Intrinsic versus Extrinsic Aging: A Histopathological, Morphometric and Immunohistochemical Study of Estrogen Receptor β and Androgen Receptor

Iman Seleit a  Ola Ahmed Bakry a  Hala Saed El Repey b  Raghdaa Ali a

Departments of a Dermatology, Andrology and STDs and b Pathology, Faculty of Medicine, Menoufiya University, Shibin El Koom, Egypt

Key Words
Estrogen receptor β · Androgen receptor · Skin aging · Immunohistochemistry

Abstract
Skin is a target organ of sex steroids which play important roles in skin health and disease. The aim of this study is to investigate the expression of estrogen receptor β (ERβ) and androgen receptor (AR) in human skin from different age groups for a better understanding of the hormonal regulation of skin aging. Using standard immunohistochemical techniques, biopsies of sun-unprotected and sun-protected skin were taken from 60 normal subjects. Sun-protected skin showed significantly higher immunoreactivity for ERβ and AR compared to sun-unprotected skin of all age groups. Significantly higher ERβ H score and percent of expression were associated with the 20–35 years age group compared to the groups that were 35–50 years and >50 years old (p < 0.02, p = 0.03, respectively) in sun-unprotected and sun-protected skin (p < 0.001, p = 0.01, respectively). AR H score showed a negative correlation with age (p = 0.04) with no significant difference in immunoreactivity in different age groups, either in sun-unprotected or sun-protected skin. There was also a significant correlation between ERβ H score and epidermal thickness in sun-unprotected (p = 0.04) and sun-protected skin (p = 0.04) in studied subjects regardless of age. The same relationships did not reach significance with AR expression. However, a significant positive correlation was detected between H scores and percent of expression of ERβ and AR in sun-unprotected (p = 0.01, p = 0.02, respectively) and sun-protected skin (p = 0.005, p = 0.02, respectively) regardless of age. In conclusion, both ERβ and AR decline gradually with intrinsic and extrinsic aging. This decline is more obvious with extrinsic aging. Further large-scaled studies are recommended to expand, validate and translate current findings to clinically significant, diagnostic and therapeutic applications. Molecular studies to investigate the probable ligand-independent action of both receptors are warranted. In addition, their gene expression patterns and associated signaling and metabolic pathways can also be tackled to provide a basis for further interventions in pathological processes that involve their dysregulation.

Introduction

During aging, skin gradually loses its structural and functional characteristics as a consequence of time per se or because of exposure to exogenous factors such as ultraviolet and infrared radiation. The effect of the endocrine
system on aging has been elucidated by several studies performed on animal and in vitro models [1–4]. Over time, important circulating hormones decline because of reduced secretion by the pituitary, adrenals and gonads or because of a concurrent disease. Amongst the numerous endocrine signals that show significant changes in blood levels with age and may directly affect the skin, androgens and estrogens play important roles [5].

Estrogen has been shown to decrease aging and wrinkling of the skin when used therapeutically [6]. It prevents aging by increasing skin thickness and dermal collagen content and strengthening the epidermal barrier [7].

There are 2 different forms of estrogen receptors, usually referred to as estrogen receptor (ER)α and ERβ, each is encoded by a separate gene (ESR1 and ESR2, respectively) [5]. ERβ is more prevalent and predominates in skin cells while ERα is scarcely found on keratinocytes, adnexa or dermal structures [8, 9].

Androgens display a distinguished role in the skin. They modulate epidermal and dermal thickness, as proven in a variety of studies measuring gender-specific differences. With advanced age, changes in the circulating androgen levels may alter not only the morphology but also key functions of the skin such as epidermal barrier homeostasis, wound-healing, sebaceous gland growth and differentiation and hair growth [10].

These effects are mediated by the ligand-binding domain of estrogen receptors or androgen receptor, and can be transmitted with similar efficiency irrespective of whether the ligand is estrogen or androgen [11]. Such interactions between estrogens and androgens may be important in the control of skin physiology [12]. Therefore, the role of the estrogen and androgen receptor subtypes in skin aging needs to be determined.

