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SERPINA3 is expressed in human adipocytes and modulated by TNF-α and vitamin B6

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Constancia de aprobación de la tesis

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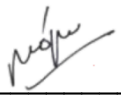

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Abstract

SERPINA3 is expressed in human adipocytes and modulated by TNF-α and vitamin B6

BACKGROUND. The *SERPINA3G* gene participates in antiadipogenesis and insulin resistance induced by TNF-α in 3T3-F442A murine cells. Since this gene has a human orthologue, *SERPINA3*, we explored if its plays similar roles in human adipose cells.

METHODS. The effects of TNF-α, retinoic acid (RA), and vitamin B6 (VIT B6) on terminal adipogenesis of primary human subcutaneous (hSA) and visceral (hVA) preadipocytes of normal-weight individuals and their *SERPINA3* expression were analyzed by quantifying the lipid accumulation by spectrophotometry and image processing and detecting adipogenic molecular markers by RT-qPCR and Western blot.

RESULTS. *SERPINA3* was detected in subcutaneous and visceral adipose depots of normal-weight individuals. TNF-α and RA increase the expression of *SERPINA3* mRNA in cultured hSA and hVA, but only TNF-α induced Serpin A3 protein expression. VIT B6 abrogated *SERPINA3* gene expression and partially canceled the anti-adipogenic effects by TNF-α in mature adipocytes.

CONCLUSIONS. Serpin A3 is expressed in human adipose tissues and modulates the antiadipogenic effects of TNF-α. These results suggest Serpin A3 could be a promissory target in the inflammatory processes linked to obesity and other adipose dysfunctions.

KEY WORDS: Serpin A3, TNF-α, retinoic acid, vitamin B6, human adipocytes, in vitro adipogenesis.

Resumen

SERPINA3 se expresa en adipocitos humanos y es modulado por TNF-α y vitamina B6

CONTEXTO. El gen *SERPINA3G* participa en la antiadipogénesis y la resistencia a insulina inducida por TNF-α en células múridas 3T3-F442A. Dado que este gen tiene un ortólogo humano, *SERPINA3*, exploramos si desempeña funciones similares en las células adiposas humanas.

MÉTODOS. Se analizaron los efectos del TNF-α, el ácido retinoico (RA) y la vitamina B6 (VIT B6) sobre la adipogénesis terminal de preadipocitos primarios subcutáneos (hSA) y viscerales (hVA) humanos en los que el mRNA de *SERPINA3* fue determinado por RT-qPCR, se cuantificó la acumulación de lípidos mediante espectrofotometría y procesamiento de imágenes y la expresión de marcadores moleculares adipogénicos mediante RT-qPCR y Western blot.

RESULTADOS. *SERPINA3* se expresa en depósitos adiposos viscerales y subcutáneos humanos de individuos con normopeso. TNF-α y RA indujeron la sobreexpresión del mRNA *SERPINA3* en adipocitos viscerales y subcutáneos humanos cultivados, pero solo TNF-α indujo la expresión de la proteína Serpina A3. La VIT B6 anuló la expresión del gen *SERPINA3* y canceló parcialmente los efectos antiadipogénicos provocados por el TNF-α en los adipocitos maduros.

CONCLUSIONES. Los tejidos adiposos humanos expresan Serpina A3, que a su vez, modula los efectos antiadipogénicos de TNF-α. Nuestros resultados sugieren que Serpina A3 es un blanco promisorio en los procesos inflamatorios relacionados con la obesidad y otras disfunciones adiposas.

Palabras clave: Serpin A3, TNF-α, ácido retinoico, vitamina B6, adipocitos humanos, adipogenesis in vitro.

1. General Introduction

1.1 Adipose tissue

Adipose tissue is a specialized connective tissue and the most prevalent in the human body. The majority of cells residing in adipose tissue are mature adipocytes, but there are several other types including preadipocytes, adipogenic progenitor cells, smooth muscle cells, pericytes, endothelial cells, and fibroblasts (Johnston & Abbott, 2022).

Until the 1980s, the adipose tissue was viewed almost exclusively as a depot for energy storage, with lipids being added or released under the influence of appropriate hormones. However, it is now known that adipose tissue plays a crucial role in the body due to its multifaceted functions. It serves as a regulator of appetite and energy expenditure, and participates in complex processes like reproduction, inflammation, and more (Sauerwein et al., 2014). For example, adipose tissue influences various metabolic pathways by secreting cytokines and adipokines which act as hormones (Castro et al., 2016).

This is important because adipose tissue is richly innervated and vascularized which means it has an impact on multiple target organs and is also capable of responding to, endocrine, immunological and metabolic signals (Mączka et al., 2024). Furthermore, in certain cases, adipose tissue has been signaled as a biomarker or predeterminant of various metabolic conditions, including obesity, diabetes, hypertension, and cardiometabolic risks (Choi et al., 2014). Additionally, the extracellular matrices of adipose tissue hold promise for tissue engineering and

regenerative medicine, particularly in soft tissue regeneration therapies (Gunawardana, 2014).

Humans have a widespread distribution of white adipose tissue (WAT). Major deposits reside in both the subcutaneous region (upper, deep, and superficial abdominal; lower, gluteal-femoral) and the visceral region (omental, mesenteric, mediastinal, and epicardial). Subcutaneous WAT (SAT), residing beneath the skin, acts as a barrier against infection, an insulator for heat retention, and a cushion against external stress. Visceral WAT (VAT), located within the trunk cavity and protected by the peritoneum and rib cage, surrounds vital organs (Kwok et al., 2016), has emerged as a key player in the development of metabolic disorders through the secretion of adipokines, such as leptin, adiponectin, and resistin (Sun et al., 2023).

In addition, VAT secretes more cytokines than subcutaneous fat, which can negatively impact insulin sensitivity (Ferreira et al., 2023). Additionally, analyzes of the methylome and transcriptome of both depots have shown that DNA methylation patterns of adipocyte depots are significantly different and the most dramatic variations in methylation occur in genes controlling development and transcription factors (Bradford et al., 2019). Another notable difference lies in the movement patterns. Subcutaneous cells move in a remarkably directed and controlled manner, constantly forming and breaking down attachments to their surroundings (focal adhesions). In contrast, visceral cells demonstrate a more random and uncoordinated movement style, with their attachments being more stable (Ritter et al., 2019).

In relation to metabolic condition and health, it has been reported that in individuals with obesity, visceral adipose tissue accumulation is associated with

insulin resistance, while variations in the type and proportions of immune cells within subcutaneous adipose tissue may play a role in determining diabetes risk (Kahn et al., 2022). Furthermore, VAT has been linked to the development of atherosclerosis and cardiovascular disease (Cesaro et al., 2023; Kataoka et al., 2023).

Although subcutaneous and visceral adipose tissues have distinct functions, some reports suggest they can also share some functional characteristics or even exhibit opposing classical functions (Kralova-Lesna et al., 2016). In addition, the cellular complexity of subcutaneous and visceral tissue demonstrates that both play a dynamic and active role in regulating energy and cellular homeostasis. Therefore, it is necessary to continue studying the regulation processes that both depots have.

1.2 Adipogenesis

Adipogenesis is the process by which precursor cells differentiate into adipocytes. This process is regulated by a complex and highly orchestrated gene expression program, hormonal and nutritional stimuli, and epigenomic regulators (Moseti et al., 2016) (Fig. 1).

The complex molecular process of adipogenesis involves many mechanisms orchestrating the transformation of adipose tissue precursors into mature adipocytes. Given this intricate system, the study of adipogenesis primarily relies on preadipocytes cell lines or preadipocytes isolated from adipose tissue (Ruiz-Ojeda et al., 2016).

The isolation of 3T3-L1 and 3T3-F442A cells from non-clonal Swiss 3T3 cells by Green and Kehinde (1974) marked the start of extensive research into the

differentiation properties of pure, clonal preadipocytes (Green & Kehinde, 1974; 1975; 1976). The 3T3-L1 cells are morphologically similar to fibroblasts but are already committed to the adipocyte lineage when exposed to prodifferentiative agents (Rosen et al., 2000).

In general terms, the adipogenic program consists of two distinct phases: the commitment phase and terminal differentiation phase. In the first, mesenchymal precursor cells limit their commitment to the adipocyte lineage under specific physicochemical signals; in this phase the cells turn into preadipocytes without significant morphological changes (Cawthorn et al., 2012; Eckel-Mahan et al., 2020). Following the commitment phase, preadipocytes undergo terminal differentiation. This phase is characterized by a series of structural and molecular changes that lead to various morphological changes allowing them to accumulate lipids (Kolodziej et al., 2019; Zhang et al., 2004).

During adipocyte differentiation, cells initially proliferate until they reach confluence, at which point most lineages undergo growth arrest, a critical first step in the differentiation process. In cultured cell lines, this arrest typically occurs due to contact inhibition and is followed by one or two rounds of mitotic clonal expansion (Tang et al., 2003). However, not all cells in the population are committed to mature adipocytes. A subset of cells remains in a quiescent, undifferentiated state, suggesting that adipogenic commitment is not uniform and may be regulated by intrinsic or extrinsic factors that modulate the differentiation potential of individual cells (Cannavino & Gupta, 2023). Subsequently, the committed cells undergo a phase or early differentiation and finally into fat cells (Chen et al., 2017).

At the molecular level, adipogenesis begins with the transient activation of the transcription factors *C/EBPβ* and *C/EBPδ* (Ambele & Pepper, 2017; Darlington et al., 1998). This is followed by the increased expression of the key adipogenic regulators PPARγ and C/EBPα. Finally, most or all genes associated with the mature fat cell phenotype are expressed, including those involved in triglyceride accumulation, e.g., glycerophosphate dehydrogenase, fatty acid synthase, acetyl CoA carboxylase, and Glut4 (Mota de Sá et al., 2017).

PPARγ

Peroxisome proliferator-activated receptor gamma (PPARγ) exists in two isoforms (PPARγ1 and PPARγ2) generated through alternative promoter usage and splicing (Mu et al., 2020). PPARγ1 is present in low amounts in many tissues; however, PPARγ2 is highly specific to adipose tissue, where it is expressed at very high levels (Sun et al., 2021). PPARγ exerts a profound influence on the expression of genes critical for proliferation, cellular differentiation, and lipid accumulation, as well as for the regulation of glucose, lipids, and cholesterol metabolism (Hernandez-Quiles et al., 2021; Sobolev et al., 2022). Additionally, clinical studies show that PPARγ dysfunction, including polymorphisms and mutations, contributes to the development of various metabolic syndrome pathologies, such as insulin resistance, obesity, dyslipidemia, and hypertension (Miao et al., 2022; Stalin et al., 2022).

