1. INTRODUCTION

1.1 SKIN AGING

1.1.1 General

Aging is a complex process that defines the changes observed throughout the organism's lifespan and cannot be defined by a single pathway or a single cause. Aging is controlled by both environmental factors and the genetic constitution of the individual (Osiewacz 1997), and has been described as a progressive decline of the ability to withdraw stress, damage and disease. Furthermore, it is characterized by an increase of degenerative and neoplastic disorders (Jazwinski 2000).

Human civilization has been always fascinated with overcoming aging and conserving eternal youth. From the ancient to the medieval ages, myths like the fountain of youth, also illustrated by the great painter Lucas Cranach the Elder, were very popular (Fig. 1.1.1).

In the course of time, the first signs of aging become evident and skin, which is the largest organ of the body accounting for 12% to 16% body weight, is the first obvious evidence of this process. Nowadays, as life expectancy is showing a steady increase (Oeppen and Vaupel 2002), the impact of aging on the function and appearance of skin is receiving growing interest. People do not only want to live longer but also remain and look healthy.

1.1.2 Skin

Skin exhibits multiple functions, among them it serves as a protective barrier between internal organs and the environment and is a complex organ with multiple cell types and structures. It is divided into three major compartments: epidermis, dermis and subcutaneous tissue.

The epidermis is the most superficial layer of the skin and is approximately 100 μm thick. The epidermis is a keratinized stratified squamous epithelium and its main function is to protect the body from harmful stimuli of the environment and diminish fluid loss. The principal cells of this region are keratinocytes which make up 95% of the epidermal cells. The epidermis is subdivided into five layers or strata, the stratum germinativum, the stratum spinosum, the stratum granulosum, the stratum lucidum and the stratum corneum in which a

keratinocyte gradually migrates to the surface and is detached in a process called desquamation.

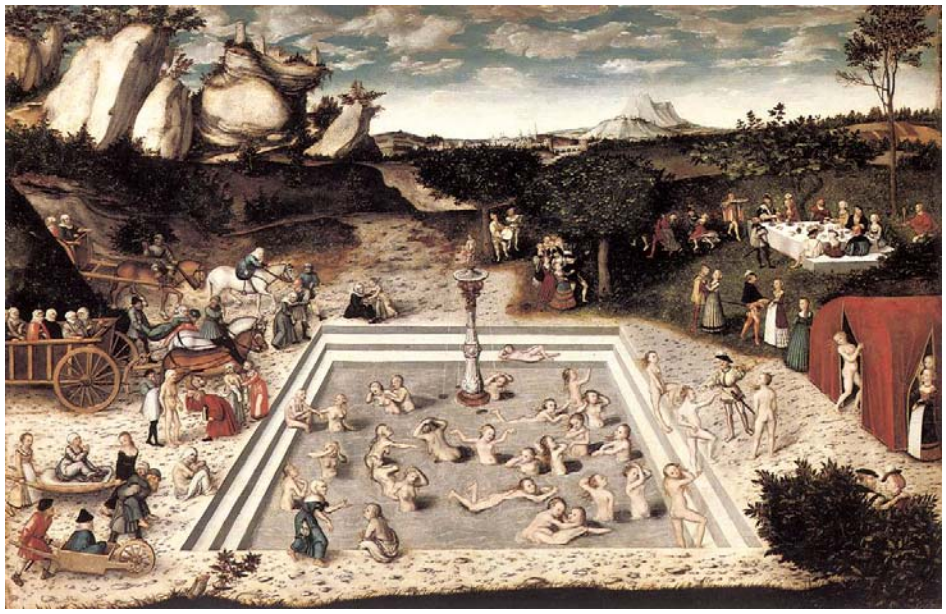

Fig. 1.1.1 "The Fountain of Youth" illustrated by Lucas Cranach the Elder (born 1472, Kronach, died 1553, Weimar). In the left half of the picture elderly women step into the waters of the magic fountain where a gradual process of rejuvenation takes place. Their old appearance disappears, their flesh becomes rosy and smooth, and when they emerge from the water in the right half of the picture they have been turned into young girls again.

The epidermis also consists of 1-2% pigment producing cells called melanocytes, dendritic Langerhans cells, which are the single most important antigens presenting cell population in the skin and Merckel cells, which may act as mechanoreceptors and are thought to have APUD-like (amino precursor uptake and decarboxylation) activity (Kanitakis 2002).

The dermis is the layer of connective tissue to which the epidermis is attached and is approximately 1-2 mm thick. The dermis assumes the important functions of thermoregulation and supports the vascular network to supply the epidermis with nutrients. It is typically subdivided into two zones, a papillary dermis and a reticular layer. It contains mostly fibroblasts which are responsible for secreting collagen, elastin, glycosoaminoglycans, proteoglycans, fibronectin and other extracellular matrix proteins that give the support and elasticity of the skin. Collagen makes up 70-80% of the weight of the dermis. It is composed mainly of glycine, proline and hydroxyproline and is one of the strongest proteins in nature.

Type I collagen is the most abundant protein in skin connective tissue, which also contains other types of collagen (III, V, VII). Newly synthesized type I procollagen is secreted into the dermal extracellular space, where it undergoes enzymatic-processing arranging itself into a triple helix configuration. The triple helix complexes associate with other extracellular matrix proteins, such as leucine-rich small proteoglycans, to form regularly arranged fibrillar structures. This process, called fibrillogenesis, results in formation of collagen bundles (Bateman and Chothia 1996) that are responsible for the strength and resiliency of the skin (Uitto 1986). Type III collagen is the second major fibrillar collagen found in the skin. It is also known as fetal collagen because of its abundance in fetal tissues. Although it is found in equal amounts to type I collagen in fetal skin, in adult skin there is a greater production of type I collagen, which results in a final ratio 6:1 type I: type III (Burgeson, Chiquet et al. 1994). Elastic fibers constitute less than 1-2% of the weight of the dermis, but they play an enormous functional role by resisting deformational forces and returning the skin to its resting shape. Elastic fibers are mainly composed of two distinct proteins, elastin and fibrillin, both produced by resident fibroblasts. Amorphous, hydrophobic, cross-linked elastin constitutes the central core of the fibers, which are surrounded by fibrillin-rich microfibrils. Fibrillin microfibrils are also found as free microfibrils arranged in bundles in the superficial dermis. Elastic fibers form a fine network that extends vertically in the dermal papillae and surrounds dermal blood vessels, while in the reticular dermis they build fibers which are much thicker and run parallel to the epidermis surrounding the larger collagen fibers. They also surround the adnexal structures (Holbrook, Byers et al. 1982; Kielty and Shuttleworth 1997).

In the dermis, immune cells are also present that are involved in defence against foreign invaders passing through the epidermis.

The dermo-epidermal junction is an undulating basement membrane that adheres the epidermis to the dermis. It is composed of two layers, the lamina lucida and lamina densa. The junction is characterized by downward folds of the epidermis called epidermal ridges or rete which interdigitate with upward projections of the dermis called dermal papillae. This structure of the dermo-epidermal junction contributes to minimizing the risk of dermo-epidermal separation by shearing forces (Bruckner-Tuderman 1993).

The subcutaneous tissue consists of fat cells that underline the connective tissue. This layer connects loosely the skin to the underlying fascia. The fat cells insulate and provide energy.

Epidermal appendages are intradermal epithelial structures lined with epithelial cells with the potential for division and differentiation. These are important as a source of epithelial cells, which accomplish re-epithelialization should the overlying epidermis be removed or destroyed in situations such as partial thickness burns, abrasions, or split-thickness skin graft harvesting. Epidermal appendages include sebaceous glands, sweat glands, apocrine glands, mammary glands and hair follicles. They often are found deep within the dermis, and in the face may even lie in the subcutaneous fat beneath the dermis. This accounts for the remarkable ability of the face to re-epithelialize even the deepest cutaneous wounds.

