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ARTICLE INFO

Keywords:
Hydroxynonenal
Lipids
Smoke
Cutaneous tissue

ABSTRACT

For its critical location, the skin represents the major interface between the body and the environment, therefore is one of the major biological barriers against the outdoor environmental stressors. Among the several oxidative environmental stressors, cigarette smoke (CS) has been associated with the development and worsening of many skin pathologies such as acne, dermatitis, delayed wound healing, aging and skin cancer. In our previous work we have demonstrated that CS is able to affect genes involved in skin cholesterol trafficking, among which SRB1, a receptor involved in the uptake of cholesterol from HDL, seems to be very susceptible to the oxidative stress induced by CS.

In the present work we wanted to investigate the presence of SRB1 in human sebocytes and whether CS can affect cholesterol cellular uptake via the redox modulation of SRB1.

By using a co-culture system of keratinocytes/sebocytes, we found that CS exposure induced a SRB1 protein loss without affecting sebocytes viability. The decrease of SRB1 levels was a consequence of SRB1/HNE adducts formation that leads to SRB1 ubiquitination and degradation. Moreover, the CS-induced loss of SRB1 induced an alteration of sebocytes lipid content, also demonstrated by cholesterol quantification in SRB1 siRNA experiments.

In conclusion, exposure to CS, induced SRB1 post-translational modifications in sebocytes and this might affect sebocytes/skin functionality.

1. Introduction

Several studies have demonstrated that more than 30% of United States population has been affected by cutaneous pathologies. Although most are not life-threatening, many skin diseases are able to influence life quality and represent a major cost for public health [1].

The tegumentary system is a complex organ that does not consists only in the cutaneous tissues, but comprises also its appendages, including glands, nails, hair. The main function of this organ system is to protect the body from the out-door insults including the environmental stressors.

Recent reports revealed that cigarette smoke (CS), ozone (O_3) and concentrated air particles (CAPs) can influence the skin physiology and its functions [2,3]. In the specific, CS has been shown to play a key role in diseases such as acne, dermatitis, delayed wound healing, aging and cancer [4–7].

CS is an heterogeneous mixture of gas, vapour and suspended solid particles, derived from the combustion of the cigarette itself and representing one of the most significant source of inhaled chemical pollutant. Among the several noxious effects, CS is able to induce cutaneous elastosis, upregulation of matrix metalloproteinases enzymes (MMPs 1-3) involved in the degradation of the connective tissues and to alter the transforming growth factor (TGF-β) pathway which is crucial for healthy wound closure [8,9].

CS noxious effects are mainly related to the reactive oxygen species (ROS) which are both present in the smoke and also induced by the interaction of CS components with biological systems. It has been demonstrated that CS leads to an oxidative stress condition in oral keratinocytes as shown by the increase of intracellular oxidants and by a significant decrease of GSH intracellular level [10]. An interesting

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study revealed that smoke is able to induce specific facial aging in identical twins with different smoking history [11].

As mentioned above, not only the skin participates to protect our body from the out-door stressors but also its appendages have an important defensive role, as it is for the sebaceous glands that have the ability to maintain the composition of the hydrolipidic film, a mixture of cholesterol, triglycerides, wax esters and squalene [14]. In addition, sebaceous glands represent the major deliver system of vitamin E to the cutaneous tissues, protecting skin from oxidative damage [12].

Indeed, sebocytes functions are more than the production of sebum and the passive formation of the cutaneous barrier; through many paracrine, endocrine and immunological mechanisms, sebaceous glands take part in many of the homeostatic physiological functions of the skin [11,14].

Sebocytes participate in the regulation of immunological functions and inflammatory processes for their ability to produce cytokines (IL-1β, IL-6, IL-8/CXCL-8, TNFα) and lipid inflammation mediators (5-LOX, LTA4 hydrolase, LTB4, PGE2), which have a key role in the pathogenesis of several inflammatory skin diseases (i.e. acne vulgaris) [14].

New etiologic models reveal that acne can develop without the colonization of pathogen microorganisms, rather can be due to other factors (androgen hormones, PPAR activation, SP-mediated stress response) that increase the production of inflammation mediators and induce hyperseborrhea [15–18]. Moreover, many subunits of cholinergic muscarinic and nicotinic receptors have been found in sebocytes at various grade of differentiation [19]. Thus it is fascinating to hypothesize that the activation of these receptors from neural or paracrine acetylcholine or from CS nicotine, can have a role in the pathogenesis of acne or other cutaneous diseases by affecting sebocytes functionality [19].

For several years our group has been studying the effects of CS on cutaneous tissue focusing the attention on Scavenger Receptor B1 (SRB1), a transmembrane receptor well known for the cholesterol uptake from high density lipoprotein (HDL) [20] and on its modulation by CS. We have demonstrated that in human keratinocytes the modification of proteins involved in cholesterol trafficking such as SRB1 and ABCA1 can lead to skin alteration [21,22]. Whereas sebaceous glands are dragged in physiologic homeostatic functions of the skin, we believe that SRB1 could have an important role also in sebocytes function although no data have yet demonstrated its presence in this particular secretory organ.

The aim of our study was to evaluate the presence of SRB1 in sebaceous glands and to investigate its susceptibility to CS-induced oxidative damage as well as its influence on cellular lipid uptake.

2. Methods

2.1. Cell culture

HaCaT cells (gift from Dr. F. Virgili) were grown in Dulbecco's modified Eagle's medium High Glucose (Lonza, Milan, Italy), supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine, as previously described [24].

SZ95 sebocytes were grown in Sebomed® (Biochrom, Berlin, Germany), supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 5 μg/L EGF (Biochrom). Cells were incubated at 37 °C for 24 hrs in 95% air/5% CO_2 until 80% confluency.

In order to reproduce cutaneous tissue closely to the real system in vitro, SZ95 sebocytes (1×10^6 viable cells/mL) and HaCaT cells (1×10^5 viable cells/mL) were co-cultured in 6 well plates (Falcon®), where HaCaT were seeded on the top of the Transwell containing a PET membrane 0,4 μm pores (BD Falcon TM). The cells were observed under an inverted microscope until co-culture cells reached a 80% density.

2.2. CS exposure and treatments

Prior to CS exposure of the co-culture cells, the culture medium was aspirated and fresh serum-free medium was added. In order to only expose HaCaT cells to CS, home made Teflon lid was put over the cell culture plates in the way that CS was only able to interact with keratinocytes and not directly with sebocytes. This allowed us to expose the cells for 50 min to CS. Control cells were exposed to filtered air for the same duration (50 min) after changing media.