C/EBP Family

CCAAT-enhancer-binding proteins (C/EBPs) belong to the basic-leucine zipper family of transcription factors expressed in different tissues; however, during adipogenesis, the expression of several C/EBP family members is tightly regulated. In differentiating preadipocyte cell lines, C/EBPβ and δ exhibit early and transient

increases in mRNA and protein levels (Cao et al., 1991; Darlington et al., 1998). In contrast, C/EBPα is induced later in the process, just before the activation of most fat cell-specific genes (Musri & Párrizas, 2012).

An amount of evidence supports a model for a transcriptional cascade regulating adipogenesis (Fig. 1). In this model, C/EBPβ and δ play a key role in inducing PPARγ expression. PPARγ, in turn, is responsible for inducing C/EBPα expression (Dutta et al., 2023; Kuri-Harcuch et al., 2019).

Together, C/EBPβ, C/EBPδ, and the transcription factor Cyclic AMP (cAMP) Response Element Binding Protein (CREB) collaborate to initiate adipogenesis. C/EBPβ and C/EBPδ constitute the first wave of transcription factors responding to adipogenic stimuli. In contrast, C/EBPα, a partner of PPARγ, belongs to the second wave and is involved in coordinating the final stages of adipocyte differentiation (Audano et al., 2022).

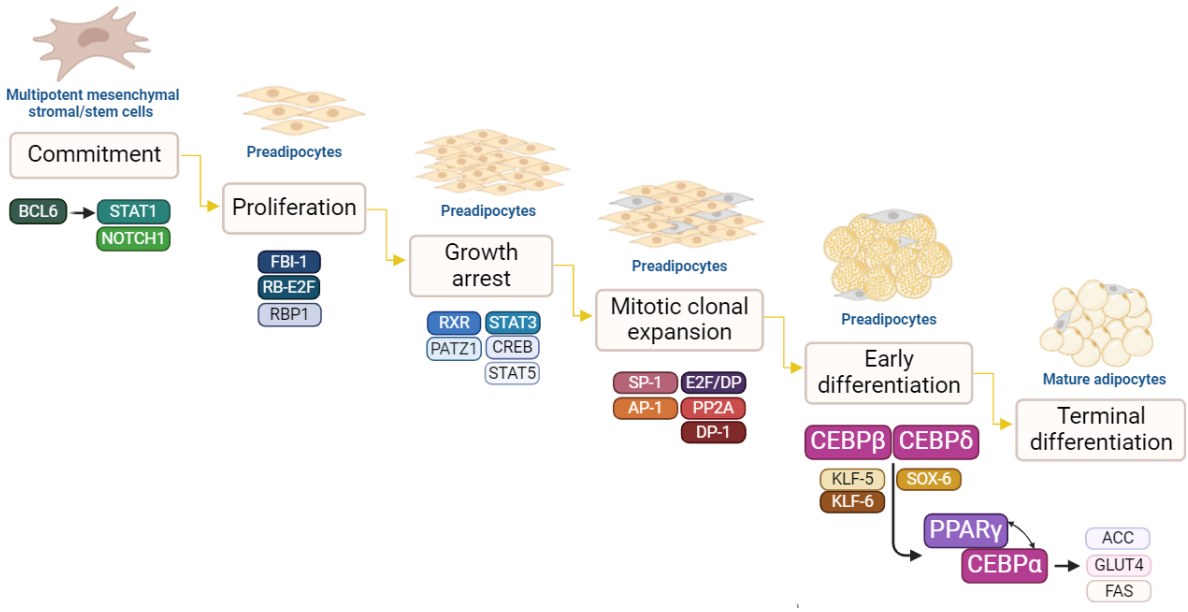

Figure 1. Cascade of events during adipogenesis
Adipogenesis is the process through which precursor cells differentiate into adipocytes. The intricate process involves numerous molecular mechanisms in which various transcription factors act as conductors, meticulously stimulating and inhibiting signaling pathways throughout this cellular commitment and differentiation. Based on data from Kuri-Harcuch et al., 2019; Audano et al., 2022; Dutta et al., 2023. Image created with BioRender.com.

1.3 Serpin superfamily of proteins

Serine protease inhibitors (Serpins) are a superfamily of proteins that regulate the activity of serine proteases (Gettins, 2002). Serpins are represented in all life kingdoms: archaea, bacteria, protist, fungi, plants and animals (Mangan et al., 2015). In humans, 36 serpins have been identified, 30 of them classified as functional protease inhibitors (Law et al., 2006; Lucas et al., 2018). According to its phylogenetic relationship, the superfamily is subdivided into nine clades (A to I) (Li et al., 2015).

Most Serpin members contain ~350–400 amino acid residues and, due to their glycosylation profile, their molecular weight varies between 40 and 100 kDa

(Irving et al., 2000; Sanrattana et al., 2019). To perform its inhibitory activity, serpins retain a classical structure of three β-sheets and 7-9 α-helices (Huntington, 2006; Mulenga et al., 2009). The structure also comprises a reactive center loop (RCL) that contains the proteinase recognition site (Marijanovic et al., 2019). The process through which the serpins carry out their inhibitory action is characterized as a suicide substrate-like inhibitory mechanism in which the proteinase initially forms a non-covalent Michaelis-like complex through its interaction with RLC residues. Upon coupling, the P1 – P ' RCL bond is cleaved, allowing the protease to covalently bind to the main chain carbonyl carbon of the P1 residue. This is carried out through a translocation to the “bottom” of the serpin; the compression and distortion generated in the proteinase leads to a kinetically-trapped covalent complex (Horvath et al., 2005; Janciauskiene, 2001; Sanrattana et al., 2019; Silverman et al., 2001).

Serpins play essential roles in multiple physiological processes, including blood coagulation, hormone transport, inflammation and complement activation, ECM maintenance and remodeling, apoptosis, microbial infection, and renal development, among others (Wang et al., 2014; Winkler et al., 2020). It is important to mention that not all serpins function as proteinase inhibitors; instead, they execute other important functions as hormone transport proteins and chaperones (Gardill et al., 2012; Köhler et al., 2020).

1.4 Serpin A3: functions and protease targets

Serpin A3 (also known as α1-antichymotrypsin) is a serine protease inhibitor composed of 423 amino acids and has a classical serpin structure consisting of eight α-helices, three β-sheets, and an active site located within a reactive center loop

(RCL) (Janciauskiene, 2001). Phylogenetically it is classified within clade A serpins that mainly include inflammatory response molecules (Lucas et al., 2018). The protein is secreted to the circulation during the acute phases of the inflammatory process, where it is required for the regulation of released leukocyte proteases (Janciauskiene, 2001). Proteolytically inhibits various serine proteases, including pancreatic chymotrypsin, mast cell chymase, staphopain C, and mainly cathepsin G (Baker et al., 2007; Travis et al., 1978; Wladyka et al., 2011). As a component of neutrophil granules, Cathepsin G regulates inflammatory responses through the activation of immune cells and its mobilization to the site of tissue damage also stimulates cytokine production and contributes to neutrophil migration due to extracellular matrix protein cleaving (Mantovani et al., 2011; Meyer-Hoffert, 2008; Zamolodchikova et al., 2020).

Serpin A3 is synthesized in a range of tissues including liver, kidneys, bronchi, brain and prostate (Kalsheker, 1996). Several studies indicate that Serpin A3 is involved in multiple physiological functions such as apoptosis, the implantation process, cell proliferation, invasion and migration, blood coagulation, wound healing, and it has also been associated with various pathologies (Kulesza et al., 2019b; Meijers et al., 2018). For instance, the first studies on the role of Serpin A3 were related to its overexpression in individuals with Alzheimer's disease and nowadays is considered a crucial player in the pathogenesis of this disease, especially in relation to the progression of cerebral amyloidosis (Abraham et al., 1988; 2001). Subsequent studies have indicated that Serpin A3 is associated with multiple cancer types, including endometrial (Wang et al., 2018; Yang et al., 2014; Zhou et al., 2019), prostate (Nguyen et al., 2018), colon (Cao et al., 2018), breast (Montel et al.,

2005; Vishnubalaji et al., 2019; Yamamura et al., 2004), ovarian (Jinawath et al., 2010), and non-small-cell lung cancer (Jung et al., 2016, 2016; Tian et al., 2016).

Serpin A3 has also been linked to other conditions such as hepatocellular carcinoma (Santamaria et al., 2013), glioblastoma (Luo et al., 2017; Miyauchi et al., 2018), cholangiocarcinoma (Chapman et al., 2012), and melanoma (Kulesza et al., 2019a; Wang et al., 2010; Zhou et al., 2016). Furthermore, recent research links Serpin A3 to the etiology ans sustainment of type 2 diabetes mellitus through inflammatory processes and oxidative stress (Cimini et al., 2019; El-Mesallamy et al., 2014; Takahashi et al., 2013).

1.4.1 Non-canonical functions of Serpin A3

Although most SERPINs are defined primarily by their protease inhibitory activities, they execute other important functions. Some serpins play a role as chaperones, as is the case of the heat-shock protein 47 (HSP47) or serpinH1, that perform key functions in the secretion and folding of fibrillar collagens (Köhler et al., 2020). An emerging role in immunity has also been recorded for intracellular serpins driven by the development of memory T cells (Ashton-Rickardt, 2012). The case of Serpin A3 is notable due to it functions not only as a serin protease inhibitor but also participates in activities such as cell migration (Zhou et al., 2017), invasion (Kulesza et al., 2019b) and proliferation (Yang et al., 2014), redox-sensitive functions (Grek et al., 2012), and promotion of chromatin condensation (Santamaria, et al., 2013).

1.4.2 Pathologies supporting functions of *SERPINA3*

Numerous advances in the role of *SERPINA3* in biological processes are about its participation in cancer pathology. It has been pointed out that the secretion of Serpin

A3 by DNA-PKcs-proficient cells allows cancer metastasis by promoting cell migration and invasion; DNA-PKcs is an important factor in DNA damage signaling (Hanamoto et al., 2013).

Likewise, STAT3 is a transcriptional factor constitutively activated in many cancers and participates in cell cycle progression, cell proliferation and tumor growth progression (Gu et al., 2020). Recently, it has been reported that Serpin A3 is one of the transcriptional targets of STAT3, the regulation through this transcription factor produces an increase in the migration of melanoma cells overexpressing *SERPINA3*, while the decreased expression of *SERPINA3* results in a lower number of invading cells (Kulesza et al., 2019b).

