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**ANDROGENETIC ALOPECIA: A POSSIBLE
TREATMENT AND A RELATIONSHIP WITH HAIR
GREYING**

**Assessment of the herbal mixture “Xiantene” for the
treatment of androgenetic alopecia and a relationship
between early hair greying and the progression of
androgenetic alopecia**

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Doctor of Philosophy

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UNIVERSITY OF BRADFORD

2010

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hair greying**

Key words; ageing, androgens, androgenetic alopecia, baldness, canities, Chinese herbs, greyness, oxidative stress, treatment, herbal extracts

Abstract

Hair plays an important role in human social and sexual communication. The androgen-stimulated, patterned loss of hair in cases of androgenetic alopecia (or common baldness) in genetically pre-disposed individuals, is associated with ageing and can cause marked psychological distress. However, it is poorly controlled. To investigate the effectiveness of daily topical application of a Chinese medicine-derived herbal mixture, Xiantene, on balding progression, two double-blind, placebo-controlled studies (3 and 12 months) were carried out on balding men using the trichogram approach. Xiantene significantly increased both the total number of hairs and those in anagen, improving the ratio of anagen:telogen hairs. This suggests that topical Xiantene increased the length of the anagen phase and may promote a cessation, or partial reversal, of the progression of androgenetic alopecia in men.

Canities, loss of scalp hair colour, is another mark of ageing. To investigate whether early greying may protect follicles from androgenetic alopecia, the extent of alopecia, assessed using the Hamilton scale, was compared between men who first became grey before, or after, 30. Both alopecia and greying increased with age in 843 men (217 European, 626 Thai) whenever they first started greying. However, men who showed greying before 30 were significantly less bald, though more grey, in both groups. Hair follicle melanocytes synthesise the pigment melanin, producing reactive oxygen species (ROS) and oxidative stress; losing melanocyte pigmentary activity, and therefore these toxic factors, appears to enable hair follicles to maintain their full size for longer, despite the androgen drive to miniaturisation.

“Fair tresses man’s imperial race ensnare
and beauty draws us with a single hair.”

Alexander Pope (1688-1744)

Acknowledgements

The help and guidance of my supervisor Professor Valerie Randall of the University of Bradford has been invaluable and is gratefully acknowledged. Thanks are also due to my colleague, Dr. Zhidao Xia, of the University of Oxford for his help and encouragement and providing the raw materials used in this study; to Ms. Helena Holičová for her help with data compilation and for typing the manuscript and to Mr. Simon Davies for assistance with data analysis. I also gratefully acknowledge the assistance of Dr. Chris Leon of the Royal Botanical Gardens, Kew with the identification of the herbal components used and for assistance with the nomenclature. Thanks are also due to Mr. Saeed Al-Adadi for providing the thin layer chromatography data; to Mr. Paul Bassett of Stats Consultancy Ltd. who verified the statistical analysis in the 3 month and 12 month trials (Chapter 3 and 4) and to Mr Edward Kent of Warwick University for checking the data in the hair greyness and androgenetic alopecia study (Chapter 5). Funding for the research from The Tri-Mill Charitable Trust and Global Beauty International Management Ltd. is also gratefully acknowledged. Finally my most grateful thanks are due to my wife and family for their endless patience and encouragement.

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List of Abbreviations

AGA	androgenetic alopecia
AR	androgen receptor
ASP	Agouti signalling protein
BCL-2	B-cell lymphoma 2
BDNF	bone-derive neurotrophic factor
BMP	bone morphogenic proteins
cAMP	cyclic adenosine 3-5-monophosphatase
CAM	cell adhesion molecules
CMPAC	Chinese Medicinal Plants Authentication and Conservation Centre
COX	cyclooxygenase
CTS	connective tissue sheath
DHEA	dehydroepiandrosterone
DHT	5 α -dihydrotestosterone
DNA	deoxynucleic acid
DOPA	dihydrophenylalanine
Eda	Ectodysplasin
Edar	Ectodysplasin receptor
EGF	epidermal growth factor
EGF-R	epidermal growth factor receptor
EPR	electron paramagnetic resonance
FGF	fibroblast growth factor
FOXN1	forkhead box protein N1

GWA	genome wide association
HGF	hepatocyte growth factor
iNOS	inducible nitric oxide synthase
IFN-γ	interferon type 2
IGF	insulin – like growth factor
IR	infra red spectroscopy
IRS	inner root sheath
K15	keratin 15
K19	keratin 19
K(D)PT	alpha-MSH-related peptide
KGF	keratinocyte growth factor
LEF	lymphoid enhancer – binding factor 1
LOX	lipoyxygenase
mRNA	messenger ribonucleic acid
MC1-R	melanocortin 1 receptor
MHRA	medical and health regulatory authority
MMP	metalloproteinases
MRE	melanogenesis regulatory enzyme
MSH	melanocyte stimulating hormone
NGF	nerve growth factor
NMR	nuclear magnetic resonance spectroscopy
OCA	oculocutaneous albinism protein
ORS	outer root sheath
PDGF	platelet derived growth factor
PKA	protein kinase A

PTG	phototrichogram
Rf	retention factor
RNA	ribonucleic acid
ROS	reactive oxygen species
RT	reverse transcriptase
SCF	stem cell factor
SEM	standard error mean
SF	scatter factor
SHBG	sex hormone binding globulin
SHh	Sonic Hedgehog
SNP	single nucleotide polymorphisms
SRD5A1	steroid 5 α -reductase type 1
SRD5A2	steroid 5 α -reductase type 2
TCM	traditional Chinese medicine
TGF	transforming growth factor
TLC	thin layer chromatography
TNF	tumour necrosis factor
TRP-1	tyrosinase-related protein 1
TRP-2	tyrosinase-related protein 2
UV	ultra violet
VEGF	vascular endothelial growth factor
Wnt	“Wingless”+”int”, signalling proteins

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1. Introduction

1.1 Hair growth

1.1.1 Hair growth overview

Hair is the important distinguishing feature of the mammals (Chase, 1954), although some insects (bees, spiders, etc.) have a complex sensory system of tactile thread-like structures, which give the insect knowledge of its surroundings and its own body position. Mammalian hair fibres produced by the keratinisation of cortical cells extrude from the hair follicles to form the coat or 'pelage', the unique feature of the mammals (Dry, 1929). Many vertebrates have developed a variety of glands and pigmented structures, derivatives of their epidermis (scales, claws, quills, nails, horns, feathers and hairs), as adaptations to changing environments. The epidermis thickens to give a topological epithelial appendage or structure (Whiting, 2004) with specialised functions: protection, insulation, hunting, flight, communication and so on. However, it is only in the mammals where 'endothermy' occurs; the maintenance of a relatively constant body temperature (Stenn & Paus, 2001) independent of that of the surrounding environment allowing a high level of activity over prolonged periods, independent of ambient temperature changes. This single ability has given the mammals their huge evolutionary success.

The overriding importance of the mammalian coat is to insulate the warm blooded mammals against heat loss (Stenn & Paus, 2001), i.e. to give them the ability to control their own environment; however, the 'pelage' has

important secondary functions. It maintains sensory support with tactile hairs and whiskers on the animal's body, allowing sensory positioning and protection and it provides sexual and social communication, the coloration and pattern of the pelage indicating camouflage or self-advertisement (Goodhart, 1960). The pelage has regenerative properties and 'determinate' growth occurs in most mammals, i.e. the coat is renewed by a periodic 'moult' (Dry, 1926). A synchronised telogen effluvium occurs and a majority of the hair follicles in specific areas of the pelage enter the non-growing phase and the hairs are shed. The new anagen hair, which may vary in colour and texture from the preceding hair fibre, forms the new pelage, the pattern of moulting varying from species to species and within successive cycles in the same species (Ling, 1970). In many mammals the moult proceeds in a 'wave' across the body in a seasonal regeneration controlled by the photoperiod – the duration of daylight – and the sexual cycle (Chase & Eaton, 1959; Ebling & Harvey, 1964). Moulting provides replacement of 'wear and tear' and the ability to allow colour and density changes to adapt to seasonal and climatic changes. The thick white winter coat of some arctic animals, for example turns to a shorter, brown coat in the summer (Flux, 1970). In man, the hair growth is non-synchronous, or 'indeterminate' and proceeds on a continuous basis (Chase, 1954), although seasonal variations in human beings are present (Courtois et al, 1996). Human scalp hair was shown to exhibit an annual cycle with 90% of hairs in the anagen growing phase in the spring and early summer, falling to 80% at the end of the summer (Randall & Ebling, 1991), confirming an environmental influence on follicular activity. In this mosaic pattern of moulting the activity of each hair

follicle is largely independent of that of its neighbour (Chase, 1954), and any connection between the sexual cycle and the hair cycle has been lost.

In some mammals the hair coat is reduced; it is almost completely missing on the naked skin of whales, it grows sparsely on the skin of elephants and naked skin appears on the buttocks of some monkeys. In man, hair, although covering almost the entire body, is drastically reduced in thickness and length and full hair growth effectively remains only on the scalp, beard, axillae and pubic areas. Small fine unpigmented vellus hairs cover the whole body except the palms, soles, lips and mucous areas; thicker, longer, pigmented terminal hairs are seen in the areas considered to be hairy, and in man hair now serves two remaining key functions; protection and communication (Randall, 2007). The head and scalp are protected from solar radiation by scalp hair (Tobin & Paus, 2001); the eyelashes protect the eyes and nasal hairs filter the inhaled air. However the roles of scalp and body hair in social and sexual communication have become paramount (Randall, 2007), and hair is a key secondary sexual characteristic differentiating male from female, adult from child and old from young. The colour, styling and presentation of scalp hair help to create the image and perception we wish to project and are crucial for social communication (Randall, 2007). The pattern of hair growth varies with age (Shah, 1957) and differs between the sexes with marked changes in hair growth, in both sexes at puberty (Marshall & Tanner, 1969; Marshall & Tanner, 1970). Loss of human scalp hair and hair colour will affect this social communication and premature hair loss, in both sexes can cause considerable psychological stress (van der Donk et al, 1991; Cash, 1992; Girman et al, 1998).

Human scalp hair is commonly lost through genetically controlled patterned androgenetic alopecia, or common baldness (Hamilton, 1951), and less commonly in cases of alopecia areata, due to an autoimmune response (Randall, 2001) or through a disturbance to the systemic hormone balance, or in response to medical or external trauma. Excess body hair may also cause considerable psychological distress (Rabinowitz et al, 1983); hirsutism, excess female body hair in the male pattern is an androgen dependent condition (Randall et al, 2007); hypertrichosis is a generalised excess hairiness in both men and women (Barth et al, 1988) caused usually by an underlying systemic condition. Excess hair, whilst distressing is relatively easily controlled by depilation, epilation, or laser treatment (Elghblawi, 2008). Hair loss however is poorly controlled due to relatively little understanding of the biology and precise mechanisms of normal and abnormal hair growth and there is a need for better treatments to control hair loss conditions.

1.1.2 Hair growth cycle

Hair consists of long keratin fibres, twisted in a rope-like structure with a central, rigid medulla and an outer protective cuticle or coating of hardened, keratinised cells giving a structure of considerable tensile strength (Wolfram & Lindeman, 1971; Steinert, 1993). The hair is produced from the hair follicle, an oblique downward indentation of the epidermis which protrudes into the dermis and encloses at its lower end the dermal papilla, a dome shaped mesenchyme derived cluster of a small number of specific cells which regulate the behaviour of the epithelial cells or keratinocytes (Oliver, 1967;

Jahoda et al, 1984; Reynolds & Jahoda, 2004), see Figure 1.1. The layer of cells immediately around the enclosed papilla constitutes the matrix from which a cone of cells differentiates, moving upward to form the hair shaft, or growing fibre structure which becomes keratinised and hardened to form the final hair structure, extruding from the skin or the scalp. The hair fibre at this point is dead tissue and it is only the growing or germinating root of the hair where the living keratinocytes are to be found. The rate of cell proliferation or mitosis, during the active phase of the hair cycle to produce the hair shaft structure, is extremely high and is comparable with that of malignant tumour growth (Stenn & Paus, 2001).

These highly active hair follicles cover the whole of the human body, except for the palms of the hands, soles of the feet, and the mucous areas; the number of hair follicles not normally increasing postnatal. The body cannot normally grow or produce new hair follicles, although this may occur after wounding or trauma (Ito et al, 2007). Follicular loss can occur in some dermal tumours and cicatricial alopecias (Dalziel & Marks, 1986) and on pre-determined scalp areas with the progression of androgenetic alopecia and senescent alopecia (Whiting, 2001). Hair growth proceeds on a cyclical basis through a repeated cycle of active follicular growth and rest phases (Chase, 1954; Kligman, 1959), orchestrated by the dermal papilla, which produces the required proliferative or inhibitory stimuli for hair growth regulation, see Figure 1.2 (Oliver, 1967; Jahoda et al, 1984; Reynolds & Jahoda, 2004). The length of each phase is determined by age, sex, genetic profile and follicular body site, and can be further influenced by systemic and external factors (Ebling & Harvey, 1964; Randall, 1994).

Figure 1.1

Hair bulb

The hair bulb surrounds the dermal papilla. Hair matrix cells divide, differentiate, ascend and are elongated and compacted into hard keratin.

The diagram shows the outer root sheath, and the 3 layers of the inner root sheath; Henle's layer, Huxley's layer and the cuticle of the inner root sheath.

(Randall, 1994)

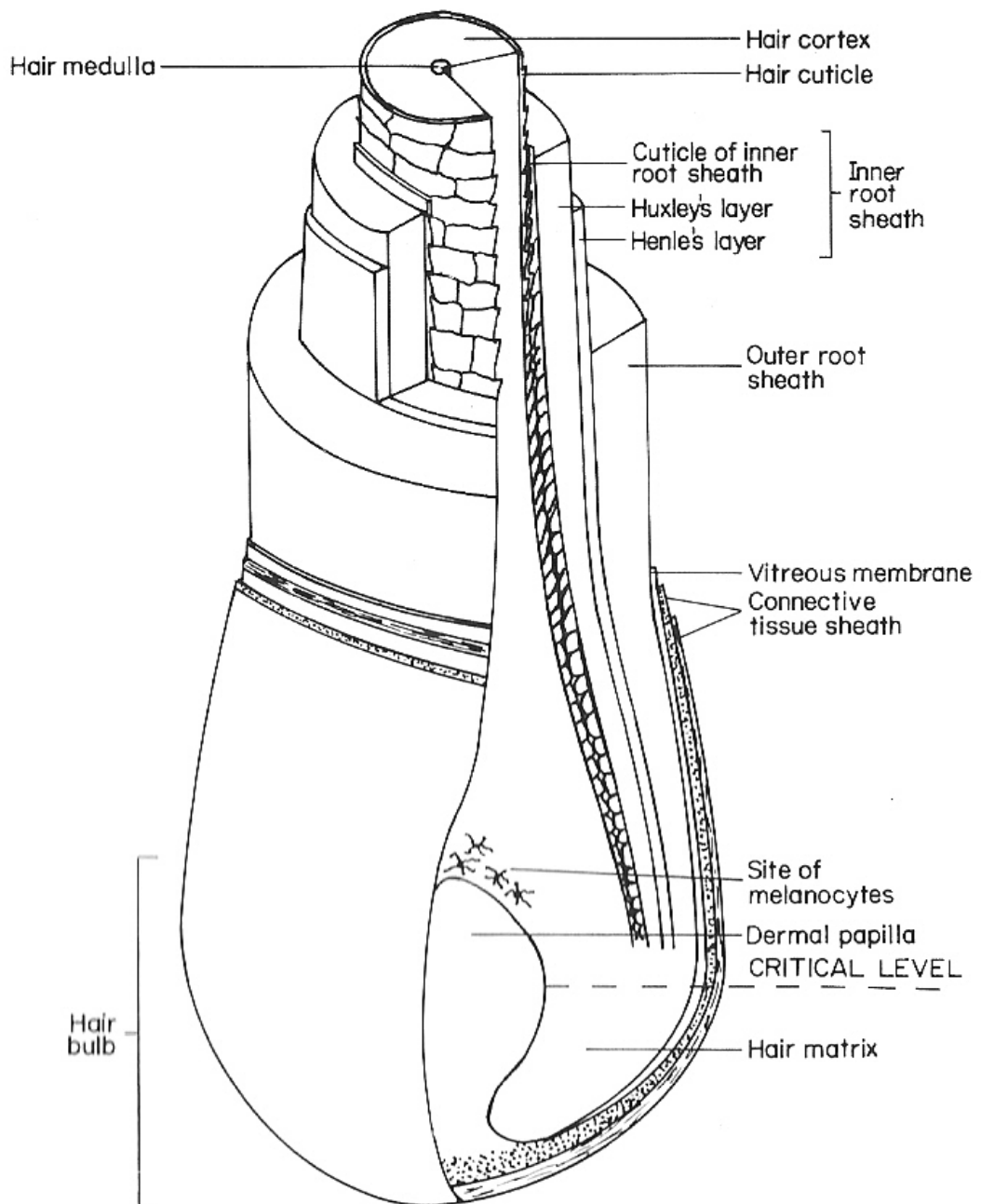
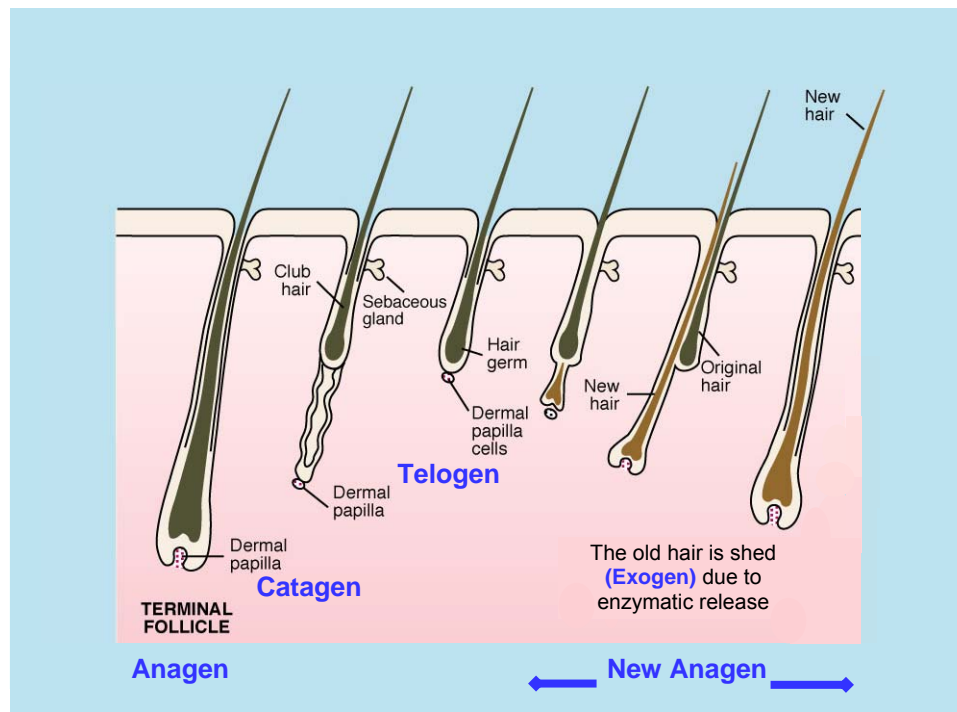


Figure 1.2

Hair growth cycle

Showing 3 main stages, anagen, catagen and telogen (Randall, 2000).

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The length of the anagen (growing) phase determines hair length and in human scalp hairs, anagen lasts from 2 to 5 years or on average 1,000 days (Chase, 1954), although some Asian women may have an extended anagen cycle for scalp hair of 7 or 8 years (personal observation), see Figure 1.3. Conversely, the hair growth cycle on the finger may be 1½-3 months (Saitoh & Sakamoto, 1970). Anagen commences with the downward growth of the follicle, enclosing the dermal papilla and accompanied by a dramatic increase in mitotic activity of the germinal cells at the base of the follicle, recreating the original morphogenesis of the follicle in fetal skin (Botchkarev & Paus, 2003). The follicle extends to its maximum length, melanin is synthesised by the melanocytes, and the new hair shaft is formed, keratinising just below the level of the sebaceous gland.

The previous resting telogen 'club' hair is ejected from the follicle and the new hair emerges at the skin surface. Anagen proceeds until the onset of the catagen (regression) phase when mitosis at the germinating matrix ceases, and the catagen phase begins (Parakkal, 1970), lasting only a few days. The dermal papilla moves upward as the hair follicle shortens and the lower section of the hair shaft becomes fully keratinised, club shaped, lacks pigment and is only partially connected to the epithelial cells as the hair follicle enters the telogen (resting) phase. The non-growing hair shaft is held in the upper section of the hair follicle in the scalp of man for approximately 100 days, hence a normal anagen/telogen of 10:1, i.e. approximately 90% (Braun-Falco & Heilgemeir, 1985), and morphogenesis of the new anagen phase is recommenced at the end of telogen.

Figure 1.3

Extended anagen (growing) phase

An Asian woman (Thailand native) exhibiting extended anagen growth phase, estimated at 7 – 8 years, assuming an average growth rate of approximately 0.37 mm/day (Munro, 1966)

Photo, author



The co-existence of the new anagen hair and the previous telogen hairs ensures that an animal is never naked and it has recently been suggested that the process of active hair shedding may constitute a separate distinct phase in the follicular cycle, for which the term “exogen” has been proposed (Stenn et al, 1996; Stenn & Paus, 2001; Van Neste et al, 2007).

Anagen has been divided into 6 sub phases anagen I – VI (Chase, 1954) see Figure 1.4. Phases I to V are mainly of the same length for all human hair follicles, independent of body site, however phase VI varies and determines the length of both the anagen phase and that of the hair shaft itself (Saitoh & Sakamoto, 1970). During early anagen the secondary hair germ, an epidermal cluster of hair cells descends with the encased dermal papilla to a pre-determined depth in the dermis at which point the growth direction reverses and proliferation and differentiation of the hair matrix cells form the upward moving hair shaft and inner root sheath structures.

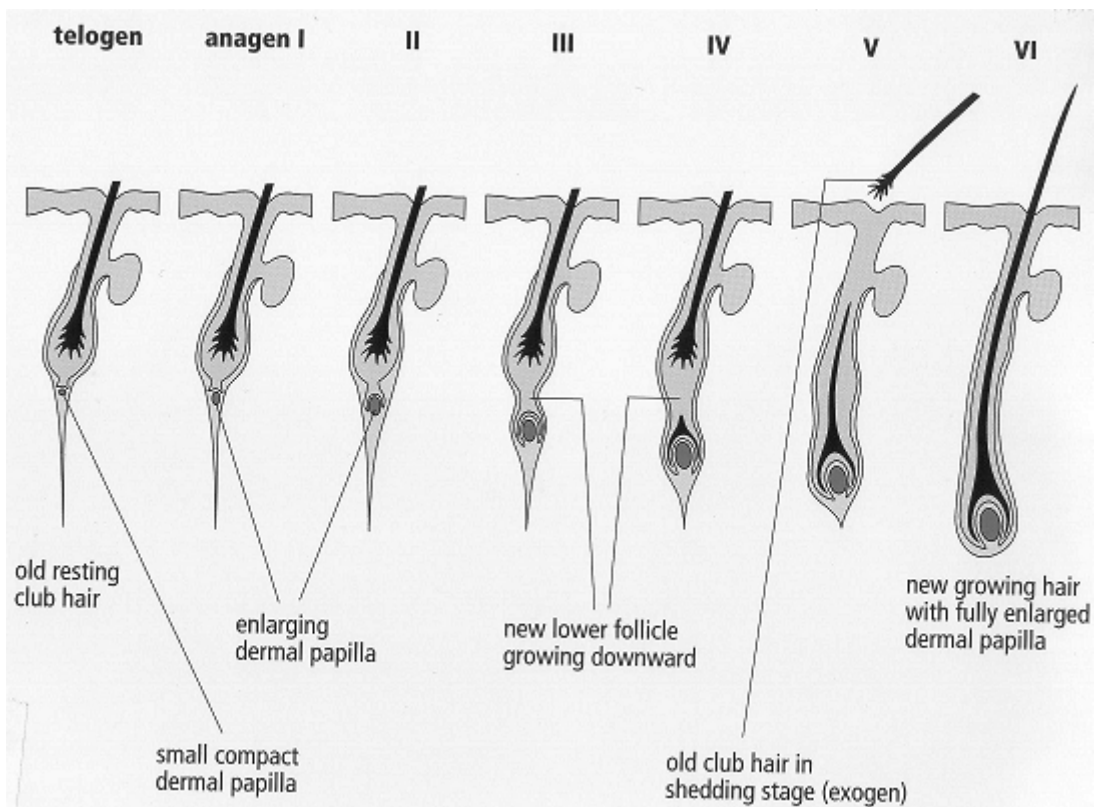
The bulge area of the isthmus provides the source of quiescent, multipotent stem cells required for this very rapid proliferation and tissue generation (Cotsarelis, 1998, Cotsarelis et al, 1999; Nishimura et al, 2005). The inductive signal for initiation of anagen may arise from the dermal papilla (Stenn & Paus, 2001) which triggers proliferation of stem cells and transient amplifying cells from the bulge region, or by a signal from the secondary hair germ which is in intimate contact with the dermal papilla (Panteleyev et al, 2001). The dermal papilla size varies according to body site (Itami et al, 1990, Randall et al, 1992) and dictates the size of the bulb, and hence the size and character of the hair produced (Ibrahim & Wright, 1982).

Figure 1.4

Hair growth cycle (anagen I – IV)

Hair growth cycle showing telogen and anagen stages I – IV (Whiting, 2004).

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The dermal papilla also changes in size during follicle life to accommodate changes in size of hair produced e.g. in response to androgens (Messenger et al, 1991). The dermal papilla sends and receives mesenchyme-epithelial signals by intimate contact with the hair matrix epithelium via its extracellular matrix (Link et al, 1990).

Anagen papilla cells, isolated and placed in non-hair bearing skin can induce epithelial hair follicle formation (Reynolds & Jahoda, 1991) and if a papilla is separated experimentally from the growing hair follicle, follicle growth ceases (Link et al, 1990) and hair follicle destruction and alopecia ensues (Pantaleyev et al, 1998). Signalling in the reverse direction also occurs and hair follicle keratinocytes produce specific growth factors which stimulate the growth of human scalp dermal cells (Warren & Wong, 1994) and epithelial-mesenchyme interactions are central to hair growth control (Hardy, 1992, Stenn & Paus, 2001).