The time and the way of exposure were chosen based on our previously published results [20,23,24] and no significant difference in the cell viability, as measured by Trypan blue exclusion, was detected between control (air) and CS treatment (data not shown). HaCaT and SZ95 cells were exposed to fresh CS in an exposure system that generated CS by burning two 3R4F Kentucky research cigarette (University of Kentucky, Kentucky, USA) (12 mg tar, 1.1 mg nicotine) using a vacuum pump to draw air through the burning cigarette and leading the smoke stream over the cell cultures as described previously by our group [24]. After the exposure (air or CS), fresh media supplemented with 10% FBS was added to the cells.

For proteasome inhibition experiment, SZ95 sebocytes were pre-treated (2 h) with MG-132 (Calbiochem, La Jolla, CA) before CS exposure with or without HDL treatment (Sigma - Aldrich®).

After treatments, cells were collected by centrifugation for the assays described below.

2.3. Cellular viability

Cell viability studies were performed by cytofluorimetric and LDH assays. The cytofluorimetric assay was performed by using the Muse Count & Viability Kit (Millipore, Corporation, Billerica, MA, USA) [25]. The amounts of LDH in the supernatant were determined and calculated according to the manufacture's instructions (EuroClone Milan, Italy), as previously described [26].

2.4. Western blot analysis

Total cell lysates were extracted in RIPA buffer containing protease and phosphatase inhibitor cocktails (Sigma–Aldrich Corp.), as described before [20]. Cells were harvested by centrifugation and protein concentration was determined by the method of Bradford (Biorad Protein assay, Milan, Italy). Samples, 30 μg of proteins, were loaded into 10% sodium dodecyl sulphate– polyacrylamide electrophoresis gels and separated by molecular size. The gels were then electro-blotted onto nitrocellulose, as previously described [27]. Membranes were incubated overnight at 4 °C with rabbit polyclonal SRB1 antibody (1:1000) (Novus Biologicals, Inc.; Littleton, CO) or rabbit polyclonal β-actin (1:1000) (Cell Signaling; Celbio, Milan, Italy). After incubation with secondary goat anti-rabbit IgG (H+L)-HRP conjugate antibody (1:10000) (BioRad, Milan, Italy), the bound antibodies were detected using chemiluminescence (BioRad, Milan, Italy). Images of the bands were digitized and the densitometry of the bands was performed using the Image-J software.

2.5. Quantitative real-time PCR

Quantitative real-time PCR was carried out as described in detail previously [26]. Briefly, total RNA was extracted, using an AURUM total RNA Mini Kit with DNase digestion (Bio-Rad), from 1×10^6 sebocytes for each experimental condition, according to the manufacturer's instructions. First-strand cDNA was generated from 1 μg of total RNA using the iScript cDNA Synthesis Kit (Bio-Rad). The primer pairs (Table 1) capable of hybridization with unique regions of the appropriate gene sequence were obtained from the Real-Time PCR GenBank Primer and Probe Database Primer Bank, RTPrimerDB.

Table 1
Primers sequencing for housekeeping and SRB1 gene, with amplified size.

RPL13A	F : 5'- ccbaggatg agcgcaagttga a- 3' R : 5'- ccacaggactagaa ca actgctta a- 3'	60.2	203	97.3	39	[48]
RPL11A	F : 5'- tgcgggaactt gcat cgc -3' R : 5'- ggg tat gcactgt gagctgc-3'	60.1	108	96.5	39	GenBank Accession NM 000975. 2
GAPDH	F : 5'- tg aag ct gggg-tggcatt g -3 R : 5'- ggctgtg gtccaggggtct -3'	60	134	94.6	39	GenBank Accession NM 002046. 3
SRB1	F : 5'- aatctgtcgcaggcat tggs -3' R : 5'- cagg aattt ggctcag gatt -3'	60.2	225	97.5	39	GenBank Accession NM 01082959.1

2.6. Immunocytochemistry

SZ95 sebocytes were maintained on coverslips at a density of $1{,}6\times10^5$ cell/mL, and after CS exposure fixed in 4% paraformaldehyde for 30 min at 4 °C, as previously described [21]. After permeation and block in BSA 5%, cells were incubated for 1 h with the following primary antibodies: rabbit polyclonal SRB1 antibody (1:100) (Novus Biologicals, Inc.); goat polyclonal 4HNE antibody (1:100) (Merck Millipore, Milano, Italy); mouse polyclonal Ubiquitin antibody (1:100) (Cell Signaling). After washing, coverslips were incubated with the fluorochrome-conjugated secondary antibodies: anti-rabbit Alexa Fluor 488 (1:100); anti-goat Alexa Fluor 568 (1:100); anti-mouse Alexa Fluor 568 (1:100) (Thermo Fisher Scientific Inc., Monza, Italy) for 1 h at room temperature in the dark. Nuclei were stained with 1 mg/mL DAPI (Molecular Probes) for 1 min after removal of secondary antibodies. Coverslips were mounted onto glass slides using anti-fade mounting medium 1,4 diazabicyclooctane in glycerine (DABCO) and examined by the Leica light microscope AF CTR6500HS (Microsystems) equipped with epifluorescence at 40x magnification. Negative controls for immunostaining experiments were performed by omitting primary antibodies. The quantification was performed using ImageJ software.

2.7. Oil Red O staining

SZ95 sebocytes were seeded on coverslips, exposed to CS and after 6 hrs incubated with 50 and 100 μg/mL HDL for 12–24 h. After changing media and washing with PBS, SZ95 sebocytes were fixed with 4% paraformaldehyde for 30 min at room temperature, washed with H_2O and 60% isopropanol. Fixed cells were stained with freshly prepared Oil Red O solution for 10 min. Extra stain was remove, samples were washed with distilled water and 60% isopropanol and incubated with Hematoxylin. Coverslips were mounted onto glass slides using glycerol-water 9:1 and than images were acquired with a Leica AF CTR6500HS microscope (Microsystems). The quantification was performed using Image-J software.

2.8. SRB1 siRNA transfection

For siRNA studies, SZ95 sebocytes were transfected with SRB1 siRNA according to the manufactured protocol (Ambion®, USA). Briefly, $1{,}6\times10^5$ cells/mL were transfected with solution containing 15 pmol SRB1 siRNA and 2 μL Lipofectamine® transfection agent. After 24 hrs the cells were treated with HDL and than fixed for Oil Red O staining at the time points previously described.

