Chapter 2
Squalene and Skin Barrier Function: From Molecular Target to Biomarker of Environmental Exposure

Boudiaf Boussouira and Dang Man Pham

Abstract The human skin naturally faces an aerial oxidative environment. The environment presents however a variable oxidative potential since enhanced by solar rays (UV, Visible) possibly combined to aerial-borne pollutants that most often act as catalysts in the different oxidative pathways. The poly-unsaturated human sebum highly present on the upper parts of the body (face, torso) is therefore a natural "receptor" of these oxidative actions. Comprised at 10–20 % within sebum, Squalene ($C_{30}H_{50}$) is not only specific to human sebum but its 6 double bonds make it a highly sensitive molecule towards various forms of Reactive Oxygen Species, singlet oxygen included, leading to different per-oxidized by-products. The latter thus appear as natural bio-markers of most oxidative actions upon the cutaneous tissue. Some mechanisms can easily be modelled in vitro, thereby demonstrating the influences of UVA rays, cigarette smoke, particulate matters or some porphyrins that are synthesized by the resident skin flora. These models allow the structures of various forms of squalene peroxides to being determined and to quantify the quenching properties of some known anti-oxidants (Carotenoids, Vitamin E). These chains of events were logically traced in vivo, by comparing the contents of Squalene and Vitamin E in the sebum of subjects living in differently polluted but close geographical locations. The oxidized state of Squalene then represents a reliable biomarker of most oxidative events induced by various environmental factors. Their possible biological impacts upon the skin physiology, which greatly remain to being documented, are discussed.

Keywords Squalene · Skin lipids · Oxidation · Barrier function

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2.1 Introduction: The Human Skin, a Constantly Adaptive Organ

The adaptation of skin to environmental changes is as old as humanity. Ethnological findings (Tishkoff and Verelli 2003; Jablonski 2004; Lucock et al. 2014) indicate that some of our far ancestors migrated from Eastern Africa 60,000 to 30,000 years ago towards Northern regions. Such moves necessarily implied drastic environmental changes to which successive genetic and phenotypic adaptations responded to various Darwinian *imperata*. Long periods of time, different sun irradiance, food/vitamins supply led numerous genetic mutations to be selected, among which those concerning skin pigmentation-related genes are rather well documented (Jablonski 2011; Basu Mallick et al. 2013; Jablonski and Chaplin 2013; Wilde et al. 2014).

With a 1.5–2 m^2 developed surface (in adults), the human skin offers a rather large exchange capacity with its close environment (Hadgraft 2001), being exogenous (sun, wind, cold, heat) or man-made (soap, water, cosmetic products, frictions). With regard to skin, the term "environment" can hardly be restricted to the sole physico-chemical conditions of the surrounding world. It includes many other factors to which skin (and body) may be exposed, all being now covered by the generic term "exposome" (Patel and Manrai 2015). This generic term comprises all factors that, over time, possibly influence the skin physiology such as food, psychological stress, medications, fatigue, smoking, alcohol consumption, etc. The notion of exposome, with regard to skin, may be even extended to the assembly (the so-called microbiome) of a resident and transient microflora (the so-called microbiome) that is permanently found on the skin surface and within the depth of the hair follicle canal (Grice and Segre 2011).

Hence, the effects of this microbiome upon the skin and, vice versa, those from the host tissue towards these micro-organisms represent constant mutual relationships.

2.1.1 Age-Related Changes

In utero, during the first two months needed for its full constitution, the human skin exposes to an aquatic and sterile medium, free from any stress. It then sets up a natural barrier to this environment, produced by already active sebaceous glands (stimulated by maternal hormones), thus delivering an important lipid-rich hydrophobic "film" (5–15 g all along the whole infant's body), called the Vernix Caseosa (Mikova et al. 2014; Visscher et al. 2015). The latter is in fact a very early sebum that nurses wipe off at the very first minutes post delivery, using sterile cotton pads.

At birth, all conditions drastically change. The skin now faces a totally different environment: dry, of a high oxygen tension (≈20 %) and non-sterile. Skin is almost

immediately colonized by a resident microflora that will permanently thrive on the skin surface all along life span (Marples et al. 1974; Baviera et al. 2014). Later, skin will be progressively exposed to sunlight (UV, Visible and Infra-red ranges). These four major elements represent its early exposome.

Progressively, age-related physiological changes make skin more prone to adapted responses to various assaults. Melanocytes have become fully operational by inducing a protecting pigmentation in the fairer skin tones (Phototypes II–III) (Fitzpatrick 1988; Chardon et al. 1991). The epidermal physiology has then set up an efficiently regulated anti-oxidant network including various enzymes (Super Oxide Dismutase, Glutathione Peroxidase) and molecules (Vitamin E, Selenium, Vitamin C) (Thiele et al. 2001). Possible contacts with exogenous allergens are normally fought by an efficient immune response, most ensured initially by the epidermal Langerhans cells (Haniffa et al. 2015).

2.1.2 Environment Exposure Changes

Since the 19th century, our aerial environment has been strongly modified with regard to growing industrialization, transporting systems, agricultural changes etc. Nowadays, pollutants of various natures and origins are, in additional to natural sources (soil erosion, volcanic eruptions, forest fires), clearly linked to human activities. These cover the increases in emitted volatiles such as CO_2, CO, NO, NO_2, O_3 (ozone), Polycyclic Aromatic Hydrocarbons (PAH's) and Particulate Matters (PM) covering a wide range in size (0.1–100 μm) and nature (Ning et al. 2006; Ding et al. 2006; Zheng et al. 2002). Combined with solar rays (UV's, Visible, Infra-Red), most of these human-related pollutants are now shown as efficient catalytic agents in many oxidizing processes (Colin et al. 1994; Tai-Long et al. 2015a, b).