A number of the molecular markers present in early anagen have been identified (Botchkarev & Paus, 2003); high levels of lymphoid enhancer factor-1 (LEF-1) are present (Zhou et al, 1995) and early anagen is associated with proteolytic enzyme production, to digest the dermis and allow the downward growth of the hair follicle (Paus et al, 1994). The growth factors epidermal growth factor (EGF) and transforming growth factor α (TGF- α) are implicated in the release of these metalloproteinases (MMP) which are expressed in the bulb and outer root sheath (ORS), but not in the dermal papilla (Weinberg et al, 1990).

Trauma or wounding will induce anagen in the area of the trauma (Li et al, 1999) and there are also a number of chemical agents which will increase

anagen, e.g. minoxidil (Burton & Marshall, 1979) and cyclosporin (Gebhart et al, 1986), or inhibit anagen e.g. glucocorticoids (Chedid et al, 1994); however oestradiol (Thornton et al, 2003; Thornton et al, 2006) and androgens (Randall, 2007) can have both inhibitory and stimulatory roles. Downward growth of the follicle, during anagen stage III (Stenn & Paus, 2001) bears similarities with the invasive downward growth of basal or squamous carcinomas, and the gene expression and mechanism may be similar (Kahari & Saarialho-Kere, 1997). During anagen stages IV and V (Figure 1.4) hair shaft and inner root sheath (IRS) construction commences, the follicle with the encased papilla having descended to its lowermost point. The ORS, which separates the hair shaft unit from the surrounding dermis partially maintains itself, independent of the bulbar matrix, by basal cell growth (Reynolds & Jahoda, 1991) and is not completely reformed with each cycle. The ORS supports the IRS and hair shaft, but is not quiescent and may play a role in hair cycle control with the ORS producing catagen inducing growth factors; Fibroblast Growth Factor (FGF) and neurotrophins (Botchkarev et al, 1998), modulating the hair cycle. The hair shaft and IRS form a complementary core of ascending hardened tissue of nearly circular cross section (Priestley, 1967). A number of molecules are expressed in the IRS during anagen, Fibroblast Growth Factor-1 (FGF-1), Transforming Growth Factor- α (TGF- α), Sonic Hedgehog (SHh), tissue inhibitors of metalloproteinases (TIMP), β -catenin (Millar et al, 1995; Millar, 2002) and their profusion and differing or contradictory functions have not been fully elucidated. At approximately the level of the sebaceous gland, the IRS “dissolves” or dissociates possibly at a signal from the sebaceous gland

(Philpott et al, 1996), and sebum production is necessary for the emergence of the distal end of the hair shaft from the surface of the epidermis (Zheng et al, 1999).

By the onset of anagen stage IV the lower portion of the hair follicle is fully reformed and full pigmented hair growth is established. Molecular control of hair follicle epidermal cells during hair growth may be effected using the Notch-pathway (Artavanis-Tsakonas et al, 1995; Schouwey & Beermann, 2008); the Notch gene family encode for transmembrane proteins and the Notch signalling pathway is a major determinant of cell fate specification (Lin et al, 2000). Notch-1 is expressed in bulb cells of neonatal mice, although not in the cells adjacent to the papilla. The Notch-receptor is expressed in differentiating cells and Notch signalling may play a part in controlling epithelial cell differentiation during hair shaft growth, maintaining the required column of keratinocytes (Kopan & Weintraub, 1993, Kimble & Simpson, 1997, Powell et al, 1998).

Anagen ends after a pre-determined period of hair growth dependent on body site and follicle physiology, when a signal of unknown origin precipitates a massive and rapid coordinated cell differentiation and apoptosis (Lindner et al, 1997, Foitzik et al, 2000, Botchkarev et al, 2000). Cell growth and pigmentation cease, the dermal papilla shrinks and is released from the bulb (Deweert et al, 1982) and the lower portion of the follicle regresses leaving an epithelial strand. The volume of the hair bulb decreases (Kligman, 1959) and the condensed papilla moves distally attached by a vascular stalk to the regressing epithelial column above to a position approximately level with the sebaceous gland. Some specialised

follicles e.g. vibrissae do not show the normal pattern of cyclical shortening and regression, but undergo a cyclical change in diameter (Young, 1980). Initiation of catagen may be triggered by the finite number of remaining transient amplifying cells (Cotsarelis et al, 1990) or by an accumulation of paracrine apoptotic factors (Paus et al, 1999) or by a combination of these and other unidentified signals. The onset of catagen is predetermined, but catagen can also be induced artificially by severe trauma (Kligman, 1959), chemotherapy and chemical trauma or by hormone treatment (Uenalan et al, 2000). There is an increase in FGF-5 expression in late anagen and in the absence of FGF-5 in mice, catagen is delayed resulting in the angora (long hair) phenotype (Herbert et al, 1994). The anagen-catagen transformation coincides with an increase in the number of deeply situated follicle mast cells (Maurer et al, 1997) which may modulate catagen progression or coordinate apoptosis of lower follicle cells (Majno & Joris, 1995). This initiating signal for synchronous programmed cell death is unidentified, but is thought to be transmitted to the epithelial cells via an intracellular route involving mainly caspase enzyme activation (Enari et al, 1998, Evan & Littlewood, 1998).

The signal may involve turning off the IGF-1 receptor, essential for follicle growth (Philpott et al, 1994) or the expression of Hepatocyte Growth Factor (HGF) or Transforming Growth Factor β 1 (TGF- β 1), both catagen promoting agents (Lindner et al, 2000, Foitzik et al, 2000). The apoptotic signal may therefore be positive i.e. activation of a death receptor and subsequent organ collapse or a negative inhibitor signal via modulation of apoptosis inhibitors which block or modulate cell death during anagen. The BCL-2 proteins are known anti-apoptotic agents (Hockenberger et al, 1993) containing apoptotic

antagonists that complex with caspases or their co-factors inhibiting cell death (Adams & Cory, 1998, Korsmeyer, 1999). During morphogenesis BCL-2 is expressed in the epithelium and the mesenchyme from the earliest stages in fetal tissue (LeBrun et al, 1993) and in the adult follicle during anagen the bulb, IRS & ORS are BCL-2 positive. In contrast the dermal papilla continuously expresses BCL-2 throughout the complete murine hair cycle (Lindner et al, 1997), even during chemically induced apoptosis using cyclophosphamide (Tobin et al, 1998). There is a progressive decline of BCL-2 expression in the epithelium at the approach to catagen (Lindner et al, 1997) and it is assumed BCL-2 protects the cells from programmed cell death. However it is unlikely that a simple control system precipitates entry into catagen and a complex multipathway is probable (Clem & Duckett, 1997; Stenn & Paus, 2001; Thompson, 1998; Alonso & Rosenfield, 2003). Transgenic BCL-2 knock-out mice exhibit largely normal hair growth, although the hair turns grey on the second coat (Veis et al, 1993).

As the follicle enters catagen the bulb keratinocytes adjacent to the dermal papilla are the first to exhibit transformation with the appearance of apoptotic clusters of groups of cells (Lindner et al, 1997) and as catagen progresses, apoptotic cells are found in all regions of the hair follicle. The lower follicle atrophies and the papilla remnants which are attached by a vascular stalk ascend to the upper follicle. The papilla may be pushed or squeezed up by contraction of the surrounding connective tissue sheath (CTS) assisted by secreted enzymes (Chase, 1954, Ito & Sato, 1990) or it may simply be pulled up by the contraction and disintegration of cells undergoing apoptosis, which exerts an upward pull as adjacent cells move to fill the gap. Catagen is a

very short and effective organ contraction period following which the upper follicle enters the telogen resting phase although there is evidence that telogen may not simply be a quiescent period and increases in enzymes (proteases) levels (Koch et al, 1998) suggest telogen may be an anagen-inhibition phase (Chase, 1954).

The reduced telogen follicle extends only into the upper dermis. At the bottom of the follicle lies a group of epithelial cells or “hair germ” which although essentially quiescent with no keratin production, carry out limited protein synthesis (Bowden et al, 1998). The papilla forms a tight cluster of fibroblasts below the telogen follicle, reduced in size, but believed to contain essentially the same cells as the corresponding anagen papilla (Wessels & Roessner, 1965, Reynolds et al, 1999). Toward the end of telogen the cells of the hair germ swell and grow downwards again, encasing the dermal papilla, and the hair follicle re-enters the anagen phase (Chase, 1954, Müller-Röver et al, 2001). The hair germ cells in late telogen express FGF-5, a regulator of the hair growth cycle (Herbert et al, 1994, Rosenquist & Martin, 1996) and oestrogen receptor expression, also a hair cycle regulator, has been found in telogen papilla cells (Oh & Smart, 1996).

The previous telogen club hair has a “shaving brush” like base attached to the remaining IRS immediately above the hair germ. The degenerated IRS remnants form a collar round the telogen shaft base (Pinkus et al, 1981), and the trichilemmal keratinous mass, anchors the shaft at a point just below the duct of the sebaceous gland prior to final ejection of the club hair, during exogen (Stenn et al, 1996). Often telogen hairs from previous growth cycles have not been shed in rodents and it is possible for there to be 2 or 3 hairs in

the same follicle with only one of the hairs actively growing (Chase & Eaton, 1959). Telogen and exogen are therefore not precisely linked and can occur independently and probably therefore have their own control systems; a proteolytic pathway has been suggested in the formation and shedding of club hairs (Ekholm et al, 1998, Lavker et al, 1998).

Anagen and telogen hairs have been shown to be firmly anchored in the hair follicle, but exogen hairs are only passively held in the hair follicle and may be distinguished by measurement of the differing cohesion forces (Van Neste et al, 2007). Telogen hairs retain cell-cell attachment to the outer root sheath, whereas exogen hairs do not and the exogen phase has been demonstrated in the murine (Milner et al, 2002) and human (Van Neste et al, 2007) hair cycles.

The exogen phase can be altered; in cases of trichostasis spinulosa, the infundibulum of the hair follicle becomes dilated and hair shafts are retained, i.e. exogen is delayed (Young et al, 1985). In cases of androgenetic alopecia the hair shaft can be shed before anagen commences i.e. there is a latent period with no shaft (Whiting, 2001), which may indicate premature exogen or an extended telogen (Courtois et al, 1996). The process of exogen completes the growth cycle of the hair shaft with the hair follicle itself continuing to cycle throughout the life of the animal unless the follicle is lost by organ deletion as in cases of androgenetic alopecia (Whiting, 2001), or the sequence of cycling is dislocated by systemic or physiological disturbances e.g. alopecia areata (Randall, 2007). It has been shown recently that new hair follicles can be constructed after wounding in adult mice (Ito et al, 2007). The new follicles were shown to form from epithelial

cells which assumed a hair follicle stem cell phenotype following trauma. The resultant hair follicles produced hair and cycled in the normal way.

1.1.3 Hair shaft structure

The mesenchyme derived dermal papilla, containing fibroblast-like cells, initiates cell division, keratinisation and hair shaft formation (Oliver, 1967; Jahoda et al, 1984 & Jahoda, 2002), see Figure 1.1, the size of the papilla and surrounding bulb being directly related to the size of hair shaft produced (Panteleyev et al, 2001). The inner root sheath (IRS) and outer root sheath (ORS) provide the funnel shaped mould through which the hair shaft emerges and determine the final shape of the hair shaft. This hair shaft shape is genetically determined and may vary from almost circular (Caucasian) to oval or flat (Negroid) with the hair produced varying from cylindrical to a twisted "ribbon" pattern and a number of specific shaft defects: monilithrix, pilli torti, pilli annulati, etc., are known (Davies & Olsen, 1996). Epidermal cells proliferate, differentiate and elongate, moving upward into the hair shaft, and human hair growth has been shown to be approximately 0.37 mm/day (Munro, 1966). Electron microscope studies (Birkbeck & Mercer, 1957) have confirmed keratinisation to be complete approximately 1 mm from the germinating root area, and to be relatively rapid, with approximately 2½ days to completion (Forslind & Swanbeck, 1966). Programmed cell death (apoptosis) occurs as the hair cells move upward; they are compacted and elongated, lose moisture and protein synthesis ceases with the eventual loss of the cell nucleus, and keratinisation is complete (Forslind et al, 1986).

The filament structure of the hair shaft has been reviewed (Steinert, 1993). The central medulla, a tube-like structure, which may be partially filled with melanin, spongy cellular matter and air spaces, is surrounded by 'whorl-like' cortical structures of straight protein filaments or macrofibrils aligned along the hair shaft axis in a twisted, helical, rope-like structure of high tensile strength and elasticity (Steinert, 1993) held together by a matrix of intercellular cement which is also composed of twisted polypeptide chains very rich in the amino acid cysteine (Johnson & Sikorski, 1965). The outer cells of the cutex, 6-10 layers deep, are flattened, plate-like and overlap to form the protective cuticle structure with an outer lipid coating, see Figure 1.1.

The chemical composition of hair is highly complex with the insolubility of the keratinous protein accounting for the very stable hair structure; and hair 55,000 years old has been identified (Dr. A. Cooper – personal communication, Oxford Hair Foundation seminar, London, 2003). The main component of hair is the protein, keratin, approximately 65-95% by weight (Ogawa & Yoshike, 1984) with water, lipids, pigment, trace elements (Passwater & Cranton, 1983) and DNA (Leanza et al, 2007) also present. Keratin contains abnormally high levels of sulphur and human hair contains approximately 5% sulphur (Forslind et al, 1986). The component amino acid units of the protein are chemically bound together by peptide linkages to form long chain polypeptides, which in keratin are arranged in an alpha-helix configuration, like a coiled spring, with three or four amino acids in each turn of the helix.

In the zone of keratinisation the polypeptide chains become connected by cross linkages between the coils of the chain and between adjacent coils. The strong S-S disulphide bond (or cystine linkage) between coils gives keratin its ladder-like molecular structure and considerable tensile strength with the weaker ionic bonds and hydrogen bonds between the coils contributing to the integrity of the structure whilst allowing elasticity of the hair (Steinert, 1993). The extruded hair shaft initially retains its integrity but may subsequently be damaged by chemical treatment or weathering (Dawber, 1977), with fragilitis crinium (split ends) common at the distal end as the cortical structure collapses. The outer cuticle layer breaks down or is lost to expose the inner cortical structure, which unravels and breaks down, losing the shaft integrity (Rushton et al, 1990).

1.1.4 Hair follicle morphogenesis

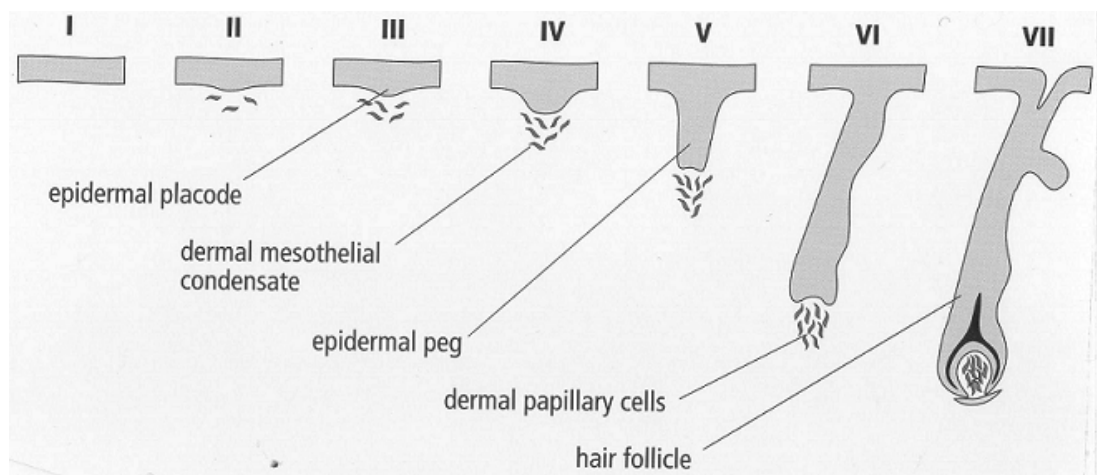
During embryogenesis, skin appendages, including the hair follicle, develop from a common origin, a thickening of the epidermis which invaginates downward into the dermis or protrudes outward to form the specialised skin structure (Mikkola, 2007) see Figure 1.5 and changes to these structures can be induced from this common genesis (Choung & Noveen, 1999). In human embryos, at approx 50 days gestation, the single cell layer of overlaying ectoderm, separated by a basement membrane from the mesenchyme, forms a placode (see Figure 1.5) a thickening of the epidermis, which descends into the mesenchyme following an initiating signal from the dermis itself (Oro & Scott, 1998, Botchkarev et al, 1999, Paus et al, 1999; Mou et al, 2006).

Figure 1.5

Embryology of hair follicle development

A thickening of the epidermis invaginates downwards into the dermis, enclosing the dermal papilla cells following which hair growth commences.

(Whiting, 2004). Reproduced with permission



The precise identity of the initiating signal is not known, but combination of dermal cells and non-hair bearing epidermis which can be induced to subsequently form hair follicles have shown that the dermis is the key activator and determines size and type of hair produced (Hardy, 1992; Reynolds et al, 1999; Alonso & Rosenfield, 2003). At least 3 key signalling pathways initiate and control placode development (Alonso & Rosenfield, 2003; Botchkarev & Paus, 2003). These are the Wnt/ β -catenin / lymphoid enhancer-binding factor (Wnt/ β -catenin/LEF-1) system, the Ectodysplasin (Eda)/Ectodysplasin receptor (Edar) and Transforming growth factor β 2 / Transforming growth factor β -receptor II (TGF- β -2/TGF- β -R II). A further 2 signalling pathways inhibit hair follicle development (Botchkarev et al, 2002; Vassar & Fuchs, 1991); BMP-2/4 signalling and EGF signalling. Targeted deletion of β -catenin (Huelsenken et al, 2001) or lymphoid enhancer-binding factor (LEF-1) (van Genderen et al, 1994), both Wnt effectors, results in sparse or absent placode development.

Active foci of cells in the dermis, the dermal papilla are enclosed by the descending hair peg and play a central controlling role in morphogenesis and subsequent hair cycling (Oliver, 1967, Jahoda, 1992). These epithelial pockets protruding into the dermis become the embryonic hair follicles forming preferentially in certain body areas before developing across the fetus in a wave-like sequence (Hardy, 1992).

The hair matrix cells at the base of the newly formed hair follicle are of ectodermal origin as are the cells of the inner and outer root sheath, although the cells of the dermal papilla and the fibroblastic cells in the connective tissue sheath are of mesodermal origin.

At 12 – 15 weeks gestational age, the hair follicle partially differentiates to form the sebaceous gland, the arrector pilli muscle and the bulge area, the site of subsequent stem cell formation (Cotsarelis et al, 1990; Waters et al, 2007). The hair matrix cells overlying the top and sides of the dermal papilla ascend in the follicle and keratinise to form the three layers of the new hair shaft (medulla, cortex and cuticle), the three layers of the inner root sheath (IRS); the cuticle, the Huxley's layer, the Henle's layer, and the outer root sheath (ORS) which coats and supports the emerging hair shaft. The inner root sheath hardens and is keratinised before the hair fibre which it encases and thus determines the resultant hair shaft characteristics (Swift, 1997). The inner cuticle layer closest to the hair shaft is only a few cells deep, interlocking with the cells of the hair cuticle, and held in intimate contact.

As the developing hair fibre ascends to the zone of keratinisation approximately at the mid-point of the follicle, intense protein synthesis occurs (Parakkal, 1969), the cells become elongated, the nucleus disintegrates and the final hair structure is formed at the completion of apoptosis. At the bulge area the inner root sheath layer desquamates and the outer root sheath keratinises to form the stratum corneum, indistinguishable from the surrounding epidermis (Sperling, 1991). At nineteen weeks the first laguno hair emerges (Holbrook & Minami, 1991) to be shed usually immediately before birth (Kligman, 1961). A second laguno synchronised shedding occurs during the first three or four months of life, following which the mosaic pattern of follicular cycling in human beings becomes established indistinguishable from follicular morphogenesis (Stenn et al, 1999).

1.1.5 Molecular controls

It is important to note that a large part of the current understanding of the molecular control of hair follicle cycling and morphogenesis is derived from animal studies, particularly rodents, and the biochemistry in human beings is not therefore necessarily the same. This thesis deals with hair biology, particularly androgenetic alopecia and hair greying in men and this section on molecular controls is therefore of interest and supplementary to and supports the main research topic.

As discussed earlier in section 1.1.2 (Hair cycling) and section 1.1.4 (Hair follicle morphogenesis) communication between epithelial and mesenchyme derived dermal cells is critical for hair follicle development and hair cycling (Stenn & Paus, 2001; Rendl et al, 2005) and therefore intercellular molecules signalling between these different cell types play key roles. The groups of signalling molecules, growth factors and molecular pathways which provide the switch-on and switch-off mechanisms to control morphogenesis and the hair cycle are a key area of research (Pittelkow, 1990; Hardy, 1992; Peus & Pittelkow, 1996; Cotsarelis, 1998, Oro & Scott, 1998; Stenn & Paus, 2001; Millar, 2002; Alonso & Rosenfield, 2003; Botchkarev & Paus, 2003; Mecklenberg et al, 2005). Results have been obtained from studying the expression patterns of candidate molecules or by experiments using transgenic or knock-out mice carrying a specific gene mutation or by analysis of results introducing candidate molecules through bead implantation into the skin. Although animal systems, particularly rodents, are not identical to human beings (e.g. the response to androgens is different), the basic regulating mechanisms are thought to be similar (Millar, 2002). Key peptide

regulators may have a stimulatory effect on cell proliferation and DNA synthesis or an inhibitory effect, using a network of positive and negative feedback loops. Regulatory molecules may be soluble growth factors, peptide hormones or other cytokines e.g. interleukins, and they exert their influence via specific cell surface receptors. Cell adhesion molecules (CAM), which are regulated in expression and activity by growth factors, also contribute to cellular communication and are membrane associated.

The mode of activity of growth factors may be: autocrine, production and reception of growth factors by the same cell; paracrine, regulating signals transmitted to neighbouring cells within the same or adjacent tissues; juxtacrine: regulation and transmission of a signal from a growth factor tethered to a producer cell; or endocrine: activation at a distant site, the signalling factor being transported via the blood. To exert its effect the growth factor is required to bind to a surface cell specific receptor forming a ligand-receptor complex. The growth factor receptor has 3 distinct spatial regions; the extracellular domain that binds the growth factor to the ligand, the transmembrane domain and the intracellular domain (Peus & Pittelkow, 1996). Receptor activation requires the ligand to bind to two neighbouring receptors or sub units to induce a conformational change transmitting a signal to the intracellular domain of the receptor complex and a cascade or series of intracellular reactions are initiated (e.g. protein phosphorylation). DNA synthesis and replication provide the specific cellular response (Fantl et al, 1993; Lemmon & Schlessinger, 1994; Millar, 2002).

Some of the growth factors are structurally or functionally similar and they will therefore be divided into main family groups.

(a) Epidermal Growth Factor Family

Epidermal growth factor (EGF), as its name suggests, stimulates growth and differentiation of the epidermis (Cohen, 1965) and it is also a mitogen for many other cell types; it can also inhibit hair growth in both mice and In human beings when injected (Moore et al, 1981, Philpott et al, 1995), but may play an important role in the initiation of anagen in mice and act as biologic switch in the hair cycle (Mak & Chan, 2003). EGF is expressed in the intermediate layers of the epidermis prior to follicle initiation in sheep (Du Cross et al, 1992) but subsequently only in the ORS adjacent to the region of fibre keratinisation. EGF receptors in rats are localised in the ORS and in the epithelial cells at the base of the hair follicle (Green & Couchman, 1984; Nanney et al, 1984). EGF receptors play a central role in the differentiation of the hair follicle and normal hair development in mice (Hansen et al, 1997).

Transforming growth factor- α (TGF- α), another family member, plays a major role in controlling follicular differentiation and keratinisation and is expressed in normal adult epidermis in human beings and mice (Coffey et al, 1987; Vassar & Fuchs, 1991). TGF- α , binds to EGF receptors to activate the tyrosine kinase pathway (Wong et al, 1989) and has a critical role in the development and differentiation of a number of tissues, including the skin and hair follicle (Derynck, 1988), stimulating keratinocyte growth, cell migration and angiogenesis (Schreiber et al, 1986); over expression of TGF- α is found in hyperproliferative skin diseases such as psoriasis (Elder et al, 1989). TGF- α is expressed in the IRS, ORS and keratogenous zone of the anagen follicle (Stenn et al, 1994). Targeted deletion of TGF- α or its receptors results in premature keratinisation of the IRS, hair follicle

abnormalities, and wavy hair in mice (Luetteke et al, 1993; Hansen et al, 1997).

(b) Fibroblast Growth Factor Family

The fibroblast growth factor (FGF) family consists of at least nine members which have a wide range of biological activities; cell growth, morphogenesis, bone growth, angiogenesis and wound healing (Klagsbrun & Edelman, 1989; Mikkola, 2008). They bind to high affinity receptors (Fornig & Gallagher, 1994) that signal via tyrosine kinase (Miyamoto et al, 1993) and to low affinity receptors. They are potent mitogens for human hair follicle keratinocytes and melanocytes (Pittelkow & Shipley, 1989) and they are key modulators of ovine follicular melanogenesis and cycling (Du Cross et al, 1993) and the proliferation of human dermal papilla cells (Katsouka et al, 1987). FGF-5 initiates entry into catagen in mice *in vivo* (Suzuki et al, 2000) and may have a paracrine role in shortening anagen during androgenetic alopecia (Hamada & Randall, 2006). Transgenic knockout mice made null for the FGF-5 gene have a prolonged anagen phase, giving long hairs and an angora-like phenotype and the FGF-5 gene is found to be mutated in angora mice (Hebert et al, 1995). Conversely, other members of the FGF family (FGF-1) (Du Cross et al, 1993) stimulate entry into catagen in sheep and the FGF family appear to be key modulators of the anagen-catagen switch.

Keratinocyte growth factor (KGF) also known as FGF-7 is a mitogen for specific keratinocytes and other epithelial cells, but not for endothelial cells (Finch et al, 1989, Finch et al, 1995). KGF is up regulated during human wound healing (Werner et al, 1992) and accelerates dermal regeneration (Pierce et al, 1994). In the hair follicle KGF is localised to the dermal papilla,

whereas the KGF-receptor is found throughout the epithelial compartment, indicating paracrine regulation and KGF is thought to be a key mediator of hair follicle growth. KGF has been shown to prolong anagen and to reduce hair loss in chemically induced alopecia (Danilenko et al, 1995).

