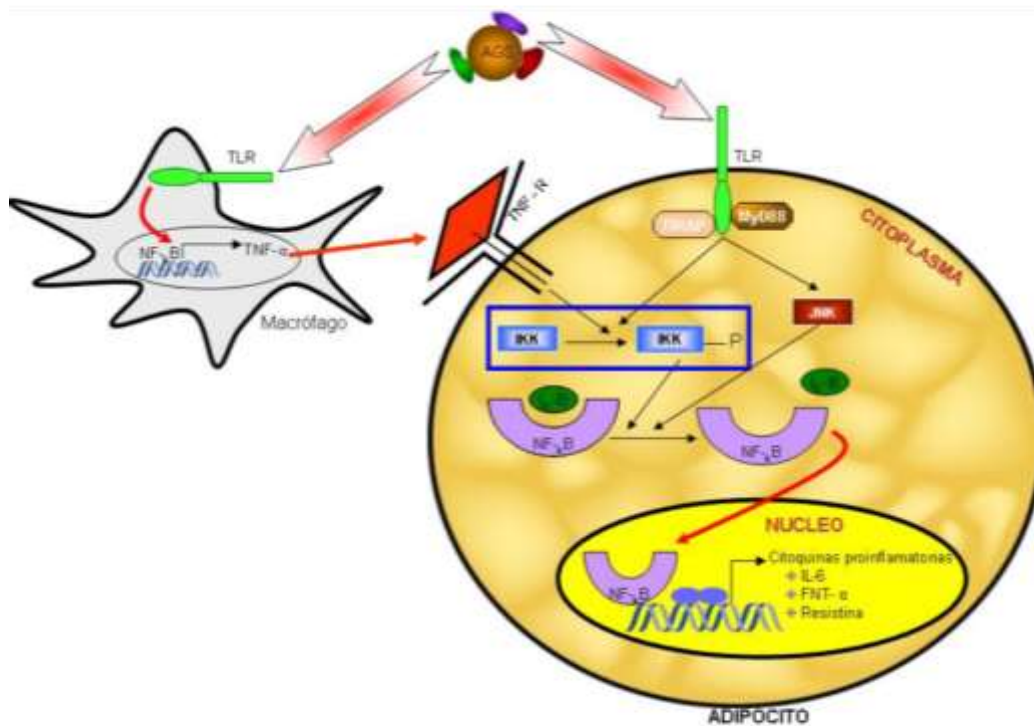

Figure 1.4. FFA binding to Toll-like receptors triggers inflammation in adipocytes and macrophages (Basain et al. 2016. *Medisan*)

These receptors are present on cells belonging to the innate immune system, like macrophages and dendritic cells, and they are activated to quickly respond to infections or tissue injury, mainly through inflammation. Although it's a beneficial mechanism to fight against pathogens and mediate tissue repair, it leads to inflammatory damage when it's excessively activated[152]. In the case of obesity, FFA, especially saturated FFA, trigger a not-needed inflammatory response by continuously activating TLR in macrophages, other immune cells, and adipocytes (Figure 1.4). Indeed, the expression of TLR was reported in adipocytes, and its expression is enhanced in obesity[152]. On the other hand, the activation of macrophages' TLR elicits the secretion of TNF-α, which, in turn, induces adipocytes to secrete inflammatory cytokines[152] and to develop insulin resistance. Both the direct activation of adipocytes' TLR and the activation of adipocytes' TNF-α receptors stimulate NF-kB, known to promote the transcription of genes that encode inflammatory cytokines (IL-1β, TNF-α, CXL8, MCP-1)[152]. Besides, the activation of NF-kB enhances the transcription of VCAM-1, which facilitates the adhesion of monocytes to the endothelium.

Moreover, when NF-kB is activated, the levels of MCP-1 are upregulated. MCP-1 regulates the migration of monocytes from the bloodstream through the vascular endothelium and the conversion to macrophages. This inflammation induced in adipocytes and ASC changes their secretion pattern, causing inflammation in the neighboring adipocytes, which, in turn, will impair lipid handling and exacerbate the metabolic imbalance[71]. Indeed, the inflammation caused by energy excess boosts the metabolic disturbance associated with obesity[32]. This response favors the fight against the pathogens, response that benefits from hyperlipidemia. That explains why the response to pathogens can modify the metabolic imbalance[153].

Insulin resistance developed in the adipocytes of subjects with obesity is the consequence of the attenuation of the insulin signaling cascade through different mechanisms[154]. On a molecular level, FFA or inflammatory cytokines like TNF-α (secreted either by macrophages or by surrounding adipocytes) induce the activation of inhibitors of kB kinase (IKK)[155] and c-Jun amino-terminal kinases (JNK)[156] pathways in adipocytes, which, in turn, prevent the activation of IRS (Figure 1.5). Specifically, JNK and IKKβ phosphorylate IRS1/2 on inhibitory serine phosphorylation sites (serine 307)[157], reducing the IRS tyrosine phosphorylation, with desensitization of insulin signaling as a result[158]. On the other hand, IL-6 lessens insulin signaling through its downstream Suppressor of cytokine signaling (SOCS) 3, which impairs the interplay between the insulin receptor and the IRS and also triggers the decomposition of IRS[149]. The reduction of the protein levels of insulin receptor and IRS, which is stimulated by the elevated insulin levels, can also lead to insulin resistance[154]. As a result of all these mechanisms, the insulin-mediated inhibition of lipolysis is impaired and increased circulating levels of non-esterified FFA are found in the bloodstream[159].

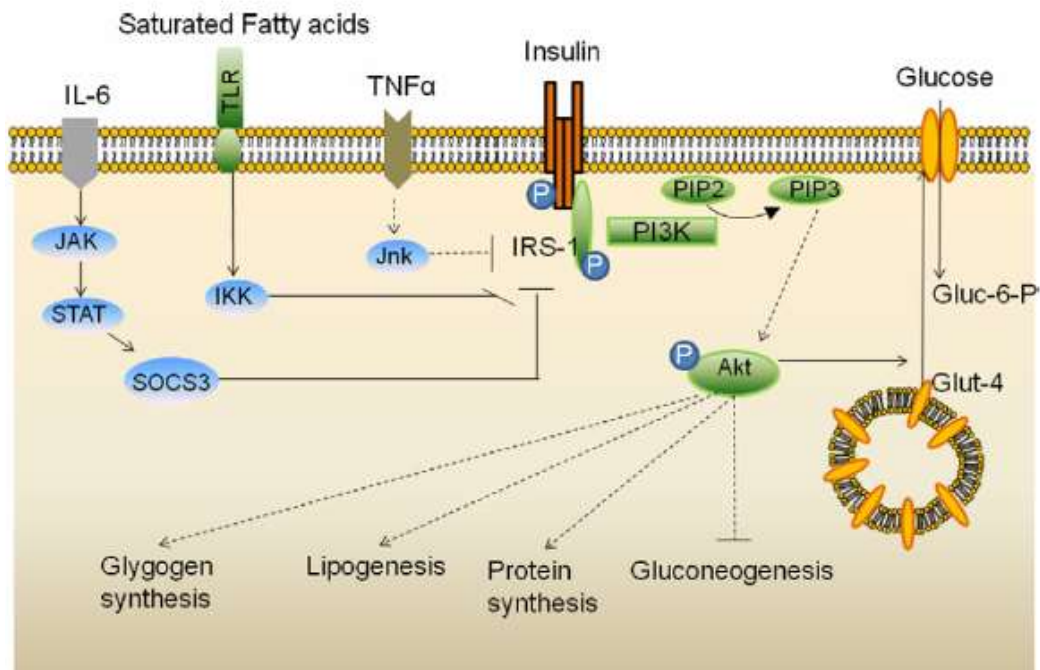

Figure 1.5. Molecular mechanisms of insulin resistance: FFA and inflammatory cytokines impair insulin signaling (Kalupahana et al. 2012. *Molecular aspects of medicine*)

The secretion of adipokines is also modified by obesity, as metabolically dysfunctional adipocytes cannot properly regulate their production of adipokines[8]. An imbalance of pro and anti-inflammatory adipokines occurs: Leptin levels are increased[9]; adiponectin levels are, however, diminished[33]. As adipose mass increases, elevated levels of leptin trigger the secretion of TNF-α, IL-6 and CCLs by macrophages. Moreover, leptin activates both macrophages and T cells[160], thus contributing to tissue inflammation and the pathogenesis of obesity complications.

1.3.2.3.Influence of immune cells on inflammation and metabolism in adipose tissue

AT metabolism significantly influences immune cell composition, with anti-inflammatory cells predominating in lean individuals and inflammatory immune cells prevalent in obese subjects[20]. Indeed, obesity changes the immune cell populations within the AT, the changes regarding the macrophage subsets being of particular interest. The infiltration of macrophages (major cells of innate immunity) in the AT is one of the principal features of obesity; the accumulation of macrophages in the AT rises from 10% in lean individuals to 40% in subjects with obesity[146]. Macrophages are one of the main drivers of obesity-related

inflammation[9]. They contribute to obesity pathophysiology by the secretion of inflammatory molecules, which decrease the insulin sensitivity of adipocytes[161]. The increase in the macrophage population within the AT comes from the excessive proliferation of AT-resident macrophages[162] and the recruitment of monocyte-derived macrophages[163]. The adipocyte secretion of chemokines like CCL2 has been proven responsible for the migration of macrophages to the AT[164]. This recruitment of additional macrophages perpetuates the inflammation in the tissue[165].

The original classification only distinguished between two macrophage phenotypes in the AT: classically-activated M1-proinflammatory (expressing TNF-α, IL-1, and IL-6) and alternatively-activated M2-anti-inflammatory (expressing IL-4, IL-10 and TGF-β)[154]. M1-proinflammatory macrophages are predominant in the AT of subjects with obesity. IFN-γ secreted by Th lymphocytes induces the polarization of macrophages towards the M1 phenotype[166]. On the contrary, the AT of lean individuals shows a considerably higher amount of M2-anti-inflammatory macrophages[167]. However, it is common knowledge that this classification may be simplistic or inaccurate, as many intermediate states coexist[168]. Mixed M1-M2 macrophages or an obesity-specific M1-macrophage (expressing proinflammatory cytokines but lacking M1-surface markers) have been described in the context of obesity[161]. These macrophages, named "metabolically activated macrophages (MMe)" differ from the classical M1-proinflammatory macrophages and originate after exposure to high levels of glucose, insulin, or palmitate[169]. The MME express CD36, ABCA1, PLIN2[163].

T and B cells are thought to be early responders to the inflammatory mediators secreted by enlarged adipocytes[170]. Hypertrophic adipocyte secretion triggers first the migration of T lymphocytes, before the recruitment of macrophages takes place[171]. Moreover, insulin resistance is not yet established when T and B lymphocytes infiltrate the AT of individuals with obesity[171]; lymphocytes might be initiators of the inflammatory response and insulin resistance that characterizes the AT of subjects with obesity[171]. The chemotactic cytokines CCL5/RANTES[172], CCL20[173], and CXCL12/SDF-1[166] secreted by adipocytes and by ASC induce the migration of lymphocytes to the AT and promote T-cell expansion. The AT of subjects with obesity is, therefore, infiltrated by large amounts of T lymphocytes due to increased proliferation and recruitment[172]. In addition, the number of T-cells correlates with BMI[173] and waist circumference[171]. T lymphocytes CM reduced the insulin sensitivity of

adipocytes and ASC by secretion of IFN-γ, shown by the lack of upregulation of lipogenic enzymes[173]. This data indicates an association between lymphocyte infiltration and insulin resistance. In other words, T-cells contribute to establishing and perpetuating inflammation and developing insulin resistance.

Regarding the specific population of lymphocytes, the number of cytotoxic CD8+ T cells and CD4+ T cells are increased in obese mice[174]. Cytotoxic T cells enhance the migration of macrophages to the AT and its activation, thus participating in the initiation and spread of inflammation throughout the tissue[175]. The number of Th1 cells is increased in the AT of subjects with obesity, showed by increased expression of Th1 typical cytokines (IFN-γ, TNF-α, RANTES, and Interleukin-2 (IL-2)) in AT lymphocytes[173]. On the other hand, Th17 lymphocytes seem to be increased in obesity. ASC reduce their differentiation into adipocytes and enhance the expression of IL-6 and IL-8 as a response to IL-17 secreted by Th17 cells[176]. Obesity leads to a loss of Tregs and regulatory B lymphocytes, known for their immunosuppressive properties, which contributes to the establishment of inflammation in the AT[101,166]. On the other hand, NK T cells are more activated in the AT of subjects with obesity[166]. Moreover, AT in the setting of obesity is also infiltrated by B cells[166].

Energy surplus or deficiency impacts immunity. Lack of nutrients can impair the correct function of the immune system and lead to infections, whereas excess nutrients activate the immune system and lead to inflammatory diseases[177]. Obesity significantly impacts immunity; it takes longer for subjects with obesity to control infection and for their wounds to heal[178]. Some of the features of this immune dysfunction related to obesity are: acceleration of thymic aging, alterations of lymphoid organ architecture, reduction of memory T cells, impairment of cell-mediated immune response, abnormal lymphoproliferative responses[178], and decline in the adipocyte production of antimicrobial peptides[18].

Of note, overweight or obese subjects have better outcomes and survival than their lean counterparts in some diseases, a phenomenon known as the “obese paradox”. It has been described that obesity might be protective in the elderly population or people suffering from certain diseases, such as myocardial infarction, heart failure, chronic obstructive pulmonary disease, and others[179]. This might be explained by the fact that BMI, the parameter used to

determine "obesity", does not consider body composition and does not reflect nutritional status. Conversely, when using other indices to define obesity (waist circumference, waist-to-hip ratio, waist-to-height ratio or body fat percentage), the protective effect associated with overweight and obese subjects disappears, suggesting abdominal adiposity is the risk factor, rather than the increase in SAT, which could be benign[180]. In addition, a low BMI may be due to undernourishment and sarcopenia. In this case, a low BMI would be detrimental and lead to decreased survival. Moreover, people affected with a chronic disease tend to lose weight as a consequence of the illness, which can lead to cachexia. Others, however, postulate overweight or obese subjects have higher amounts of cardioprotective adipokines[181].

1.3.2.4.Contribution of SAT and VAT to systemic inflammation

Obesity disrupts the homeostasis of both SAT and VAT, leading to hypertrophy of the adipocytes, tissue hypoxia, and adipocyte necrosis, followed by macrophage accumulation and escalated inflammation[182]. The contribution of SAT and VAT to systemic inflammation in an obesity context is an area of active investigation.

While VAT expansion has traditionally been associated with obesity-induced inflammation and related metabolic and cardiovascular disorders, recent evidence indicates that obesity also disrupts the homeostasis of SAT[109]. Studies have substantiated that SAT contributes significantly to obesity-related inflammation, with a higher correlation between the inflammatory status of SAT-resident macrophages and BMI that VAT macrophages. This supports the hypothesis that the systemic status of an individual may be more accurately reflected by SAT than VAT[109].

Although macrophage recruitment is higher in VAT than in SAT, coinciding with greater adipocyte necrosis in VAT[183], macrophage infiltration in SAT is better correlated with insulin resistance markers compared to VAT[184,185]. Some researchers report higher macrophage infiltration in SAT of metabolically unhealthy lean subjects compared to healthy lean individuals, with negligible differences in VAT[186]. These findings may partly elucidate why certain normoweight individuals exhibit metabolic disorders, positioning SAT as an early detector of metabolic alterations[186]. Consistent with these observations, weight loss has been found to reduce inflammation, especially in the SAT depot[187,188]. Obesity has also been linked to increased fibrosis in SAT, more so than in VAT, as indicated

by higher levels of extracellular matrix proteins in the SAT secretome[189].

From an adipocyte perspective, while VAT-derived adipocytes may secrete more inflammatory cytokines compared to those from SAT[12], the contribution of subcutaneous adipocytes to systemic inflammation should not be discounted. In fact, IL-6 levels in SAT (but not in VAT) have been found to correlate with serum levels[190].

Taken together, these findings suggest that the status of SAT may be a more accurate reflection of an individual's systemic metabolic health.

1.4. IMMUNOMETABOLISM

Immunometabolism, a field at the intersection of immunology and metabolism, has emerged as a pivotal area of investigation in recent years. This field explores the profound influence of metabolic processes on the behavior and functions of immune cells and, reciprocally, how immune responses shape cellular metabolism.

In resting cells in aerobic conditions, mitochondrial oxidative phosphorylation (OXPHOS) is the predominant pathway. Glycolysis transforms glucose into pyruvate, which, in turn, is converted into acetyl-CoA. The acetyl-CoA enters the TCA cycle, obtaining NADH and FADH2, which will donate electrons in the mitochondrial electron transport chain to obtain 38 ATP[191].

Metabolic reprogramming is the change in cellular bioenergetics to support increased and immediate energy requests. The paradigm of metabolic reprogramming is the Warburg effect in tumor cells, which allows these cells to undergo rapid proliferation and survival, thanks to aerobic glycolysis' quick energy supply. The Warburg effect is the switch in metabolism by which tumor cells (with high rates of proliferation) obtain energy mainly through glycolysis in aerobic conditions instead of using oxidative phosphorylation OXPHOS, which is the regular metabolic pathway when oxygen is available[192]. In these cells, glucose undergoes glycolysis, and the resulting pyruvate is mainly converted into lactate, a pathway usually adopted by cells when the conditions are anaerobic. Although this pathway is much less efficient than oxidative phosphorylation (2 ATP), it is a quicker metabolic route to obtain ATP, and cells with high energetic demands (like tumor cells) tend to prefer it[191].

Since Warburg described this effect in cancer cells in 1927, other metabolic reprogramming cases have been described. Immune cells undergo metabolic reprogramming at the onset of inflammation, requiring metabolic restructuring to restore cellular homeostasis. During inflammation, macrophages initially employ aerobic glycolysis to swiftly produce ATP, followed by a late adaptation phase relying on lipolysis and fatty acid oxidation to facilitate the macrophages' anti-inflammatory response (M2) (Figure 1.6)[193]. Thus, macrophages adopt aerobic glycolysis as their primary metabolic pathway to switch to the M1-inflammatory phenotype[194]. Specifically, for macrophage activation, the stabilization of the Hypoxia-inducible factor 1α (HIF-1α) promoted by NF-kB is necessary to increase the expression of glucose transporters and glycolysis enzymes[195]. The route of the pentose

phosphate pathway (PPP), the production of lactate, the glutaminolysis, and the lipid synthesis are enhanced. At the same time, TCA cycle is disrupted with an elevated release of citrate and succinate[191]. Glycolysis provides energy to meet the high energy demands but also intermediates for anabolic pathways required for growth, differentiation, or activation[191]. The accumulation of specific metabolites as a result of the activation of these metabolic pathways enhances the expression of inflammatory genes and the secretion of inflammatory molecules through signaling modulation, post-transcriptional modification, post-translational changes, or epigenetic regulation[194]. Macrophage functions such as phagocytosis, cytokine secretion, and ROS production are impaired if this metabolic switch to glycolysis doesn't occur, illustrating the intimate link between metabolism and immune function[194].

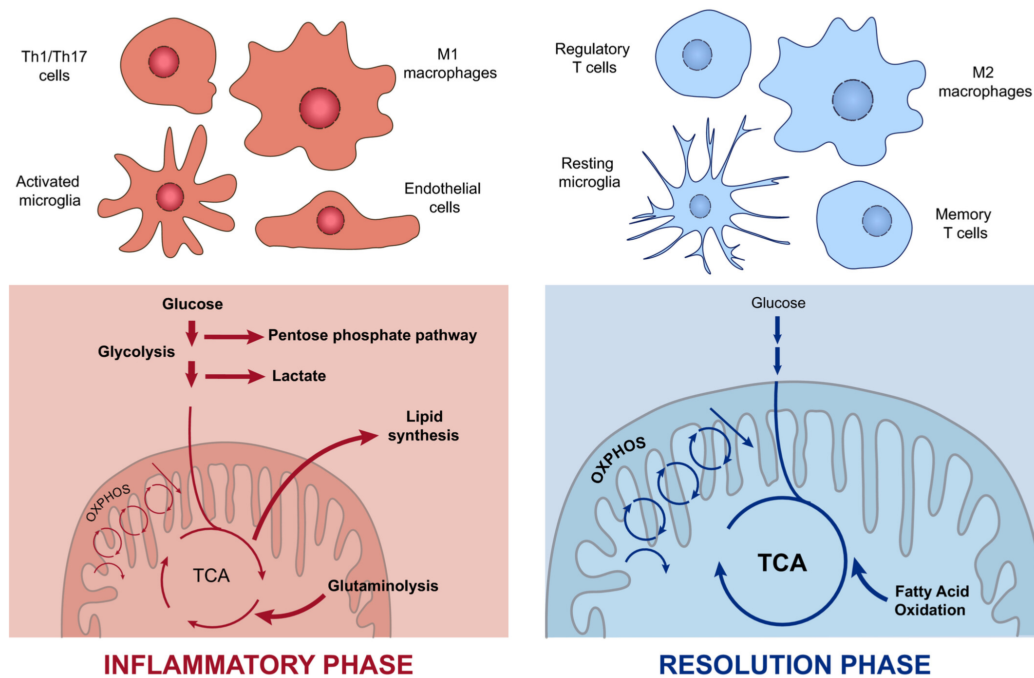

Figure 1.6. Metabolic profile of proinflammatory M1 macrophages and anti-inflammatory M2 macrophages (Soto-Heredero et al. 2020. *FEBS journal*)

However, the metabolic requirements of MSC oppose those of macrophages[196]. Macrophages depend on fatty acid oxidation to give an M2-response to resolve inflammation[197]. In contrast, MSC rely on aerobic glycolysis and lactate production to appropriately act as immunosuppressive cells (Figure 1.7)[196]. In essence, the secretion of immunomodulatory factors (PGE2, IDO, TFG-β) by MSC is sustained by aerobic glycolysis,

probably because this metabolic pathway fuels the carbon source and the energy necessary for the synthesis of these molecules[198]; just like it provided metabolites and energy for cytokine and chemokine synthesis in macrophages[194].

Mechanistically, the activation of HIF-1α and the induction of glycolysis that comes with it in MSC is fundamental to develop their immunosuppressive functions, such as inhibition of T-cell proliferation[199]. Decreasing glycolysis rates impairs MSC' immunomodulatory function[200]. Moreover, when HIF-1α is silenced in MSC, the inhibition of T-cell proliferation is reduced due to the switch to OXPHOS[199]. In addition, studies have demonstrated that MSC-secreted lactate is responsible for the polarization of macrophages towards the M2 γ) or activation of TLRs by FFA are inducers of HIF-1α expression in MSC and potentiate their immunomodulatory properties[196,202]. HIF-1α might be a key factor mediating the plasticity of MSC; the presence of inflammatory mediators in the environment stimulates the anti-inflammatory properties of MSC via stimulation of HIF-1α expression[196].

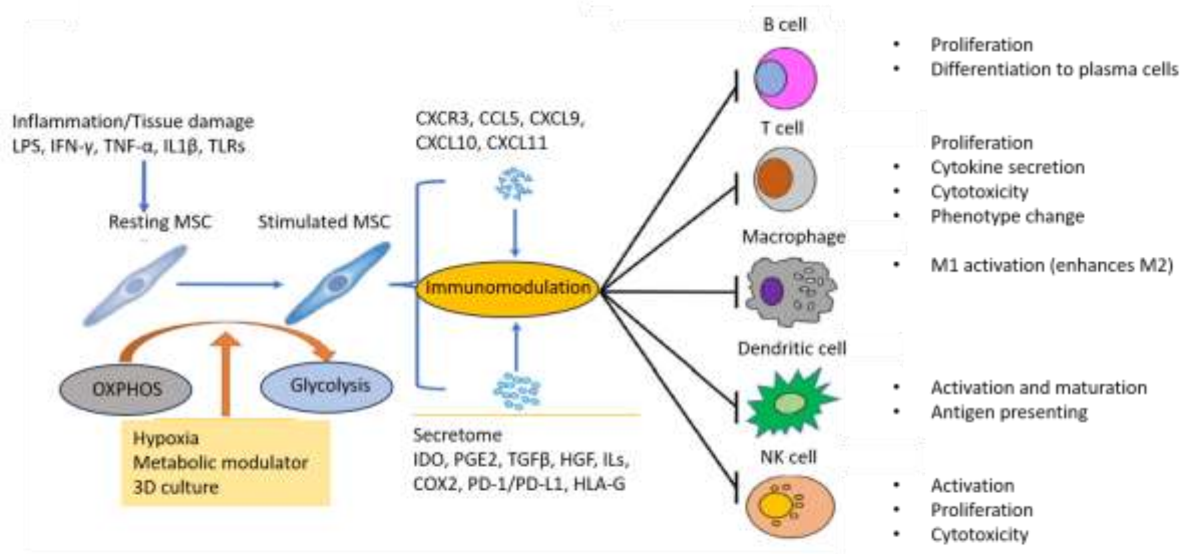

Figure 1.7. Metabolic switch to glycolysis to support MSC immunomodulatory properties (Yuan et al. 2019. *Frontiers in immunology*)

1.5. SIRTUINS

1.5.1. DEFINITION AND MOLECULAR ACTIVITY

Sirtuins are a family of Nicotinamide adenine dinucleotide (NAD)-dependent class III histone deacetylases and ADP-ribosyltransferases, known for their ability to deacetylate both acetyl groups and long-chain fatty acid acyl groups. They are homologous to yeasts' Silencing information regulator 2 (Sir2), protein regulator of chromatin structure and genome stability[203]. The sirtuin family consists of seven members (SIRT1-7), each highly conserved, with SIRT1 being the most conserved[11]. The sirtuin family members are distributed across different cellular compartments: the nucleus, the cytoplasm, and the mitochondria. More specifically, SIRT1, SIRT6, and SIRT 7 are primarily located in the nucleus, where they regulate the transcription of genes and DNA repair (Figure 1.8)[203]. SIRT2 is located in the cytoplasm and controls glucose and lipid metabolism, while SIRT 3, SIRT4, and SIRT5 are located in the mitochondria, modulating mitochondrial enzymes activity[204]. We focus our study on SIRT1 and SIRT6 as they have been described to be the most relevant in aging[205] (see next point for detailed information). SIRT6 is notable for its ability to deacetylate histone H3 at lysine 9 and 56[206], while SIRT1 demonstrates a broader range of deacetylation targets, including lysine residues in histones as well as other proteins such as p53[207], Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1 Alpha (PGC-1α), and Forkhead Box Protein 01 (Fox01)[208].

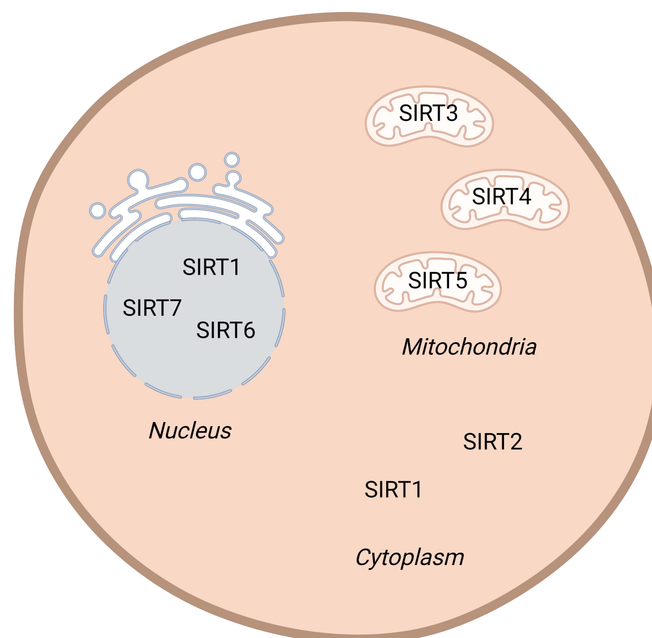

Figure 1.8. Sirtuins subcellular localization.
Created with *BioRender.com*

1.5.2. FUNCTIONS: GATEKEEPERS OF TISSUE HOMEOSTASIS

Extensive biological roles are recognized for the sirtuins, which ensure tissue equilibrium at different levels.

1.5.2.1.Guardians of genome stability

Exposure to environmental factors (ultraviolet (UV) radiation or ionizing radiation) and products of normal cellular processes (ROS, reactive nitrogen species, reactive carbonyl species, etc) induces DNA damage. In eukaryotic cells, four different mechanisms to respond to DNA damage have been described: base-excision repair (BER) and nucleotide-excision repair (NER) for the repair of single-strand breaks; homologous recombination (HR) and non-homologous end joining (NHEJ) for the repair of double-strand breaks[209]. Due to their nuclear localization, SIRT1 and SIRT6 are critical facilitators of DNA repair[203]. SIRT6 is responsible for preserving genomic stability and forestalling DNA damage[210], participating in BER and NHEJ[209]. On the other hand, SIRT1 ensures the maintenance of telomere length[211] and activates damage-respond pathways like NER, NHEJ, and HR[209].

1.5.2.2.Senescence modulators and lifespan enhancers

Caloric restriction remains the only known intervention capable of extending lifespan, with SIRT1 being an important mediator of this effect in mammals[212]. Observations reveal that SIRT1 knockout mice fail to exhibit lifespan extension under caloric restriction, indicating SIRT1's necessity for lifespan extension through caloric restriction[213]. It seems to play a role in stress-induced lifespan reduction, as demonstrated in metabolically-stressed mice fed a high fat diet (HFD)[214]. On the other hand, SIRT6 deficiency leads to accelerated aging[210], which emphasize the influence of SIRT6 on longevity.

1.5.2.3.Regulators of energy metabolism: energy status sensors

Sirtuins are considered energy status sensors that modulate energy metabolism in response to exercise and dietary changes, particularly in states of energy depletion. Among the various substrates regulating sirtuin activity, NAD+ is crucial due to its role in redox reactions, rendering situins as NAD+ sensors. Sirtuins identify alterations in energy and redox status and coordinate responses to dietary changes by activating corresponding mechanisms[205]. Their role in inducing metabolic adaptations to counter cellular metabolic stress warrants their title as guardians of tissue homeostasis[205]. SIRT1, and particularly

SIRT6, modulate DNA transcription by repressing the transcription of glycolysis-related genes. SIRT1 activates PGF1α by acetylation, repressing glycolytic gens and subsequently increasing glucose plasma levels[215]. SIRT1 and SIRT6 also reduce glycolysis by suppressing HIF-1α[212], a transcription factor that promotes the transcription of glycolysis enzymes and glucose transporters (GLUT)[216].