Sebaceous glands or *holocrine glands* are found over the entire surface of the body except the palms, soles and dorsum of the feet. They are largest and most concentrated in the face and scalp where they are the sites of origin of acne. The normal function of sebaceous glands is to produce and secrete sebum, a group of complex oils including triglycerides and fatty acid breakdown products, wax esters, squalene, cholesterol esters and cholesterol (Ramasastry, Downing et al. 1970; Nikkari, Schreibman et al. 1974; Downing, Stewart et al. 1987; Thody and Shuster 1989). Sebum lubricates the skin to protect against friction and makes it more impervious to moisture.

1.1.3 Classification of skin aging

Skin aging is a complex biological phenomenon. Skin, like all other organs, undergoes chronological aging, which depends on the passage of time per se. Chronological aging, otherwise called intrinsic or endogenous aging, is influenced by genetics, hormonal changes and metabolic processes, which appear at advanced age (Rittie and Fisher 2002). Endogenous skin aging can be viewed on non UV-exposed areas of the body and can be considered as model of the aging process taking place in internal organs (Uitto 1986).

Besides, as skin is in direct contact with the environment, it is constantly influenced by various environmental factors (Fisher, Kang et al. 2002), including ionizing and non-ionizing irradiation, air pollution, natural deleterious gases (e.g. ozone and high concentrations of oxygen), smoking, invasion of pathogenic bacteria, viruses, xenobiotics and mechanical stress [extrinsic aging] (Evelson, Ordonez et al. 1997; Podda, Traber et al. 1998; Kohen 1999). Among them UV-irradiation is the most fundamental one, as it can damage skin to such an extent, that makes it seem prematurely aged (photoaging). This premature aging process,

typically found on sun-exposed areas, is cumulative with sun exposure and affects more individuals of skin phototypes I and II.

1.1.4 Characteristics of exogenously aged skin

In contrast to the gradual atrophy of endogenously aged skin, the superposition of enviromental factors especially of UV-irradiation on skin results in massive morphological alterations mainly of the dermis.

The most prominent histopathologic change is the accumulation of abnormal elastic tissue in the mid and deep dermis called solar elastosis (Fig.1.1.4.1A). UV-irradiation induces the elastin gene transcriptional activity by a fourfold increase in elastin promoter activity (Bernstein, Chen et al. 1996) and decreases the fibrillin-1 expression resulting in heavy deposition of elastic fibers, which are dystrophic and truncated. In addition to elastin and fibrillin, normal elastic fibers also appear to contain versican, a large chondroitin-sulfate proteoglycan, which in areas of solar elastosis shows increased expression by immunohistochemical staining (Bernstein, Fisher et al. 1995). Moreover, an increase of a lysine-derived cross-link compound, desmosine, has been demonstrated in photoaged skin (Gonzalez, Moran et al. 1999). On these altered elastic fibers exogenous substances may deposit such as lysozyme, which correlates with basophilic degeneration of the fibers (Suwabe, Serizawa et al. 1999). This abnormal elastic tissue replaces the normal matrix composed mainly of collagen and is almost always separated from the epidermis by a border zone of normal appearing collagen with or without elastic fibers.

Cutaneous photoaging has been also correlated to increased levels of glycosaminoglycans (Bernstein, Underhill et al. 1996). The presence of glycosaminoglycans within the solar elastotic material in sun-damaged skin might be expected from versican deposition on elastic fibers, as versican has a hyaluronic acid-binding domain. Glycosaminoglycans are normally distributed diffusely in the dermis between collagen bundles, and they serve as a major source of hydration in skin, binding water to promote movement of nutrients and cellular metabolites. Although sun-damaged skin contains increased amounts of glycosaminoglycans, they may be associated with the abnormal elastotic material and thus be unable to function in a similar way to glycosaminoglycans in sun-protected skin.

Changes in the collagen content of sun-damaged skin also accompany elastic fiber alterations. Immunohistochemical staining of collagen fibers has demonstrated a sparse distribution in

areas of solar elastosis, in contrast to the normally dense, almost confluent organization of collagen fibers found in the superficial dermis (Bernstein, Chen et al. 1996). Collagen type I has been found to be diminished in photoaged skin (Griffiths, Russman et al. 1993; Trautinger, Mazzucco et al. 1994). Data suggest that the decrease in collagen content in photoaged skin is due to increased collagen degradation, whereas the collagen production remains the same (Varani, Spearman et al. 2001). The enzymatic capacity for extracellular degradation resides in fibroblasts and in inflammatory cells which are increased. Various matrix-metalloproteinases, serine and other proteases are responsible for the breakdown of connective tissue components such as collagen and are induced *in vitro* and *in vivo* by UVA- and UVB-irradiation (Scharffetter-Kochanek, Wlaschek et al. 1993; Brenneisen, Oh et al. 1996; Fisher, Datta et al. 1996; Fisher, Wang et al. 1997). Furthermore, collagen turns up with a basophilic appearance (basophilic degeneration) (Scharffetter-Kochanek, Brenneisen et al. 2000) and it has been shown that the proportion of collagen type III is increased in photodamaged skin (Plastow, Lovell et al. 1987).

Apart from changes in the organization of the structural components of the dermis, also the resident fibroblast of the dermal connective tissue reveals characteristic features in the photoaged skin. The fibroblasts adopt a stellate phenotype and at the ultrastructural level reveal a highly activated rough endoplasmic reticulum indicating an increased biosynthetic activity (Uitto 1986).

Furthermore, an increase in mast cells and neutrophils have been reported in photoaged skin (Lavker and Kligman 1988; Kligman and Murphy 1996; Scharffetter-Kochanek, Brenneisen et al. 2000) showing an inflammatory response to UV-irradiation. The vascular walls of postcapillary venules and of arterial and venous capillaries are thickened by the peripheral addition of a layer of basement membrane-like material. The veil cells, which are intimately related to these layers, often have dilated cisternae of rough endoplasmic reticulum containing electron dense material (Braverman and Fonferko 1982).

The dermo-epidermal junction is also a target for UV-induced skin changes. Fibrillin appears significantly truncated and depleted in the upper dermis in comparison to sun-protected skin and there is a marked loss of fibrillin-positive structures (Watson, Craven et al. 2001). Similarly, keratinocytes expression of type VII collagen, which forms the anchoring fibrils at the dermo-epidermal junction, is reduced in sun-exposed skin areas, confirming data showing

that the number of anchoring fibrils is significantly reduced in photoaged skin. Reduced content of collagen VII may contribute to wrinkles by weakening the bond between dermis and epidermis (Contet-Audonneau, Jeanmaire et al. 1999).

Recent studies have also revealed that UV-irradiation does not only affect the dermis but may also cause alterations in the homeostasis of the epidermis and specifically in the biology of keratinocytes (Bosset, Bonnet-Duquennoy et al. 2003). The homeostasis of the epidermis is a critical balance between proliferation, differentiation, desquamation and apoptosis. Chronic sun exposure impairs all of these processes.

Sun-exposed skin typically shows a thickened epidermis (Kligman 1989), something which can be explained as a consequence of a chronic wound-like condition and a chronic attempt to repair. The stratum corneum is mostly affected and appears thickened resulting from the faulty degradation of the corneocytes desmosomes. *In vivo*, involucrin protein, a differentiation marker normally expressed by irreversibly differentiated keratinocytes in the stratum corneum, has been found to have an increased expression in sun-damaged skin (Bosset, Bonnet-Duquennoy et al. 2003). This observation speaks for the fact that the differentiation process of keratinocytes is impaired by UV-irradiation. In addition, in basal epidermal cells, b1 integrin cell surface expression was greatly reduced with a parallel downregulation in b1 integrin mRNA (Bosset, Bonnet-Duquennoy et al. 2003). B1 integrins are transmembrane receptor heterodimers and are responsible for the connection of the basal keratinocytes with each other and with the basal membrane. There are mainly two types of b1 integrins, a2b1 and a3b1 which interact with extracellular matrix proteins such as fibronectin, laminin 1 and 5, and collagen type I and IV. Integrins are used as markers of the keratinocyte proliferation and their adhesion to extracellular matrix. These data suggest that proliferation and adhesion of aged keratinocytes in sun-exposed skin are significantly impaired.