Its relationship with the processes of cell migration and invasion has also been studied in colon cancer. It has been observed that the knockdown of *SERPINA3* in KM-12L4 and HT-29LMM colon cancer cell lines results in a decrease in migration and invasiveness of these cells compared to control cells. Accompanying this effect, a decrease in the expression of Mmp-2 and Mmp-9, two members of the family of matrix metalloproteinases that function as indicators for invasion or metastasis in colon cancer, was also observed (Cao et al., 2018). Likewise, it has been pointed out that alterations in the concentration of Serpin A3 in human colorectal cancer tissue could function as a biomarker of disease progression, especially since the expression of Serpin A3 tended to be higher in disseminated cancer than in localized cancer (Dimberg et al., 2011). In this line, a meta-analysis of *in vitro* microarray studies and proteomic data suggests that Serpin A3 is a strong prognostic candidate for colorectal cancer metastasis (Long et al., 2016).

On the other hand, in endometrial cancer, a positive expression of *SERPINA3* was found in 80% of the cancer group cases and none in the control group. In addition, the expression of *SERPINA3* was associated with the degree of tumor differentiation, especially in those cases with medium and low-grade differentiation (Wang et al., 2018). For its part, an upregulated *SERPINA3* expression is present in recurrent ovarian carcinoma, and its participation could be related to the activation of inflammatory networks (Jinawath et al., 2010).

Breast cancer research has also addressed the role of *SERPINA3* in the development of this pathology. In this way, microarray analysis of primary breast cancer biopsies pointed to *SERPINA3* as a gene that co-clusters with estrogen receptor (ER) (Dressman et al., 2001). Classification based on ER status comprises ER-positive and ER-negative, of which more than three quarters are ERα-positive in breast cancer patients. *SERPINA3* expression is present in high levels in ER1α-positive samples and on the contrary, at low levels in ER1α-negative breast cancer tissue samples (Craig et al., 2004; Hua et al., 2018). Likewise, it has been shown that the levels of mRNA expression of *SERPINA3* could be considered as predictors of response to adjuvant hormone therapy in patients with human breast cancer, since *SERPINA3* is an estrogen-inducible gene and is associated with good prognosis in hormone receptor-positive in these patients (Yamamura et al., 2004). Research along the same lines has reported that the mechanism by which the transcription of *SERPINA3* is dependent on estrogen receptor in human breast cancer cells is mediated by the recruitment of PLK1, a key regulator of cell division, to estrogen-sensitive elements (ERE) that are present in promoter regions of the *SERPINA3* gene after hormonal stimulation (Wierer et al., 2013). Besides, further

investigations to determine the interaction of breast cancer cells and adipocytes indicated that the co-culture of both MCF-7 cells and 3T31-L1 adipocytes or MCF-7 cells and adipocyte-conditioned medium (ACM), produced a significant up-regulation of *SERPINA3* in cancer cells compared to those in regular growth medium as controls, which could show some type of adipose regulation through Serpin A3 in cancer cells (Nickel et al., 2018).

1.4.3 *SERPINA3*: a novel regulator of cell differentiation and proliferation across diverse cell types

The involvement of *SERPINA3* in the process of adipogenesis has been also addressed. In the 3T3-F442A cell line, the murine ortholog *SERPINA3g* is induced by tumor necrosis factor-alpha (TNF-α) during the stages of the differentiation and participates in the insulin-resistance caused by this cytokine (Salazar-Olivo et al., 2014).

Likewise, the study of novel genes that participate in the early adipogenesis of the 3T3-L1 cell line establishes that serpins A3c, A3n, and AdamTs15 are highly expressed in conditioned medium compared with the induction medium, suggesting that the secretion of these proteins by adipocytes could play a role during 3T3-L1 differentiation (Choi et al., 2020). In fact, *SERPINA3c* has been identified as one of the adipocyte-secreted proteins during adipogenesis in 3T3-L1 adipocytes (Wang et al., 2004; Zvonic et al., 2007). Moreover, *SERPINA3c* is highly expressed in mice adipose tissue and its participation is critical in the early stages of adipogenesis, mainly during mitotic clonal expansion via insulin growth factor 1 and by inhibiting serine proteases responsible for degrading integrin α5, thus preventing the

attenuation of adipose differentiation (Choi et al., 2020). In the same way, microarray analysis revealed that the *SERPINA3c* gene is upregulated in small proliferative adipocytes (SPA) compared with stromal vascular cells (non-adipocyte cells). SPA cells express adipocyte-specific genes and have a proliferative capacity suggesting they may be intermediate cells in the course of adipogenesis (Hanamoto et al., 2013; Kajita et al., 2013; Taguchi et al., 2020).

It has also been reported that during hemopoietic progenitor cells mobilization, a decrease in *SERPINA1* and *SERPINA3* expressions occur at mRNA and protein levels. This produces an accumulation of the serine proteases required for the maintenance of hematopoietic progenitor cells in bone marrow, which ultimately determines their fate and participation in cell proliferation and survival (Winkler et al., 2005). A screening for hallmarks of mesenchymal stem cell differentiation into articular chondrocytes found that *SERPINA1* and *SERPINA3* are induced during *in vitro* chondrogenesis (Boeuf et al., 2008; Meyer et al., 2016).

Concerning *SERPINA3n*, this inhibitor abolishes the function of differentiated osteoblasts, which could finally impact osteoblastic bone formation. However, in the early stage of osteoblastic differentiation, the overexpression of *SERPINA3n* does not appear to affect the differentiation of mesenchymal cell into osteoblasts (Ishida et al., 2019). Furthermore, histological analysis showed that *SERPINA3g*-expressing cells have been observed in the trabecular bone region of the bone marrow, where the hematopoietic stem cell microenvironment needed to maintain blood cell production is located. Therefore, *SERPINA3n* expression could be related to processes of self-renewal, proliferation, and cellular differentiation (Mizukami et al., 2008).

Participation of *SERPINA3* in cell proliferation or growth has also been reported in cancer cells. For example, *SERPINA3* has emerged as a factor with *in vitro* proliferative effects in human colon cancer (HT-29) cells; its participation could be related to the activation of growth pathways by ribosomal protein S6 and Akt phosphorylation (Meijers et al., 2018). It has been shown that knockdown of serpinA3 expression arrested cell cycle at G2/M phase leading to proliferation inhibition of endometrial cancer cell lines; this serpin can control cell viability through AKT phosphorylation and activation of the ERK1/2 pathway, whose nuclear translocation allows the progression of the G1 to the S phase (Brunet et al., 1999; Yang et al., 2014). Moreover, microarray analysis and telomere length studies identified *SERPINA3* as the strongest determinant of liver hepatocellular carcinoma cell invasion, migration, and proliferation (Ko et al., 2019). Additionally, it has been suggested that *SERPINA3* could be a factor that allows monitoring *in vitro* hepatogenesis since it has been found in a hypomethylated state in hepatocellular carcinoma and non-tumor hepatic tissue, while a hypermethylated state occurs in human embryonic stem cells as in human induced pluripotent stem cells (Kim et al., 2011).

1.4.4 Metabolic disorders and *SERPINA3*

Obesity is a persistent ailment distinguished by the accumulation of fat mass. Today, obesity represents a significant challenge to global public health, given that approximately 800 million people globally are impacted by it (World Health Organization, 2022). The main concern about obesity is its association with other metabolic diseases such as type 2 diabetes mellitus (T2D), hypertension,

cardiovascular diseases, and some types of cancer (Al-Goblan et al., 2014; Bhushan et al., 2018; López-Jiménez & Cortés-Bergoderi, 2011; Stone et al., 2018). Multiple causes underpin the pathogenesis of obesity, including hereditary components, physiological processes, external circumstances, and communal determinants. For example, a range of research suggests that adipose tissue plays a role in the progression of obesity. This is because it doesn't solely serve as an energy storage site, but also functions as a gland that secretes various chemical messengers, including cell-communication molecules, signaling and regulatory proteins, and enzymes, which can influence how cells and biological structures function (Booth et al., 2016; Kershaw & Flier, 2004; Longo et al., 2019; Trayhurn, 2005; Wright & Aronne, 2012). Furthermore, obesity is recognized as a condition significantly driven by inflammation. Consequently, extensive research has investigated the production of inflammatory proteins, like tumor necrosis factor and various cytokines, within adipose tissue (Hamid et al., 2016; Samaras et al., 2010; Schmidt et al., 2015). Nevertheless, to gain a comprehensive understanding of how adipose tissue contributes to related diseases, it's necessary to investigate other genes and proteins that might significantly influence how obesity is regulated.

A prime example is the Serpin A3 protein (Baker et al., 2007). Recently, significant interest has grown around *SERPINA3* due to its established connection with obesity and T2D. For instance, increased concentrations of *SERPINA3* have been found in the plasma of animal models exhibiting T2D, leading to its consideration as a potential circulating biomarker in diabetic retinopathy. (Takahashi et al., 2013), and a urine biomarker in diabetic patients (Riaz, 2015). *SERPINA3* has also been identified as a precursor of T2D in patients with abdominal obesity (Kim

et al., 2019). Likewise, significantly higher levels of *SERPINA3* have been reported in diabetic patients compared to healthy subjects (Takahashi et al., 2017) and its presence has been noted in the proteomic profile of individuals with obesity (Al-Daghri et al., 2017).

1.5 Towards understanding the human adipogenic network: exploring the role of SERPINA3

As noted above, adipogenesis is the process through which precursor cells, preadipocytes, differentiate and engage in sustaining energy homeostasis as mature adipocytes (Sarjeant & Stephens, 2012). Multiple factors control this process, from endocrine hormones, activation, and repression of cell signaling pathways to growth factors, cytokines, and adipokines, among others (Tang & Lane, 2012). One of these molecules is TNF-α, a proinflammatory cytokine that has been associated with various metabolic diseases such as obesity, T2D, and insulin resistance (Alzamil, 2020). During adipose differentiation of murine cell lines, TNF-α acts as an inhibitor of adipogenesis (Song et al., 2013). The mechanism involved appears to implicate blocking or decreasing the expression of transcription factors essential for the promotion of adipogenesis such as PPARγ and C/EBPα (Kudo et al., 2004; Meng et al., 2001). It has also been reported that TNF-α is overexpressed, both at the mRNA and protein level, in adipose tissue of humans and animals with obesity (Al-Shukaili et al., 2013; Krogh-Madsen et al., 2006), and that it is able to interfere with the expression patterns of various molecules associated with obesity and T2D (Kirchgessner et al., 1997; Wu et al., 2016).

One of these molecules is Serpin A3, a protein of the serine protease inhibitor

superfamily (Baker et al., 2007). As noted, SERPINA3 gene and protein plays various roles at the cell and physiology levels and it is also important to highlight that the protein is present on the proteomic profile of individuals with obesity (Al-Daghri et al., 2017) and significantly higher levels of *SERPINA3* have been reported in individuals with T2D than in healthy subjects (Takahashi et al., 2017).