This work investigates the expression of ERβ and androgen receptor (AR) in sun-unprotected and sun-protected human skin from different age groups.

### Materials and Methods

#### Studied Population

This study was carried out on 60 normal subjects, who were collected from the Plastic Surgery Outpatient Clinic, Menoufiya University Hospital during the period from March to September 2014. The study protocol was approved by the Ethical Committee, Menoufiya Faculty of Medicine. Signed informed consents were obtained from all participants prior to enrolment. This was also in accordance with the Helsinki Declaration of 1975 (revised in 2000). Clinical data describing patients’ demographics (age and gender) were documented.

### Exclusion Criteria

All subjects with ≥1 diseases that may affect AR or ER expression were excluded, e.g. congenital adrenal hyperplasia, polycystic ovary (syndrome), breast cancer and androgen- or estrogen-producing tumors. Patients receiving hormone replacement therapy, oral contraceptives, topical estrogen or androgen therapy were also excluded. All selected subjects had a sedentary lifestyle.

### Skin Biopsies

Two 5-mm punch skin biopsies were taken from every subject under 2% lignocaine local anesthesia; 1 from the dorsum of the hand (sun-unprotected skin) and the other from the buttock (sun-protected skin).

All biopsies were submitted to the Pathology Department, Menoufiya Faculty of Medicine. They were fixed in 10% neutral buffered formalin, dehydrated in ascending grades of ethanol, followed by immersion in xylene and then impregnated in paraffin. Serial 5-μm-thick sections were taken from each block. One slide was stained with hematoxylin and eosin (H&E) for routine histopathological examination. Other sections were mounted on positively-charged slides for immunohistochemical staining.

#### Histopathological Evaluation

H&E-stained sections were examined under a light microscope for assessment of the following: hyperkeratosis, hypergranulosis, epidermal thickness, flattening of dermal-epidermal junction, dilated blood vessels and the presence of inflammatory cells, melanophages and solar elastosis.

#### Quantitative (Morphometric) Assessment of Epidermal Thickness

One section per patient was used for quantitative assessment. One field per section (×40) was captured using a Leica DMI B2/11888111 microscope equipped with a Leica DFC450 camera using the Leica C PLAN 4 × 0.10. The distance from the outermost surface of the epidermis (excluding the stratum corneum) and the dermal-epidermal junction was measured (in micrometers) at 3 different locations per section using image software, and averaged per section.

#### Immunohistochemical Staining

Serial sections were cut from the paraffin-embedded blocks with subsequent steps of deparaffinization and rehydration in xylene and a graded series of alcohol, respectively. Antigen retrieval was performed by boiling in 10 ml citrate buffer (pH 6.0) for 20 min, followed by cooling at room temperature. The slides were incubated overnight at room temperature using PBS with the following antibodies:

- Mouse monoclonal antibody raised against AR, supplied by Thermo Fisher Scientific Anatomical Pathology (Fremont, Calif., USA, code MS-443P0). It is supplied as a 0.1-ml concentrate that was diluted by 1:25 according to the manufacturer’s instructions.
- Rabbit polyclonal antibody raised against ERβ, supplied by Thermo Fisher Scientific Anatomical Pathology (code RB-10658-P1). It is supplied as 1-ml concentrate that was diluted by 1:50 according to the manufacturer’s instructions.

All slides were deparaffinized using xylene and then rehydrated in decreasing concentrations of ethanol. Antigen retrieval using microwave heating (for 20 min in 10 mmol/citrate buffer, pH 6.0)
after inhibition of endogenous peroxidase activity (hydrogen peroxidase for 15 min) was used. The primary antibody was applied to the slides and incubated overnight at room temperature in a humidity chamber. Sections were then washed with PBS, incubated with the secondary antibody for 15 min and followed by a PBS wash. Finally, the detection of bound antibody was accomplished using a modified, labeled avidin-biotin (LAB) reagent for 20 min, then a PBS wash. A 0.1% solution of diaminobenzidine (DAB) was used for 5 min as a chromogen. Slides were counterstained with Mayer’s hematoxylin for 5–10 min.