Regarding lipid homeostasis, SIRT1 promotes lipolysis and fat mobilization during fasting[212,217]. SIRT1's inhibition of PPARG diminishes adipocyte differentiation, reducing adiposity and fat storage[11]. Nutrient availability correlates with diminished SIRT1 activity, amplifying PPARG and increasing adiposity[11].

Published data supports that caloric restriction leads to beneficial effects by mechanisms involving the sirtuins[205]. Accordingly, studies have demonstrated that SIRT1 protects against HFD-induced obesity[218,219].

1.5.2.4. Regulators of inflammation

Sirtuins modulate the metabolic switch from aerobic glycolysis to fatty acid oxidation in macrophages, which is necessary for homeostasis restoration[205]. SIRT1 and SIRT6 are critical in facilitating the glycolysis to fatty acid oxidation transition in macrophages following TLR4 stimulation[193]. An increase in SIRT1 and SIRT6 is necessary to obtain M2 macrophages[205]. By repressing PPARG, SIRT1 shifts metabolism from glycolysis towards fatty acid oxidation[197]. This metabolic switch is vital for inflammation resolution; hence, sirtuin deficiencies hinder metabolic reprogramming and, consequently, inflammation resolution, resulting in chronic inflammation[205].

Additionally, reduction of sirtuins is directly associated with enhanced inflammation. SIRT1 deacetylates NFkB and affects promoters of inflammatory genes, preventing their transcription[220].

1.5.3. SIRTUINS' ROLES IN AGING AND OBESITY

Chronic inflammation, a common characteristic of aging and obesity, has been linked to a decline in NAD+ and Sirtuins levels in the AT[205]. On the other hand, dysregulation of glucose and lipid metabolism has been associated with a reduction in SIRT/NAD+ activity[221].

As previously mentioned, out of the seven known sirtuin isoforms, SIRT1 and SIRT6 are particularly relevant to aging[205]. As aging progresses, NAD+ levels decrease and HIF-1α increase, independent of oxygen levels[222]. Under normal oxygen conditions, glycolysis prevails, mirroring the Warburg effect observed in cancer cells, before cited. This age-related pseudohypoxic state leads to metabolic changes and diminished cellular function. A reduction of SIRT1 levels intensifies this process[222]. Indeed, the replenishment of NAD+ could restore mitochondrial function in aged mice through SIRT1[222]. Moreover, the absence of SIRT6 in mice leads to loss of subcutaneous fat, lymphopenia, and severe metabolic disturbances, mirroring the aging process[210].

Research has demonstrated reduced sirtuin levels in the WAT of individuals with obesity[220]. Decreased levels of SIRT1 were found in the SAT of obese women[223]. In parallel, mice on a HFD have shown reduced SIRT1 levels in the AT[224]. Studies analyzing the SAT and VAT of obese women revealed diminished SIRT1 expression[225]. Other studies reported the AT of subjects with obesity had decreased SIRT6 protein levels compared to their lean counterparts[226]. Interestingly, weight loss seems to restore the SIRT1 and SIRT6 levels in the SAT[227].

Emerging data indicates changes in metabolism as a response to environmental factors regulate immune cells' pro or anti-inflammatory fate. SIRT1 and SIRT6, as regulators of glycolysis and fatty acid oxidation through OXPHOS, could mediate this switch in cell metabolism. The reduced expression of SIRT1 and SIRT6 found in Aging and obesity could underlie changes in immune cell function as a response to the inflammatory and hypoxic AT associated with aging and obesity.

HYPOTHESIS AND OBJECTIVES

2. HYPOTHESIS AND OBJECTIVES

Given that

- Both aging and obesity commonly feature low-grade and chronic systemic inflammation.
- AT dysfunction significantly contributes to the metabolic disruptions associated with obesity and aging.
- ASC, considered primary contributors to the AT secretome, function as adipocyte precursors, regenerators of tissue, and immune response regulators.
- Alterations in metabolic processes have been linked to inflammation.

We hypothesized that the low-grade systemic inflammation observed in physiological processes like aging and pathological conditions like obesity is partly due to metabolic changes within AT cells. Specifically, we propose that the ASC of elderly subjects or individuals with obesity undergo metabolic changes that trigger the secretion of inflammatory mediators contributing to tissue inflammation.

2.1. Main objective

Investigate the impact of aging and obesity on the functional properties and metabolism of ASC from SAT, highlighting both similarities and differences and potential interaction effects. Further, elucidate possible mechanistic pathways through which metabolic changes in adipocyte precursors because of obesity or aging, induce inflammation and functional modification.

2.2. Specific objectives

2.2.1. Study 1

- Explore how obesity and aging affect the ASC' proliferative and differentiative capacities, glucose metabolism, and secretion pattern of inflammatory and anti-inflammatory mediators.
- Examine the expression of nutrient-sensing molecules SIRT1 and SIRT6 in the context of obesity and aging, and the interaction between glycogen deposition, sirtuins, and inflammation in ASC.

2.2.2. Study 2

- Investigate the effect of aging and obesity on the immunomodulatory properties of ASC
- Assess the impact of aging and obesity on the APC marker HLA-II
- Explore a potential relationship between the metabolic status of the ASC and their immunomodulatory properties
- Investigate sirtuins as potential modifiers of the immunomodulatory properties of ASC

METHODS

3. METHODS

3.1. Study subjects

Subjects were recruited at the University Hospital Joan XXIII and Santa Tecla Hospital (Tarragona, Spain) following the tenets of the Helsinki Declaration. The corresponding hospital ethics committees reviewed and approved the study and written informed consent was obtained from all participants before they entered the study. SAT biopsies were obtained from donors undergoing nonacute surgical interventions, such as hernia or cholecystectomy, in a scheduled routine surgery. Donors were classified as adult (>20 and <65 years) or elderly (≥65 years) based on their age, and as lean (BMI<25 kg/m^2) or obese (BMI≥30 kg/m^2) based on their BMI, following World Health Organization criteria. This classification led to the formation of four groups: lean adult (LA), lean elderly (LE), obese adult (OA), and obese elderly (OE). The anthropometric and biochemical variables from the cohort are presented in Table S.1. Patients with cancer, diabetes, and inflammatory chronic diseases were excluded from the study. The *n* corresponding to the subsample used for each experiment is specified in the figure legends.

3.2. Isolation and culture of human adipose-derived mesenchymal stromal cells

Human adipose-derived mesenchymal stromal cells (hASC) were isolated from SAT biopsies as described [75,228]. In brief, SAT was washed extensively with PBS to remove debris and treated with 0.1% collagenase in PBS and 1% bovine serum albumin for 1 h at 37ºC with gentle agitation. Digested samples were centrifuged at 300 × g at 4ºC for 5 min to separate adipocytes from stromal cells. The cell pellet containing the stromal fraction was resuspended in stromal culture medium consisting of DMEM/F12, 10% fetal bovine serum (FBS), and 1% antibiotic/antimycotic solution and then placed into a flask. The flask was placed in an incubator at 37ºC with 5% CO_2 and 21% O_2. At 24 h after seeding, the flask was washed with PBS, and the medium was replaced. As the hASC had already adhered to the flask, the PBS wash removed only non-adherent cells. The culture medium was replaced every 48–72 h. After seven days of incubation, when cells had achieved 90% confluence, the primary cultures of hASC at P0 were harvested with trypsin-EDTA, and aliquots of 1×10^6 cells were cryopreserved in liquid nitrogen until required. The AT-cell number ratio was defined as the ratio of the number of cells obtained at P0 per gram of AT digested. CM was

collected at P3–7 after 24 h in culture using a minimum concentration of hASC of 10,000 cells/cm^2 and was centrifuged at 400 × g for 5 min.

3.3. Immunophenotyping

To verify the isolation of hASC, we assessed the immunophenotypic profile of undifferentiated hASC populations using a panel of positive and negative surface markers that identify hASC according to the quantitative criteria established by the International Society of Cell Therapy (ISCT) and the International Federation for Adipose Therapeutics and Science (IFATS). Briefly, 2×10^5 hASC were incubated with a panel of primary antibodies (CD34, CD73, CD90, CD105, CD14, CD31, CD45) and then analyzed by flow cytometry using 405-nm, 488-nm, and 633-nm excitation on the FACS ARIA III cytometer (BD Biosciences, San Jose, CA). All experiments were performed in cells at P3–7. Flow cytometry analysis of cell marker expression was consistent with the minimum criteria defined for hASC. Accordingly, cells were positive for the surface markers CD73, CD90, and CD105 and negative for CD34, CD14, CD31, and CD45. No significant differences were detected between groups (Table S.2.).

3.4. hASC proliferation assays

3.4.1. MTT assay. hASC proliferation was determined by a standard colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide (MTT) reduction assay (Sigma-Aldrich, Madrid, Spain). In brief, 1.6×10^3 hASC per well were seeded in a 96-well plate. Two MTT assays were performed 24 h (day+1) and 7 days (day+7) after seeding. The proliferation rate was calculated as the difference in absorbance between day+7 and day+1, measured with a spectrophotometer at 540 nm.

3.4.2. CTV assay. Flow cytometric analysis of intracellular Cell Trace Violet (CTV) (Invitrogen, Eugene, OR) was used to measure hASC proliferation based on dye-dilution of CTV-labeled cells throughout the course of 48 h.

3.5. Multilineage differentiation capacity

To assess the differentiation capacity of hASC, we used specific conditions to trigger cell differentiation to the adipocyte, osteocyte, and chondrocyte lineage, as described [229]. Intracellular lipid enrichment in mature adipocytes was measured by Oil Red O staining; calcium depots in osteocytes were assessed by Alizarin Red staining; and glycosaminoglycan precipitation in chondrocytes was analyzed by Alcian Blue 8GX

staining. Differentiated cells were observed in a bright-field microscope (Zeiss Axio Vert A1; Carl Zeiss AG, Oberkochen, Germany). Relative gene expression of adipogenic osteogenic and chondrogenic markers in hASC was analyzed by real-time polymerase chain reaction.

3.6. hASC migration capacity

The migratory capacity of hASC was analysed using a Transwell system (8-µm pore polycarbonate membrane (Corning Costar)), as previously described[75,230]. In summary, the lower chamber wells were filled with DMEM/F12 and without FBS. hASC (8×10^4 in 0.2 mL) were added to the upper chamber and incubated for 24h at 37ºC. We collected the medium of the lower chamber and counted the cells using an automatic cell counter (Bio-Rad TC10, Hercules, CA).

3.7. Isolation of human peripheral blood mononuclear cells and human lymphocytes

Human Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Hypaque gradients (Amersham Bioscience). Lymphocytes were purified from PBMC by magnetic cell sorting using CD14-microbeads (MiltenyiBiotech); the CD14 negative fraction was kept[231]. Each biologically independent sample was obtained from a pool of three human samples.

3.8. Isolation and culture of human monocytes

Human PBMC were isolated using Ficoll-Hypaque gradients (Amersham Bioscience). Monocytes were purified from PBMC by magnetic cell sorting using CD14-microbeads (MiltenyiBiotech); the CD14-positive fraction was kept[231].

3.9. Peripheral blood mononuclear cells proliferation assay

PBMC in RPMI 1640 medium with 10% FBS were seeded at 2×10^5 /mL in a 96-well plate and incubated at 37ºC, in 5% CO_2, with 0,1 mL of 24h-CM from hASC of the different four cohorts. 72h after seeding, the number of cells was counted with an automatic cell counter (Bio-Rad TC10, Hercules, CA). At least an n=4 per cohort in triplicate was used to perform this experiment.

3.10. human T lymphocytes and human monocytes migration capacity (chemotaxis test)

The migratory capacity of T lymphocytes and monocytes in the presence of 24h-CM from hASC was analysed using a 24-well-Transwell system (5-mm pore polycarbonate membrane (Corning Costar)), as previously described[75]. In summary, the lower chambers were filled with 250 µL of 24h-CM and 250 µL of DMEM/F12 without FBS. T lymphocytes or monocytes (2x 10^5 in 0.2 mL of DMEM/F12 without FBS) were added to the upper chamber and incubated for 4h at 37ºC. Migrated cells from the lower chamber were counted using an automatic cell counter (Bio-Rad TC10, Hercules, CA). Data shown corresponds to at least an n=4 per cohort; the experiment was performed in duplicate.

3.11. Macrophage polarization assays

Human PBMC-derived macrophages Mϕ were obtained by culturing CD14+ cells in RPMI medium in a 24-well plate supplemented with human recombinant M-CSF (50 ng/ml; PeproTech) for 7 d at 37 °C, refreshed every two days. Macrophages were then incubated with CM of the different groups in the cohort for 6h. RNA was extracted, and the gene expression of pro and anti-inflammatory genes was assessed.

3.12. APC properties

Antigen-presenting cell marker expression was assessed after cells 2×10^5 hASC were stained with antibodies against human leukocyte antigen–DR isotype (HLADR), CD86, and CD40 and then analyzed by flow cytometry using 405-nm, 488-nm, and 633-nm excitation on the FACS ARIA III cytometer (BD Biosciences, San Jose, CA). All experiments were performed in cells at P3–7. The expression of these markers was also determined in hASC that had been incubated for 16h with Ovoalbumin (OVA) (5µg/mL).

Gene expression of APC markers was determined in hASC with and without incubation with OVA.

3.13. Cytokine secretion

We used pools of 24-h CM from hASC of different donors (at least an n=5 was used for each group), of which 500-µL samples were filtered using a 3-k pore filter and centrifugated at 14000 × g for 5 min. A total of 100 µL was used in duplicate for each cohort on the RayBio Human Cytokine Antibody Array 5 (G series, cat# AAH-CYT-G5-8, RayBiotech Life,

Norcross, GA; www.raybiotech.com) and the array was sent to the manufacturer for scanning. Fluorescence signal intensities were measured on the Innopsys InnoScan (Carbonne, France) laser fluorescence scanner. Normalization was performed by defining one of the two sub-arrays of the LA group as a reference. The image of the array is shown in Figure S.1.

3.14. Gene expression analysis

Total RNA was isolated from hASC using the RNeasy Lipid Tissue Mini Kit (Qiagen Science, Hilden, Germany). RNA was transcribed to cDNA with random primers using the Reverse Transcription System (Applied Biosystems, Foster City, CA). Amplification was performed on a 7900HT Fast Real-Time PCR System using the TaqManR Gene Expression Assays hydrolysis probes (Applied Biosystems) (Figure S.2.). Results were calculated using the comparative threshold cycle (Ct) method (2-ΔΔCt) normalized to the expression of the housekeeping gene *18S* (Hs 03928985_g1) or cyclophilin 1A (*PPIA*) and expressed relative to the control condition, which was set to 1. Two technical duplicates were performed for each biological replicate.

Figure S.2. Human gene expression analysis

Gene symbol-Assay ID	Gene nane
Senescence markers	
CDKN2A-Hs00923894_m1	Cyclin Dependent Kinase Inhibitor 2A
GLB1-Hs01035168_m1	Galactosidase Beta 1
TP53-Hs01034249_m1	Tumor Protein P53
Adypocyte differentiation markers	
FABP4-Hs01086177_m1	Fatty Acid Binding Protein 4
FASN-Hs01005622_m1	Fatty Acid Synthase
LPL-Hs00173425_m1	Lipoprotein Lipase
PLIN1-Hs00160173_m1	Perilipin 1
PPARG-Hs01115513_m1	Peroxisome Proliferator Activated Receptor Gamma
Osteocyte differentiation markers	
ALPL-Hs01029144_m1	Alkaline Phosphatase
COL1α1-Hs00164004_m1	Collagen Type I Alpha 1 Chain
RUNX2-Hs01047973_m1	RUNX Family Transcription Factor 2
SPP1-Hs00959010_m1	Secreted Phosphoprotein 1/Osteopontin
Condrocyte differentiation markers	
COL1A1-Hs00164004_m1	Collagen Type I Alpha 1 Chain
COMP-Hs00164359_m1	Cartilage Oligomeric Matrix Protein
Metabolism markers	
GYS_1-Hs00157863_m1	Glycogen Synthase 1
GBE_1-Hs00609186_m1	1,4-Alpha-Glucan Branching Enzyme 1
HK2-Hs00606086_m1	Hexokinase 2
LDHb-Hs00929956_m1	Lactate Dehydrogenase B
OGDH-Hs01081865_m	Alpha-ketoglutarate dehydrogenase
PDK4-Hs01037712_m1	Pyruvate dehydrogenase kinase, isozyme 4
PFKM-Hs00175997_m1	Phosphofructokinase M
PPP1R3C-Hs00193642	Protein Phosphatase 1 Regulatory Subunit 3C/ Protein Targeting To Glycogen (PTG)
PYGL-Hs00958087_m1	Glycogen Phosphorylase L
SDH_b-Hs01042482_m1	Succinate dehydrogenase b
SLC2A1/GLUT1-Hs00892681_m1	Solute Carrier Family 2 Member 1/Glucose Transporter Type 1
SLC2A3/GLUT3-Hs00892681_m1	Solute Carrier Family 2 Member 3/Glucose Transporter Type 3
Inflammation markers	
CCL2-Hs00234140_m1	C-C Motif Chemokine Ligand 2
CCL3-Hs00234142_m1	C-C Motif Chemokine Ligand 3
IL1B-Hs01555410_m1	Interleukin 1 Beta
IL6-Hs00174131_m1	Interleukin 6
TNFA-Hs00174128_m1	Tumor necrosis factor alpha

Results were calculated using the comparative Ct method and expressed relative to the expression of the housekeeping genes cyclophilin 1A (PPIA) (Hs04194521_s1) and 18S (Hs03928985_g1)

Gene symbol-Assay ID	Gene nane
Antiinflammatory markers	
IL10-Hs00961622_m1	Interleukin 10
KLF4-Hs00358836_m1	Krüppel-like factor 4
MRC1-Hs07288635_g1	Mannose receptor c-type 1
PPARG-Hs01115513_m1	Peroxisome proliferator-activated receptor
Antigen presentation markers	
CD40-Hs01002915_g1	Cluster of differentiation 40
CD74-Hs00269961_m1	Cluster of differentiation 74
CD80-Hs01045161_m1	Cluster of differentiation 80
CD86-Hs01567026_m1	Cluster of differentiation 86
CTSB-Hs00947439_m1	Cathepsin B
CTSS-Hs00175407_m1	Cathepsin S
HLA-DMb-Hs00157943_m1	Human Leucocyte Antigen DM
HLA-DRb1-Hs04192464_mH	Human Leucocyte Antigen DR
Sirtuins	
SIRT1-Hs01009005_m1	Sirtuin 1
SIRT6-Hs00213036_m1	Sirtuin 6

3.15. Protein expression analysis

Cells were lysed and homogenized in Mammalian Protein Extraction Reagent (M-PER™; ThermoFisher Scientific, Waltham, MA) containing a protease and phosphatase inhibitor cocktail (Sigma-Aldrich). The BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL) was used to determine protein concentration. Equal amounts of protein were separated on SDS-PAGE gels, transferred to Immobilon membranes (Merck Millipore, Burlington, MA), and blocked. Antibodies diluted 1/1000 against p-5'AMP-activated protein kinase (AMPK) (THR172 (40H9)) (2535; Cell Signaling Technology [CST], Danvers, MA), p-Glycogen synthase (GS) (SER641) (3891; CST), p-Glycogen synthase kinase 3 (GSK3) α/β (SER21/9) (9331; CST), Glycogen branching enzyme (GBE) (AB617523; Abcam, Cambridge, UK), GFP (8371-2; Clontech, Palo Alto, CA), protein targeting to glycogen (PTG) (SC-6582; Santa Cruz Biotechnology, Santa Cruz, CA), p-Liver-type Glycogen phosphorylase (PYGL) (S15) (AB227043; Abcam), SIRT1 (2310, CST), SIRT6 (AB88494; Abcam), were used to perform immunoblot analysis. GAPDH (MA5–15738; Sigma-Aldrich) was a loading control. Protein bands were detected with anti-rabbit (NA934; GE Healthcare, Chicago, IL) or anti-chicken (ab131366; Abcam) peroxidase-conjugated secondary antibodies, diluted 1/2000. Immunoreactive bands were visualized using a SuperSignal West Femto chemiluminescent substrate (Pierce), and images were captured on an "iBrightCL1000 image System". ImageJ software (NIH) was used to quantify the intensity of the bands.

3.16. Glycogen immunofluorescence

The monoclonal anti-glycogen antibody used for the immunofluorescence was generously provided by Dr. Otto Baba [232]. hASC grown on coverslips were fixed with 4% (w/v) paraformaldehyde, rehydrated with 2% (v/v) fish skin gelatin, and permeabilized with 0.2% Triton X-100 before incubation with 5% (v/v) goat serum. Subsequently, cells were incubated overnight at 4ºC with the monoclonal mouse anti-glycogen antibody in PBS containing 1% goat serum. Coverslips were washed with PBS and incubated for 1 h at room temperature with an Alexa Fluor 568 conjugated goat anti-mouse antibody (1:100) and then mounted with ProLong Gold Antifade Reagent containing 40,6-diamidino-2-phenylindole (DAPI) (Invitrogen). Microscopy was performed with a Leica DM 4000B fluorescence microscope (Leica Microsystems, Wetzlar, Germany), and images were captured with a Leica DFC 300 FX camera (Leica Microsystems).

3.17. Glycogen colorimetric assay

Glycogen levels were measured in hASC using the Glycogen Colorimetric Assay Kit (BioVision Inc., Milpitas, CA). In total, 3×10^5 hASC were homogenized with 200 µL of water on ice, and the assay was performed following the manufacturer's instructions. A glucose background control was determined and then subtracted from the glycogen readings.

3.18. Adenoviral transduction

Cells were infected one day after seeding with an adenovirus expressing murine PTG (Ad-PTG) or GFP (Ad-GFP) under the control of the CMV promoter [233]. The adenovirus expressing PTG or GFP (used as a control) was diluted in Opti-MEM Reduced Serum Medium (Gibco, ThermoFisher Scientific) at 1/200 and 1/4000, respectively, prior to use. Adenoviral infection was carried out for 2 h at 37ºC using a multiplicity of infection of 50. The medium containing the adenovirus was then removed and replaced with a standard culture medium. Two days after infection, culture medium and hASC were collected and frozen.

3.19. SIRT1 and SIRT6 silencing

Silencing consumables were all from Horizon Discovery (Cambridge, UK). Cells seeded at 10,000 cells/cm^2 were transfected with human SIRT1 short interfering RNA (siRNA) (003540) or SIRT6 siRNA (013306) or a control (On-Target Plus Non-targeting Pool, number 001810). siRNA (5 µM) and Dharmafect Transfection Reagent were diluted 1/20 and 1/25, respectively, in Opti-MEM Reduced Serum Medium (Gibco; ThermoFisher Scientific) and incubated for 5 min at room temperature. The same amount of each solution was mixed carefully and incubated for 20 min at room temperature. The final solution of siRNA at a concentration of 0.125 µM was added to hASC, and medium containing serum was added to the wells. hASC were incubated at 37ºC with 5% CO_2 for 72–96 h.

3.20. Statistical analysis

Statistical analysis was performed with GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA). For *in vitro* data, experimental results were presented as mean ± SEM from 3–5 independent donors for each experiment. Statistical significance was tested by parametric two-way analysis of variance (ANOVA), when four groups were analyzed, and by the parametric Student's unpaired t-test when two groups were analyzed. For the gain or loss of function studies, the parametric Student's paired t-test was used. Correlations were tested by

Pearson's correlation analysis. General linear model and multiple linear regression analyses were employed to exclude sex as a confusing factor in the differences in proliferation found between groups.

RESULTS

4. RESULTS

Study 1

Influence of the subject phenotype regarding age and BMI on the hASC features and functions. Metabolic reprogramming as a potential cause of the dysfunction governing ASC in aging and obesity.

Glycogen accumulation in adipocyte precursors from elderly and obese subjects triggers inflammation via SIRT1/6 signaling

Terrón-Puig M, Huber-Ruano I, Sabadell-Basallote J, Ejarque M, Núñez-Roa C, Maymó-Masip E, Jorba R, Serena C, Vendrell J, Fernández-Veledo S.

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Impact Factor **11.005** (2021) **7.8** (2022)

Study 2

Aging and obesity modulation of the immunomodulatory properties of ASC

4.1. Study 1

Influence of the subject phenotype regarding age and BMI on the hASC features and functions. Metabolic reprogramming as a potential cause of the dysfunction governing ASC in aging and obesity.

Donors used in this work were classified as adult (>20 and <65 years) or elderly (≥65 years) based on their age, and as lean (BMI<25kg/m2) or obese (BMI≥30 kg/m2) based
on their BMI, following World Health Organization criteria. This classification led to the formation of four groups: lean adult (LA), lean elderly (LE), obese adult (OA), and obese elderly (OE). The anthropometric and biochemical variables from the cohort are presented in Table S.1.

Table S.1. Anthropometric and biochemical variables from the cohorts used to obtain human adipose-derived mesenchymal stromal cells (hASCs)

	Lean Adult	Lean Elderly	Obese Adult	Obese Elderly
n	29	16	30	8
Sex (male/female)	12/17	10/6	17/13	0/8
Age (years)	45.3±9.8[b,d]	70.9±6.7[a,c]	48.7±8.5[b,d]	72±4.7[a,c]
BMI (kg/m²)	23.6±2.7[c,d]	24.7±2.3[c,d]	34.3±4.5[a,b]	33.3±3.5[a,b]
Glucose (mmol/L)	4.86±0.97	4.81±1.13	5.43±0.99	5.82±0.94
Total Cholesterol (mmol/L)	4.69±0.93	5.25±1.19	5.14±1.19	5.20±0.59
HDLc (mmol/L)	1.46±0.48	1.54±0.34	1.20±0.37	1.45±0.41
LDLc (mmol/L)	2.68±0.90	3.26±0.85	3.19±0.98	3.02±0.49
Triglycerides (mmol/L)	1.18±0.70[c]	1.09±0.32[c]	1.89±0.82[a,b]	1.60±0.54

Abbreviations
BMI: body mass index; HDLc: high-density lipoprotein cholesterol; LDLc: low-density lipoprotein cholesterol. Results are given as mean±SD. ANOVA followed by post hoc Bonferroni was used to compare means between groups: [a] *P* < .05 *vs* Lean Adult; [b] *P* <.05 *vs* Lean Elderly, [c] P<.05 vs Obese Adult; [d] P<:05 vs Obese Elderly

To verify the isolation of hASC, we assessed the immunophenotypic profile of undifferentiated hASC populations using a panel of positive and negative surface markers that identify hASC according to the quantitative criteria established by the International Society of Cell Therapy (ISCT) and the International Federation for AdiposeTherapeutics and Science (IFATS) (Table S.2.).

Table S.2. Immunophenotypic profile of undifferentiated human adipose-derived mesenchymal stromal cells (hASCs) isolated from adult and elderly individuals[a]

Surface markers	Adult		Elderly	
	%	MFI	%	MFI
CD34	0.52±0.52	100±67	0.71±0.63	130±117
CD73	92.63±3.15	6187±2985	93.29±5.57	7825±3533
CD90	89.42±8.21	11775±8203	89.27±9.19	12022±11674
CD105	70.28±17.15	2722±2061	78.69±14.66	2318±1280
CD14	0.33±0.28	115±40	0.25±0.27	96±25
CD31	0.54±0.37	188±81	0.56±0.58	234±157
CD45	0.29±0.21	121±69	0.28±0.37	132±62.10

Human adipose-derived mesenchymal stromal cells obtained from subcutaneous adipose tissue biopsies from Adult and Elderly donors were stained with the panel of antibodies described and analyzed by flow cytometry using 405-nm, 488-nm and 633-nm excitation on the FACSAREA III cytometer (BD). Values are reported as the mean ±SD and means were compared between groups with the Student's unpaired t-test:
[a] $P<.05$ *vs* Lean Adult
Abbreviation: MFI, mean fluorescence intensity (arbitrary units)

4.1.1. Obesity and aging differentially impact the proliferative capacity of hASC

Obesity is known to influence hASC plasticity[107]; however, whether aging also has an impact is unclear. We first examined the growth and proliferation of hASC from donors stratified by age and BMI into the following groups: adult (>20 and <65 years) or elderly (≥65 years), and lean (BMI<25 kg/m^2) or obese (BMI≥30 kg/m^2), and the isolated hASC were divided into the following four groups on this basis: lean adult (LA), lean elderly (LE), obese adult (OA) and obese elderly (OE).

Following our previous results demonstrating that obese-derived hASC have a higher proliferation rate than their lean-derived counterparts[228], we found that the AT-cell number ratio (number of proliferating hASC at passage 0 per gram of digested AT) was significantly higher in the OA group than in the LE group (Figure 4.1A). By contrast, a lower AT-cell number ratio was found in the groups of elderly subjects compared with the LA group independently of obesity (Figure 4.1A). Analysis of proliferation assessed by MTT reduction (Figure 4.1B) and by flow cytometric analysis of intracellular CTV dilution (Figure 4.1C) confirmed the negative effect of aging on hASC proliferation, even in a background of obesity, suggesting that aging has a predominant impact over obesity for proliferation. We employed a general linear model to question whether proliferation (measured by MTT reduction) was significantly different between groups after adjusting for sex. This analysis revealed significant differences in MTT values between the four groups ($p<0.001$) independent of sex ($p=0.490$). We also used multiple linear regression analysis (stepwise forward selection procedures) to evaluate the potential role of age, sex, and BMI as independent factors associated with proliferation (MTT assay). Notably, age was the main determinant of MTT values (R2=0.304; ß=-0.552, $p<0.001$).

Finally, examination of senescence-related protein markers in cell extracts revealed an overall trend for greater expression in hASC from elderly subjects (Figure 4.1D), which was significant for Galactosidase Beta 1 (GLB1) expression.