The relationship of *SERPINA3* with TNF-α has been documented during the *in vitro* administration of TNF-α to lung cells, which produces an increase in the expression of SERPINA3, this action requires the presence of glucocorticoid receptors and the soluble TNF-α receptor type 1 (Lannan et al., 2012). In *in vivo* and *in vitro* models, the cytokines IL-1β and TNF-α, respectively, induce the transcription and translation of SERPINA3 (Machein et al., 1995; Morihara et al., 2005). It has also been reported that the expression of the *SERPINA3* gene is strongly modulated by proinflammatory cytokines such as IL-1 and IL-6 (Baker et al., 2007; Kordula et al., 1998).

Furthermore, the involvement of TNF-α and *SERPINA3* in the adipogenic process has been reported in murine preadipocytes of the 3T3-F442A line in which TNF-α induces the expression of *SERPINA3g*, the murine orthologue of *SERPINA3*, independently of the state of cell differentiation. Silencing of *SERPINA3g* in this cell line suggests that serpin participates in the antiadipogenic effect of TNF-α on preadipocytes and in cytokine-induced insulin resistance in mature adipocytes (Salazar-Olivo et al., 2014).

Despite previous findings in murine preadipocytes, neither the involvement of *SERPINA3* in the course of *in vitro* human adipogenesis nor the effects of TNF-α on

SERPINA3 expression during this process have been explored.

1.6 Rationale

The absence of the *SERPINA3g* gene in humans implies a knowledge gap regarding its role in human adipose metabolism and adipogenesis. Therefore, the study of *SERPINA3*, its human orthologue, aims to elucidate the molecular mechanisms in *SERPINA3* mRNA and protein expression during human adipogenesis and adipocyte function, which could address this critical knowledge gap in our understanding of adipocyte cell development and metabolism in humans.

Furthermore, since *SERPINA3* appears to be modulated by TNF-α, a known cytokine with pro-inflammatory effects, the present study could lay the foundation for understanding its function in inflammation-linked adipose dysfunction.

In addition, this study focuses on *SERPINA3* expression in both subcutaneous and visceral human adipocytes primary cultures; therefore, to analyze how *SERPINA3* function differs in both depots could provide valuable insights into the regulation of fat storage and dysfunction in specific adipose tissues.

In general, this research aims to provide a more comprehensive understanding of the adipose tissue physiology and the mechanisms governing adipogenesis and, given the relationship of both with *SERPINA3*, it could contribute to elucidating its mechanism of action and therapeutic utility in *SERPINA3*-related metabolic conditions.

2. Materials and Methods

2.1 RT-PCR for human adipose tissue samples

Subcutaneous and visceral adipose tissue samples were obtained from fourteen healthy, non-pregnant volunteers (seven woman, seven men) undergoing abdominal surgery at Hospital Militar Regional de San Luis Potosí. All participants provided written informed consent, and the study protocol was approved by the Hospital's Ethics Committee. Inclusion criteria were: age 20-45 years, normal weight defined by a body mass index (BMI) of 18.5-24.9 kg/m² according to World Health Organization criteria (WHO, 2024), and no prior diagnosis of cancer or HIV. Exclusion criteria were current pregnancy and use of medication known to alter metabolism or significantly affect body weight, including antipsychotics.

Total RNA was extracted from subcutaneous and visceral adipose tissue using TRIzol®. RNA was quantified by UV spectrophotometry at 260 nm. For reverse transcription, 1 µg of total RNA was combined with 0.5 µg of oligo(dT), 200 U reverse transcriptase, 25 U RNAsin, 0.5 mM dNTPs, and 1x RT buffer in a 25 µL reaction, incubated at 42 °C for 90 min. Negative controls without reverse transcriptase were included. *hGAPDH* was used as an internal control.

cDNA was amplified using *SERPINA3* and *hGAPDH* primers (see Table 1). PCR was performed with 500 ng cDNA, 0.2 µM primers, 0.2 µM dNTPs, 1.5 mM $MgCl_2$, 1.25 U Taq polymerase, and 1× buffer in a 15 µL volume. Cycling conditions were: 95 °C for 5 min; 33 cycles of 95 °C for 30 s, 58.4 °C for 1 min, and 72 °C for 1 min; final extension at 72 °C for 5 min. *SERPINA3* products (322 bp) were visualized on 1.5% agarose gels stained with ethidium bromide

2.2 Adipose differentiation

Human preadipocytes isolated from inguinal subcutaneous tissue (hSA) and from visceral adipose tissue (hVA) from a 20-month-old male patient treated for syndactyly were cultured in a humidified atmosphere of free gas exchange at 37 °C with L15 medium supplemented with 5% calf serum, 10% fetal bovine serum and antibiotics (penicillin 80 U/mL and streptomycin 80 μg/mL) (Basal Medium; BM). When cells reached 80% and 100% confluence in BM medium, or BM supplemented with 10 ng/mL TNF-α or 10 μM retinoic acid, total RNA was extracted from the proliferating and quiescent culture stages, respectively.

For the stages of freshly committed cells to adipose differentiation and mature adipocytes, confluent cells were induced to differentiate with adipogenic medium (AM; L15 medium supplemented with 10% SFB, 1 μM insulin, 33 mM biotin, 17 μM calcium pantothenate, 100 nM cortisol, 5 μg/mL transferrin, 2 nM triiodothyronine, 100 nM dexamethasone, 500 μM isobutyl-methyl-xanthine, 1 μM rosiglitazone, and antibiotics), or else, with adipogenic medium supplemented with 10 ng/mL TNF-α or 10 μM retinoic acid (RA, used as anti- adipogenic control) for 4 days, at which time RNA corresponding to the stage of cells freshly committed to adipose differentiation was obtained. Parallel cultures remained in maintenance medium (MM; L15 medium supplemented with 10% SFB, 1 μM insulin, 33 mM biotin, 17 μM calcium pantothenate, 100 nM cortisol, 5 μg/mL transferrin, 2 nM triiodothyronine and antibiotics) for 14 days for subsequent RNA extraction corresponding to the mature or terminally differentiated adipocyte stage. During each stage, parallel cultures were treated with non-adipogenic medium (NAM; L15 medium supplemented with 5% calf

serum, 10% fetal bovine serum, and antibiotics) as undifferentiated controls.

2.3 Oil red O staining

hSA and hVA preadipocytes were seeded into 24-well plates and subjected to adipose differentiation following the design of the previous experiment. After 14 days, cells were fixed with 3.5% formaldehyde and incubated for 30 min at 4 °C. Excess formaldehyde was removed, oil red dye was added and treated cultures were kept at room temperature for 24 hours. After this time the excess dye was eliminated, and lipid accumulation was observed under an inverted microscope (Leica) at 20x magnification. Subsequently, the dye was extracted using 1 mL isopropanol for 10 minutes at room temperature and the absorbance of the eluate was determined at 510 nm in the spectrophotometer (SmartSpec Plus).

2.4 RT-qPCR for cell culture samples

Total RNA from hSA and hVA preadipocytes in proliferating condition (80% confluence in BM in the presence or absence of 10 ng/mL TNF-α or RA 10 μM), quiescent (100% confluence in BM in the presence or absence of 10 ng/mL TNF-α or RA 10 μM), freshly committed to differentiation (induced for 4 days with AM in the presence or absence of 10 ng/mL TNF-α or RA 10 μM), and mature adipocytes (pre-adipocytes induced for 4 days with AM in the presence of 10 ng/mL TNF-α or RA 10 μM and kept in MM for an additional 14 days) were extracted using TRIzol®. RNA was quantified by spectrophotometry at 260 nm. For reverse transcription, 1 μg total RNA was mixed with 0.5 μg Oligo(dT), 200 U reverse transcriptase, 25 U RNAsin, 0.5 mM dNTPs, and 1× RT buffer in a 25 μL reaction, incubated at 42 °C for 90 min.

Expression levels of adipose differentiation marker genes and *SERPINA3* were assessed by quantitative PCR (qPCR) at each stage of adipose differentiation (proliferating preadipocytes, quiescent preadipocytes, adipocytes freshly committed to differentiation, and mature adipocytes). SYBR Green-based qPCR was performed on a Rotor-Gene 6000 real-time PCR system. The qPCR reaction was prepared in a total volume of 20 µL containing 10 µL of 1X SYBR Green master mix (Applied Biosystems®), 1 µL of cDNA at 100 ng/µL, 1.2 µL of 0.6 µM oligonucleotides and 7.8 µL of Milli-Q® water. A negative control was used by replacing the cDNA with Milli-Q® water. The sequences of the oligonucleotides used are shown in Table 1. Analysis of the relative mRNA expression for the selected genes was performed by $2^{-\Delta\Delta Ct}$ method using β-actin as the constitutive gene.

Table 1. Oligonucleotides used in RT-qPCR

mRNA	Sequences
β-actin	Fw. GGACCTGACTGACTACCTCAT Rv. CGTAGCACAGCTTCTCCTTAAT
PPARγ	Fw. GCCTGCATCTCCACCTTATTA Rv. ATCTCCACAGACACGACATTC
C/EBPα	Fw. GATAACCTTGTGCCTTGGAAATG Rv. GAGGCAGGAAACCTCCAAATA
SERPINA3	Fw. CCAACGTGGACTTCGCTTTC Rv. CTCTTGGCATCCTCCGTGAA
hGAPDH	Fw. GAAGGTGGTGAAGCAGGCGT Rv. ATGTGGGCCATGAGGTCCACCA

2.5 Cell proliferation assay

hSA and hVA cells were seeded in 24-well plates at a density of $5x10^3$ cells/well in BM. After 6 hours the cells were exposed to different concentrations (100 µM, 250

µM and 500 µM) of Vitamin B6 (VIT B6) (pyridoxal 5'-phosphate; SIGMA p3657-1G). VIT B6 was dissolved in DMSO as a stock solution and dissolved in culture medium according to the concentrations indicated without exceeding 0.1% DMSO.

The proliferation of hSA and hVA cells was determined by direct counting in a Neubauer chamber at 24, 48, 72, and 96 hours. The medium was changed every other day.

2.6 Adipose differentiation in the presence of VIT B6

hSA and hVA cells were subjected to adipose differentiation following the design of the previous differentiation experiment with the addition of two more groups. For the stages of freshly committed cells and mature adipocytes, confluent cells were induced to differentiation with adipogenic medium or else, with adipogenic medium supplemented with 100 µM VIT B6, or with 100 µM VIT B6 plus 10 ng/mL TNF-α or 10 µM RA for 4 days. Parallel cultures remained in a maintenance medium for 14 days until reaching the mature stage. Simultaneous cultures were maintained with basal medium as undifferentiated controls.