2.2 Specificities of the Stratum Corneum

The present chapter mostly focuses on the various effects of an oxidative environment upon the facial skin surface. The latter is indeed a privileged skin site for assessing the impacts of some environmental assaults for the following reasons:

- It is usually (in common with hands) the most constantly exposed skin region to the external environment.
- It is a skin site that gathers highly functional appendages (apocrine and eccrine Sweat glands, Sebaceous glands). Hence, many epidermal by-products (horny cells, sebum, epidermal lipids, peptides and amino acids, salts, organic acids, urea, water, etc.) are daily found at its surface, exposed to environment.

2.2.1 A Cornified Protecting Barrier Covered by Sebum

The Stratum Corneum (SC) comprises stacked flat dead cells (corneocytes) of usually 15–20 μm in thickness, embedded in a lipid-rich medium within intercellular spaces (the so-called brick and mortar organization), as shown by Fig. 2.1.

The SC is a powerful and vital barrier that ensures a wide array of defensive functions (Elias 2005). It is constantly produced by the epidermal cells (keratinocytes) renewal and their progressive keratinization process that ends up with natural desquamation, as single cells. A normal epidermis shows in fact a rather low mitotic index (about 10 %), i.e. the ratio of dividing cells at a given moment. In other words, epidermis possesses a high potential in speeding up the renewal of keratinocytes. The latter then allows the SC thickness (number of cell layers) to being adapted by an increase in cell layers according to needs (cut, wound, burn, frictions).

The Stratum Corneum, through both multilayer corneocytes and inter-cellular lipid medium, ensures a dual barrier function. Internally, it controls the Trans Epidermal Water Loss (TEWL, 5–10 g m^{-2} h^{-1}). The latter parameter is a precious marker of SC cohesion and thickness, since rapidly elevated in the case of a loose, damaged or thinned SC (Rawlings and Matts 2005; Rawlings and Leyden 2009). Externally, SC controls the flux of exogenous substances, acts as a thermo-insulating tissue and is an efficient shield against UV rays.

As a mantle exposed to various environments, the SC surface is daily covered by sebum (and traces of sweat according to the thermo-regulation function). Depending on the skin sites, the density of sebaceous glands (and eccrine glands) and consequently the amount of sebum (and sweat) presents some local variations.

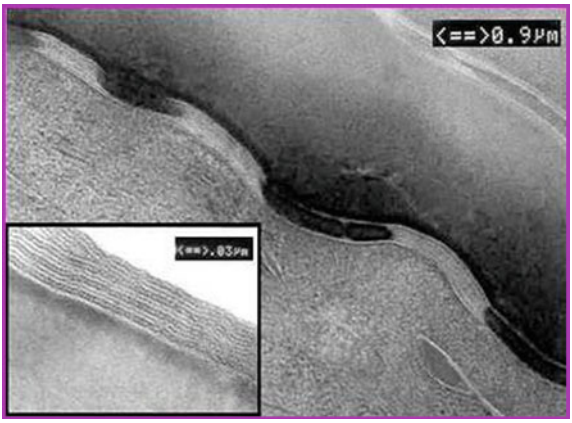

Fig. 2.1 Transversal section of the Stratum Corneum showing its organization in multi layered corneocytes, separated by an inter-cellular lipid medium (*zoomed section*). The corneodesmosomes (in *black*) ensure the attachments between corneocytes. The progressive degradation of corneodesmosomes drives the desquamation process that further delivers corneocytes as single cells in normal conditions (Courtesy of A. Potter, A.M. Minondo, F. Fiat. Life Sciences, L'Oréal Research and Innovation)

2.2.2 The Human Sebum

2.2.2.1 Quantitative Aspects

This complex lipid mixture is constantly produced by the Sebaceous Glands (Bernard and Saint-Léger 2000) and delivered within the follicular canal under the disintegration (holocrine process) of their cells (sebocytes). Excreted to the skin surface from the follicular ostia (about 250 per cm^2), at a rate ranging 0.4–2.5 μg cm^{-2} min^{-1} according to gender, age, ethnics, circadian rhythms. Sebum further spreads over the skin surface to reach an equilibrium level (the so-called casual level) of some 50–300 μg cm^{-2} which could be reached within a few hours post cleaning (Saint-Leger et al. 1982). The human face (surface ≈ 500 cm^2) appears then daily covered by 25–150 mg of sebum, in addition to admixed lipids of epidermal origin (Cholesterol, Ceramides, Triglycerides). On face, the ratio of Sebum to Epidermal lipids is about 97/3 (Wilkinson 1969). Such a lipid mantle then represents a "film" of a theoretical thickness of 3–10 μm, hence greatly facilitating exchanges with the environment.