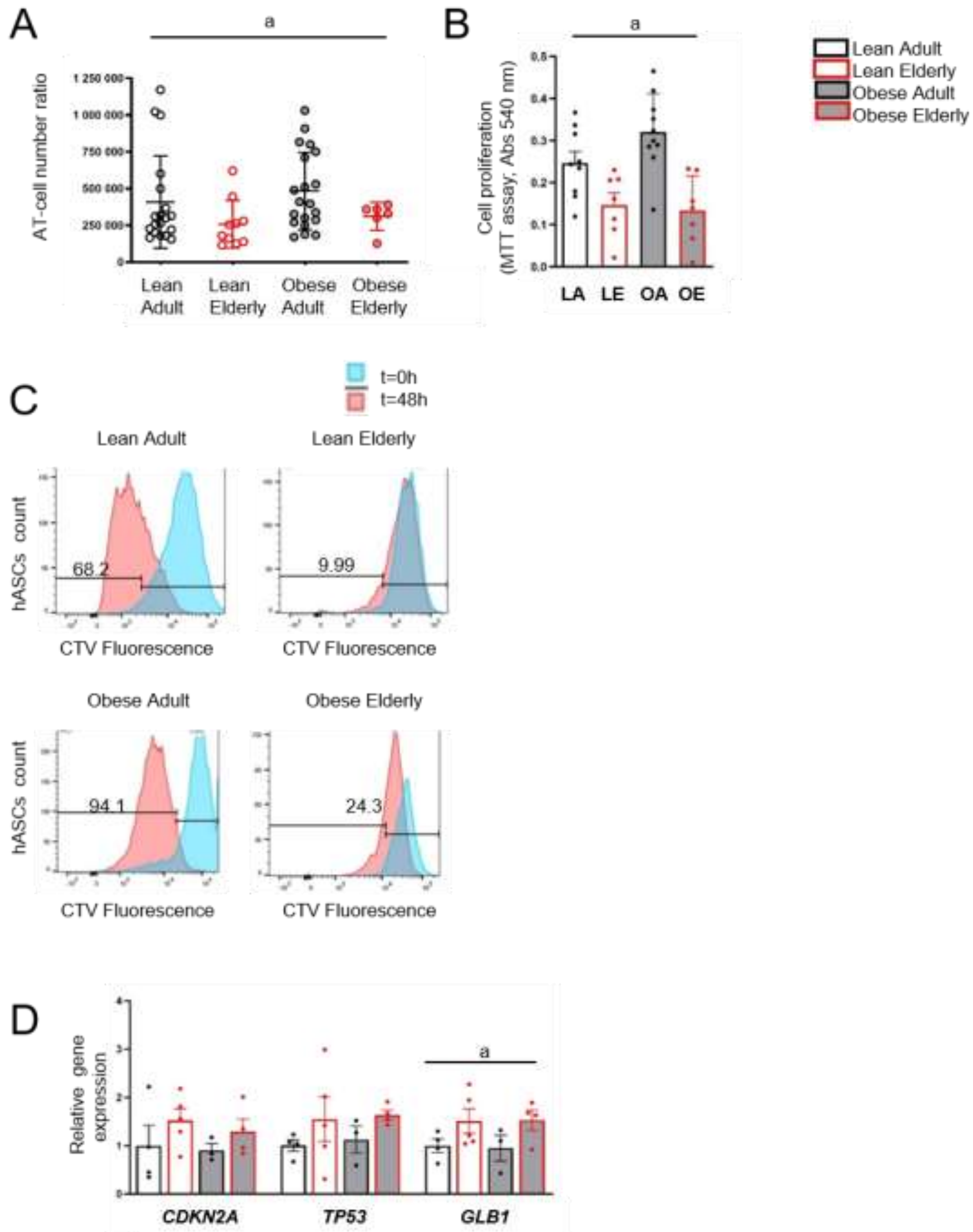

Figure 4.1. hASC modified by aging or obesity show differences in proliferation.

a) AT-cell number ratio in all groups (n=6–20). b,c) Proliferation measured by MTT reduction after 7 days in culture (b) (n=7–10) and CTV dye dilution after 48 h of incubation (c) (n=1). d) Gene expression of the senescence markers CDKN2A, TP53 and GLB1 in hASC (n=3–5).

4.1.2. Obesity and aging reduce the differentiation capacity of hASC

We next analyzed the influence of aging on the multilineage differentiation potential of hASC. Cells isolated from elderly subjects with or without obesity were significantly impaired in their capacity to differentiate into adipocytes, as revealed by Oil Red O staining of neutral lipids (Figure 4.2A) and by the gene expression of common adipogenic markers (*PPARG, FABP4, LPL, PLIN1,* and *FASN*) (Figure 4.2B). Similar results were obtained when we analyzed osteogenic differentiation in the different groups, as shown by the lower amount of calcium deposits stained with Alizarin Red (Figure 4.2C) and a trend for lower expression of osteogenic markers (*ALP, COL1A1, SPP1,* and *RUNX2*) (Figure 4.2D). By contrast, no differences were found in chondrogenic differentiation between the different hASC groups, as measured by Alcian Blue 8GX staining (Figure 4.2E) and gene expression of chondrogenic markers (*COMP* and *COL1A1*) (Figure 4.2F).

With the caveat that obesity might bolster some aspects associated with aging, our results suggest that obesity and aging differentially influence hASC properties.

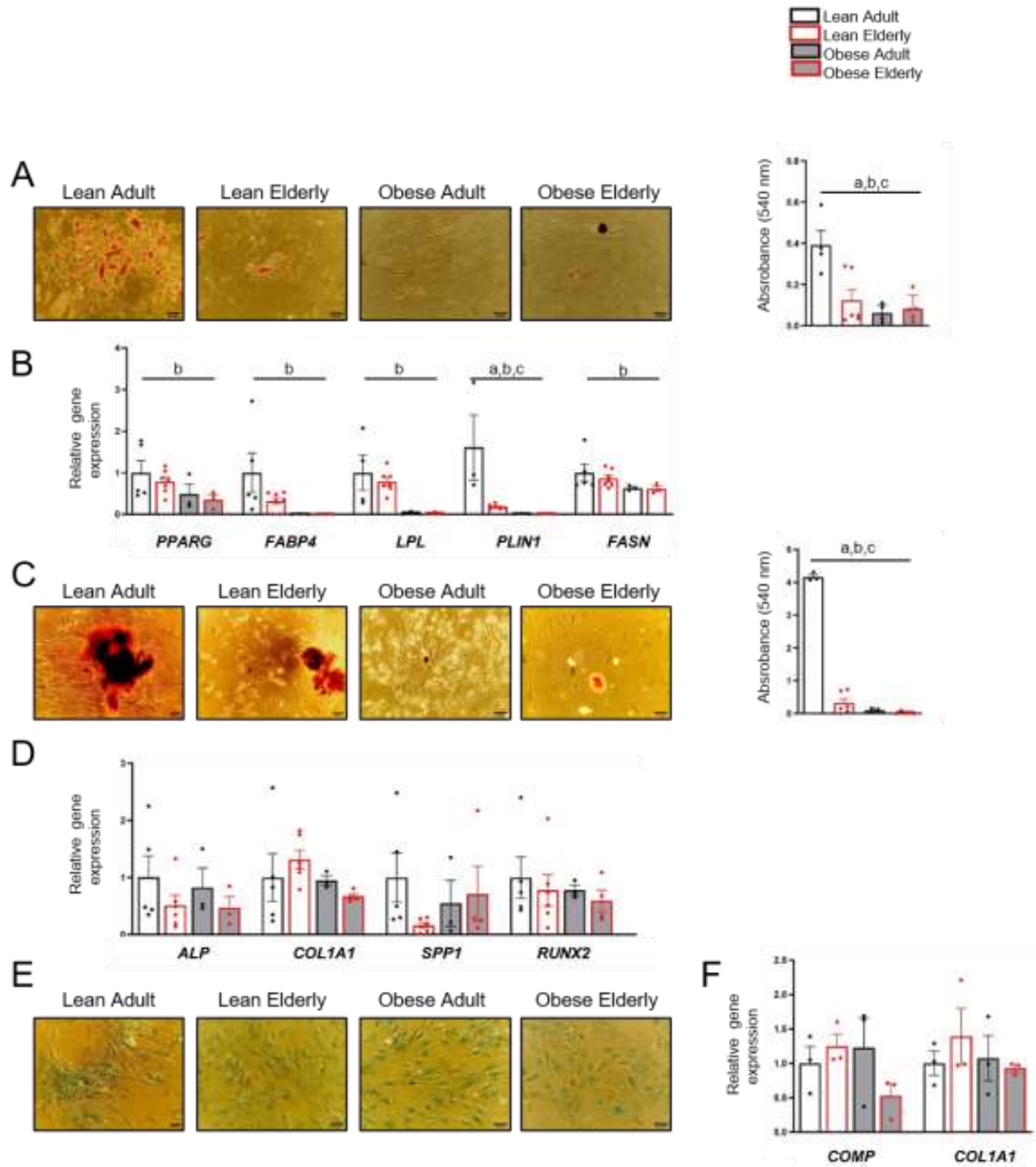

Figure 4.2. hASC modified by aging or obesity limit the differentiation potential of hASC. a,b) Representative images and quantification of Oil Red O staining (a) (n=4–6) and relative gene expression of adipogenic (*PPARG, FABP4, LPL, PLIN1,* and *FASN*) markers (b) (n=3–7) in adipocytes differentiated from hASC. c,d) Representative images and quantification of Alizarin Red staining (c) (n=3–6) and relative gene expression of osteogenic (*ALP, COL1A1, SPP1,* and *RUNX2*) markers (d) (n=3–6) in osteocytes differentiated from hASC. e,f) Representative images of Alcian Blue 8GX staining (e) and relative gene expression of chondrogenic (*COMP* and *COL1A1*) markers (f) (n=3) in chondrocytes differentiated from hASC. All images were taken at ×200 magnification; scale bar 100 µm. Data are shown as mean ± SEM; results of two-way ANOVA were age $p<0.05$ (a), BMI $p<0.05$ (b), interaction between age and BMI $p<0.05$ (c). Abbreviations: LA, Lean Adult; LE, Lean Elderly; OA, Obese Adult; OE, Obese Elderly

4.1.3. Obesity and aging dysregulate glucose metabolism in hASC

We used RNA expression profiling of hASC to study the impact of obesity and aging on metabolic-related genes. We found that the expression of several glucose metabolism-related genes was higher in hASC from elderly subjects than in adult-derived hASC, which was augmented by obesity (Figure 4.3A). We also found a significant positive correlation between age and markers of glucose transport (*Solute carrier family 2 member 1 (SLC2A1), Solute carrier family 2 member 3 (SLC2A3)) and metabolism (Hexokinase 2 (HK2), Phosphofructokinase M (PFKM)), tricarboxylic acid cycle (Succinate dehydrogenase b (SDHB), 2-oxoglutarate dehydrogenase (OGDH)) and glycogen metabolism (Glycogen synthase (GYS), PYGL, GBE))* (Figure 4.3B). The glycolytic phenotype was more pronounced in hASC from elderly subjects (and was amplified by obesity) and was characterized by a significantly higher secretion of lactate and succinate, which are markers of aerobic glycolysis[234] and mitochondrial stress[235], respectively (Figure 4.3C, D). Lactate and succinate release by hASC positively correlated with age and BMI (Figure 4.3C, D). Although we found that both obesity and aging promoted a glycolytic phenotype, the specific upregulation of glycogenic enzymes (*GYS, PYGL,* and *GBE1*), which are known to be expressed in adipocytes[236], was evident in the groups of elderly subjects (LE and OE) (Figure 4.3A).

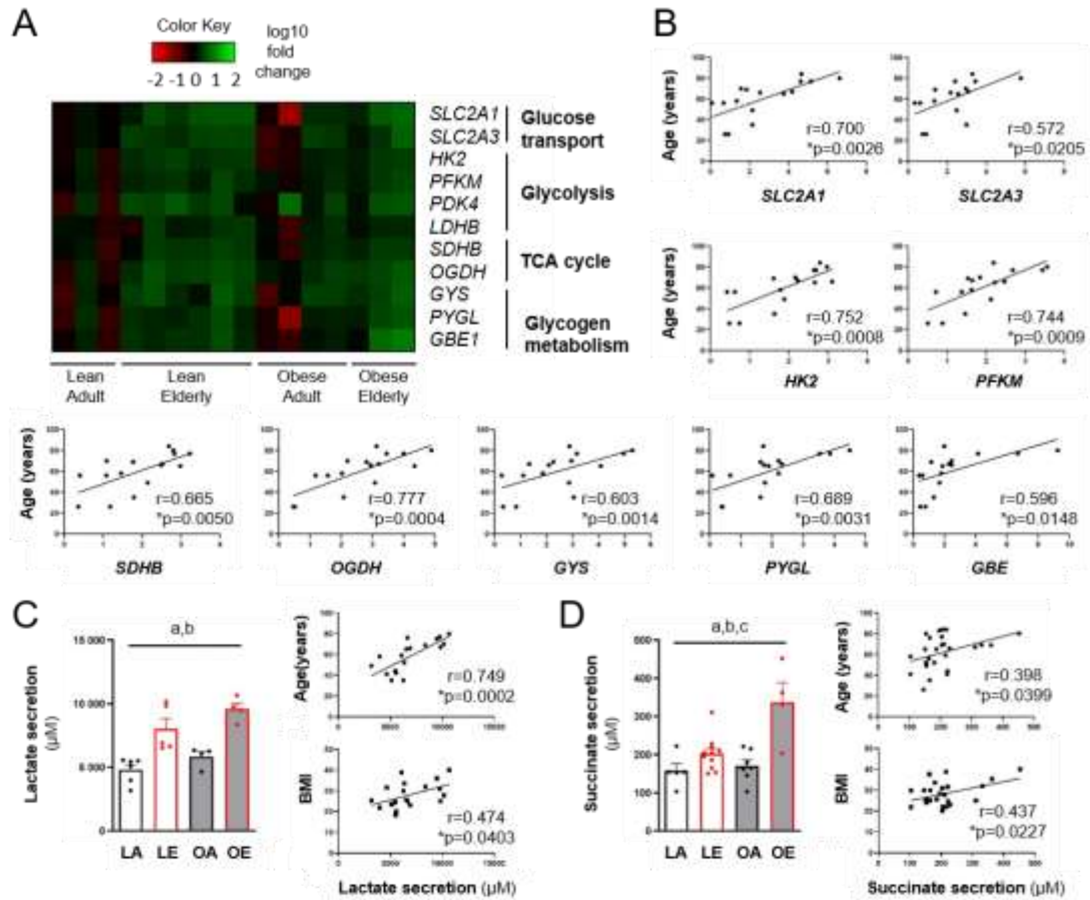

Figure 4.3. Obesity, but particularly aging, promote glucose utilization by glycolytic pathways

a) Gene expression heatmap of glucose transporters (*SLC2A1, SLC2A3*), glycolytic markers (*HK2, PFKM, PDK4*), TCA enzymes (*LDHB, SDHB, OGDH*), and glycogen synthesis and degradation enzymes (*GYS, PYGL* and *GBE1*) (n=3–6). b) Positive correlation between age and expression of *SLC2A1, SLC2A3, HK2, PFKM, SDHB, OGDH, GYS, PYGL,* and *GBE1* determined by Pearson's correlation analysis. c) Lactate secretion of hASC cultured for 24 h and correlation analysis with age and body mass index (BMI) (n=4–6). d) Succinate secretion of hASC cultured for 24 h and correlation analysis with age and BMI (n=4-11). Data are shown as mean ±SEM; results of two-way ANOVA were age $p<0.05$ (a), BMI $p<0.05$ (b), interaction between age and BMI $p<0.05$ (c). Correlations were determined by Pearson's correlation analysis. Abbreviations: LA, Lean Adult; LE, Lean Elderly; OA, Obese Adult; OE, Obese Elderly

As glycogen synthesis is mainly regulated at the protein level, we used immunoblotting to examine different proteins regulating this pathway. GS, the rate-limiting enzyme in glycogen synthesis, exists in an active (dephosphorylated) and an inactive (phosphorylated) form. The LE group showed significantly lower phosphorylated (p)-GS levels (inactive GS), which mirrors a higher activity than the LA group (Figure 4.4A). Results also revealed significantly higher p-GSK3 (inactive form of GSK3) levels in hASC isolated from the LE

group (Figure 4.4A), which agrees well with the activated GS. Moreover, hASC isolated from the LE group showed higher levels of the glycogen targeting subunit PTG. No differences were found in the protein levels of the active form of glycogen phosphorylase (p-PYGL), which metabolizes glycogen, or in GBE, which mediates glycogen branching (Figure 4.4A). Moreover, the expression of p-AMPK, which has also been inversely related to glycogen levels[237], was lower in hASC from the LE group. Correlation analysis showed that p-GS and p-GSK3 protein abundance correlated negatively and positively, respectively, with age (Figure 4.4B, C), suggesting that aging might promote glycogenesis in hASC. This finding was supported by the evident increase in glycogen content in hASC from obese and elderly subjects, as measured by indirect immunofluorescence (Figure 4.4D). A quantitative colorimetric assay of glycogen (Figure 4.4E) indicated that age was the main factor influencing glycogen content. Overall, our data reveal that both obesity and aging enhance glucose utilization in hASC by glycolytic and glycogenesis pathways.

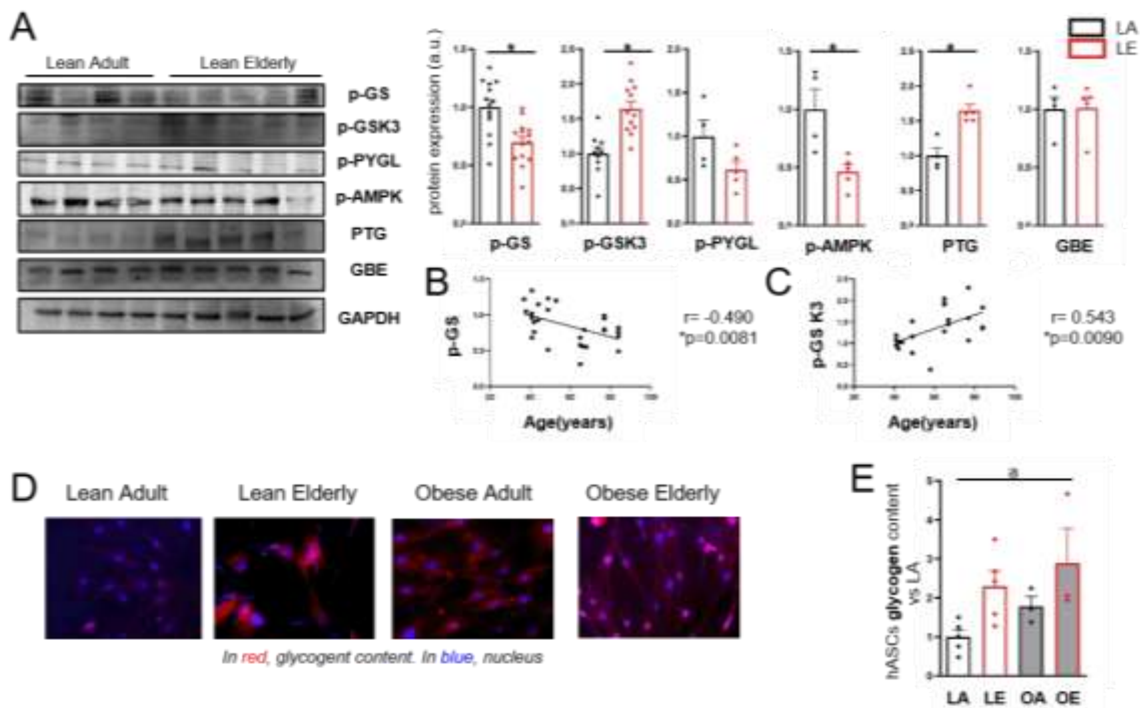

Figure 4.4. Obesity, and especially aging, promote glucose utilization in glycogenesis pathways

a) Representative immunoblots and densitometry of p-GS, p-GSK3, p-PYGL, p-AMPK, PTG, and GBE protein expression in hASC from adult and elderly lean donors (n=4–5). b) Correlation between p-GS protein expression and age. c) Correlation between p-GSK3 protein expression and age. GAPDH was used as a loading control. Densitometry analyses are presented in arbitrary units (a.u). Data are shown as mean ± SEM from three independent experiments; two-tailed unpaired Student's t test, $p<0.05$ (*). Correlations were determined by Pearson's correlation analysis. d,e) Glycogen content was determined by indirect immunofluorescence (d) and by a quantitative colorimetric assay (e) (n=3–5). Data are shown as mean ± SEM; results of two-way ANOVA were age $p<0.05$ (a), BMI $p<0.05$ (b), interaction between age and BMI $p<0.05$ (c). Abbreviations: LA, Lean Adult; LE, Lean Elderly; OA, Obese Adult; OE, Obese Elderly

4.1.4. HIF-1α, driver of both glycolysis and glycogen synthesis, is upregulated in hASC from aged and obese individuals

Upon the observation that in hASC from obese and aged settings glycolysis and glycogen formation appear to be the predominant pathways of metabolism, we decided to assess the gene expression of *HIF1A*, as it's a transcription factor for glycolytic and glycogen synthesis enzymes[238]. As expected, the levels of *HIF1A* were upregulated in the LE group but especially in the OA and OE groups (Figure 4.5A), suggesting obesity might promote a stronger upregulation of HIF-1α in hASC and probably in other cells in the AT.

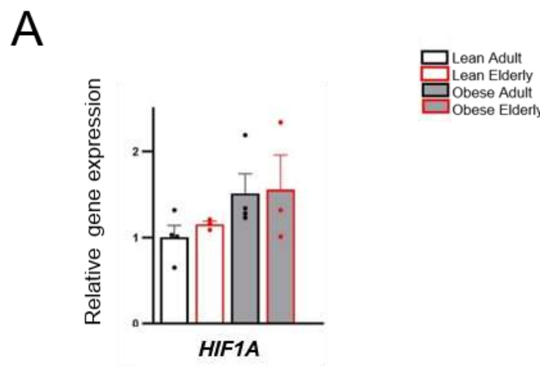

Figure 4.5. HIF1A levels are upregulated in obesity

a) Gene expression of HIF1A was assessed. Data are shown as mean ± SEM; results of two-way ANOVA were age $p<0.05$ (a), BMI $p<0.05$ (b), interaction between age and BMI $p<0.05$ (c)

4.1.5. Obesity exacerbates the inflammatory phenotype of hASC in elderly subjects

Metabolic reprogramming toward glycolysis is known to be associated with inflammatory states[194]. In this context, we recently reported a link between glycogen accumulation and proinflammatory cytokine expression in human AT[236]. In line with these studies and with our previous finding of an inflammatory phenotype in hASC from obese donors[75], we noted that the expression of the proinflammatory markers *IL1B, IL6,* and *CCL2* was higher in hASC from obese adults than from lean adults (Figure 4.6A). Likewise, age was a major determinant for the expression of the three cytokines. Moreover, a synergistic effect of age and BMI was found for the gene expression of *IL1B* and *IL6*, achieving statistical significance for *CCL2* (Figure 4.6A). To better understand the effect of aging and obesity on the inflammatory phenotype of hASC, we used a multiplexed cytokine array (Figure S.1) to interrogate the hASC secretome (including chemokines, inflammatory cytokines, and angiogenesis- and senescence-related factors). As shown in Figure 4.6B, the cytokine secretion pattern of the CM of hASC differed among groups, with the most pronounced changes found in hASC of elderly subjects (both lean and obese). From the 62 cytokines measured, we focused on those 26 cytokines with a significant increase of ≥1.5-fold-change over hASC from the LA group in at least one of the groups (Figure 4.6C). A Venn diagram of the 26 cytokines (Figure 4.6D) showed that the increase in the abundance of inflammatory cytokines in the hASC-CM of the OE group was due to aging rather than obesity, leading us to conclude that aging has a greater influence than obesity on the establishment of a proinflammatory secretome. Nonetheless, a group of five cytokines required the presence of both conditions (aging and obesity) for a significant increase in secretion. Of note, leptin—a classic adipokine—was exclusively increased in the hASC-CM of the OA group, suggesting that aging counteracts the impact of obesity on leptin secretion. This fits well with previous data describing a negative correlation between leptin plasma levels and age in subjects with obesity[239]. Conversely, the abundance of the chemoattractant Chemokine (C-C motif) ligand 11 (CCL11)/ Eotaxin-1 in hASC-CM was lower in a background of obesity only, and increased markedly in the hASC-CM of the OE group (Figure 4.6B, C, D). Indeed, a synergistic induction of chemokine secretion as a response to proinflammatory cytokines has been previously described[240]. To identify whether the presence of both conditions (aging and obesity) could trigger the synergistic induction of some chemokines, we assessed the 23 cytokines secreted by the OE group with a significant ≥1.5-fold-change increase for those with double the sum of the rise of the LE and OA groups. Three chemokines met this criterion: CCL5, CCL7, and CCL11 (Figure

4.6E). Overall, our data demonstrate that obesity and aging differentially impact the secretory properties of hASC.

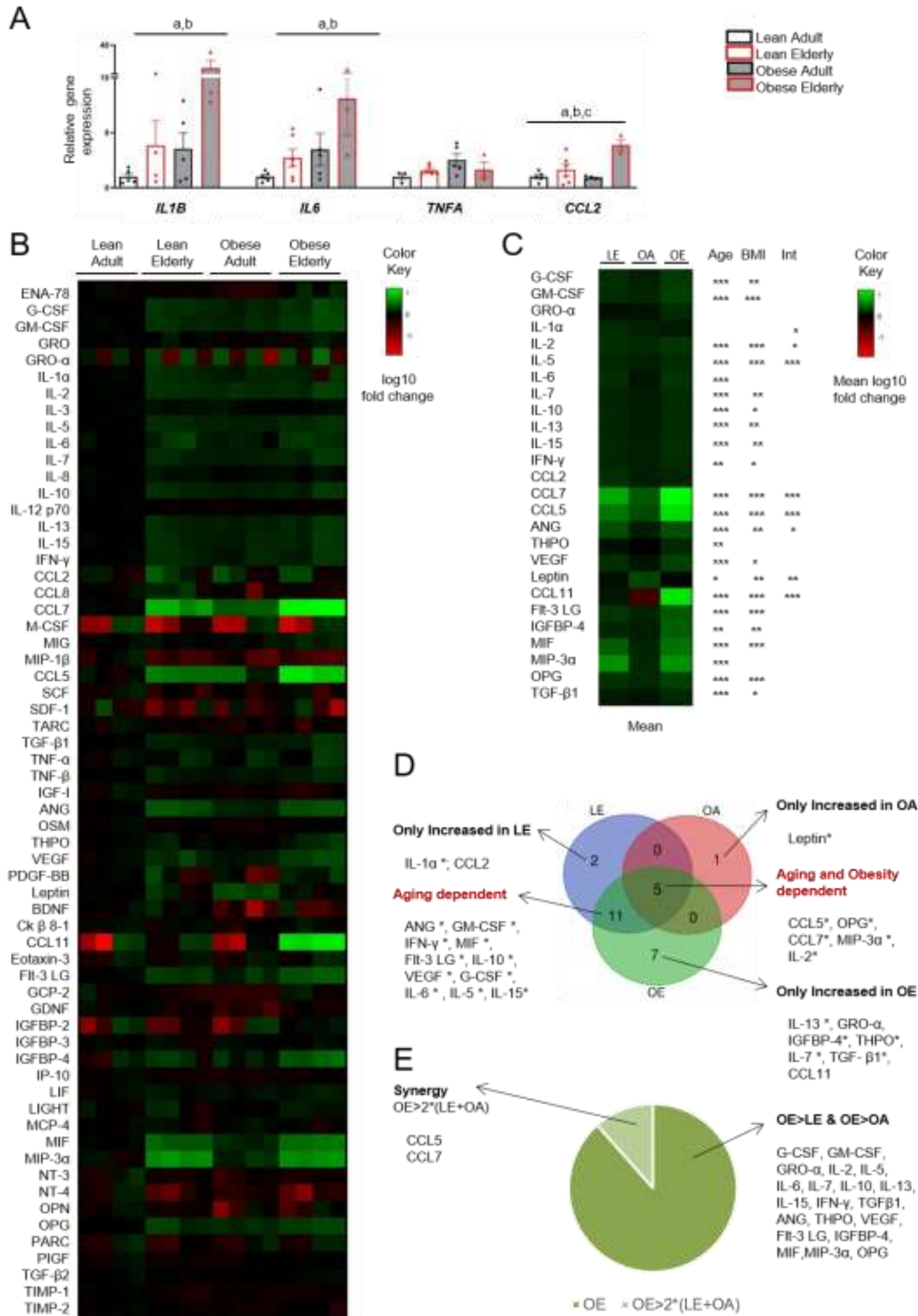

Figure 4.6. Changes in the cytokine secretion phenotype in hASC from elderly donors are exaggerated by obesity

a) Relative gene expression of inflammatory markers (*IL1B, IL6, TNFA* and *CCL2*) in hASC from all groups (n=3–6). Data are shown as mean ± SEM; results of two-way ANOVA were age $p<0.05$ (a), BMI $p<0.05$ (b), interaction between age and BMI $p<0.05$ (c). b) Heatmap of the 62 cytokines secreted by hASC of all four groups, expressed as the log10 of the fold change *versus* hASC of the lean adult (LA) group (n=5–10). c) Heatmap of the 26 cytokines secreted by hASC of the lean elderly (LE), the obese adult (OA), and/or the obese elderly (OE) groups showing ≥1.5-fold greater levels than those of the LA group, expressed as the log10 of the mean fold change *versus* the LA group. Results of two-way ANOVA were age $p<0.05$, BMI $p<0.05$, interaction between age and BMI $p<0.05$. d) Venn diagram of the 26 cytokines shown in c. e) Of the 23 cytokines secreted by hASC from the OE group with ≥1.5-fold greater levels than those of the LA group, synergy was established for those in this group that showed double the sum of the increase in LE and OA *versus* LA groups. Abbreviations: LA, Lean Adult; LE, Lean Elderly; OA, Obese Adult; OE, Obese Elderly

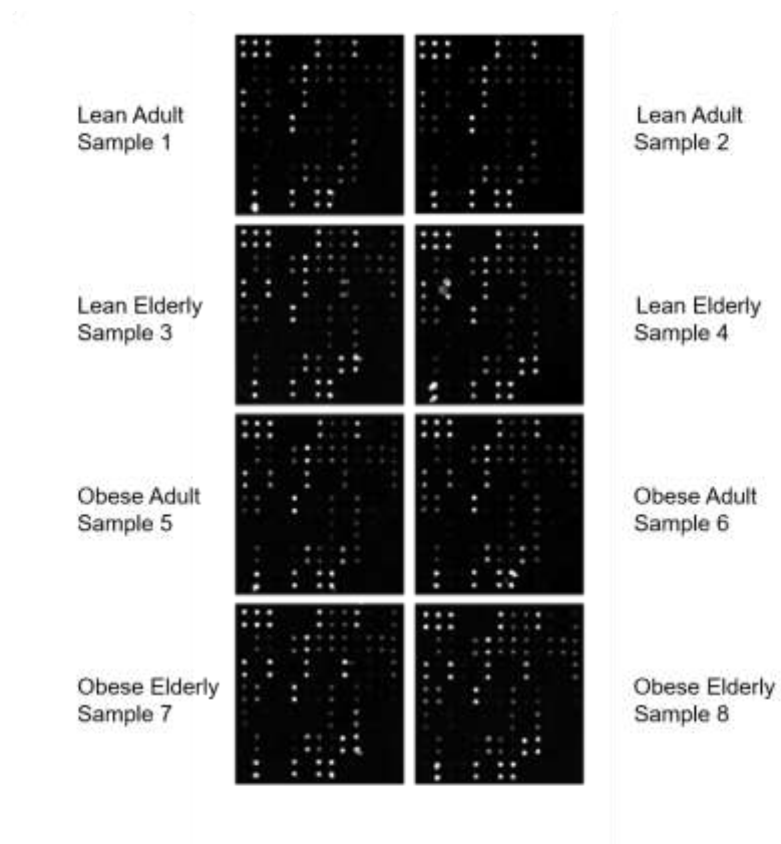

Figure S.1. Raybio human cytokine antibody array

4.1.6. The aged- and obesity-related inflammatory states of hASC are associated with an antagonistic cross-talk between glycogen deposition and SIRTs

Sirtuins are key metabolic sensors involved in the pathophysiology of inflammatory-related processes, including aging and obesity[205], and both obesity[225] and aging[241] have been associated with a reduction in SIRT1 activity in AT. Similarly, published data point to a reduction in SIRT6 protein levels in the AT of people with obesity[226]. Thus, we analyzed the expression of SIRT1 and SIRT6 in hASC from the different groups of donors, finding a downregulation of both in the elderly (Figure 4.7A) and obese (Figure 4.7B) groups, without significant differences at the mRNA level (data not shown). Correlation analysis showed that SIRT1 and SIRT6 expression in hASC correlated negatively with age (Figure 4.7C), whereas only SIRT1 correlated negatively with BMI (Figure 4.7D).