2.9. Total cellular cholesterol determination

After treatments, total cholesterol levels in sebocytes were determined using a commercially available assay kit (Cell Biolabs, Inc. San Diego, CA, USA), following manufacturer's instructions. This is a fluorometric assay, which involves the extraction of total cholesterol with chloroform/isopropanol/NP-40. Briefly, 50 μl of extracted sample, diluted 1:50 in 1X assay diluent, were loaded into a 96-well plate in triplicate and then treated with a cholesterol reaction reagent that includes cholesterol esterase, cholesterol oxidase, horseradish peroxidase and a fluorescence probe. After an incubation of 45 min at 37°C, the fluorescence signal was read at 530 nm (excitation) and 590 nm (emission) with a microplate reader (TECAN – infinite M200). Sample cholesterol concentrations were determined by interpolation from a standard curve. For normalization, the protein concentration was determined by Bradford method and the data are presented as μg of cholesterol per mg of protein in cell extracts.

2.10. Immunoprecipitation of SRB1

The antibody for SRB1 (5 μg) (Thermo Fisher Scientific Inc., Waltham, MA USA) was pre-coupled to 50 μL of magnetic Dynabeads Protein G (Novex, Life Technologies). Excess antibody was washed by placing the tube on a DynaMag™ magnet and removing the supernatant. Then, cell protein extracts (500 μg) were incubated with the antibody-coated beads for 10 min at RT. After washing, the immunocomplexes were mixed with reducing sample buffer, boiled and analyzed by SDS/PAGE and immunoblotting with 4HNE antibody (Millipore Corporation, Billerica, MA, USA).

2.11. Immunohistochemistry

Human skin tissues for research purposes were collected from patients whose informed consent was obtained in writing according to the policies of the Ethics Committee of the European Institute of Oncology and regulations of Italian Ministry of Health.

The tissues were fixed in 10% NBF (neutral-buffered formalin) for 24 hrs at room temperature. Sections (4 μm) were deparaffinized in xylene and rehydrated in alcohol gradients. After dewaxing, sections were incubated overnight at 4 °C with anti-SRB1 (Novus Biologicals, Inc.; Littleton, CO). Then slides were washed three times with PBS and endogenous peroxidase was blocked with 3% hydrogen peroxide in absolute methyl alcohol for 30 min at room temperature. Finally, the slides were incubated with EnVision+System-HRP (DAKO, Glostrup, Denmark) for 45 min at room temperature. The reaction products were stained with diaminobenzidine (DAB), counterstained with Mayer's Hematoxylin and after drying were mounted with Eukitt mounting medium.

2.12. Statistical analysis

For each of the variables tested, two-way analysis of variance (ANOVA) was used. A significant effect was indicated by a P-value <0.05. Data are expressed as mean ± S.D. of triplicate determinations obtained in 5 independent experiments.

3. Results

3.1. SRB1 expression in human sebaceous glands

To evaluate the presence of SRB1 in sebaceous glands, immunohistochemistry analysis was performed in samples from human skin biopsies. As shown in Fig. 1, a strong SRB1 cytoplasmic positivity was observed in sebaceous glands. Moreover SRB1 presence was further confirmed also in basal keratinocytes, as previously reported by our group [22].

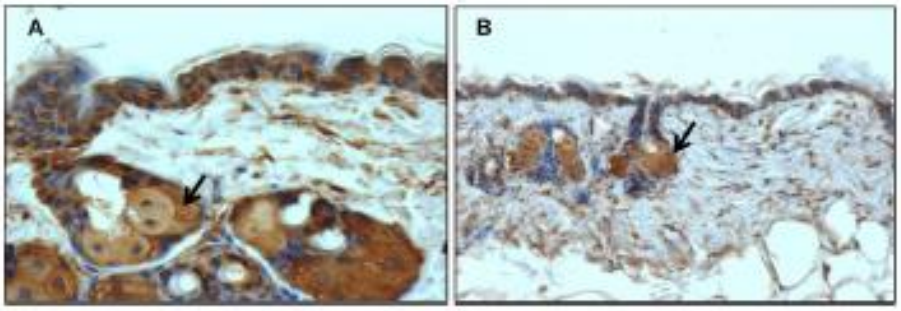

Fig. 1. Immunohistochemistry of human skin biopsies. The anti SRB1 antibody stains the cytoplasm of the cells of sebaceous glands in normal human skin biopsies. DAB, Original Magnification ×400 (A) and ×200 (B).

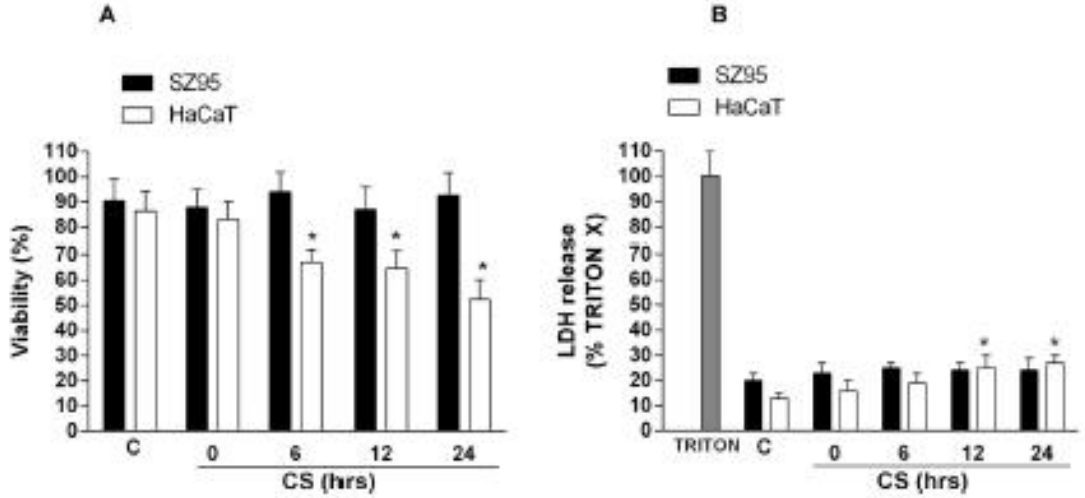

Fig. 2. Keratinocytes/sebocytes viability after CS exposure. The cytotoxicity of the two different cell lines was measured by cytofluorimetric assay (A) and by LDH assay (B). Cells treated with TritonX represent 100% of cell death (100% of LDH release from the cells). Data are expressed as mean ± SD from five independent experiments *p <0.05.