2.2.2.2 Qualitative Aspects

The human sebum is an oil, unlike that, waxy, of most animals. This fluid behavior mostly results from a high proportion of mono and poly unsaturated lipid chains that, by nature, are highly sensitive to oxidization process. At a native state (within the sebaceous glands), sebum initially comprises a mixture of 3 lipid classes: Triglycerides (TG) 60 %, Wax esters 25 % and Squalene 15 %. Later, excreted sebum will transform. TG's are hydrolyzed by lipases emitted by the resident and

Table 2.1 Description of the major classes (approximate figures) and properties of lipids within sebum daily present on face

Lipid class	Number of C atoms	Unsaturated chains (iso and ante-iso methyl branched)	Relative concentrations present at the skin surface	Linked to
Triglycerides (TG)	30–60	66 % (2/3)	0–60 %	Sebaceous glands
Free fatty acids (FFA)	10–20	66 % (2/3)	60–0 %	Lipases/microflora, S.C hydrolases
Wax esters (WE)	20–60	50 % (1/2)	25 %, stable, untransformed	Sebaceous glands
Squalene (SQ)	30	100 %	10–20 %	Sebaceous glands and oxidization processes

The ratio TG/FFA is a precious reflection of the metabolic activity of the skin microflora and may greatly vary between individuals and regimen (e.g. intake)

lipophilic microflora, yielding Free Fatty Acids of 10–20 carbon chain lengths at the skin surface (Nicolaides 1974). Table 2.1 summarizes the major events of the sebum transformation steps and their major effectors or origins.

2.3 Squalene (SQ), a Key Element

2.3.1 A Biological Human Curiosity

Squalene is a specific marker of human sebum since absent in the sebum of almost all mammalian species (Lindholm and Downing 1980). As a readily precursor of Cholesterol and since rapidly transformed, it is present in almost all cells at minute amount. The major exception remains the case of the liver of the Squalidea family (its derived name) where large amount of SQ are found. The human sebaceous glands do not achieve a complete synthesis of Cholesterol and thus liberate pure squalene. The human sebaceous glands therefore clearly diverge from those of all animals where sebum is almost uniquely Cholesterol or Sterol-based, (e.g. Lanolin in sheep).

2.3.2 Structure/Properties of SQ

Squalene is a triterpene of the general formula $C_{30}H_{50}$ (see Fig. 2.2) that comprises 6 non-conjugated double bonds, making this compound one of the most unsaturated lipids.

It is a transparent oil, of a specific gravity of 0.855, fluid under normal conditions (Fusion T° = −20 °C). As most lipids, it is readily soluble in organic solvents and totally insoluble in water.

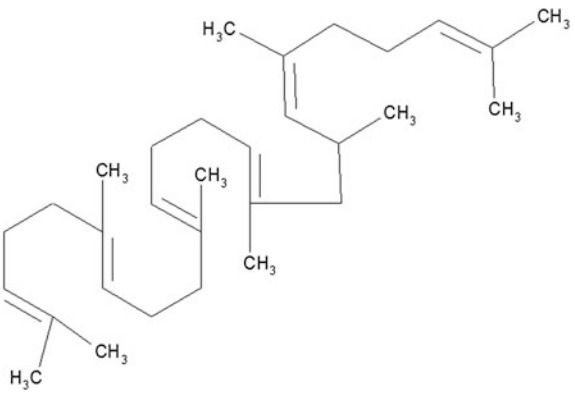

Fig. 2.2 Simplified chemical structure of squalene. Such representation illustrates how, when cyclized, squalene generates the future sterol ring

2.3.3 Squalene, a Strong Acceptor of All Forms of Oxygen

Such a richly unsaturated level naturally makes squalene highly prone to oxidization processes. The latter phenomenon was early described (Chapman 1923) showing that, when completely oxidized, squalene can absorb oxygen up to ¼ of its weight. However, squalene is highly sensitive to singlet oxygen ($^1O_2$), a very reactive oxidative species, that could be generated by various ionizing sources. This Singlet Oxygen rapidly reacts with the double bonds of squalene (Leong et al. 1976; Miquel et al. 1989; Petrick and Dubowski 2009). Yielding families of squalene peroxydes (SQOOH) and, to a lesser extent, squalene hydroxides (SQOH) (Ekanayake Mudiyanselage 2003). A slower but progressive oxidization can however be obtained by simply exposing a thin film of pure squalene to an ambient air free from singlet oxygen. In days, regular increases of its oxidized forms concomitant to decreased values of pure squalene are observed. Chemically speaking, all these chain-reaction processes lead to the addition of "ene" types of mechanisms into which the 6 electron-rich carbon double bonds (C=C) play a central role. Such finding was later confirmed (Saint-Leger et al. 1986; Tochio et al. 2009).

Sebum extracted from forehead, analyzed by liquid chromatography with UV and Light Diffracted Detector shows the presence of squalene and also squalene peroxides (SQOOH) and squalene hydroxides (SQOH). Further works using LC/MS (Thiele et al. 2003) confirmed that levels of squalene monohydroperoxides were strongly increased under low doses of UV exposures.

An alternative analytical method to quantify SQ and SQOOH was early developed in our laboratories and currently used, allowing low amount of SQOOH forms to being detected. Post solvent extraction and filtration, squalene peroxides are quantified by ultra-performance liquid chromatography (UPLC), on reversed phase, coupled with atmospheric pressure chemical ionization (APCI) tandem mass spectrometry (MS/MS) on positive mode (UPLC-APCI-MS/MS). Residual squalene (i.e. non-oxidized) is quantified on the same run with PDA detection at 205 nm, using pure squalene as standard. Under such conditions, the Limit Of Detection (LOD) and Limit Of Quantification (LOQ) of squalene monohydroperoxides are 10 and 50 ng ml^{-1}, respectively, together with an acceptable reproducibility (coefficient of variation <10 %). LOD and LOQ for residual squalene are 0.1 µg and 1 µg ml^{-1}, respectively. These limits allow very low amount of SQOOH and squalene to be determined on a freshly collected sebum (basal values) since slightly (per)oxidized before its excretion over the skin surface.

