1.1 SKIN

The skin is the largest organ of the human body. It is a complex epithelial and mesenchymal tissue comprising a multilayered stratified epidermis, adnexal structures such as hair follicles, sweat glands and sebaceous glands, a dermis containing collagen and elastic fibres, and underlying subcutaneous fat.

The skin is often said to be the largest organ in the body, comprising 16% of total body weight. The word ‘integument’ is derived from the Latin integere, meaning to cover. The skin consists of two layers, the epidermis and the dermis\textsuperscript{1, 2}.

Skin is the outermost organ of the body so it is first organ to be effected by change in external environment and hence it plays most important part in body defense mechanism. The skin functions as a first line of defense against invading microorganisms.

The mechanisms by which it is able to do this include the production of antimicrobial peptides, resident epidermal Langerhans cells, and transient epidermal T-cells.
Table 1.1 Skin Components and Functions³

<table>
<thead>
<tr>
<th>Cell type/component/system</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melanocytes</td>
<td>Synthesize pigment for protection from UV radiation¹⁸,¹⁹</td>
</tr>
<tr>
<td>Keratinocytes</td>
<td>Cell signaling, barrier function, mechanical protection, cytokine production,</td>
</tr>
<tr>
<td>Collagen</td>
<td>ECM component</td>
</tr>
<tr>
<td>Langerhans</td>
<td>Antigen presentation Cell(APC)</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>Synthesis and degradation of ECM</td>
</tr>
<tr>
<td>Subcutaneous fat</td>
<td>Thermoregulation, energy storage</td>
</tr>
<tr>
<td>Elastin</td>
<td>ECM component</td>
</tr>
<tr>
<td>Dermal vascular bed</td>
<td>Thermoregulation</td>
</tr>
<tr>
<td>T-Cells</td>
<td>Transient in epidermis</td>
</tr>
<tr>
<td>Sensory afferent</td>
<td>modalities include touch, vibration, temperature, pressure, pain and itch and transmit stimuli to the central nervous system.</td>
</tr>
</tbody>
</table>

1.1.1 The Colour of the Skin⁴

Normal skin colour is determined by a number of chromophores, the most important of which is melanin. Besides melanin, other chromophores that contribute significantly to skin colour include haemoglobin (in both the oxygenated and reduced state) and carotenoids.

Racial and ethnic differences in skin colour are related to the number, size, shape, distribution and degradation of melanin-laden organelles called melanosomes. These are produced by melanocytes and are transferred to the surrounding epidermal keratinocytes.

Two types of melanin pigmentation occur in humans. The first is constitutive skin colour, which is the amount of melanin pigmentation that is genetically
determined in the absence of sun exposure and other influences. The other is *facultative* (inducible) skin colour or ‘tan’, which results from sun exposure. Increased pigmentation can also be due to endocrine, paracrine and autocrine factors.

**Table 1.2 Fitzpatrick Classification of Skin Phototypes.**

<table>
<thead>
<tr>
<th>Skin type</th>
<th>Sun sensitivity pigmentary response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td>Fair skin, always burns and never tans</td>
</tr>
<tr>
<td>Type II</td>
<td>Fair skin, burns easily, tans little and with difficulty</td>
</tr>
<tr>
<td>Type III</td>
<td>Fair skin, burns minimally, tans gradually</td>
</tr>
<tr>
<td>Type IV</td>
<td>Olive skin, burns minimally, always tans easily</td>
</tr>
<tr>
<td>Type V</td>
<td>Dark skin, rarely burns, tans deeply</td>
</tr>
<tr>
<td>Type VI</td>
<td>Very dark to black skin, never burns, tans deeply</td>
</tr>
</tbody>
</table>

1.1.2 Melanin Pigment

Melanin Pigments produced by melanocytes are deposited throughout Epidermis, which is determinant of skin color. However disturbances in the amount and distribution of melanin pigments might ultimately provide clues to several diseases.