3.2. CS exposure did not affect sebocytes viability

As shown in Fig. 2A, CS was able to decrease keratinocytes viability after 6 hrs of CS exposure in co-culture system where only HaCaT where directly exposed to CS. The viability dropped by 40% in keratinocytes after 24 hrs CS exposure. On the other hand, CS did not affects sebocytes viability. These data were confirmed by the LDH assay, and only keratinocytes showed an increased of LDH release at 12 and 24 h after CS exposure (Fig. 2B).

3.3. CS exposure decreased SRB1 protein levels in sebocytes

Next, we evaluated whether SRB1 protein level was affected by CS exposure in SZ95 sebocytes. As shown in Fig. 3, SRB1 protein levels decreased 12 h after CS exposure, reaching the maximum decrease at 24 h time point (reduction of 75% compared with control). SRB1 protein decrease was not a consequence of sebocytes death since their viability was not affected by CS (see Fig. 2).

3.4. CS exposure increased SRB1 gene expression

To investigate whether the decrease of SRB1 protein was a consequence of the transcriptional modulation, SRB1 gene expression was determined. Fig. 4 shows a time dependent increase of SRB1 mRNA level starting 6 hrs after CS exposure (20%). This effect was even more enhanced at the later time points (12 and 24 hrs) with circa 2 fold increase.

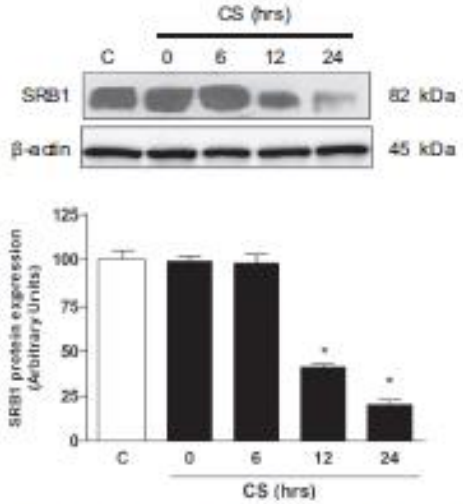

Fig. 3. SRB1 protein expression after CS exposure in human sebocytes. Cells were exposed to CS for 50 min, then harvested at different time points (0–24 h) and the protein expression was measured by Western blot. Representative Western blot of five different experiments is depicted in the top panel. Quantification of the SRB1 bands is shown in the bottom panel. Data are expressed as mean ± SD from five independent experiments *p <0.05. β-actin was used as a loading control.

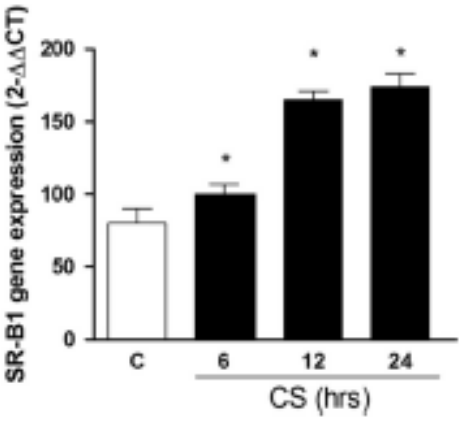

Fig. 4. SRB1 gene expression after CS exposure in human sebocytes. mRNA was extracted from SZ95 sebocytes after CS exposure and the gene expression was measured by Real Time PCR. Data are expressed in $\%2^{-\Delta\Delta CT}$, in arbitrary units as mean ± SD from five independent experiments *p <0.05.

3.5. CS exposure induced HNE/SRB1 adducts

Many toxic effects of CS can be linked to the generation of aldehydes, such as 4-hydroxynonenal (HNE). Therefore, we have evaluated HNE protein adducts levels in sebocytes exposed to CS by using immunocytochemical analysis. As shown in Fig. 5, there was an increase of HNE protein adducts levels under CS exposure (visualized by red stain). This increase was already evident 6 hrs after the exposure to CS. Then, we evaluated whether the formation of HNE protein adducts induced by CS could affect SRB1. As shown in Fig. 5, after CS exposure, the levels of HNE increased dramatically (red color, left column) with a concomitant decrease of SRB1 (green color, central column). The co-localization (yellow) appreciable in the right column showed the presence of HNE adducts on SRB1. We also confirmed their interaction using immunoprecipitation assay, as it is shown in the right panel of Fig. 5. The levels of HNE adducts in the immunoprecipitated SRB1 increased significantly after CS exposure (3 fold) and even further at 6 hrs time point (4 fold) (Fig. 5).

3.6. CS exposure induced Ubiquitin/SRB1 adducts and SRB1 degradation via proteasome

A consequence of a cellular oxidative damage is the activation of the proteasome machinery to remove oxidized damaged proteins. Therefore we evaluated whether CS exposure induced ubiquitination in SZ95 sebocytes. Immunocytochemistry (Fig. 6A) showed an increase of Ubiquitin (Ub) expression (red color, left column) and a decrease of SRB1 (green color, central column) after CS exposure. The co-localization (yellow) appreciable in the right column showed the presence of Ubiquitin and SRB1 (right column yellow), suggesting that CS is able to induce the formation of Ub/SRB1 adducts. To confirm the proteasome involvement in SRB1 degradation after CS exposure, we next treated sebocytes with the proteasome inhibitor MG-132. CS exposed cells in the presence of MG132 did not show changes in SRB1 levels (Fig. 6B).

3.7. CS exposure decreased lipid content

Since one of the most important role of SRB1 is cholesterol uptake by HDL, we investigated whether the decrease in SRB1 protein levels by CS exposure could have any effect on lipid content in sebocytes. As shown in Fig. 7A, when cells were treated with different concentrations of HDL (50 and 100 μg/mL), their lipid content decreased by 20% after 12 and 24 h CS exposure. This result was also confirmed by the determination of intracellular cholesterol levels using a more sensitive assay (Fig. 7B).

As a proof of concept, we performed SRB1 silencing experiments in sebocytes. As shown in Fig. 8A and Fig. 8B, SZ95 sebocytes knocked out for SRB1 and treated with HDL showed a significant decreased (circa 20%) of lipid content, indicating a role of this receptor on cellular lipid uptake.