2.3.4 Squalene and the Resident Oxidative Skin Microflora

Within the depth of the follicular canal, porphyrins are synthesized and excreted by *Propionibacteria* spp. (Cornelius and Ludwig 1967; Fuhrhop et al. 1980). These compounds strongly absorb in the 360–450 nm range (UVA and Visible),

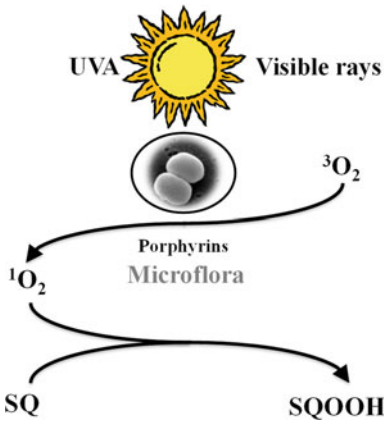

Fig. 2.3 Simplified scheme of the chain of reactions induced by photo-catalytic porphyrins, yielding singlet oxygen ($^1O_2$) that further reacts with squalene (SQ) to generate SQOOH forms

according to their structures, and generate Singlet Oxygen ($^1O_2$) from oxygen (Ekanayake Mudiyanselage 2003). This explains why squalene (per)oxides are naturally found within the sebum of most subjects.

This presence of porphyrins could also explain the reason why a high level of SQOOH is found within the comedones of acneic subjects (Motoyoshi 1983; Saint-Leger et al. 1986).

In brief, squalene, before facing additional external oxidative environments, is already- and partly-oxidized. The simplified scheme in Fig. 2.3 illustrates such chain of events.

This scheme allows a better understanding of the possible impact of Vitamin E (α tocopherol) since, supplied by food, it is eliminated through the sebaceous gland metabolism and further excreted within sebum (Thiele et al. 1999). A follicular canal enriched with Vitamin E is therefore likely more prone to inhibit such chain reactions, that can be quenched by well-known singlet oxygen scavengers such as Carotenoids, Vitamin E, Butyl Hydroxy Toluene (BHT) etc. as exposed later in this chapter.

The scheme also indicates how UVA sunscreens may efficiently slow down these oxidative pathways through controlling the penetration of UVA rays within the depth of the follicular canal, in agreement with previous findings (Fourtanier et al. 2006; Battie et al. 2014).

2.3.5 Squalene Facing Singlet Oxygen Released by Porphyrins

As previously mentioned, porphyrins are prone to generate singlet oxygen under UVA exposure. This can be easily demonstrated by simple preliminary assays. First, under UVA exposure, a methanolic solution of pure squalene (Sigma Aldrich, ref S3626) leads to a rather low SQOOH/SQ ratio whereas the methanolic extract of

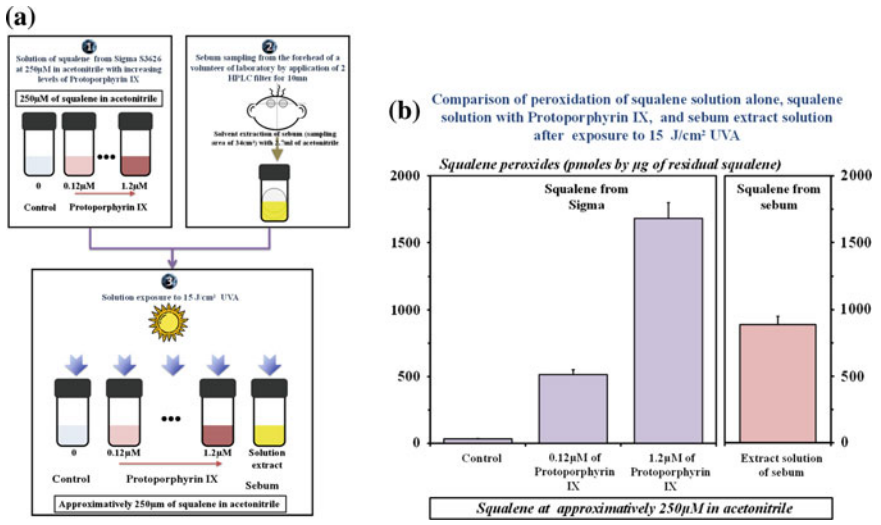

Fig. 2.4 **a** Examples of experimental protocols using either pure squalene and Protoporphyrin IX or squalene from sebum without addition of Protoporphyrin IX. All samples were exposed to 15 J/cm^2 UVA. **b** Ratios of SQOOH/SQ obtained under the two protocols exposed in (**a**)

a sebum collected from a human forehead (that contains traces of porphyrins) leads, in same conditions of UVA exposure, to a much higher SQOOH/SQ ratio, as shown by Fig. 2.4a, b.

Second, adding increased concentrations of a porphyrin, Protoporphyrin IX (Sigma Aldrich, ref P8293) to a methanolic solution of pure squalene shows a dose dependent increase in the generation of SQOOH forms under UVA exposure, as illustrated by Fig. 2.5a, b, at least within the studied concentrations of porphyrin IX.

Figure 2.5a, b illustrate the linear dependence of generated SQOOH with increasing amount of squalene and a fixed amount of Protoporphyrin IX or, at a constant concentration of squalene, with increasing amount of Protoporphyrin IX.

The latter assays indicate that the follow up of SQOOH forms is a precious indicator of an oxidative stress driven by singlet oxygen and obviously paves the road to in vitro testing of known or candidate molecular scavengers of this reactive form of oxygen (see next paragraph).