**Albanism:** Genetic abnormality caused by deficiency in melanin biosynthesis.  
**Hypo pigmentation:** Albanism which manifests as hypo pigmentation is associated with sensitivity to UV radiation and predisposition to skin cancer.  
**Hyper pigmentation:** Abnormal accumulation of melanin pigments is responsible for hyper pigmentation including Melasma, Freckles, and Senile lentigens.

1.1.3 Melanocytes and Melanogenesis

Melanocytes (MCs) develop from melanoblasts that are derived from ectodermal neural crest cells. They are present in the basal layer of the epidermis in a ratio of 1:30 keratinocytes (KCs). Distribution varies in different body parts and there is a reduction with age.
Racial differences in skin colour are not so much due to a difference in number of melanocytes present but rather degree of melanin/melanosome synthesis that occurs. Although MCs constitute <1% of epidermal cells, they and their products account for virtually all visible pigmentation.

Important functions include cosmesis, barrier against ionizing radiation and scavenger of cytotoxic radicals.

- The exact chemical structure of melanin is not clear. It is produced via phenylalanine, tyrosine, DOPA, dopaquinone and, depending on presence of cysteine, coverts to either eumelanin (brown/black) or pheomelanin (yellow/red). The process is facilitated by copper containing enzyme tyrosinase that is bi-functional. The biosynthesis of melanosomal components is facilitated by the golgi-endoplasmic reticulum lysosomal system. It then either develops into eumelanin or if cysteine is high, into pheomelanin. KCs phagocytose melanin laden dendritic tips of melanocytes, and the skin pigmentation that results depends on amount of melanin transferred.

- There are 5 forms of melanocortin receptors, MC1-R to MC5-R, which are a sub-family of G protein-coupled receptors. MC1-R is found in melanocytes and keratinocytes, and expression is stimulated by UVA, - MSH and ACTH. MC1-R variation is the major determinant of red hair (loss of function). 30 allelic variants have been described.

- Dosage effect of MC1-R variation on skin types and hair colours are well recognized. Studies have shown that over-representation of “red-hair alleles” is associated with increased risk of both melanoma and non-melanoma skin cancer. This is related to increased production of photosensitizing or potentially mutagenic pheomelanin.
1.1.4 Melanocyte Biology

Melanocytes of skin, hair follicle, inner ear and uveal tract of the eye (iris, ciliary body, and choroid) originate from the neural crest. During development, these melanoblasts migrate to the dermis along the dorsolateral pathway of the neural tube. They ultimately emigrate into the epidermis and differentiate into dendritic melanocytes, residing in the hair follicles and interfollicular epidermis.

The retinal pigment epithelium (RPE) is formed from the outer layer of the neuroectodermal optic cup that in turn is derived from the developing forebrain of the embryo. Melanin production continues postnatally only in melanocytes of the epidermis and the RPE.

Table 1.3: Main Types of Epidermal Melanin Pigments.

<table>
<thead>
<tr>
<th>Type</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eumelanins</td>
<td>Brown or black nitrogenous pigments, insoluble in all solvents, which arise by oxidative polymerization of 5,6-dihydroxyindoles derived biogenetically from tyrosine</td>
</tr>
<tr>
<td>Phaeomelans</td>
<td>Alkali-soluble pigments, ranging from yellow to reddish-brown; most of them contain sulphur in addition to nitrogen and arise by oxidative polymerization of cysteinyldopa via 1,4-benzothiazine intermediates</td>
</tr>
<tr>
<td>Trichochromes</td>
<td>A variety of sulphur-containing phaeomelanic pigments with a well-defined structure, characterized by a Δ2,2′-bi(1,4-benzothiazine) chromophore</td>
</tr>
</tbody>
</table>

1.1.5 Epidermal Melanin Unit

Melanin synthesis takes place in distinct cytoplasmic organelles known as melanosomes located in melanocytes, which inhabit the stratum basale of the epidermis.