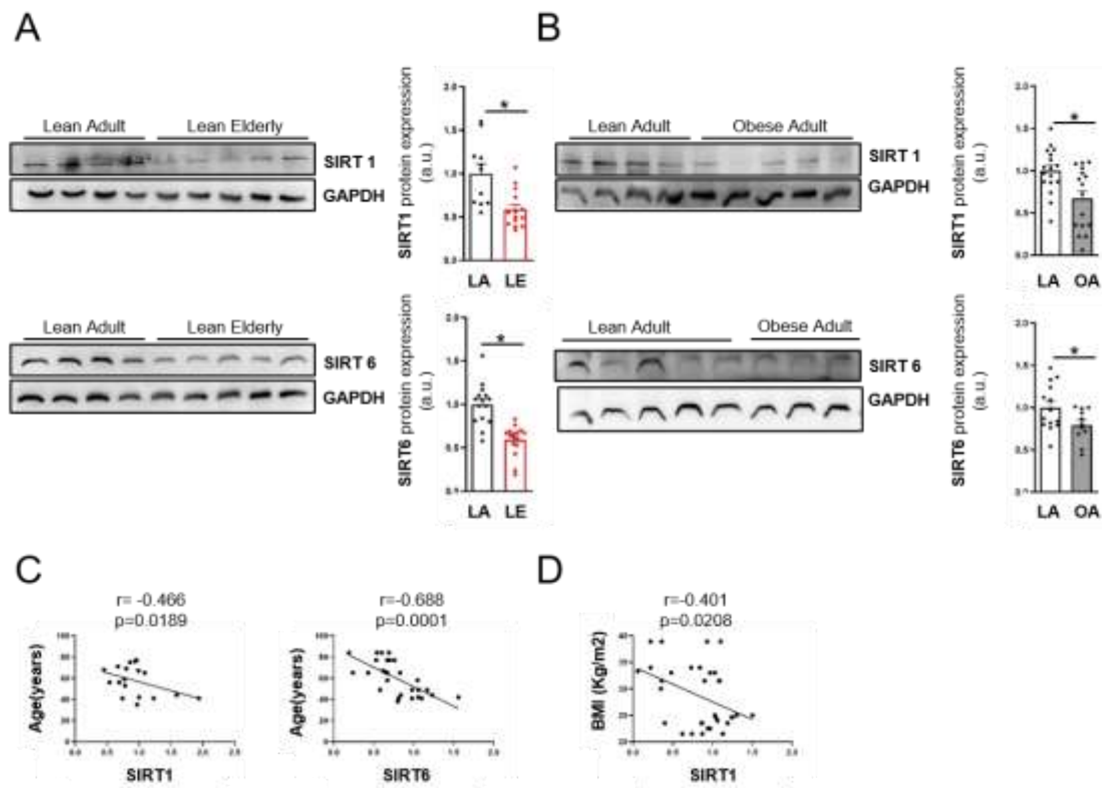

Figure 4.7. SIRT1 and SIRT6 protein levels are diminished in hASC from inflamed obese and aged nixes

a,b) Representative immunoblots and densitometry analysis of SIRT1 and SIRT6 protein expression in hASC of lean adult (LA) and lean elderly (LE) (a) and LA and obese adult (OA) (b) groups (n=3–5). Data are shown as mean ± SEM from three independent experiments; two-tailed unpaired Student's t test, $p<0.05$ (*). c,d) Correlation analysis between SIRT1 or SIRT6 expression and age (c) and between SIRT1 and body mass index (BMI) (d). Correlations were determined by Pearson's correlation analysis. Abbreviations: LA, Lean Adult; LE, Lean Elderly; OA, Obese Adult

To test for a link between the decline in SIRT1 and SIRT6 expression and the acquisition of a proinflammatory profile in elderly-derived cells, we used siRNA to independently knockdown the expression of *SIRT1* and *SIRT6* in control (LA-derived) hASC. Gene expression analysis of inflammatory markers showed that both *SIRT1* (Figure 4.8A) and *SIRT6* (Figure 4.8B) knockdown resulted in the upregulation of several proinflammatory genes, including a significant upregulation of *IL1B*. In addition, *SIRT1* downregulation resulted in a significant increase in *TNFA* expression (Figure 4.8A). The inflammatory phenotype of control hASC induced by *SIRT1* knockdown was accompanied by a significant increase in *HK2* and *PFKM* expression (Figure 4.8C), whereas *SIRT6* knockdown in control hASC resulted in a significant increase in *HK2*, *PDK4,* and *SLC2A1* expression (Figure 4.8D).

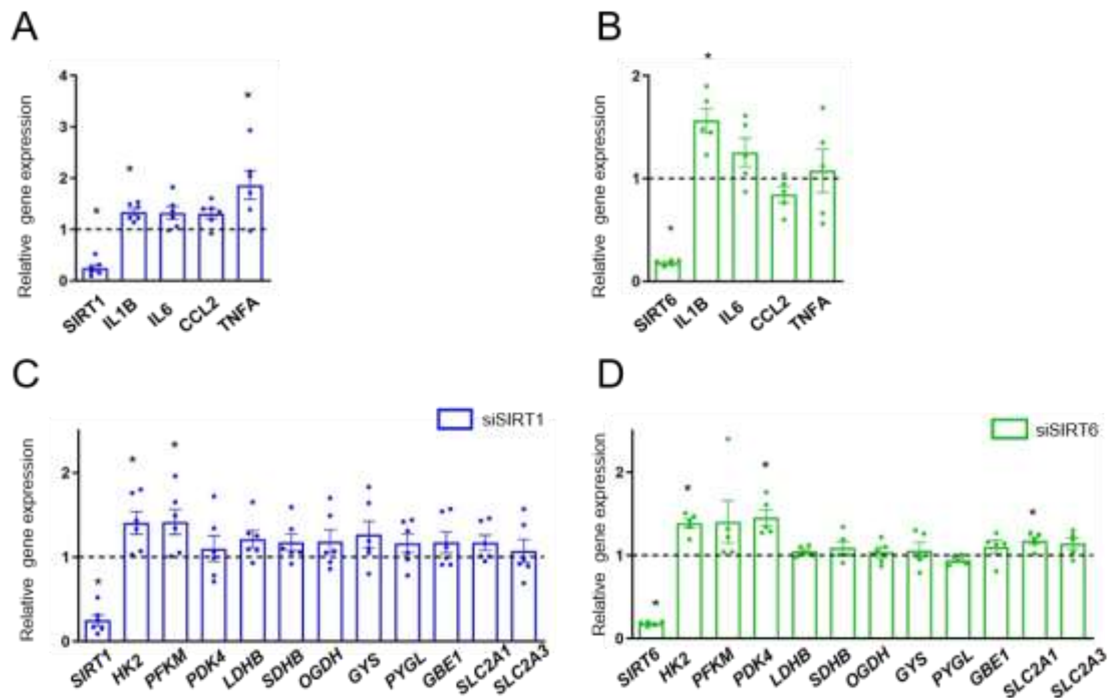

Figure 4.8. SIRT1 and SIRT6 silencing triggers inflammation and enhances glycolysis in hASC

a,b) Relative gene expression of *SIRT1* (a) and *SIRT6* (b) and inflammatory markers (*IL1B, IL6, CCL2,* and *TNFA*) in lean adult (LA) hASC silenced with siSIRT1 (a) (n=6) or siSIRT6 (b) (n=5) *versus* control hASC. c,d) Relative gene expression of *SIRT1* (c) or *SIRT6* (d) and glycolytic markers (*HK2, PFKM, PDK4*), tricarboxylic acid cycle enzymes (*LDHB, SDHB, OGDH*), glycogen synthesis and degradation enzymes (*GYS, PYGL,* and *GBE1*) and glucose transporters (*SLC2A1, SLC2A3*) in siSIRT1 (c) (n=6) or siSIRT6 (n=5) hASC (d) *versus* control hASC. Control values are arbitrarily set to 1.0 (dotted line). Data are shown as mean ± SEM; Two-tailed paired Student's t test, $p<0.05$ (*)

Searching for a molecular mechanism underlying SIRT downregulation associated with obesity and aging, we explored metabolic reprogramming as a potential cause. Specifically, we forced glycogen deposition in control hASC using an adenoviral transduction system overexpressing PTG[236,242]. As expected, glycogen levels were markedly higher in Ad-PTG-hASC than in control Ad-GFP-hASC overexpressing GFP (Figure 4.9A), consistent with decreased p-GS expression (Figure 4.9B). We also observed an increase in proinflammatory gene marker expression (*IL1B, IL6, CCL2,* and *TNFA*) in PTG-overexpressing cells, which was significant for *IL1B* and *IL6* expression (Figure 4.9C), supporting a link between glycogen and inflammation[236]. Notably, SIRT1 and SIRT6 expression was significantly lower in Ad-PTG-hASC than in control cells (Figure 4.9B), indicating that the downregulation of SIRT1 and SIRT6 in hASC from elderly and obese donors could be, at least partly, a consequence of glycogen deposition. Contrastingly, no differences in protein expression were found for p-AMPK (Figure 4.9B), ruling it out as the upstream interface for SIRT1 and SIRT6 downregulation, at least in these cells. We assessed cell proliferation and adipocyte differentiation to explore the functional impact of glycogen deposition on hASC function. hASC overexpressing PTG showed a significant decrease both in proliferation, as determined by MTT reduction (Figure 4.9D), and in adipocyte differentiation capacity, as revealed by quantitative Oil Red O staining of neutral lipids (Figure 4.9E), and by gene expression of the adipogenic markers *FABP4*, *LPL* and *PLIN1* (Figure 4.9F).

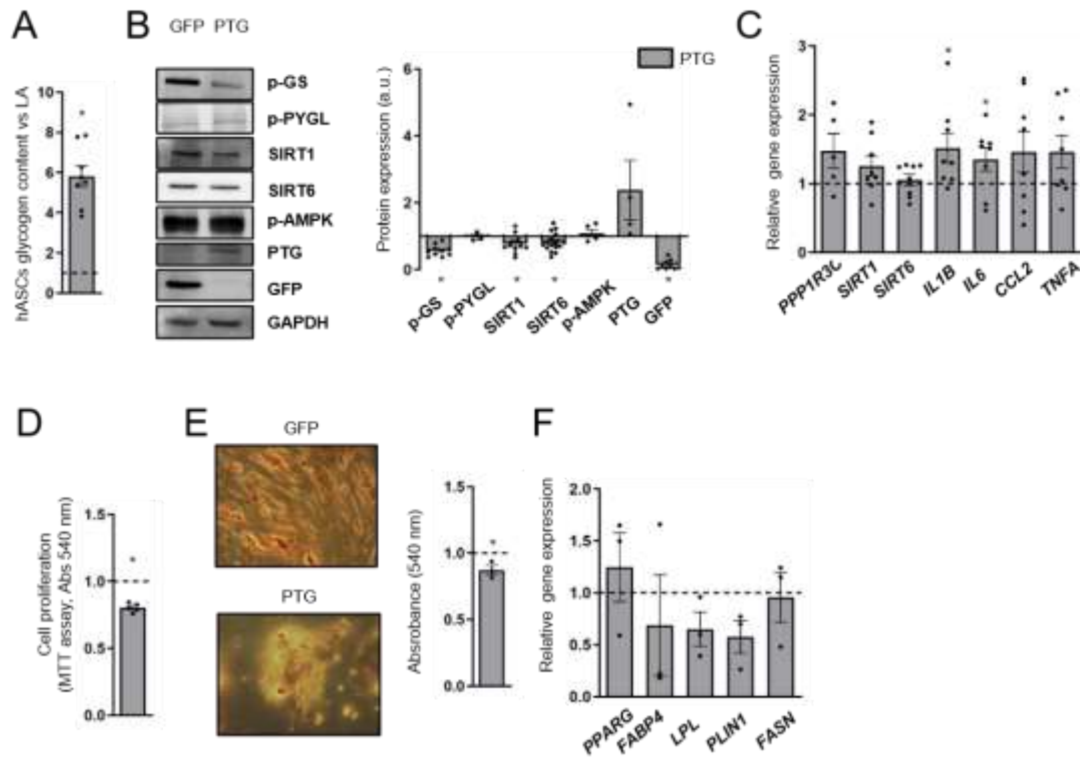

Figure 4.9. Enhancing glycogen deposition in hASC reduces SIRT1 and SIRT6 protein expression

a–c)PTG was overexpressed in hASC using an adenoviral transduction system. a,b) LA hASC overexpressing PTG *versus* those overexpressing GFP (control) were analyzed for glycogen deposition (a) (n=8) and p-GS, p-PYGL, SIRT1, SIRT6, p-AMPK, PTG and GFP protein expression (b) (n=3–16). c) Gene expression of *PPP1R3C, SIRT1, SIRT6,* and inflammatory markers (*IL1B, IL6, CCL2,* and *TNFA*) was determined in hASC overexpressing PTG *versus* those overexpressing GFP (control) (n=5–8). d–f) Proliferation determined by MTT assay (d) and adipocyte differentiation analyzed by Oil Red O staining (e) and gene expression of adipocyte differentiation markers (f) were assessed in hASC upon PTG overexpression *versus* those overexpressing GFP (control). Control values are arbitrarily set to 1.0 (dotted line). Data are shown as mean ± SEM; Two-tailed paired Student's t test, $p<0.05$ (*)

Finally, we measured glycogen accumulation in cells silenced for *SIRT1/6* to explore a potential feedback loop between SIRT1/6 and glycogen metabolism. Both *SIRT1* and *SIRT6* knockdown led to a decrease in glycogen content in hASC without any significant changes to the inactive form of GS (p-GS) or the active form of PYGL (p-PYGL) (Figure 4.10A–B, D-E), indicating that metabolic dysregulation and glycogen accumulation precedes SIRT1/6 downregulation in aged cells. Moreover, a trend for a decrease in p-AMPK protein

expression was found upon *SIRT1* silencing (Figure 4.10A), and a significant decrease in p-AMPK levels was found upon *SIRT6* downregulation (Figure 4.10D). These data suggest that p-AMPK could be downstream of SIRT1 and SIRT6, mediating the increased inflammation that occurs in response to *SIRT1/6* downregulation. Finally, no differences in proliferation were found in hASC silenced for *SIRT1* (Figure 4.10C) or *SIRT6* (Figure 4.10F). Overall, our data point to a glycogen-SIRT1/6 axis as a putative driver of age-related inflammation in hASC.

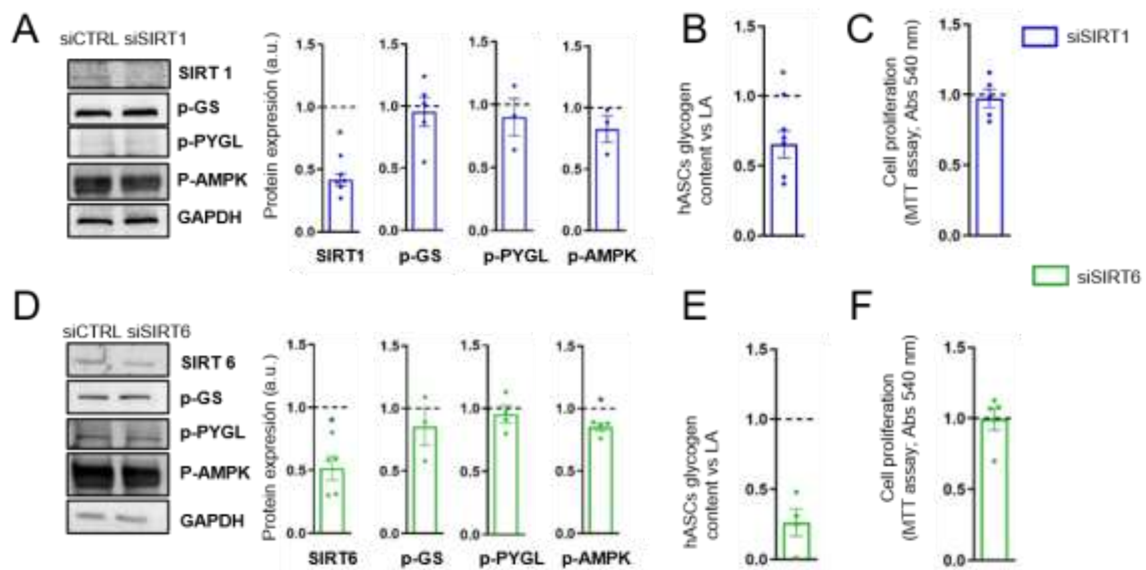

Figure 4.10. Reduction of SIRT1 and SIRT6 expression enhances glycogen content and reduces p-AMPK levels

a–c) Representative immunoblots and densitometric analysis of SIRT1, p-GS, p-PYGL, and p-AMPK (a) (n=3–6), glycogen content (b) (n=6) and proliferation (c) in hASC transfected with siSIRT1 *versus* control hASC. d–f) Representative immunoblots and densitometric analysis of SIRT6, p-GS, p-PYGL, and p-AMPK (d) (n=3–5), glycogen content (e) (n=4) and proliferation (f) in hASC transfected with siSIRT6 *versus* control hASC. For immunoblots, GAPDH was used as the loading control. Densitometric analyses are presented in arbitrary units (a.u.). Control values are arbitrarily set to 1.0 (dotted line). Data are shown as mean ± SEM; Two-tailed paired Student's t test, $p<0.05$ (*)

4.2. Study 2

Aging and obesity modulation of the immunomodulatory properties of hASC

4.2.1. Aging and obesity enhance the migration capacity of hASC

hASC isolated from LE, OA, and OE display increased migration capacity. When placed in the upper chamber of a Transwell system with an 8-µm pore polycarbonate membrane, the number of hASC that were able to go through the membrane and reach the lower chamber was higher when the hASC were isolated from obese or aged environments (Figure 4.11A). In other words, the hASC from obese and aged individuals have a significantly increased migratory potential, facilitating one of the functions of these cells, which is reaching the site of injury. Interestingly, obesity is the factor that boosts the most the migratory capacity of hASC.

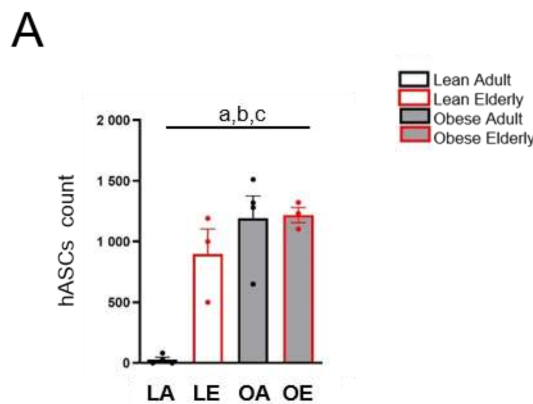

Figure 4.11. Migration of hASC

a) hASC migration after 24h of incubation was assessed by counting the cells in the lower chamber of a transwell system (8-µm pore) with an automatic cell counter. Data are shown as mean ± SEM; results of two-way ANOVA were age $p<0.05$ (a), BMI $p<0.05$ (b), interaction between age and BMI $p<0.05$ (c)

4.2.2. Obesity boosts the immunosuppressive capacity of hASC

Suppressing T-cell proliferation is an important immunomodulatory property attributed to hASC[96]. To study if low-grade inflammatory environments like those found in the AT of obese and elderly individuals stimulate or lessen this ability of hASC, we conducted an experiment where PBMC proliferation was determined after 72h incubation with 24h-CM from the different groups in the cohort. hASC CM from the LE but particularly from the

Obese groups was more capable of suppressing T-cell proliferation than the CM of lean individuals (Figure 4.12A). Elderly and obese nixes are significantly stimulating the immunosuppressive capacities of hASC when compared to their lean counterparts, which goes under the knowledge that MSC's immunomodulatory activities need to be licensed by stimulation such as inflammation or hypoxia[243]. Nonetheless, obesity was the main determinant influencing T-cell proliferation suppression, independently of age. In conclusion, hASC isolated from inflamed environments, like those found in aging and obesity, inhibit T-cell proliferation by molecules in their secretome to a higher degree than their lean counterparts.

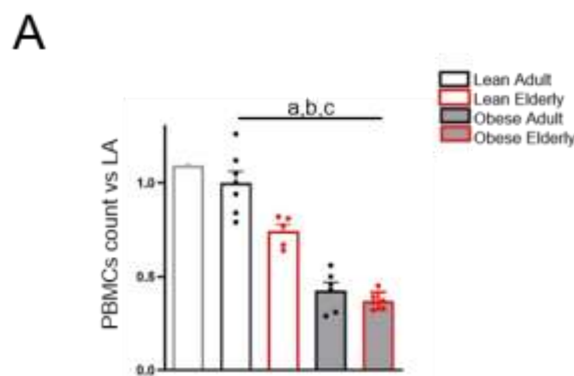

Figure 4.12. hASC capacity to suppress T-cell proliferation is strongly enhanced by obesity
a) PBMC proliferation was assessed after 72h-incubation with 24h-CM from hASC of the different groups. Data are shown as mean ± SEM; results of two-way ANOVA were age $p<0.05$ (a), BMI $p<0.05$ (b), interaction between age and BMI $p<0.05$ (c)

4.2.3. Aging and obesity increase T-cell and monocyte chemotaxis

MSC are known to act as chemoattractants by secreting chemotactic cytokines known as chemokines. Thus, they can induce the migration of lymphocytes and monocytes from the blood towards the AT where hASC are[89]. They recruit leucocytes in a paracrine manner; this is why we though chemotactic assays with the 24h-CM of hASC from the different groups would help us assess the influence of the subject phenotype on the chemotactic properties of hASC. 24h-CM from elderly and obese backgrounds induced the migration of monocytes and lymphocytes to the lower chamber of a transwell system (5-mm pore polycarbonate membrane) more strongly than the CM from lean adult hASC (Figure 4.13A, B). The effect of the hASC secretome on the migration of monocytes seems to be

significantly higher than the effect on lymphocyte migration. In conclusion, the CM of elderly and obese hASC triggers more the migration of lymphocytes and, specially, that of monocytes, when compared to their lean counterparts, probably by the increased secretion of chemokines, demonstrated in previous experiments in this thesis.

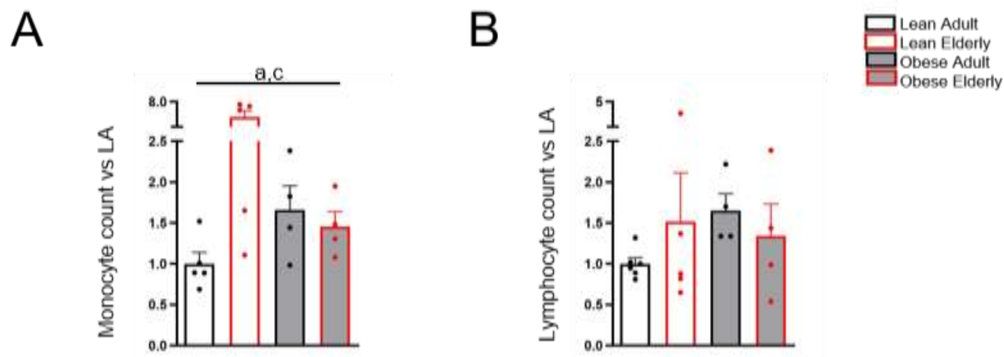

Figure 4.13. Lymphocyte and Monocyte chemotaxis is stimulated by hASC from aged and elderly environments

a) Human T Lymphocytes and human Monocytes migration to hASC-CM from LE, OA, and OE is higher when compared to the CM of their lean counterparts. Data are shown as mean ± SEM; results of two-way ANOVA were age $p<0.05$ (a), BMI $p<0.05$ (b), interaction between age and BMI $p<0.05$ (c)

4.2.4. Incubation with the CM from hASC obtained from an aged or obese milieu makes macrophages adopt a mixed-M2 phenotype

To determine if hASC from obese and elderly nixes can modify macrophage phenotype in a paracrine manner, human PBMC-derived macrophages Mϕ were incubated for 6h with hASC-CM of the different groups in the cohort. The culture of macrophages with the CM from hASC of obese and elderly groups induced a switch towards a mixed-M2 phenotype, shown by the increased expression of the M2-anti-inflammatory markers *IL10 and KLF4*, achieving significance for *PPARG* (Figure 4.14A). On the other hand, analysis of the gene expression of the M1-inflammatory markers *IL6*, *IL1B*, *MCP1,* and *CCL3* demonstrated a trend for lower expression in macrophages incubated with CM from elderly and obese groups, which was significant for *CCL3* (Figure 4.14A). Finally, a trend for a higher expression of *TNFA* was revealed for macrophages incubated with CM from obese groups (Figure 4.14A). In conclusion, the secretome of hASC from obese and aged nixes seems to

polarize macrophages towards the M2 phenotype predominantly, but still shows an increased expression of some inflammatory markers like *TNFA*. This goes in accordance with previous results of our group indicating that CM from obese-derived hASC promotes the obtention of hybrid macrophages that exhibit both M1 and M2-phenotype features, promoting a protumoral microenvironment[244].

In an attempt to determine if SIRT1 and SIRT6 expression in hASC could modify their secretome and modulate the polarization of macrophages, we cultured human PBMC-derived macrophages Mϕ for 6h with CM from SIRT1 or SIRT6-silenced hASC. Then we assessed the gene expression of M1 and M2 gene expression markers. Intriguingly, we found opposite effects for SIRT1 and SIRT6. The silencing of SIRT1 in hASC enhanced the expression of all the M2 markers (*IL10, PPARG, MRC1, KLF4*) and showed no change in the expression of inflammatory markers (Figure 4.14B). In other words, our results reveal that enhanced expression of SIRT1 in hASC could be promoting the M1 macrophage phenotype. These results agree with previous data postulating that SIRT1 overexpression in macrophages promoted the polarization of macrophages towards the M1 phenotype, helping the immune reaction against the tumor[245]. Nonetheless, SIRT6-silenced hASC promote the polarization of macrophages towards the M1 phenotype, shown by increased expression of *TNFA*, *IL6,* and *ILB* and a slight decrease in anti-inflammatory markers (Figure 4.14C). The findings align with published data that points to SIRT6 levels in AT as drivers of macrophage polarization towards the M2[246].