With photoaging, both hypermelanosis and hypomelanosis are observed. Pigmentary alterations such as mottling, ephelides, pigmented actinic and seborrheic keratoses, pigmented basal cell carcinomas and stellate pseudoscars are often observed as a result of the dysfunction of melanocytes (Castanet and Ortonne 1997; Bosset, Bonnet-Duquennoy et al. 2003). Clinically, photoaged skin is characterized by deep wrinkles, laxity, roughness, sallowness, increased fragility, blister formation, pigmentary changes, teleangiectases, impaired wound healing and benign and malignant growth (Fig.1.1.4.2).

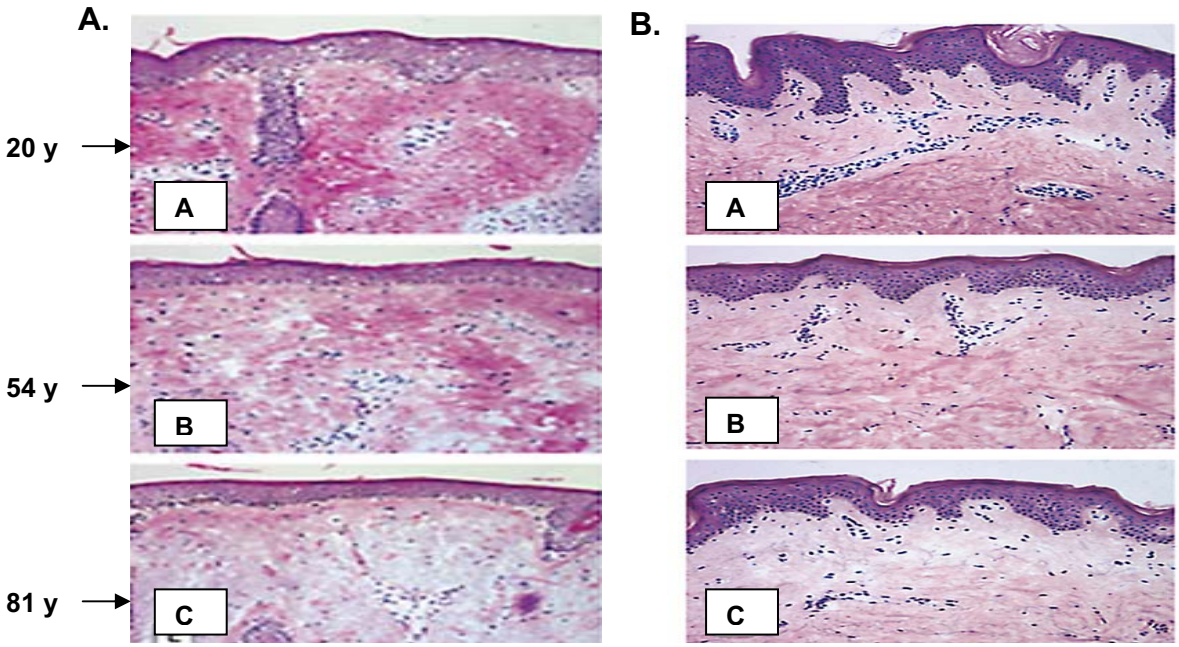

Fig. 1.1.4.1 Representative skin samples of 3 healthy subjects (ages given at left) obtained from chronically sun-exposed skin, where increasing dermal photodamage and reduced rete edge formation is demonstrated (A), and from sun-protected skin (B) where the moderate thinning of the epidermis is demonstrated (A, B, and C; staining with hematoxylin-eosin [H&E]) (Chung, Yano et al. 2002).

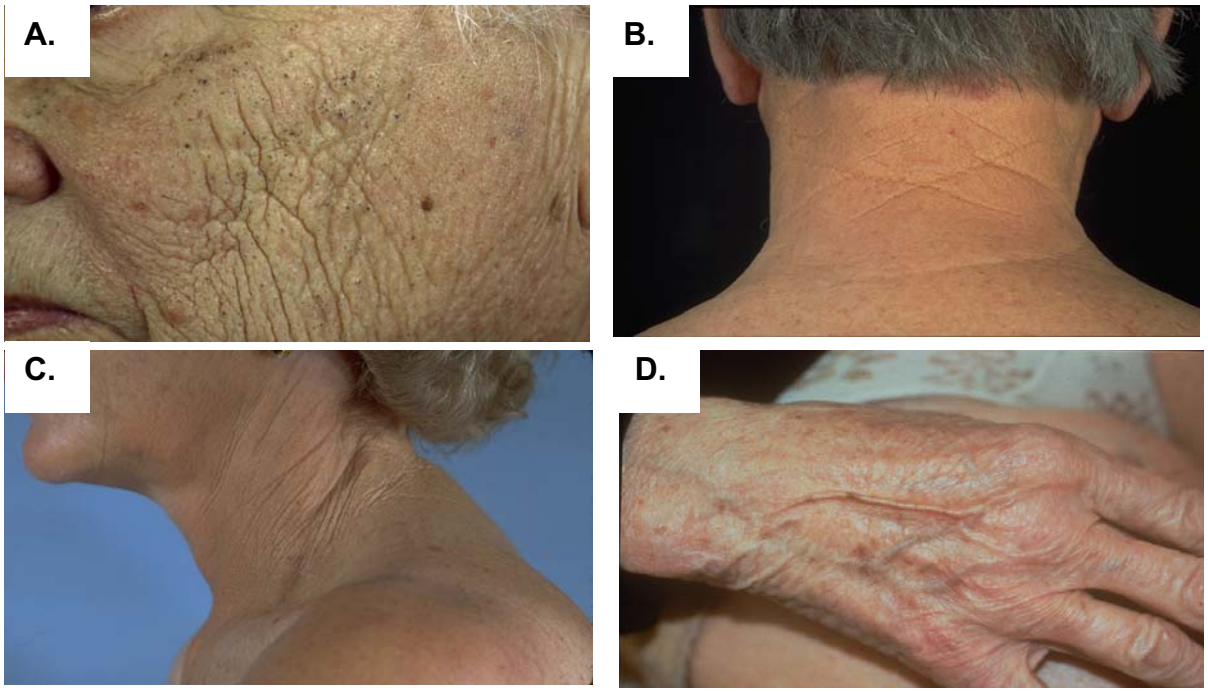

Fig.1.1.4.2 Exogenously aged skin in UV-exposed areas such as face (A), neck (B, C) and back of the hand (D)

1.1.5 Characteristics of endogenously aged skin

During endogenous aging skin gradually loses its structural and functional characteristics. Anatomically, the epidermis undergoes thinning by 10–50% between the age of 30 and 80 (Moragas, Castells et al. 1993; Lock-Andersen, Therkildsen et al. 1997), although the number of the cell layers remains conserved. Atrophy seems to affect mostly the stratum spinosum, whereas neither stratum corneum nor stratum granulosum seem to be affected (Lavker 1979).

Histologically, the most pronounced changes occur within the basal cell layer, stratum germinativum. Basal cells display an increased heterogeneity in size with an overall increased volume (Bregegere, Soroka et al. 2003). These changes are sometimes called 'epidermal dyscrasia' and they are accentuated in photodamaged skin (West 1994). Functionally, epidermal dyscrasia is characterized by decreased mitotic activity, increased duration of the cell cycle and migration time from the basal cell layer to the stratum corneum by 50% (Bauer, Crombag et al. 1980; Grove and Kligman 1983; Engelke, Jensen et al. 1997). Moreover, the intradermal villous cytoplasmic projections of basal layer keratinocytes are lost in aged skin (Lavker 1979; Lavker, Zheng et al. 1987).

Although the stratum corneum is unaltered in thickness, there is apparently a slow replacement of neutral lipids, adversely affecting the barrier function (Lavker 1979; Yaar and Gilchrest 2001). A clear flattening of the dermo-epidermal junction occurs resulting in a decrease in surface contact area by approximately 35% (Moragas, Castells et al. 1993).