4. Discussion

The present study represents the continuation of our previous work [22] in which we have analyzed the mechanisms involved in SRB1 modulation in human keratinocytes by CS. We wanted also to extend this study to sebocytes, since these cells play a key role in protecting the skin from the outdoor stressors by their ability to produce lipids incorporated to sebum and also to maintain skin in its physiological healthy conditions. In addition, SRB1 is involved in cholesterol cellular trafficking and, therefore, its exogenous and endogenous modulation in sebocytes is of importance for skin health.

SRB1 in sebaceous glands was not yet investigated and this is the first work detecting its presence in sebocytes. Certainly, the first step of our study was to localize this receptor in human skin with focus on sebocytes. The presence of SRB1 in sebaceous glands is in line with the suggestion by Thiele et al. [12] that this organ is the main responsible for vitamin E secretion in skin and indeed, among the several functions related to SRB1, its involvement in lipophilic antioxidant trafficking, such as Vitamin E and A, has also been demonstrated [28].

CS contains circa 5000 toxic and carcinogenic compounds (carbon monoxide, benzene, acetaldehyde, methanol, hydrocyanic acid, ammonium, acrolein, formaldehyde, tar, vinyl chloride, 2-naphthylamine, nicotine) [29] and its noxious effect is not limited to the respiratory and cardiovascular systems, but it is now well documented that also cutaneous tissues are a target of its adverse effects [30,31]. Because of its location, cutaneous tissue is directly exposed to CS during expiration, and indirectly, through the absorption of its toxic compounds from respiratory epithelium that can then reach the skin by blood flow. CS exposure has a fundamental impact on cutaneous aging, leading to the so called "smokers face", which underline the particular features including, but not limited to, darker skin color (index of toxicity), deep wrinkles and gauntness with prominence of the underlying bony contours [4].

CS exposure leads, from one side, to the production of free radicals with the consequent oxidative biological tissue damage [2,32,33], and from the other side, induced the decrease of cutaneous antioxidant concentration such α-tocopherol [34] and the alteration of lipid film composition in which human keratinocytes are immersed [21,22,34]. Among several lipid components of the skin, cholesterol is essential for cell membranes and normal skin functions [22,35]. SRB1 is one of the main players that are involved in the selective uptake of tissues cholesteryl ester (CE) [36] and its presence in both keratinocytes and sebocytes makes this receptor as one of the main skin cholesterol players.

Sebocytes are localized in the dermis forming one of the ancillary skin organs, the sebaceous gland. Recent studies revealed that sebaceous glands are not only a passive "memento" with the unique function to produce sebum, but they are also responsible for the physical-chemical barrier function against the out-door environment, contributing to the skin protection against external insults and acting as the "brain" of the skin at the neuronal-immune-endocrine level [19].

To mimic the cutaneous morphology, characterized by the epidermal keratinocytes in contact with the underlying dermis, where sebaceous glands are localized, our studies were performed in keratinocytes/sebocytes co-culture. SRB1, as previously demonstrated [22], is highly expressed in human epidermis, and for the first time we also demonstrated here its presence in sebaceous glands. CS exposure induced a decrease of keratinocyte viability in a time dependent manner, in contrast sebocytes viability was not affected by CS. This