2.7 Lipid droplet counting

Micrographs taken from cultures in the presence of the different treatments were analyzed through the implementation of a specific algorithm using MATLAB. The algorithm was developed to estimate the number of lipid droplets present in each of the cultures in their respective treatments. Two images per treatment were analyzed for both hSA and hVA, and a total of 56 images were examined. The algorithm parameters were adjusted to dilation, erosion, opening, closing and binary

conversion functions of the image to identify each lipid droplet more accurately. Subsequently, 10 images were randomly selected from the total to verify that the number provided by the algorithm was close to the manual count of lipid droplets in the same images. This count was performed through the ImageJ software for image processing and analysis. Subsequently, the sigma value calculated for both droplet counting methods was 0.004105. Since the higher the sigma value the greater the difference between the tests, the value obtained indicates that the difference between the two averages in terms of standard deviation is negligible.

2.8 Human adipose tissue samples

Paired cDNA samples from visceral adipose tissue and subcutaneous adipose tissue corresponding to 19 patients undergoing cholecystectomy were obtained. A database was accessed with the values corresponding to the biochemical parameters of this population for subsequent analysis. The metabolic obesity condition of the participants was determined according to their body mass index (BMI): BMI between 18.5 and 24.9 for normal weight and ≥ 30 for obesity (WHO, 2024).

2.9 RT-qPCR for human adipose tissue samples

qPCR reactions were prepared in 20 µL volumes containing 10 µL of 1X SYBR Green master mix (Applied Biosystems®), 1 µL cDNA, 1.2 µL of 0.6 µM primers, and 7.8 µL Milli-Q® water.. A negative control was used by replacing the cDNA with Milli-Q® water. The sequences of the oligonucleotides used in this analysis are shown in Table 1. PCR reactions were performed in triplicate. Melt curve analysis was performed in each run to ensure the specificity of the amplified product, and a no-

sample negative control (NTC) was included in each reaction. The relative expression analysis for the selected genes was performed by the $2^{-\Delta\Delta Ct}$ method using β-actin as the constitutive gene.

3. Statistical analysis

Cell culture data are expressed as the mean ± standard deviation of three separate experiments. Statistical analysis of data obtained by spectrophotometry, densitometry, and RT-qPCR was performed by ANOVA followed by a Tukey test. In all cases, differences were considered significant when $p \leq 0.05$, and the results obtained were plotted using GraphPad Prism 7.

3. Results

3.1 TNF-α and retinoic acid block terminal adipogenesis in subcutaneous and visceral human preadipocytes

We previously showed that *SERPINA3g* participates in the antiadipogenesis and insulin resistance induced by TNF-α in murine 3T3-F442A adipose cells. To characterize the role of this serpin or some of its human orthologs during *in vitro* human terminal adipogenesis we established primary cultures of subcutaneous (hSA) and visceral (hVA) human preadipocytes and tested on them the antiadipogenic effects of TNF-α, a proven inductor of *SERPINA3g* expression in murine cells, and retinoic acid, also an antiadipogenic agent which does not induce the *SERPINA3g* expression (Salazar-Olivo et al., 2014). hSA or hVA cells maintained on basal medium (BM; Fig. 2A) remained fibroblastic in appearance and did not accumulate lipid droplets in their cytoplasm. On the contrary, cells induced

with adipogenic medium and kept in the maintenance medium for 14 days showed the mature adipocyte phenotype and a high amount of lipid accumulation characteristic of the differentiated stage (AM; Fig. 2A).

The presence of TNF-α 10 ng/mL or RA 10 μM in the adipogenic medium blocked the lipid accumulation in hSA cells by 60% and 78% respectively, and by 93.93% and 94.06 % in hVA cells, as determined by Oil red O staining (Fig. 2B, C). In both cell lineages, the antiadipogenic effects of TNF-α and RA were mediated by the underexpression of *PPAR*γ and *C/EBPα* genes (Fig. 2D, E).

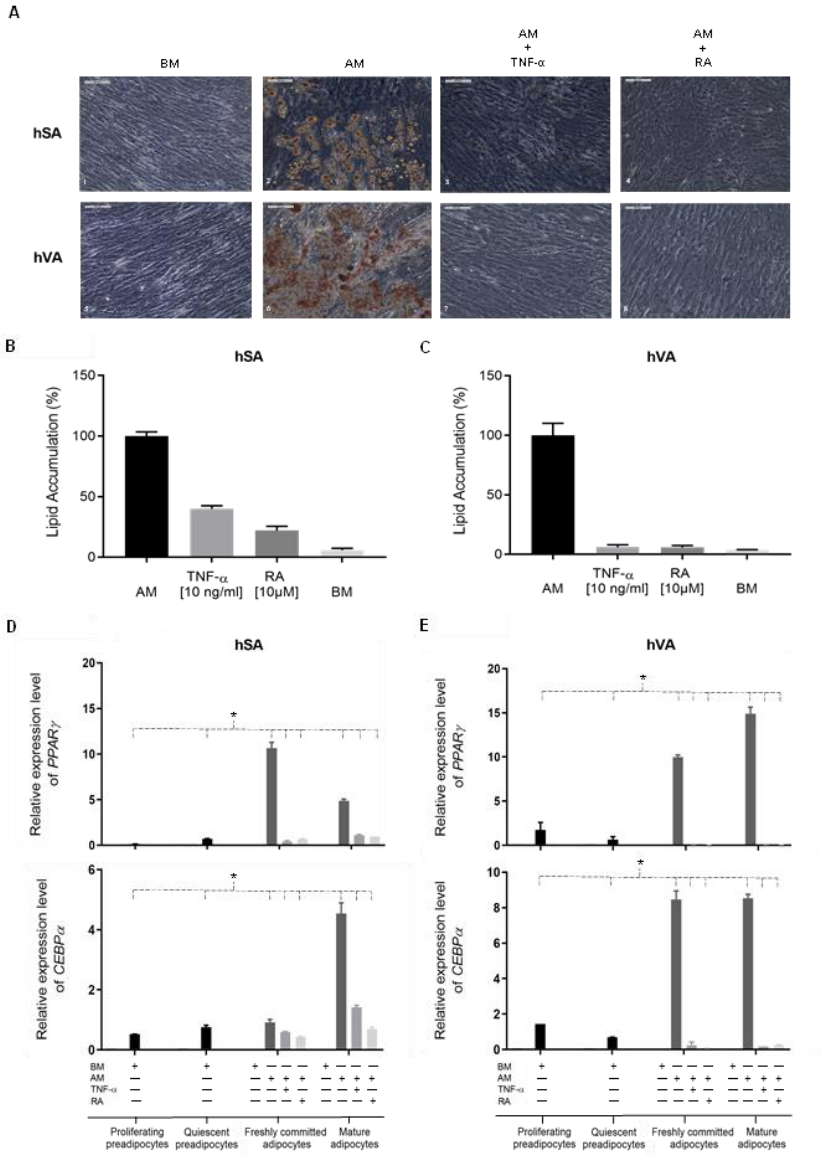

Figure 2. Effects of TNF-α and retinoic acid on terminal adipogenesis of human subcutaneous (hSA) and visceral (hVA) preadipocytes

Terminal adipogenesis of human subcutaneous (hSA) or visceral (hVA) preadipocytes was induced in the absence or he presence of 10ng/mL TNF-α or 10 μM retinoic acid (RA). Fourteen days after induction cultures were fixed with formaldehyde and stained with Oil Red O (A) to quantify the lipid accumulation (B). The expression levels of adipogenic markers *PPARγ* and *CEBPα* were quantified using total RNA from hSA (C) and hVA (D) cells in proliferating and quiescent preadipocytes, as well as from freshly committed and mature adipocytes. β-actin was used as a constitutive gene. Asterisks indicate statistically significant differences according to an ANOVA test between their respective treatments followed by a Tukey test to determine which groups differ from each other ($p < 0.05$). BM, Basal Medium; AM; Adipogenic Medium. Adapted from Guzmán-Herrera et al., 2025.

3.2 TNF-α and retinoic acid induced the overexpression of *SERPINA3* gene in hSA and hVA cells

SERPINA3g gene is not present in humans. Its human ortholog *SERPINA3* is highly expressed in the liver (Horvath et al., 2004), but its expression in adipose tissues has only been documented for epicardial fat (Zhao et al., 2020). Therefore, SERPINA3 expression was first assessed by RT-PCR in subcutaneous and visceral adipose tissue samples from normal-weight donors (n = 6; 3 females, 3 males). All samples, except one female subcutaneous sample, showed a 322 bp band corresponding to SERPINA3 (Fig. 3A). Despite the assay was not quantitative, SERPINA3 expression appeared stronger in visceral than in subcutaneous samples.

Then, we analyzed the expression of *SERPINA3* along the hSA and hVA terminal adipogenesis, in the absence or the presence of TNF-α or RA. Our analyses showed that proliferative and quiescent preadipocytes of both cell types expressed low levels of *SERPINA3* mRNA, which were induced 3.86-fold (hSA) and 3.30-fold (hVA) by TNF-α but not RA in quiescence. has cells maintained in BM during the experiment (deep quiescence) expressed similar levels of *SERPINA3* mRNA than freshly committed adipocytes (Fig. 3B). In contrast, adipogenesis but not deep quiescence increased 6.13-fold the expression of *SERPINA3* in hVA cells (Fig. 3C).

SERPINA3 gene increased 5.9-fold (hSA) and 2.98-fold (hVA) in freshly committed adipocytes, and 14.3-fold (hSA) and 3.2-fold (hVA) in mature adipocytes, concerning proliferating or quiescent preadipocytes. Furthermore, the presence of TNF-α induced a 2.1-fold (hSA) and 2.4-fold (hVA) *SERPINA3* overexpression in freshly committed cells and a 3.3-fold (hSA) and 7.3-fold (hVA) in mature adipocytes

concerning AM. In the case of RA, its application increased *SERPINA3* expression 1.09-fold (hSA) and 1.95-fold (hVA) in freshly committed adipocytes and a 2.8-fold (hSA) and 4.5-fold (hVA) in mature adipocytes concerning AM (Fig. 3B, C).