**Interpretation of Immunohistochemical Results**

A brown nuclear staining in any number of cells was considered positive in the studied normal skin biopsies [13, 14].

The epidermis (keratinocytes) and dermis (adnexa, endothelial cells, fibroblasts and inflammatory cells) were assessed separately for the expression of AR and ERβ being either positive or negative.

The epidermis was also assessed for:

- Intensity of the stain: mild (+), moderate (++) or strong (+++).
- Expression percentage: positive cells were counted and given a percentage over 200 basal cells of the whole section at a ×200 magnification [15].
- Histo-score (H score): H score was calculated in all positive specimens according to the following equation [16]:

  \[ H \text{ score} = 1 \times \% \text{ of mildly stained cells} + 2 \times \% \text{ of moderately stained cells} + 3 \times \% \text{ of strongly stained cells}. \]

**Statistical Analysis**

Data were collected, tabulated and statistically analyzed using a personal computer with SPSS v22 (SPSS Inc., Chicago, Ill., USA). Fisher’s exact test was used for the comparison of qualitative variables in 2 × 2 tables when the expected cell count of >25% of cases was <5. The χ² test was used to study the association between 2 qualitative, normally distributed variables. The Mann-Whitney U test was used for the comparison between 2 groups not normally distributed and having quantitative variables. Spearman’s coefficient was used to measure the correlation between 2 quantitative variables. Differences were considered statistically significant when \( p < 0.05 \).

### Table 1. Comparison between H&E changes in sun unprotected and sun protected skin

<table>
<thead>
<tr>
<th>Variable</th>
<th>Studied cases (n = 60)</th>
<th>Test</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>sun-unprotected skin</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>Hyperkeratosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>28</td>
<td>46.7</td>
<td>2</td>
</tr>
<tr>
<td>Negative</td>
<td>32</td>
<td>53.3</td>
<td>58</td>
</tr>
<tr>
<td>Hypergranulosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>10</td>
<td>16.7</td>
<td>1</td>
</tr>
<tr>
<td>Negative</td>
<td>50</td>
<td>83.3</td>
<td>59</td>
</tr>
<tr>
<td>Flattening of DEJ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diffuse</td>
<td>14</td>
<td>23.3</td>
<td>7</td>
</tr>
<tr>
<td>Focal</td>
<td>1</td>
<td>1.7</td>
<td>2</td>
</tr>
<tr>
<td>Negative</td>
<td>45</td>
<td>75.0</td>
<td>51</td>
</tr>
<tr>
<td>Dilated blood vessels</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>24</td>
<td>40</td>
<td>10</td>
</tr>
<tr>
<td>Negative</td>
<td>36</td>
<td>60</td>
<td>50</td>
</tr>
<tr>
<td>Inflammation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>17</td>
<td>28.3</td>
<td>10</td>
</tr>
<tr>
<td>Negative</td>
<td>43</td>
<td>71.7</td>
<td>50</td>
</tr>
<tr>
<td>Melanophages</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Negative</td>
<td>60</td>
<td>100</td>
<td>60</td>
</tr>
<tr>
<td>Solar elastosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>23</td>
<td>38.3</td>
<td>8</td>
</tr>
<tr>
<td>Negative</td>
<td>37</td>
<td>61.7</td>
<td>52</td>
</tr>
<tr>
<td>Epidermal morphometry</td>
<td>Mean ± SD</td>
<td>230.90±66.47</td>
<td>160.34±61.22</td>
</tr>
<tr>
<td>Range</td>
<td>88.68 – 372.24</td>
<td>86.1 – 354.22</td>
<td></td>
</tr>
</tbody>
</table>

DEJ = Dermal-epidermal junction; SD = standard deviation. * Significant. a χ² test. b Mann-Whitney U test.
Results

The study group consisted of 60 normal subjects whose age ranged from 20 to 66 years (39.17 ± 11.27 years). There were 39 males (65%) and 21 females (35%); females included 14 (66.7%) premenopausal and 7 (33.3%) postmenopausal subjects. They were divided according to age: group I = 20–35 years, group II = 35–50 years and group III = >50 years.