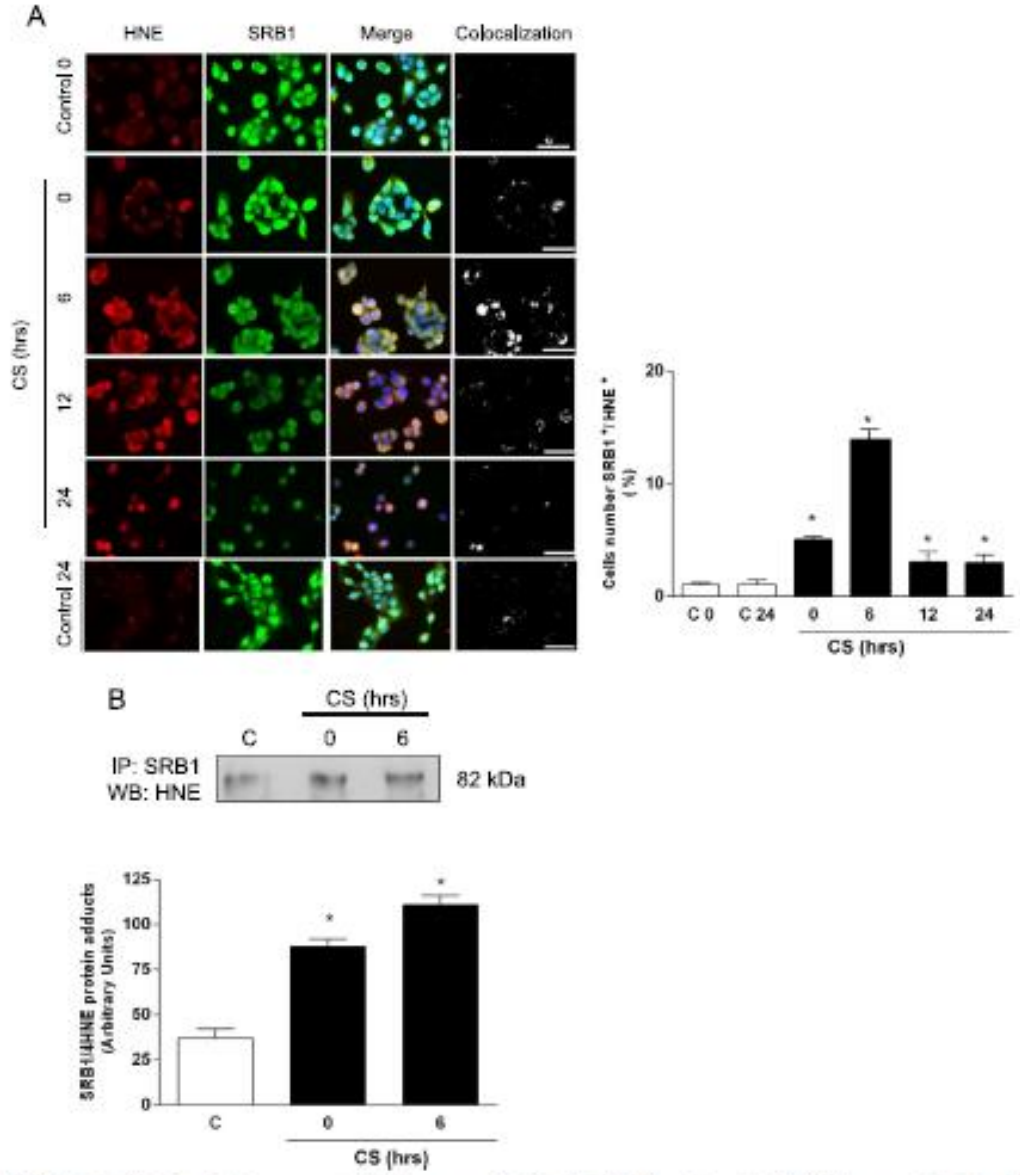

Fig. 5. A. HNE/SRB1 protein adducts after sebocytes exposure to CS. Cells were exposed to CS and fixed at different time points (0–24 h). Immunocytochemistry of SZ95 sebocytes shows localization of HNE–adducts (left column, red color), SRB1 (central column, green color) and HNE/SRB1 adducts (right column, yellow color). Images are merged in the right panel and the yellow color indicates overlap of the staining. Quantification is shown in the right panel. Data are expressed as mean ± SD from five independent experiments: *p <0.05. B. HNE/SRB1 protein adducts after sebocytes exposure to CS. Cells were exposed to CS, harvested at different time points (0–6 h) and SRB1 was immunoprecipitated. Western blot shows increased levels of SRB1/HNE adducts after CS exposure. Quantification of SRB1/HNE adducts is shown in the bottom panel. Data are expressed as mean ± SD from five independent experiments: *p <0.05.

finding was not surprising because only keratinocytes were directly exposed to CS, whereas sebocytes were covered and isolated by a home made Teflon lid. Therefore, it is possible to hypothesize that CS exposure is able to trig a cascade of effects from the keratinocytes to the cells localized in the deeper layer of the skin, including the sebocytes; this effect is comparable to that of ozone exposure that can affect target organs, like skin and lung, not directly, but via the generation of oxidative lipids that are present in the *stratum corneum* and in the lining fluid, respectively. These bioactive compounds can themselves trig a series of events including the release of proinflammatory mediators [37], the activation of endogenous ROS production (ie. NADPH oxidases [22]), and the modulation of transcriptional factors [37].

The decreased sebocytes SRB1 levels by CS are mainly due to post-transcriptional oxidative modifications. Indeed, our data showed that there was a significant increase in SRB1 mRNA expression and this can be a consequence of the protein loss, that can induce a positive feedback at the gene transcription levels, aiming to replenish the decrease protein.

CS is one of the most toxic insults to which skin can be exposed and it is surprising that passive smoking can even be more toxic than active smoking, precisely for its chemical composition [38]. CS can affect

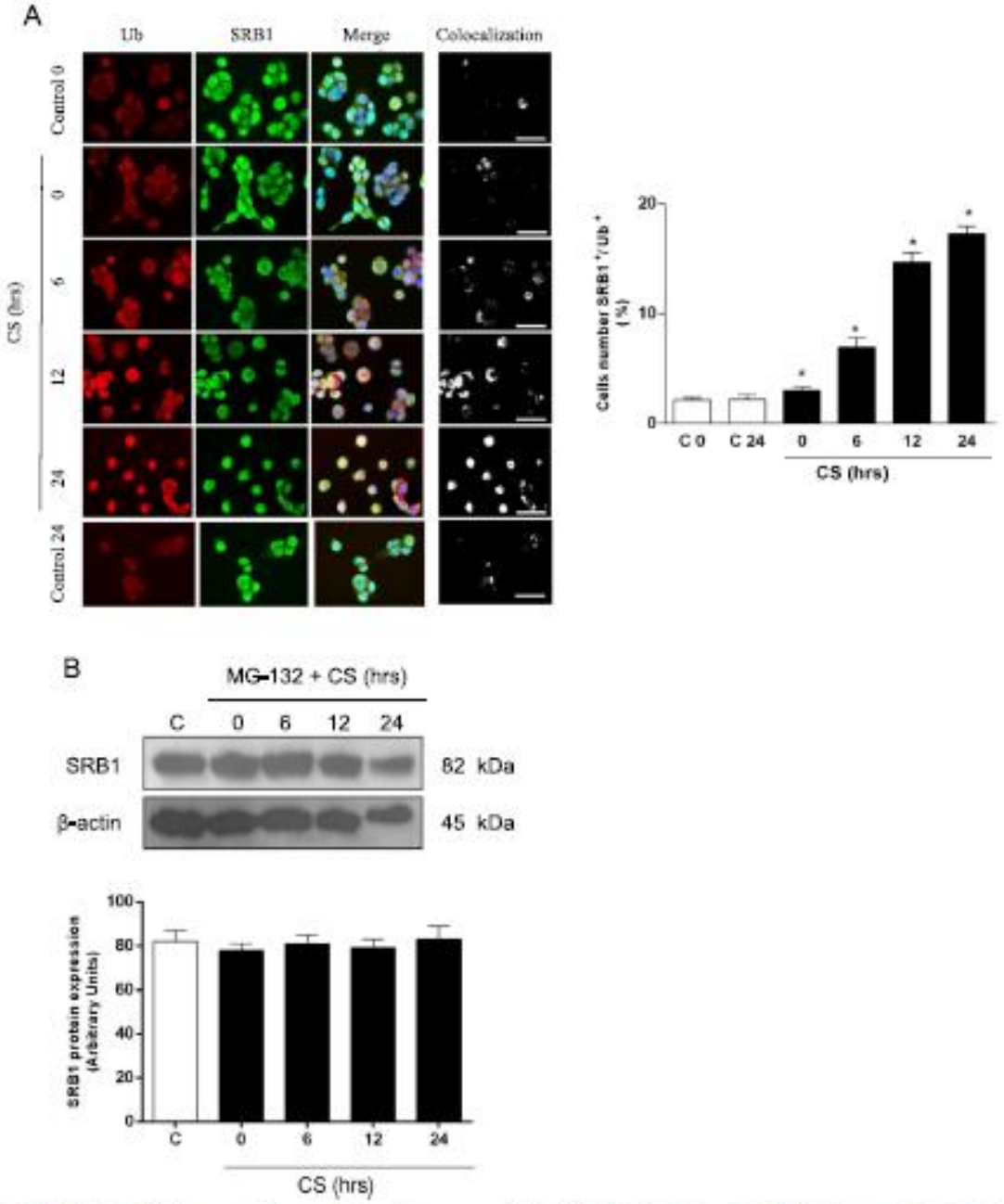

Fig. 6. A. Ubiquitin/SRB1 adducts after CS exposure in human sebocytes. Cells were exposed to CS and fixed at different time points (0–24 h). Immunocytochemistry of SZ95 sebocytes showing localization of UB–adducts (left column, red color), SRB1 (central column, green color) and UB/SRB1 adducts (right column, yellow color). Images are merged in the right panel and the yellow color indicates overlap of the staining. Quantification is shown in the right panel. Data are expressed as mean ± SD from five independent experiments *p < 0.05. B. SRB1 protein expression after proteasome inhibition and CS exposure in human sebocytes. Cells were pretreated with MG-132 (proteasome inhibitor), exposed to CS for 50 min, harvested at different time points (0–24 h) and protein expression was measured by Western blot. The Western blot data shown in the top is representative of five different experiments. Quantification of the SRB1 bands is shown in the bottom panel. Data are expressed as mean ± SD from five independent experiments *p < 0.05. β-actin was used as loading control.