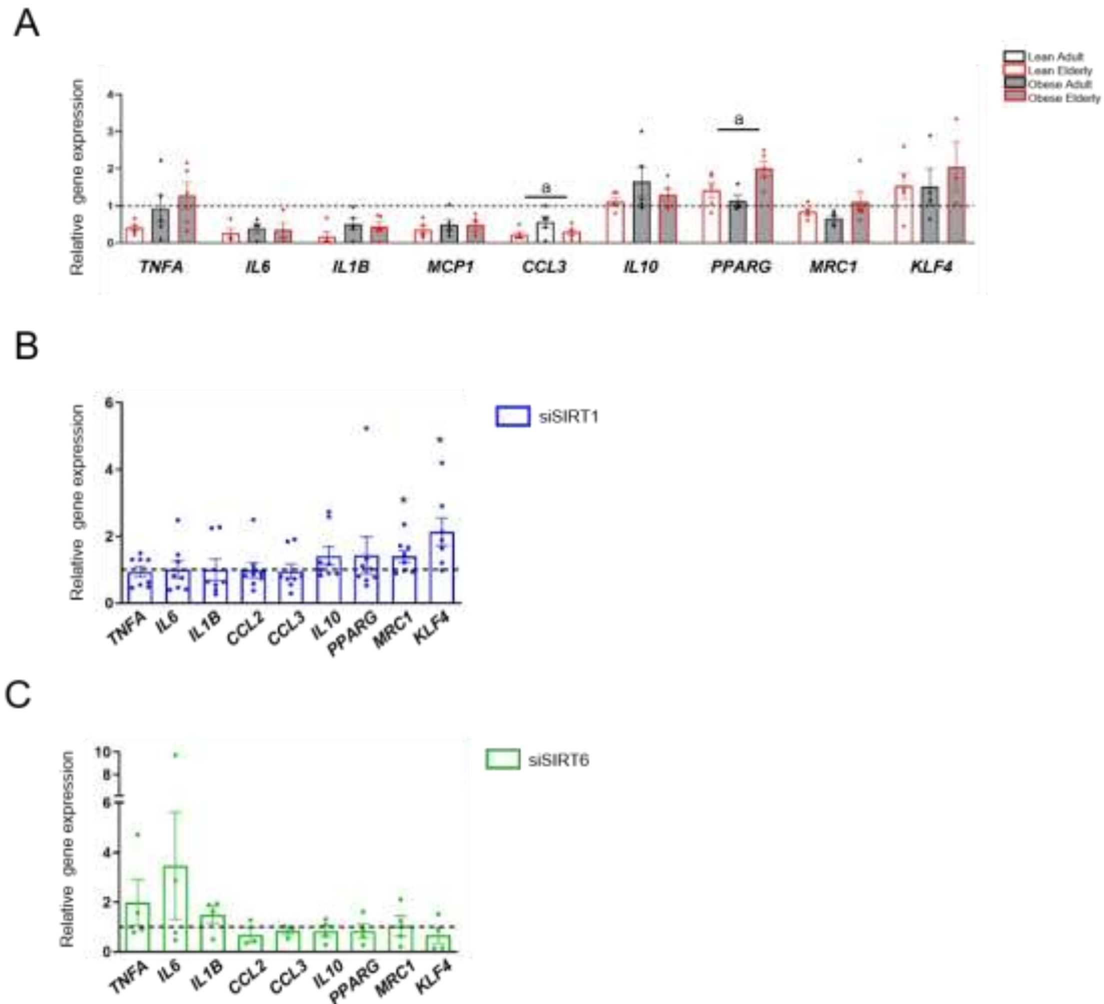

Figure 4.14. Aged and obese settings enhance hASC's capacity to polarize macrophages towards a mixed-M2 phenotype. Reduced expression of SIRT1 in hASC triggers their ability to turn macrophages towards the anti-inflammatory phenotype

a) M1 and M2 gene expression markers of human PBMC-derived macrophages Mϕ incubated for 6h with CM from hASC of the different groups in the cohort. LA group values are arbitrarily set to 1.0 (dotted line). Data are shown as mean ± SEM; results of two-way ANOVA were age $p<0.05$ (a), BMI $p<0.05$ (b), interaction between age and BMI $p<0.05$ (c) b) M1 and M2 gene expression markers of human PBMC-derived macrophages Mϕ incubated for 6h with CM from SIRT1-silenced hASC *versus* control. Control values are arbitrarily set to 1.0 (dotted line). Data are shown as mean ± SEM; Two-tailed paired Student's t test, $p<0.05$ (*). c) M1 and M2 gene expression markers of human PBMC-derived macrophages Mϕ incubated for 6h with CM from SIRT6-silenced hASC *versus* control. Control values are arbitrarily set to 1.0 (dotted line). Data are shown as mean ± SEM; Two-tailed paired Student's t test, $p<0.05$ (*).

4.2.5. The expression of APC is potentiated when hASC are isolated from chronically inflamed settings

We used flow cytometry and PCR to establish if hASC can act as APC and determine if an inflammatory environment determined by age or BMI triggers this function. We found that the expression of APC markers (HLA-DR, CD40, and CD86) was increased on the surface of hASC after the incorporation of OVA (antigen to be presented) (Figure 4.15A, B). Moreover, the expression of the HLA-DR was higher in hASC from LE, OA, and OE (Figure 4.15A, B) and the gene expression of this marker was also upregulated (Figure 4.15C). Intriguingly, it was revealed that the upregulation of the surface expression of the costimulatory molecules CD40 and CD86 only occurred in hASC from obese environments (Figure 4.15A, B). Overall, our results prove that hASC are able to express larger quantities of MHC-I and MHC-II on their surface when isolated from inflammatory settings like those found in aging and obesity. Obesity seems to be a key factor for the increase in the surface expression of the costimulatory molecules needed for antigen presentation. Nonetheless, these results should be corroborated by functional assays.

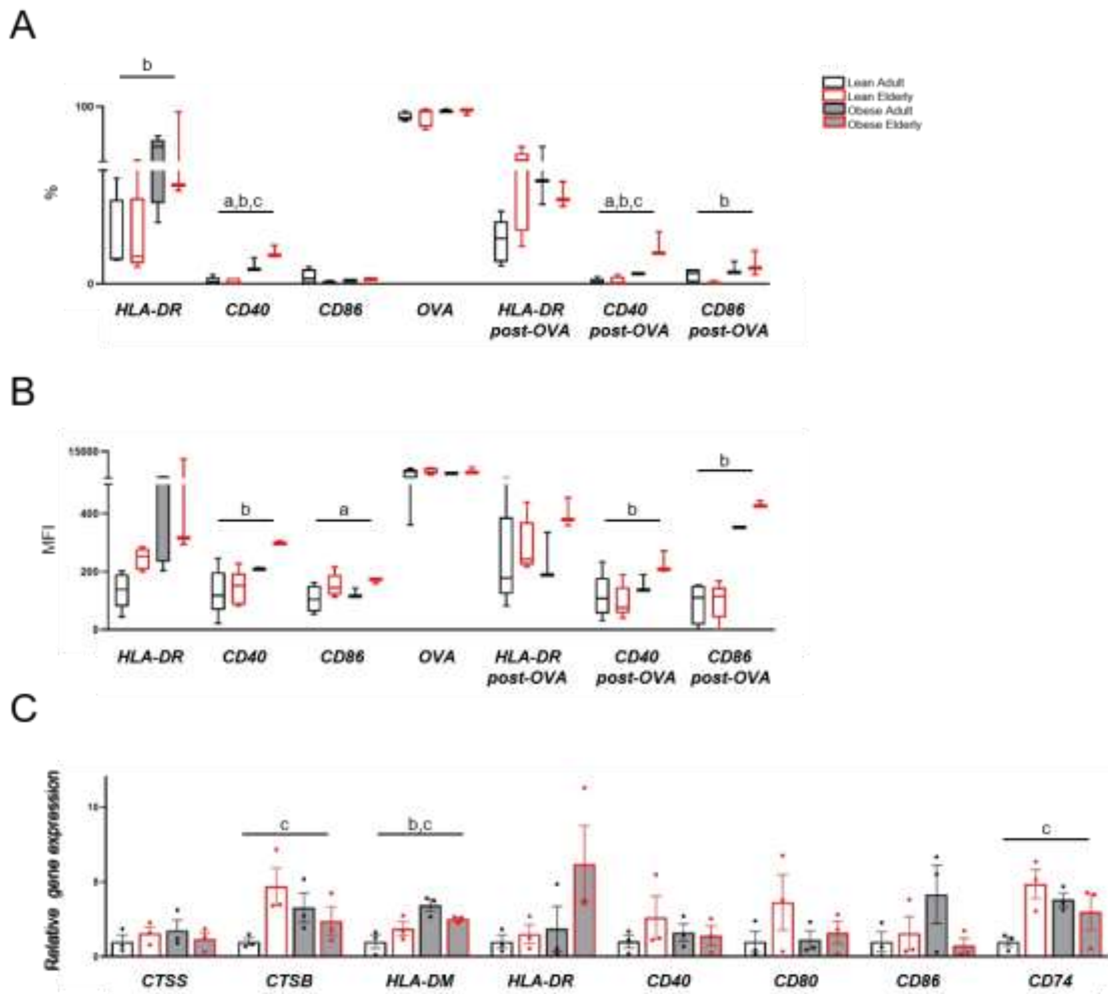

Figure 4.15. hASC as APC

a-b) Expression of antigen-presentation surface markers in hASC from the different groups with and without incubation with OVA, expressed in % (a) and as MFI (b). c) Gene expression of antigen processing and antigen presentation markers was assessed. Data are shown as mean ± SEM; results of two-way ANOVA were age $p<0.05$ (a), BMI $p<0.05$ (b), interaction between age and BMI $p<0.05$ (c)

DISCUSSION

5. DISCUSSION

Similarities have been established between obesity and aging regarding AT[107], serving as a primary driver of the chronic, low-grade systemic inflammation associated with these conditions. While comprising only a small fraction of the total cellular components of AT, adipocyte precursors from the SVF are now acknowledged as a significant source of cytokines and chemokines[70]. Our research elucidates the complex mechanisms driving adipocyte precursor dysfunction in the context of aging and obesity; a metabolic transition towards glycolysis and glycogen synthesis appears to correlate with the secretion of chemotactic and proinflammatory mediators, as we previously identified in mature adipocytes[236], alongside the secretion of specific anti-inflammatory cytokines with immunomodulatory effects. The modifications in AT properties induced by obesity and aging prime hASC to perform their inherent immunomodulatory functions, with glucose metabolism identified as a crucial factor in this process.

Our results confirm the participation of glycogen in the downregulation of SIRT1 and SIRT6, positioning them as influencers of inflammation and regulators of the immunomodulatory functions of hASC, hence highlighting a deep connection between metabolic and immune pathways.

5.1. SAT: A Principal sensor of metabolic alterations

Although VAT is recognized for its substantial role in precipitating obesity-related inflammation and instigating metabolic disorders, emerging data suggest a disruption in VAT and SAT homeostasis within an obesogenic context[109]. Recent research has discovered a robust correlation between proinflammatory macrophages in SAT and systemic metabolic impacts, such as insulin resistance[109], reinforcing the theory that the condition of SAT, rather than VAT, more accurately mirrors the systemic state of an individual. Various studies have documented an increased infiltration of macrophages in SAT depots of lean subjects with metabolic syndrome compared to metabolically healthy, lean counterparts, with virtually no differences observed in VAT[186]. These findings might partially clarify why certain normal-weight individuals are metabolically unhealthy and implicate SAT as the primary sensor of metabolic alterations[186]. This is congruent with studies indicating that weight reduction aids in diminishing inflammation, especially in SAT[188]. Consequently, it can be hypothesized that SAT status provides a more precise representation of systemic metabolic health. Future

research is required to ascertain whether the present study's findings in hASC from SAT are generalizable to hASC from VAT depots.

5.2. Proliferative and differentiation capabilities of hASC under obese and aged conditions

An inflammatory environment, instigated by excessive nutrients or aging, alters AT properties and impacts the cells' functionality. It can precipitate senescence, halt proliferation, or cause defective differentiation[247]. The evaluation of hASC altered by aging or obesity revealed that aging may nullify the proliferative advantage of hASC in an obesogenic setting. Conversely, obesity and aging impaired the differentiative potential of hASC. These observations align with the impeded differentiation potential documented in senescent MSC[248] and with previous research from our laboratory, which demonstrated an augmented proliferative capacity[228] and a decline in adipocyte differentiation capacity in ASC from obese patients[228]. Some controversy persists regarding ASC proliferation rates in an obese environment, as some researchers suggest obesity diminishes the proliferation capacity of ASC[73,249]. These discrepancies in the literature may be attributable to the obesity status of patients within cohorts, as ASC from morbidly obese patients in advanced stages may exhibit decreased proliferation capacity compared to patients in the early stages of obesity.

5.3. Impact of age and BMI on the predominance of specific metabolic pathways

hASC modified by aging or obesity shared several metabolic hallmarks. As previously described in an obesogenic context[228], hASC from elderly subjects displayed a glycolytic phenotype with increased levels of lactate secretion, which is similar to that reported in human fibroblasts[250] and hematopoietic stem cells[251] from elderly people. Indeed, many studies have described a clear link between glycolysis and inflammatory response (reviewed in[194]). Moreover, the blockage of the mitochondrial respiration chain concomitant with glycolysis that occurred in hASC from obese and elderly backgrounds was shown by the release of the TCA cycle metabolite succinate.

5.4. Obesity and aging-driven glycogen mishandling at fault for hASC dysfunction

The present study provides evidence of a causal association between glycogen accumulation and inflammation in adipocyte precursors. This highlights our prior findings on adipocytes[236], proving that hASC compelled towards glycogen deposition via an adenovirus-

mediated overexpression of PTG[242] manifested a significantly amplified inflammatory response.

Abnormal glycogen storage (beyond liver and skeletal muscle) has been described in neurodegenerative diseases (Lafora disease)[252] and in inflammatory-related pathological conditions such as obesity[236], diabetic retinopathy[253] and rheumatoid arthritis[254]. Thus, glycogen metabolism has been recently described as an important event in macrophage-mediated inflammatory responses[255]. This work shows that glycogen deposition in hASC reduces their capacity to proliferate and differentiate, suggesting that glycogen mishandling alters hASC function. Consistent with our results in hASC modified by aging, enhanced glycogen levels have been described in the senescent liver[256] and hematopoietic stem cells in an aging context[251]. We found that dysfunctional hASC from elderly and obese individuals had enhanced glucose conversion to glycogen and an aberrant use of glycolytic pathways, two features that seem to be related.

5.5. Disturbance of glucose metabolism as a potential cause of SIRT1/SIRT6 decreased levels

The association between metabolic dysregulation and the diminution of SIRT/NAD activity has been extensively documented[224]. Additionally, sirtuins have been proposed as crucial in the maintenance of tissue homeostasis and the mitigation of inflammation[205]. In agreement with previous data showing no significant differences in *SIRT1* and *SIRT6* expression in hASC from SAT depots of people with obesity[257], we found no changes across the hASC groups; however, we found a significant decrease in SIRT1 and SIRT6 protein expression in the elderly and obese groups, underlining the important role of post-transcriptional and post-translational regulation of sirtuins[212]. Low protein levels of SIRT1 have been previously reported in the AT of obese mice[224], and low SIRT6 protein levels were reported in the AT of obese patients[226]. Similarly, SIRT1 protein expression was reduced in the AT of aged mice[258]. hASC overexpressing PTG displayed a significant reduction of SIRT1 and SIRT6 protein expression, revealing the glycogen that accumulates in hASC from aged and obese subjects could be a potential driver of the decrease in SIRT1 and SIRT6 levels.

Furthermore, our data supports a link between SIRT1/6 downregulation and inflammation in adipocyte precursors, as previously demonstrated for macrophages[259]. SIRT1/6 knockdown in hASC increased the gene expression of the inflammatory markers (*IL1B,*

TNFA). Correspondingly, evidence indicates that a reduction in the expression of SIRT1[260] and SIRT6[226] in AT precipitates inflammation. In addition, the knockdown of SIRT1/6 reduced glycogen deposition. This indicates SIRT1/6 might promote glycogen synthesis and rules it out as the upstream of the glycogen accumulation found in hASC from aged and obese individuals, where SIRT1/6 levels were low. Recent literature presents SIRT6 as a glycogenolysis inhibitor and a glycogen synthesis promoter[261]. Therefore, we establish glycogen could be the upstream of SIRT1/6 and the responsible for changes in their expression. This decrease in SIRT1 and SIRT6 protein levels was associated with a concurrent downregulation of p-AMPK in elderly people. Moreover, this decline in SIRT1/6 translated into a reduction of the p-AMPK protein levels, which is acknowledged for its role in mitigating inflammation in AT[237] and for its involvement in the replenishment of glycogen following periods of fasting[262]. Therefore, we hypothesize that a decrease in p-AMPK might be responsible for the enhanced inflammation and the impaired recovery of glycogen levels following *SIRT1*/*SIRT6* silencing.

Given the bidirectional interplay between glycogen and AMPK, we investigated the protein expression of p-AMPK in hASC isolated from the LE group. Our findings revealed a decrease in the levels of p-AMPK expression. However, no significant differences in p-AMPK expression were observed between hASC overexpressing PTG and control Ad-GFP hASC. This suggests glycogen accumulation is not the primary causative factor leading to AMPK inactivation in this context. It is important to note that previous research conducted by our team in adipocytes[236] and by others in fibroblast-like synoviocytes[254] has demonstrated the role of glycogen accumulation in AMPK inactivation. Consequently, these results indicate that AMPK may serve as a downstream target of SIRT proteins, at least in hASC.

5.6. Conditions with dysfunctional adipose tissue as modifiers of the immunomodulatory traits of hASC

It is now known that hASC have important immunomodulatory properties beyond their potential to differentiate into adipocytes. Analogous to other AT immune cells, hASC might also control tissue remodeling in response to specific challenges such as overnutrition and aging. We and others have demonstrated that obesity disturbs this dual function of hASC[75], reflected in a local proinflammatory phenotype and the inability to store triglycerides in AT properly. Therefore, the reduced proliferation and differentiation of hASC isolated from the

elderly impairs the expansion of SAT necessary to respond to energy excess adequately. The AT expansion through the differentiation of hASC into new adipocytes (termed hyperplasia) is a counteracting mechanism to prevent lipids from being stored in other organs in response to chronic positive energy balance. Elderly subjects commonly present with elevated levels of serum FFA, dyslipidemia, insulin resistance, and a fat redistribution towards ectopic depots[263]. These are likely consequences of a dysfunctional AT with hASC with reduced adipocyte differentiation capacity. We propose that, just like it occurs in obesity, the hostile environment associated with aging induces significant changes in the immunomodulatory properties of hASC, primarily to respond to the inflammation in the tissue and at the expense of differentiation potential.

5.7. Suppression of T-cell proliferation by hASC from obese and aged settings

Aged but especially obese environments stimulate one of the immunosuppressive properties of hASC: the suppression of T-cell proliferation. This may be explained by the fact that the inflamed microenvironment surrounding hASC in the AT of elderly and obese individuals triggers the inherent capacity of these cells to suppress lymphocyte proliferation to attenuate inflammation. Nonetheless, the suppressive impact on T-lymphocytes was more pronounced in hASC derived from an obese milieu, indicating that inflammation and other environmental factors linked to the pathophysiology of obesity contribute to the activation of hASC characteristics. This agrees with previous data showing that the metabolic imbalance (without inflammation) is enough to initiate the display of the ASC' immunosuppressive properties. It has been postulated that SAT ASC obtained from an obesogenic setting are initially influenced by the metabolic disturbances induced by obesity, followed by the subsequent onset of inflammation resulting from this metabolic dysfunction[101].

Studies regarding the effect of obesity on the immunomodulatory properties of ASC show different results. While some establish that obesity prevents ASCs from suppressing T lymphocyte proliferation because their capacity as immunosuppressors is reduced by obesity[75], others postulate that the suppression of T-cell proliferation is enhanced in the presence of obesity[101].

Moreover, it has been reported that an obesogenic context has an early effect on ASC, particularly regarding T-lymphocyte suppression. SAT ASC of HFD-fed mice have been described to display an enhanced T-cell suppression compared to SAT ASC of standard diet-

fed mice; however, differences using ASC from a VAT origin were only found after 14 weeks of HFD and were less efficient[101]. This agrees with previous data that establishes SAT as the first sensor of metabolic imbalance. Indeed, the reduced number of lymphocytes and macrophages on SAT from obese, compared to VAT, may be explained by the enhanced suppressive activity of SAT ASC compared to their VAT counterparts. As T lymphocytes infiltrated in the AT recruit and activate macrophages[166], the enhanced capacity of ASC from SAT to suppress T-cell proliferation implies a reduction in the recruitment of macrophages[166]. This aligns with the observed increased infiltration of macrophages in VAT[183]. In addition, ASC from obese individuals have been shown to reduce, more strongly than ASC from lean, Th1 cell cytokine secretion and Tc cytolytic activity in PHA-activated PBMC[178]. The mechanism seems to be the increase of the Programmed death-ligand 1 (PD-L1) (activator of inhibitory receptors in lymphocytes) on the surface of MSC[50], monocytes and adipocytes[178], which mediates a reduction in TNF-α and IL-2 lymphocyte secretion. This increase in the surface expression of PD-L1 in ASCs seems to be mediated by IFN-γ[178].

There is also controversy in the literature regarding how the inflammatory milieu affects their capacity to suppress lymphocyte proliferation and polarize macrophages[264,265]. Some authors established that inflammation is a signal that triggers an ASC response[266]; although, this inherent capacity of ASC is not triggered until a certain threshold of inflammation around the ASC is reached[266].

Few studies have been published regarding the effect of obesity on human ASC immunomodulatory capacities; however, the impact of inflammation on ASC has been explored in many articles. Crop et al. demonstrated that ASCs cultured in the presence of proinflammatory cytokines (IFN-γ, TNF-α, and IL-6) showed enhanced suppression of PHA-stimulated PBMC proliferation and that of alloreactive PBMC in a mixed lymphocyte reaction[266]. An IDO blocker prevented this effect, indicating that ASC-secreted IDO mediates the ASC immunosuppressive potential[266]. Moreover, increased expression of IDO by ASC from obese subjects was also reported in other studies[267]. In other words, inflammation significantly impacts ASC function, stimulating the immunosuppressive capacities of ASC.

The increased lymphocyte proliferation suppression showed by ASC from elderly people and people with obesity agrees with the increased protein expression of IL-10 and TGF-β

found in their CM. Although hASC isolated from obese or aged niches secreted chemotactic factors (CCL5, CCL7, CCL20) to the media that were contributing to the migration of leucocytes to the AT, they also secreted substantial amounts of anti-inflammatory cytokines known for their T-cell proliferation suppressing capacities, like IL-10 and TGF-β. Indeed, this is consistent with data indicating the immunosuppressive action of MSC is the sum of two processes: the first one being the secretion of chemokines to recruit T-cells to the tissue where ASC have homed and, the second, the secretion of molecules like Nitric oxide (NO) to suppress T-cell proliferation[94]. This work should be completed by studying the levels of IDO and PGE2, molecules that also hold T-cell suppressing properties. The key to regeneration induced by ASC relies on their interaction with immune cells, which is not yet fully understood.

While certain authors have proposed that chronic inflammation leads to a reduced release of immunosuppressive factors resulting in immune activation[113], alternate viewpoints suggest that chronic inflammation triggers immunosuppression in NK and T-cells[268], thereby creating an immunosuppressive environment that promotes the development of cancer. Perhaps it is necessary to shift the perspective and consider that the secretion of ASC anti-inflammatory proteins might be inadequate to counteract the persistent inflammation within the tissue[95]. Despite the enhanced capacity of hASC to suppress T-cell proliferation, T lymphocyte proliferation rates in the AT of obese and elderly individuals are higher than in the AT of lean individuals. This suggests that the effect of hASC is insufficient to counteract the expansion of T-cells induced by the underlying pathology. In other words, chronic inflammation demands a response that the ASC present in the tissue cannot provide enough; thereby, the resolution of inflammation is never achieved. However, this does not imply that chronic inflammation fails to elicit an immunosuppressive effect from ASC. Even though acute inflammation induces a more robust immunosuppressive response from ASC; in chronic inflammation, ASC still exert an immunosuppressive action, albeit not as pronounced as in acute inflammation. Nonetheless, it is stronger than the response observed in ASC that have not been stimulated or licensed. Indeed, the excess of proinflammatory mediators in AT found in chronic inflammation-conditions (like obesity) triggers the anti-inflammatory cellular response[268]. This aligns with our results concerning the immunosuppressive activity of hASC in obese and aged environments.

Moreover, our results agree with the widely accepted statement that the immunocompetence of obese individuals is compromised, as they have a lower capacity to cope with infections[269]. Obesity is characterized by the coexistence of chronic inflammation and immunosuppression that constrains the ability to fight against infections[205] and cancer[264]. Besides, the higher immunosuppressive properties of hASC isolated from obese niches help us understand why chronic inflammation can contribute to tumor development. The suppressor effect of hASC on lymphocyte proliferation impairs the action of lymphocytes against the tumor[264]. Thus, proinflammatory cytokines present in the AT of obese subjects induce ASC to undertake immunosuppressive activities, activating immune checkpoint pathways to limit tissue inflammation. However, they fail to accomplish this goal and actively create an ideal environment for cancer development, given the essential role of cellular immunity against tumors[264].

5.8. Chemotactic properties of hASC isolated from obese and aged microenvironments

ASC's paracrine effects include immunomodulatory properties, and chemoattractant and trophic activities. Both ASC's hypoxic preconditioning and ASC's stimulation with inflammatory mediators increase the expression and secretion of growth factors such as VEGF and ANG[270]. Significant higher levels of these cytokines were found in the secretome of hASC from elderly and obese individuals; BMI and age, but especially age, were revealed to be factors associated with the increased release. Besides, many chemoattractants showed significantly elevated levels in the supernatants of hASC isolated from elderly or obese subjects. That was the case for CCL5, a chemokine that promotes T-lymphocytes and monocytes migration to the inflammatory sides in the AT[271], and also for the CCL7, a chemokine with monocyte and lymphocyte chemoattractant properties[272]. Both aging and obesity were determinants in the levels of secretion of CCL5 and CCL7, and they even showed synergy for CCL5 and CCL7. Conversely, aging was identified as the primary factor influencing the secretion of CCL20, a T-lymphocyte recruiter to AT[108]. Leptin secretion was affected by both aging and obesity but in opposite ways; while obesity increased leptin secretion, aging constrained it, even when both conditions coexist. Aging impairs the mechanism by which obesity induces leptin secretion. In this line, Isidori et al.[239] described a negative correlation between leptin plasma levels and age in subjects with obesity. As expected, CCL11 secretion was increased in the hASC from elderly subjects. Indeed, this cytokine is called the aging factor[273]. Surprisingly, in the group where only obesity was a

contributing factor, the secretion of CCL11 remained low. However, in the group characterized by aging and obesity, a synergistic increase in CCL11 secretion was observed.

In our hands, after the chemotactic assays, we observed that the secretome of hASC from the elderly and obese helps recruit lymphocytes and monocytes to the AT more efficiently than hASC from LA individuals. This goes in accordance with the increased protein expression of chemotactic cytokines, like CCL5, CCL7, and CCL11, observed in the CM of obese and elderly individuals. It is noteworthy that hASC are recognized for their role as chemoattractants. They can initiate the migration of leukocytes to their specific locations. Before that, ASC have already migrated to the site of injury, and once situated, they secrete chemotactic cytokines to facilitate the arrival of lymphocytes and monocytes, which aid in resolving tissue damage. In the case of obesity and aging, the inflammation present in the tissue simulates a disrupted blood vessel or injured tissue. The resident hASC in the AT of these individuals respond as if the scenario were identical, recruiting lymphocytes and monocytes to the already inflamed adipose tissue, thereby perpetuating the inflammatory response.

There is agreement in the literature on the monocyte and T lymphocyte chemotaxis induced by ASC in an inflammatory context. This environment enhances the recruitment of monocytes and T lymphocytes by ASC, perpetuating an inflammatory state in the AT[111,274]. ASC placed in inflammatory conditions increase the expression of T lymphocytes attractants like C-X-C motif chemokine 9 (CXCL9)/ Monokine induced by gamma interferon (MIG), CXCL10 and CXCL11 and of monocyte attractants as CCL2, CCL5, CCL7[266].

5.9. Changes in the secretion pattern due to aging and obesity

It is known that various stimuli trigger changes in the AT secretory pattern in obese and aged individuals towards a more proinflammatory phenotype[275]. Both aging and obesity boosted the overall proinflammatory status of hASC but with different patterns. Assessment of the hASC secretome revealed that aging had a more significant impact than obesity. On the one hand, as mentioned earlier, the lymphocyte and monocyte-recruiting cytokines levels secreted by hASC from obese and aged environments exceeded those secreted by control LA hASC. On the other hand, the secretion of traditional inflammatory cytokines like IL-2, IL-6, and IFN-γ by hASC increased in those isolated from aged or obese settings. These cytokines are usually present in chronic diseases. Although their primary role is to potentiate

inflammation, increasing evidence points to a dual role, where they also contribute to the resolution of inflammation. That is the case of IFN-γ, which intensifies the inflammatory response once the inflammation cascade is initiated but then stimulates different mechanisms to limit inflammation and walk to resolution, such as induction of Tregs[276]. The same happens for IL-2, a major immune activator (it induces the proliferation of activated T-cells) but also stimulates the generation of Tregs[277]. Finally, IL-6 is known to mediate AT macrophage accumulation in obesity; nevertheless, recent literature establishes that the CM of IL-6-overexpressing MSC displayed enhanced immunosuppressive capacity[278].

5.10. Macrophage polarization by hASC from obese and elderly niches

Incubation of human PBMC-derived macrophages Mϕ with CM obtained from hASC of both obese and elderly individuals revealed an interesting ability of hASC to induce a shift in macrophage phenotype towards a mixed-M2 phenotype. Macrophages exposed to hASC-derived CM from elderly or obese subjects exhibited increased expression of anti-inflammatory markers, namely *IL10*, *KLF4*, and *PPARG*. Simultaneously, the expression of proinflammatory markers associated with the M1 phenotype, such as *IL6*, *IL1B*, *MCP1*, and *CCL3*, showed a declining trend. These findings suggest that hASC from these specific contexts possess immunosuppressive properties.

We classified this altered macrophage phenotype as mixed-M2 due to the upregulation of the inflammatory molecule *TNFA*. Interestingly, these results align with a previous study conducted by our research group[244], which demonstrated the generation of mixed macrophages, displaying both M1 and M2 characteristics, in response to hASC-derived CM from obese subjects. These mixed macrophages were found to contribute to a pro-tumoral environment.

The polarization of macrophages towards the M2-anti-inflammatory phenotype is an essential feature of ASCs. ASCs isolated from the VAT of obese mice showed enhanced capacity to suppress TNF-α expression by M1-macrophages; however, they were insufficient to compensate for other factors that stimulate the secretion of TNF-α in the tissue[101]. Nonetheless, there’s controversy regarding macrophage polarization, as other authors defend that ASC isolated from lean individuals can switch the polarization of the M1 macrophage towards the M2. In contrast, ASC from obese subjects shows no effect.

These authors postulate that obesity prevents ASCs from polarization macrophages towards the M2 phenotype[267].

We hypothesize that alterations in hASC secretion resulting from obesity and aging are pivotal in influencing the macrophage phenotype. This phenomenon promotes a mixed-M2 phenotype and the heightened suppression of T-cell proliferation. hASC derived from both obese and elderly environments exhibit a comprehensive immunosuppressive response. However, they cannot counteract the reinforcement of the M1 phenotype induced by other stimuli, such as the formation of crown-like structures following adipocyte death[101].

5.11. hASC might act as APC

We postulate, first, that hASC can function as APC in particular situations like it has been demonstrated for Crohn's disease[279]. Although known by their immune-privileged status that includes low expression of both MHC-I and MHC-II, hASC can indeed increase the expression of these molecules on their surface. In LA subjects, these levels are kept low. However, gene expression analysis, but particularly cytometry analysis, proved that hASC isolated from elderly or obese individuals display an upregulation of the MCH-II HLA-DR.

The subject's phenotype appears to influence the ability of hASC to present antigens significantly. Specifically, only hASC derived from obese individuals demonstrated an enhanced surface expression of the critical costimulatory molecules CD40 and CD86, which are indispensable for antigen presentation. In contrast, hASC isolated from LE individuals exhibited a low surface expression of these molecules. These findings suggest that hASC from obese subjects are more prone to antigen presentation compared to their counterparts from LE individuals. Moreover, the changes induced by obesity in the AT from which these cells are derived differ from those triggered by aging and have distinct effects on the functions of hASC.