The number of melanocytes decrease by 8–20% per decade after the age of 30 (Gilchrest, Blog et al. 1979). In addition, considerable heterogeneity of their morphologic and functional characteristics develops. Foci of activated melanocytes forming lentigines ('liver' spots) are seen aside groups of small, inactive melanocytes manifested as guttate amelanosis (Breathnach, Nazzaro-Porro et al. 1991). The inactive melanocytes are particularly abundant in hair follicles, the reason why graying of hair occurs (Ortonne 1990). Melanin, a chromophore produced by melanocytes, contributes to a big extent to skin fluorescence, which correlates with age, increasing about 2% per year and can serve as a useful marker of endogenous aging *in vivo* (Na, Stender et al. 2001).

The number of Langerhans cells decreases significantly from approx. 1200 mm^{-2} in young skin to approx. 800 mm^{-2} in elderly subjects (Bhushan, Cumberbatch et al. 2002). Langerhans cells undergo morphological alterations comprising less dendrite formation and reduced

antigen trapping capacity. More importantly, aged Langerhans cells seem to be functionally impaired, which may explain diminished cutaneous immune function in the elderly (Grewe 2001).

The dermis is characterized by a gradual loss of its thickness. A general atrophy of the extracellular matrix appears, accompanied by a decrease in cellularity especially of the fibroblasts (Varani, Spearman et al. 2001). There are reduced levels and disintegration of collagen and elastic fibers (Braverman and Fonferko 1982; Uitto 1986). This may be attributed either to decreased rate of synthesis, increased rate of degradation, or both. Exogenous substances such as amyloid P tend to deposit on the elastic fibers in the papillary dermis (Suwabe, Serizawa et al. 1999). Moreover, the vessels become abnormally thin. The veil cells are either absent or decreased in number with progressive aging and there is a decrease in their synthetic activity, which correlates with the appearance of abnormally thin walled vessels (Braverman and Fonferko 1982).

Clinically, intrinsically aged skin appears thin, finely wrinkled, smooth, dry, unblemished, sallow and pale, with some loss of elasticity (Fig.1.1.5). The skin becomes more fragile and vulnerable to damage by abrasion, can easily form non-healing ulcers (Ashcroft, Herrick et al. 1997) and is more sensitive to irritating environmental factors and allergens. The time required to heal an epidermal wound increases approximately 50% (Eaglstein 1989). Hair loss occurs more easily and the perspiration ability is affected.

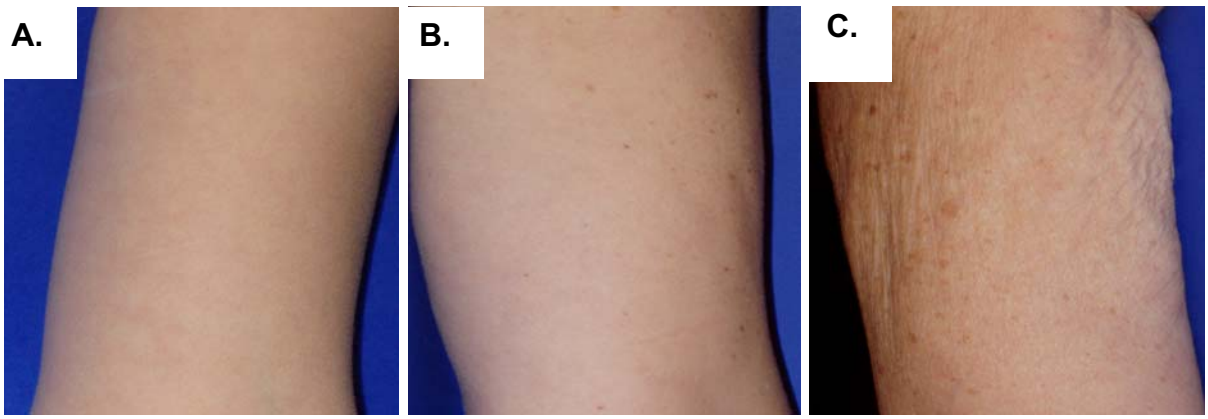

Fig.1.1.5 Intrinsically aged skin in the inner non UV-exposed area of the arm. Photos taken from a 10- (A), 45- (B) and 70- (C) year-old person.

1.1.6 Pathomechanisms of endogenous skin aging

While exogenous skin aging has been extensively studied, the pathomechanisms of endogenous skin aging and the role of hormonal deficiency on it remains far less clear. Some of the theories regarding the generation of endogenous skin aging support cellular senescence (Hayflick 1965) and decreased proliferative capacity (Schneider and Mitsui 1976; Gilchrest 1983; Dimri, Lee et al. 1995), decrease in cellular DNA repair capacity, loss of telomeres with advancing age (Allsopp, Vaziri et al. 1992; Smith and Pereira-Smith 1996; Bodnar, Ouellette et al. 1998; Kosmadaki and Gilchrest 2004), point mutations of extranuclear mtDNA (Michikawa, Mazzucchelli et al. 1999), which may be associated to increased oxidative stress (Miquel 1998) and increased frequency of chromosomal abnormalities (Benn 1976; Ly, Lockhart et al. 2000) and gene mutations.

1.1.6.1 Cellular senescence

The theory of cellular senescence describes the observed loss of the cells proliferative potential after a limited number of cell divisions (Hayflick 1965). According to this theory, cells possess a 'biological clock', which signals the end of their replicative life span, and as a consequence, they cannot be stimulated to enter the S1 phase by physiological mitogens, arresting at the G1 phase. This process can be partly explained by the selective repression of growth regulatory genes.

Studies on keratinocytes (Gilchrest 1983), fibroblasts (Schneider and Mitsui 1976; Mets, Bekaert et al. 1983; Cristofalo and Pignolo 1993) and melanocytes (Gilchrest, Vrabel et al. 1984; Medrano, Yang et al. 1994) have revealed that they all show an age-associated decrease in cumulative population doublings. Fibroblasts, for instance, taken from a normal human tissue go through only about 25-50 population doublings when cultured in a standard mitogenic medium. Towards the end of this time, proliferation slows down and finally stops and the cells enter a state from which they never recover.

The reduction in proliferative capacity of skin derived cells in culture from old donors and patients with premature aging syndromes, and the accumulation *in vivo* of senescent cells with altered patterns of gene expression (Dimri, Lee et al. 1995) also support the theory of cellular senescence.

1.1.6.2 Telomere shortening and senescence

The telomere hypothesis of cellular aging (Harley 1991) proposes that loss of telomeres due to incomplete DNA replication and absence of telomerase provides a mitotic clock that signals cycle exit, limiting the replicative capacity of the somatic cell (Allsopp, Vaziri et al. 1992). Human telomeres consist of repeats of the sequence TTAGGG/CCCTAA at chromosome end, which are not replicated in the same manner as the rest of the genome but instead are synthesized by the enzyme telomerase (Greider and Blackburn 1989; Feng, Funk et al. 1995; Lingner, Hughes et al. 1997). By mechanisms that remain unclear, telomerase also promotes the formation of protein cap structures that protect the chromosome ends. Telomerase is active in germline cells and in humans, telomeres in these cells are maintained at about 15 kilobase pairs (kbp). In contrast, telomerase is not expressed in most human somatic cells like skin cells (Kim, Piatyszek et al. 1994; Shay and Bacchetti 1997). As a result, their telomeres become 50-100 nucleotides shorter with every cell division, and their protective protein caps progressively deteriorate. Eventually, after many cell generations, DNA damage occurs at chromosome ends. The damage activates a p53-dependent cell-cycle arrest that resembles the arrest caused by other types of DNA damage.
The lack of telomerase in most somatic cells has been proposed to help protect humans from the potentially damaging effects of runaway cell proliferation, as occurs in cancer. Telomere loss is thought to control entry into senescence (Harley, Futcher et al. 1990; Hastie, Dempster et al. 1990; Chang and Harley 1995; Wright and Shay 1995; Weng, Granger et al. 1997).