2.3.5.1 Effect of Some Anti-oxidants

With regard to the high sensitivity of the analytical technique, the in vitro tests exposed above can be applied for determine the amount of the decreased SQOOH forms induced by five common anti-oxidant molecules. Figure 2.6a, b illustrate the various amplitudes of their effects, outlining (and confirming) the very high potency

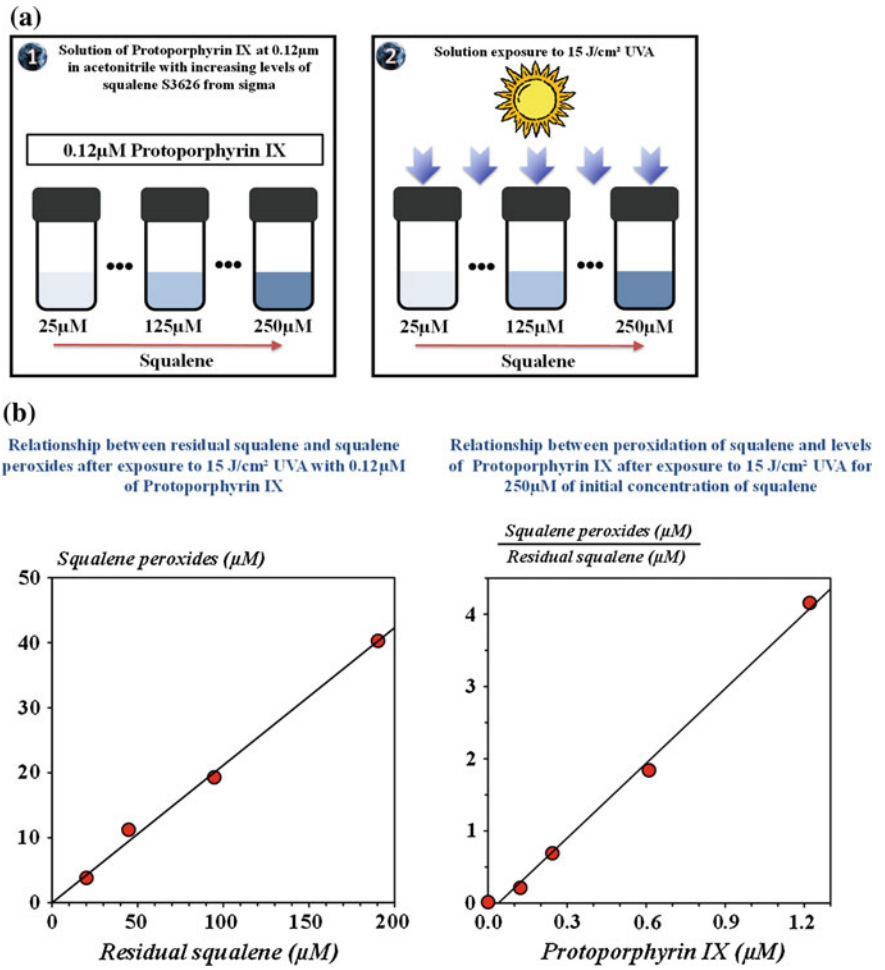

Fig. 2.5 **a** Global protocols used to precise the dose dependence of SQOOH forms with squalene and Protoporphyrin IX. **b** Dose responses of generated SQOOH forms with different amount of squalene and/or Protoporphyrin IX

of β Carotene to inhibit the peroxidization of squalene, as a potent scavenger of singlet oxygen, a property shared by most Carotenoids (Hosaka et al. 2005).

2.3.5.2 SQOOH Properties

The easiness in preparing, in vitro, oxidized forms of squalene, under the above methodologies given as examples, allows their major characteristics to be precised. It has to be kept in mind that other oxidizing methods can be used, such as exposing