ASCs from LA express low/very low HLA class I and II levels, respectively[48]. When ASC are cultured in a medium containing proinflammatory cytokines, the expression of HLA class I increases slightly (6-fold), with a dramatic increase in HLA class II expression (144-fold)[50,266]. The expression of CD40 is also upregulated in the presence of IFN-γ[50]. Thus, inflammatory conditions induce ASC to express surface markers of professional APC[50]; however, this doesn't translate into an alloreactive lymphocyte response[51], perhaps because

the enhanced lymphocyte suppression capacity showed by inflammatory-primed hASC impairs the cell to act as APC, as they are opposing processes.

Although we have established that inflammation induces some of the intrinsic advantageous capacities of hASC, like migration to the site of injury and suppression of T-lymphocyte proliferation, inflammation may also be harmful, as when a certain threshold is hit, it might turn immunosuppressive hASC into immune-activating cells. Further research needs to be developed to determine the surface expression level of these molecules that will enable hASC to present antigen. These levels should be compared to traditional APC like macrophages, and functional studies should also be performed to demonstrate that hASC are presenting antigens.

In summary, age but, more importantly, BMI might be triggering factors for hASC to act as APC; nonetheless, a certain inflammatory threshold needs to be reached, or a particular level of specific cytokines in the environment surrounding hASC needs to be achieved for them to act as APC. The higher expression of these molecules may underlie the development of APC capacities, although further functional tests are needed to prove it. Perhaps acquiring the APC properties is concurrent with a progressive loss of the immunosuppressive and trophic capacities traditionally attributed to hASC.

5.12. Influence of inflammation on the immunomodulatory properties of hASC

Inflammation should not always be perceived as detrimental, as it plays a crucial role in initiating an anti-inflammatory response mediated by hASC and other immunosuppressive cells[265,280]. Indeed, soluble factors in the microenvironment surrounding the hASC may influence their in vivo function. In other words, the host tissue microenvironment may enhance or reduce their action. hASC can be primed by an inflammatory milieu, meaning the surrounding inflammation signals their activation[243]. They act as immunosuppressors after detecting excessive inflammation, explaining why they are thought to be important determinants in the resolution of inflammation and, in turn, in tissue regeneration.

Pre-activation with different inflammation molecules stimulates lymphocyte proliferation suppression by hASC[243,266]. Therefore, it should not be a surprise that CM of hASC from inflammatory nixes (obese and aged subjects) have enhanced lymphocyte proliferation suppression capacities and promote macrophage polarization towards a mixed-M2

phenotype. Aging and obesity, both characterized by a dysfunctional AT[107], promote an inflammatory milieu that will activate the hASC present in the tissue to counteract the established inflammation of these conditions.

Much literature exposes the importance of inflammatory licensing in stimulating hASC immunosuppressive function. MSC can be pre-activated in vitro using inflammatory factors to elicit an immunosuppressive response before their administration to the patient[243]. This strategy to improve the effects of MSC mimics a physiological inflammatory microenvironment that occurs in vivo in many conditions and that triggers the immunomodulatory actions of MSC.

TNF-α, upregulated in ischemic and damaged tissue; IL-1β, mainly produced by macrophages in inflamed tissues; and INF-γ are some inflammatory cytokines that can prime MSC[198]. For instance, under the influence of chemotactic factors, TNF-α-primed MSC show more substantial migration capacity than control MSC[281,282]. Stimulation of MSC with TNF-α induces M2 macrophage polarization[283]. Furthermore, this priming stimulates them to secrete VEGF and HGF to enhance the protection of the endothelium[282] and IL-6 and IL-8 to promote vascularization[284]. On the other hand, IL-1β-primed MSC show increased immunosuppressive and migration capacities in treating DSS-induced colitis[285]. Moreover, macrophage polarization towards M2 is triggered more effectively by exosomes from IL-1β primed MSC than from control MSC[286]. When MSC are primed with IFN-γ, they inhibit more strongly alloantigen T-cell proliferation[287] and PBMC proliferation[50,288] compared with naïve MSC, particularly by the secretion of IDO and upregulation of the surface marker PD-L1. In addition, IFN-γ licensing enhances the migratory capacity of MSC[288].

5.13. Effect of metabolism on hASC immunomodulatory properties

It has been established that AT has a role as an endocrine organ[1] besides its function as an energy reserve. In the same way, metabolism within the AT is not only beneficial for the storage and mobilization of lipids but has emerged as a signal regulating cell function. In other words, stimuli capable of producing changes in metabolism will be able to modify cell function. Focusing on glucose metabolism, it has been proved that glycolysis and pyruvate's shunt into lactate support MSC's immunosuppressive and regenerative properties[198]. Studies have reported the conversion of pyruvate into lactate in MSC (at the expense of acetyl-CoA) potentiates the suppression of T-cell proliferation[289] and induces the secretion of growth factors promoting tissue repair.

Inflammation stimuli like IFN-γ induce a switch from oxidative phosphorylation towards glycolysis. This transition is necessary to respond to the energy demands of the secretion of immunosuppressive factors to inhibit T-cell proliferation[196]. Indeed, the expression of IDO and PGE2 in MSC is enhanced after IFN-γ-induced glycolysis[196]. Inflammatory-primed MSC show significant increases in HIF-1α that proved essential for T-cell proliferation suppression[199]. Hypoxic preconditioning, like inflammation, promotes MSC's regenerative and immunosuppressive properties through glycolysis stimulation after HIF-1α stabilization[290].

We hypothesize that, as previously described for myeloid-derived suppressor cells (MDSC)[291291], hypoxic and inflamed niches like those found in the AT of obese and elderly individuals stimulate the immunosuppressive capacities of hASC by adopting glycolysis as their primary pathway for energy obtention. One of the mechanisms that could be involved is the induction of the expression of HIF-1α. TNF-α and IFN-γ primed MSC increase HIF-1α levels[199], revealing that two of the common features of aging and obesity (a hypoxic and inflamed AT) converge in the same pathway. The upregulation of HIF-1α aimed to help the cell survive in the hypoxic environment (by promoting the transcription of genes related to angiogenesis, vascularization, cellular proliferation, survival, and energy metabolism)[292] also stimulates the immunosuppressive action of hASC. Specifically, studies have proved that HIF-1α increases PD-L1 in MDSCs, which suppresses T-cell proliferation and T-cell function[291] by interacting with PD-1 in the surface of T-cells and enhancing the secretion of IL-6 and IL-10 in MDSCs[293]. Moreover, HIF-1α silencing in MSC impaired the suppression of T-cell proliferation because of the decline in glycolysis[199]. Consistent with these results, upregulation of HIF-1α in MSC increased the expression of the immunosuppressive molecules IDO and PD-L1[294]. Moreover, dental MSC induced the polarization of macrophages towards the M2 phenotype when HIF-1α was overexpressed[295].

Regarding our results, on the one hand, we proved that the levels of HIF-1α were increased in hASC that came from obese and elderly individuals; on the other hand, some of the genes induced by HIF-1α were upregulated. Among the downstream of HIF1α that we found upregulated in hASC from obese and aged settings, there are glucose transporters (SLC2A1/GLUT1, SLC2A3/GLUT3) and glycolytic enzymes (HK2, PFKM, PDK4, LDH). Moreover, lactate levels were increased in the supernatants of hASC from aged and obese

individuals due to the increased glycolytic flux. HIF-1α promotes the translation of glycolytic genes and genes encoding enzymes for glycogen formation (HK2, Phosphoglucomutase 1 (PGM-1), GYS, GBE1 and Protein Phosphatase 1 Regulatory Subunit 3 (PPP1R3C))[238]. Therefore, it is unsurprising that hASC isolated from hypoxic environments and with elevated levels of *HIF1A*, like those brought by obesity and aging, showed a metabolism based on glycolysis and glycogen storage. The glycogen accumulation in hASC was demonstrated by the upregulation of the genes *HK2*, *GYS*, and *GBE1* by the decreased protein levels of p-GS (the inactive form of GS) and enhanced levels of p-GSK3 (the inactive form of GSK3), which both translate into increased GS activity, a key enzyme in the glycogen synthesis.

Moreover, glycogen targeting IFI and a quantitative colorimetric glycogen assay also proved to increased glycogen content. In addition, these cells with increased glycolytic flux and glycogen deposition had an increased capacity to suppress T-cell proliferation, probably mediated by the increased secretion of IL-10, IL-6, and TGF-β revealed in the protein array of the hASC secretome. Furthermore, glycolytic hASC from obese and elderly settings could polarize macrophages to a mixed-M2 phenotype, thus emphasizing the immunosuppressive features a glycolytic phenotype brings.

In conclusion, our hypothesis suggests that, in hASC derived from obese and elderly individuals, which originate from hypoxic and inflamed environments, the upregulation of HIF1α leads to a shift from OXPHOS to glycolytic metabolism[296]. This metabolic alteration ultimately enhances the immunosuppressive capabilities of hASC. The observed metabolic reprogramming in hASC may also be responsible for changes in their secretome, which in turn affects the immunomodulatory capacity of hASC. Like inflammatory priming or exposure to a hypoxic environment, the metabolic preconditioning of hASC could emerge as another, and potentially superior, approach to augment the immunosuppressive properties of hASC.

5.14. SIRT-1 inflammatory and immunosuppressive roles

Abundant literature has established the anti-inflammatory role of SIRT1 in AT in the context of chronic inflammation by preventing the recruitment of macrophages towards the AT[260] and by repressing the gene expression of inflammatory genes (*IL1B*, *IL6*, *TNFA,* and *CCL2*) in adipocytes through suppression of the NF-kB signaling[297,298]. Indeed, here we

demonstrated the knockdown of SIRT1 in hASC led to an increased expression of inflammatory cytokines. SIRT1 is also relevant in the resolution of acute inflammation as it directs the metabolic reprogramming that switches from glycolysis to fatty acid oxidation in macrophages[205].

On the other hand, MSC undergo a metabolic shift from fatty acid oxidation to glycolysis to exert their immunosuppressive functions[196]. Upregulation of SIRT-1 in MSC has been linked to reduced immunosuppression[200], which is consistent with the fact that SIRT1 upregulation causes a decrease in glycolysis activity. Accordingly, recent evidence suggests that SIRT1 acts as an anti-tumoral molecule in MSC[200], with its mechanism involving proinflammatory actions. SIRT1 appears to induce an anti-tumoral environment by reducing immunosuppression around the tumor. Specifically, SIRT1 limits the activity of immunosuppressive cells like MSC[200], allowing T-cells to proliferate and become activated, and enabling macrophages to acquire an M1-tumor-repressing phenotype to combat the tumor.

Studies have reported that MSC overexpressing SIRT1 maintain their chemotactic properties and the secretion of chemokines but drastically reduce the secretion of iNOS, known to suppress T-cell proliferation under inflammatory conditions[200]. Consequently, the overexpression of SIRT1 in MSC leads to increased CD8+ T cells due to the diminished MSC-mediated suppression of T-cell proliferation, while immune cell recruitment remains unchanged[200]. In other words, the downregulation of SIRT1 in MSC (as observed in obese and aged hASC) would lead to an increased suppression of T-cell proliferation.

Our findings align with these results, as the CM from SIRT1-knockdown hASC polarized macrophages towards a mixed-M2 phenotype, indicated by increased expression of M2 markers (*IL10*, *PPARG*, *MRC1*, *KLF4*) and unaltered expression of inflammatory markers (*TNFA*, *IL6*, *ILB*, *CCL2*, *CCL3*). This suggests that SIRT1 upregulation may alter the secretion pattern of hASC, facilitating the polarization of macrophages towards the M1 phenotype, consistent with the anti-tumoral properties associated with SIRT1[245]. Consequently, the low expression of SIRT1 observed in hASC from obese and aged environments may contribute to macrophage polarization towards a mixed-M2 phenotype.

Although further research is needed to ascertain whether SIRT1 knockdown in hASC also enhances the suppression of T-cell proliferation, we have already demonstrated that hASC from obese and aged contexts exhibit reduced SIRT1 levels, concurrent with increased immunosuppressive capacities and a predominantly glycolytic phenotype. Therefore, we propose that the decreased SIRT1 levels found in elderly and obese hASC elevate the expression of glycolytic genes, thus promoting the immunosuppressive properties of hASC.

In conclusion, we hypothesize that SIRT1 may play a crucial role in modulating the immunomodulatory properties of hASC and serve as a molecular link between energy status and immune responses, further highlighting the intricate interplay between metabolism and inflammation.

5.15. Metabolic reprogramming, immunomodulation, and sirtuins

Aging and obesity-related metabolic reprogramming (predominance of aerobic glycolysis and glycogen deposition) may be responsible for changes in the secretome of hASC. On the one hand, the release of some inflammatory and chemotactic cytokines would be potentiated, contributing to the inflammation of the AT and enhancing the migration of lymphocytes and monocytes to the AT. On the other, the glycolytic switch would allow an immunosuppressive response of hASC, mediated by the secretion of anti-inflammatory cytokines like IL-10, TGF-β, and probably other cytokines like IDO and PGE2, not evaluated in this study. Indeed, TGF- β is known to be part of the SASP and responsible for some of the immunosuppressive activities carried out by MSC: suppression of PMBCs proliferation, inhibition of NK cells and cytotoxic T-lymphocyte, and rise in Tregs[299]. In summary, in the context of aging and obesity, the perpetuation of inflammation in AT is aggravated by hASC secretion, but, at the same time, the immunomodulatory capacities of hASC are being stimulated by the inflammation surrounding them.

Nutrient-sensing molecules like sirtuins, at the crossroads of metabolism and inflammation, regulate the inflammation status of the cell and might be partially responsible for the interaction of hASC with other immune cells. To what degree this interaction extends remains to be resolved. SIRT1 and SIRT6 may have opposite effects on the regulation of macrophage polarization[245,246]. The macrophage polarization pattern of CM from SIRT1 knockdown hASC seemed to mimic more closely that of CM from obese and aged individuals. Whether SIRT1 or SIRT6 effect prevails remains to be determined, although

SIRT1 prevalence appears more likely. Their influence may be only partial, as many other molecules might be involved in the modulation of macrophage polarization.

Our research highlights the potential role of sirtuins as key mediators connecting cellular metabolism and function. To ascertain the practical implications of our findings, further in vivo studies will be necessary. These investigations will help determine whether reversing the metabolic shift from glycolysis to fatty acid oxidation, reducing glycogen levels, or restoring SIRT1/6 levels in hASC can restore their adipocyte differentiation function and prevent the undesired accumulation of lipids in inappropriate tissues, thereby mitigating local and systemic inflammation. Alternatively, if our goal is to maximize the immunosuppressive capabilities of hASC, maintaining low levels of SIRT1 might be necessary, even if it comes at the expense of their differentiation potential. These two functions may be mutually exclusive, and enhancing one may compromise the other. The significance of SIRT1 in augmenting the immunomodulatory capacities of hASC warrants further investigation. Manipulating hASC metabolism in vitro could emerge as a novel approach to enhance their therapeutic efficacy.

CONCLUSIONS

6. CONCLUSIONS

- Aging negatively affects hASC proliferation and adipocyte differentiation, while obesity increases hASC proliferation but impairs their adipocyte differentiation capacity.

- Both aging and obesity shift hASC metabolism from oxidative phosphorylation towards glycolysis and glycogenesis and reduce SIRT1/6 protein expression.

- Aging and obesity modify the secretion pattern of hASC, with a concomitant enhancement of inflammatory-chemotactic and anti-inflammatory cytokines.

- Aging and obesity enhance the immunosuppressive properties of hASC, with enhanced T-cell proliferation, chemotaxis for T-cells and monocytes, and macrophage polarization towards a mixed-M2 phenotype.

- The improvement of the immunosuppressive traits of ASC in the context of aging and obesity is accompanied by an upregulation of glycolytic pathways, a boosting of glycogen deposition and a reduction of SIRT1/6.

- The dysregulation of glycogen metabolism in hASC contributes to diminished adipocyte differentiation, heightened inflammation and decreased SIRT1/6 protein expression.

- The downregulation of SIRT1 and SIRT6 in hASC increases glycolysis, enhances inflammation, but impairs glycogen accumulation, indicating glycogen deposition precedes the reduction in SIRT1/6 levels.

- SIRT1 knockdown in hASC stimulates macrophages to adopt of a mixed-M2 phenotype, mirroring the behavior of hASC from elderly subjects and individuals with obesity.

- These findings underscore the specific impact of obesity and aging on hASC function, highlighting metabolic changes and immunosuppressive alterations that may have clinical implications in the context of obesity-related disorders and age-related conditions.

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ANNEX

8. ANNEX

8.1. Scientific articles published during the doctoral studies

8.1.1. Manuscript with scientific content from the thesis

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WILEY

Glycogen accumulation in adipocyte precursors from elderly and obese subjects triggers inflammation via SIRT1/6 signaling

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Abstract
Dysfunctional adipocyte precursors have emerged as key determinants for obesity- and aging-related inflammation, but the mechanistic basis remains poorly understood. Here, we explored the dysfunctional adipose tissue of elderly and obese individuals focusing on the metabolic and inflammatory state of human adipose-derived mesenchymal stromal cells (hASCs), and on sirtuins, which link metabolism and inflammation. Both obesity and aging impaired the differentiation potential of hASCs but had a different impact on their proliferative capacity. hASCs from elderly individuals (≥65 years) showed an upregulation of glycolysis-related genes, which was accompanied by increased lactate secretion and glycogen storage, a phenotype that was exaggerated by obesity. Multiplex protein profiling revealed that the metabolic switch to glycogenesis was associated with a pro-inflammatory secretome concomitant with a decrease in the protein expression of SIRT1 and SIRT6. siRNA-mediated knockdown of *SIRT1* and *SIRT6* in hASCs from lean adults increased the expression of pro-inflammatory and glycolysis-related markers, and enforced glycogen deposition by overexpression of protein targeting to glycogen (PTG) led to a downregulation of SIRT1/6 protein levels, mimicking the inflammatory state of hASCs from elderly subjects. Overall, our data point to a glycogen-SIRT1/6 signaling axis as a driver of age-related inflammation in adipocyte precursors.

KEYWORDS
adipose-derived mesenchymal stromal cells, Aging, glycogen, glycolysis, inflammation, obesity, SIRT1, SIRT6

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1 | INTRODUCTION

An increase in life expectancy and an aging population, together with the unabated epidemic of obesity, represent one of the greatest health challenges facing our society. Obesity and aging are interrelated and influence each other, which worsens the economic and social burden of these conditions. The risk of obesity increases with age and, conversely, obesity can accelerate aging and increase the risk of early mortality (Tzanetakou et al., 2012). It is now appreciated that aging (a physiological state) and obesity (a pathological state) share similar biological hallmarks including metabolic dysregulation, weakened immunity, and systemic inflammation, which are all pathological phenotypes that occur with dysfunctional adipose tissue (AT) (Pérez et al., 2016).

AT is considered one of the largest and more plastic organs, with important immune and endocrine functions beyond energy storage (Stolarczyk, 2017). Obesity and aging are known to disturb AT metabolism as a consequence of AT expansion or senescence, respectively, leading to local inflammation and, ultimately, to a systemic and chronic state of inflammation (Mancuso & Bouchard, 2019). The mechanistic basis of these disturbances remains, however, unclear. Enhanced glycolytic flux and glycogen deposition appear to be overlapping metabolic abnormalities under certain aging (Wiley & Campisi, 2016) (Seo et al., 2008) and obesity (Serena et al., 2016) (Ceperuelo-Mallafré et al., 2016) contexts. Along this line, we recently reported a causal relationship between glycogen deposition in adipocytes and inflammation (Ceperuelo-Mallafré et al., 2016).

Within AT, human adipose-derived mesenchymal stromal cells (hASCs) function as precursors of differentiated adipocytes and also have important immunoregulatory properties that are crucial for tissue homeostasis (Maria Spaggiari & Moretta, 2013). Both obesity (Pachón-Peña et al., 2016) and aging (Turinetto et al., 2016) compromise the adipogenic potential of hASCs and modify their immune function (Zhang et al., 2021) (Serena et al., 2016). Interestingly, there is increasing evidence that hASCs are predominantly responsible for the changes in the secretory profile of AT that are induced by obesity (Siklova-Vitkova et al., 2012) (Chung et al., 2006). This raises the question that the secretion of cytokines and chemokines in the context of aging or obesity might induce an inflammatory response in neighboring adipocytes, impairing lipid handling (Chung et al., 2006).

First discovered as NAD+-dependent epigenetic regulators in yeast, sirtuins have emerged as bioenergetic sensors at the crossroads of metabolism and inflammation and are considered as crucial gatekeepers of tissue homeostasis during stress responses (Vachharajani et al., 2016). Failure of these systems to recover can lead to chronic inflammatory diseases. Of note, both obesity (Song et al., 2013) and aging (Khanh et al., 2018) have been associated with a fall in SIRT1 activity in AT, which in turn has been related to aberrant inflammation (Gillum et al., 2011).

In the present study, we sought to investigate the dysfunctional AT in relation to age and obesity in adults, focusing on the metabolic and inflammatory status of hASCs as key drivers of AT homeostasis. We report a new signaling pathway through which changes in the metabolic profile of hASCs as a consequence of obesity and aging regulate inflammation. Specifically, we found that hASCs from elderly and obese subjects exhibit an aberrant glycolytic flux concomitant with enhanced conversion of glucose to glycogen, which drives a pro-inflammatory phenotype via a SIRT1/6-dependent mechanism.

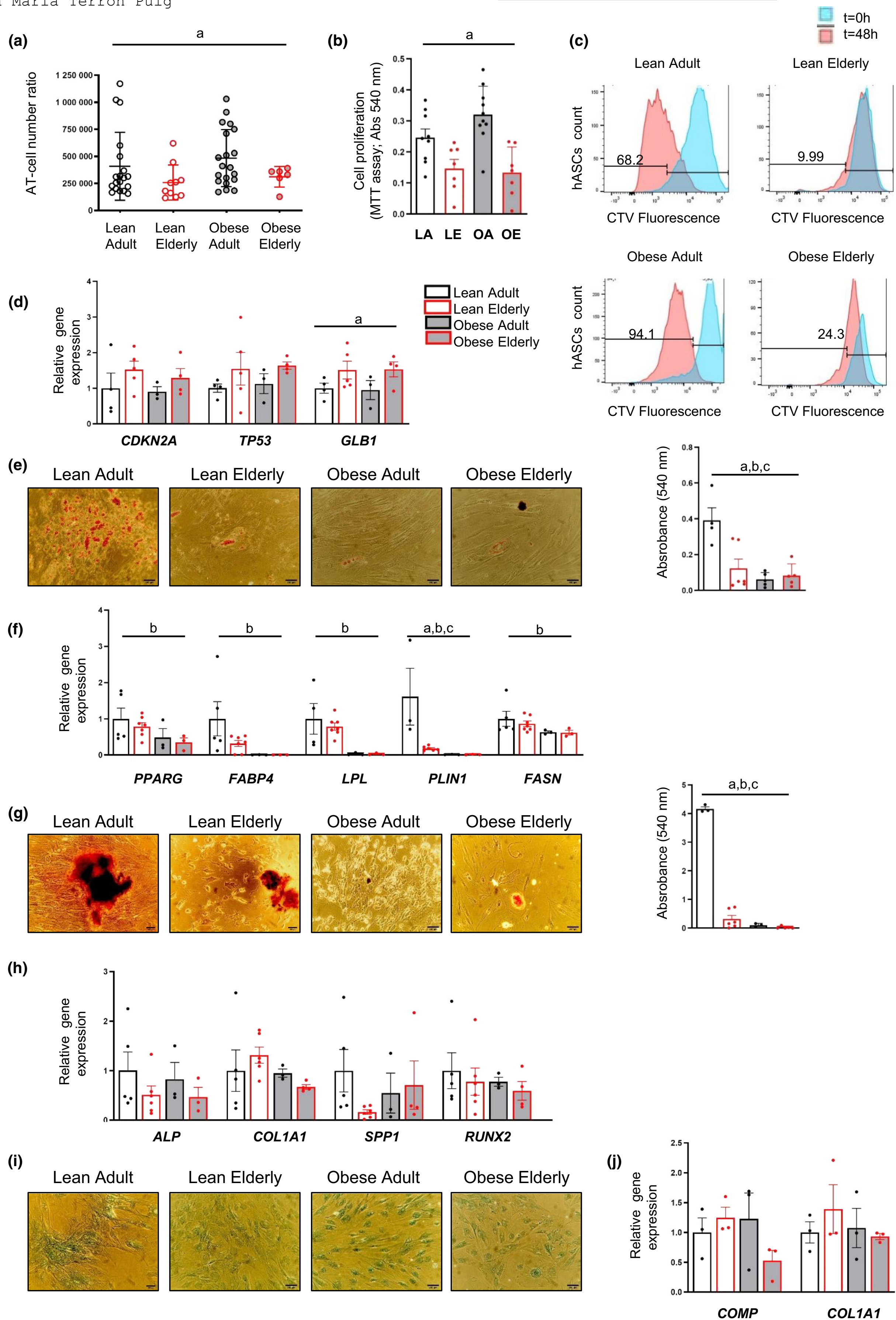
2 | RESULTS

2.1 | Obesity and aging differentially impact the proliferative but not the differentiation capacity of hASCs

Obesity is known to influence hASC plasticity (Pérez et al., 2016); however, whether aging also has an impact is less clear. We first examined the growth and proliferation of hASCs from donors stratified by age and body weight index (BMI) into the following groups: adult (>20 and < 65 years) or elderly (≥65 years), and lean (BMI < 25 kg/m^2) or obese (BMI≥30 kg/m^2), and the isolated hASCs were divided into the following four groups on this basis: lean adult (LA), lean elderly (LE), obese adult (OA), and obese elderly (OE).

In accordance with our previous results demonstrating that obese-derived hASCs have a higher proliferation rate than their lean-derived counterparts (Pachón-Peña et al., 2016), we found that the AT-cell number ratio (number of proliferating hASCs at passage [P]0 per gram of digested AT) was significantly higher in the OA group than in the LE group (Figure 1a). By contrast, a lower AT-cell number ratio was found in the groups of elderly subjects compared with the LA group independently of obesity (Figure 1a). Analysis of proliferation assessed by MTT reduction (Figure 1b) and by flow cytometric analysis of intracellular Cell Trace Violet (CTV) dilution (Figure 1c) confirmed the negative effect of aging on hASC proliferation, even in a background of obesity, suggesting that aging has a predominant

FIGURE 1 hASCs modified by aging or obesity show differences in proliferation and differentiation capacity. (a) Adipose tissue (AT)-cell number ratio in all groups ($n = 6–20$). (b,c) Proliferation measured by MTT reduction after 7 days in culture (b) ($n = 7–10$) and CTV dye dilution after 48 h of incubation (c) ($n = 1$). (d) Gene expression of the senescence markers *CDKN2A*, *TP53*, and *GLB1* in hASCs ($n = 3–5$). (e,f) Representative images and quantification of Oil Red O staining (e) ($n = 4–6$) and relative gene expression of adipogenic (*PPARG*, *FABP4*, *LPL*, *PLIN1*, and *FASN*) markers (f) ($n = 3–7$) in adipocytes differentiated from hASCs. (g,h) Representative images and quantification of Alizarin Red staining (g) ($n = 3–6$) and relative gene expression of osteogenic (*ALP*, *COL1A1*, *SPP1*, and *RUNX2*) markers (h) ($n = 3–6$) in osteocytes differentiated from hASCs. (i,j) Representative images of Alcian Blue 8GX staining (i) and relative gene expression of chondrogenic (*COMP* and *COL1A1*) markers (j) ($n = 3$) in chondrocytes differentiated from hASCs. All images were taken at ×200 magnification; scale bar 100 µm. Data are shown as mean ± SEM; results of two-way ANOVA were age $p < 0.05$ (a), BMI $p < 0.05$ (b), interaction between age and BMI $p < 0.05$ (c). Abbreviations: LA, lean adult; LE, lean elderly; OA, obese adult; OE, obese elderly


(a)
a
AT-cell number ratio
1 250 000
1 000 000
750 000
500 000
250 000
0
Lean Adult
Lean Elderly
Obese Adult
Obese Elderly
(b)
a
Cell proliferation (MTT assay; Abs 540 nm)
0.5
0.4
0.3
0.2
0.1
0.0
LA
LE
OA
OE
(c)
t=0h
t=48h
Lean Adult
Lean Elderly
hASCs count
68.2
9.99
CTV Fluorescence
CTV Fluorescence
Obese Adult
Obese Elderly
94.1
24.3
CTV Fluorescence
CTV Fluorescence
(d)
Relative gene expression
a
Lean Adult
Lean Elderly
Obese Adult
Obese Elderly
CDKN2A
TP53
GLB1
(e)
Lean Adult
Lean Elderly
Obese Adult
Obese Elderly
Absrobance (540 nm)
a,b,c
(f)
Relative gene expression
b
b
b
a,b,c
b
PPARG
FABP4
LPL
PLIN1
FASN
(g)
Lean Adult
Lean Elderly
Obese Adult
Obese Elderly
Absrobance (540 nm)
a,b,c
(h)
Relative gene expression
ALP
COL1A1
SPP1
RUNX2
(i)
Lean Adult
Lean Elderly
Obese Adult
Obese Elderly
(j)
Relative gene expression
COMP
COL1A1

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effect over obesity for proliferation. We employed a general linear model to question whether proliferation (measured by MTT reduction) was significantly different between groups after adjusting for sex. This analysis revealed significant differences for MTT values between the four groups ($p < 0.001$) independent of sex ($p = 0.490$). We also used multiple linear regression analysis (stepwise forward selection procedures) to evaluate the potential role of age, sex, and BMI as independent factors associated with proliferation (MTT assay). Notably, we found that age was the main determinant of MTT values ($R2 = 0.304$; $ß = -0.552$, $p < 0.001$). Finally, examination of senescence-related protein markers in cell extracts revealed an overall trend for greater expression in hASCs from elderly subjects (Figure 1d), which was significant for GLB1 expression.