Melanosomes originate from the endosomal compartment. The mature melanin-filled melanosomes travel along the dendrites and are transferred to the neighboring keratinocytes. In keratinocytes melanosomes are translocated over the nucleus where they protect the DNA from solar ultra-violet (UV) radiation.
Thus, keratinocytes bearing the internalized melanosomes divide and move to the upper layers of the epidermis, causing the skin to look visibly pigmented. This association between one melanocyte and approximately 36 keratinocytes is defined as the epidermal melanin unit (EMU).

### 1.1.6 Melanosome Formation

There are four successive stages in melanosome formation. Stage I “premelanosomes” are electron-lucent, membrane-delimited spherical structures with variable amounts of poorly organized internal membranes and no melanin.

They are similar in morphology to early multivesicular endosomes. In stage II, the melanosomes acquire an elliptical shape with intralumenal fibers that run the length of the organelle in an organized array, producing a striated appearance. In stage III, there is a regular periodic deposition of electron-opaque melanin on these fibers, resulting in blackened and thickened striations and finally in stage IV, the melanosome is completely melanised such that all the intralumenal structure is obscured by the melanin.

These striations likely function to detoxify eumelanin intermediates, to sequester and concentrate melamins, and in epidermal melanocytes to prevent diffusion of melanin during transfer to keratinocytes. Integral membrane protein Pmel17 (also known as gp100) is preferentially enriched in premelanosomes. Tyrp1, and likely tyrosinase and Tyrp2, are enriched in later stage, pigmented melanosomes. Melanosomes have a low internal pH of 4 – 4.5. The more highly melanized melanosomes appear to have a lower internal pH. Individual skin types differ in their ability to produce mature fully melanized, melanosomes in addition to the ratio of eumelanin to pheomelanin.

### 1.1.7 Activity of Tyrosinase

Tyrosinase, an enzyme catalyzing the rate limiting step for the biosynthetic pathway of melanin pigments, is widely distributed in nature.

- It is responsible for coloring of Skin, Hair& Eyes in animals.

- It catalyzes two distinct reaction of conversion of tyrosine to Dopa (tyrosine hydroxylase activity; tyrosine,3,4-dihydroxyphenylanaline,
Introduction

The enzyme oxidizes phenols and diphenols using a catalytic mechanism.

That depends on the presence of Copper atoms at the active site.

Dopaquinone produced by Tyrosinase is nonenzymatically converted to dopachrome, which is acted upon by an isomerase producing dihydroxyindoles.

Melanin pigments are eventually produced by further oxidation and polymerization of indoles.

Melanins are complex polymers derived from tyrosine and other intermediates, which are converted through a multistep process of oxidative and complexation reactions to brown black eumelanins and yellow-red pheomelanins, which create the diversity of coloration observed across the human population.

The regulation of melanin production is very complex and involves more than 80 genes. The synthesis is regulated by various extracellular signaling components that trigger a signal transduction cascade. There is also evidence that fibroblasts participate in this signaling. While the baseline state of melanin in each individual’s skin is dictated by genetic composition, internal and external triggers such as aging and UV exposure can lead to significant alterations in net synthesis of the melanins.
1.2 PIGMENTATION$^{6,7}$

Pigmentation is the most visible phenotypic characteristic in nature. Among the various pigments, melanin is widely distributed in living organisms such as bacteria, fungi, plants, animals and human beings. Human skin color is determined principally by the amount, type and distribution of melanin in the epidermis. It is the consequence of communication between and cooperation of two distinct cell types in the epidermis, the melanocyte and keratinocyte. Melanins are heterogeneous polyphenol-like biopolymers with a complex structure, synthesized in melanosomes within the melanocytes.
1.2.1 Pigmentary Skin Disorders

In the initial evaluation of pigmentary disorders are: the nature of the pigmentary disorder i.e. hyper, hypo or depigmentation, the cause of the pigmentation i.e. is the pigmentation due to melanin or other pigments, morphology, areas involved, textural change of involved skin, size, colour, gender, age of onset, family history, response to sun exposure, progression of lesion, associated congenital anomalies, associated medical problems and the presence of any offending drugs. These features allow for a working classification that enables the dermatologist to reach a rational diagnosis or differential diagnosis, to prognosticate and for proper subsequent management (which includes patient and perhaps even genetic counseling, treatment and prevention of possible future complications).