(c) Transforming Growth Factor- β Family

Transforming growth factor- β (TGF- β) is critically involved in cell production and proliferation during embryogenesis in both rabbits and humans (Porrás-Reyes et al, 1993, Paus et al, 1997) and in wound healing (Massague, 1990; Atkins et al, 2006); TGF- β inhibits growth of normal human epithelial cells, including hair follicle keratinocytes (Philpott et al, 1990), but stimulates proliferation of human fibroblastic or mesenchymal cells (Pittelkow et al, 1988). TGF- β expression is dependent on hair cycle phasing and it is expressed immediately before entry into catagen (Seilberg et al, 1995). TGF- β was shown to induce catagen in mouse (Soma et al, 2003) and human (Soma et al, 2002) hair cycles. TGF- β negatively controls proliferative activity of follicular epithelial cells and has key regulatory roles in murine and human hair growth (Blessing et al, 1993; Foitzik et al, 1999; Foitzik et al, 2000; Hibino & Nishiyama, 2004). TGF- β was found to be up regulated by androgens in human balding frontal dermal papilla cells, and transforming growth factor (TGF- β) is a strong candidate for inhibiting keratinocyte differentiation in androgenetic alopecia (Hibino & Nishiyama, 2004; Hamada & Randall, 2006). TGF- β was not similarly up regulated in human non-balding dermal papilla cells (Inui et al, 2002). At least 3 isomers are known, TGF- β 1, TGF- β 2 and TGF- β 3.

Bone morphogenic proteins (BMP) are members of the TGF- β superfamily and BMP 2 and BMP 4 are morphogenic regulatory factors, expressed during murine hair follicle morphogenesis (Lyons et al, 1990) and cycling (O'Shaughnessy et al, 2004). BMP 4 is transiently expressed in early murine mesenchymal condensation (Jones et al, 1991) preceding hair follicle formation and may modulate the dermal signal inducing germ formation. BMP 2 is expressed in the epidermal placode, and in mature follicles BMP 2 expression coincides with the cessation of follicle cell proliferation (Wozney et al, 1988; Massague & Chen, 2000).

(d) Insulin-Like Growth Factor (IGF) Family

IGF-I & IGF-II, the primary members of the insulin-like growth factor family, both have a substantial amino acid homology to insulin and are probably the key regulators of human hair cell activity and hair follicle cycling (Philpott et al, 1994, Weger & Schlake, 2005). Both are synthesised in the body by a wide range of tissues. IGF-II is more prominently expressed in fetal tissue, whereas IGF-I persists at higher levels in adult tissue and, uniquely among growth factors, plasma contains substantial levels of IGF-I suggesting endocrine influence. Transgenic mice that over express IGF-1 are reported to have accelerated rates of whisker growth (Su et al, 1999). IGF-1 has a ten fold greater follicle growth potential than IGF-II, but both entities are powerful mitogens and important regulators of hair growth and may be major controlling influences on both the human and murine hair cycle (Philpott et al, 1994; Su et al, 1999). IGF-I and IGF-II are potent suppressors of follicle entry into catagen. IGF-I stimulates replication of both mesenchymal and epithelial cells, increases human hair growth *in vitro* and in the absence of the IGF

signal, DNA synthesis and cell replication cease. IGF-I expression in the dermal papilla cells of human beard follicles is stimulated by androgens which may mediate their hair follicle activity via IGF (Itami et al, 1995). Interestingly IGF-I expression in human dermal papillae cells correlates with therapeutic efficacy of finasteride in androgenetic alopecia (Tang et al, 2003).

(e) Other important growth factors

Nerve growth factor (NGF) is synthesised in developing skin (Davies et al, 1987) and is expressed in both epithelial and mesenchymal compartments. NGF and brain-derived neurotrophic factor (BDNF) both members of the neurotrophin family, control morphogenesis of the follicular papilla (Botchkarev et al, 1999). NGF has neurotrophic and morphogenic properties; it exerts an inhibitory influence during murine anagen (Paus et al, 1994) but stimulates proliferation of keratinocytes in telogen. NGF-receptor levels are maximum during early murine anagen, in the bulge region, but expression declines in late anagen and catagen and hair cycling is dependent on NGF and NGF-receptor expression (Paus et al, 1994), and neurotrophins are key regulators of murine hair follicle morphogenesis and cycling (Peters et al, 2002).

Hepatocyte growth factor/Scatter factor (HGF/SF) acts as a mitogen or a morphogen, and also influences cell motility (Montesano et al, 1991). HGF/SF is secreted by mesenchyme derived cells and exerts a paracrine influence on neighbouring epithelial cells (Matsumoto & Nakamura, 1992) and has been shown to promote human hair growth *in vitro* (Jindo et al, 1994, Shimaoka et al, 1995). The exact role of HGF/SF in the hair follicle is

unknown, but HGF/SF and its receptor have been shown to be key mediators of mesenchymal-epithelial interaction in murine hair follicle morphogenesis and cycling (Lindner et al, 2000).

Vascular Endothelial Growth Factor (VEGF) is a highly specific mitogen for murine endothelial cells via specific cell surface receptors (Plate et al, 1992; Kim et al, 1993). However VEGF and VEGF receptors are localised to the dermal papilla cell during anagen with decreased expression during catagen and telogen (Lachgar et al, 1995). VEGF is an autocrine growth factor for dermal papilla cells (Lachgar et al, 1996). Platelet Derived Growth Factor (PDGF) is found in some endothelial cells and keratinocytes and binds to specific cell surface receptors. PDGF is implicated in many biological processes including morphogenesis and wound healing (Meyer-Ingold & Eichner, 1995). PDGF is present in the hair matrix and PDGF receptors are localised in the dermal papilla (Ponten et al, 1994). Its precise role is not known but it may activate and mediate proliferation of dermal papilla cells and possibly modulate the hair cycle and hair growth (Karlsson et al, 1999). Tumour Necrosis Factor- α (TNF- α) plays an inhibitory role in hair follicle cycling and has a role in catagen induction and human hair follicle degradation (Philpott et al, 1996).

In summary over 100 growth factors or molecular entities have been identified as modulating morphogenesis or cycling of the pilosebaceous unit and the inductive focus, the dermal papilla, sends and receives its inductive signals using mesenchyme-epithelium reactions in a highly complex network (Stenn & Paus, 2001; Millar, 2002; Alonso & Rosenfield, 2003).

1.1.6 Stem cells

Most mammalian organs and tissues essentially cannot self-renew and eventually undergo a terminal period of increased molecular disorder, senescence and death (Hayflick, 2007). However, some organs and tissues do retain the ability to renew and to affect repair after wounding or trauma and the source of this regenerative ability is a reservoir of undifferentiated, quiescent, multipotent specialised cells or stem cells. The capacity of stem cells to differentiate into a broad range of different types, and therefore the potential for the development of adult stem cell therapy has driven a very considerable amount of stem cell research (Miller et al, 1993; Morrison et al, 1997; Cotsarelis et al, 1999; Blanpain et al, 2004; Nishimura et al, 2005; Hoffman, 2006; Waters et al, 2007; Fuchs, 2008).

Embryonic stem cells function only during a very limited period of ontogenesis (Pera et al, 2000) but some mammalian systems retain their own lineages of stem cells; brain, liver (Morrison et al, 1997), muscles, cornea (Schermer et al, 1986) and some epithelial structures, like the epidermis or hair follicle, are good examples of self-renewing tissues. Stem cells can also be re-programmed to produce different stem cells (Bjornson et al, 1999; Morrison, 2001) and murine hair follicle stem cells cultured *in vitro* have been shown to produce a full range of skin tissue; skin cells, follicle cells and sebaceous gland cells (Blanpain et al, 2004). Corneal stem cells can also be induced to proliferate and form hair follicles (Ferraris et al, 2000; Pearton et al, 2005). Hair follicle stem cells when grafted onto “nude” mice produce normal hair density (Blanpain et al, 2004). Additionally this

transdifferentiation and interconversion is modulated by the surrounding tissue (Fuchs & Segre, 2000; Seale & Rudniski, 2000; Ferraris et al, 2000).

Hairs and epithelial cells are constantly shed and renewed and the proliferative pool of keratinocytes required is generated by epithelial stem cells (Cotsarelis et al, 1999; Tumber et al, 2004), and also by hair follicle dermal stem cells after wounding (Jahoda & Reynolds, 2001; Gharzi et al, 2003). Stem cells are undifferentiated, very slow cycling and form a latent reservoir with a high proliferative potential which in response to an inductive signal (i.e. post-trauma or the hair follicle entry into anagen) differentiate to form further stem cells and also transient amplifying cells which actively divide to replace keratinocytes lost during apoptosis. Each stem cell in the hair follicle provides a flow of cells and is described as an “epithelial proliferative unit” (Koloda et al, 1998; Cotsarelis et al, 1999; Fuchs, 2008).

Most hair follicle stem cells reside in a reservoir located at approximately the bulge area or isthmus and this is thought to be the ultimate source of both epidermal and hair follicle keratinocytes (Cotsarelis et al, 1990; Kobayashi et al, 1993; Bickenbach & Chism, 1998; Akiyama et al, 2000; Waters et al, 2007). These cells proliferate only briefly during early anagen; they are quiescent throughout the rest of the hair cycle (Lyle et al, 1998) and are thought to survive for the lifetime of the animal (Morris & Potten, 1999). Location of stem cells may differ between follicle types; pelage follicle stem cells are located at the bulge area whereas vibrissae (whisker) stem cells occur at the base of the follicle, although in both structures they effectively occur at the lowermost point of the permanent follicle. The bulge area is a prominent feature in human fetal skin, but is less easily identified in adults

and the arrector pilli attachment site is not necessarily an exact location (Wilson et al, 1994; Lyle et al, 1998). This slow cycling concentration of stem cells in the bulge area may also be the site of origin for some tumours. Genetic variations may accumulate preferentially in these cells and the hair follicle is a prime source of skin cancers (Miller et al, 1993; Jih et al, 1999). The bulge area of the ORS may also be the target for agents involved in various scarring and non-scarring alopecias; if the bulge area remains intact during alopecia, hair growth may re-commence, as in alopecia areata reversal or it may be permanently damaged and re-growth will not be possible i.e. systemic lupus erythematosus and pseudopelade.

Stem cells are found to be biochemically different from the surrounding keratinocytes, and mesenchyme cells; they have a higher keratin content and they are rich in EGF-R and other receptors, with convoluted nuclei reflecting their proliferative potential (Cotsarelis et al, 1999). Keratin expression patterns correlate well with levels of cell differentiation and are used as a marker to define cells in the same state of differentiation (Lane et al, 1991; Schirren et al, 1997). Keratin 15 (K15) and keratin 19 (K19) are both markers for stem cells (Michel et al, 1996) and keratin expression patterns can be used to locate stem cell colonies in some species, although identification is less clear in human follicles. Many basal cell carcinomas and trichoepitheliomas arise in the bulge and are K15 positive, suggesting they originate from hair follicle stem cells (Jih et al, 1999). Loss of stem cell K15 expression may be an early sign of transition from stem cell to transient amplifying cell (Michel et al, 1996). Using K19 expression patterns, two distinct populations of stem cells have been identified in the hair follicle, each

inductively independent; one population in the bulge area and a second population in the hair germ at the base of the hair follicle (Como et al, 2000). These two populations have unrelated cell lineages (Kamimura et al, 1997) and are of dual origin (Ghazizadeh & Taichman, 2001) and must therefore have multiple progenitor cells. It is suggested that the bulge stem cells are the source of the descending ORS structure whilst the hair germ stem cells are the source of keratinocytes to assemble the ascending hair shaft and IRS (Akiyama et al, 2000). During anagen III the bulge derived ORS pushes the hair follicle downward, simultaneously cell proliferation from the germ derived hair shaft and IRS pushes the assembled structure in an upward direction (Reyholds & Jahoda, 1991; Oshima et al, 2001). Transgenic “hairless” mouse skin in which the dermal papilla is stranded deep in the dermis and does not ascend shows a downward cellular string similar to an ORS, but does not exhibit an IRS or hair shaft (Panteleyev et al, 1998). The dermal papilla must ascend to the bulge and be aligned with it or the hair growth cycle will not recommence and a new hair will not be formed (Cotsarelis et al, 1990). Labelling experiments show that bulge derived cells can migrate down the ORS to the lowermost part of the follicle in both vibrissae and pelage follicles (Oshima et al, 2001; Roh et al, 2005), and become the source of the epithelial proliferative unit (Taylor et al, 2000; Ghazizadeh & Taichman, 2001). During mature anagen these virtually quiescent and bulge derived cells concentrate to form a “lateral disc”, at the base of the follicle, in intimate contact with the dermal papilla.

This distinct group of apoptosis resistant epithelial cells at the base of the follicle can induce a new hair follicle without reference to the bulge region.

The murine hair follicle when transplanted maintains its original intrinsic rhythm over approximately the first hair cycle (Ebling & Harvey, 1964) and then acquires the features of the surrounding cycling follicles. The hair germ appears to initiate the entry into anagen signal via the dermal papilla, whilst in subsequent hair cycles the stem cells are modified by the surrounding tissue and adopt the neighbouring cycling sequence. It has been proposed therefore that there is a “pre-determination” of hair follicle genesis, which is conditioned by the preceding hair cycle (Panteleyev et al, 2001).

In summary, the dual origin of the hair follicle stem cell lineages and the timing of recruitment of stem cells suggest that the ascending portion of the hair follicle (hair shaft and IRS) arises not from bulge located stem cells, but from stem cells in the secondary hair germ, the bulge stem cells contributing only to the descending ORS during anagen. The lower hair follicle disintegrates during catagen, but the lateral disc of cells survives apoptosis and is pulled upward with the dermal papilla to the bulge region, transforming into a secondary hair germ and reacquiring stem cell characteristics (Ferraris, 2000). The secondary hair germ initiates the primary signal to the dermal papilla and anagen is initiated stimulating the activity of bulge located stem cells and the cycle recommences (Waters et al, 2007).

1.2. Hair pigmentation

1.2.1 Description

Hair colour in human beings ranges from white, yellow and red through to black, however most human beings have black hair (Eiberg et al, 2008). The

pigmentation serves no purpose and is purely decorative, but provides crucial sexual and social communication, although it is possible that there was an earlier evolutionary purpose (Bertazzo et al, 1996; Slominski et al, 2004). In the furry mammals (canine, murine, feline etc.) pigmentation and patterning of the pelage provides communication and camouflage with the ability to adapt pelage pigmentation to seasonal changes (Flux, 1970).

In some mammals e.g. Syrian golden hamster (Quevedo, 1961) the underlying epidermis is also pigmented, but in others it is unpigmented e.g. mouse, domestic animals, chimpanzees (Post et al, 1975). In human beings the epidermis is pigmented, the amount of pigmentation correlates approximately to latitude or racial origin i.e. the lower the degree of latitude the higher the level of pigmentation. Epidermal pigmentation is stimulated by, and provides protection from, UV exposure; the pigment melanin absorbs UV radiation, although too much melanin in the skin interferes with vitamin D3 synthesis (Jablonski & Chaplin, 2000). Scalp hair colour does not correspond well with latitude nor does it necessarily correspond to skin colour. Several ethnic groups have black hair but white skin e.g. people of Chinese descent, other ethnic groups e.g. people of Thai or South Asian descent have black hair with brown skin, although the epidermis is less pigmented under the black scalp hair and the skin becomes pigmented when hair is lost as in male pattern alopecia (personal observation). Active melanocytes (melanin producing cells) are only present in the exposed areas (Post et al, 1975). People of African or Caribbean descent tend to have highly pigmented black hair and skin. In people of North European descent the hair is often blonde or not fully pigmented until puberty (Sunderland, 1956; Allende, 1972). The

unusually wide diversity of hair colour of Northern European people (brown, blonde and red) is pronounced and has been linked to polymorphism of the melanocortin 1 receptor (MCI –R) gene (Rees, 2000). It has been suggested that an earlier genetic mutation affecting the OCA 2 gene, which codes for protein P, involved in the production of melanin, gave rise to reduced melanin synthesis and human beings with blue eyes, fair skin and blond hair (Eiberg et al, 2008).

1.2.2. Melanin synthesis

Epidermal and follicular pigmentation are provided by the pigment, melanin, the production of which is under complex genetic control (Hearing & Tsukamoto, 1991; Slominski et al, 2005). Tyrosine is the key precursor for melanogenesis and is converted by the copper containing enzyme tyrosinase to L-dihydrophenylalanine (DOPA) which is then oxidised to dopaquinone (Prota, 1995), a key intermediate in the production of the two main melanin types; pheomelanin and eumelanin. The synthesis of epidermal and follicular pigmentation is similar sharing identical chemical pathways, crucially however epidermal melanin can be in continuous production, stimulated further by UV exposure or trauma, whereas follicular melanin production is cyclical and is exactly linked to the hair cycle (Slominski et al, 2005). Hair is actively pigmented only in the anagen phase (Slominski & Paus, 1993; Slominski et al, 2005) and pigment synthesis is absent in catagen and telogen. During embryogenesis, after construction of the hair follicles, melanoblasts (potential melanin producing cells) migrate from the neural crest (Barsh & Cotsarelis, 2007) to all body regions specifically to the dermis

and into the epidermis from where some enter the maturing hair bulb to become active melanocytes. Mutations in the transcription factor FOXN 1 which causes the nude phenotype in mice, characterised by a lack of visible hair, also contributes to hair colour by marking which cells are to receive pigment from the melanocytes (Weiner et al, 2007). The presence and densities of embryonic melanocytes has been determined (Holbrook, 1989) and melanogenic melanocytes have been shown to be present at all stages following the appearance of the hair germ (Peters et al, 2002).

1.2.3 Melanin transfer

Melanogenesis (the synthesis of the pigment melanin) in the hair occurs only in the hair bulb (Slominski et al, 2005), where highly specialised organelles, the melanosomes within the melanocytes produce pigment granules of melanin. Melanosome structure correlates with the type of melanin produced; eumelanosomes are elliptical and have a fibrillar matrix whereas pheomelanosomes are predominantly smaller and spherical and have a vesicular globular matrix (Slominski et al, 2005). The melanosomes locate to the dendritic tips of the melanocytes and when completely melanised (full of melanin) are transferred to the hair follicle keratinocytes. Melanosome transport may involve direct inoculation (Yamamoto & Bhawan, 1994), pinching off the inserted melanocyte dendrite tip; membrane fusion from melanocyte to keratinocyte or possibly the release of the melanosome into the intercellular space. Melanin granules are incorporated mainly into the cortex of the developing hair shaft (aligned parallel to the axis of the hair shaft itself) with a smaller number in the sponge like hair medulla, with very

few or none in the cuticle or inner root sheath. Hair colour in all mammals depends on the levels and types of melanins synthesised (Hearing & Tsukamoto, 1991) and also on the degree of effective melanin transfer to the hair shaft keratinocytes (Granholm et al, 1990, Vale, 2003).

Melanosomes in the same cell can produce either type of melanin (pheomelanin or eumelanin) but not both simultaneously (Oyehaug et al, 2002). Eumelanin is a black-brown pigment insoluble in all solvents produced by a multistep transformation from the common precursor dopaquinone. Pheomelanin is a red-brown alkali soluble pigment produced by the initial conversion of dopaquinone to cysteinyl-dopa and then by stages to pheomelanin, which is sulphur containing (Prota, 1995). Oxymelanins are similar to pheomelanins but contain no sulphur (Prota, 1988). Further types of melanins may be present by enzymatic oxidation of other agents (serotonin, melatonin) catalysed by tyrosinase (D'Ischia et al, 1991; Bertazzo et al, 1994). It has been proposed that the ratio and relative concentrations of the amino acids cysteine and glutathione are implicated in the regulatory switch between pheo and eumelanin synthesis (Jimbow et al, 1992). Cysteine transported through the melanosome membrane forms the intermediate cysteinyl-dopa producing pheomelanin (Potterf et al, 1999). High concentrations of glutathione lead to synthesis of eumelanin, whereas low concentration of glutathione produce pheomelanin (Slominski et al, 2005). The speed and specificity of melanin synthesis is controlled by a group of melanogenesis regulatory enzymes (MRE); however the availability and concentration of tyrosinase is the central component for the initiation and

regulation of melanogenesis (Pawalek & Korner, 1982, Schallreuter & Wood, 1999, Westerhof, 2006).

1.2.4 Melanocytes and melanin structure

All melanins have a broadly similar organic C-C or C-N ring structure but they have different chemical and physical properties which allow melanin identification. Both exhibit chemiluminescence, but only eumelanin has a stable paramagnetic state giving a specific electron paramagnetic resonance (EPR) spectrum which allows identification of the melanin type (Seal et al, 1982).

Black and brown hair contains high levels of principally eumelanin pigment and these follicles have the largest number and density of melanocytes of any hair type. Red hair contains mainly pheomelanin pigment, deposited in irregular patches, whereas blonde hair contains smaller granules of mixed, poorly melanised eu & pheomelanins with often only the melanosomal matrix visible. Grey or white hair has little or no pigmentation with a reduced number of hair follicle melanocytes and decreased melanosome activity (Como et al, 2004). Melanocyte distribution in the hair bulb has been determined and hair follicle melanocytes have been cultured (Tobin et al, 1995, Tobin and Bystryn, 1996). Two subtypes of functionally different melanocytes have been identified; pigmented dendritic melanocytes in the infundibulum and hair bulb and amelanotic non-dendritic melanocytes possibly transient in the outer root sheath and the middle and lower follicle (Narisawa et al, 1997). The amelanotic melanocytes are believed to be precursors for the dendritic ones (Nishimura et al, 2002; Nishimura et al,

2005). Hair bulb melanocytes are active only during anagen II-VI (Sugiyama et al, 1995) although tyrosinase synthesis occurs during early anagen (Slominski et al, 1991), accompanied by an increase in size and number of melanocytes. There is no tyrosinase synthesis during catagen and telogen and pigmented melanocytes lose their dendrites, become amelanotic and disappear from the hair bulb (Slominski and Paus, 1993). The fate of bulb melanocytes during catagen and telogen and the source of melanocytes as the new bulb invaginates downward into the dermis remains unclear (Tobin et al, 1999; Tobin & Paus, 2001). The melanocytes may survive in an undifferentiated form or they may undergo apoptosis during catagen and are lost (Lindner et al, 1997, Tobin et al, 1998) to be replenished in early anagen from a pool of undifferentiated melanocyte stem cells residing in the hair follicle bulge. These quiescent melanocyte stem cells residing in the bulge receive signals at entry into anagen to produce both transient amplifying cells which differentiate into active melanocytes and other dormant melanocyte stem cells which return to the niche (Nishimura et al, 2002). Recently it has been shown that there are 2 distinct melanocyte subpopulations in the hair follicle exhibiting differing patterns of apoptosis and survival during catagen (Sharov et al, 2005). The follicular melanocytes do not proliferate during catagen and melanocytes from the outer root sheath or dermal papilla; repopulate the secondary hair germ in late catagen.

1.2.5 Melanogenesis control systems

Complex multi-layer molecular control systems regulate mammalian melanogenesis (Slominski et al, 2004), in a series of parallel control systems

with no simple linear control sequence. Extensive murine coat mutation experiments have shown that over 150 coat mutations can be engineered and some of the large numbers of genes involved in active pigmentation have been identified (Jackson 1994, Westbrook et al, 2001, Vale, 2003). Approximately 150 alleles spread over 90 loci have been determined (Nakamura et al, 2002) synthesising a multitude of protein products acting as enzymes, structural proteins, transcription regulators, transporters, receptors and growth factors with numerous functions and target cells (Hearing, 1999). Tyrosinase gene expression level appears similar across human racial groups (Fuller et al, 2001) and human melanogenic activity levels depends mainly on post-translational pathways, especially the effective processing of tyrosinase (Iozumi et al, 1993).

Mutation in the *c-kit* gene results in piebaldism (Spritz, 1994) and in mice, inhibition or “knock out” of the *c-kit* gene results in the growth of unpigmented hair (Nishikawa et al, 1991) and the tyrosine kinase receptor *c-kit* activated by stem cell factor (SCF) has a positive key role in murine melanocyte embryogenesis (Nishikawa et al, 1991, Hemesath et al, 1998), its presence is required for melanoblast proliferation and migration. Kit/SCF interaction is vital for melanocyte activity (Steel et al, 1992) and disruption of this pathway interferes with migration, differentiation and survival of melanocytes (Peters et al, 2002). Endothelin 3 and receptor B is another signalling pair essential for mammalian melanocyte development (Lahav et al, 1998; Alhaidari et al, 1999). An array of enzymes mediate human and murine melanogenesis, principally tyrosinase, tyrosinase-related protein I (TRP I) and tyrosinase-related protein II (TRP II) and the relative ratios may control the switch

between eumelanogenesis and pheomelanogenesis (Bentley et al, 1994 and Ganss et al, 1994). Other melanogenesis stimulating proteins have been identified; the peptide hormone, melanocyte stimulating hormone (MSH) has a regulatory role in melanogenesis and may also regulate the switch between production of eu and pheomelanin (Suzuki et al, 1999). Recently the synthetic alpha-MSH-related peptide, K(D)PT showed human hair re-pigmentation stimulation, increasing the amount of melanin in the hair follicle after pre-treatment with Interferon type II (IFN- γ) and demonstrating a possible anti-greying hair pathway (Meyer et al, 2008).

Agouti signalling protein (ASP) is a key modifier and is an inhibitor of melanogenesis in animal, but not human hair, although it may modify intensity and end product of melanogenesis (Barsh et al, 2000), and the hormones serotonin (McEwan & Parsons, 1987) and melatonin (Valverde et al, 1995) have an anti-melanogenic effect in some mammals, possibly by interference in the regulation of apoptosis and proliferation of melanocytes (Slominski et al, 2003). Melatonin is known to be synthesised in the hair follicle and may have a functional role in hair cycle control (Fisher et al, 2008). Melanin pigment itself may also modulate melanocyte behaviour (Donatien & Orlow, 1995). Intracellular signalling pathways have positive and negative roles with cAMP a critical positive factor in the regulation of melanogenesis (Pawalek, 1976, Pawalek, 1979). cAMP is activated by protein kinase A (PKA) and stimulates melanin proliferation and also melanocyte differentiation and proliferation (Korner & Pawalek, 1977, Park & Gilchrest, 1999). Abnormal pigmentation arises either from mutations in gene sequences or from defects in the subsequent melanogenic pathway.