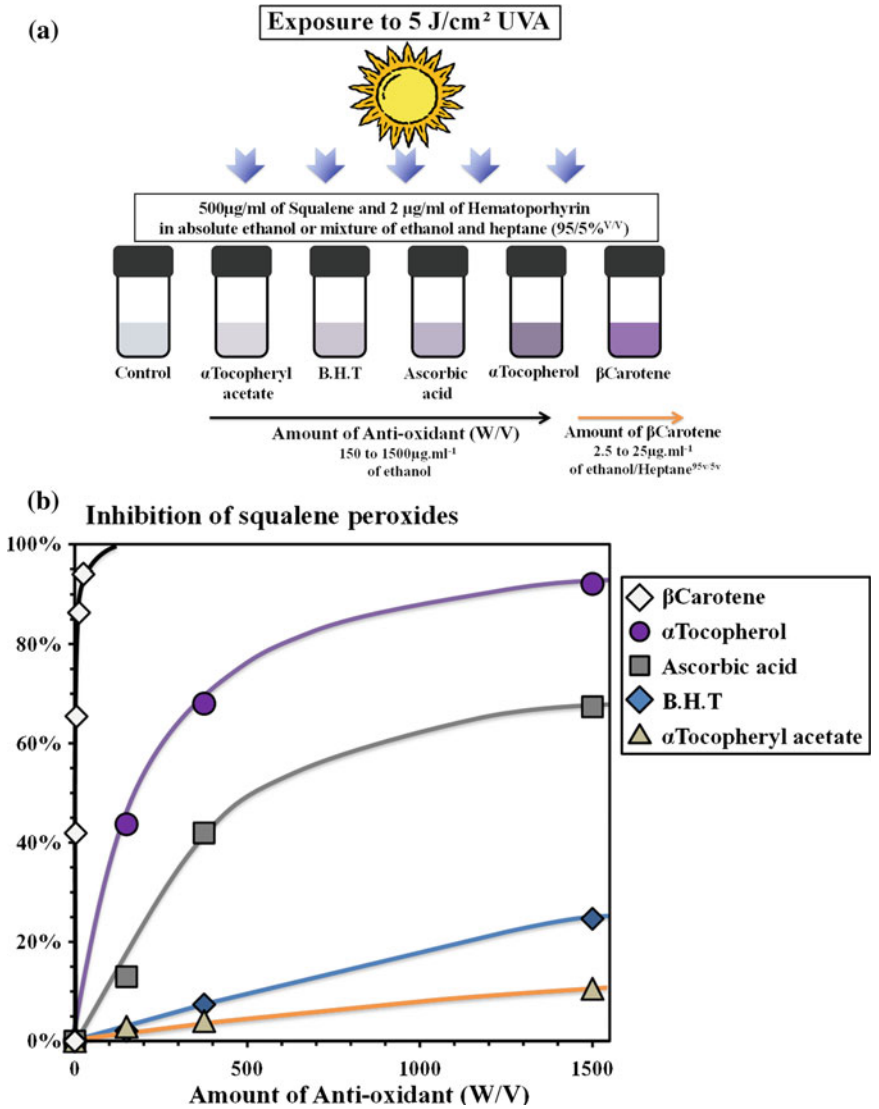

Fig. 2.6 **a** General protocol used for determining the efficacy of some common anti-oxidant molecules. **b** Effects of some anti-oxidant molecules upon the genesis of SQOOH from squalene in the presence of porphyrin and exposure to UVA. β-Carotene shows a high anti-oxidant activity whereas the acetate form of vitamin E brings the lower activity

squalene to Ozone (O_3), a marker of aerial pollution, according to a previously quoted work (Petrick and Dubowski 2009). Little is known, however, whether such a procedure leads to a similar chemical family of SQOOH forms.

- In our experimental conditions, the production of SQOOH's is UVA dose-dependent.
- The minimum UVA dose at which such SQOOH production starts being detected corresponds to a reasonable (not extreme) sun exposure of the skin at zenith time during a European summer climatic condition.
- Chemical instability is the hallmark of most peroxides. Despite, SQOOH forms appear relatively stable under the experimental conditions exposed above and can be kept at −20 °C under Nitrogen without significant losses. However, high UVA doses lead to their transformation/disappearance.
- Oxidizing different amounts of squalene by a same dose of UVA leads to similar SQOOH/SQ ratios.

These reasons are then crucial in the establishment of in vitro testing, in the need of adopting adequate and realistic conditions such as UV doses, initial SQ amount, respective ratio with a photocatalytic agent etc.

2.4 Squalene as a Reliable Bio-marker of an Oxidative Environment

Squalene then appears as a privileged molecule in studies dealing with oxidization-related processes for the following reasons:

- Easily oxidizable under mild conditions.
- Its oxidized by-products (SQOOH, SQOH) can be detected at very low amount.
- Apart from using pure squalene, human sebum as a source of squalene is an easy alternative model that better integrates or mimics the actual in vivo situation.

Indeed (i) it is of an easy collection (forehead), (ii) it is constantly renewed by the skin, and (iii) it comprises physiological components (porphyrins, unsaturated fatty acids).

A recent paper from our group (Pham et al. 2015) explored and illustrated some possible technical approaches for assessing the rate, amplitude and specificity of some factors (UVA, aerial pollutants) at enhancing the (per)oxidization of squalene, either in a pure form or present within human sebum. Their results, summarized below, suggest that the latter could be a reliable bio-marker of the impacts of aerial pollution upon human skin, of an easy collection, i.e. adapted to various protocols of in vivo studies.

2.4.1 In Real Life (In Vivo) Conditions

Two comparable in vivo studies were carried out by our group of research in 2000 and 2008 in Mexico city/Mexico and Shanghai/P.R. China regions, respectively (Nguyen et al. 2015a, b). In both cases, half of subjects under study (a total of 348

Table 2.2 Record of some pollution markers

Average of 8 h	O_3 ($\mu g\ m^{-3}\ h^{-1}$)	NOx ($\mu g\ m^{-3}\ h^{-1}$)	SO_2 ($\mu g\ m^{-3}\ h^{-1}$)	PM_{10} ($\mu g\ m^{-3}$)
Mexico city (2000)	170 ± 46	109 ± 26	N.A	N.A
Shanghai center (2007)	N.A	75 ± 25	100 ± 35	86 ± 30

N.A Not addressed

Table 2.3 Distribution of subjects under study in the 4 different Mexican and Chinese locations

Regions	Number of subjects	Women (average age)	Men (average age)
Mexico city	96	56 (32y ± 15)	40 (30y ± 12)
Cuernavaca	93	57 (34y ± 13)	36 (31y ± 15)
Shanghai/center	79	40 (33y ± 12)	39 (36y ± 10)
Chongming	80	40 (35y ± 10)	40 (35y ± 10)

women and men of comparable ages) were recruited as residing in city center whereas the other half were living in a close surrounding (<100 km, Cuernavaca in Mexico, Chongming in China) that is much less daily exposed to aerial pollution. The records of Air Pollution Indexes issued by local official bureaus confirm higher contents of pollution markers (NO, NO_x, SO_2, PM, O_3) in the atmosphere of both city centers (Table 2.2).

Table 2.3 summarizes the composition of the four cohorts of studied subjects.