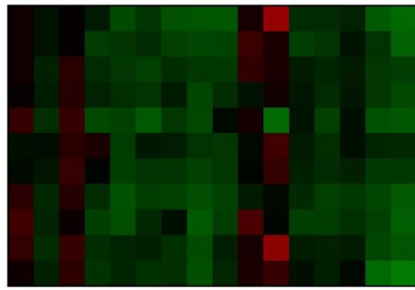
We next analyzed the influence of aging on the multilineage differentiation potential of hASCs. Cells isolated from elderly subjects with or without obesity were significantly impaired in their capacity to differentiate into adipocytes, as revealed by Oil Red O staining of neutral lipids (Figure 1e) and by the gene expression of common adipogenic markers (*PPARG*, *FABP4*, *LPL*, *PLIN1*, and *FASN*) (Figure 1f). Similar results were obtained when we analyzed osteogenic differentiation in the different groups, as shown by the lower amount of calcium deposits stained with Alizarin Red (Figure 1g) and a trend for lower expression of osteogenic markers (*ALP*, *COL1A1*, *SPP1*, and *RUNX2*) (Figure 1h). By contrast, no differences were found in chondrogenic differentiation between the different hASC groups, as measured by Alcian Blue 8GX staining (Figure 1i) and gene expression of chondrogenic markers (*COMP* and *COL1A1*) (Figure 1j). With the caveat that obesity might bolster some aspects associated with aging, our results suggest that obesity and aging differentially influence hASC properties.

2.2 | Obesity and aging dysregulate glucose metabolism in hASCs

We used RNA expression profiling of hASCs to study the impact of obesity and aging on metabolic-related genes. We found that the expression of several glucose metabolism-related genes was higher in hASCs from elderly subjects than in adult-derived hASCs, and this was augmented by obesity (Figure 2a). We also found a significant positive correlation between age and markers of glucose transport (*SLC2A1 and SLC2A3*) and metabolism (*HK2 and PFKM*), tricarboxylic acid cycle (*SDHB*, *OGDH*), and glycogen metabolism (*GYS*, *PYGL*, *and GBE1*) (Figure 2b). The glycolytic phenotype was more pronounced in hASCs from elderly subjects (and was amplified by obesity) and was characterized by a significantly higher secretion of lactate and succinate, which are markers of aerobic glycolysis (Garcia-Alvarez et al., 2014) and mitochondrial stress (Weinberg et al., 2000), respectively (Figure 2c,d). Lactate and succinate release by hASCs positively correlated with age and BMI (Figure 2c,d). Although we found that both obesity and aging promoted a glycolytic phenotype, the specific upregulation of glycogenic enzymes (*GYS*, *PYGL*, and *GBE1*), which are known to be expressed in adipocytes (Ceperuelo-Mallafré et al., 2016), was evident in the groups of elderly subjects (LE and OE) (Figure 2a). As glycogen synthesis is mostly regulated at the protein level, we used immunoblotting to examine different proteins regulating this pathway. Glycogen synthase (GS), the rate-limiting enzyme in glycogen synthesis, exists in an active (dephosphorylated) and an inactive (phosphorylated) form. The LE group showed significantly lower phosphorylated [p]-GS levels (inactive GS), which mirrors a higher activity, than the LA group (Figure 2e). Results also revealed significantly higher p-GSK3 (inactive form of GSK3) levels in hASCs isolated from the LE group (Figure 2e), which agrees well with the activated GS. Moreover, hASCs isolated from the LE group showed higher levels of the glycogen targeting subunit protein targeting to glycogen (PTG). No differences were found in the protein levels of the active form of glycogen phosphorylase (p-PYGL), which metabolizes glycogen, or in glycogen branching enzyme (GBE), which mediates glycogen branching (Figure 2e). Moreover, the expression of p-AMPK, which has also been inversely related to glycogen levels (Bijland et al., 2013), was found to be lower in hASCs from the LE group. Correlation analysis showed that p-GS and p-GSK3 protein abundance correlated negatively and positively, respectively, with age (Figure 2f,g), suggesting that aging might promote glycogenesis in hASCs. This finding was supported by the evident increase in glycogen content in hASCs from both obese and elderly subjects, as measured by indirect immunofluorescence (Figure 2h). A quantitative colorimetric assay of glycogen (Figure 2i) indicated that age

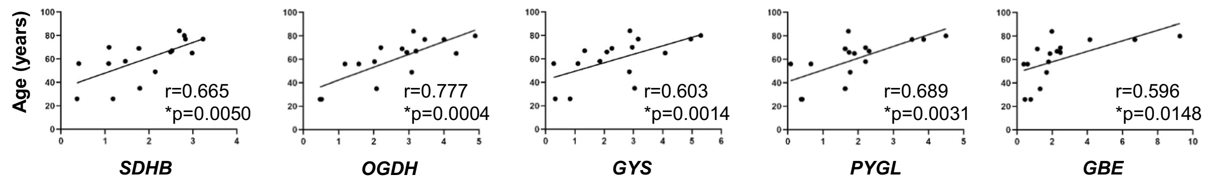
FIGURE 2 Obesity, but particularly aging, promote glucose utilization by glycolytic and glycogenesis pathways. (a) Gene expression heatmap of glucose transporters (*SLC2A1 and SLC2A3*), glycolytic markers (*HK2*, *PFKM*, *and PDK4*), tricarboxylic acid cycle (TCA) enzymes (*LDHB*, *SDHB*, *and OGDH*) and glycogen synthesis and degradation enzymes (*GYS*, *PYGL*, and *GBE1*) ($n = 3–6$). (b) Positive correlation between age and expression of *SLC2A1*, *SLC2A3*, *HK2*, *PFKM*, *SDHB*, *OGDH*, *GYS*, *PYGL*, and *GBE1* determined by Pearson's correlation analysis. (c) Lactate secretion of hASCs cultured for 24 h and correlation analysis with age and body mass index (BMI) ($n = 4–6$). (d) Succinate secretion of hASCs cultured for 24 h and correlation analysis with age and BMI ($n = 4–11$). Data are shown as mean ± SEM; results of two-way ANOVA were age $p < 0.05$ (a), BMI $p < 0.05$ (b), interaction between age and BMI $p < 0.05$ (c). Correlations were determined by Pearson's correlation analysis. (e) Representative immunoblots and densitometry of p-GS, p-GSK3, p-PYGL, p-AMPK, PTG, and GBE protein expression in hASCs from adult and elderly lean donors ($n = 4–5$). (f) Correlation between p-GS protein expression and age. (g) Correlation between p-GSK3 protein expression and age. GAPDH was used as a loading control. Densitometry analyses are presented in arbitrary units (a.u). Data are shown as mean ± SEM from three independent experiments; two-tailed unpaired Student's *t* test, $p < 0.05$ (*). Correlations were determined by Pearson's correlation analysis. (h,i) Glycogen content was determined by indirect immunofluorescence (h) and by a quantitative colorimetric assay (i) ($n = 3–5$). Data are shown as mean ± SEM; results of two-way ANOVA were age $p < 0.05$ (a), BMI $p < 0.05$ (b), interaction between age and BMI $p < 0.05$ (c). Abbreviations: LA, lean adult; LE, lean elderly; OA, obese adult; OE, obese elderly

(a)

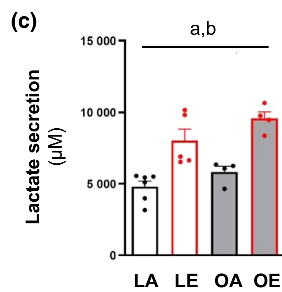


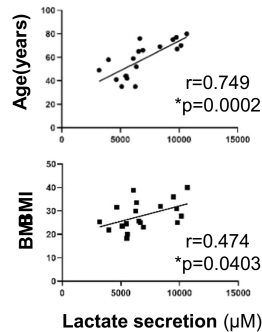
(b)

Age (years)
r=0.700 *p=0.0026 SLC2A1
r=0.572 *p=0.0205 SLC2A3
r=0.752 *p=0.0008 HK2
r=0.744 *p=0.0009 PFKM

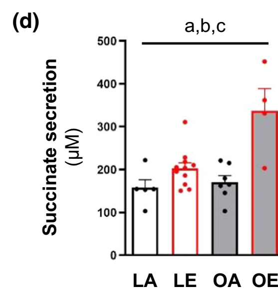


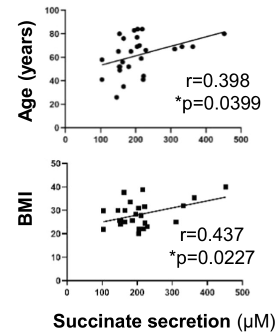
(c)



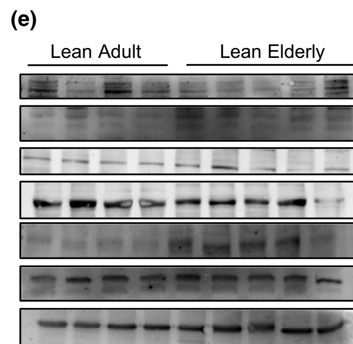


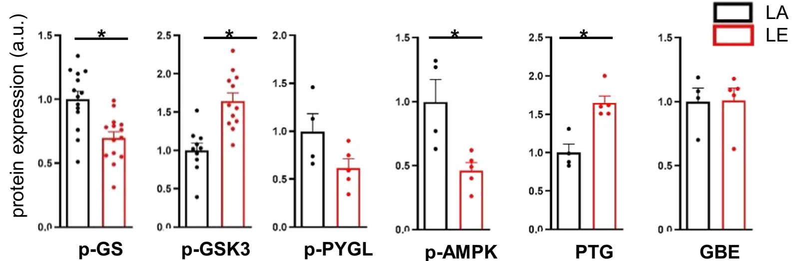
(d)



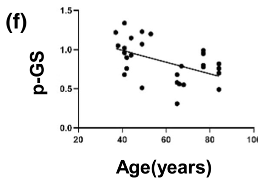


(e)

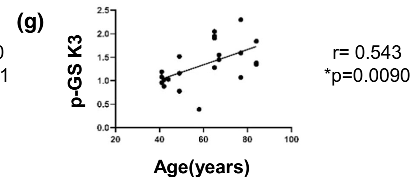




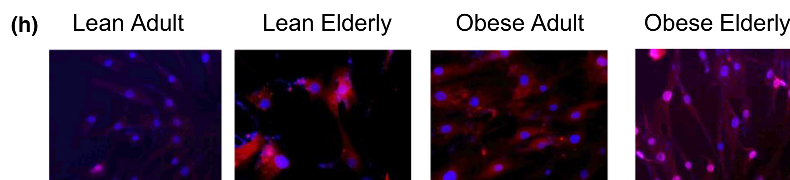
(f)



(g)

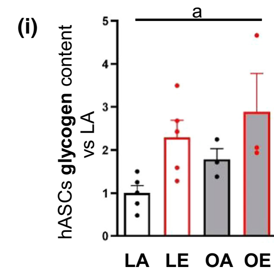


(h)



In red, glycogent content. In blue, nucleus

(i)



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was the main factor influencing glycogen content. Overall, our data reveal that both obesity and aging enhance glucose utilization in hASCs by glycolytic and glycogenesis pathways.

2.3 | Obesity exacerbates the inflammatory phenotype of hASCs from elderly subjects

Metabolic reprogramming toward glycolysis is known to be associated with inflammatory states (Soto-Heredero et al., 2020). In this context, we recently reported a link between glycogen accumulation and pro-inflammatory cytokine expression in human AT (Ceperuelo-Mallafré et al., 2016). In line with these studies and with our previous finding of an inflammatory phenotype in hASCs from obese donors (Serena et al., 2016), we noted that the expression of the pro-inflammatory markers *IL1B*, *IL6*, and *CCL2* was higher in hASCs from obese adults than from lean adults (Figure 3a). Likewise, age was found to be a major determinant for the expression of the three cytokines. Moreover, a synergistic effect of age and BMI was found for the gene expression of *IL1B* and *IL6*, achieving statistical significance for *CCL2* (Figure 3a). To better understand the effect of aging and obesity on the inflammatory phenotype of hASCs, we used a multiplexed cytokine array (Figure S1) to interrogate the hASC secretome (including chemokines, inflammatory cytokines, and angiogenesis- and senescence-related factors). As shown in Figure 3b, the cytokine secretion pattern of the conditioned medium (CM) of hASCs differed among groups, with the most pronounced changes found in hASCs of elderly subjects (both lean and obese). From the 62 cytokines measured, we focused on those 26 cytokines with a significant increase in ≥1.5-fold change over hASCs from the LA group in at least one of the groups (Figure 3c). A Venn diagram of the 26 cytokines (Figure 3d) showed that the increase in the abundance of inflammatory cytokines in the hASC-CM of the OE group was due to aging rather than to obesity, leading us to conclude that aging has a greater influence than obesity on the establishment of a pro-inflammatory secretome. Nonetheless, a group of five cytokines required the presence of both conditions (aging and obesity) for a significant increase in secretion. Of note, leptin––a classic adipokine––was exclusively increased in the hASC-CM of the OA group, suggesting that aging counteracts the impact of obesity on leptin secretion. This fits well with previous data describing a negative correlation between leptin plasma levels and age in subjects with obesity (Isidori et al., 2000). Conversely, the abundance of the chemoattractant CCL11 in hASC-CM was lower in a background of obesity only, and increased markedly in the hASC-CM of the OE group (Figure 3b,c,d). Indeed, a synergistic induction of chemokine secretion as a response to pro-inflammatory cytokines has been previously described (Gouwy et al., 2005). To identify whether the presence of both conditions (aging and obesity) could trigger the synergistic induction of some chemokines, we assessed the 23 cytokines secreted by the OE group with a significant ≥1.5-fold change increase for those with double the sum of the increase in the LE and OA groups. Three chemokines met this criterion: CCL5, CCL7, and CCL11 (Figure 3e). Overall, our data demonstrate that obesity and aging differentially impact the secretory properties of hASCs.

2.4 | The aged- and obesity-related inflammatory states of hASCs are associated with an antagonistic cross-talk between glycogen deposition and SIRTs

Sirtuins are key metabolic sensors involved in the pathophysiology of inflammatory-related processes including aging and obesity (Vachharajani et al., 2016), and both obesity (Song et al., 2013) and aging (Khanh et al., 2018) have been associated with a reduction in SIRT1 activity in AT. Similarly, published data point to a reduction in SIRT6 protein levels in the AT of people with obesity (Kuang et al., 2017). Thus, we analyzed the expression of SIRT1 and SIRT6 in hASCs from the different groups of donors, finding a downregulation of both in the elderly (Figure 4a) and in the obese (Figure 4b) groups, without significant differences at the mRNA level (data not shown). Correlation analysis showed that both SIRT1 and SIRT6 expression in hASCs correlated negatively with age (Figure 4c), whereas only SIRT1 correlated negatively with BMI (Figure 4d).

To test for a link between the decline in SIRT1 and SIRT6 expression and the acquisition of a pro-inflammatory profile in elderly derived cells, we used short interfering RNA (siRNA) to independently knockdown the expression of *SIRT1* and *SIRT6* in control (LA-derived) hASCs. Gene expression analysis of inflammatory markers showed that both *SIRT1* (Figure 5a) and *SIRT6* (Figure 5b) knockdown resulted in the upregulation of several pro-inflammatory genes, including a significant upregulation of *IL1B*. In addition, *SIRT1* downregulation resulted in a significant increase in *TNFalpha* expression (Figure 5a). The inflammatory phenotype of control hASCs induced by *SIRT1* knockdown was accompanied by a significant increase in *HK2* and *PFKM* expression (Figure 5c), whereas *SIRT6* knockdown in control

FIGURE 3 Changes in the cytokine secretion phenotype in hASCs from elderly donors are exaggerated by obesity. (a) Relative gene expression of inflammatory markers (*IL1B*, *IL6*, *TNFA*, and *CCL2*) in hASCs from all groups ($n = 3–6$). Data are shown as mean ± SEM; results of two-way ANOVA were age $p < 0.05$ (a), BMI $p < 0.05$ (b), interaction between age and BMI $p < 0.05$ (c). (b) Heatmap of the 62 cytokines secreted by hASCs of all four groups, expressed as the log10 of the fold change versus hASCs of the lean adult (LA) group ($n = 5–10$). (c) Heatmap of the 26 cytokines secreted by hASCs of the lean elderly (LE), the obese adult (OA) and/or the obese elderly (OE) groups showing ≥1.5-fold greater levels than those of the LA group, expressed as the log10 of the mean fold change versus the LA group. Results of two-way ANOVA were age $p < 0.05$, BMI $p < 0.05$, interaction between age and BMI $p < 0.05$. (d) Venn diagram of the 26 cytokines shown in c. (e) Of the 23 cytokines secreted by hASCs from the OE group with ≥1.5-fold greater levels that those of the LA group, synergy was established for those in this group that showed double the sum of the increase in LE and OA versus LA groups. Abbreviations: LA, lean adult; LE, lean elderly; OA, obese adult; OE, obese elderly

(a)

Lean Adult
Lean Elderly
Obese Adult
Obese Elderly

Relative gene expression

40
10
5
0

a,b
a,b
a,b,c

IL1B
IL6
TNFA
CCL2

(b)

Lean Adult
Lean Elderly
Obese Adult
Obese Elderly

ENA-78
G-CSF
GM-CSF
GRO
GRO-α
IL-1α
IL-2
IL-3
IL-5
IL-6
IL-7
IL-8
IL-10
IL-12 p70
IL-13
IL-15
IFN-γ
CCL2
CCL8
CCL7
M-CSF
MIG
MIP-1β
CCL5
SCF
SDF-1
TARC
TGF-β1
TNF-α
TNF-β
IGF-I
ANG
OSM
THPO
VEGF
PDGF-BB
Leptin
BDNF
Ck β 8-1
CCL11
Eotaxin-3
Flt-3 LG
GCP-2
GDNF
IGFBP-2
IGFBP-3
IGFBP-4
IP-10
LIF
LIGHT
MCP-4
MIF
MIP-3α
NT-3
NT-4
OPN
OPG
PARC
PIGF
TGF-β2
TIMP-1
TIMP-2

Color Key
1
0
-1
log10 fold change

(c)

	LE	OA	OE	Age	BMI	Int
G-CSF				***	**	
GM-CSF				***	***	
GRO-α						
IL-1α						*
IL-2				***	***	*
IL-5				***	***	***
IL-6				***		
IL-7				***	**	
IL-10				***	*	
IL-13				***	**	
IL-15				***	**	
IFN-γ				**	*	
CCL2						
CCL7				***	***	***
CCL5				***	***	***
ANG				***	**	*
THPO				**		
VEGF				***	*	
Leptin				*	**	**
CCL11				***	***	***
Flt-3 LG				***	***	
IGFBP-4				**	**	
MIF				***	***	
MIP-3α				***		
OPG				***	***	
TGF-β1				***	*	

Mean

Color Key
1
0
-1
Mean log10 fold change

(d)

LE
OA
OE
2
0
1
5
11
0
7

Only Increased in LE

IL-1α *; CCL2

Only Increased in OA

Leptin*

Aging dependent

ANG *, GM-CSF *, IFN-γ *, MIF *, Flt-3 LG *, IL-10 *, VEGF *, G-CSF *, IL-6 * , IL-5 *, IL-15*

Aging and Obesity dependent

CCL5*, OPG*, CCL7*, MIP-3α *, IL-2*

Only Increased in OE

IL-13 *, GRO-α, IGFBP-4*, THPO*, IL-7 *, TGF- β1*, CCL11

(e)

Synergy
OE>2*(LE+OA)

CCL5
CCL7

OE>LE & OE>OA

G-CSF, GM-CSF, GRO-α, IL-2, IL-5, IL-6, IL-7, IL-10, IL-13, IL-15, IFN-γ, TGFβ1, ANG, THPO, VEGF, Flt-3 LG, IGFBP-4, MIF,MIP-3α, OPG

OE
OE>2*(LE+OA)

hASCs resulted in a significant increase in *HK2*, *PDK4*, and *SLC2A1* expression (Figure 5d).

Searching for a molecular mechanism underlying SIRT downregulation associated with obesity and aging, we explored metabolic reprogramming as a potential cause. Specifically, we forced glycogen deposition in control hASCs using an adenoviral transduction system overexpressing PTG (Newgard et al., 2000) (Ceperuelo-Mallafré et al., 2016). As expected, glycogen levels were markedly higher in Ad-PTG-hASCs than in control Ad-GFP-hASCs overexpressing GFP (Figure 5e), which was consistent with a decrease in

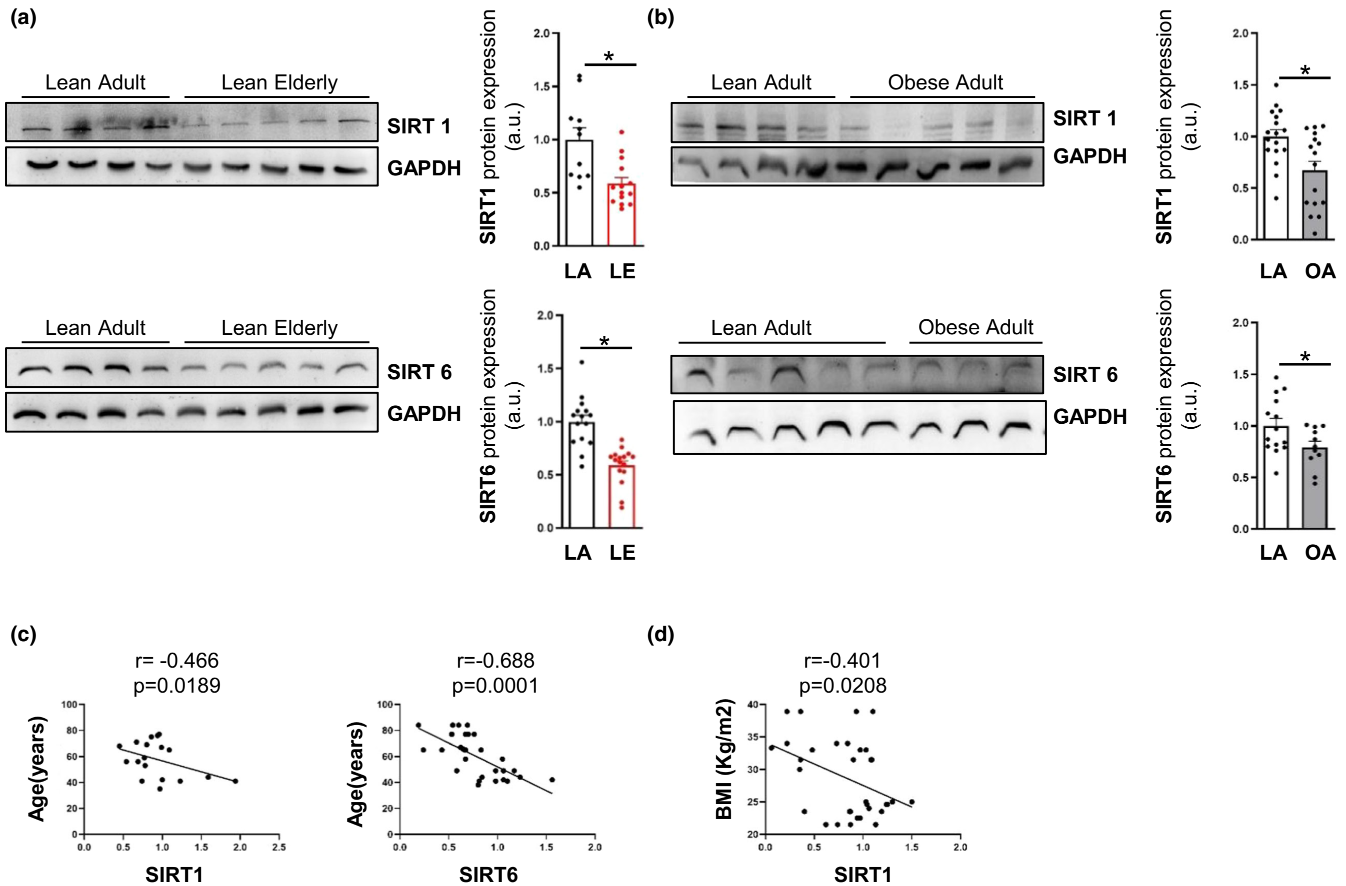
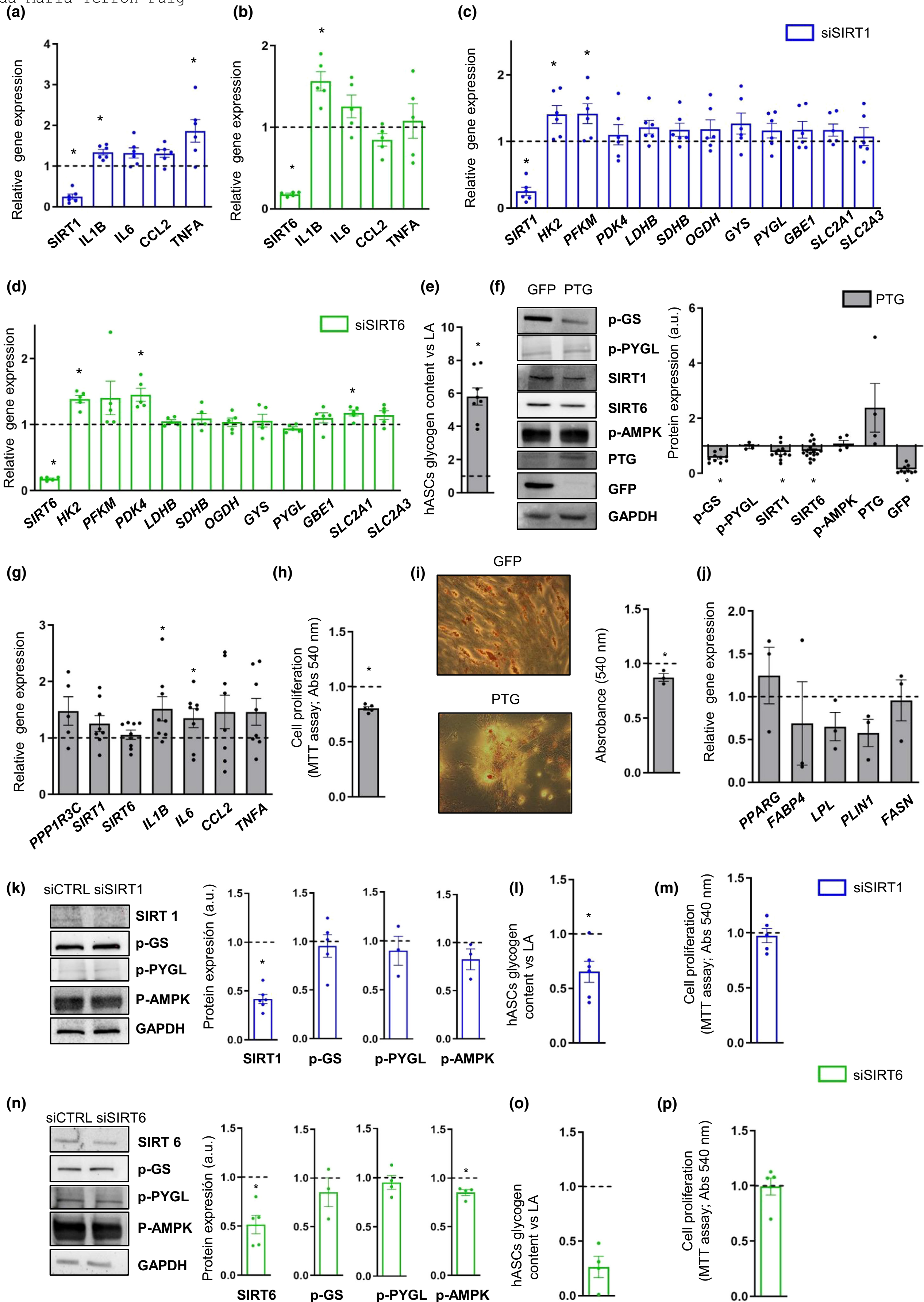


FIGURE 4 Age- and obesity-related inflammatory status of hASCs negatively associates with SIRT1 and SIRT6 expression. (a,b) Representative immunoblots and densitometry analysis of SIRT1 and SIRT6 protein expression in hASCs of lean adult (LA) and lean elderly (LE) (a) and LA and obese adult (OA) (b) groups ($n = 3$–5). Data are shown as mean ± SEM from three independent experiments; two-tailed unpaired Student's t test, $p < 0.05$ (*). (c,d) Correlation analysis between SIRT1 or SIRT6 expression and age (c) and between SIRT1 and body mass index (BMI) (d). Correlations were determined by Pearson's correlation analysis. Abbreviations: LA, lean adult; LE, lean elderly; OA, obese adult; OE, obese elderly

FIGURE 5 SIRT1 and SIRT6 silencing triggers inflammation and enhances glycolysis in hASCs, whereas enhancing glycogen deposition reduces SIRT1 and SIRT6 protein expression. (a,b) Relative gene expression of *SIRT1* (a) and *SIRT6* (b) and inflammatory markers (*IL1B*, *IL6*, *CCL2*, and *TNFA*) in lean adult (LA) hASCs silenced with siSIRT1 (a) ($n = 6$) or siSIRT6 (b) ($n = 5$) versus control hASCs. c,d) Relative gene expression of *SIRT1* (c) or *SIRT6* (d) and glycolytic markers (*HK2*, *PFKM*, *and PDK4*), tricarboxylic acid cycle enzymes (*LDHB*, *SDHB*, *and OGDH*), glycogen synthesis and degradation enzymes (*GYS*, *PYGL*, and *GBE1*) and glucose transporters (*SLC2A1 and SLC2A3*) in siSIRT1 (c) ($n = 6$) or siSIRT6 (d) ($n = 5$) hASCs versus control hASCs. (e–g) protein targeting to glycogen (PTG) was overexpressed in hASCs using an adenoviral transduction system. (e,f) LA hASCs overexpressing PTG versus those overexpressing GFP (control) were analyzed for glycogen deposition (e) ($n = 8$) and p-GS, p-PYGL, SIRT1, SIRT6, p-AMPK, PTG, and GFP protein expression (f) ($n = 3$–16). (g) Gene expression of *PPP1R3C*, *SIRT1*, *SIRT6* and inflammatory markers (*IL1B*, *IL6*, *CCL2*, and *TNFA*) was determined in hASCs overexpressing PTG versus those overexpressing GFP (control) ($n = 5$–8). (h–j) Proliferation determined by MTT assay (h) ($n = 5$) and adipocyte differentiation analyzed by Oil Red O staining (i) ($n = 3$) and gene expression of adipocyte differentiation markers (j) ($n = 3$) were assessed in hASCs upon PTG overexpression versus those overexpressing GFP (control). (k–m) Representative immunoblots and densitometric analysis of SIRT1, p-GS, p-PYGL and p-AMPK (k) ($n = 3$–6), glycogen content (l) ($n = 6$) and proliferation (m) ($n = 5$) in hASCs transfected with siSIRT1 versus control hASCs. (n–p) Representative immunoblots and densitometric analysis of SIRT6, p-GS, p-PYGL and p-AMPK (n) ($n = 3$–5), glycogen content (o) ($n = 4$) and proliferation (p) ($n = 5$) in hASCs transfected with siSIRT6 versus control hASCs. For immunoblots, GAPDH was used as the loading control. Densitometric analyses are presented in arbitray units (a.u.). Data are shown as mean ± SEM; Two-tailed paired Student's t test, $p < 0.05$ (*)


(a)
(b)
(c)
siSIRT1
Relative gene expression
SIRT1
IL1B
IL6
CCL2
TNFA
SIRT6
HK2
PFKM
PDK4
LDHB
SDHB
OGDH
GYS
PYGL
GBE1
SLC2A1
SLC2A3
(d)
siSIRT6
(e)
hASCs glycogen content vs LA
(f)
GFP PTG
p-GS
p-PYGL
SIRT1
SIRT6
p-AMPK
PTG
GFP
GAPDH
PTG
Protein expression (a.u.)
(g)
PPP1R3C
(h)
Cell proliferation (MTT assay; Abs 540 nm)
(i)
GFP
PTG
Absrobance (540 nm)
(j)
PPARG
FABP4
LPL
PLIN1
FASN
(k)
siCTRL siSIRT1
SIRT 1
p-GS
p-PYGL
P-AMPK
GAPDH
Protein expresión (a.u.)
p-AMPK
(l)
(m)
siSIRT6
(n)
siCTRL siSIRT6
SIRT 6
(o)
(p)

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p-GS expression (Figure 5f). We also observed an increase in pro-inflammatory gene marker expression (*IL1B*, *IL6*, *CCL2*, and *TNFalpha*) in PTG-overexpressing cells, which was significant for *IL1B* and *IL6* expression (Figure 5g), supporting a link between glycogen and inflammation (Ceperuelo-Mallafré et al., 2016). Notably, SIRT1 and SIRT6 expression was significantly lower in Ad-PTG-hASCs than in control cells (Figure 5f), indicating that the downregulation of SIRT1 and SIRT6 in hASCs from elderly and obese donors could be, at least partly, a consequence of glycogen deposition. Contrastingly, no differences in protein expression were found for p-AMPK (Figure 5f), ruling it out as the upstream interface for SIRT1 and SIRT6 downregulation, at least in these cells. To explore the functional impact of glycogen deposition on hASC function, we assessed cell proliferation and adipocyte differentiation. hASCs overexpressing PTG showed a significant decrease both in proliferation, as determined by MTT reduction (Figure 5h) and in adipocyte differentiation capacity, as revealed by quantitative Oil Red O staining of neutral lipids (Figure 5i), and by gene expression of the adipogenic markers *FABP4*, *LPL*, and *PLIN1* (Figure 5j). Finally, we measured glycogen accumulation in cells silenced for *SIRT1/6* to explore a potential feedback loop between SIRT1/6 and glycogen metabolism. Both *SIRT1* and *SIRT6* knockdown led to a decrease in glycogen content in hASCs without any significant changes to the inactive form of GS (p-GS) or to the active form of PYGL (p-PYGL) (Figure 5k,l,n,o), indicating that metabolic dysregulation and glycogen accumulation precedes SIRT1/6 downregulation in aged cells. Moreover, a trend for a decrease in p-AMPK protein expression was found upon *SIRT1* silencing (Figure 5k), and a significant decrease in p-AMPK levels was found upon *SIRT6* downregulation, (Figure 5n). These data suggest that p-AMPK could be downstream of SIRT1 and SIRT6 and linked to the increase of inflammation in response to *SIRT1/6* downregulation. Finally, no differences in proliferation were found in hASCs silenced for *SIRT1* (Figure 5m) or *SIRT6* (Figure 5p). Overall, our data point to a glycogen-SIRT1/6 axis as a putative driver of age-related inflammation in hASCs.