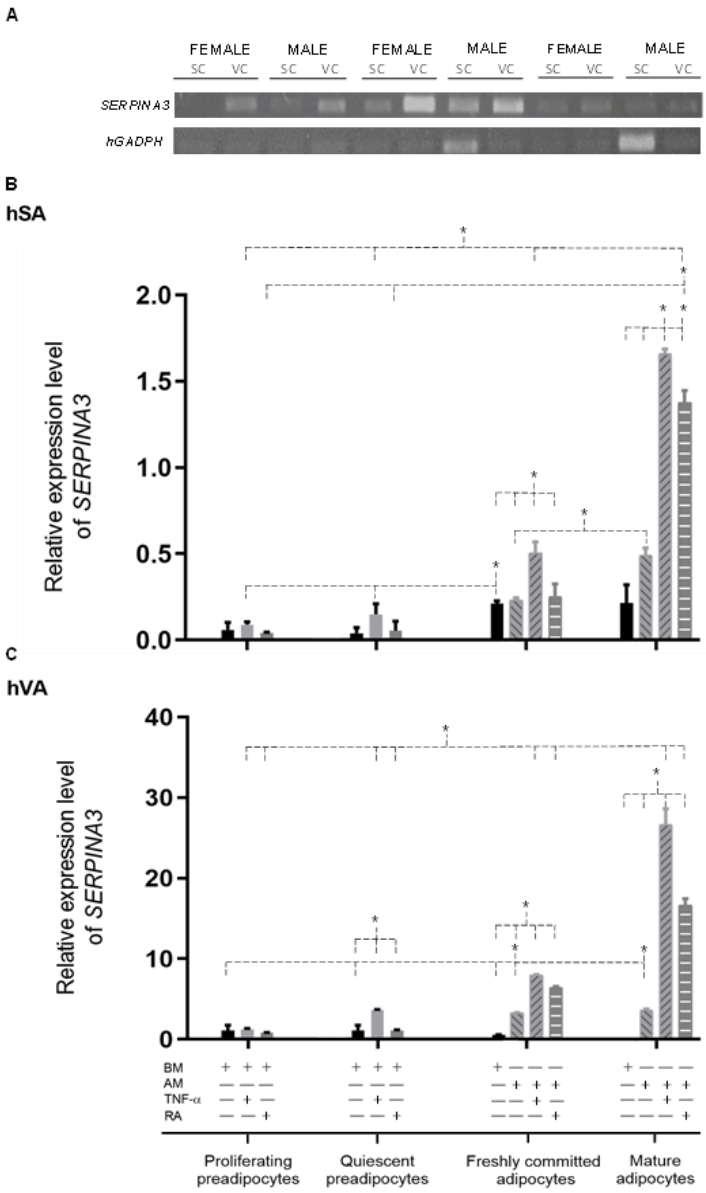

Figure 3. Expression of *SERPINA3* in human adipose tissues and cells

Total RNA (1 µg), isolated from either subcutaneous (SC) or visceral (VC) human adipose tissue samples, underwent analysis via RT-PCR to detect specific *SERPINA3* mRNA. *hGAPDH* served as a normalization standard, and the absence of reverse transcriptase as a negative control. The resulting amplified fragments were observed on 1.5% agarose gels stained with ethidium bromide (A).*SERPINA3* expression in hSA (B) or hVA (C) cells was analyzed by RT-qPCR in proliferating and quiescent preadipocytes as well as in freshly induced and mature adipocytes, cultured in the presence or absence of 10 ng/mL TNF-α or 10 µM RA. β-actin was used as the constitutive gene. Asterisks indicate statistically significant differences according to an ANOVA test between their respective treatments followed by a Tukey test to determine which groups differ from each other ($p < 0.05$). BM, Basal Medium; AM; Adipogenic Medium. Adapted from Guzmán-Herrera et al., 2025.

3.3 Vitamin B6 decreases SERPINA3 expression in early and late hSA and hVA adipocytes

Our results showing that TNF-α and RA induce *SERPINA3* overexpression in freshly committed and mature hSA and hVA adipocytes prompted us to explore if decreasing *SERPINA3* expression would eliminate the antiadipogenic effects of the cytokine and the retinoid.

An early report showed that vitamin B6 (pyridoxal phosphate; VIT B6) suppresses *SERPINA3* expression in rat colon cells and human colon cancer HT-29 cells (Yanaka, 2011a), and a recent bioinformatic analysis showed that VIT B6 strongly interacts with this serpin (Wang et al., 2023). Therefore, we examined the effect of VIT B6 on *SERPINA3* expression along the adipogenic pathway under the influence of TNF-α and RA.

To determine VIT B6 concentrations assayable on human adipose cells we evaluated three VIT B6 concentrations on hSA and hVA cell proliferation. Analyses showed that 100 μM and 250 μM VIT B6 did not significantly affect the cell growth of both cell types (Fig. 4A, B); in contrast 500 μM VIT B6 exerted a clear cytostatic effect on both cell types (Fig. 4A, B). Therefore, 100 μM VIT B6 was used in subsequent expression analyses.

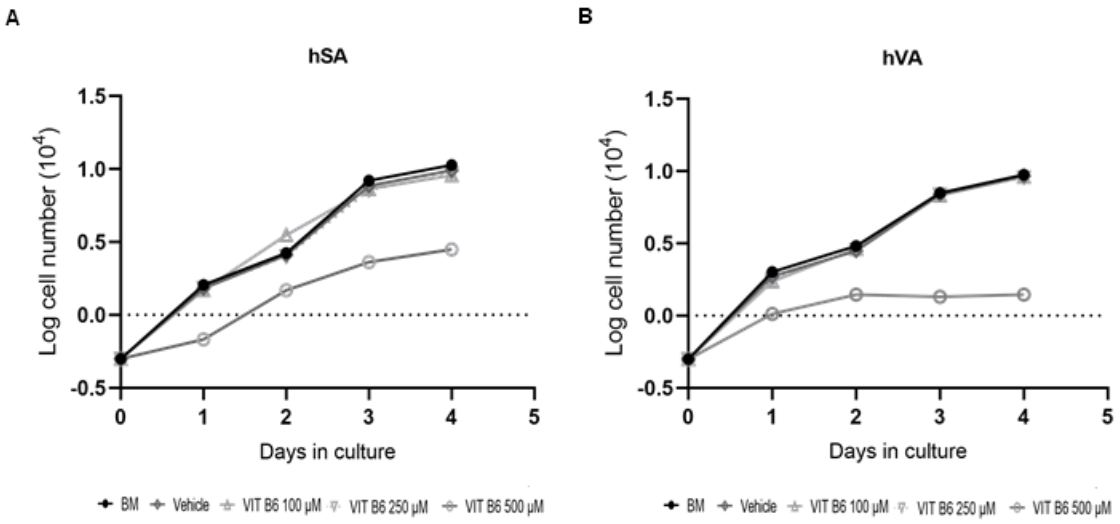

Figure 4. Effect of Vitamin B6 on hSA and hVA cell proliferation

Preadipocytes hSA (A) or hVA (B) were seeded in 24-well plates (0.5 x 10^4 cells/well) with basal medium (BM). After 6 h, cultures were refed with BM or BM added with VIT B6 at 100 µM, 250 µM, or 500 µM and maintained with media changes every other day. BM added with DMSO 0.1% was included as vehicle control. Cell counts were performed in triplicate with a hemacytometer at 24, 48, 72, and 96 h.

We then analyzed the effect of VIT B6 on the overexpression of *SERPINA3* induced by TNF-α or RA during hSA and hVA terminal adipogenesis. As previously shown (Fig. 3), TNF-α increased *SERPINA3* expression 2.46-fold (hSA) and 2.23-fold (hVA) in freshly committed adipocytes and 2.91-fold (hSA), and 7.11-fold (hVA) in mature cells, respect to AM in the same stages. RA also significantly increased *SERPINA3* expression 1.85-fold only in freshly committed hVA, and 2.37-fold (hSA) and 4.62-fold (hVA) in mature adipocytes, concerning AM. *SERPINA3* overexpression of induced by TNF-α or RA was abrogated by VIT B6. In early adipocytes, VIT B6 decreased *SERPINA3* expression 1.62-fold (hSA) and 3.56-fold (hVA), while in mature adipocytes, it decreased *SERPINA3* expression 3.27-fold (hSA) and 32.63-fold (hVA) respect to AM (Fig. 5A, B).

To explore if the changes we observed at the level of gene expression also occurred in protein expression, we assessed the levels of SERPINA3 protein by Western blot in mature adipocytes under different treatments. Our results show that SERPINA3 protein expression was induced by terminal adipogenesis 4.85-fold in hSA and 1.86-fold in hVA. In both cases, VIT B6 mostly (hSA) or totally (hVA) abrogated this induction. Our results also show that TNF-α induced SERPINA3 overexpression of 1.34-fold (hSA) and 1.93-fold (hVA) compared to AM. This protein overexpression was also totally abrogated by VIT B6.

Contrary to TNF-α, *SERPINA3* gene overexpression induced by RA in the two cell types did not result in the protein overexpression (Fig. 5C, D).