- Measurements of skin colour are essential in the overall documentation of pigmentary skin disorders as such measurements allow for progression / improvements to be tracked by the attending dermatologist. Such measurements can be performed in various ways, these include: visualization, photography under natural light or UV, photography with computerized image analysis, spectrophotometry or tristimulus colorimetry.

- There has been much progress in pigment cell research in the last 2 decades. Rapid progress is expected especially with advances in genomics and proteomics. Pigmentary disorders offer many research opportunities. The genetic basis of more diseases will be unraveled in the near future. Genetic engineering may become useful in therapy and prevention of some diseases. Understanding the mechanisms of post-inflammatory pigmentation and controlling these pathways for the benefit of patients will be one of the great challenges for dermatologists in the future.
1.2.2 Pathology of Pigmentary Skin Disorders

Histopathology may assist the dermatologist in the evaluation of pigmentary skin disorders. A histopathological approach to the common pigmentary skin disorders is as follows:

A. Disorders of hypopigmentation

In hypopigmentary skin disorders, melanin deposition is decreased and melanocytes present are normal, decreased or totally absent. Hypopigmentation may result from the following:

i. Abnormal migration/differentiation of melanoblasts – piebaldism

ii. Destruction of melanocytes – vitiligo

iii. Reduced tyrosinase activity – ocular cutaneous albinism type 1A

iv. Abnormal structure of melanosomes – Progressive macular hypomelanosis

v. Reduced melanization and/or numbers of melanosomes – albinism.

vi. Reduced transfer to keratinocytes – postinflammatory leukoderma.

B. Disorders of hyperpigmentation

In hyperpigmentary skin disorders, melanin deposition is increased and melanocytes present may be normal or increased. Hyperpigmentation may result from the following:

i. Increased melanin deposition

   a. Generalized – Addison’s, ACTH and MSH producing tumours, drugs, sun-exposure.

   b. Localized – freckles, melasma, melanosis.

ii. Increased melanocytes

   a. Lentigenes – lentigo simplex, actinic lentigo, LEOPARD syndrome.

iii. Pigmentary incontinence – Post-inflammatory hyperpigmentation, macular amyloidosis.

iv. Hyperpigmentation with epidermal changes
   a. Lentigo – lentigo simplex, actinic lentigo
   b. Reticulate pigmented dermatosis – Dowling-Degos, Kitamura’s.

v. Reactive pigmentation and melanocytic colonization – Solar lentigo, seborrhoeic keratosis, melano-acanthoma, basal cell carcinoma, solar keratosis, adnexal tumours.

1.3 SKIN WHITENING\textsuperscript{11,12}

Conventionally what we mean by whitening is the elimination of the melanin that acts as a self defense mechanism for the human skin against the exposure to Ultraviolet light. The meaning of the expression “whitening”, however, has evolved to mean far more. It entails inhibiting the further synthesis of melanin. Functional, natural cosmetics are in vogue recently, which carries the skin whitening function, for example, retinal and collagen. Cosmetics that contain the retinal, which is vitamin A, is quite good for removing freckles and stretch marks by retarding the skin’s aging process and reliving it of pigmentation. Whitening cosmetics that contain vitamin C, on the other hand, are popular because they are effective in whitening and wrinkle elimination.

Products that help enhancing the skin’s immunity are also recommended with best skin permeation rate. It is important not to forget that those products are just supplementary for the inhibiting pigmentation. Mainly the skin whitening ingredients works in three ways –

- By absorbing the UV rays, thus preventing the sun from darkening your skin.

- By reducing the production of melanin, the skin pigment found in your skin which is responsible for skin darkening.
Most skin whiteners currently in the market contain ingredients—Hydroquinone, Ascorbic acid, Kojic acid, Glycyrrhetinic acid acts as direct inhibitors of tyrosinase, the enzyme in the skin pigment cells which produce melanin.