Mutation in the tyrosinase gene gives rise to impaired or no tyrosinase activity and albinism results where hair, skin and eye pupils have no pigmentation. Lack of melanosomes or reduced melanosome size presents as vitiligo of the epidermis and whitening patches, piebaldism of the hair (Comings & Odland, 1966, Wankowitz-Kalinska, 2003) and in cases of alopecia areata spontaneous reversal of hair growth is not always accompanied by full pigmentation, indicating impaired melanogenesis (Paus et al, 1995, McDonagh & Messenger, 1996).

1.2.6 Loss of scalp hair colour

Melanocytes remain active for between 7 and 15 hair cycles (Peters et al, 2002) until melanogenesis ceases or is seriously impaired and the gradual dilution of pigment with age leads to grey hair or canities. Hair greyness may be variable in degree and age of onset but correlates with age and is a prime marker of the ageing process (Bulpitt et al, 2001). Premature canities of the scalp hair may appear in the second or third decade, although the fourth decade would be normal for the onset of canities; it is proposed that 50% of caucasians are 50% grey by the age of 50 (Keogh & Walsh, 1965). It has been suggested that premature canities may be linked to low bone density (Morton et al, 2007), or to thyroid disease (Wright, 1986), or to pernicious anaemia (Dawber, 1970). All hair pigmentation is usually lost by the age of 70 (Keogh & Walsh, 1965) and loss of hair pigmentation is usually permanent, although partial reversion has been reported (Tobin & Cargnello, 1992; Shetty, 1995)

Greying or loss of scalp hair colour occurs in both sexes and in all races, usually starting in the parietal areas spreading gradually to the vertex and whole scalp, affecting the occipital area last (Boas & Michelson, 1932; Straile, 1964; Keogh & Walsh, 1965). The greying progress usually takes approximately 15 years to affect the full scalp (Burch et al, 1971). Melanocytes are lost through cell senescence (cellular ageing) or self-destruction by apoptosis and the free radical theory of ageing, proposes that reactive oxygen species (ROS), generated by bulbar melanocytes in the synthesis of melanin induce “oxidative stress” (Tobin & Paus, 2001). Reactive oxygen species cause damage to the nuclear and mitochondrial DNA resulting in an accumulation of mutations. Hydrogen peroxide even in low doses can induce senescence in cultured fibroblasts (Hu et al, 1995) and the link between oxidative stress and longevity has been demonstrated in mice protected against oxidative stress which showed 30% longer life spans than unprotected controls (Migliacco et al, 1999). Melanocytes in grey hair are often highly vacuolated (Westerhof, 1997), a cellular response to increased oxidative stress, and may resemble apoptosis debris. These melanocytes contain fewer and smaller melanosomes, finally suffering degenerative changes and disappearing (Como et al, 2004; Nishimura et al, 2005) and the melanocyte reservoir may experience genetically regulated exhaustion after the average grey free life span of 45 years. However it has been shown that some hair follicle melanocytes remain in senile white hair and these can be induced to pigment in culture (Tobin et al, 1995) raising the theoretical possibility of canities reversal. Additionally there may be dislocation of melanocyte–keratinocyte transfer and melanin granules may

be present in melanosomes, albeit reduced in number, but not incorporated into the keratinocytes (Tobin & Paus, 2001).

BCL-2 is a human proto-oncogene which is a known apoptosis inhibitor (Veiss et al, 1993, Hockenbergen et al, 1993). BCL-2 deficient mice turn grey with second coat hair growth (Nakayama et al, 1994) and BCL-2 appears necessary to prevent melanocyte stem cells undergoing uncontrolled apoptosis during entry into the dormant state (Veiss et al, 1993, Hockenbergen et al, 1993). It has been shown using transgenic murine coat mutations that hair graying is probably due to incomplete maintenance of melanocyte stem cells in the bulge region (Steingrimsson et al, 2005), a process rapidly accelerated by BCL-2 deficiency (Nishimura et al, 2005), rather than loss of melanocytes from external cytotoxicity following melanin synthesis (Johnson and Jackson, 1992, Veiss et al, 1993). Hair colour may also be influenced by androgens (Slominski et al, 2004) which were shown to increase feather pigmentation in mallards (Hasse et al, 1995) and have been used as a treatment for generalised vitiligo of the skin (Muto et al, 1995). Conversely they were shown to reduce hair colour in androgenetic alopecia by inhibiting dermal papilla stem cell factor (SCF), disrupting melanocyte pigmentation in the hair bulb (Randall et al, 2008).

1.3 Hormonal regulation of hair growth

1.3.1 Androgens

Androgens, the male sex steroid hormones, are the main regulators of human body hair growth and a connection between the testes and hair growth was noted by Aristotle. Androgens, *in utero*, determine the normal

male phenotype, and during childhood scalp hair, eyelashes and eyebrows grow normally with no androgens present. The appearance of male and female sex hormones at puberty determine libido, muscle mass, skeletal shape and induce the appearance of the secondary sexual characteristics. Androgens alter the body hair growth pattern in both sexes into well defined patterns (Marshall & Tanner, 1969; Marshall & Tanner, 1970) and the appearance of body hair in both sexes is a marker for the endocrine changes at puberty (Reynolds, 1951).

In response to androgen stimulation, the vellus hairs of the axillae and pubis, which are fine, soft, poorly pigmented and with no central medulla, are replaced by thicker, medullated terminal hairs with greater pigmentation. The follicles of the male beard are changed from fine vellus hair production to terminal hair production (Reynolds, 1951), and in cases of androgenetic alopecia the reverse process occurs in defined areas of the scalp (Hamilton, 1942; Hamilton, 1960). The growth of the eyelashes remains unchanged, beard growth is fully established at around 30 years of age (Raynolds, 1951), while ear canal hair growth is not established until 50 years of age (Hamilton, 1946). The differing and contradictory hair growth responses to androgens present a paradox (Randall, 2007). Successive hairs from the same follicle are therefore able to change in length, diameter and colour as, for example, in case of androgenetic alopecia (Hamilton, 1951). It is probable that the same hair follicle is capable of producing laguno, vellus and terminal hairs, in successive hair cycles (Kligman, 1959) allowing these changes to occur.

1.3.2 Androgen synthesis

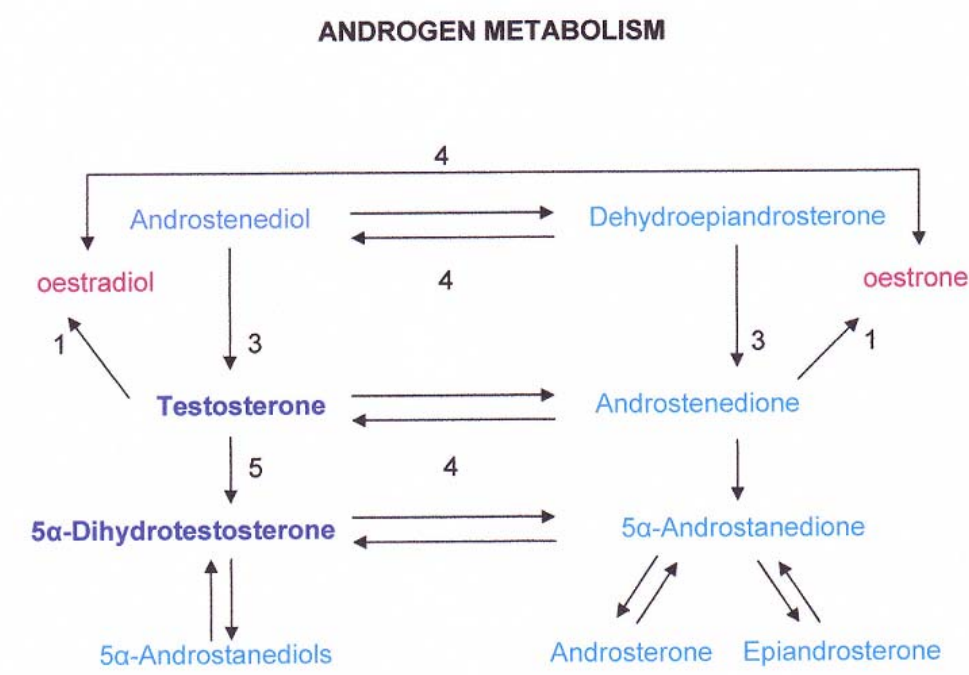
Androgens are synthesised in the body by enzyme cleavage from pregnenolone, a 21 carbon substrate to give a C-19 complex with a C-17 ketone, distal ring. This family of weak androgens, characterised by their affinity for androgen receptors, includes dehydroepiandrosterone (DHEA) which can be converted into testosterone, the key circulating androgen in man (see Figure 1.6), and a potent androgen with great affinity for the androgen receptor. Additionally, testosterone can be metabolised in many tissues, including the skin, into 5 α -dihydrotestosterone (DHT) by the enzyme 5 α -reductase. DHT is five times more potent than testosterone itself based on its affinity for androgen receptors (Fang et al, 1969; Mainwaring, 1969; Rosner et al, 1992) and it has been implicated in the pathogenesis of several disorders including benign prostatic hyperplasia, hirsutism and androgenetic alopecia. Enzymatic pathways in the skin can remove testosterone and DHT by conversion to weaker 17-ketosteroids or androstenedione and testosterone can be metabolised by other enzymes including aromatase to oestrogenic compounds including oestrone and 17 β -oestradiol (Kaufman, 1996).

Systemic androgen levels in women are low, weaker androgens, such as DHEA and androstenedione acting as the precursors; however elevated androgen levels in women can produce hirsutism, excess hair growth in the male pattern, as found in cases of polycystic ovaries (Ferriman & Gallwey, 1961; Conway et al, 1989). Hirsutism can also be idiopathic in nature with a case reported of hirsutism on one side of the body (Jenkins & Ash, 1973).

Figure 1.6

Androgen metabolic pathways in the skin

(Randall, 2000) Reproduced with kind permission.



- KEY**
- Weak androgens
 - Potent androgens – act via the androgen receptor
 - Oestrogens – act via the oestrogen receptor
- 1 - Aromatase
2 - 17 α -Hydroxylase
3 - 3 β -Hydroxysteroid Dehydrogenase
4 - 17 β -Hydroxysteroid Dehydrogenase
5 - 5 α -reductase

Mutation of the 5 α -reductase gene produces people with 5 α -reductase deficiency having normal testosterone levels and greatly reduced DHT levels. The phenotype in men with this deficiency is partially feminised with body hair in the female pattern and no incidence of androgenetic alopecia or benign prostatic hyperplasia, whilst women are phenotypically normal (Imperato–McGinley et al, 1974; Walsh et al, 1974). Therefore, DHT rather than testosterone itself appears to mediate the progression of benign prostatic hyperplasia, beard and other male characteristic hair growth including androgenetic alopecia.

Two isoforms of the enzyme 5 α -reductase have been identified; type 1 and type 2 (Anderson & Russell, 1990; Anderson et al, 1991; Harris et al, 1992; Russell & Wilson, 1994) and these are differentially expressed in body tissues. Type 1 5 α -reductase, which has an optimum pH of 7.5 for the reduction of testosterone to DHT, is the predominant form in the skin and sebaceous glands and is expressed normally in patients with 5 α -reductase deficiency syndrome. Type 2 5 α -reductase which has a lower optimum reduction pH of 5.0, is decreased or absent in patients with 5 α -reductase deficiency, and is the predominant enzyme in genito-urinary tissues. Type 2 tissue activity has been found in the beard, chest skin, liver, seminal vesicles, prostate, testes and foreskin. Men with 5 α -reductase deficiency have not been reported to go bald and finasteride, a 5 α -reductase type 2 inhibitor, can stimulate hair to regrow in young men with androgenetic alopecia. Type 2 5 α -reductase is also expressed in normal scalp skin briefly after birth, followed by an absence of expression of 5 α -reductase until puberty, when type 2 expression appears and predominates through adult

life. The purpose of this brief role is unknown, but some form of imprinting of the immature hair follicles has been suggested (Thigpen et al, 1993). Type 2 5 α -reductase post puberty determines beard growth and the progression of androgenetic alopecia but it is not required for axillary and pubic hair growth (Randall, 2007; Randall, 2008).

1.3.3 Androgen action at the hair follicle

Androgens, in common with all steroid hormones, mainly exert their influence and physiological action by binding to unique intracellular receptors to form hormone-receptor complexes, which activate or modulate transcription and processing, stimulating production of specific proteins (Figure 1.7).

The reaction is reversible and the circulating levels of free androgen are in equilibrium with androgens bound to the carrier proteins, principally sex hormone binding globulin. Androgens can also act independently of the intracellular androgen receptors by interaction with c-AMP providing a second messenger pathway (Nakhla et al, 1995; Rosner et al, 1992), though this is considered to be a very minor route. The current hypothesis (Randall, 1994; Randall, 2007) for androgen action proposes that androgens in the blood stream enter the hair follicle via the capillaries in the dermal papilla and bind to specific androgen receptors inside the dermal papilla cells of androgen sensitive follicles, sometimes having first been converted to DHT which also binds to the appropriate receptors (see Figure 1.8). The hormone androgen receptor complex alters the gene expression of the dermal papilla cells, altering the production of paracrine factors resulting in changed activity of neighbouring keratinocyte and melanocyte cells.

Figure 1.7

Androgen action at cell level

Androgens inside the cell bind to specific androgen receptors and are metabolised to the more potent androgen 5 α -dihydrotestosterone which binds more strongly to the androgen receptor (Randall, 1994). Reproduced with kind permission.

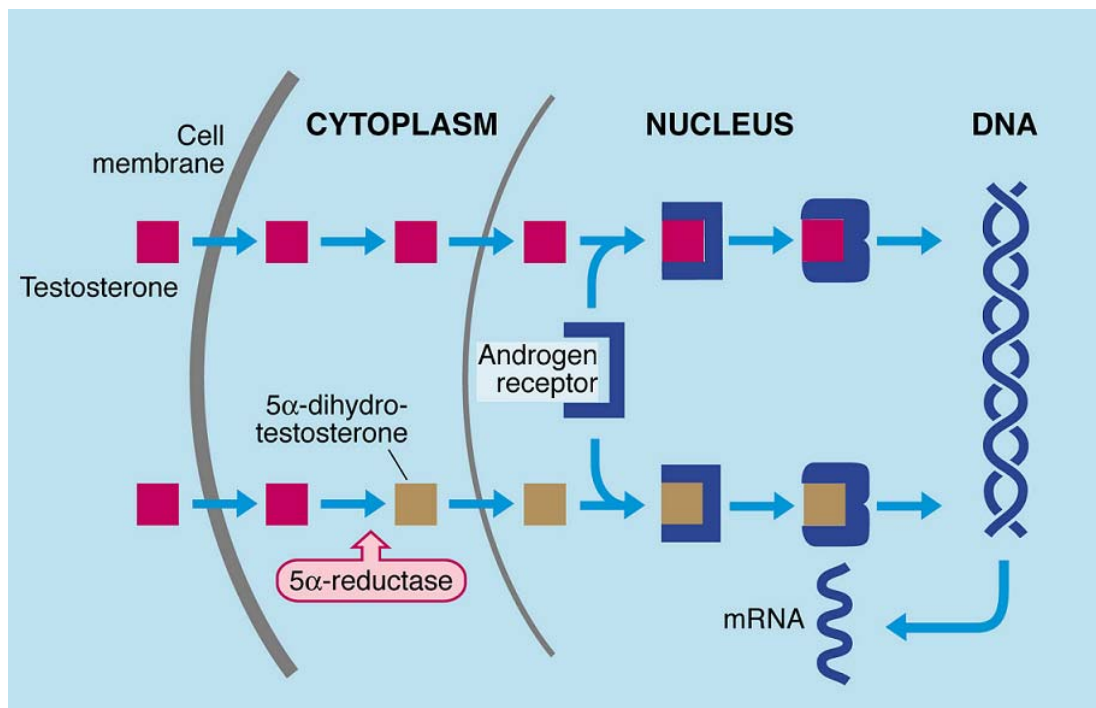
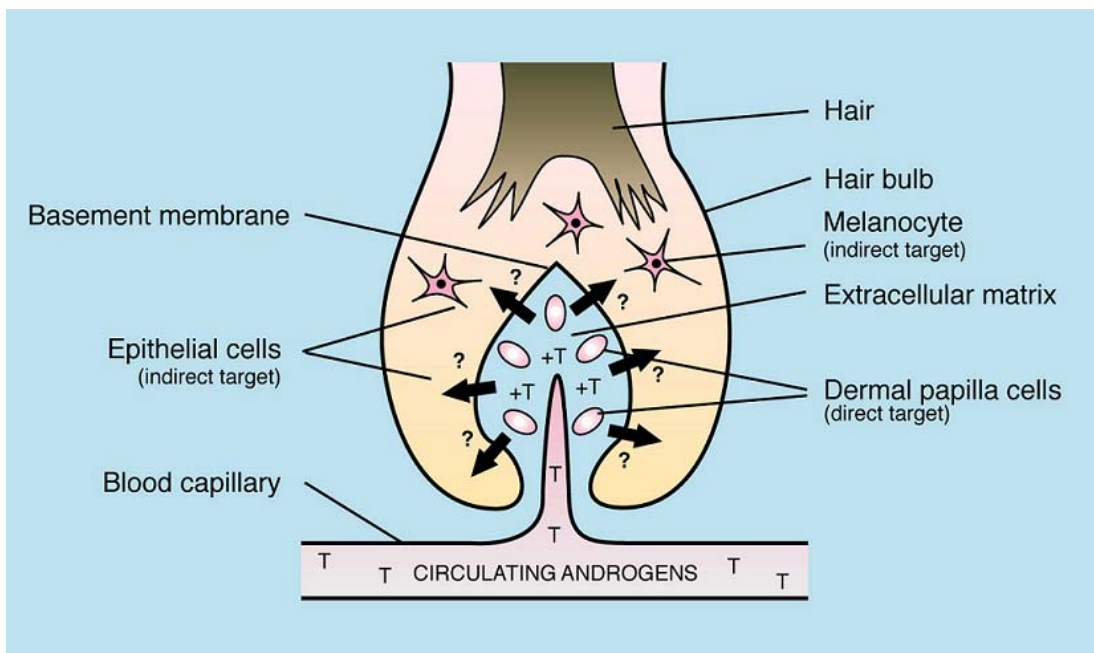


Figure 1.8

Androgen action in the hair follicle

Androgens in the blood enter the hair follicle via capillaries in the dermal papilla (Randall, 2007). Reproduced with kind permission.



In summary, androgens are not required for the production of terminal scalp hair or the other protective terminal hair seen in childhood e.g. eyelashes and eyebrows, but they are required for the expression of adult body hair. DHT is required for beard, chest and supra-pubic hair, but testosterone alone is sufficient for pubic and axillary hair in both sexes. DHT is required for the development of patterned hair loss in men.

1.3.4 Oestrogens

Oestrogens, the female sex hormones influence hair growth and also appear to be able to influence the hair cycle in rodents (Oh & Smart, 1996) and human beings (Moverare et al, 2002; Thornton et al, 2003). Growth of pubic hairs in male and female infants, aged 4 months to 2 years, was reported following the accidental application of a dermal cream containing oestrogen (Beas et al, 1969). However it is now known that oestrogens cannot promote sexual hair growth in the absence of functional androgen receptors (Thornton et al, 2002).

Oestrogen, like testosterone, binds to a nuclear hormone receptor and oestrogen receptors are expressed in the hair follicle, localised in the dermal papilla and outer root sheath (Oh & Smart, 1996), it is now known that there are two separate oestrogen receptor types, alpha and beta, each of which is thought to play a specific role in hair follicle growth regulation (Couse et al, 1997; Thornton et al, 2003; Thornton et al, 2006). The extended anagen phase during pregnancy followed by a diffuse telogen effluvium post partum is an established clinical phenomenon (Lynfield, 1960). The hormones of pregnancy appear to maintain anagen with oestrogen and prolactin the prime

candidates. Human hair follicles have receptors for both 17β -oestradiol (Thornton et al, 2006) and prolactin (Foitzik et al, 2006). However, 17β -oestradiol has also been shown to inhibit hair growth and precipitate entry into catagen in rodents (Smart et al, 1999; Ohnemus et al, 2005), the opposite of the effect seen in pregnancy. Inhibitory and stimulatory effects have been demonstrated on non-balding scalp follicles (Conrad et al, 2004) and stimulatory effects on male frontal scalp follicles (Kondo et al, 1990). The enzyme aromatase may play a role as it can reversibly metabolise androgens (4-androstenedione and testosterone) into oestrogens (oestrone and oestradiol). Aromatase may lower the local levels of androgens by shunting the oestrogen pathway as hair follicle aromatase is expressed in the outer root sheath and at higher levels in non-balding than balding hair follicles; suggesting that conversion of androgen to oestrogen in occipital follicles may protect against androgen-derived hair loss (Sawaya & Price, 1997). The role of oestrogens in hair growth has been reviewed (Ohnemus et al, 2006).

1.3.5 Other hormonal regulators of hair growth

The seasonal coat changes in many mammals are under photoperiod control, regulated by light and temperature (Lincoln & Richardson, 1998). These changes relate to serum prolactin levels (Gebbie et al, 1999; Hunt & Rose, 2002) and a 'prolactin-pelage' axis has been proposed (Alonso & Rosenfield, 2003) activated by the pituitary gland. Prolactin receptors have been located in the outer root sheath of the hair follicle and in the sebaceous gland and increased serum prolactin levels precipitate hair follicle entry into

catagen and a subsequent telogen shedding (Thompson et al, 1997; Nixon et al, 2002; Foitzik et al, 2006) mirroring the hair fall seen post partum in women. Targeted deletion of prolactin receptors in mice leads to a shorter anagen phase and an increased rate of hair cycling (Craven et al, 2001) and excess prolactin stimulation of adrenal androgen production may be a factor in hirsutism (Glickman et al, 1982). A number of other hormones are also known to cause changes to the normal hair cycle pattern when they are not present at optimum levels. Clinically the most common problems arise from the thyroid hormones which can affect hair growth, resulting in reversible alopecia and poor hair condition in cases of both hypothyroidism and hyperthyroidism (Credille et al, 2001). The thyroid hormone acts by binding to thyroid receptors (type α & β) which are found localised in the outer root sheath and dermal papilla (Ahsan et al, 1998). However targeted deletion of both thyroid receptors α and β in mice did not obviously affect hair growth (Aoki et al, 2000).

In summary an array of hormones and hormone receptors are implicated in hair growth; however the androgens remain the key regulators of most human hair growth post-puberty.

1.4. Androgenetic alopecia

1.4.1 Description

The replacement of pigmented terminal hairs by smaller pale vellus hairs in a progressive, distinctive pattern usually on the crown and frontal areas post puberty characterises androgenetic alopecia and produces a slow degenerative change in the hair growth of the scalp (Hamilton, 1951;

Rushton et al, 1983) (Figure 1.9). This condition is referred to as common baldness, male pattern and female pattern alopecia or androgenetic alopecia.

The scalp begins to show the characteristic bi-temporal recession and movement of the front hair line in men, with thinning of the frontal and crown areas, proceeding in pre-determined stages, leading ultimately to complete baldness in some cases. The occipital and parietal areas do not usually exhibit hair loss (see Figure 1.9). Women have a different pattern of hair loss, the hairline is retained and diffuse thinning occurs behind the hairline on the frontal and crown areas, and the occipital areas may also be affected (Venning & Dawber, 1988). Complete baldness in women is unusual, although it is possible for some women to experience hair loss in the male pattern (Hamilton, 1951). The hair loss patterns are described as “Hamilton” for men, modified by Norwood, and “Ludwig” for women (Hamilton, 1951; Ludwig, 1977; Norwood, 1975). The length of the anagen phase of the hair cycle begins to decrease in the affected areas of the scalp, producing hairs of successively reduced length, colour and diameter, i.e. the length of the anagen phase is severely reduced. Miniaturisation continues until follicular activity ceases and atrophy of the hair follicle occurs.

The incidence of androgenetic alopecia is high in male Caucasians approaching 100% (Hamilton, 1951), with wide genetic variations, as low as 50% in Japanese and Afro-Caribbean men (Setty, 1970). The incidence of androgenetic alopecia is lower in women, approximately 10% in pre-menopausal women, where chronic telogen effluvium is more common, approximately 30% usually due to nutritional imbalance (Rushton, 2002).

Figure 1.9.A

Pattern of androgenetic alopecia in men

Androgens cause a gradual inhibition of hair growth in pre-disposed individuals. Bi-temporal recession spreads backwards joining thinning areas on the vertex to give a bald crown (after Hamilton, 1951).

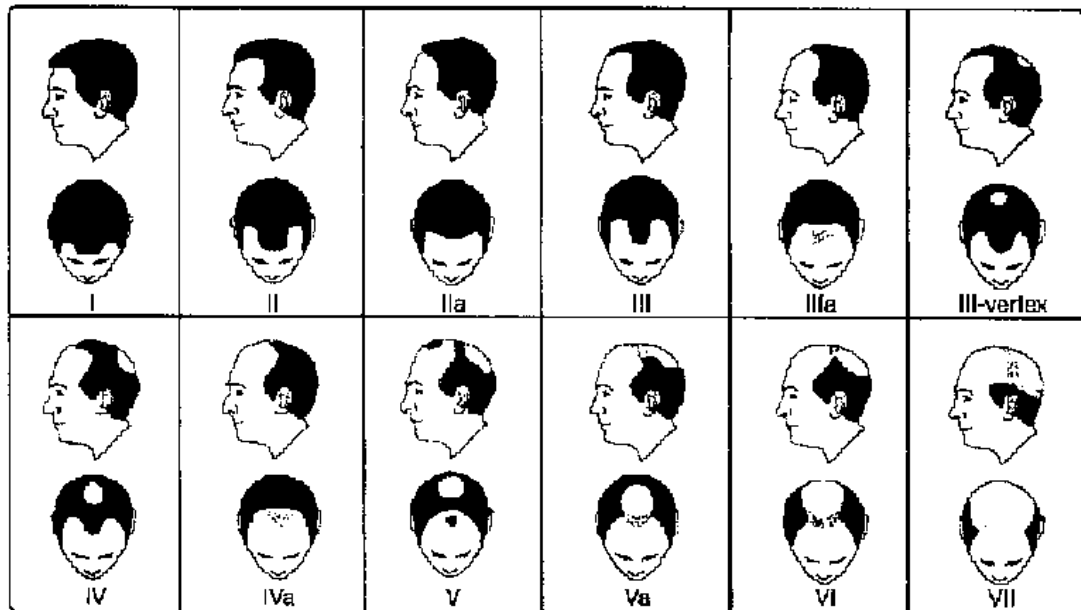
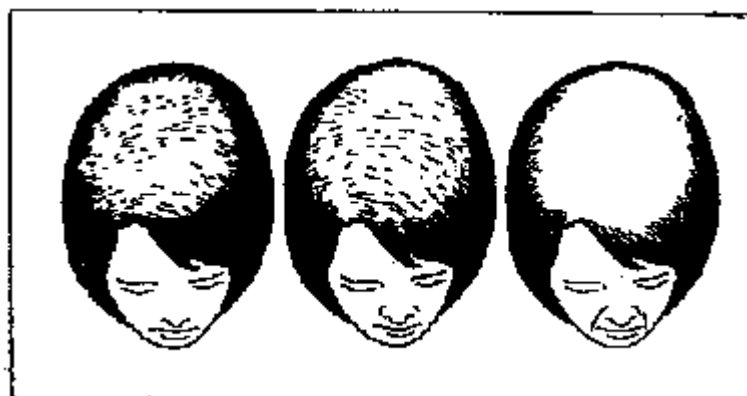


Figure 1.9.B

Pattern of androgenetic alopecia in women

Androgenetic alopecia is less common in women. The front hair line is normally retained and a general thinning on the vertex gradually becomes more pronounced until the vertex becomes bald (after Ludwig, 1977).