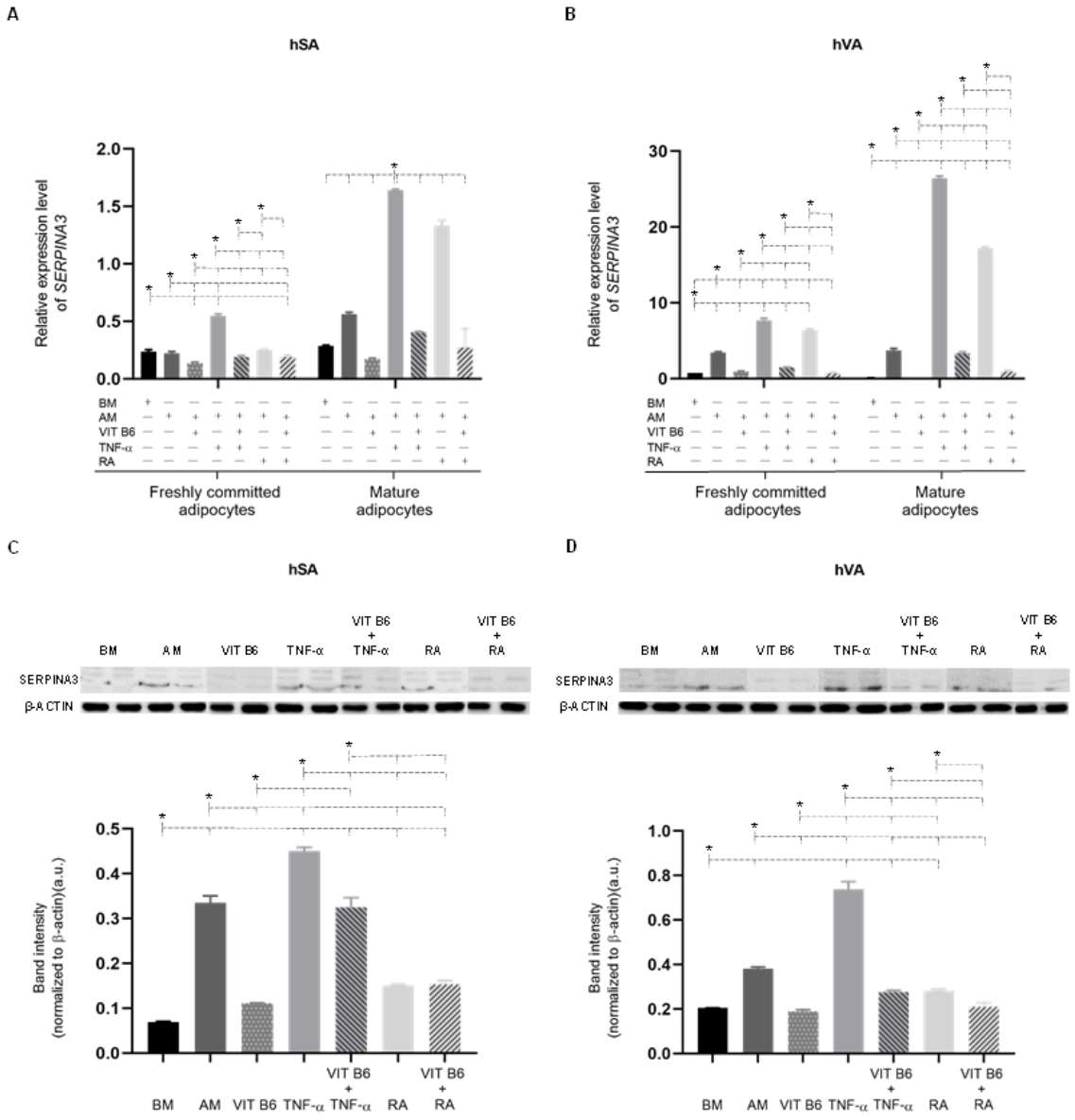

Figure 5. Effect of Vitamin B6 on the expression of *SERPINA3*

The expression levels of *SERPINA3* were analyzed by RT-qPCR in hSA (A) or hVA (B) freshly committed and mature adipocytes induced to differentiation in the absence or the presence of 10 ng/mL TNF-α, 10 μM RA, and 100 μM VIT B6 using β-actin as the constitutive gene. The whole cell protein of mature hSA (C) and hVA (D) adipocytes under the same treatments was analyzed by SDS-PAGE and Western blot to detect the presence of SERPINA3 protein. The bands detected for SERPINA3 in each treatment were quantified by densitometry and normalized to β-actin. The two lanes in each treatment correspond to samples from two separate experiments. Asterisks indicate statistically significant differences according to an ANOVA test between their respective treatments followed by a Tukey test ($p < 0.05$). BM, Basal Medium; AM; Adipogenic Medium. Taken from Guzmán-Herrera et al., 2025.

3.4 Vitamin B6 partially reverses the antiadipogenic effect of TNF-α in mature adipocytes

Next, we evaluated the effect of VIT B6 on the antiadipogenic effect of TNF-α, the only one of the two adipogenic blockers assayed that induced SERPINA3 mRNA and protein overexpression. As expected, the presence of TNF-α in AM blocked lipid accumulation in both hSA and hVA mature adipocytes (Fig. 6A, 4 and 4'; Fig. 6B, 9 and 9') while VIT B6 did not significantly affect adipose differentiation, as the presence of lipid droplets under this treatment (Fig. 6A, 3 and 3'; Fig 6B, 8 and 8') was like those present in AM treatment (Fig. 6A, 2 and 2'; Fig. 6B, 7 and 7´). In contrast, addition of VIT B6 to AM + TNF-α reversed partially (hSA; Fig. 5A, 5 and 5') or markedly (hVA; Fig. 6B, 10 and 10') the blocking of lipid accumulation by TNF-α.

We quantified the above observations with a MATLAB-generated algorithm allowing the estimation of lipid droplets in each treatment. The statistical analysis of data obtained after the execution of the algorithm showed that TNF-α significantly reduced lipid accumulation in mature hSA (99.98%) and hVA (100%) concerning AM (Fig. 6C, D). In contrast, the addition of VIT B6 to the TNF-α treatment significantly increased lipid accumulation in mature hSA (12.92%) and hVA (19.03%), indicating that VIT B6 partially abrogates TNF-α inhibition of lipid accumulation (Fig. 6C, D).

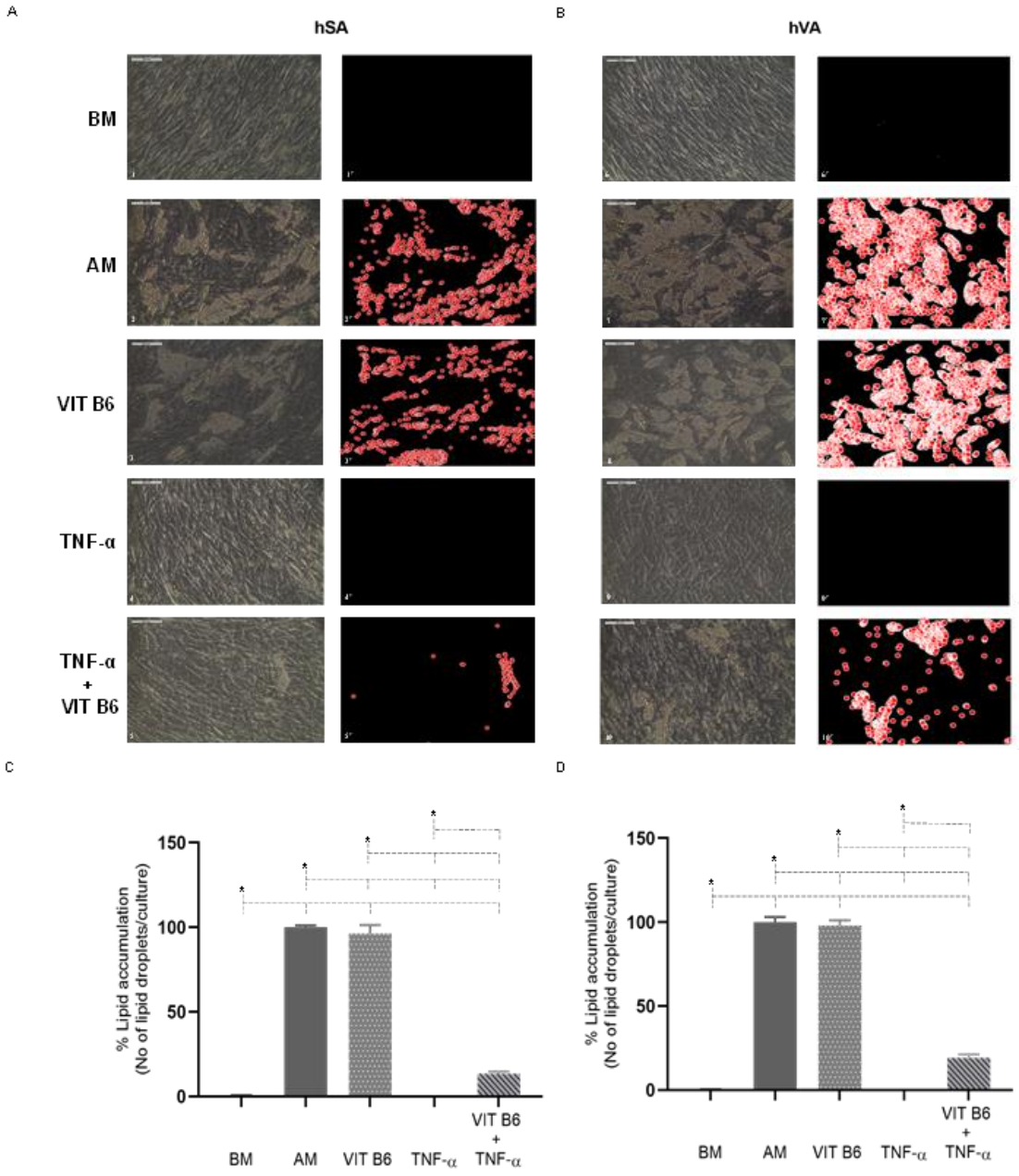

Figure 6. Effect of TNF-α and VIT B6 on hSA and hVA cells differentiation and MATLAB-based algorithm for counting lipid droplets

Human subcutaneous (hSA) and visceral (hVA) preadipocytes cultured in basal medium (BM) until confluence were induced with adipogenic medium (AM) in the absence or presence of 10 ng/mL TNF-α or 100 μM VIT B6. After 14 days postinduction, micrographs of cultures were processed through an algorithm generated in MATLAB (shown in red) to estimate the number of lipid droplets by treatment (C, D). Asterisks indicate statistically significant differences according to an ANOVA test between their respective treatments followed by a Tukey test ($p < 0.05$). BM, Basal Medium; AM; Adipogenic Medium. Adapted from Guzmán-Herrera et al., 2025.

3.5 Differential expression and metabolic correlation of *SERPINA3* according to adipose depot and obesity status

Finally, in order to delve into the physiological role of *SERPINA3* according to adipose depot and metabolic status, *SERPINA3* gene expression levels were analyzed in paired samples of subcutaneous and visceral adipose tissue corresponding to individuals with normal weight or obesity. The study population was composed of 8 patients with an average age of 49.12 ± 12.68 years, 2 men and 6 women with an average BMI of 23.92 ± 1.12 so they were classified as normal weight; and 11 patients with an average of 36.63 ± 6.65 years old, 2 men and 9 women with an average BMI of 40±7.42 classified as obese. RT-qPCR analyses showed that dividing the population with obesity according to class I, II, or III, and comparing it with the with normal weight group, *SERPINA3* expression is significantly higher in individuals with class I obesity compared with normal weight individuals. There were also significant differences between class I and class III obese individuals (Fig. 7A).

A more thorough exploration of the subcutaneous and visceral adipose tissue revealed that differences in *SERPINA3* expression were observed only within visceral adipose tissue. Contrary to our expectations, *SERPINA3* levels are significantly higher in visceral adipose tissue of normal-weight individuals than in obese individuals (Fig. 7B).

To shed light on the function of *SERPINA3* in metabolism, we investigated the correlation between *SERPINA3* expression and a diverse array of biochemical parameters in the study population. The correlation matrix between *SERPINA3* expression levels and biochemical parameters of individuals with normal weight and

obesity for each type of adipose depot indicated that in the normal weight group there is a significant negative correlation between *SERPINA3* expression and urea levels only in VAT. Likewise, a positive correlation was identified in the same adipose depot between the cholesterol/HDL ratio and *SERPINA3* expression. In the obesity subgroup, it was found that as *SERPINA3* expression increases, high-sensitivity C-reactive protein, cholesterol, and LDLc levels decrease significantly only in SAT (Fig. 7C). These data suggest that *SERPINA3* can play an elemental role not only in the metabolic processes of individuals with obesity but also in individuals with normal weight. Furthermore, its role could be differential depending on the type of adipose depot, SAT, or VAT.