3 | DISCUSSION

Parallels have been drawn between obesity and aging with respect to AT (Pérez et al., 2016), a major contributor to the low-grade systemic inflammation characteristic of these conditions. Although representing only a fraction of the cells that comprise the AT, adipocyte precursors from the stromal vascular fraction are now regarded as a major source of cytokines and chemokines (Siklova-Vitkova et al., 2012). Our study provides new insight into the mechanisms governing the dysfunctioning of adipocyte precursors in response to aging and obesity, which activate a metabolic switch to glycogen synthesis that appears to be related to a pro-inflammatory secretory profile, as we previously reported in adipocytes (Ceperuelo-Mallafré et al., 2016). In the same line, glycogen metabolism has been recently described as an important event in macrophage-mediated inflammatory responses (Ma et al., 2020) and in fibroblast-like synoviocyte-mediated inflammation (Shi et al., 2018). Our data not only confirm the involvement of glycogen as a driver of inflammation, but also identify SIRT1 and SIRT6 as mediators of this pathogenic event.

While visceral AT (VAT) is known to actively contribute to obesity-related inflammation and to the development of metabolic disorders, there is emerging evidence for a disruption of both VAT and subcutaneous AT (SAT) homeostasis in an obesogenic context (Lesna et al., 2017). Moreover, recent studies point to a strong correlation between pro-inflammatory macrophages in SAT and systemic metabolic effects such as insulin resistance (Lesna et al., 2017), supporting the hypothesis that SAT and not VAT status better reflects the systemic state of the subject. Some authors have also reported higher macrophage infiltration in SAT depots of lean subjects with metabolic syndrome when compared to lean healthy individuals, with nearly no differences found in VAT (Moreno-Indias et al., 2016). These findings could, in part, explain why some normoweight subjects are metabolically unhealthy and point to the SAT depot as the primary detector of metabolic changes (Moreno-Indias et al., 2016). This is consistent with studies showing that weight loss helps to reduce inflammation particularly in SAT (ClÉment et al., 2004). It is, therefore, conceivable that SAT status better reflects systemic metabolic health. Further work will be needed to determine whether the results obtained in the present study can be extrapolated to hASCs from VAT depots.

The characterization of hASCs modified by aging or obesity revealed that aging could nullify the proliferative advantage of hASCs conferred by an obesogenic background. By contrast, both obesity and aging clearly compromised the differentiation potential of hASCs. These findings are in accord with previous studies from our laboratory (Pachón-Peña et al., 2016), and with the disrupted differentiation potential reported in senescent mesenchymal stromal cells (Turinetto et al., 2016). Concomitant with these functional alterations, both aging and obesity boosted the overall pro-inflammatory status of hASCs, but with different patterns. Assessment of the hASC secretome revealed that aging had a more significant impact than obesity, which in turn might boost the aging phenotype. It is known that various types of stimuli trigger changes in the AT secretory pattern in obese and aged individuals toward a more pro-inflammatory phenotype, which in the case of aging is commonly referred to as the senescence-associated secretory phenotype (SASP) (Tominaga, 2015). Of note, we also observed an increase in anti-inflammatory cytokines (e.g., IL-10 and TGF-β1) in the CM of hASCs modified by aging or obesity, which fits with the finding that age-related inflammation is linked to the presence of some anti-inflammatory factors in the hASC niche (Zhang et al., 2021).

hASCs modified by aging or obesity shared several metabolic hallmarks. As previously described in an obesogenic context (Serena et al., 2016), hASCs from elderly subjects display a glycolytic phenotype, which is similar to that reported in human fibroblasts (Wiley & Campisi, 2016) and hematopoietic stem cells (Poisa-Beiro et al., 2020) from elderly individuals. Indeed, many studies have described a clear link between glycolysis and inflammatory response

(reviewed in (Soto-Heredero et al., 2020)). We demonstrate here a causal relationship between glycogen deposition and inflammation in adipocyte precursors, extending our previous findings in adipocytes (Ceperuelo-Mallafré et al., 2016), and showing that hASCs with forced glycogen deposition by an adenovirus overexpressing PTG (Newgard et al., 2000) have a significantly elevated inflammatory response. Abnormal glycogen storage (beyond liver and skeletal muscle) has been described in neurodegenerative diseases (Lafora disease) (Nitschke et al., 2018) and in inflammatory-related pathological conditions such as obesity (Ceperuelo-Mallafré et al., 2016), diabetic retinophathy (Gardiner et al., 2015), and rheumatoid arthritis (Shi et al., 2018). We show that glycogen deposition in hASCs reduces their capacity to proliferate and differentiate, indicating that glycogen mishandling alters hASC function.

Consistent with our results in hASCs modified by aging, enhanced glycogen levels have been described in senescent liver (Seo et al., 2008) and in hematopoietic stem cells in an aging context (Poisa-Beiro et al., 2020). We found that dysfunctional hASCs from elderly and obese individuals have enhanced glucose conversion to glycogen and an aberrant use of glycolytic pathways, two features that seem to be related.

Metabolic dysregulation has long been associated with a reduction in SIRT/NAD activity (Chalkiadaki & Guarente, 2012), and sirtuins have been postulated as sentinels of tissue homeostasis and suppressors of inflammation (Vachharajani et al., 2016). In agreement with previous data showing no significant differences in *SIRT1* and *SIRT6* expression in hASCs from subcutaneous AT depots of individuals with obesity (Mariani et al., 2016), we found no changes across the hASC groups; however, we found a significant decrease in SIRT1 and SIRT6 protein expression in the elderly and obese groups, underlining the important role of post-transcriptional and post-translational regulation of sirtuins (Houtkooper et al., 2012). Low protein levels of SIRT1 have been previously reported in the AT of obese mice (Chalkiadaki & Guarente, 2012), and low SIRT6 protein levels were reported in the AT of obese patients (Kuang et al., 2017). Similarly, a reduction in SIRT1 protein expression was found in the AT of aged mice (Gong et al., 2014). Our data support a link between SIRT1/6 downregulation and inflammation in adipocyte precursors, as has been previously demonstrated for macrophages (Yoshizaki et al., 2010). Likewise, it is known that a decrease in the expression of SIRT1 (Gillum et al., 2011) and SIRT6 (Kuang et al., 2017) in AT leads to inflammation. Concomitant with the decline in SIRT1 and SIRT6 levels was a downregulation of p-AMPK in the elderly group. In addition, a decline in SIRT1/6 translated into a reduction in p-AMPK protein levels, known to inhibit inflammation in AT (Bijland et al., 2013) and to be responsible for the restoration of glycogen after fasting (Cantó et al., 2010). Therefore, we hypothesize that a decrease in p-AMPK might be responsible for the enhanced inflammation and the impaired recovery of glycogen levels that occurs following *SIRT1*/*SIRT6* silencing. Given that the inter-play between glycogen and AMPK is bidirectional, we explored p-AMPK protein expression in hASCs isolated from the LE group, finding a reduction in its levels. However, no significant differences in p-AMPK expression were found between hASCs overexpressing PTG and control Ad-GFP hASCs, suggesting that glycogen accumulation is not the main driver of AMPK inactivation in this context, although this has been described by us in adipocytes (Ceperuelo-Mallafré et al., 2016), and by others in fibroblast-like synoviocytes (Shi et al., 2018). These results points to AMPK as a downstream target of SIRT proteins, at least in hASCs.

It is now known that hASCs have important immunomodulatory properties beyond their potential to differentiate into adipocytes. Analogous to other AT immune cells, hASCs might also control tissue remodeling in response to specific challenges such as overnutrition and aging. We and others have demonstrated that obesity disturbs this dual function of hASCs (Serena et al., 2016), which is reflected in both a local pro-inflammatory phenotype and in the inability to properly store triglycerides in AT. We propose that similar to what occurs in obesity, the hostile environment associated with aging induces significant changes in hASCs primarily to respond to the inflammation in the tissue, at the expense of differentiation potential. Therefore, the reduced proliferation and differentiation of "elderly" hASCs impairs the expansion of SAT necessary to adequately respond to energy excess. The expansion of AT through differentiation of hASCs into new adipocytes (termed hyperplasia) is a counteracting mechanism to prevent lipids being stored in other organs, in response to chronic positive energy balance. Elderly subjects commonly present with elevated levels of serum-free fatty acids, dyslipidemia, insulin resistance (Pararasa et al., 2015) and a redistribution of fat toward ectopic depots, which are likely consequences of a dysfunctional AT with hASCs with reduced adipocyte differentiation capacity.

Overall, our study reveals a novel pathway linking metabolic dysfunction to inflammation in adipocyte precursors, particularly in the context of aging and obesity (figure 6, graphical abstract). While glycogen accumulation has previously been described as a driver of inflammation in other cells and pathologies, this is the first time that SIRT1 and SIRT6 have been reported as mediators between the two processes. Perpetuation of inflammation in AT would be aggravated by a metabolic switch in hASCs to aerobic glycolysis, which enhances glycogen deposition. Our work points to sirtuins as possible mediators linking both features. Further studies in vivo will be required to determine whether switching back to fatty acid oxidation, reducing glycogen levels or restoring SIRT1/6 levels in hASCs might prevent local and systemic inflammation.

4 | EXPERIMENTAL PROCEDURES

4.1 | Study subjects

Subjects were recruited at the University Hospital Joan XXIII and at Santa Tecla Hospital in accordance with the tenets of the Helsinki Declaration. The corresponding hospital ethics committees reviewed and approved the study and written informed consent was obtained from all participants before entering the study. SAT

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biopsies were obtained from donors undergoing nonacute surgical interventions, such as hernia or cholecystectomy, in a scheduled routine surgery. Donors were classified as adult (>20 and < 65 years) or elderly (≥65 years) based on their age; and as lean (BMI < 25 kg/m^2) or obese (BMI≥30 kg/m^2) based on their BMI, following World Health Organization criteria. This classification led to formation of four groups lean adult (LA), lean elderly (LE), obese adult (OA), and obese elderly (OE). The anthropometric and biochemical variables from the cohort are presented in Table S1. Patients with cancer, diabetes and inflammatory chronic diseases were excluded from the study. The *n* corresponding to the subsample used for each experiment is specified in the figure legends.

4.2 | Isolation and culture of human adipose-derived mesenchymal stromal cells

hASCs were isolated from SAT biopsies as described (Serena et al., 2016; Pachón-Peña et al., 2016). In brief, SAT was washed extensively with PBS to remove debris and treated with 0.1% collagenase in PBS and 1% bovine serum albumin for 1 h at 37°C with gentle agitation. Digested samples were centrifuged at 300 × *g* at 4°C for 5 min to separate adipocytes from stromal cells. The cell pellet containing the stromal fraction was resuspended in stromal culture medium consisting of DMEM/F12, 10% fetal bovine serum (FBS), and 1% antibiotic/antimycotic solution and then placed into a flask. The flask was placed in an incubator at 37°C with 5% CO_2 and 21% O_2. At 24 h after seeding, the flask was washed with PBS and the medium was replaced. As the hASCs had already adhered to the flask, the PBS wash removed only non-adherent cells. Culture medium was replaced every 48–72 h. After 7 days of incubation, when cells had achieved 90% confluence, the primary cultures of hASCs at P0 were harvested with trypsin–EDTA, and aliquots of 1×10^6 cells were cryopreserved in liquid nitrogen until required. The AT-cell number ratio was defined as the ratio of the number of cells obtained at P0 per gram of AT digested. CM was collected at P3–7 after 24 h in culture using a minimum concentration of hASCs of 10,000 cells/cm^2 and was centrifuged at 400 × *g* for 5 min.

4.3 | Immunophenotyping

To verify the isolation of hASCs, we assessed the immunophenotypic profile of undifferentiated hASC populations using a panel of positive and negative surface markers that identify hASCs according to the quantitative criteria established by the International Society of Cell Therapy (ISCT) and the International Federation for Adipose Therapeutics and Science (IFATS). Briefly, 2×10^5 hASCs were incubated with a panel of primary antibodies (CD34, CD73, CD90, CD105, CD14, CD31, and CD45) and then analyzed by flow cytometry using 405-nm, 488-nm, and 633-nm excitation on the FACS ARIA III cytometer (BD Biosciences, San Jose, CA). All experiments were performed in cells at P3–7. Flow cytometry analysis of cell marker expression was consistent with the minimum criteria defined for hASCs. Accordingly, cells were positive for the surface markers CD73, CD90, and CD105 and negative for CD34, CD14, CD31, and CD45. No significant differences were detected between groups (Table S2).

4.4 | Proliferation assays

4.4.1 | MTT assay

hASC proliferation was determined by a standard colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) reduction assay (Sigma-Aldrich, Madrid, Spain). In brief, 1.6×10^3 hASCs per well were seeded in a 96-well plate. Two MTT assays were performed at 24 h (day+1) and 7 days (day+7) after seeding. The proliferation rate was calculated as the difference in absorbance between day+7 and day+1, measured with a spectrophotometer at 540 nm.

4.4.2 | CTV assay

Flow cytometric analysis of intracellular CTV (Invitrogen, Eugene, OR) was used to measure hASC proliferation, based on dye dilution of CTV-labeled cells over the course of 48 h.

4.5 | Multilineage differentiation capacity

To assess the differentiation capacity of hASCs, we used specific conditions to trigger cell differentiation to the adipocyte, osteocyte, and chondrocyte lineage, as described (Bunnell et al., 2008). Intracellular lipid enrichment in mature adipocytes was measured by Oil Red O staining; calcium depots in osteocytes were assessed by Alizarin Red staining; and glycosaminoglycan precipitation in chondrocytes was analyzed by Alcian Blue 8GX staining. Differentiated cells were observed in a bright-field microscope (Zeiss Axio Vert A1; Carl Zeiss AG, Oberkochen, Germany). Relative gene expression of adipogenic osteogenic and chondrogenic markers in hASCs was analyzed by real-time polymerase chain reaction (Figure S2).

4.6 | Cytokine secretion

We used pools of 24 h CM from hASCs of different donors (at least an *n* = 5 was used for each group), of which 500 μL samples were filtered using a 3-k pore filter and centrifugated at 14000 × *g* for 5 min. A total of 100 μl was used in duplicate for each cohort on the RayBio Human Cytokine Antibody Array 5 (G series, cat# AAH-CYT-G5-8, RayBiotech Life; www.raybiotech.com), and the array was sent to the manufacturer for scanning. Fluorescence signal intensities were measured on the Innopsys InnoScan (Carbonne,) laser fluorescence

scanner. Normalization was performed by defining one of the two sub-arrays of the LA group as a reference. The image of the array is shown in supplementary data (Figure S1).

4.7 | Gene expression analysis

Total RNA was isolated from hASCs using the RNeasy Lipid Tissue Mini Kit (Qiagen Science,). RNA was transcribed to cDNA with random primers using the Reverse Transcription System (Applied Biosystems,). Amplification was performed on a 7900HT Fast Real-Time PCR System using the TaqManR Gene Expression Assays hydrolysis probes (Applied Biosystems) (Figure S2). Results were calculated using the comparative threshold cycle (Ct) method ($2^{-\Delta\Delta Ct}$) normalized to the expression of the housekeeping gene *18S* (Hs 03928985_g1) or cyclophilin 1A (*PPIA*) and expressed relative to the control condition, which was set to 1. Two technical duplicates were performed for each biological replicate.

4.8 | Protein expression analysis

Cells were lysed and homogenized in Mammalian Protein Extraction Reagent (M-PER™; ThermoFisher Scientific,) containing a protease and phosphatase inhibitor cocktail (Sigma-Aldrich). The BCA Protein Assay Kit (Pierce Biotechnology,) was used to determine protein concentration. Equal amounts of protein were separated on SDS-PAGE gels, transferred to Immobilon membranes (Merck Millipore,) and blocked. Antibodies diluted 1/1000 against p-AMPK (THR172 [40H9]) (2535; Cell Signaling Technology [CST]), p-GS (SER641) (3891; CST), p-GSK3 α/β (SER21/9) (9331; CST), GBE (AB617523; Abcam), GFP (8371–2; Clontech), PTG (SC-6582; Santa Cruz Biotechnology),

p-PYGL(S15) (AB227043; Abcam), SIRT1 (2310, CST), SIRT6 (AB88494; Abcam), were used to perform immunoblot analysis. GAPDH (MA5–15738; Sigma-Aldrich) was used as a loading control. Protein bands were detected with anti-rabbit (NA934; GE Healthcare,) or anti-chicken (ab131366; Abcam) peroxidase-conjugated secondary antibodies, diluted 1/2000. Immunoreactive bands were visualized using a SuperSignal West Femto chemiluminescent substrate (Pierce) and images were captured on a "iBrightCL1000 image System." ImageJ software (NIH) was used to quantify the intensity of the bands.

4.9 | Glycogen immunofluorescence

The monoclonal anti-glycogen antibody used for the immunofluorescence was generously provided by Dr. Otto Baba (Baba, 1993). hASCs grown on coverslips were fixed with 4% (w/v) paraformaldehyde, rehydrated with 2% (v/v) fish skin gelatin, and permeabilized with 0.2% Triton X-100 prior to incubation with 5% (v/v) goat serum. Subsequently, cells were incubated overnight at 4°C with the monoclonal mouse anti-glycogen antibody in PBS containing 1% goat serum. Coverslips were washed with PBS and incubated for 1 h at room temperature with an Alexa Fluor 568 conjugated goat anti-mouse antibody (1:100) and then mounted with ProLong Gold Antifade Reagent containing 40,6-diamidino-2-phenylindole (DAPI) (Invitrogen). Microscopy was performed with a Leica DM 4000B fluorescence microscope (Leica Microsystems), and images were captured with a Leica DFC 300 FX camera (Leica Microsystems).

4.10 | Glycogen colorimetric assay

Glycogen levels were measured in hASCs using the Glycogen Colorimetric Assay Kit (BioVision Inc.,). In total, 3×10^5 hASCs were homogenized with 200 µl of water on ice, and the assay was performed following manufacturer's instructions. A glucose background control was determined and then subtracted from the glycogen readings.

4.11 | Adenoviral transduction

Cells were infected 1 day after seeding with an adenovirus expressing murine PTG (Ad-PTG) or GFP (Ad-GFP) under the control of the CMV promoter (Gasa et al., 2000). The adenovirus expressing PTG or GFP (used as a control) was diluted in Opti-MEM Reduced Serum Medium (Gibco, ThermoFisher Scientific) at 1/200 and 1/4000, respectively, prior to use. Adenoviral infection was carried out for 2 h at 37°C using a multiplicity of infection of 50. The medium containing the adenovirus was then removed and replaced with standard culture medium. Two days after infection, culture medium was collected, and hASCs were collected and frozen.

4.12 | SIRT1 and SIRT6 silencing

Silencing consumables were all from Horizon Discovery. Cells seeded at 10,000 cells/cm^2 were transfected with human SIRT1 siRNA (003540) or SIRT6 siRNA (013306) or a control (On-Target Plus Non-targeting Pool, number 001810). siRNA (5 µM) and Dharmafect Transfection Reagent were diluted 1/20 and 1/25, respectively, in Opti-MEM Reduced Serum Medium (Gibco; ThermoFisher Scientific) and incubated for 5 min at room temperature. The same amount of each solution was mixed carefully and incubated for 20 min at room temperature. The final solution of siRNA at a concentration of 0.125 µM was added to hASCs and medium containing serum was also added to the wells. hASCs were incubated at 37°C with 5% CO_2 for 72–96 h.

4.13 | Statistical analysis

Statistical analysis was performed with GraphPad Prism 8 (GraphPad Software Inc.,). For in vitro data, experimental results

were presented as mean ± SEM from 3 to 5 independent donors for each experiment. Statistical significance was tested by parametric two-way analysis of variance (ANOVA), when 4 groups were analyzed, and by the parametric Student's unpaired t test when two groups were analyzed. For the gain or loss of function studies, the parametric Student's paired *t* test was used. Correlations were tested by Pearson's correlation analysis. General linear model and multiple linear regression analyses were employed to exclude sex as a confusing factor in the differences in proliferation found between groups.

AUTHOR CONTRIBUTIONS

M.T–P. contributed to conception and design, provision of study material or patients, collection and/or assembly of data, conduction of experiments, data analysis and interpretation, manuscript writing, and final approval of the manuscript. I.H-R. contributed to conception and design, data analysis and interpretation, and final approval of the manuscript J.S-B., M.E., E.M-M., and C.N-R. conducted the experiments. C.S. contributed to data analysis and interpretation. J.V. contributed to financial support, administrative support, and final approval of the manuscript. S. F-V. contributed to conception and design, financial support, administrative support, data analysis and interpretation, manuscript writing, and final approval of manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

CONSENT TO PARTICIPATE

Informed consent was obtained from all subjects involved in the study. Subjects were recruited by the Endocrinology and Surgery departments at the University Hospital Joan XXIII. The cells were stored in a tissue biobank registered at the National Register of Biobanks (registration number #C.0003609).

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CHARACTERIZATION OF ADIPOSE MESENCHYMAL STROMAL CELLS (ASC) IN AGING AND OBESITY
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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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8.1.2. Manuscripts non-related to the thesis

Impaired succinate response to a mixed meal in obesity and type 2 diabetes is normalized after metabolic surgery

Diabetes Care. 2020 Oct;43(10):2581-2587. doi: 10.2337/dc20-0460. Epub 2020 Jul 31.

Authors: Brenno Astiarraga, Laia Martínez, Victoria Ceperuelo-Mallafré, Gemma Llauradó, **Margarida Terrón-Puig**, M Mar Rodríguez, Anna Casajoana, Silvia Pellitero, Ana Megía, Núria Vilarrasa, Joan Vendrell, Sonia Fernández-Veledo

Journal: ***Diabetes Care***

Impact factor: **17.2** (2021)

Rank by discipline: 6/146 Endocrinology and metabolism (D1)

Microbial signature in Adipose Tissue of Crohn's disease patients

J Clin Med. 2020 Jul 31;9(8):2448. doi: 10.3390/jcm9082448.

Authors: Carolina Serena, Maribel Queipo-Ortuño, Monica Millan, Lidia Sanchez-Alcoholado, Aleidis Caro, Beatriz Espina, Margarita Menacho, Michelle Bautista, Diandra Monfort-Ferré, **Margarida Terrón-Puig**, Catalina Núñez-Roa, Elsa Maymó-Masip, M Mar Rodriguez, Francisco J Tinahones, Eloy Espin, Marc Martí, Sonia Fernández-Veledo, Joan Vendrell

Journal: ***Journal of clinical medicine***

Impact factor: **5.0** (2021)

Rank by discipline: 55/172 Medicine, General and internal (Q2)

Adipose stem cells from patients with Crohn's disease show a distinctive DNA methylation pattern

Clin Epigenetics. 2020 Apr 6;12(1):53. doi: 10.1186/s13148-020-00843-3.

Authors: Carolina Serena, Monica Millan, Miriam Ejarque, Alfonso Saera-Vila, Elsa Maymó-Masip, Catalina Núñez-Roa, Diandra Monfort-Ferré, **Margarida Terrón-Puig**, Michelle Bautista, Margarita Menacho, Marc Martí, Eloy Espin, Joan Vendrell, Sonia Fernández-Veledo

Journal: ***Clinical epigenetics***

Impact factor: **7.3** (2021)

Rank by discipline: 20/175 Genetics and heredity (Q1), 53/245 Oncology (Q1)

Crohn's disease disturbs the immune properties of human adipose-derived stem cells related to inflammasome activation

Stem Cell Reports. 2017 Oct 10;9(4):1109-1123.doi: 10.1016/j.stemcr.2017.07.014.

Authors: Carolina Serena, Noelia Keiran, Ana Madeira, Elsa Maymó-Masip, Miriam Ejarque, **Margarida Terrón-Puig**, Eloy Espin, Marc Martí, Natalia Borruel, Francisco Guarner, Margarida Menacho, Antonio Zorzano, Monica Millan, Sonia Fernández-Veledo, Joan Vendrell

Journal: ***Stem Cell Reports***

Impact factor: **7.3** (2021)

Rank by discipline: 8/29 Cell and tissue engineering (Q2), 56/195 Cell biology (Q2)

Survivin, a key player in cancer progression, increases in obesity and protects adipose tissue stem cells from apoptosis

Cell Death Dis. 2017 May 18;8(5):e2802.doi: 10.1038/cddis.2017.209.

Authors: Miriam Ejarque, Victòria Ceperuelo-Mallafré, Carolina Serena, Gisela Pachón, Yaiza Núñez-Álvarez, **Margarida Terrón-Puig**, Enrique Calvo, Catalina Núñez-Roa, Wilfredo Oliva-Olivera, Francisco J Tinahones, Miguel Angel Peinado, Joan Vendrell, Sonia Fernández-Veledo

Journal: ***Cell Death & Disease***

Impact factor: **9.7** (2021)

Rank by discipline: 36/195 Cell biology (Q1)

8.2. Posters, oral communications and oral presentations presented during the doctoral studies

Glycogen signature as an aging inflammatory

Tarraco-Malacca V Workshop. An update on new players in obesity and type 2 diabetes (Diamet, IISPV. Tarragona. 25-27 January 2018)

Oral presentation

Metabolic studies in adipose stem cells: drawing parallels between obesity and aging

II Seminari científic IISPV 2018 (IISPV. Tarragona. 12 February 2018)

Oral presentation

Células madre mesenquimales derivadas de tejido adiposo en el envejecimiento

VI Encuentro Malacca-Tarraco Workshop (IISPV, IBIMA. Málaga. 23-24 April 2019)

Oral presentation

A Senescent hASC impaired cytokine secretion could be linked to glycogen accumulation

X Reunión anual Ciberdem 2019 (CIBERDEM. ISCIII. Mataró. 6-8 November 2019)

Poster

Age and obesity induce glycogen accumulation and inflammation in adipocyte precursors via SIRT1 and SIRT6

XII Reunión anual Ciberdem 2021 (CIBERDEM. ISCIII. Mataró. 3-4 November 2021)

Poster

Glycogen deposition found in elderly-derived adipocyte precursors impacts cell function and promotes inflammation

Cellular aging and metabolism symposium (Universidad de A Coruña. A Coruña. 18-20 July 2022)

Poster

Efecto diferencial de la obesidad y el envejecimiento sobre los efectos inmunomoduladores las células madre mesenquimales de tejido adiposo

XVIII Congreso Nacional de la SEEDO. (SEEDO. Barcelona. 16-18 November 2022)

Oral communication