Non-invasive samplings, using cotton pads imbibed with an ethanol water solution 70/30 v/v solution or adhesive D'Squame® stripping were performed on various sites of the faces of all subjects. Prior to samplings, some instrumental measurements were performed on the same facial locations (skin pH, Sebum Excretion Rate, Skin colour, Skin hydration) using standardized techniques. From cotton pads, following extraction by methanol, analytical assays of total lipids, squalene, Vitamin E, Cholesterol, lactic acid were carried out whereas the adhesive D'Squame® disks allowed collected proteins, ATP and interleukin (IL1α) residual content to be analyzed. All technical details can be found in the two previously quoted publications from Nguyen et al. For practical reasons (un-availability of equipment, methods still in development at these periods), the SQOOH forms could not unfortunately be analyzed.

Overall, most results of these two studies converge and showed significant differences in many parameters between a polluted environment and a less polluted one, in both countries and independent of gender. With regard to squalene and lipids, two major and significant ($p < 0.01$) findings were as follows:

– Squalene content (versus total lipids) much decreased (by approximately 50 %) in a polluted environment, suggesting that its (per)oxidized forms increased by a comparable extent.

– The ratio Vitamin E/Squalene strongly decreased in a polluted environment (by almost 90 %). In other words, a possible protective action towards (per)oxidization of squalene becomes abolished by an environmental pollution. Such decrease in Vitamin E (likely unrelated to differences in Vitamin E intakes by such close subjects) is in agreement with a previous work (Thiele et al. 1997) showing how ozone may deplete Vitamin E.

These results confirm an oxidative boosting impact of polluted environments.

2.4.2 *Possible Influences of Other Factors from a Polluted Aerial Environment*

On a practical basis, sampling skin surface lipids on the face is easily performed non-invasively and the regular sebum excretion affords a constant supply of a "fresh" sebum/squalene, as control of ulterior oxidative events. The latter may be driven by various factors present in an aerial polluted environment, susceptible (or not) to generate singlet oxygen from O_2 through UV irradiance. Some of these are listed in Table 2.4, showing that their implication of some airborne pollutants in the Squalene oxidization process largely remains to being explored.

In real life conditions, assessing the actual impact(s) of UVA and Visible rays, shown as important (direct or indirect via Porphyrins) inducers of oxidizing agents, is a rather difficult task since airborne particles, fumes of all kinds in a heavily polluted environment, shield (filter out) almost all sun rays. This paradoxical situation creates ambiguities when aiming at evaluating the relative contributions of solar rays and airborne pollutants in the oxidization processes of squalene. Whatsoever, from a protective aspect, applications of UVB-UVA sunscreens, intakes of natural anti-oxidants (Vitamin E in vegetable oils, Vitamin C in fruits) seem being logical measures. With regard to environment, it has to be kept in mind that indoor conditions more concentrate some volatile oxidizing compounds than an outdoor environment. This is particularly relevant to PAHs that are generated by cigarette smoke as previously mentioned, confirmed by our in vitro approach

Table 2.4 Summary of some major factors susceptible to enhance the (per)oxidization of squalene

Airborne compounds/aerial pollution	Pro-oxidant action/SQ
Ozone (O_3)	Yes. Depletes vitamin E and reacts with vitamin C to generate singlet oxygen
Particulate matters (PM)	Probable but still unexplored Influence of size and content (heavy metals)?
Volatile organic and non organic compounds (NO, SO_2, NO_2, CO, PAH's, aldehydes)	Probable but still unexplored, PAH's excepted
Metallic atoms (Ni, Cd, Pb, Fe)	Probable but still unexplored

exposed above. Apart from volatile elements, the biological consequences of possible contacts between skin and particulate matters still remain largely unknown. A recent work (Tai Long 2015a, b), using pig skin in vivo, indicates that such contacts induce changes in both structural elements and functions of the SC, thereby modifying the skin absorption of drugs.

2.4.3 *Mimicking, In Vitro and/or Ex Vivo, the Impact of Some Environmental Factors upon Squalene Oxidization*

There are many variants and applied purposes (effect of anti-oxidants) that appear versatile and sensitive enough for assessing the impact of UV exposure, aerial or solid compounds upon the oxidization of the oxygen-sensitive (and naturally present) squalene molecule.

Squalene samples:

Two complementary approaches can be used, as previously described (Pham et al. 2015).

(a) Using a squalene standard solution as a model for studying the effect of oxidative processes. This option obviously allows a full control of various in vitro testing, by adding possible effector molecules of a known structure.
(b) Sebum could be collected from skin (face or forehead are the most easily accessible skin sites) using non-invasive procedures (contacts of the skin surface with polytetrafluoroethylene (PTFE) disks or cotton pad wipes, for instance). Sebum collected could be used directly as a thin film (adsorbed in the PTFE disks) or as solution extracted from cotton pad. In this case, squalene is surrounded with other compounds present in sebum (unsaturated fatty acids, porphyrins). In all cases, the determination of basal SQOOH within the collected sebum is paramount since, as mentioned above, excreted sebum is already partially and weakly (per)oxidized. This point is fundamental when collecting, in vivo, a sebum that has been exposed to various conditions (UV, Pollutants).

Stress exposures and further analysis:

In a thin support like PTFE disks, samples of collected human sebum were placed into chamber with a quartz window to allow UVA exposures. To simulate different forms of aerial stress, cigarette smoke (2 puffs as example) or an aerosol mixture (gases or PM) can fill the volume of the quartz chambers.