Histopathological Findings
The results of H&E-stained sections are demonstrated in table 1 and online supplementary figure 1 (see www.karger.com/doi/10.1159/000446662 for all online suppl. material).

Morphometric Findings
Quantitative assessment of epidermal thickness is demonstrated in table 1. Epidermal thickness decreased

Table 2. Immunohistochemical expression of ERβ and AR in sun-unprotected and sun-protected skin

<table>
<thead>
<tr>
<th>Variable</th>
<th>Studied cases (n = 60)</th>
<th>ERβ</th>
<th>AR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>sun-unprotected</td>
<td>sun-protected</td>
<td>test</td>
</tr>
<tr>
<td><strong>Epidermis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Keratinocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>54 (90)</td>
<td>56 (93.3)</td>
<td>0.44b</td>
</tr>
<tr>
<td>Negative</td>
<td>6 (10)</td>
<td>4 (6.7)</td>
<td></td>
</tr>
<tr>
<td>Pattern</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytoplasmic</td>
<td>36 (66.7)</td>
<td>21 (37.5)</td>
<td>9.37b</td>
</tr>
<tr>
<td>Nucleocytoplasmic</td>
<td>18 (33.3)</td>
<td>35 (62.5)</td>
<td>5 (11.1)</td>
</tr>
<tr>
<td>Intensity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>19 (35.2)</td>
<td>9 (16.1)</td>
<td>7.94b</td>
</tr>
<tr>
<td>Moderate</td>
<td>30 (55.6)</td>
<td>33 (58.9)</td>
<td>25 (55.6)</td>
</tr>
<tr>
<td>Strong</td>
<td>5 (9.3)</td>
<td>14 (25)</td>
<td></td>
</tr>
<tr>
<td>Percentage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>55.74±22.76</td>
<td>66.88±18.89</td>
<td>2.60a</td>
</tr>
<tr>
<td>Range</td>
<td>10–90</td>
<td>30–95</td>
<td></td>
</tr>
<tr>
<td>H score</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>102.87±66.56</td>
<td>144.82±68.05</td>
<td>3.36a</td>
</tr>
<tr>
<td>Range</td>
<td>20–270</td>
<td>30–270</td>
<td></td>
</tr>
<tr>
<td><strong>Dermis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endothelium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>60 (100)</td>
<td>60 (100)</td>
<td></td>
</tr>
<tr>
<td>Fibroblasts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>3 (5)</td>
<td>5 (8.3)</td>
<td>0.54c</td>
</tr>
<tr>
<td>Negative</td>
<td>57 (95)</td>
<td>55 (91.7)</td>
<td></td>
</tr>
<tr>
<td>Sweat glands</td>
<td>(n = 36)</td>
<td>(n = 31)</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>3 (8.3)</td>
<td>3 (9.7)</td>
<td>0.04c</td>
</tr>
<tr>
<td>Negative</td>
<td>33 (91.7)</td>
<td>28 (90.3)</td>
<td></td>
</tr>
<tr>
<td>Hair follicles</td>
<td>(n = 34)</td>
<td>(n = 30)</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>5 (14.7)</td>
<td>5 (16.7)</td>
<td>0.05c</td>
</tr>
<tr>
<td>Negative</td>
<td>29 (85.3)</td>
<td>25 (83.3)</td>
<td></td>
</tr>
<tr>
<td>Sebaceous glands</td>
<td>(n = 17)</td>
<td>(n = 20)</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>1 (5.9)</td>
<td>5 (25)</td>
<td>2.47c</td>
</tr>
<tr>
<td>Negative</td>
<td>16 (94.1)</td>
<td>15 (75)</td>
<td></td>
</tr>
</tbody>
</table>

* Values denote n (%), unless otherwise indicated. SD = Standard deviation.* Significant.

a Mann-Whitney U test. b χ² test. c Fisher’s exact test.
gradually with age both in sun-unprotected and sun-protected skin (data not shown in tables).