tissues by its ability to induce cellular redox imbalance that leads to membrane peroxidation and formation of toxic molecules, such as highly reactive α,β-unsaturated aldehydes of which HNE is one of the most reactive ones. This aldehyde is able to form covalent protein binding with aminoacid residues, such as lysine, histidine and cysteine, leading to the alteration of protein function [39]. In our experimental procedure, CS was able not only to induce an increase of HNE protein adducts levels, but also a co-localization between HNE and SRB1 was detected, showing that SRB1 can be one of the protein targets of this aldehyde. In addition, the increased levels of HNE protein adducts has been correlated with both skin aging and inflammation [40].

During an oxidative event, cells can eliminate damaging or oxidized proteins by the proteasome machinery [41], as a consequence of protein ubiquitination, a post-translational modification that consists in covalent binding of ubiquitin to the protein target. We found an increased ubiquitin expression after CS in sebocytes, that co-localized

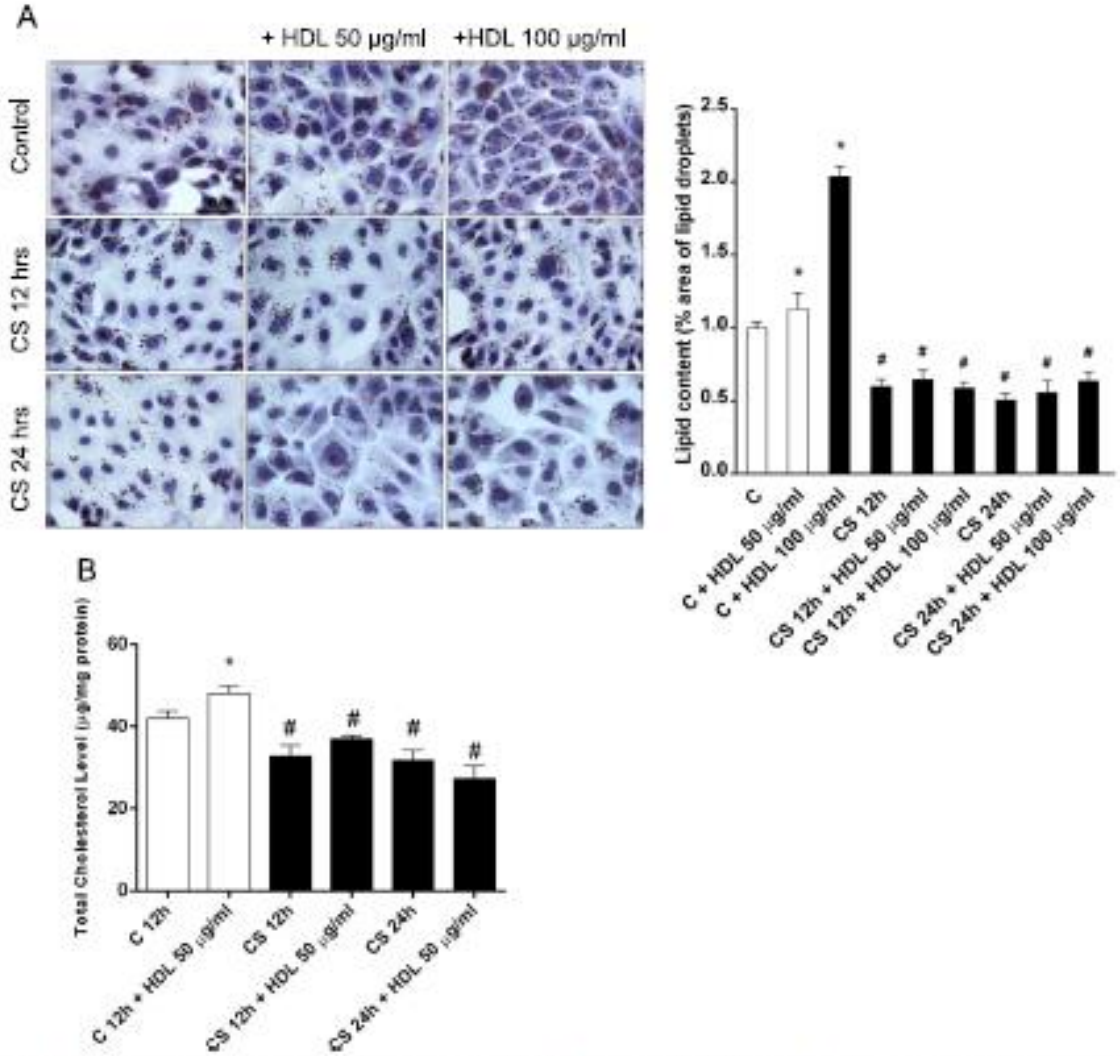

Fig. 7. A. Sebocyte lipid content after CS exposure. Images on the left represented the Oil Red staining which shows lipid drops into human sebocytes after the treatment with HDL and CS exposure. Scale Bar, 50 µm. Quantification of the cholesterol uptake HDL-mediated is shown in the right panel. Values are the area of the lipid drops (%). Data are expressed as mean ± SD from five independent experiments; *p<0.05 vs C; # vs the respective C w or w/o HDL. B. Sebocytes total cholesterol level after CS exposure. Cells were exposed to CS, treated with HDL and then total cholesterol levels were measured into human sebocytes at different time points. Data are normalized with protein concentration and expressed in µg/mg protein, as mean ± SD from five independent experiments; *p<0.05 vs C 12 h; # vs C 12 h w or w/o HDL.

with SRB1, suggesting that CS is able to induce SRB1 ubiquitination. The involvement of the proteasome machinery in the modulation of SRB1 protein levels was confirmed by the use of the proteasome inhibitor MG132, which was able to prevent SRB1 loss. This effect is in line with the one induced by CS in human keratinocytes, suggesting that both epithelial cell types, sebocytes and keratinocytes, respond similarly to CS insults regarding SRB1 levels. Therefore, CS exposure might decrease SRB1 levels in several skin cells promoting a more intensive and disseminate effect in the skin.