Post menopause the incidence of androgenetic alopecia is higher and 37% of women have a marked recession in the male pattern (Venning & Dawber, 1988). In many cases hair loss and baldness in human beings can cause psychological stress particularly when this shift is premature (Cash, 1992).

Some of the other primates also exhibit androgenetic alopecia: orang-utans, chimpanzees, gorillas and stump-tailed macaques (Rhodes et al, 1994), although both sexes are thought to pattern in the male form. The genetic influence in the progression of androgenetic alopecia has been confirmed (Hamilton, 1951; Setty, 1970); the pattern of inheritance may suggest an autosomal dominant trait with variable penetration (Küster & Happle, 1984; Bergfeld, 1995; Ellis & Harrap, 2001; Birch & Messenger, 2001).

Men with complete androgen insensitivity syndrome, i.e. without functional androgen receptors, do not exhibit androgenetic alopecia indicating that androgens and effective androgen receptors are a prerequisite for balding to occur (Imperato-McGinley et al, 1974). The enzyme, 5 α -reductase, appears to be involved in androgenetic alopecia; higher 5 α -reductase activity has been found in plucked, balding scalp follicles, compared with non-balding follicles (Schweikert & Wilson, 1974), and men with 5 α -reductase deficiency have not been reported to go bald (Wilson et al, 1993) although this may occur in women with this deficiency (Cousen & Messenger, 2010). Also finasteride, a 5 α -reductase (type 2) inhibitor, has been used orally to halt progression or partially reverse androgenetic alopecia in stump-tailed macaques (Rhodes et al, 1994) and in human beings (Kaufman et al, 1998; Rushton et al, 2002). More recently dutasteride, a type 1 and type 2 5 α -

reductase inhibitor has also been found to be effective in the partial reversion of androgenetic alopecia (Olsen et al, 2006).

Conversely, androgens stimulate increased hair growth in adult males of some animals, including the mane of the lion (West & Packer, 2002), and the deer (Randall et al, 1994) and the human beard (Randall et al, 1993). Changed hair growth in adult males would seem logical to distinguish a dominant breeding male particularly the oldest leading animal. A distinguishing feature could be useful to identify the leader, such as the silver (i.e. grey) back of the oldest male gorilla or the largest mane of the male deer and the bald crown of the older adult man in human beings would be an effective marker for an alpha male. The male lion with the largest darkest mane would be the preferred mate for a lioness (West & Packer, 2002), however recent research on lions has shown that the mane of some male lions is fullest and darkest when the lion is actually past his prime breeding age (approx. 4-5 years) and that lionesses would favour a mate with less hair; suggesting that hair and the absence of hair are useful sexual markers (Gnoske et al, 2006).

1.4.2 Pathogenesis

Androgenetic alopecia proceeds with a shortening of the anagen (growing) phase, and progressive follicular miniaturisation in the pre-determined areas of the scalp (Rushton et al, 1991; Kaufman et al, 2008). The normal telogen phase on the scalp remains unchanged lasting approximately three months (Kligman, 1959), and the anagen:telogen ratio change can be detected by unit area, trichogram measurement (Rushton et al, 1983). There may also be

a time lag following cessation of telogen (and shedding of the previous club hair) before anagen recommences when the follicle is without a developing hair (Guarrera & Rebora, 1996; Whiting, 2001) this 'no-hair' lag period may last up to 6 months and the term "kenogen" has been proposed (Rebora, 2004). The rate of hair growth in successive anagen phases may also be reduced in androgenetic alopecia (Whiting, 2001) and the length of the lag phase may increase (Van Neste, 2008).

Follicular miniaturisation and reversion of terminal to vellus hair follicles proceeds during androgenetic alopecia (Rushton et al, 1991) and ultra structural studies have shown that the early stages of androgenetic alopecia are accompanied by progressive fibrosis of the perifollicular sheath (Whiting, 1990; Jaworsky et al, 1992; Whiting, 1993) with inflammation and fibroplasia present. It has been proposed that an immune system mechanism or related deficiency may initiate or modulate the progression of androgenetic alopecia (Jaworsky et al, 1992) and immunomodulatory therapy e.g. cyclosporin has been used in the treatment of androgenetic alopecia (Picascia & Roenick, 1988). Inflammatory cells may infiltrate the region of the follicular bulge with possible cytotoxicity to the stem cell population, impairing normal follicular cycling and resulting in miniaturisation and eventual permanent hair loss (Jaworsky et al, 1992). Conversely reversible hair loss e.g. alopecia areata is found to be associated with lymphatic infiltration of the bulbar matrix, as opposed to inflammation around the infundibular epithelium, and the condition is reversible (McDonagh & Messenger, 1996). The gradual miniaturisation of the hair follicle in androgenetic alopecia produces smaller, less pigmented hair and the sebaceous gland (also an androgen dependent

tissue) becomes enlarged with the scalp having an oily and greasy appearance (Kligman, 1988). The hairs produced are progressively shorter (Rushton et al, 1991), and miniaturisation of the follicle can be seen histologically (Whiting, 1993) as the process proceeds over several follicular cycles. The arrector pili muscle also reduces in size, but this reduction proceeds more slowly (Maguire & Kligman, 1963). Electron microscopy studies of hair shaft structure in common baldness showed no abnormality (Puccinelli et al, 1968), and no abnormality in chemical composition of the hair shaft has been found (Salamon, 1971). Miniaturisation continues until cessation of hair growth and the scalp area appears bald. The miniaturised quiescent hair follicles lay dormant and the unsupported nerve network is coiled, twisted and truncated (Klemp et al, 1989). Whiting has proposed (Whiting, 2001) that the miniaturisation process may not be a smooth, lengthy transition but an abrupt, large-step process as a direct result of reduction in cell number and hence size of the dermal papilla. Other researchers (Van Neste, 2002) have found evidence that the transition to miniaturisation is a gradual process. It is suggested that the reduced length of the anagen phase represents the initial stages of androgenetic alopecia resulting in thick but shorter hairs and that miniaturisation and the production of thin hairs represents the final stages of the condition leading eventually to no visible hairs and baldness (Van Neste, 2006).

The genetic influence on the progression of androgenetic alopecia is well established (Hamilton, 1942; Hamilton, 1951; Setty, 1970) and studies have been made to identify candidate genes. Using gene association studies comparing single nucleotide polymorphisms (SNP) between high incidence

and low incidence groups based on androgen related candidate genes (5 α -reductase genes [SRD5A1 and SRD5A2], aromatase genes, IGF-I receptor genes, Y chromosome and androgen receptor genes) (Sinclair, 2006) a significant difference was found in the frequency of a single base change in the coding region of the AR gene between the 2 groups. Almost the entire young bald group had a particular variant of the AR gene indicating the importance of AR gene polymorphism in the development of androgenetic alopecia (Ellis et al, 2002; Hillmer et al, 2005). The location of the AR gene on the X chromosome supports maternal transmission of androgenetic alopecia (Sinclair, 2006).

Recently a new gene-hunting technique GWA (genome wide association) has been used to search for gene variants linked to androgenetic alopecia (Hillmer et al, 2008; Richards et al, 2008). A highly significant association was shown for 5 SNPS (single nucleotide polymorphisms) on chromosome 20, indicating therefore both paternal and maternal influence on the progression of AGA. No interaction was found with the already known X chromosome androgen receptor also implicated in AGA (maternal inheritance only) and this new locus on chromosome 20 may, surprisingly, indicate a new androgen independent pathway in the pathology of androgenetic alopecia, the mechanism for which is unknown.

1.4.3 Role of androgens

The central role of androgens in the progression of androgenetic alopecia has been known since earliest times when it was observed that eunuchs did not go bald and that the testes were associated with maleness. The first

systematic correlation was made by Hamilton who showed that men castrated before puberty did not go bald and that eunuchs treated with testosterone therapy subsequently developed androgenetic alopecia, a process arrested when therapy was withheld (Hamilton, 1942). More specifically, males born with androgen receptor deficiency exhibit no androgen effects post puberty and appear as phenotype women with no external testes and no axillary, pubic or body hair growth (Kutten et al, 1979), and no androgenetic alopecia (Quigley, 1998). In cases of androgenetic alopecia in men, circulating androgen levels are not elevated (Phillipou & Kirke, 1981), and normal androgen levels trigger the follicular regression in the genetically determined areas. In women, raised androgen levels do appear to be related to hair loss (Georgala et al, 1986) in women with an inherited pre-disposition. Androgens and androgen receptors mediate both positive signals to prolong anagen in beard, chest, nose and ear follicles etc. and negative signals to shorten anagen in androgenetic alopecia. Epithelial (ectoderm derived) and dermal (mesoderm derived) interactions control and coordinate the hair cycle (Millar, 2002; Rendl et al, 2005) and mesenchyme-derived dermal papilla cells are found to have differing levels of androgen receptors dependent on body site. Dermal papilla cells from the beard, axillary and frontal scalp areas contain specific low capacity high affinity androgen receptors, whereas fewer androgen receptors were present in the cells from androgen independent occipital scalp hair follicles (Choudry et al, 1992; Randall et al, 1992; Hibberts et al, 1998). Also beard dermal papilla cells have higher levels of 5 α -reductase activity than occipital scalp, pubic or axillary cells *in vitro* (Itami et al, 1990; Thornton et al, 1993; Hamada et al,

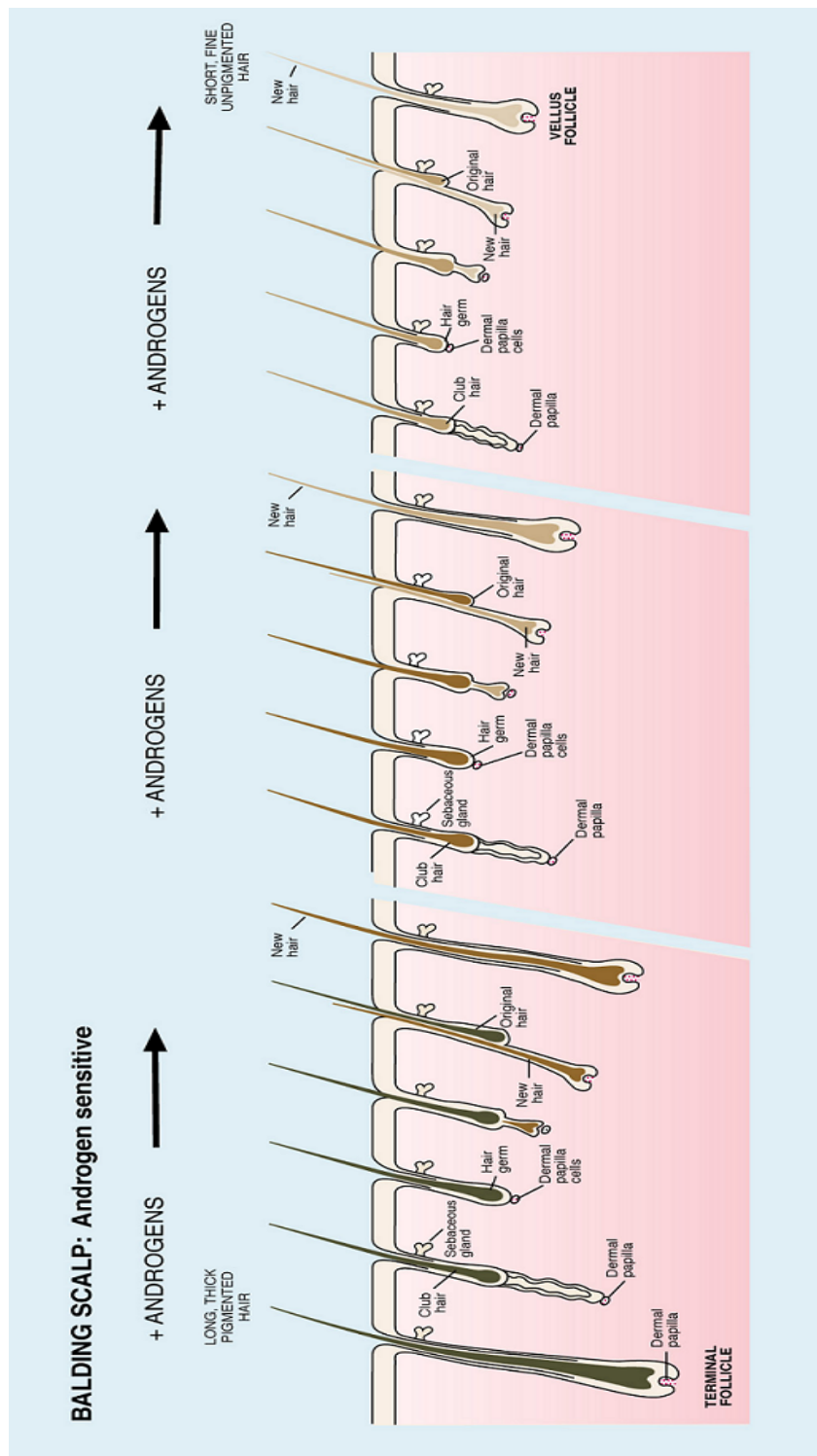
1996) and this has been identified as type 2 5 α -reductase. The expression of mRNA encoding for the androgen receptor in dermal papilla cells was found to be site specific and to be strongly expressed in the beard, frontal scalp and axillary areas (Asada et al, 2001). Also type 1 5 α -reductase expression is found to be common for all dermal papilla types whereas type 2 5 α -reductase is limited to beard and frontal scalp dermal papillae. The progression of androgenetic alopecia appears to require the expression of type 2 5 α -reductase, and for functional androgen receptors to be present in dermal papillae cells. Dermal papilla cells are the primary target cells for androgen activity and they mediate signals to follicular epithelial cells via a paracrine mechanism (Randall, 2007). Co-culture experiments using beard (and axillary) dermal papilla cells and outer root sheath cells showed that androgen significantly stimulated outer root sheath cells proliferation suggesting that dermal papilla cells produce androgen-dependent diffusible growth factors. IGF-I has been identified as a candidate diffusible growth factor (Itami et al, 1995) in promoting hair growth (see Figure 1.10).

As discussed in section 1.1.5 IGF-I is a paracrine or autocrine growth factor present in many organs and tissues whose expression is usually limited to mesenchymal cells. IGF-I stimulates human hair growth *in vitro* and prevents premature entry of the follicle into catagen (Philpott et al, 1994). Conversely, co-culture experiments using dermal papilla cells from frontal scalp area with outer root sheath cells showed androgen induced inhibition of outer root sheath cells proliferation suggesting androgen-dependent soluble factors from dermal papilla cells are involved in hair growth suppression (Obana et al, 1997; Pan et al, 1999; Inui et al, 2003; Itami & Inui, 2005).

Figure 1.10

Androgen action in human hair follicles

Transforming growth factor- β 1 (TGF- β 1) is an androgen – dependent paracrine mediator for androgenetic alopecia (Randall, 2000). Reproduced with kind permission.



Similarly, factors secreted by balding dermal papilla cells have been shown to inhibit the growth of other dermal papilla cells and delay the onset of anagen in mice *in vivo* (Hamada & Randall, 2006). TGF- β 1, a soluble growth factor, is known to inhibit hair growth and to induce catagen (Shiple et al, 1986; Foitzik et al, 2000; Liu et al, 2001).

TGF- β 1 and TGF- β 2 were found to be up-regulated by androgen in bald frontal dermal papilla cells but not in non-bald dermal papilla cells (Inui et al, 2003). TGF- β 1 may therefore mediate the signal from androgenetic alopecia dermal papilla cells for hair growth suppression.

Differential gene sensitivity to androgens in dermal papilla cells from different follicles causing the production of different paracrine factors may partially explain the differing and converse response of body and scalp hair follicles to the same androgen stimulation. It suggests a possible therapeutic root for the treatment of androgenetic alopecia.

1.4.4 Treatment

The ideal treatment for androgenetic alopecia could be developed after isolation of the specific gene or set of genes responsible, but the high incidence has prevented this so far. A number of drug therapies are available, but the genetic nature of the condition makes effective treatment very difficult, and a reduction of the rate of progression represents a realistic expectation.

Anti-androgens, which block the binding of androgens to the androgen receptor, are not the first drug of choice as they would tend to block all systemic androgens with consequent loss of masculinity in men, and

possible feminisation of a male fetus in women. However, a number of these anti-androgens, e.g. spiro lactone, cyproterone acetate, have been used for the treatment of female androgenetic alopecia and they may have some clinical effect on progression (Vexiau et al, 2002).

There has also been some success with systemic and topical oestrogen treatment, although the use is restricted to women. Oestrogens have an indirect anti-androgenic effect by increasing the production of sex hormone binding globulin (SHBG) and giving a decrease in bioactive testosterone, prolonging the anagen cycle, and inhibiting sebum secretion (Winkler, 1969; Moretti et al, 1977). The topical use of oestrogens in the treatment of female pattern alopecia has been found to be helpful (Conrad & Paus, 2004).

Inhibition of 5 α -reductase and hence inhibition of 5 α -dihydrotestosterone synthesis offers a more elegant, selective and practical approach and a number of these blocking agents are known, a number of which occur naturally. The best researched drug for this application is finasteride (Kaufman et al, 1998; Rushton et al, 2002), a 5 α -reductase type 2 inhibitor, used originally for the treatment for benign prostatic hyperplasia, and shown in clinical trials to have a hair growth effect. The conversion of testosterone to dihydrotestosterone is blocked, the anagen phase is extended, the lag phase may shorten and the miniaturisation of the hair follicle is delayed (Van Neste, 2006). Finasteride is used as a systemic treatment administered orally at a dose of 1mg per day, although used at 5mg per day for prostatic disorders. Three double blind, placebo controlled trials totalling 1,879 men aged 18-41 years with mild to moderate androgenetic alopecia showed

reduced hair loss and increased scalp coverage, increasing the length and diameter of existing miniaturised hairs (Kaufman et al, 1998). The beneficial effect is lost within twelve months if treatment is discontinued due to the underlying genetic pre-disposition with the androgen receptors and source of androgens still present. It was not effective in the treatment of post-menopausal women (Price et al, 2000) and it is contra-indicated in women of childbearing age due to possible birth defects of the male fetus. Some side effects (less than 2%) have been reported in men, including reduced libido, erectile dysfunction and reduced ejaculate volume.

More recently, it was shown that dutasteride, a dual type 1 and type 2 5 α -reductase inhibitor maybe effective in the treatment of male pattern hair loss (Olsen et al, 2006). In a study of 416 men, between the ages of 21 and 45 years exhibiting androgenetic alopecia and treated with dutasteride, finasteride or placebo it was found that dutasteride significantly increased target area hair count compared with placebo and that dutasteride was superior to finasteride (Olsen et al, 2006). Although the trial was limited to 24 weeks, the results suggest that type 1 and type 2 5 α -reductase may be important in the progression of androgenetic alopecia in men.

A topical application, using a transdermal approach is probably the most useful to reduce potential side effects and to control the delivery point possibly using liposome technology allowing delayed release of actives into the hair bulb itself (Zulli & Suter, 1997; Vogt et al, 2006). Minoxidil is the best known of the topical applications and was groundbreaking when discovered in the 1970s, the first time androgenetic alopecia had been shown to be partially reversible. Minoxidil is a vasodilator (Olsen et al, 1985) originally

used orally for the treatment of hypertension which had an unacceptable side effect of stimulating hypertrichosis. A topical preparation was developed for the treatment of androgenetic alopecia and in some cases alopecia areata. Minoxidil was found to increase the length of the anagen phase of the hair cycle and was effective in partially reversing the miniaturisation process; the appearance of more terminal hairs gave a measurable cosmetic response (Olsen et al, 1985). Approximately 25-30% of men in a study of 2,294 balding men treated with minoxidil 2% or 3% had moderate re-growth (Olsen, 1989) apparent after 4-6 months which plateaued after approximately one year. Cessation of treatment resulted in loss of the re-grown hair within 3-4 months. The response to minoxidil treatment in cases of alopecia areata is controversial (Fielder, 1992; Epstein, 1991) especially as spontaneous reversal can be a feature of this condition. Clinically minoxidil treatment for androgenetic alopecia was found to be useful, but not ideal. The re-growth was in most cases modest, and the new hair was lost when treatment ceased. The mechanism for minoxidil has been unclear for many years. Minoxidil is a vasodilator and the increased blood and nutrient supply to the dermal papilla would seem beneficial. Minoxidil is now known to be able to act as a potassium channel opener within the hair follicle itself (Davies et al, 2005), independent of the vasodilator action (Shorter et al, 2007). Countless other remedies have been proposed for the treatment of androgenetic alopecia (Proctor, 1999), and probably cover most of the spectrum of the material world. There is often a body of anecdotal supporting evidence but only limited clinical data, but an open mind is always essential when reviewing treatments. The topical route is the preferred option to reduce

systemic effects, reduce dose and focus point of delivery at the hair follicle and particularly the dermal papilla and it has been shown recently (Hoffman, 2002) that genes can be selectively targeted in hair matrix cells using a liposome carrier system and transdermal injection; suggesting a different therapeutic route.

There are without doubt other agents available that could have a physiological effect and could be used to control, or partially reverse the progression of androgenetic alopecia; traditional Chinese medicine and Ayurvedic medicine are documented systems of health care that could provide such active agents. The ultimate treatment for androgenetic alopecia and certainly the most effective is the employment of a surgical procedure for the rearrangement of the hair follicles on the scalp. The response to androgens is intrinsic to the individual hair follicle (Randall, 1994) and this site-specificity forms the basis of cosmetic hair transplant surgery. The dermis of the fronto-parietal region of the quail chick develops from the neural crest during embryogenesis, whereas the occipital-temporal areas of the scalp develop from the mesoderm (Ziller & Smith, 1982), if this is mirrored in the human body it may help to explain the differing response to androgens in cases of androgenetic alopecia. During human embryogenesis the mesoderm derived dorsal (occipital) covering is formed before the ectoderm derived ventral (fronto-parietal) area and their sensory nerve innervation differs. Sensory nerves can stimulate or inhibit target tissue activity by secreting differing neuropeptides (Paus et al, 1997) which may account for this differing response.

Recent advances in stem cell research have shown that hair follicle dermal papilla cells can be isolated and stimulated to produce cultured dermal papillae which can then be transplanted back into the donors scalp (Teumer & Cooley, 2005). The process is not yet available commercially, but could ultimately lead to the 'holy grail' of unlimited hair re-growth.

1.5. Traditional Chinese Medicine (TCM)

The Taoist hermits in their ancient search for the elusive elixir of life found that immortality was only spiritual, but their investigation of all animal, vegetable and mineral derivatives in their quest provided the therapeutic benefits on which traditional Chinese medicine is based (Weiyi Yang, 1998). This knowledge of herbs was recorded as early as 4th Century B.C. and the first *Classic of Materia Medica* containing details of 252 botanical extracts appeared around 220 AD. The Imperial Medical School was founded in 624 AD and their first pharmacopoeia (The newly revised *Materia Medica*) was published in 659AD containing references to 844 herbs. Over 500 items are listed in *The coloured atlas of Chinese materia medica* (Dunmu & Jiangbo, 1995), although only 300 or so are used in general practice and traditionally the whole plant or parts of it are used instead of a concentrated extract. The plants are also used in combination for a maximum synergistic effect (Yanchi Liu & Zanwen, 1998), and the use of the whole or part of the plants and using them in combination provides particular difficulties for researchers and clinicians due to a technically uncertain and variable starting point for clinical research. Additionally these products cannot usually be patented meaning that there is limited economic incentive to perform detailed clinical trials.

However, many herbal remedies contain powerful ingredients and their efficacy awaits further research. The “Herbalome Project” (De-An, 2008) at the Shanghai Institute of Materia Medica is probably the most ambitious project, attempting to identify active compounds and toxic contaminants in over 10,000 TCM herbs and animal tinctures. TCM is the treatment of choice and often the only recourse for many of the people in Asia.

A herbal tea “Yin Zhi Huang” made from the plant, *Artemisia capillaris*, was proved in controlled experiments to be effective in the treatment of jaundice (Huang et al, 2004). The active constituent, 6,7-dimethyl-esculetin, was identified and shown to stimulate the constitutive androstane receptors (CARs) on the surface of liver cells, key regulators in the control of jaundice. In 2004 the World Health Organisation symposium declared that the Chinese herb “*Artemisia annua L*” (or Qing Hao [Sweet Wormwood]) was now the number one drug of choice in the treatment of malaria. *Artemisia* has been found to be highly effective against malaria parasites including multi-drug resistant ‘*plasmodium falciparum*’ and against chloroquine resistant malaria (Eckstein-Ludwig et al, 2003). The unique anti-malarial properties of *Artemisia annua L* and related compounds are the focus of considerable current research.

Recently it was shown that *Cucubita ficifolia* (Siam pumpkin or shark fin melon) fruit extract promoted regeneration of damaged pancreatic cells in diabetic rats (Xia & Wang, 2007), boosting levels of insulin-producing beta cells and insulin in the blood. The protective effect of Siam pumpkin is thought to be due to the presence of antioxidants and D-chiroinositol, a

mediator of insulin activity, offering a possible oral treatment to assist in the treatment of diabetes type 1.