**Immunohistochemical Expression of AR and ERβ**

ERβ epidermal expression was positive in 90 and 93.3% of sun-unprotected and sun-protected skin, respectively. The pattern was either cytoplasmic or nucleocytoplasmic and intensity varied from mild to strong. Dermal ERβ showed variable reactivity in the examined fibroblasts in sun-unprotected and sun-protected skin. ERβ was expressed in basal and differentiated sebocytes and in hair follicle structures including the inner and outer root sheaths, bulge region and dermal papilla. Detailed ERβ expression in examined specimens is demonstrated in table 2 and figure 1.

AR epidermal expression was positive in 75 and 93.3% of sun-unprotected and sun-protected skin, respectively. The pattern was cytoplasmic or nucleocytoplasmic. Intensity of expression varied from mild to moderate in sun-unprotected skin and from mild to strong in sun-protected skin. Dermal AR showed positive immunoreactivity in basal and differentiated sebocytes and in hair follicle structures including the inner and outer root sheaths, bulge region and dermal papilla. AR showed negative immunoreactivity in endothelial cells, fibroblasts and sweat glands both in sun-unprotected and sun-protected skin. Detailed AR expression in the examined specimens is demonstrated in table 2 and figure 2.

Fig. 1. a–f ERβ expression in sun-protected skin. a Strong nucleocytoplasmic expression in epidermis (20-year-old male). b Moderate nucleocytoplasmic expression in epidermis (40-year-old male). c Mild nucleocytoplasmic expression in epidermis while both dermal fibroblasts (thick arrows) and endothelial cells (thin arrows) were ERβ-negative (55-year-old female). d Positive cytoplasmic expression in hair follicle (42-year-old female). e Mild positive cytoplasmic expression in sweat glands (42-year-old male). f Positive expression in dermal fibroblasts (arrows) and overlying epidermis (27-year-old male). g–i ERβ expression in sun-unprotected skin. g Strong nucleocytoplasmic ERβ expression in epidermis (32-year-old male). h Focal mild cytoplasmic expression (arrows) in basal layer of epidermis (65-year-old male). i Mild cytoplasmic expression in hair follicle (40-year-old female). Immune peroxidase. ×400, except for d, g, i: ×200.
Comparison between Immunohistochemical Expression of ERβ and AR in the Epidermis and Dermis in Sun-Unprotected and Sun-Protected Skin in the Different Age Groups

Estrogen Receptor β

Nucleocytoplasmic pattern (p = 0.002), strong intensity (p = 0.02), higher H score (p = 0.001) and higher percent of expression (p = 0.009) were significantly associated with sun-protected compared to sun-unprotected skin (table 2).

ERβ epidermal H score and percent of expression showed a gradual decline from the 20–35 years group to the 35–50 years group, both in sun-unprotected and sun-protected skin. Significantly higher H score and percent of expression were associated with the 20–35 years group (p < 0.02, p = 0.03, respectively) in sun-unprotected and sun-protected skin (p < 0.001, p = 0.01, respectively; fig. 3).

Androgen Receptor

Sun-protected skin showed significantly higher keratinocyte expression (p = 0.006), stronger intensity (p ≤ 0.001), a higher percent of expression (p = 0.006) and a higher H score (p ≤ 0.001). Cytoplasmic pattern was significantly associated with sun-unprotected skin (p = 0.049; table 2).

There was no significant difference in ERβ immunoreactivity between premenopausal and postmenopausal females in sun-unprotected and sun-protected skin (data not shown in tables).

Higher H score was significantly demonstrated in sun-protected skin in the 20–35 years group (p = 0.02), 35–50 years group (p = 0.04) and >50 years group (p = 0.03) than in sun-unprotected skin of the corresponding age groups (fig. 4).