1.1.6.3 Genes and mutations

The mechanisms, which seem to be associated with aging are complex (Guarente and Kenyon 2000; Robert and Robert 2003). Recent studies on models such as the yeast *Saccharomyces cerevisiae* (Jazwinski 1999), the nematode *Caenorhabditis elegans* (Johnson, Henderson et al. 2002), the fly *Drosophila melanogaster* (Rogina, Reenan et al. 2000; Tatar, Kopelman et al. 2001; Arking, Buck et al. 2002), the mouse *Mus musculus* (Kuro-o, Matsumura et al. 1997) and humans (Yu, Oshima et al. 1996) show that single gene mutations can contribute to the initiation of aging and induce premature aging syndromes. However, there are no special genes that can cause aging-associated damages. The manifestation of aging is mostly due to the failure of maintenance and repair mechanisms (Partridge and Gems 2002; Rattan 2004).

Studies on human keratinocytes have demonstrated altered expression of growth-regulating molecules with age; there is an increase of the baseline expression of the differentiation-associated genes like SPR2 and interleukin 1 receptor antagonist (Gilchrest, Garmyn et al. 1994) and EGF binding and receptor phosphorylation is reduced and thought to be the result of age related changes in a critical downstream signaling element (Yaar, Eller et al. 1995).
In senescent fibroblasts, genes like the c-fos proto-oncogene (Seshadri and Campisi 1990), the helix-loop-helix Id-1 and Id-2 genes (Hara, Yamaguchi et al. 1994) and components of the E2F transcription factor (Dimri, Hara et al. 1994; Good, Dimri et al. 1996) have been shown to be downregulated, and negative growth regulators are overexpressed including the p21 and p16 inhibitors of cyclin dependent protein kinases (Noda, Ning et al. 1994). Other changes seen in senescent skin fibroblasts include increased expression of IL-1 and of the EGF-like cytokine heregulin that modulates the growth and differentiation (Jenkins 2002). Moreover, elastin gene expression is markedly reduced after the age of 40–50, as determined by mRNA steady state levels (Uitto 1979).
Futhermore, recent studies indicate that endogenous and exogenous aging may share some fundamental pathways, and may have some common mediators. Photoaging is thought to be the superposition of UV-irradiation from the sun on intrinsic aging (Fisher, Kang et al. 2002).
Some of the similarities are changes in the MAP kinase signaling pathways, like decreases in ERK-dependent MAP kinase activity and increases in stress-activated JNK and p38 kinase (Chung, Kang et al. 2000), which result in reduced cell proliferation, differentiation, and cell survival (Xia, Dickens et al. 1995) and enhanced growth arrest, apoptosis and stress-related responses (Xia, Dickens et al. 1995; Verheij, Bose et al. 1996). As a consequence of the stress-activated MAP kinase pathways, the expression of c-jun and c-Jun N terminal kinase – an upstream activator of c-jun, is elevated in aged compared with young skin (Chung, Kang et al. 2000). As c-jun is a constituent of the transcription factor AP-1, AP-1 is also elevated and subsequently the AP-1 regulated connective tissue degrading enzymes MMP-1 (interstitial collagenase), MMP-3 (stromelysin 1) and MMP-9 (gelatinase B). Parallely, there is an observed reduction in the expression of tissue inhibitors of metalloproteinases (West, Pereira-Smith et al. 1989; Millis, Hoyle et al. 1992; Wick, Burger et al. 1994). Another common feature is the increased insoluble degraded collagen and the reduction of type I and III

procollagen synthesis, which may result from the impaired TGFß signaling pathway (Zeng, McCue et al. 1996; Mori, Hatamochi et al. 1998).

Ly et al measured mRNA levels in fibroblasts isolated from young, middle-aged, and elderly patients with progeria and found chromosomal pathologies that lead to misregulation of key structural, signaling, and metabolic genes associated with the aging phenotype (Ly, Lockhart et al. 2000).

Moreover, four mutations in the gene responsible for Werner's syndrome were identified. This gene expresses a mutated putative helicase, the product of the Werner's syndrome gene, suggesting that defective DNA metabolism may be responsible for the complex process of aging (Yu, Oshima et al. 1996).

All this evidence reveals the complexicity of skin aging and disputes the causative role of these gene alterations in aging.

1.1.6.4 The mitochondrial DNA (mtDNA) theory

Genetic damage and instability outside the nuclear genome has been suggested to contribute to aging (Wallace 2001). The mtDNA synthesis takes place near the inner mitochondrial membrane, where the sites of formation of reactive oxygen species (ROS) are and the fact that mtDNA lacks excision and recombination repair, has made many investigators to believe that cumulative damage of the mtDNA may play a key role in the pathogenesis of the aging phenotype (Miquel 1998; Michikawa, Mazzucchelli et al. 1999).

Examination of human fibroblast mtDNA in aged individuals revealed point mutations at specific positions in the control region for replication. Notably, a T414G transversion was found in a significantly higher proportion of persons older than 65 years of age when compared with younger persons. These results lent support to the notion that cumulative damage to mtDNA during life contributes to the aging process (Ly, Lockhart et al. 2000).

1.1.6.5 The free radical theory

According to the free radical theory, or otherwise called the oxidative stress theory, one of the major and important contributions to skin aging comes from excess ROS, which are produced as a consequence of aerobic metabolism (Harman 1992). Skin is exposed to production of ROS both from endogenous and exogenous sources. Endogenous sources are the enzymes-xanthine oxidase and nitric oxide synthase, the cells-neutrophils and pathological processes,

such as ischemic and post ischemic events, diseases, psoriasis and cancer inflammation. To exogenous sources belong pollutants (cigarette smoke, gases in the atmosphere, ozone, chemicals acids, herbicides and UV-irradiation). Intrinsic skin aging is affected mostly by endogenous sources.

Skin possesses many defensive mechanisms in order to reduce the production of ROS from internal sources. For example, the activity of enzymes which indirectly produce oxygen metabolites can be altered (xanthine oxidase modulation). There is a repair system consisting of enzymes and small molecules (Shigenaga and Ames 1991; Ames, Shigenaga et al. 1993; Lopez-Torres, Shindo et al. 1994; Beckman and Ames 1997), antioxidant enzymes, such as catalase and peroxidase, and low molecular weight antioxidants, such as tocopherols, ascorbic acid, NADH and carnosine, which can donate an electron and then scavenge ROS (Kohen 1999). However, it has been shown that all ROS levels rise and anti-oxidant activity declines with advancing age (Hu, Forsey et al. 2000; Kohen and Gati 2000).

Excess ROS production leads to accumulation of cellular damage (Sohal and Weindruch 1996; Hensley and Floyd 2002), which includes oxidation of DNA resulting in mutations, oxidation of proteins resulting in reduced function and oxidation of membrane lipids leading to reduced transport efficiency and altered transmembrane signaling, processes, which have as consequence the aging phenotype.

Moreover, accumulative evidence suggest that ROS play a crucial role not only in extrinsic but also in intrinsic aging by participating in multiple MAP kinase pathways, which induce AP-1 and in turn the signal cascade, already mentioned above (*see* 1.1.6.3 Genes and mutations).

The free radical theory has been also supported by the fact that strategies that reduce metabolism and the production of ROS, such as reduced food intake, or overexpression of superoxide dismutase and catalase, can extend lifespan of experimental animals like *Drosophila melanogaster* (Orr and Sohal 1994).

1.2 HORMONAL AGING

1.2.1 General

System-based theories of aging suggest that alterations of the endocrine system with advancing aging lead to deterioration of the organism and the aging process (Snowdon 1990). The importance of hormones and their metabolism on aging has been also displayed by recent studies performed on animal models (Bartke, Coschigano et al. 2001; Gems and Partridge 2001; Simon, Shih et al. 2003; Tatar, Bartke et al. 2003). This is not surprising as age-specific processes in humans such as puberty and menopause are also controlled hormonally. Serum levels of hormones decline with age in female and male individuals (Gray, Feldman et al. 1991; Simon, Preziosi et al. 1992; Corpas, Harman et al. 1993; Tenover 1997; Yen and Laughlin 1998; Roshan, Nader et al. 1999; Miller 2001; Russell-Aulet, Dimaraki et al. 2001) (Fig.1.2.1). This is due to decreased functional reserve of the endocrine organs as a result of aging per se or secondary to an intercurrent disease.