A number of plant extracts and mixtures have been used in traditional Chinese medicine in the treatment of androgenetic alopecia (Reid, 1995), and several proprietary “over the counter” products have been marketed. “Xiantene” is a complex mixture of traditional Chinese botanical derivatives, which experiments showed could stimulate normal human hair follicle growth *in vitro* (Dr. Z. Xia, personal communication). Xiantene is thus a good example of Chinese medicine to investigate for its potential action *in vivo* in the treatment of androgenetic alopecia.

1.6. Aims

The purpose of this study is to investigate two aspects in the understanding and treatment of androgenetic alopecia.

A novel traditional Chinese medicine (TCM) topical mixture Xiantene will be tested in a preliminary (three months) and extended (12 months) consecutive double blind trials in subjects with androgenetic alopecia. The trials will determine whether any naturally occurring factors in the herbal mixture Xiantene would have an effect on hair growth.

Secondly, a possible relationship between two age related aspects of hair growth, canities (hair greyness) and androgenetic alopecia (patterned hair loss) will be investigated. The purpose of this study will be to investigate whether the early onset of canities delays the progression of androgenetic alopecia in men.

2. Preliminary investigation into the effects of herbal mixture Xiantene on hair growth in people with androgenetic alopecia

2.1 Aim

The purpose of this pilot study was to investigate whether naturally occurring hair growth factors in the herbal mixture Xiantene (based on eight herbs used in traditional Chinese medicine, see Sections 1.5 and 4.2) would have any effects on hair growth in cases of androgenetic alopecia when applied topically. Earlier work (Dr. Xia, Oxford, personal communication) had shown that Xiantene could stimulate normal human hair follicle growth *in vitro*.

2.2 Experimental design

A preliminary investigation of the effects of topical applications of the herbal mixture Xiantene on hair growth variables in people with androgenetic alopecia were investigated in a small group of men and women for three months. Half the subjects were given a control vehicle and half the Xiantene lotion. Neither the subjects nor the investigator were aware which subjects were given the Xiantene. Hair growth parameters were measured using the unit area trichogram method (Rushton et al, 1983) and a photographic record of each subject's scalp was also made. The parameters to be measured were: total number of hairs, number of anagen hairs, anagen hair pigmentation (on a digitalised scale) and anagen hair shaft diameter.

2.3 Materials and methods

2.3.1 Subjects

A total of 35 subjects (31 men and 4 women) were invited to enter a three month trial of Xiantene (Xiantene I, see chapter 4) in the treatment of androgenetic alopecia, recruited at random by newspaper advertising. Subject ages ranged from 22 to 63 years. All subjects were examined by a qualified trichologist (the author) to confirm the presence and stage of androgenetic alopecia using the Hamilton grading scale for men (Hamilton, 1951) or Ludwig scale for women (Ludwig, 1977) and to eliminate those with any underlying pre-condition. Subjects were asked to sign a consent form and a disclaimer form and were verbally instructed and given a written standardised washing procedure (see Appendix I). The trial was conducted for a 3 month period, commencing in June and finishing in August.

2.3.2 Application of Xiantene

The experiment was conducted on a double-blind, vehicle-controlled basis with half the subjects using Xiantene and the remainder using a vehicle. The vehicle was prepared using 0.25% w/v caramel (Univar Ltd., Essex, UK), 20% w/v denatured ethyl alcohol (Alcohols Ltd., London, UK) and made up to 100% w/v with tap water. Neither researcher nor subject knew which treatment any participant received as this was identified by code number only. The test lotion was applied using a pump spray head provided with the 30 ml plastic bottle of lotion (S. Murray & Co Ltd., Woking, UK), to the crown area only. Subjects were instructed to apply a single pump, approximately 1ml, of the spray each night, and given a demonstration by the author before

commencing the trial. Subjects were seen by the same examiner (the author) throughout the trial and were required to present for examination each month. Each subject was thus expected to be seen four times during the three month study.

2.3.3 Assessment of hair growth

A photographic record was made at the beginning and end of the three month period. Each subject's crown area was photographed, using a tripod mounted Contax SLR camera with a 55 mm, 2.8 macro lens, fitted with a Sunpack ring flash attachment, at a distance of 30 cms (see Figure 2.1).

Assessments of changes in the total number of hairs, the number of anagen hairs, anagen hair pigmentation and anagen hair diameter were made using the unit area trichogram method (Rushton et al, 1983). A template was prepared from rigid plastic sheet, as per the Rushton method, with three circles of 8 mm diameter (area 50.7 mm²) precision cut in a triangular pattern (see Figure 2.2A). This was used to outline the areas of epilation on the crown approximately 20 mm below the centre point of the crown and approximately 10 mm right of the centre, delineated with an "Edding 1800 Profipen (0.5)" indelible artist's pen. Subjects with oily scalps had the crown area degreased with alcohol before the scalp was marked.

All visible hairs in one of the circumscribed areas up to, and including, the outer pen marks were epilated using 10 cm surgical nasal forceps (Solingen, Germany) and an illuminated magnifying glass (magnification and manufacturer unknown). Validation of the methodology is given in Appendix II.

Figure 2.1

Photographic equipment used to record subjects crown area.

A photographic record was made using a Contax SLR 35 mm roll film camera fitted with a 55 mm 2.8 macro lens with a Sunpack ring flash attachment, at a distance of 30 cm.



Figure 2.2.A.

Template used to define area of epilation.

Template was prepared with 3 circles of 8 mm diameter (area 50.7 mm²) precision cut in a triangular pattern to outline the area of epilation on the crown.

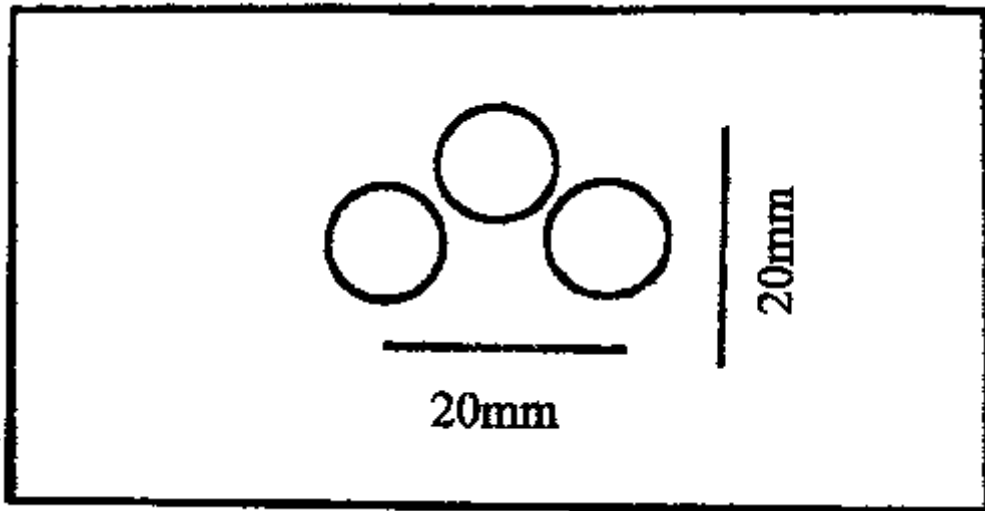
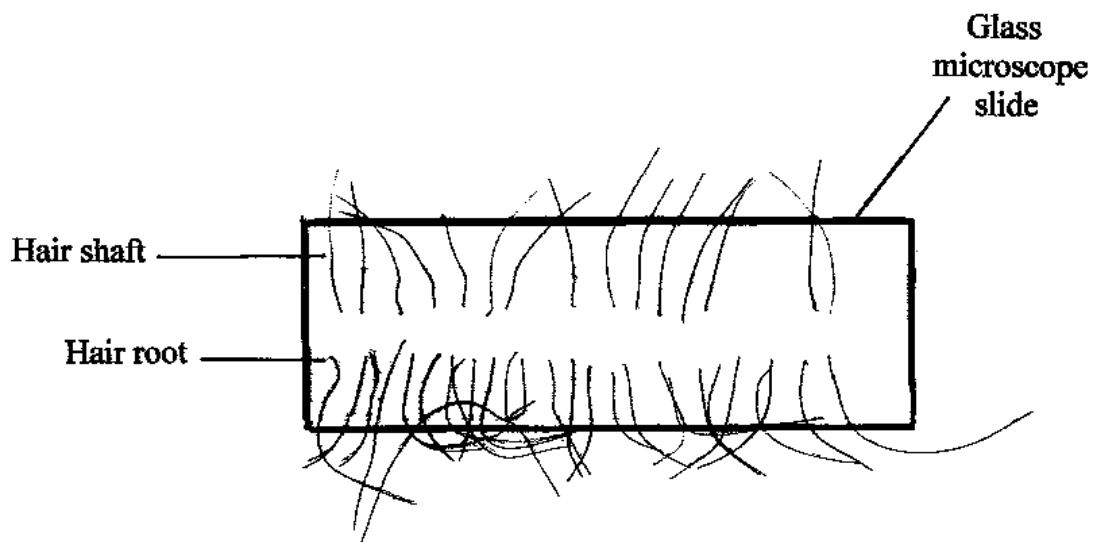


Figure 2.2.B.

Plucked hairs mounted on microscope slide

Hairs were epilated and mounted at the root end on a microscope slide using transparent adhesive tape (Sellotape), photo, (author) actual size



The hairs were mounted at the root end on a glass microscope slide using transparent adhesive tape (Sellotape) prior to measurements being made (see Figure 2.2B). After 3 months of treatment with Xiantene or vehicle, hair samples were collected in an identical manner in an adjacent area of the crown using the circle provided by the template.

2.3.4 Hair measurements

Hair samples were examined using a light microscope (Labor Lux S, Zeiss, Germany) and the numbers of hairs counted (magnification x40, i.e. x10 eyepiece with x4 lens). Anagen and telogen hairs were distinguished by their typical morphology (Chase, 1954) (see Figures 2.3 and 2.4) and the images were collected by a video camera (800 x 600 pixels) connected to an IBM personal computer and analysed by image analysis software Optimas 5.2 (Meyer Instruments, Texas USA). Hair diameter measurements were obtained from anagen hairs by taking the average of three diameter measurements between 0.2mm and 1mm above the hair root using the computer imaging cursor (Figure 2.5). This was chosen as the area in which the final cross section and pigmentation of the emergent shaft were fully determined, but before any environmental or cosmetic damage to the shaft had occurred.

The level of pigmentation of the shaft was assessed by computer imaging using the area morphometry macro and Optimas 5.2 (Meyer Instruments, Texas USA) software for analysis.

Figure 2.3

Typical anagen and telogen hairs distinguished by their morphology.

Anagen hairs exhibited soft, variably shaped, pigmented ends with tissue remnants and cellular matter. Telogen hairs exhibited a hard end with a rounded “shaving brush” appearance and loss of pigment.



Typical hair in anagen phase (magnification x40)



Typical hair in telogen phase (magnification x40)

Figure 2.4

Non-typical anagen or telogen hairs.

Hairs with non-typical morphology were re-examined by two independent assessors and classified by consensus.

(A) Non-typical hair root morphology (magnification x40)

Pigmented soft folded end with tissue remnant, classified as anagen



(B) Non-typical hair root morphology (magnification x40)

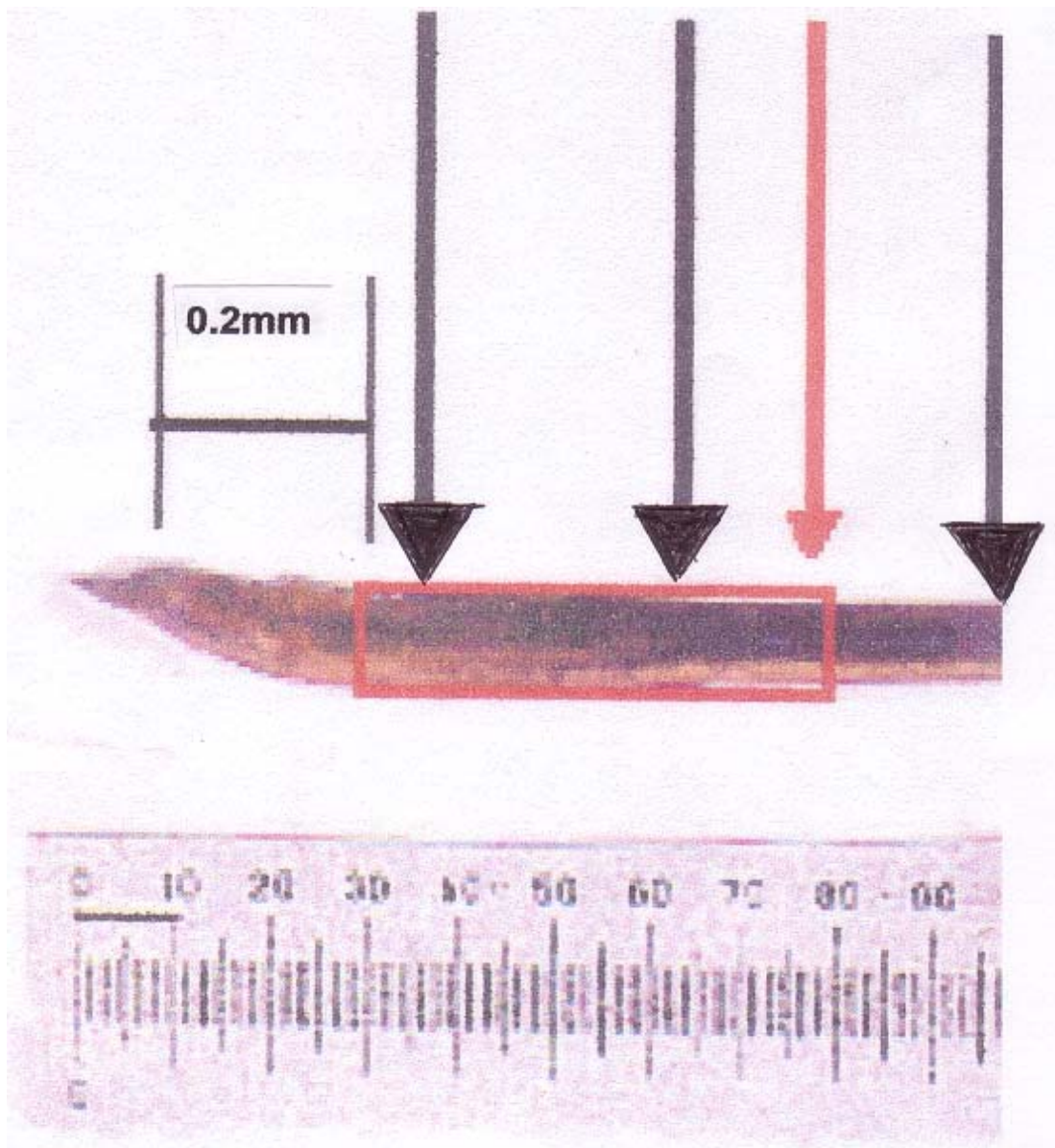
This is a broken hair suggesting that it was well anchored in the follicle and therefore classified as telogen



Figure 2.5

Anagen hair shaft measurement (magnification x 25).

Anagen hair shaft diameter measurements were obtained from the average of 3 diameter measurements between 0.2mm and 1mm from the hair root end.



Anagen hair analysis

Black arrows show 3 random points for diameter measurement. The red arrow and square show the area for pigmentation measurement.

A computer derived standard oblong template was constructed and superimposed on the hair fibre between 0.2 mm and 1mm above the hair root and the mean degree of pigmentation in the area covered was measured. The level of pigmentation was digitalised and scored from 0-250, 0 being white and 250 black. The help of Dr. Zhidao Xia of the University of Oxford, in the measurement of hair parameters and in the compilation of the data, is gratefully acknowledged. The two sets of data, Xiantene and vehicle, were de-coded after data analysis, and all measurements were tabulated and expressed in graphical form. Mean values were calculated as mean \pm standard error mean (SEM). Normal distribution was verified (Paul Bassett, personal communication) and paired Student's t-tests were used for comparison between groups before and after treatment. Results where $p < 0.05$ were taken as significantly different.

2.4 Results

2.4.1. Subjects

Thirty-five subjects commenced the experiment, half using Xiantene and half using the vehicle. Twenty six were re-examined at the end of the first month, 19 at the end of the second month and 12 completed the full 3 month study. This included 6 subjects treated with Xiantene and 6 subjects using the vehicle. The methodology was validated (see Appendix II) and the coefficient of variation of the drawing of the circles on the scalps was found to be 4.94%.

Since no women completed the study, they were excluded from all the results including the pre treatment values. The high drop out rate was

unexpected, but importantly, equal numbers of treated and untreated subjects completed the trial.

The age distribution of the 31 male participants who started and 12 who completed the trial are shown in graphical form (Figure 2.6). The mean age of all subjects commencing the study was 34.9 ± 1.6 years (mean \pm SEM) and the mean age of subjects completing the study was higher at 39.0 ± 3.7 years. The majority (68.0 %) of the younger group (20-40 years) failed to complete the trial; conversely most of the older group (66.7 %) (41-70 years) did manage to complete the trial.

The extent of baldness as indicated by the Hamilton grading scale was also plotted (Figure 2.7). The Hamilton types ranged from 2 to 5 both before and after the study. The mean Hamilton type was 3.1 ± 0.2 for those who did not complete the trial and higher, at 3.7 ± 0.2 for subjects completing the trial, $p=0.02$. The subjects who completed the trial were significantly more bald than the subjects who did not finish the experiment.

When the extent of baldness was related to age there was no significant difference between the commencement and completion of the study in the average age of the subjects at any particular extent of balding (Figure 2.8).

Importantly the mean ages of the two study groups completing the trial did not vary significantly; mean age of the Xiantene group was 37.2 ± 4.9 years and the vehicle group 40.8 ± 6.0 years, $p = 0.65$, allowing a valid comparison of the effects of the Xiantene and vehicle treatments (see Figure 2.9).

Similarly the extent of balding as measured on the Hamilton grading scale did not vary significantly between the two groups; Xiantene 3.8 ± 0.3 and vehicle 3.7 ± 0.3 , $p = 0.72$, confirming the validity of the comparison.

Figure 2.6

Age distribution of male subjects initially enrolled and completing the Xiantene pilot study.

Subjects were invited to enter a 3 month trial of topical Xiantene in the treatment of androgenetic alopecia. Half of the subjects used Xiantene and half of the subjects used a vehicle.

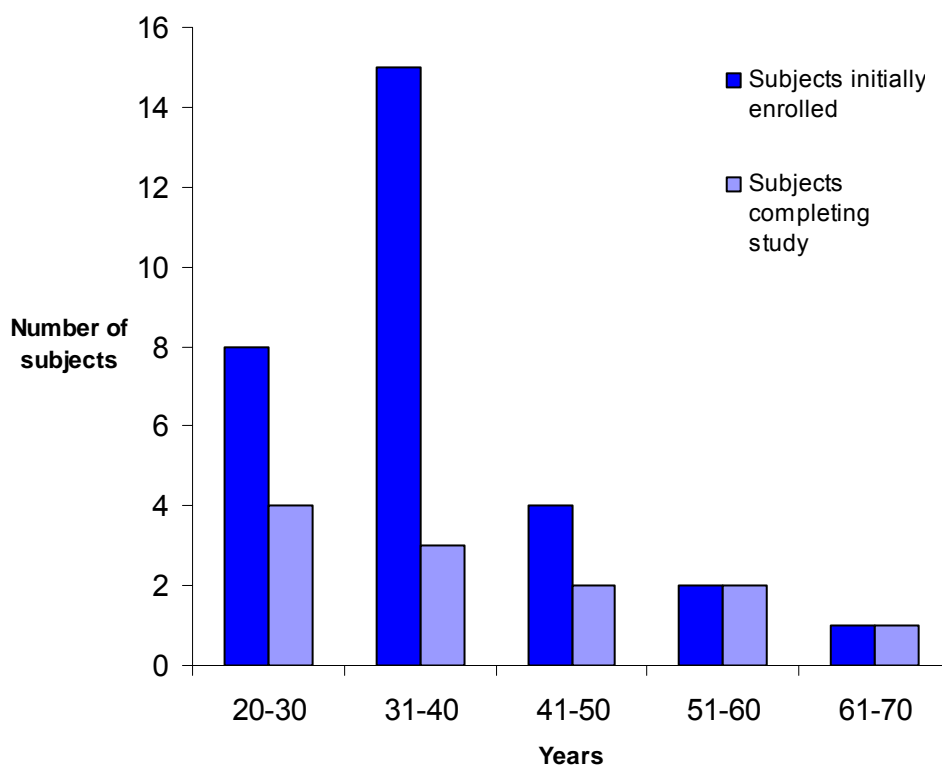


Figure 2.7

The extent of balding assessed using the Hamilton scale in subjects initially enrolled and completing the pilot study.

Subjects were invited to enter a 3 month trial of Xiantene in the treatment of androgenetic alopecia. Half of the subjects used Xiantene and half of the subjects used a vehicle.

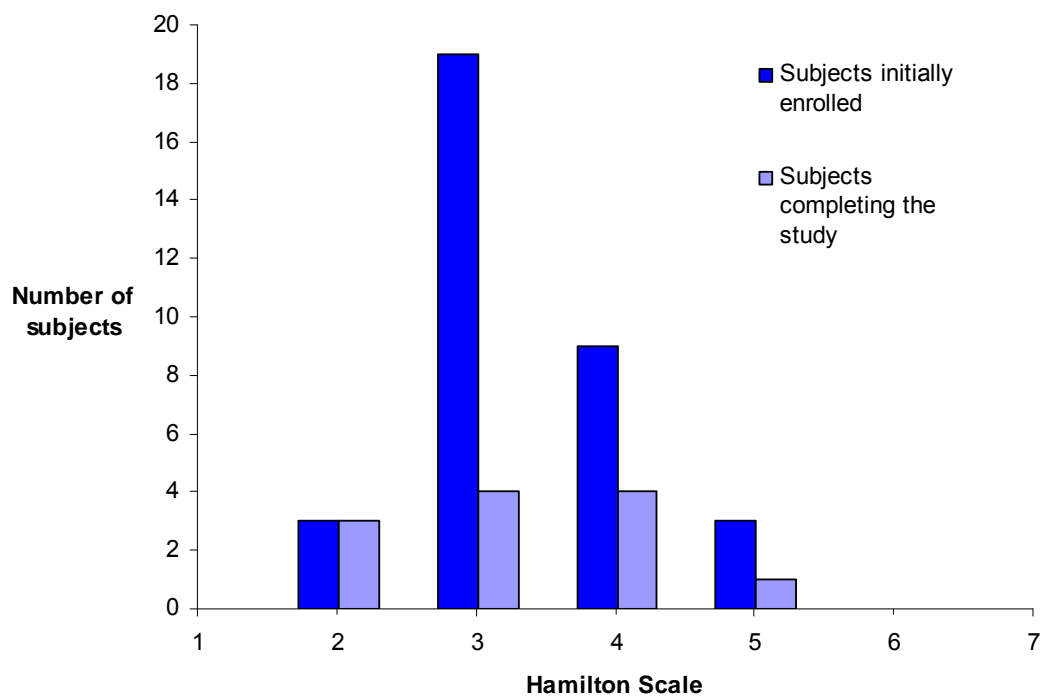


Figure 2.8

Comparison of the mean age of subjects for each Hamilton stage of baldness between subjects initially enrolled and subjects completing the pilot study.

Subjects were invited to enter a 3 month trial of Xiantene (n=35) in the treatment of androgenetic alopecia. Half of the subjects used Xiantene (n=18) and half of the subjects used a vehicle (n=17), values are mean \pm SEM.

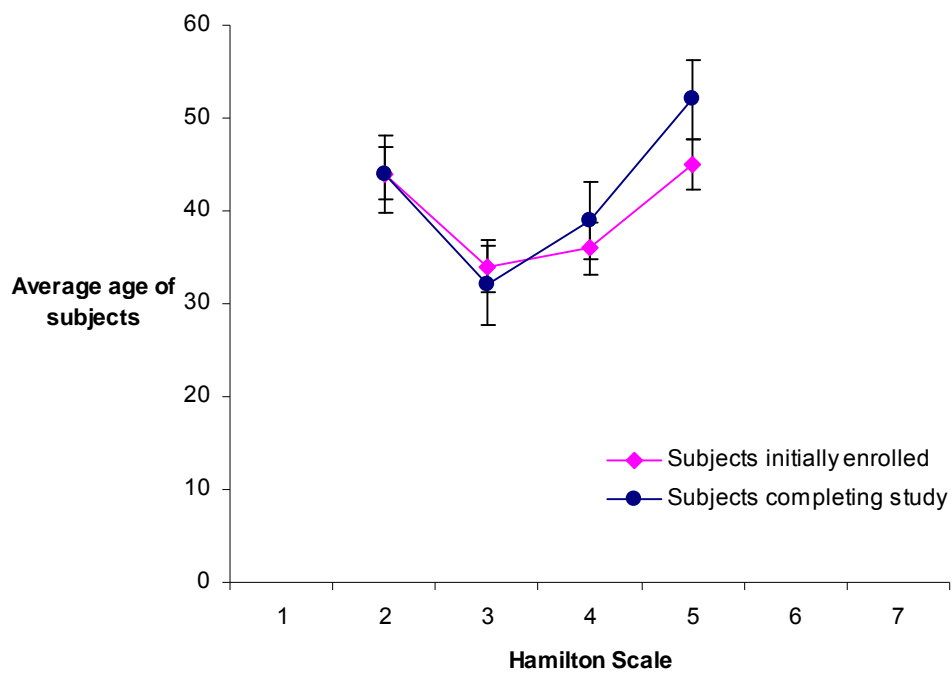
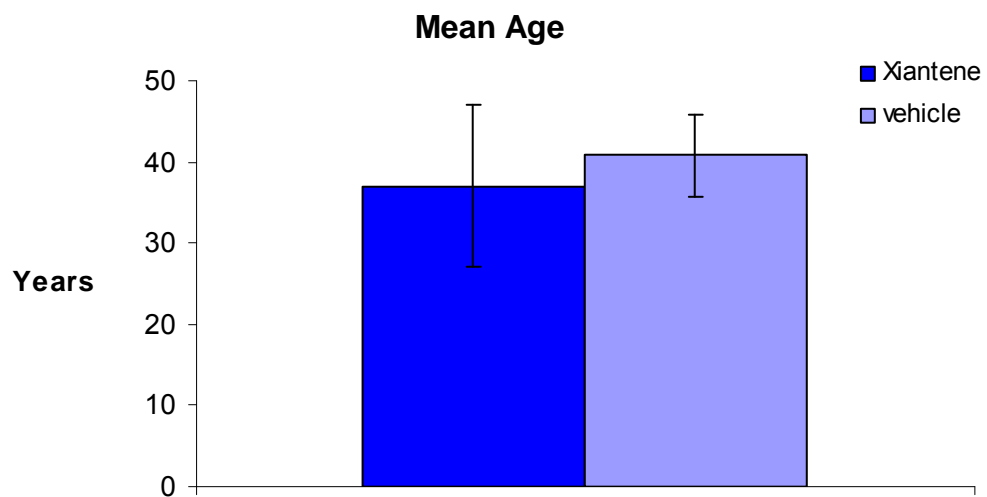


Figure 2.9

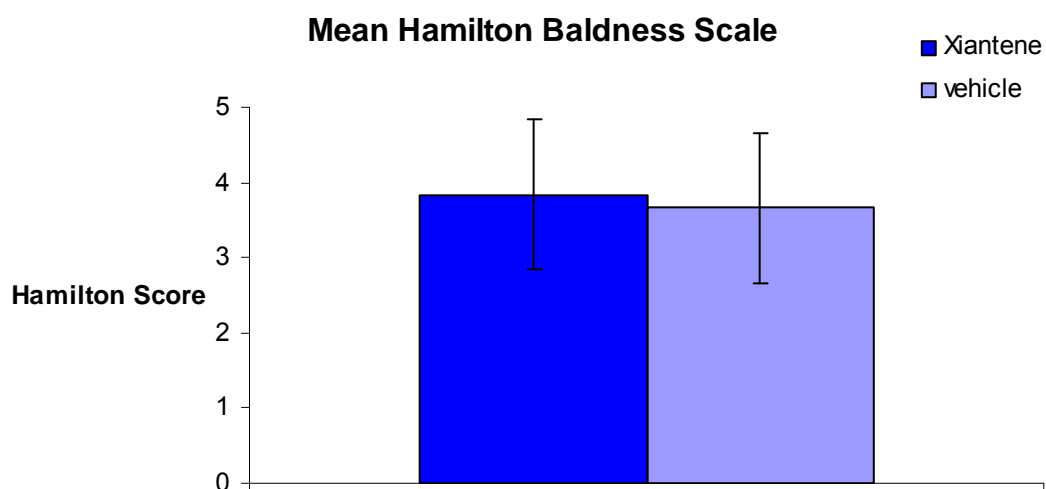
There was no difference in the age or extent of balding at the beginning of the study in subjects completing the study treated with vehicle or Xiantene.