There was no significant difference in ERβ immunoreactivity between premenopausal and postmenopausal females in sun-unprotected and sun-protected skin (data not shown in tables).

Higher H score was significantly demonstrated in sun-protected skin in the 20–35 years group (p = 0.02), 35–50 years group (p = 0.04) and >50 years group (p = 0.03) than in sun-unprotected skin of the corresponding age groups (fig. 4).

No significant difference was noted in AR immunoreactivity in different age groups in sun-unprotected or sun-protected skin. There was also no significant differ-
ence in AR immunoreactivity between premenopausal and postmenopausal females in sun-protected skin (data not shown in tables).

Higher epidermal H score was significantly demonstrated in sun-protected skin in the 20–35 years group ($p \leq 0.001$), 35–50 years group ($p = 0.03$) and >50 years group ($p = 0.03$) than in sun-unprotected skin of the corresponding age groups (fig. 4).

**Comparison between ERβ and AR Expression in Male and Female Subjects**
No significant difference was detected in ERβ and AR expression in male and female subjects in sun-unprotected or sun-protected skin (online suppl. tables 1, 2).

**Correlation between ERβ and AR H Scores and Percentage of Expression and Age**
There was a significant negative correlation between age and ERβ percentage of expression ($r = -0.35$, $p = 0.020$).

**Fig. 3.** Comparison of immunohistochemical expression of ERβ in epidermis in different age groups. In sun-unprotected skin, a higher percentage of expression (a) and higher H score (b) were significantly associated with the age group 20–35 years. In sun-protected skin, a higher percentage of expression (c) and higher H score (d) were significantly associated with the age group 20–35 years.

**Fig. 4.** a, b Comparison between immunohistochemical expression of ERβ and AR in sun-unprotected and sun-protected skin in different age groups. Higher H score was significantly associated with sun-protected skin in all 3 age groups for both ERβ and AR.
0.009) and between age and ERβ H score (r = –0.33, p = 0.02) in sun-unprotected and in sun-protected skin (r = –0.50, p ≤ 0.001 and r = –0.41, p = 0.002, respectively; fig. 5). A significant negative correlation was detected between age and AR H score in sun-protected skin (r = –0.27, p = 0.046; fig. 5).

Relationship between AR and ERβ Expression and Morphometric Data
There was a significant positive correlation between H score of ERβ and epidermal thickness in sun-unprotected and sun-protected skin (p = 0.04 for both) in the studied subjects regardless of age and in the >50 years group (p = 0.04 for both; table 3). The same relationship did not
reach significance with AR expression in sun-unprotected and sun-protected skin (data not shown in tables).

**Correlation between ERβ and AR H Scores and Percentage Values in Sun-Unprotected and Sun-Protected Skin**

A significant positive correlation was noted between ERβ and AR H scores and percentage values in the studied subjects regardless of age (r = 0.38, p = 0.01 and r = 0.36, p = 0.02, respectively) in sun-unprotected and sun-protected skin (r = 0.39, p = 0.005 and r = 0.31, p = 0.02, respectively; fig. 5).

**Discussion**

In this work, keratinocytes showed positive ERβ expression in both sun-unprotected and sun-protected skin, mainly in the basal layer with variable suprabasal immunoreactivity. These results were consistent with previous studies [9, 17]. Estradiol can stimulate both epidermal keratinocyte proliferation and DNA synthesis [18]. Kanda and Watanabe [19] demonstrated that estradiol suppresses apoptosis in keratinocytes by promoting Bcl-2 expression and induces the expression of cyclin D2, an important cell-cycle regulatory protein [20]. Estrogen may also play a role in the stratum corneum barrier function [21–24].