Skin being a target for a plethora of hormones and also a peripheral endocrine organ by itself (Zouboulis 2000) can be affected to a big extent by the decline of hormones. For instance, menopause in females, which is characterized by a sudden decline of sex-specific hormones, is associated with a rapid worsening of skin structure and functions, which can be at least partially repaired by hormone replacement therapy or local estrogen treatment (Brincat 2000). Improvement of epidermal skin moisture, elasticity and skin thickness (Fuchs, Solis et al. 2003), enhanced production of surface lipids (Sator, Schmidt et al. 2001), reduction of wrinkle depth, restoration of collagen fibers (Schmidt, Binder et al. 1996) and increase of the collagen III/I ratio (Affinito, Palomba et al. 1999) have been reported. However, the role of this multiple hormone lack in the skin aging process remains to be determined.

1.2.2 Growth hormone (GH) and insulin-like growth factor-I (IGF- I)

GH synthesis is directed by the hGH-N gene on chromosome 17. It is expressed in the anterior pituitary in the somatotroph cells and encodes the principal growth hormone molecule, a 22 kDa, 200 amino acid single-chain peptide with two internal disulfide bonds. Growth hormone secretion is pulsatile, principally under the stimulatory influence of the hypothalamus via GH-releasing hormone, and is inhibited by somatostatin also produced in hypothalamus.

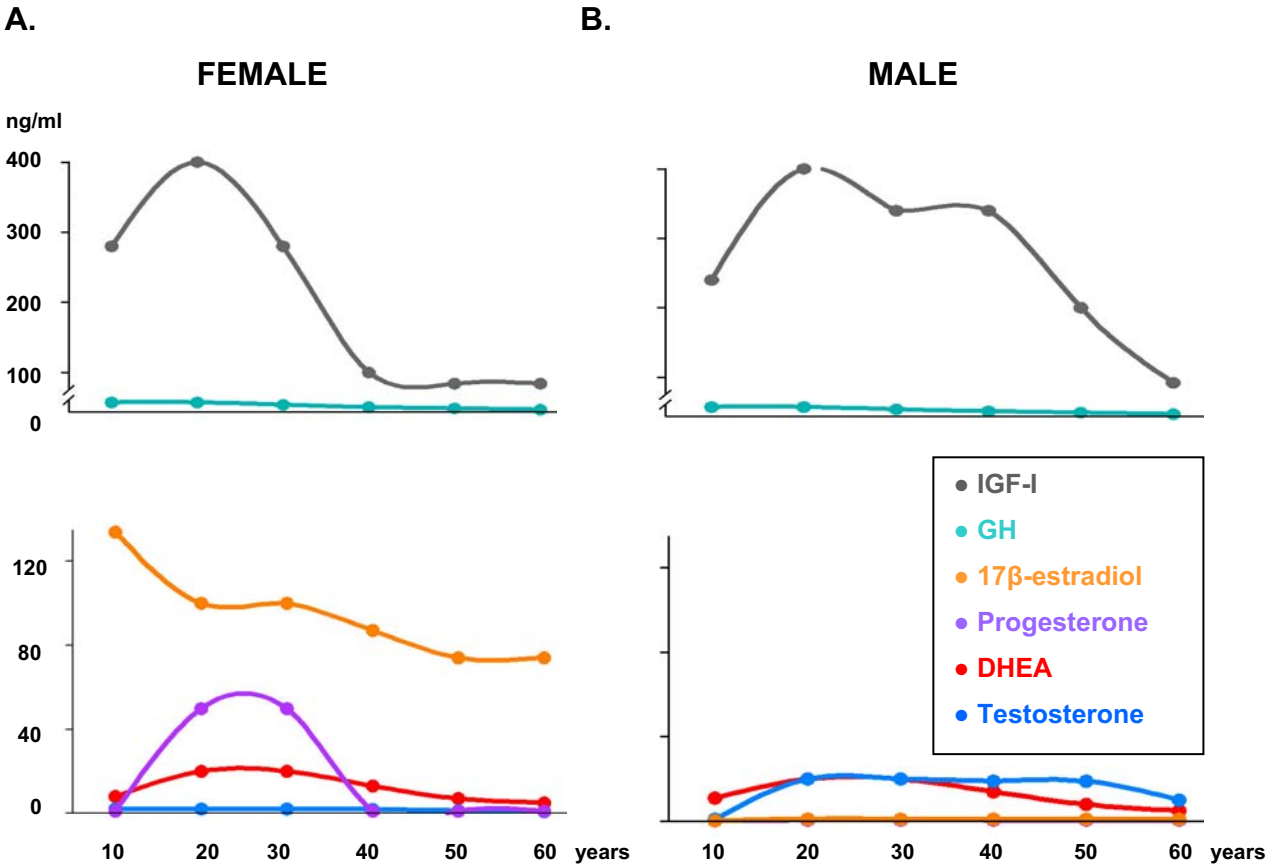

Fig.1.2.1 Circulating hormone levels in women (A) and men (B) during life (Zouboulis C, Makrantonaki E. In: Krutman J, Gilchrest B (eds) Skin aging. Springer, New York, Heidelberg)

GH acts by binding to the extracellular portion of a specific growth hormone receptor (GHR), which is an integral member of the cytokine receptor family. GHR has been shown to be transcribed via at least two different promoters, resulting in GHR1A and GHR1B. Both GHR1A and 1B are expressed in liver, whereas GHR1B is also expressed in muscle, uterus, and ovary tissues (Amit, Youdim et al. 2000; Liu, Carroll et al. 2000). The intracellular portion of the receptor activates JAK2 tyrosine kinase, which stimulates a phosphorylation cascade in the signal transducing activators of transcription (STAT) pathway, which in turn leads to binding of STAT proteins to DNA and protein synthesis (Carter-Su and Smit 1998). Some of the actions are direct and others are mediated via IGF-I.

GH secretion is relatively stable during childhood, increases during adolescence and decreases gradually during adulthood. This age-related decline in GH secretion involves a

number of changes in the GH axis, including decreased serum levels of IGF-I and decreased secretion of GH-releasing hormone from the hypothalamus. The cause of the normal age-related decrease in GH secretion is not well understood, but is thought to result in part from increased secretion of somatostatin, the GH-inhibiting hormone, or from an age-related decrease in the number and size of somatotrophs. Moreover, the GH response to GH-releasing hormone is attenuated with advancing age.

GH acts on virtually all tissues of the body. GH stimulates epiphyseal bone growth and also maintenance of bone after epiphyseal closure, is anabolic for muscle (Kostyo, Hotchkiss et al. 1959), stimulates lipolysis (Fain, Kovacev et al. 1965), decreases body fat (Salomon, Cuneo et al. 1989) and increases gluconeogenesis. It can also cause resistance to the action of insulin (Weaver, Monson et al. 1995). Decline of GH with aging may impair all the functions mentioned above.

IGF-I is produced primarily by hepatocytes, but also by many other cells including chondrocytes, osteoblasts, epithelial breast cells, granulosa cells, melanocytes and dermal fibroblasts (D'Ercole, Stiles et al. 1984; Adashi, Resnick et al. 1985; Han, Hill et al. 1987; Tavakkol, Elder et al. 1992), where it may act in an autocrine or paracrine manner. It is a 7.6 kDa, 70 amino acid polypeptide single-chain molecule with three internal disulfide bonds.

IGF-I binds to IGF-I receptor (IGF-IR), which signals through a tyrosine kinase. IGF-IR is a 350 kDa heterotetrameric glycoprotein composed of two exracellular α subunits, which form the ligand-binding domain and two β subunits, which contain a short extracellular and transmembrane segment and a larger intracellular segment, and transmit the ligand-induced signal. The subunits are linked by two disulfide bonds. The cytoplasmic protein tyrosine kinase is activated by IGF-I binding to the extracellular domains of IGF-IR and causes its conformational change and its autophosphorylation. Kinase activation in turn stimulates an intracellular cascade of molecular interactions involving multiple signaling pathways, such as the PI3-kinase and ERK pathway, which result in protecting the cells from apoptosis, promoting cell growth and proliferation, regulating cell adhesion and motility and inducing differentiation (Sussenbach, Steenbergh et al. 1992; Romano 2003). The receptor-ligand interactions in the IGF system are modulated by the IGF-binding proteins (IGFBPs).