Subjects were invited to enter a 3 month trial of Xiantene in the treatment of androgenetic alopecia. Half of the subjects used Xiantene and half of the subjects used a vehicle. Values are mean \pm SEM for 6 men in each group.

(A) Ages prior to the start of the study



(B) Extent of balding prior to the start of the study



2.4.2 Hair measurements before treatment

The following measurements only refer to those who completed the study.

The total number of hairs within the prescribed circle (50.7 mm²) and the number of hairs in anagen were recorded and plotted for each individual subject before, and after, treatment and the two groups, vehicle and Xiantene compared. Validation of methodology, see Appendix II.

The mean total number of hairs per 50.7 sq mm before treatment did not differ significantly ($p = 0.80$) between the control 57.1 ± 8.2 and Xiantene treated 60.0 ± 7.1 groups (Figure 2.10A). There was also no significant difference ($p = 0.76$) between the mean number of anagen hairs per 50.7 sq mm before treatment between the control (39.8 ± 4.8) and Xiantene treated (42.2 ± 5.5) groups (Figure 2.10B).

Further there was no significant difference in anagen hair diameter pre-treatment; vehicle group $73.8 \pm 4.3 \mu\text{m}$, Xiantene group $78.3 \pm 6.4 \mu\text{m}$, $p=0.58$ or in the level of hair pigmentation; vehicle group 175.9 ± 11.5 units, Xiantene group 167.2 ± 13.5 units, $p=0.63$.

2.4.3 Hair measurements after treatment

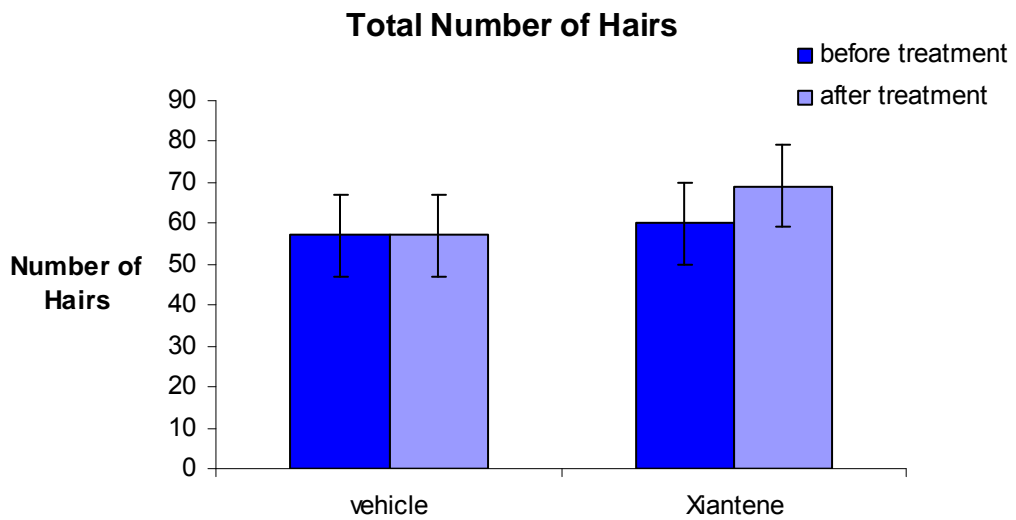
Each individual treated with Xiantene showed an increase in the total number of hairs epilated after treatment (Figure 2.11). This was in marked contrast to the control subjects where only 3 showed an increase i.e. subject numbers P4, P9 and P31, and 3 a decrease (see Figure 2.11). The vehicle group showed no significant difference ($p = 0.98$) when the mean number of hairs before (57.1 ± 8.2) and after treatment (57.0 ± 6.2) were compared.

Figure 2.10

Comparison of the number of hairs and the number of anagen hairs, in the treated and control groups before and after treatment.

Results are the mean \pm SEM for men treated with vehicle (n=6) or Xiantene (n=6) for 3 months. Men with androgenetic alopecia used topical Xiantene (1 ml) or a vehicle daily. All hairs were plucked from a circle of 50.7 mm² area on the crown before and after 3 months treatment. Values are mean \pm SEM.

(A) Total number of hairs



(B) Number of anagen hairs

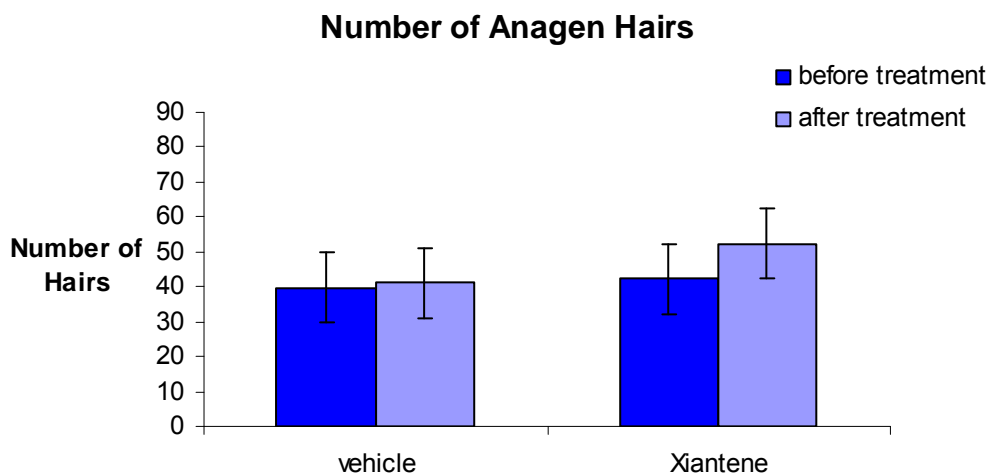
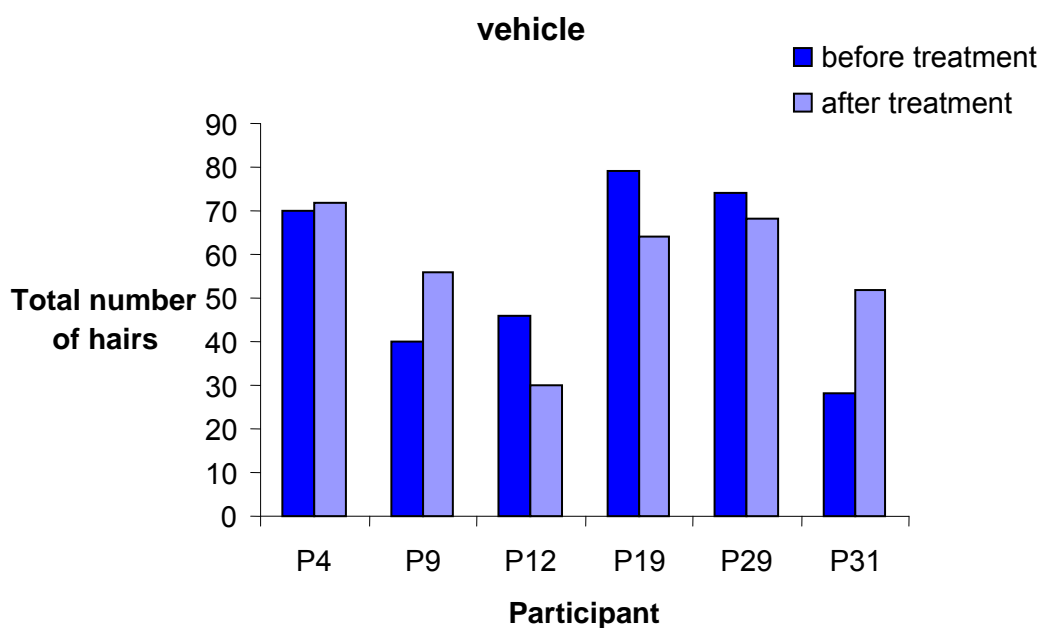
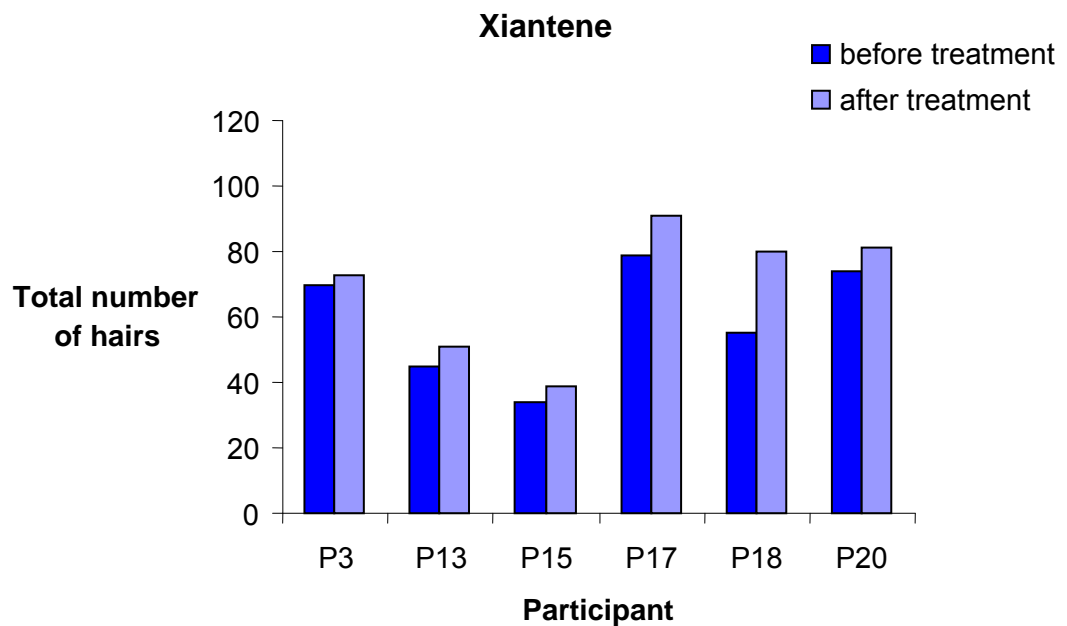


Figure 2.11

Total number of hairs before and after treatment in individual subjects using Xiantene or vehicle.

Men with androgenetic alopecia used topical Xiantene (1ml) or a vehicle daily. All hairs were plucked from a circle of 50.7 mm² area on the crown before and after 3 months treatment.



However, the Xiantene group showed a significant increase ($p = 0.045$) in the mean total number of hairs from 60.0 ± 7.1 before, increasing to 69.2 ± 8.1 after treatment (see Figure 2.10.A.), an increase of 15.33%.

The effect of Xiantene treatment on the number of anagen hairs was also investigated. Each subject treated with Xiantene also showed an increase in the number of anagen hairs epilated after treatment, whereas the control subjects only 3 showed an increase, i.e. subject numbers P4, P9 and P31 and 3 showed a decrease (see Figure 2.12). There was no significant alteration in the mean number of anagen hairs over 3 months for the vehicle group from 39.8 ± 4.8 to 41.2 ± 3.6 ($p = 0.84$), but the Xiantene group showed a very highly significant increase in the mean number of anagen hairs from 42.2 ± 5.5 to 52.2 ± 6.1 , $p = 0.00033$ (Figure 2.10B). Treatment with Xiantene had increased the number of anagen hairs in the six subjects by 23.71%.

The number of telogen hairs before and after treatment were also counted. Neither the vehicle group with a mean number of telogen hairs of 17.3 ± 4.8 before treatment and 15.8 ± 3.9 after treatment, ($p = 0.36$) nor the Xiantene group 17.8 ± 2.0 before treatment and 17.0 ± 3.0 after treatment ($p = 0.81$) showed any significant change in the numbers of telogen hairs.

Hair diameter measurements

Changes in anagen hair diameter were much more variable than the other parameters in both the control and treated groups (Figure 2.13).

Figure 2.12

The number of anagen hairs before, and after, treatment in subjects using Xiantene or vehicle.

Men with androgenetic alopecia used topical Xiantene (1ml) or a vehicle daily. All hairs were plucked from a circle of 50.7 mm² area on the crown before, and after, 3 months treatment.

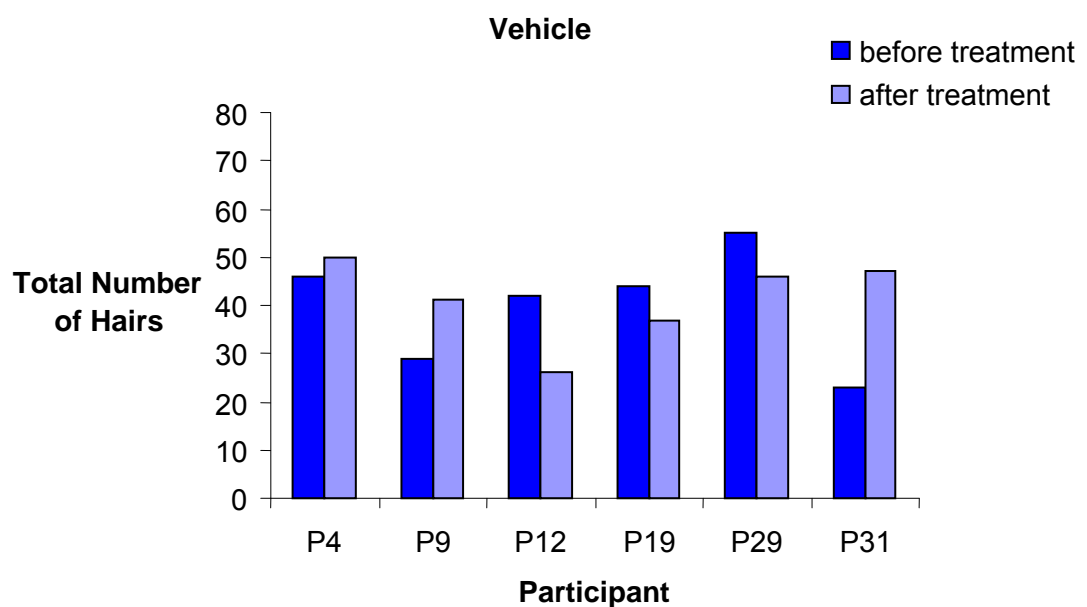
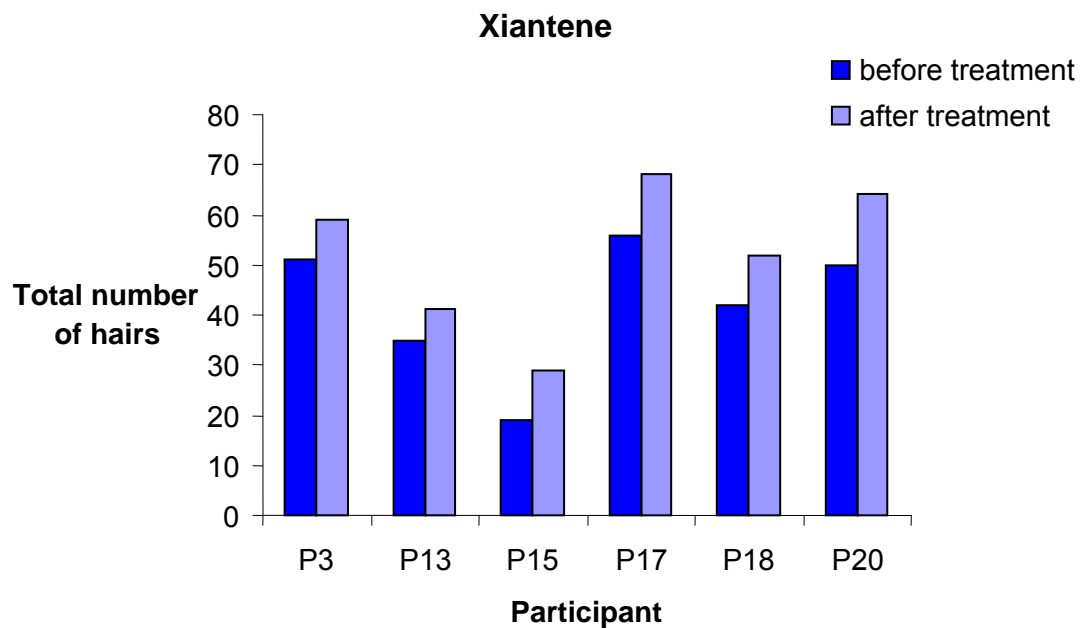
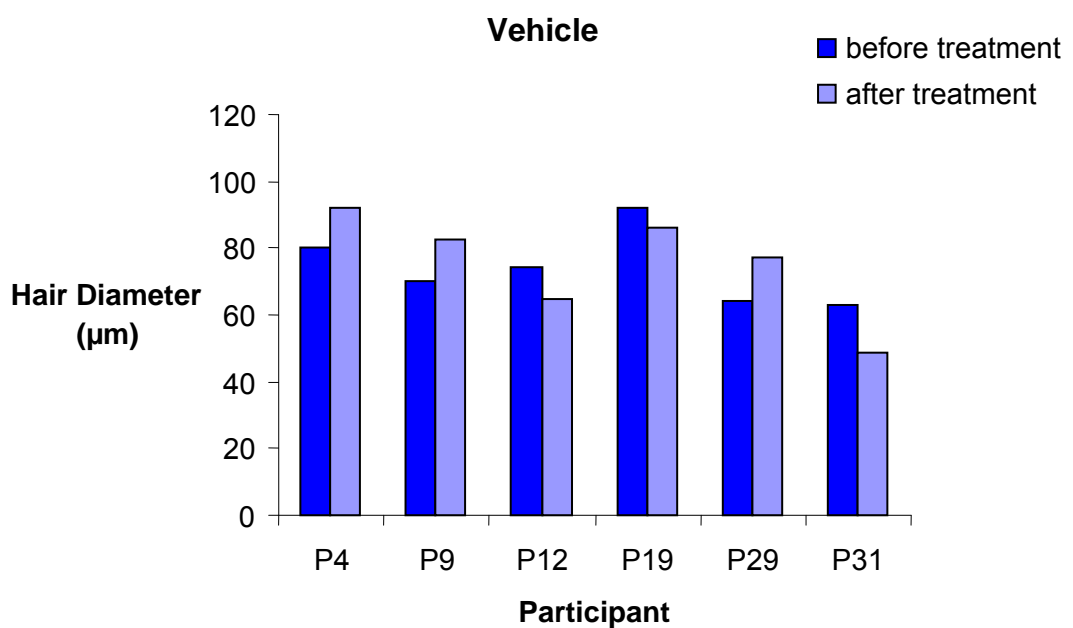
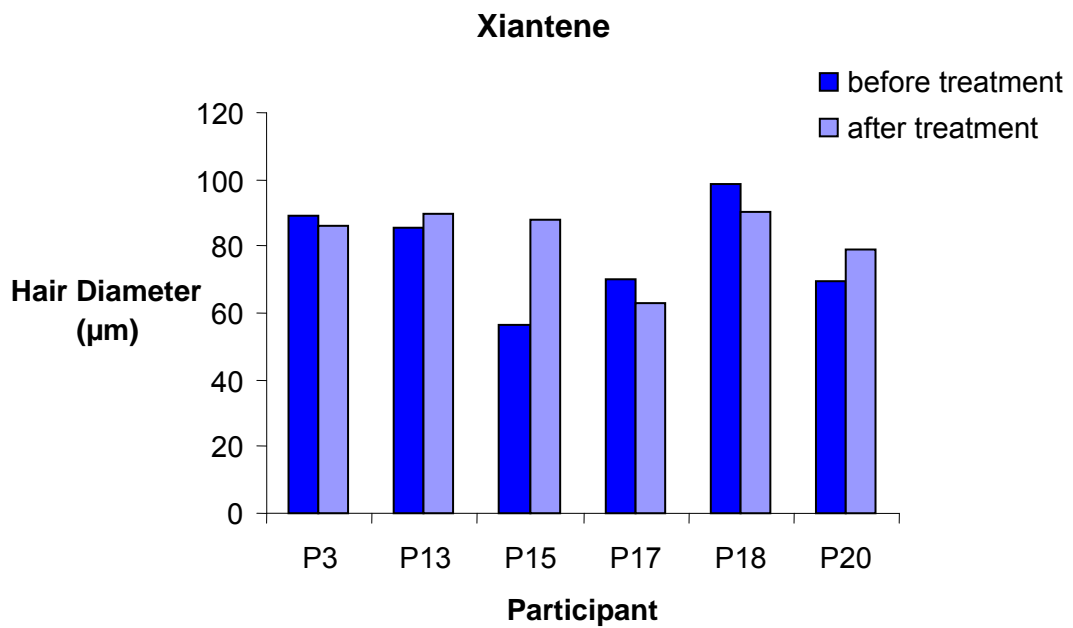


Figure 2.13

Mean hair shaft diameters of anagen hairs before and after treatment in subjects using Xiantene or vehicle.

Men with androgenetic alopecia used topical Xiantene (1ml) or a vehicle daily. All hairs were plucked from a circle of 50.7 mm² area on the crown before and after 3 months treatment.



Hair diameter measurements of hairs in the anagen phase, before and after treatment, showed no significant change in mean hair shaft diameter for either the vehicle group ($p = 0.78$) or the Xiantene group ($p = 0.51$) after 3 months, see Figure 2.13.

Xiantene group mean diameters were $78.3 \pm 6.4 \mu\text{m}$ before treatment and $82.6 \pm 4.3 \mu\text{m}$ after treatment; vehicle group $73.8 \pm 4.3 \mu\text{m}$ before treatment and $75.3 \pm 6.5 \mu\text{m}$ after treatment, (see Figure 2.14.A). The largest hair diameter recorded was $98.60 \mu\text{m}$ and the smallest hair diameter measurement record was $38.49 \mu\text{m}$ (subject P12, before treatment with vehicle); all other hair diameter measurements were above the $40 \mu\text{m}$ threshold, proposed by Rushton (Rushton et al, 1991).

Anagen hair pigmentation measurements

The Xiantene group showed slightly increased mean hair pigmentation after treatment, 175.5 ± 13.8 units compared to pre-treatment 167.2 ± 13.5 units, ($p = 0.01$). This contrasted with the control group where there was no significant change in mean hair pigmentation between pre-treatment 175.9 ± 11.5 and post-treatment 165.9 ± 13.5 , $p = 0.09$, see Figures 2.14. B. and 2.15. Some staining of the scalp was reported by 2 people from the Xiantene group.

Photographic record

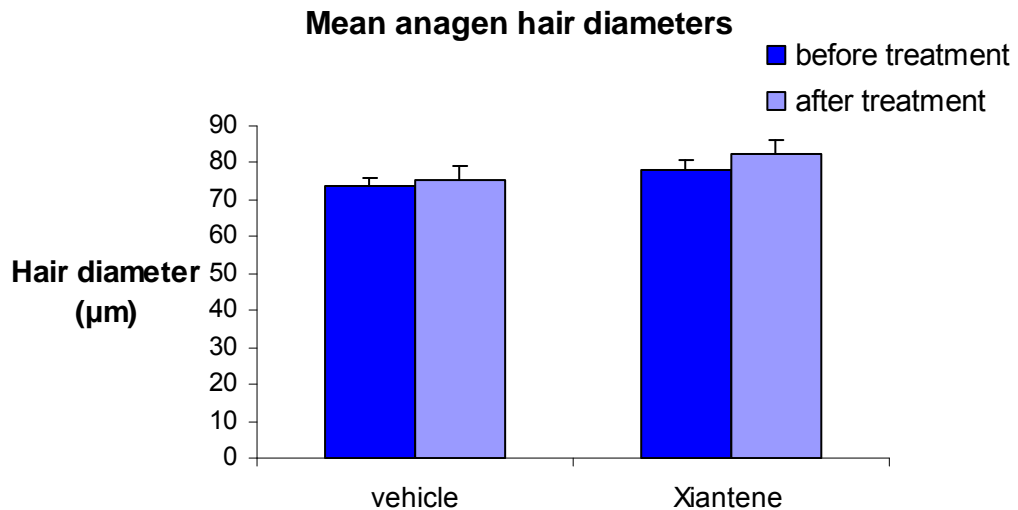
A photographic record was made of each subject's crown area at the beginning and end of the trial to allow a cosmetic assessment to be made of hair growth.

Figure 2.14

Comparison of the mean anagen hair diameter measurements and the mean anagen hair pigmentation measurements in the treated and control groups before and after treatment.