The positive ERβ immunoreactivity in normal skin fibroblasts, noted here, was similarly reported before [25, 26]. ERβ expression in fibroblasts, the major producers of type I collagen, strongly suggests that estrogen directly affects fibroblast biology through receptor-mediated effects [27]. This is brought through promoting fibroblast transforming growth factor β (TGF-β) production [28]. TGF-β is a multifunctional cytokine that stimulates fibroblast proliferation in the dermis, induces the synthesis and secretion of the major extracellular matrix proteins and downregulates the expression of proteolytic enzymes, such as collagenase and stromelysin [29]. Consistent with this view, it has been reported that cultured mouse dermal fibroblasts increase collagen synthesis in response to estrogen [30, 31].

In this study, ERβ immunoreactivity was detected in basal and differentiated sebocytes. Similar findings were reported previously [10]. Studies using cultured human sebaceous glands have shown that physiological levels of estradiol significantly decrease lipogenesis without affecting the rate of cell division [32].

Our results also showed positive ERβ expression in hair follicle structures including the inner and outer root sheaths, bulge region and dermal papilla. The bulge region contains the stem cells for hair follicle keratinocytes [33]. Therefore, these results may provide evidence that estradiol mediates its effects on hair follicle keratinocytes through the direct regulation of stem cells; this suggests an important role for estrogens in the regulation of hair growth. The role of estrogens in the regulation of eccrine sweat glands which express ERβ is still unknown [10]. Therefore, the positive immunoreactivity that we detected requires further investigation in order to be clarified.

We found that ERβ H score and percentage of expression showed a gradual decline with advancing age. This is in agreement with Inoue et al. [34], who showed a sig-
significant decrease in its epidermal expression in subjects >70 years old. In addition, ERβ H score values and percentage of expression were negatively correlated with age in sun-unprotected and sun-protected skin. A significant positive correlation between ERβ H score and epidermal thickness in sun-unprotected and sun-protected skin was also detected.

It is well known that, with aging, there is a decline in the level of sex hormones including estrogens [35]. This hormonal decline is accompanied by significant changes within the skin [36]. Skin cellular renewal becomes sluggish, collagen production is reduced and its distribution is altered, resulting in thinning of the epidermal and dermal layers and the ability of the skin to maintain its barrier function and its hydration, strength and elasticity suffer as a result [37, 38]. From our results, we come to the conclusion that not only estrogen hormone but also its functioning receptor, ERβ, is downregulated with age.

Cerdà et al. [39] showed that the number of ERs declines with the onset of menopause in women. However, there was no significant difference between pre- and post-menopausal women in our work. This may be due to the small number of studied female subjects.

We detected positive epidermal AR expression in sun-unprotected and sun-protected skin, and this was mainly in the basal layer with variable suprabasal immunoreactivity. These results agree with previous studies [40]. The expression of AR in the epidermis indicates that keratinocytes are target cells for androgen action on the skin. The role of androgens on the activity of keratinocytes remains to be clarified. However, the presence of AR-expressing keratinocytes within the pilosebaceous ducts supports the hypothesis that androgens can directly influence keratinization [41]. It has been stated that AR can modulate the proliferation and differentiation of epithelial cells of the epidermis and adnexa, the functional activity of dermal fibroblasts [42] and dermal angiogenesis [43].

Androgens have strong effects on hair growth and appear to act through AR and type II 5α-reductase on dermal papilla cells [44]. AR gene polymorphisms have been associated with hirsutism in women [45] and androgenetic alopecia in men [46]. Dermal papilla cells appear to mediate the growth-stimulating signals of androgens by releasing growth factors that act in a paracrine fashion on the other cells of the follicle [47]. However, overexpression of the AR promotes androgen-induced premature senescence of dermal papilla cells [48].

Androgens cause the enlargement of hair follicles in androgen-dependent areas, but in the scalp follicles of susceptible men, paradoxically, androgens foster miniaturization and shortage of hair in the anagen stage that commonly leads to baldness [49]. These contradictory effects may be explained by genetically determined differences in the response of the papilla cells to androgens in different body areas [27]. Nevertheless, the positive expression of both AR and ERβ in the hair follicle structures including the inner and outer root sheaths, bulge region and dermal papilla, that we detected, requires further investigation to determine whether androgen metabolites, acting over ERβ, affect follicular dermal papilla cells, as occurs in the mouse prostate [9, 50].