1.4 INTRODUCTION TO ASPASOMES$^{22,23}$

1.4.1 Ascorbyl Palmitate

Ascorbyl Palmitate is a highly bioavailable, fat-soluble derivative of ascorbic acid. Ascorbyl palmitate possesses all the benefits of vitamin C, but unlike the water-soluble form, is able to be stored in the lipid cell membrane until the body is ready to put it to use. Vitamin C offers a wide range of support for the human body. It is a potent antioxidant and free radical scavenger supporting cellular and vascular health. Vitamin C has been reported to promote nitric oxide activity as well as to help maintain healthy platelet function. It supports the body’s defense system by enhancing white blood cell function and activity, and increasing interferon levels, antibody responses, and secretion of thymic hormones.

Furthermore, this antioxidant has histamine lowering properties and increases lymphocyte formation. It is also essential for the formation and maintenance of intercellular ground substance and collagen, important for joint health. Vitamin C aids in the absorption of iron and the formation of red blood cells and converts folic acid to its active forms. Ascorbyl palmitate is derived from corn dextrose fermentation and palm oil.

1.4.2 Aspasome

Aspasome is a ascorbyl palmitate vesicle with biological activity, Ascorbyl palmitate (ASP) was explored as bilayer vesicle forming material. It formed vesicles(Aspasomes) in combination with cholesterol and a negatively charged lipid (dicetyl phosphate).

Ascorbyl palmitate is a Ascorbic acid esters which is amphiphilic in nature and studies delineating their surface active properties. It is capable to suppress
pigmentation of the skin and decomposition of melanin, it can be used to whiten the skin. It also improves elasticity of the skin by promoting the formation of collagen. Ascorbyl palmitate is more stable than ascorbic acid. Its lipophilic character is beneficial for its skin penetration.

Aspasomes were prepared by film hydration method under nitrogen atmosphere in which 200 µmol of lipid mixture (ascorbyl palmitate: cholesterol in varied molar ratio with dicetyl phosphate included at 10 mol% of the total lipid) was dissolved in 9ml of chloroform and 1ml of methanol in a round bottom flask and was kept under reduced pressure in rotary evaporator at 50°C till it formed a thin dry film on the walls of the flask. The dried thin lipid film was hydrated with 10 ml of phosphate buffered saline (PBS, pH 7.4) containing drug substance. The vesicles were then sonicated for 2 min using ultrasonicator on which power was set at 50% of maximum output. The Aspasomes were stored in nitrogen-purged vials.

1.5 INTRODUCTION OF HYDROQUINONE
1.5.1 Chemical and Physical Data:
1.5.2 Nomenclature: 1,4-Benzenediol
1.5.3 Synonym: Benzoquinol
1.5.4 Chemical and Physical properties of the pure substance:
a) **Description:** Hexagonal Prisms  

b) **Boiling-Point:** 287° C  

c) **Melting-Point:** 172.3° C  

d) **Solubility:** Soluble in water, ethanol and diethyl ether.  

e) **Vapour Pressure:** 532 Pa at 150° C; relative vapour density (air=1), 3.81  

f) **Flash-point:** 165° C, closed cup  

g) **Conversion factor:** $\text{mg/m}^3 = 4.5 \text{ ppm}$  

1.5.5 **Production and Use:** In 1992 world production of hydroquinone was approximately 35 thousand tonnes (WHO 1994). Hydroquinone is used as photographic developer (with black and white film), a dye intermediate, a stabilizer in paints, varnishes, motor and fuel, an oils, an antioxidant for fats and oils, an inhibitor of polymerization and in the treatment of skin hyperpigmentation.

1.5.6 **Occurrence:**

a) **Environmental Occurrence:**  
Hydroquinone is both a natural and anthropogenic compound. It occurs naturally as a conjugate with $\beta$-D-glucopyranoside in the leaves bark and fruit of a number of plants, espicially the ericaceous shrubs such as cranberry, cowberry, bearberry. It maybe released to the enviornment as a fugitive emission, during its production, formulation and use as a chemical intermediate, photographic chemical and stabilizer. Users of skin-bleaching formulations may be exposed to hydroquinone.