Solvent extraction of sebum or pure squalene from the Teflon Disks is further carried out, using methanol as solvent. Following filtration, the extract is analyzed

through the method exposed earlier. In brief, these technical approaches can offer in vitro, or ex vivo methods to evaluate oxidative effects upon skin lipids from various aerial environments.

2.5 Biological Consequences of Squalene (Per)oxides on the Skin

Many findings from previous works converge. As previously mentioned, the link between Sun exposure and comedogenesis (onset of acneic lesions) was early suggested and the pivotal role of squalene in such a process was further specified (Chiba et al. 2000; Ottaviani et al. 2006). Squalene (per)oxides were shown active mediators in the development of inflammatory acne (Picardo et al. 1991; Ottaviani et al. 2010). At the cellular level, squalene monohydroperoxide was shown depleting glutathione, an important compound within the natural anti-oxidant cellular system (Chiba et al. 2001). Topical applications of squalene monohydroperoxide onto the skin of hairless mice enhanced the skin roughness and induced a wrinkling process (Chiba et al. 1999, 2003). Little is known, however, whether same effects can occur on the human skin as a response. When applied onto the skin of guinea pigs, squalene peroxides led to a hyperpigmentation via the release of prostaglandin E_2 by keratinocytes (Ryu et al. 2009). In humans, oxidized surface lipids are viewed as potent inflammatory mediators in many skin afflictions such as pityriasis versicolor or seborrheic dermatitis (De Luca and Valacchi 2010). A very recent paper (Oyewole and Birch-Machin 2015) examines the mediating role of UVR-oxidized lipids as activators of NALP3 inflammasome (Nod Like Receptor Proteins).

2.6 Perspectives/Conclusion

The in vitro and in vivo data presented here indicate that squalene may be considered as a reliable biomarker of the impact of some pollution-related oxidative processes upon the human skin. Although other skin lipids (unsaturated fatty acids, cholesterol) might be used as markers of oxidative events, their oxidized forms may pose technical limits in detection, stability or specificity. The analytical determination of oxidized forms of squalene affords a reliable detection of very low signals of an oxidative environment, i.e. prone to record subtle impacts of oxygen-driven assaults upon the skin.

An external environment implies the combination of many different elements, gaseous and/or solid, at variable concentrations according to external, changing and often uncontrollable conditions (weather, air-ventilation, time of the day, geographical location). This aspect makes it hardly possible, in real life condition, to assess the precise contribution of each given element in the formation of (per) oxidized forms of squalene.

However, although squalene is particularly sensitive to singlet oxygen, it appears clear that the SQOOH/SQ index shall be viewed as a global signal since induced by various oxidative mechanisms (and agents).

On the one hand, as sebum is constantly renewed by the skin and easily collected from the skin surface, squalene and its oxidized forms could be used to record a short term and low external oxidative stress such as the one induced by low doses of UVA (2.5 J/cm^2). On the other hand, with regard to peroxides instability, our own experience on repeated exposures to oxidative environment, such as UV, has revealed that these do not lead to a progressive accumulation of SQOOH forms onto the skin surface. Longer exposures probably need to record other—and possibly secondary—oxidative radical side events (induced or not by SQOOH) such as carbonyl adducts on SC proteins, as example.

Such considerations call for the complementary and practical in routine uses of in vitro models where an aerial environment can be more easily controlled, by introducing a given gaseous element (Ozone, NO, SO_2) at realistic dosages. To such perspective, the use of reconstructed skin techniques (Marionnet et al. 2010, 2014; Duval et al. 2012) is possibly a valuable approach since also allowing contacts with PM or lipids, squalene included. These reconstructed tissues, that can be used for safety or efficacy purposes, offer structures and functions of much similarities with those of real skin, even allowing genes activated or shut-down by a given compound or electro-magnetic waves to being detected (Marionnet et al. 2012; Cottrez et al. 2015). These precious investigative tools have been proven reproducible and some are now introduced within the legal frame of U.E., as alternative testing methods to animals. In addition, they offer a wide pattern of applications since possibly composed by different cell types, i.e. extended to other tissues than skin alone. For instance, the impact of a given pollutant upon a reconstructed cornea (Skinethic, France) may help to decipher the mechanisms involved in ocular irritation, a frequent symptom that occurs during a period of heavy aerial pollution (Wieslander and Norbäck 2010; Novaes et al. 2010). The availability of in vitro techniques of hair growth (Thibaut et al. 2003; Collin et al. 2006) may well contribute to explore the possible and specific impacts of some pollutants upon the hair follicle physiology.

These available in vitro models seem much complementary to in vivo experiments such as those exposed above, by describing skin parameters that specifically reflect the impact of a given air pollutant. In addition, future in vivo experimental protocols should include, in the next future, non-invasive measurements offered by (bio)physical technics. Their possible contributions (e.g. Skin Imaging under UVA or IR rays, Photo-acoustics, Skin Fluorescence recorded by using Confocal laser Microscopy or Multi-photon microscopy etc.) should obviously be initially explored on the skin of subjects who are daily exposed to differently polluted indoor or outdoor environments. The combination of all possible non-invasive techniques is likely a pre-requisite for better evaluating the actual cumulative impacts of this complex aerosol upon the human skin and their related possible side effects. Meanwhile, from a skincare viewpoint, the use of UVB-UVA sunscreens, anti-oxidant enriched formulations, together with efficient and well-tolerated

cleansing products offer practical preventive and corrective actions against undesired—and possibly deleterious—oxidative events that daily assault and challenge the cutaneous tissue.

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