It is well accepted that testicular and adrenal androgens decline progressively with aging in men [51, 52]. However, in women, this issue is controversial and has been under much debate [53, 54]. In this work, AR expression was negatively correlated with age but did not show a meaningful difference across the different age groups or between pre- and postmenopausal females. Further, larger-scale studies are recommended.

In this study, ERβ and AR showed higher expression in sun-protected than in sun-unprotected skin. This finding has not been commented on in similar studies and its significance is not clear. The effect of UV radiation on sex steroids and their receptor expression has been proved in animals but not in humans [55]. However, the downregulation noted in sun-unprotected skin with advancing age may provide evidence that deficient receptor expression occurs in both the intrinsic and extrinsic aging processes. This finding corresponds to the fact that skin aging is a degenerative process whereby alterations due to the passage of time (chronological/intrinsic aging) are superimposed with effects produced by environmental factors, the most important of which is UV radiation [56, 57].

It is worthy to note that sex steroid receptors proved to have ligand-independent actions [58]. Questions now arise. Does the downregulation of AR and ERβ, as we have demonstrated, have further unknown hormonal-independent effects that play a role in the deterioration of the skin with aging? Does ERβ downregulation have a role in hair aging, especially in females? Further molecular in vitro and in vivo studies are required to demystify these issues.

ERs and AR are known to exhibit nuclear immunoreactivity [59]. In our study, the nucleocytoplasmic pattern of both ERβ and AR was detected. It was postulated that androgens and other sex steroids may affect cells via mechanisms that do not involve gene transcription/expression, but the activation of cytoplasmic signaling pathways that affect cell survival and/or modulate other functions.
growth factor signaling [60, 61]. Parker [62] concluded that ERs can move between the nucleus and the cytoplasm, but under normal conditions they are predominantly located in the nucleus. Pelletier and Ren [12] detected nucleocytoplasmic ERβ immunoreactivity in ducts of eccrine sweat glands. Heemers and Tindall [63] concluded that AR, in its inactive form, is located in the cytoplasm of the cell in complex with heat-shock proteins. Upon ligand binding, AR homodimerizes and translocates to the nucleus where it binds to the specific sequences on the DNA known as androgen response elements. It may also recruit cofactors (proteins) that either enhance or reduce AR transactivation [64].

The lack of significant differences between male and female subjects regarding AR and ERβ expression that we noted needs to be confirmed by additional large-scale studies investigating different sites on the body. The regional differences in receptor density or activity in both genders have not been investigated before.

Our work showed a significant positive correlation between ERβ and AR H scores and expression percentage in both sun-unprotected and sun-protected skin. This is in line with the hypothesis that tissues traditionally thought to be responsive to one class of steroids, contain receptors for other classes, and that steroid receptors can cross-talk with one another [65, 66].

From the aforementioned findings, both ERβ and AR decline gradually with intrinsic and extrinsic aging. This decline is more obvious with extrinsic aging. Further large-scale studies are recommended to be able to expand, validate and translate the current findings to clinically significant diagnostic and therapeutic applications. Studies are also needed to clarify the physiological role of androgens and AR on keratinocytes. With new investigative techniques, perhaps now is the time to readdress many of the outstanding questions regarding their role in skin aging. Molecular studies to investigate the probable ligand-independent action of both receptors are also warranted. In addition, gene expression patterns and associated signaling and metabolic pathways of ERβ and AR can also be tackled to provide a basis for further interventions in pathological processes that involve their dysregulation.

Statement of Ethics
Taking samples was done after obtaining written consent from the patients and control subjects.

Disclosure Statement
The authors have no conflicts of interest that are directly relevant to the content of this study. No sources of funding were used to conduct this study or prepare the manuscript.

References


Hibberts NA, Howell AE, Randall VA: Balding hair follicle dermal papilla cells contain high levels of androgen receptors than those from non-balding scalp. J Endocrinol 1998;133:141–147.