1.5.7 **Studies of Cancer in Human:**  
Pifer et.al (1995) reported a cohort mortility study of 879 workers at a plant Tennessee (United States) plant in which hydroquinone was manufactured and used over several decades. Job history records were linked to extensive industrial hygiene data and expertise to estimate cumulative exposure to hydroquinone. Average hydroquinone dust levels ranged from 0.1 to 6.0 mg$^3$, with levels over 2mg/m$^3$ for most of the period of operation of the plant. Mean employment duration was 13.7 years and mean follow-up from first exposure was 26.8 years. Relative risk eliminates (Standarized Mortality Ratios) SMRs for this cohort were derived by
comparison with the general population at Tenessee as well as occupational cohort not exposed to hydroquinone.

The SMRs for all causes of death combined (n=168) was significantly below 1.0 as was the SMR causes for all kind of death (n=33). Only two sites colon (n=5) and lung (n=14) had more than three observed cases. More site specific MRs were well below 1.0. The results were similar for both population comparison.

The dose-response analyses of selected cancer sites did not reveal any meaningful trend of heterogeneity.

1.5.8 Toxic Effect:
The toxicity of hydroquinone reviewed (WHO, 1994)

  a) On humans no data were available on the working group
  b) Experimental systems: Long term feeding of hydroquinone to rats led to aplastic anaemia, liver cord-cell atrophy and ulcer of gastric mucosa, Aingle high dose was reported to induce renal tubule necrosis.(IARC, 1977)

In the carcinoenecity study (United States National Toxicology Program, 1989) , nephropathy was observed in nearly all male and female rats of all dosed groups and vehicle controls. The nephropathy was characterised by degeneration and regeneration of tubule epithelium, atrophy and dilation of some tubules, hyaline casts in the tubule lamina, glomerulosclerosis, interstitial fibrosis and chronic inflammations. In males the nephropathy was more severe in high-dose (50 mg/kg bw per day) group, while in female no dose-dependence was observed. Nephropathy was observed also in males in 13 week studies.

1.5.9 Hydroquinone Use as Skin Lightening Agents (SLA): SLAs are used to treat hyper pigmentation. Dermatologists, Pharmacists, applied botanists and allied heath care researchers have discovered and undertaken various in-vitro and in-vivo studies on SLA. Hydroquinone, its derivatives, several plant extracts, either monotherapy or in combination are tried to achieve the skin-lightening effect and are being used in cosmetics. Each approach has its advantages and disadvantages with adverse side effects. It is analyzed the latest developments of SLA with reference to in vivo studies and clinical trials. Tyrosinase inhibitors play a vital role as a SLA. SLA mostly act as tyrosinase inhibitors and tyrosinase is a potential target in the search for a newer SLA.
Hydroquinone and topical steroids: benefits and side effects. The gold-standard SLA is hydroquinone. Hydroquinone covalently binds to histidine or interacts with copper at the active site of tyrosinase. Besides tyrosinase inhibition, alteration of melanosomes functions, depletion of glutathione, generation of reactive oxygen species, and subsequent oxidative damage of membrane lipids and proteins may play a role in the hypopigmenting effect of hydroquinone.

Hydroquinone is very unstable and rapidly gets oxidized. It can induce erythema, skin irritation, contact dermatitis, and permanent skin depigmentation. Its cytotoxic effects prevent its usage in cosmetics. Exogenous ochronosis, cataract, pigmented colloid millium, sclera and nail pigmentation, loss of skin elasticity, and impaired wound healing of skin are the adverse effects of hydroquinone. Ochronosis commonly presents as asymptomatic blue-black macules on the malar areas, temples, inferior cheeks, and neck. Chronic usage of cosmetics containing this SLA exudes an offensive fish odor in the sweat that is described as "fish odor syndrome." This is due to excretion of a chemical, trimethylamine in the breath, urine, sweat, saliva, and vaginal secretions. Phenols, resorcinol, and quinine are also associated with ochronosis.