Serum levels of IGF-I have been reported to increase from birth to puberty, followed by a slow decline through adulthood. This reduction is correlated with the progressive decline of

GH with advancing age (Bennett, Wahner et al. 1984). The reduction of GH and IGF-I with aging is also called somatopause (Fig.1.2.2).

1.2.3 Sex steroids

Steroidogenic organs include the adrenal cortex, the gonads and the placenta. All of these tissues use cholesterol as the precursor for the formation of charasteristic steroid hormones. Cholesterol synthesis and steroid hormone synthesis from cholesterol has been confirmed that also occur in the epidermis and the sebaceous glands (Menon, Feingold et al. 1985; Smythe, Greenall et al. 1998).

Steroids are lipophilic molecules and enter cells by passive diffusion. Within a target cell, the steroid binds to its receptor with high affinity. Before the steroid binds to the receptor, the receptor is associated with heat shock proteins (hsp). Under physiological conditions binding of a steroid to the receptor dissociates hsp. After the binding, the receptor-steroid complex can then homodimerize and bind to the hormone response element (HRE), which results in activating the transcription of target genes.

Among the numerous endocrine signals that affect the skin, sex steroids -androgens and estrogens- play a predominant role. The sex steroids secretion is under the stimulatory influence of luteinizing hormone (LH) and follicle stimulating hormone (FSH), both derived from the pituitary and regulated by a decapeptide, gonadotropin-releasing hormone (GnRH) synthesized in the hypothalamus. Sex steroids can be distinguished by the carbon numbers, C-19 being androgens, C-18 being estrogens and C-21 being progestenoids.

1.2.3.1 Androgens

Androgens can be classified into two categories: adrenal androgens [androstenedione and 11β-hydroxyandrostenedione, dehydroepiandrosterone (DHEA) and dehydroepiandrosterone-sulphate (DHEA-S)] and gonadal androgens (testosterone).

The direct biological activity of the adrenal androgens is minimal, and they function primarily as precursors for peripheral conversion to the active androgen hormones testosterone and dihydrotestosterone (5α-DHT). In males with normal gonadal function, the conversion of adrenal androstenedione to testosterone accounts for less than 5% of the production rate of this hormone and thus the physiologic effect is negligible.

In females, the adrenal substantially contributes to total androgen production by the peripheral conversion of androstenedione. In the follicular phase of the menstrual cycle, adrenal precursors account for two-thirds of testosterone production and one-half of 5α-DHT production. During midcycle, the ovarian contribution increases and the adrenal precursors account for only 40% of testosterone production.

Skin and its appendages, including hair follicles, sebaceous glands and eccrine/apocrine glands have been shown to possess all the necessary enzymes required for androgen synthesis and metabolism and skin can be responsible for the development of hyperandrogenism-associated conditions and diseases, such as seborrhea, acne, hirsutism and androgenetic alopecia (Chen, Thiboutot et al. 2002). Furthermore, several functions of the human skin can be affected by androgens, such as sebaceous gland growth and differentiation, hair growth, epidermal barrier homeostasis and wound healing.

DHEA

DHEA and its sulfated form DHEA-S are mainly produced by the human adrenal gland and in small amounts by the gonads. DHEA has three possible mechanisms of action. One is as a specific receptor, although no receptor for DHEA has been reported. The second is the conversion of DHEA into a more active sex steroid such as testosterone or estradiol in the peripheral tissues, after which it is bound to the androgen receptor or estrogen receptor. This mechanism is known as an ‘intracrine action’. The last possibility is that the hydrophobic DHEA molecule may alter the cell function after binding to macromolecules such as enzyme proteins (Nawata, Yanase et al. 2004). The secretion of DHEA is episodic and displays a diurnal rhythm (Rosenfeld, Rosenberg et al. 1975).

DHEA and DHEA-S are the most abundant steroids in the human plasma having serum concentrations of the order of 10^{-8} and 10^{-6} M, respectively. In adult men and women, serum DHEA-S levels are 100 to 500 times higher than those of testosterone and are 1,000 to 10,000 times higher than those of estradiol (Labrie, Belanger et al. 1998). The concentrations in serum reach a peak between the ages of 25 and 30 years and thereafter decline steadily, so that by the age of 60, serum concentrations are only 5-10% of corresponding values in young adults (Orentreich, Brind et al. 1984). This decline in women appears to be primarily a function of age and seems to be unrelated to menopause status (Burger, Dudley et al. 2000).

In addition, the maximal responses of DHEA to adrenocorticotropin hormone (ACTH) or corticotropin releasing hormone (CRH) in older adults are also significantly lower than they are in young men and women, whereas the secretory response of glucocorticoids to these modulators is not reduced in aging (Parker, Slayden et al. 2000).

Changes of DHEA with age have been related with altered immune response (Lucas, Ahmed et al. 1985), declines in cognitive ability (Flood and Roberts 1988), bone mass density (Hollo, Feher et al. 1970), libido (Bachmann, Bancroft et al. 2002) and increase in the incidence of cardiovascular diseases and atherosclerosis in men (Ishihara, Hiramatsu et al. 1992).

TESTOSTERONE

In males, over 95% of the testosterone is secreted by the testicular Leydig cells; the remainder is derived from the adrenals. In addition to testosterone, the testes secrete small amounts of the potent androgen 5α-DHT. In females, small amounts of testosterone are also produced by the ovary and are released into the circulation as precursors to estrogen synthesis as well as to act on peripheral tissues. The secretion of testosterone displays a diurnal rhythm (Faiman and Winter 1971).

Approximately 40%–50% of circulating testosterone in men is bound with high affinity to sex hormone binding globulin (SHBG) (Dunn, Nisula et al. 1981). This portion of the circulating total testosterone is not readily available to target tissues, whereas testosterone bound to albumin and free testosterone (1%–3%) are available to target tissues (Pardridge 1981). The albumin-bound and free testosterone are referred to as the bioavailable or weakly bound testosterone.

The effects of testosterone and 5α-DHT are mediated by binding to nuclear androgen receptors. The androgen receptor (AR) is a member of the steroid superfamily of ligand-dependent transcription factors. *In vitro*, 5α-DHT binds to the AR with greater affinity than testosterone does and the 5α-DHT/androgen receptor complex appears to be more stable (Anderson and Liao 1968).

Serum testosterone levels in men decrease with age (Gray, Feldman et al. 1991; Harman, Metter et al. 2001). It has been shown that 20% of healthy men in their sixties and 30% of men in their seventies have lower testosterone levels than 97.5% of healthy 20 - 45-y-old men (Harman, Metter et al. 2001). Feldman et al found that total testosterone levels decreased by

0.8% per year while bioavailable testosterone fell by 2% per year and SHBG levels increased by 1.6% per year (Feldman, Longcope et al. 2002). These changes are due in part to a reduction in the number of Leydig cells in the testes that produce testosterone.
Symptoms and findings of testosterone deficiency are similar to those associated with aging. They include loss of energy, depressed mood, decreased libido, erectile dysfunction, decreased muscle mass and strength, increased fat mass, frailty, osteopenia, and osteoporosis (Hijazi and Cunningham 2005). The decline of testosterone in men which is accompanied by androgen deficiency symptoms and signs is called androgen deficiency in the aging male (ADAM), partial androgen deficiency in the aging male (PADAM), or aging-associated androgen deficiency (AAAD) (Fig.1.2.2).