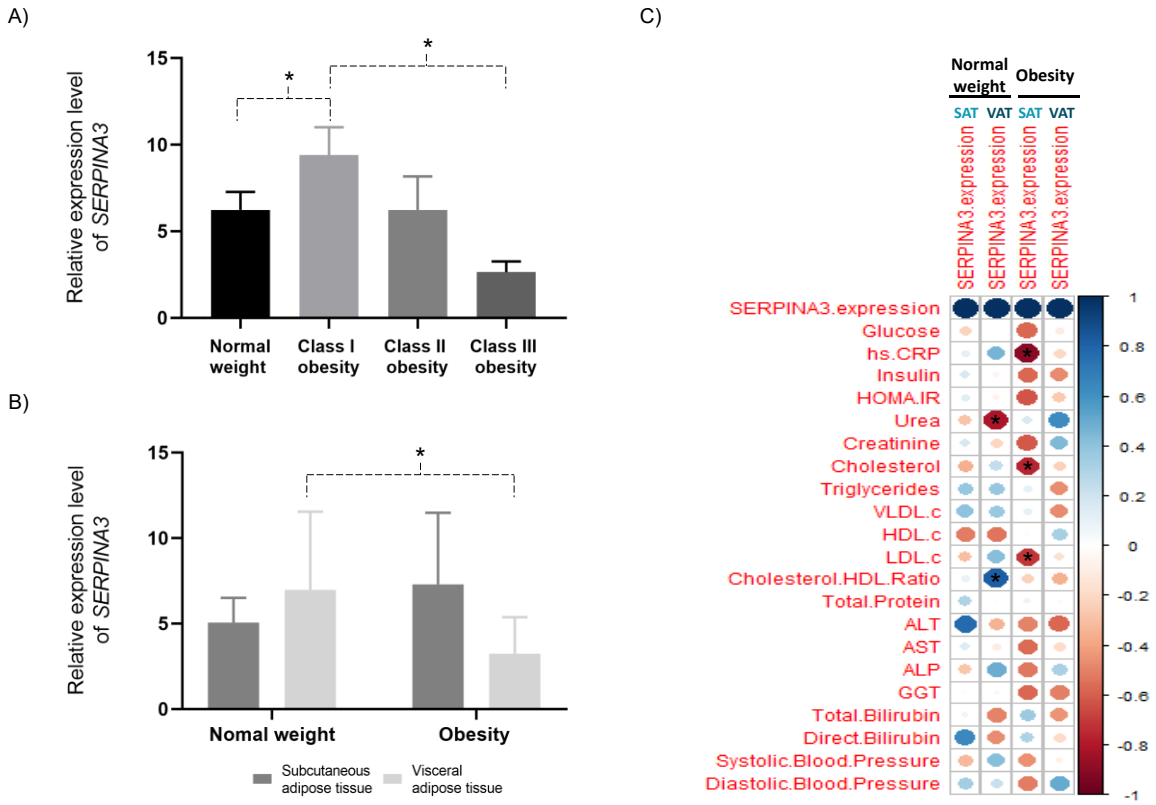

Figure 7. Analysis of *SERPINA3* expression in SC and VC adipose tissue samples and its correlation with biochemical parameters

Paired samples of subcutaneous and visceral adipose tissue from a population of patients with normal weight or obesity were processed by RT-qPCR to compare the expression of *SERPINA3* in both groups (A) and by type of adipose depot (B). Subsequently, correlation analyzes were carried out between the expression of *SERPINA3* and biochemical parameters obtained from the same study population (C). Asterisks represent statistically significant differences. Hs.CRP, High-Sensitivity C-reactive Protein; VLDL.c, Very-Low-Density Lipoprotein; HDL.c, High-Density Lipoprotein; LDL.c, Low-Density Lipoprotein Cholesterol; ALT, Alanine Transaminase; AST, Aspartate Aminotransferase; ALP, Alkaline Phosphatase; GGP, Gamma-Glutamyl Transferase.

4. General Discussion

Here we documented that Serpin A3, also known as alpha-1-antichymotrypsin, a serine protease inhibitor, is expressed in human subcutaneous and visceral adipose tissue and by freshly committed and mature cultured adipocytes derived from both depots. We also demonstrate that *SERPINA3* expression is induced by TNF-α at both mRNA and protein levels in committed and mature adipocytes, and that vitamin B6 abrogates this induction. Notably, the abrogation of *SERPINA3* induction by TNF-α due to vitamin B6 is accompanied by the partial reversal of the antiadipogenic effect of TNF-α on both adipose cell types.

Previous work from our laboratory showed that *SERPINA3G* gene expression in the cell 3T3-F442A murine cell line is induced by TNF-α and its silencing cancels the antiadipogenic and insulin resistance effects induced by the cytokine (Salazar-Olivo et al., 2014b). In concordance with this finding, recent work has documented that *SERPINA3G* upregulation inhibits lipogenesis in 3T3-L1 adipocytes (Lee et al., 2022).

However, as the *SERPINA3G* gene is not present in humans, the results obtained in murine cells could not be directly extrapolated to human adipose cells. Therefore, to gain an insight on the role of *SERPINA3* in human adipose tissue metabolism, we established primary cultures of subcutaneous (hSA) and visceral (hVA) human preadipocytes and tested on them the antiadipogenic effects of TNF-α, a proven inductor of *SERPINA3g* expression in murine cells, and RA, also an antiadipogenic agent which does not induce the *SERPINA3g* expression (Salazar-Olivo et al., 2014). TNF-α and RA significantly reduced the lipid accumulation in

these primary preadipocytes in agreement with previous results obtained on diverse adipose *in vitro* model systems (Campos et al., 2016; Castro-Muñozledo et al., 2003; Chae & Kwak, 2003; Jack et al., 2022).

Serpin A3 is a serine protease inhibitor encoded by the *SERPINA3* gene, a human ortholog of murine *SERPINA3g*. Serpin A3 inhibits various serine proteases including pancreatic chymotrypsin, mast cell chymase, staphopain C, and mainly cathepsin G (Baker et al., 2007; Wladyka et al., 2011). *SERPINA3* is highly expressed in the liver (Horvath et al., 2004), but its expression in adipose tissues had only been documented for epicardial fat (Zhao et al., 2020). We demonstrated by RT-PCR that *SERPINA3* gene is expressed in subcutaneous and visceral adipose tissue samples obtained from normal-weight donors. Despite being non-quantitative, the assay indicated higher SERPINA3 expression in visceral compared to subcutaneous samples, suggesting that *SERPINA3* could exhibit a differential expression in different human adipose depots, as has been documented for other genes and proteins (Mathur et al., 2022; Raajendiran et al., 2020). Our additional RT-qPCR and Western blot results showed that visceral adipocytes exhibit significantly higher *SERPINA3* mRNA and protein expression than subcutaneous adipocytes. Further studies are needed to address if such differential expression participates in the metabolic differences among adipose depots.

Our results also show that TNF-α induces *SERPINA3* overexpression in both cell types. These results are consistent with previous studies showing the ability of TNF-α to modulate SERPINA3 expression in both *in vitro* and *in vivo* models (Al-Daghri et al., 2017; Lannan et al., 2012; Machein et al., 1995).

It has been reported that 500 μM VIT B6 suppresses the expression of *SERPINA3* in rat colon and in HT-29 cells stimulated with TNF-a (Yanaka et al., 2011a). We observed that at this concentration VIT B6 affects the viability of human preadipocytes, while lower (100 μM and 250 μM) concentrations do not affect their viability. Treatment with 100 μM VIT B6 did not alter the differentiation of hSA and hVA preadipocytes into mature adipocytes as measured by lipid accumulation analysis through image processing. Our result contrasts with that of Yanaka et al. (2011b) that 100 μM vitamin B6 promotes adipogenesis in the 3T3-L1 adipocytes. This difference could respond to the different model systems used and emphasize the importance of using human experimental systems to compare the results obtained on non-human models.

The absence of an intrinsic adipogenic effect of VIT B6 on human preadipocytes correspond with our result showing that VIT B6 treatment decreases *SERPINA3* mRNA and protein expression in both hSA and hVA cells. This finding suggests a potential role for vitamin B6 in counteracting the anti-adipogenic effects of TNF-α, possibly by regulating molecular pathways involved in PPARγ-mediated target gene expression, as observed in 3T3-L1 adipocytes (Yanaka, et al., 2011b). Further studies are needed to elucidate the specific mechanisms underlying this interaction.

Notably, pyridoxal phosphate, the functional form of VIT B6 used in our experiments, was identified as a potential ligand for Serpin A3. The potential interplay between VIT B6 and *SERPINA3* that we described may hold significant implications for comprehending and treating metabolic disorders characterized by

dysregulated lipid metabolism.

Adipogenesis is a serpin-rich process, since numerous serine protease inhibitors participate in the regulation of adipose metabolism (Zvonic et al., 2007) by diverse and even contrasting mechanisms and effects. While the results reached on this issue have been obtained mostly in murine model systems, our results using primary human adipose cell cultures provide strong evidence for the relevance of these mechanisms to human physiology.

In relation to adipose tissue samples, we observed that *SERPINA3* expression is higher in individuals with class I obesity than in individuals with normal weight. This fact suggests that *SERPINA3* expression may be modulated by obesity severity. When comparing the expression according to the adipose depot, we observed a clear difference only in visceral adipose tissue where, interestingly, the expression of *SERPINA3* was statistically higher in the normal weight group; however, more detailed analyzes of *SERPINA3* expression with biochemical parameters showed a statistically significant correlation depending on the type of depot analyzed, suggesting that, as well as we observed *in vitro*, there could be a depot-specific regulation in *SERPINA3* expression in human adipose tissue. Despite that, it is necessary to continue studying the effects of *SERPINA3* expression in human adipose tissue through a greater number of samples in each group, as well as other molecular analyzes to explain the relationship of *SERPINA3* with biochemical parameters not only in individuals with obesity but also in individuals with normal weight.

5. Conclusions

Our findings show that SERPINA3 is expressed in human subcutaneous and visceral adipose tissue, and its expression is upregulated during adipogenesis by TNF-α in quiescent preadipocytes, freshly committed, and mature adipocytes.

Vitamin B6 completely abolishes *SERPINA3* overexpression in both types of adipose cells and partially reverses the antiadipogenic effect of TNF-α.

Our results suggest that Serpin A3 plays a significant role in adipose tissue metabolism and is a promising target in the inflammatory processes linked to obesity and other adipose dysfunctions.

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