The loss of SRB1 can affect the ability of cells to uptake lipids and, as suggested, also vitamin E [28]. Indeed, SRB1 KO animals have shown to have very large HDL particles, increased plasma levels of cholesterol and very low levels of tissue tocopherol [42].

Our results confirm the role of SRB1 on cellular lipid uptake. In fact sebocytes exposed to CS and then treated with several doses of HDL, showed impairment in lipid cellular uptake that can consequently affect the skin barrier functionality.

This study suggests a possible role of SRB1 in acne pathogenesis induced by CS. The literature, has reported a role of CS in acne development [7]. For instance, an interesting study highlighted the connection between smoking and post-pubertal non inflammatory acne (APAA) and the increase in sebum peroxidation as well as the decrease of vitamin E [43]. Among the numerous compounds present in CS there are also the polycyclic aromatic hydrocarbons (PAHs), several of which have been identified to have chloracnegenic potential [44,45]. Shelley and Kligman showed a localized acne in healthy subjects with daily topical application of penta- and hexachloronaphthalene, further demonstrating the inherently acnegenic potential of these organic compounds [46]. Among its several functions, SRB1 plays a role in bacterial recognition [26,47]. Therefore, it is possible that the loss of SRB1 in sebocytes could lead to the alteration of the sebaceous gland lipid composition, impairment in skin bacterial recognition and decreased antioxidants (vitamin E) levels. Together with the increased oxidative stress induced by CS, skin can become more susceptible to damage and infections.

In conclusion, we provided evidences for sebocytes being a CS target affecting cholesterol uptake from HDL due to the loss of SRB1 protein via post-translational modifications, leading to the alteration of sebum lipid composition and to the impairment of protective sebum function against oxidative environmental insults, such as CS. Although SRB1 is not the unique protein involved in the cholesterol pathway, these results represent a new direction towards a better understanding

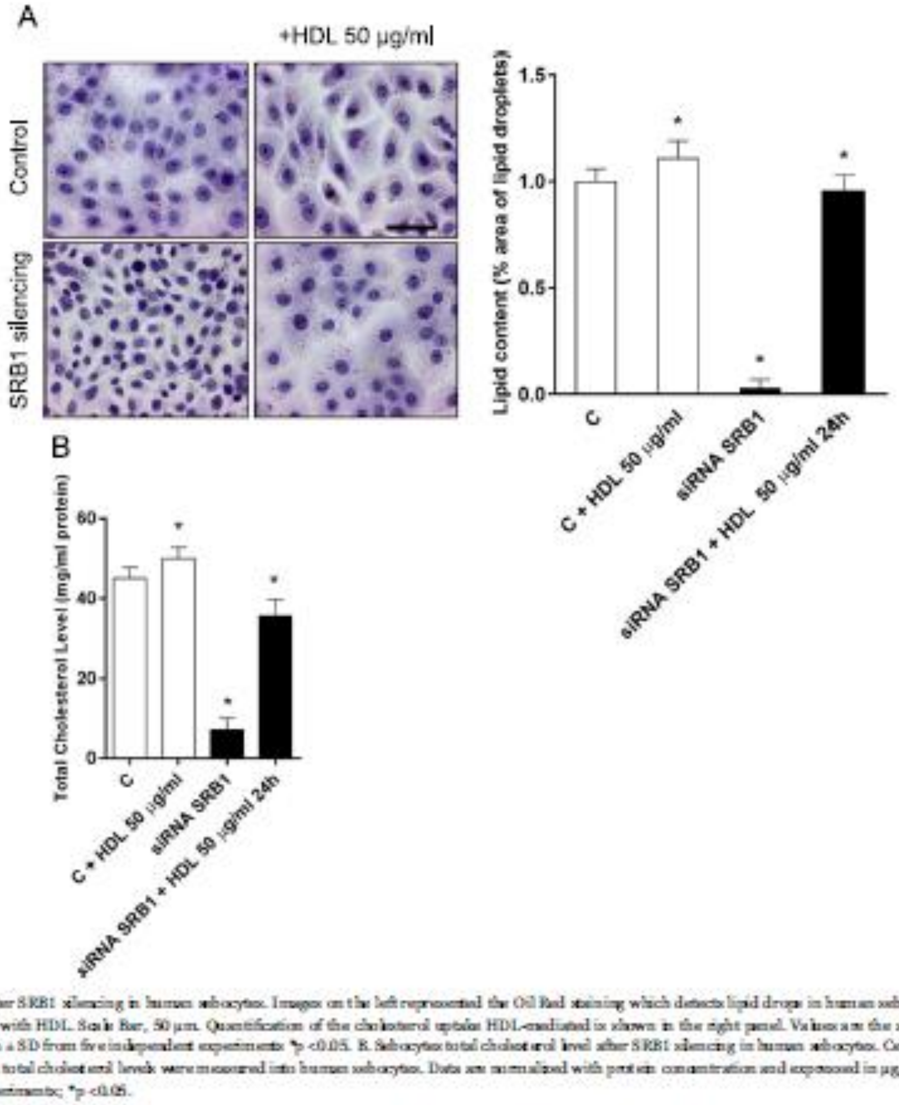

Fig. 8. A. Lipid content after SRB1 silencing in human sebocytes. Images on the left represented the Oil Red staining which detects lipid drops in human sebocytes. Cells were silenced for SRB1 and then treated with HDL. Scale Bar, 50 µm. Quantification of the cholesterol uptake HDL-mediated is shown in the right panel. Values are the area of the lipid drops (%). Data are expressed as mean ± SD from five independent experiments; $^{*}p < 0.05$. B. Sebocytes total cholesterol level after SRB1 silencing in human sebocytes. Cells were silenced for SRB1, treated with HDL and then total cholesterol levels were measured into human sebocytes. Data are normalized with protein concentration and expressed in µg/mg protein, as mean ± SD from five independent experiments; $^{*}p < 0.05$.

of the molecular modifications affecting sebocytes that occur in pathologies linked to CS exposure and lipid profile alterations.

Acknowledgments

The authors would like to thank Dr. Alessandro Trentini, Dr. Angela Pignatelli and Mr. Andrea Margutti for technical support.

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