1.2.3.2 Estrogens

Estrogens are required for the normal maturation of the female. They stimulate the maturation of the gonads as well as the secondary sex charasteristics. The ovary is normally the major source of estrogens, although the conversion of androgen precursors in other tissues such as skin is clinically important after the menopause. In males, small quantities of estrogens such as estradiol, estrone and progesterone are produced by the Leydig cells of the testes. However, estradiol is not derived only by direct secretion from the testes but also by conversion in peripheral tissues of estrogen precursors secreted both by the testes and the adrenals. Thus, about 80% of the circulating concentrations of estradiol is derived from peripheral conversion.
Skin is one of the peripheral endocrine organs also responsible for the estrogen production in both genders. After immunohistochemical examination it was shown that the aromatase, which catalyzes three consecutive hydroxylation reactions converting C-19 androgens to C-18 estrogens is expressed in the outer rooth sheath of anagen and terminal hair follicles, in sebaceous glands, in keratinocytes and fibroblasts (Chen, Thiboutot et al. 2002).
Estrogens are derived from androstenedione and testosterone. Estrone is obtained from androstenedione and estradiol from testosterone. Estradiol has approximately ten times greater estrogenic activity than estrone and is bound in plasma by SHBG and albumin.
The effects of estrogens are mediated by binding with two nuclear estrogen receptors, ERα and, the currently found, ERβ (Kuiper, Enmark et al. 1996). Estradiol shows a high affinity for both receptors.

In women, estrogen levels decline rapidly at menopause as a result of the loss of ovarian follicles (Smyth, Gosden et al. 1994), whereas in men the levels of estrogens remain unchanged (Fig.1.2.1).

The fall-off in ovarian production of estrogens tends to accelerate skin aging, bone and vascular aging. Low levels of estrogens have been also correlated with profound effects on various compartments of the skin (Uitto 1986; Castelo-Branco, Rovira et al. 1996). There is also evidence, that lack of estrogens plays an important role in the osteoporosis in men (Eastell and Lambert 2002) and systemic application of phyto-estrogens has been shown to reduce the clinical symptoms of the benign prostate hypertrophy (Gambacciani, Ciaponi et al. 2001). Furthermore, decline of estrogens has been associated with alterations of brain functions such as cognition, learning and memory, neuroprotection, mood and affective behavior, and locomotor activity (Chakraborty and Gore 2004).

PROGESTERONE

Progesterone is an intermediate product in the biosynthetic pathway of estrogens. The ovary produces and secretes large amounts of progesterone during the luteal phase of the cycle. After entering the cell, progesterone binds to the cytoplasmic progesterone receptor and the resulting complex translocates to the nucleus (Guiochon-Mantel, Delabre et al. 1996). Progesterone has affinitity for transcortin, albumin and orosomucoid. The effects of progesterone on the reproductive organs include glandular development of the breasts and the cyclic glandular development of the endometrium. Moreover, progesterone exhibits important metabolic effects in other organs and tissues, producing changes in carbohydrate, protein, and lipid metabolism. The absence of progesterone increases the impact of the androgens on the sebaceous glands, the body hair and hair on the head (de Lignieres 1991).

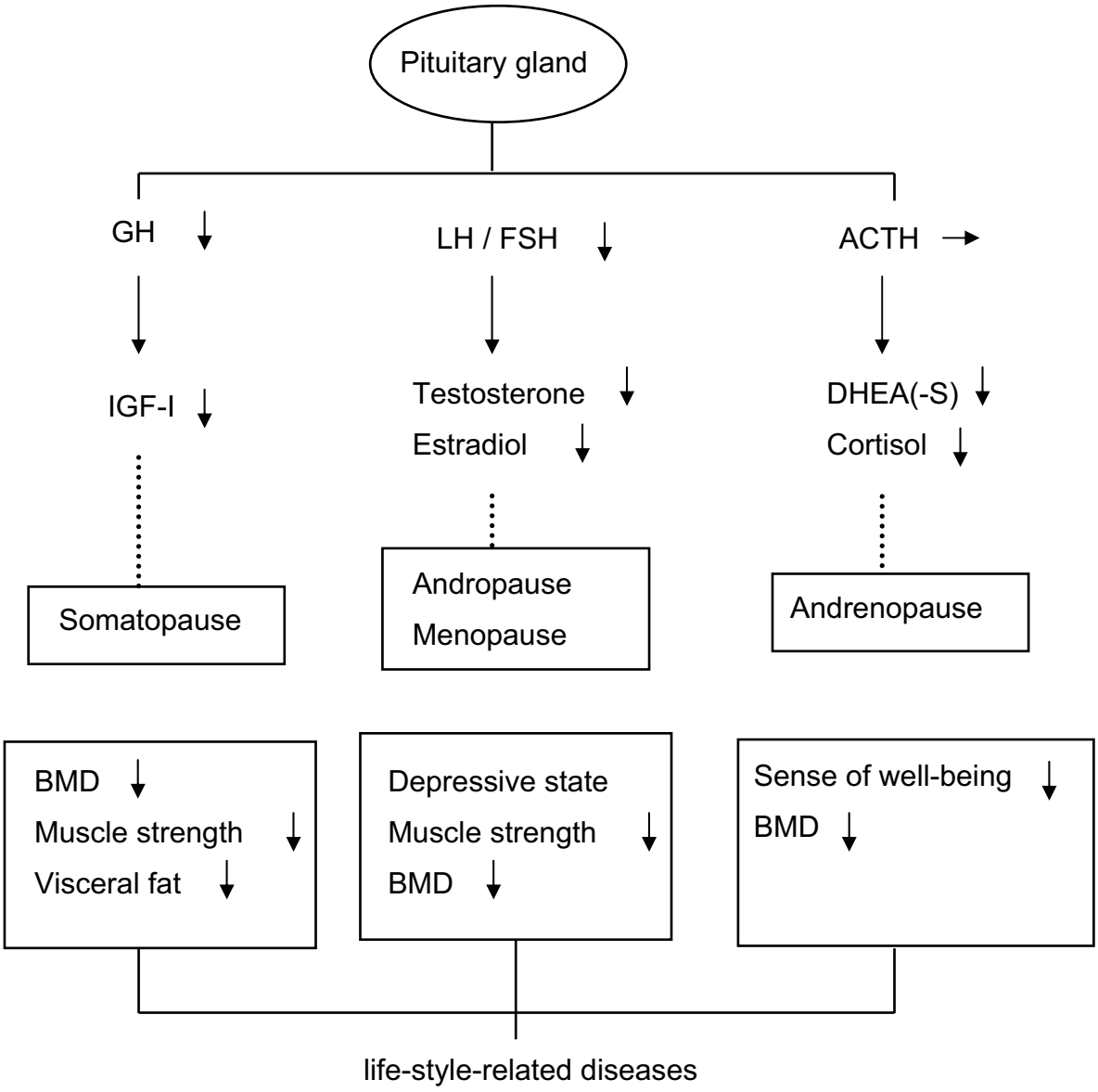

Fig.1.2.2 Decline of growth and steroid hormones with advanced age and resulting effects (Nawata, Yanase et al. 2004)

1.3 AIM OF THE STUDY

The main focus of this study was to elucidate the molecular events that take place with hormonal aging on skin cells by demonstrating the effects of GH, IGF-I, 17β-estradiol, progesterone, testosterone and DHEA at age- and sex-specific levels in combination and as single agents on human sebocytes and fibroblasts.

In depth, the aim of this work was:

1. to examine the expression of growth factor and steroid receptors in human skin cells by means of RT-PCR, Western blotting and immunocytochemistry;
2. by measuring the cell proliferation, lipid production and cytotoxicity to investigate whether human sebocytes and human fibroblasts show differential biological behavior by exposure to a hormone environment, consisting of IGF-I, GH, 17β-estradiol, progesterone, testosterone and DHEA corresponding to levels circulating in 20- and 60-y-old males and females, respectively;
3. to investigate whether there is an altered expression pattern of two age-associated genes, c-Myc and fibronectin by means of Northern and Western blotting after exposure of sebocytes to the two different hormone environments corresponding to 20- and 60-y-old males and females;
4. to map human genes in the treated sebocytes, which are associated with hormonal aging and hormonally induced diseases, using a cDNA microarray composed of 15,529 cDNAs from known and novel genes;
5. and to demonstrate the effects of IGF-I, GH, 17β-estradiol, progesterone, testosterone and DHEA at levels similar to those circulating in 20- and 60-y-old males and females as single agents on the biological behavior of sebocytes and fibroblasts.

The findings should allow a better view into the world of the hormonally affected genes and pathways in human cells and illustrate what is the level of hormone involvement in the aging process.