Results are the mean \pm SEM for men treated with vehicle (n=6) or Xiantene (n=6) for 3 months. Men with androgenetic alopecia used topical Xiantene (1 ml) or a vehicle daily. All hairs were plucked from a circle of 50.7 mm² area on the crown before and after 3 months treatment. Values are mean \pm SEM

(A) Mean hair diameter



(B) Mean anagen hair pigmentation

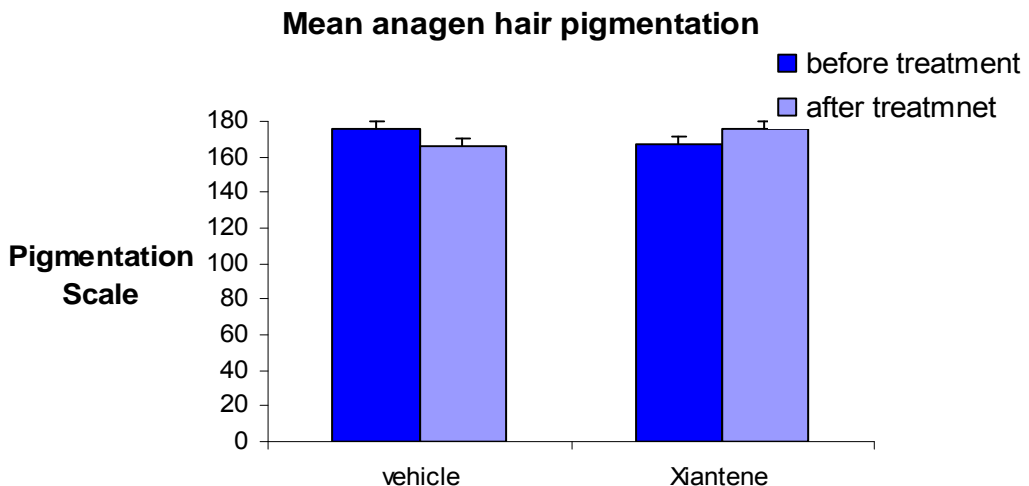
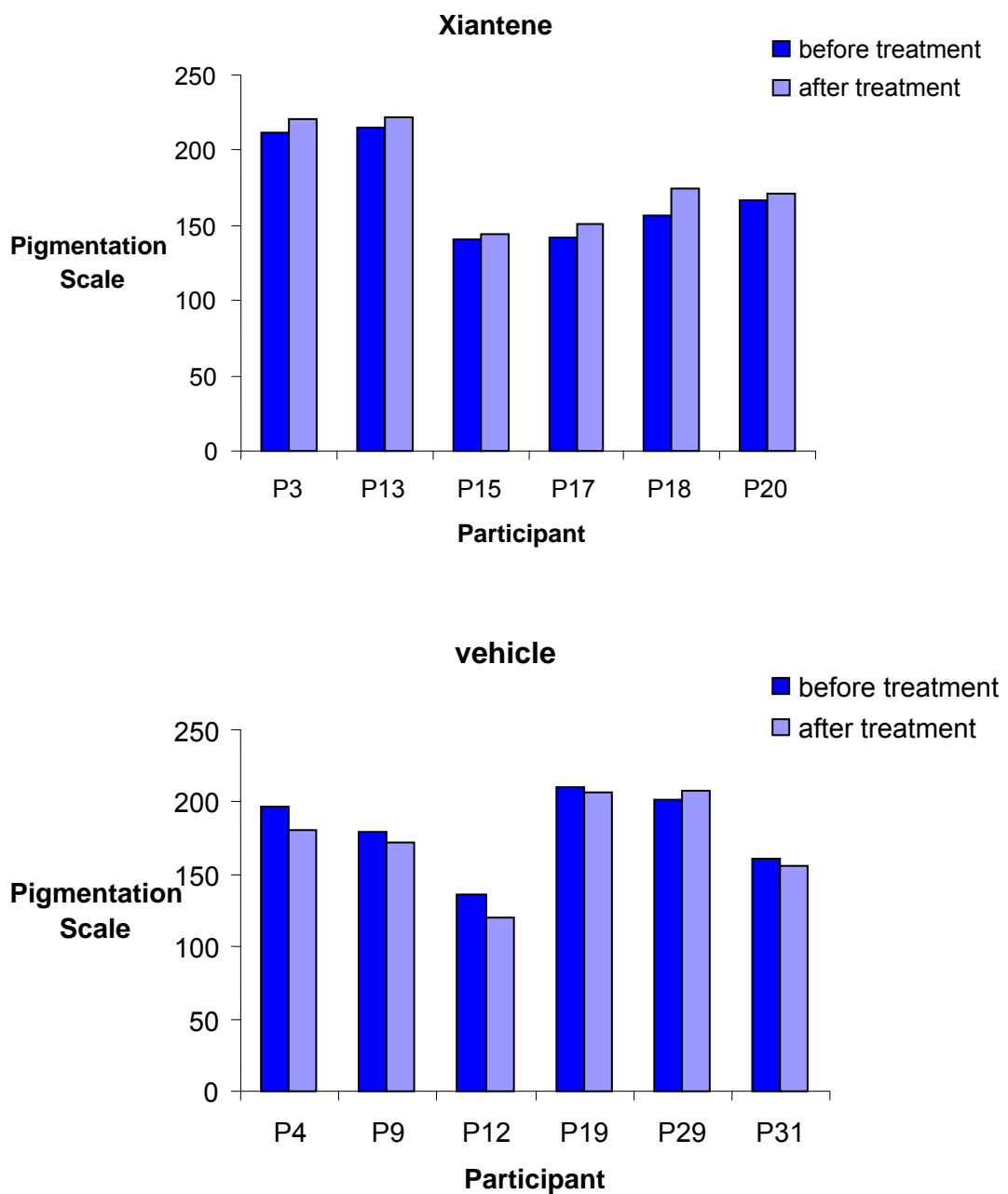


Figure 2.15

Mean pigmentation of anagen hairs before and after treatment in subjects using Xiantene or vehicle.

Men with androgenetic alopecia used topical Xiantene 1ml (n=6) or a vehicle (n=6) daily. All hairs were plucked from a circle of 50.7 mm² area on the crown before and after 3 months treatment. Pigment was assessed using a digitalised scale, 0 = white, 250 = black.



The ultimate test for any hair loss treatment should be the eventual improvement to the cosmetic appearance of the scalp hair in the treated area. The Xiantene group subjects did not show any dramatic improvement overall, although three of the subjects did appear to show possible improvement after treatment (see Figure 2.16). The vehicle group subjects did not show any changes (see Figure 2.17). Neither group showed any visible deterioration, probably due to the short 3 months period of the study.

2.5 Discussion

2.5.1 Methodology

Methods of evaluating hair growth have been reviewed extensively (Rushton & James, 1986; Rushton et al, 1993; Van Neste, 2002; Van Neste, 2003; Chamberlain & Dawber, 2003) and may be divided into 3 main groups. “Invasive” using scalp biopsies sectioned vertically and horizontally to assemble a “follicugram” to provide follicle length and anagen:telogen ratios (Whiting, 1993). “Semi-invasive” methods, principally the unit area trichogram where the hairs are epilated from a small area of the scalp and examined to give the required hair parameters (Barman et al, 1965; Rushton et al, 1983).

This has been further developed into the phototrichogram (PTG) method in which magnified sequential photographs of the shaved scalp area, possibly dyed are examined over a period of days to determine anagen and telogen hair numbers, the analysis of which has been enhanced by computer assisted imaging (Van Neste & Trúeb, 2006).

Figure 2.16

Photographs of subject before and after treatment with Xiantene.

Men with androgenetic alopecia used topical Xiantene 1 ml daily for a 3 month trial.



Figure 2.17

Photographs of subject before and after treatment with vehicle.

Men with androgenetic alopecia used topical vehicle 1 ml daily for a 3 month trail.



Thirdly there are “non-invasive” techniques based on questionnaires for subject and researcher assisted by macro photography of the scalp and a scoring system which is constructed from this data.

The unit area trichogram was used in this experiment to provide samples which could be repeatedly measured and assessed for parameters which can be accurately measured (validation see Appendix II) and does not allow overestimation of the number of hairs. The method has the draw back of being quite painful and possibly unacceptable for the subject and the technique is time consuming; however it is well suited to the relatively small subject numbers in this experiment. Large scale hair growth studies now favour the phototrichogram (PTG) method of assessment which has been shown to be of high accuracy and reliability (Van Neste et al, 2006), but care must be taken not to underestimate the total hair density due to the difficulty of identifying vellus, unpigmented hairs which may remain undetected (Van Neste, 2004). The PTG method does not allow assessment of hair diameters.

2.5.2 Subjects

The total of 35 people who commenced the pilot study were reduced to 12 at the end of the trial. This high drop out rate of 65.7% was disappointing, probably enhanced by a combination of extremely heavy rain and a central London tube strike on the final days. The number of people who completed the trial was therefore small, however subject numbers were equal for both the Xiantene treated and control groups. No women completed the trial and only data relating to male subjects was used in this study. The age

distribution (see Figure 2.6) of those starting and those completing the trial indicates that the highest drop out rate was amongst the youngest men, particularly in the 20-40 years age range where only 32.0 % of subjects completed the course. Subjects in the 41-70 years age range, where 66.7 % completed the course, were either more conscientious and/or more concerned about their hair loss. Interestingly, the major drug hair loss trials (Olsen et al, 1985; Kaufman et al, 1998) tend to be based on the younger age ranges (18-41 years) for which a greater hair growth recovery might be possible. In this study it proved difficult to retain those younger subjects with possibly the greater potential to respond to treatments. Subjects with the lower Hamilton stages were also less likely to complete the trial.

Analysis of the Hamilton baldness stages (Hamilton, 1951) of those completing and those not completing the study (see Figure 2.7) were significantly different ($p=0.02$) and confirms the loss of 77.77% of Hamilton stage 3 group where any hair growth response might have been most easily seen. The comparison of mean age of subject for each Hamilton grading scale of baldness shows that the Hamilton type 2 group have a higher mean age than the Hamilton type 3 group indicating possibly that the trial has attracted some older men with a good head of hair. It is not uncommon (personal observation) for older men with good heads of hair to be particularly interested in hair growth and willing participants in hair growth experiments. See also Chapter 5 where the same phenomenon was observed. Men with good heads of hair appear to be proud of their natural good fortune and want to maintain it.

2.5.3 Pre-treatment data

Before treatment started both groups of men who completed the trial, Xiantene and control, shared similar parameters and were well matched. The mean ages; Xiantene 34.5 ± 2.3 years; vehicle 35.3 ± 2.4 years (see Figure 2.6) compared well and were not significantly different, ($p = 0.80$). Likewise the extent of baldness using the Hamilton scale is similar; Xiantene score 3.3 ± 0.2 , vehicle 3.4 ± 0.2 (see Figure 2.7), $p = 0.71$. In normal men not suffering from androgenetic alopecia, there is no significant difference in hair density between the occipital area, 311 ± 11 hairs per cm^2 and the frontal area, 312 ± 15 hairs per cm^2 (Rushton et al, 1983). Analysis of the pre-treatment parameters in this trial indicates that the total number of hairs per sq cm found here, on the crown 117.8 ± 13.9 Xiantene group and 112.2 ± 16.2 in the vehicle group, are lower than other published data; Rushton's group (Rushton et al, 1983) reported 175 ± 6 hairs/sq cm on the frontal or vertex area of men suffering from androgenetic alopecia, although this group were younger, with a lower mean age of 24 years. Other researchers (Olsen et al, 1985) found a lower comparable hair count of 58.6 hairs/sq cm for the frontal/vertex area in men with androgenetic alopecia, although this later photographic counting technique may not have evaluated the total vellus hair count (James & Rushton, 1986). The mean number of anagen hairs per sq cm (83.8 ± 10.9 , 71.1% of the total) in the Xiantene group and (78.0 ± 9.4 , 69.5% of the total) in the vehicle group recorded is as expected lower than published data of $85.4 \pm 1.5\%$ for the androgen insensitive occipital area (Rushton et al, 1983) but higher than the figure for the androgen sensitive frontal area which was found to be $57.3 \pm 5.5\%$ (Rushton et al, 1983). It has

been proposed based on histological observations that more than 80% of the hairs in the anagen phase is normal: more than 20% of the hairs in the telogen phase, i.e. an anagen:telogen ratio of less than 4:1 is consistent with androgenetic alopecia (Bergfeld, 1989). The lower percentage of hairs in the anagen phase in this experiment indicates a more advanced stage of patterned hair loss and confirms androgenetic alopecia with a correspondingly reduced total number of hairs. The mean hair diameters before treatment of $78.3 \pm 6.4 \mu\text{m}$ for the Xiantene treated group and $73.8 \pm 4.3 \mu\text{m}$ (see Figure 2.14.A) for the vehicle group compare well and are in good agreement with $69 \pm 3 \mu\text{m}$ recorded by Rushton's group for hair from the occipital area of balding men (Rushton et al, 1983) but higher than the $54 \pm 3 \mu\text{m}$ found for the frontal/vertex area. Overall the pre-treatment measurements confirmed that the subjects were showing the characteristics of androgenetic alopecia i.e. they were suitable for study.

2.5.4 Comparison of pre-treatment and post-treatment data

The Xiantene and vehicle groups completing the trial were well matched. The mean ages compare well and are not significantly different; Xiantene group 37.2 ± 4.9 years, vehicle group 40.8 ± 6.0 years (see Figure 2.6), ($p = 0.65$). Similarly the extent of baldness for the two groups does not differ significantly, ($p = 0.72$) (Xiantene group Hamilton type 3.8 ± 0.3 , vehicle group Hamilton type 3.7 ± 0.3 , see Figure 2.7). The equal subject numbers and the non-significant differences in the key parameters allow valid comparison between the Xiantene treated group and the vehicle control group completing the 3 month study (see Figures 2.9, 2.10.A and 2.10.B).

Examination of the vehicle group shows that there were no significant changes in the key parameters: number of hairs, number of anagen hairs, hair shaft diameter and hair shaft pigmentation when pre and post treatment values were compared (see Figures 2.10.A, 2.10.B 2.14.A and 2.14.B). Possible changes could have been expected in a balding population over time (Rushton et al, 1991; Kaufman et al, 2008); additionally seasonal changes could also have been expected (Randall & Ebling, 1991). The lack of significant change in any of the four hair growth parameters in the vehicle treated group probably reflects the short duration of the study as it was carried out only for 3 months. It is now generally accepted that any hair growth study should be conducted for a minimum of 12 months to allow for seasonal variations (Randall, 2008).

Treatment with the herbal mixture Xiantene affected several hair follicle parameters. The total number of hairs per unit area in the Xiantene treated group was increased significantly ($p = 0.045$) by 47.38% (see Figure 2.10.A). This increase could be a reflection of the stimulation of vellus hairs to terminal hairs with a consequent increase in the number of “meaningful” hairs (Rushton et al, 1983) i.e. the number of hairs that can be seen and plucked. “Meaningful” hairs have been defined as those with a diameter of 40 μm or more (Rushton et al, 1983) i.e. hairs below 40 μm diameter did not contribute to the aesthetic appearance of the scalp. Other treatments e.g. minoxidil (Olsen, 1985), finasteride (Kaufman et al, 1998) and dutasteride (Olsen et al, 2006) have also been shown to increase the numbers of meaningful hairs per unit area. It is unlikely that Xiantene treatment would actually increase the number of hairs by any other mechanism, particularly in

the short time involved. The human scalp is believed to have its full complement of hair follicles soon after birth (Dry, 1926) with loss but not increase in hair follicles reported in later life (Hamilton, 1951; Whiting, 2001). Xiantene would appear to have increased the number of meaningful hairs during the 3 month study by stimulating vellus hair follicles into making larger hairs, or in view of short time scale, more likely stimulating anagen in “empty” follicles where the hair has been shed due to exogen (Stenn et al, 1996; Van Neste et al, 2007) while the follicle was resting. The 3 month period of the study is unlikely to be long enough for the follicle to go through all the processes of replacing a hair.

The increase in the number of anagen hairs of the Xiantene group was very highly significant $p = 0.00033$, (Figure 2.10.B) with a mean increase in anagen hairs for the Xiantene group of 23.70%. There was no corresponding increase in the vehicle group $p = 0.84$, (Figure 2.10.B). The mean number of telogen hairs showed no significant difference in either group after treatment; (Xiantene $p = 0.81$, vehicle $p = 0.36$). The increased numbers of anagen hairs, while the number of telogen hairs remains unchanged suggests that the increase in overall numbers of hairs is due to increased numbers of visible hairs in the anagen phase. This may suggest that Xiantene treatment is stimulating vellus follicles, as vellus follicles spend much less time in anagen (Chase, 1954) than terminal hairs. A reduction of the number of hairs in the anagen phase, along with miniaturisation of the follicles is one of the characteristics of androgenetic alopecia (Bergfeld, 1989; Whiting, 2001). Histological observations (Bergfeld, 1989), indicate that androgenetic alopecia is characterised by: a decrease in the anagen: telogen ratio

(normally 80:20), a decrease in the number of hair follicles, miniaturisation and involution of the hair follicle; and compacting of the dermal connective tissue resembling scar formation. It is proposed that miniaturisation of the hair follicle is the key feature of androgenetic alopecia and further that the process may not proceed in a gradual progression, but as a series of large step processes (Whiting, 2001). It was found (Whiting, 2001) that in cases of androgenetic alopecia, the anagen phase may eventually be reduced from 2 or 3 years to 1 month, but the telogen phase remained constant at approximately 3 months, causing a reversed anagen:telogen ratio and further that there may be a time lag after the shedding exogen phase (Van Neste, 2007) of up to 6 months before the appearance of the new anagen hair. The rate of hair growth was found to be reduced in androgenetic alopecia (Whiting, 2001) corresponding to a reduced dermal papilla size which could be seen histologically.

Finasteride was found to partially reverse miniaturisation of the hair follicle in subjects with androgenetic alopecia (Kaufman et al, 1998, Van Neste, 2006) although the beneficial effect was gradually lost within 12 months if the treatment was discontinued (Kaufman et al, 1998). Minoxidil similarly promoted the entry of follicles into, and prolonged the length of anagen, in cases of androgenetic alopecia (Olsen et al, 1985, Olsen, 1989), something also observed in this 3 month study of treatment with Xiantene. It is possible Xiantene has increased the length of the anagen phase in subjects with androgenetic alopecia. The increase in the total number of hairs (see Figure 2.10.A) suggests that new hair has been added i.e. an increase in the number of meaningful hairs (Rushton et al, 1983). This reversal and increase

in the number of hairs in the anagen phase (see Figure 2.10.B) of the hair cycle is significant in any evaluation of herbal mixture Xiantene and suggests that further investigations are warranted.

2.5.5 Hair diameter measurements

The mean anagen hair diameters recorded before treatment (Xiantene group $78.3 \pm 6.4 \mu\text{m}$, vehicle group $73.8 \pm 4.3 \mu\text{m}$, see Figure 2.14.A) compare well with earlier work. Subjects with androgenetic alopecia were found to have a mean hair diameter of $69 \pm 3 \mu\text{m}$ in the occipital region for men and $66 \pm 4 \mu\text{m}$ for women (Rushton et al, 1983). Young women not suffering from hair loss were found to have a larger hair diameter, $73.69 \pm 11.71 \mu\text{m}$ than mature women, $68.63 \pm 5.41 \mu\text{m}$ and post menopausal women, $59.01 \pm 10.08 \mu\text{m}$ who complained of hair loss (Van Neste, 2004). There was no significant alteration in this experiment in mean hair diameter in either group after 3 months of treatment. It is the gradual miniaturisation of the hair shaft and eventual atrophy of the hair follicle which characterises androgenetic alopecia (Bergfeld, 1989; Whiting, 2001), therefore stopping the decrease might be expected. It is equally possible that any change in hair diameter may not be detectable in the short duration of this pilot trial. An increase in the number of hairs less than $40\mu\text{m}$ diameter in subjects with androgenetic alopecia was found to be of diagnostic significance (Rushton et al, 1983). If a number of smaller hairs have been increased to “meaningful” hairs (Rushton et al, 1983) there would be an increase in the number of hairs with small diameters and the mean hair diameter therefore would actually decrease. Rushton studied hair diameters in subjects with androgenetic alopecia

(Rushton et al, 1983) and found that earlier work on the diameters of human scalp hair (Barman et al, 1965; Jackson et al, 1972) may not have differentiated between the major and minor axes. Hair shaft diameter measurements may also be complicated by swelling due to humidity and also variation in diameter along the length of the hair shaft (White & Stam, 1949), possible variation of diameter of individual hair fibres was also observed by other researchers (Rushton et al, 1983). The cross sections of hair shafts will depend on genetic background (see section 1.1.3) although all subjects in this study were Caucasians and in this experiment no distinction was made between major and minor axes. Interestingly it has also been observed (Rushton et al, 1983) that in the progression of androgenetic alopecia a large percentage of hairs were found of normal diameter but of restricted growth (less than 3 cm) i.e. the hairs were shorter indicating a shorter anagen (growth) phase.

2.5.6 Anagen shaft pigmentation

The increase in anagen hair pigmentation of the Xiantene group compared with those of the control was significant, ($p = 0.012$) (see Figure 2.14.B). Hair pigmentation is known to decrease with age as tyrosinase activity in the hair bulb melanocytes reduces following accumulating reactive oxygen species (ROS) damage (Van Neste & Tobin, 2004). Increased melanogenesis of the anagen hair shaft might be expected if a partial cessation or reversal of androgenetic alopecia had been achieved. The increased pigmentation level is therefore consistent with an extended anagen phase and with increased mitosis and melanogenesis and this phenomenon of increased pigmentation

post treatment has been reported by other researchers (Olsen et al, 1985; Rushton et al, 1989). However, it is also possible that the increased pigmentation recorded may be due to staining of the hair shaft by the test material; a third of the subjects using Xiantene had reported some staining of the scalp. Further, Xiantene contains *Isatidis indigotica fort* (see Chapter 4) which is a pre-cursor for the dye indigo. On the other hand the herb *Polygonum multiflorum* also present in Xiantene and used internally as a decoction (hot aqueous herbal extract) has been claimed to darken grey hair (Reid, 1995), see Chapter 4. The Chinese name for this herb “Ho Shou Wu” derives from the ancient king (Ho) whose head (Shou) of white hair turned black (Wu) when he used the herb, hence the name “Ho Shou Wu” (Reid, 1995). However it is not possible to determine the cause of the pigmentation and this observation of increased pigmentation using Xiantene is interesting but cannot be relied upon.

2.6 Conclusion

These results showed that topical lotion Xiantene might possibly increase the total number of hairs and may increase the percentage of anagen hairs in a small group of men with androgenetic alopecia in a 3 month period, compared with controls, where no significant increases were observed. This could indicate that Xiantene may promote a cessation or partial reversion in the progression of androgenetic alopecia. Therefore, Xiantene treatment for androgenetic alopecia merits further investigation. An extended study over a longer period with a larger subject number is necessary to strengthen these observations.

3. Investigation into the effects of 12 months application of herbal mixture Xiantene on hair growth in men with androgenetic alopecia

3.1 Aim

The purpose of this study was to extend the results from the earlier 3 months preliminary investigation (see Chapter 2), by investigating whether naturally occurring hair growth factors in the herbal mixture Xiantene would have any effects on hair growth in cases of androgenetic alopecia when applied topically for a period of 12 months.

3.2 Experimental design

The effects of topical application of the herbal mixture Xiantene in cases of androgenetic alopecia were studied in a larger group of men over a period of 12 months to prevent any possibility of seasonal changes affecting the results (Orentreich, 1969; Randall & Ebling, 1991; Courtois et al, 1996). The trial, similar in design to the shorter 3 month trial (see chapter 2), was conducted on a double blind basis, half the participants being treated with Xiantene whilst the remainder used a vehicle. The parameters measured included the total number of hairs, the number of anagen hairs and the anagen:telogen ratio in a specific area of the crown. Hair samples for each group were collected using the unit area trichogram method (Rushton et al, 1983). The changes in parameters over the 12 month period were then compared for the two groups, Xiantene and vehicle. Validation of the methodology, see Appendix II.

3.3 Materials and methods

3.3.1 Subjects

A total of 48 male subjects were recruited using local advertising for a 12 month trial of herbal mixture Xiantene in the treatment of androgenetic alopecia. Advertisements were placed in the Brabant House Clinic of Natural Medicine in Thames Ditton, Surrey for 6 weeks asking for male volunteers for a one year trial of a topical herbal mixture in the treatment of common baldness, part of a research programme supervised by the University of Bradford. No payment was offered to trial participants, but the study was 'end loaded' with the offer of a magnum of champagne for all subjects completing the 12 month trial, in an effort to reduce the high drop out rate experienced in the earlier 3 month trial (see chapter 2). Subject ages ranged from 24 years to 85 years. All subjects were examined by a qualified trichologist (the author) at the beginning and end of the trial to confirm androgenetic alopecia and to eliminate any other underlying hair or scalp condition. Subjects were asked to sign a consent form confirming informed consent and confirming that they were in good health although it was not possible to check their health status. They also signed a disclaimer form and were given a standardised washing procedure (see Appendix I). No subject had used any treatment for balding in the 12 months prior to the study. Ethical permission was obtained from the University of Bradford Ethical Committee.

3.3.2 Application of Xiantene

Fifty per cent of the subjects were given herbal lotion Xiantene (Xiantene II, see chapter 4) and the remainder given a vehicle (see Section 2.3.2 chapter

2), and asked to use 1 ml of solution daily on the crown area. Neither subjects nor researcher were able to distinguish between the two lotions, which were identified by code number only. The results were de-coded after analysis of the data.

3.3.3 Assessment of hair growth

The assessment of hair growth followed the same procedure as the initial investigation, see chapter 2 section 2.3.3.

3.3.4 Hair measurements

The hair measurements followed the same procedure as the initial investigation, see Section 2.3.4, chapter 2, except that two people (author and Ms Helena Holicova) independently verified the number of hairs and the morphological identification. All hairs were counted and broken hairs were classified as telogen. Classification was repeated if necessary until concurrence was achieved. Hair diameters and hair pigmentation were not measured for the 12 month trial subjects as hair diameters had shown no change in the 3 month trial and hair pigmentation measurements were not considered meaningful due to the colour of the vehicle. All data are expressed as mean value \pm S.E.M. (Standard Error Mean). The normality of the distribution was determined by producing both histograms and normal plots and examining the distribution values; paired Student's t-tests were used for comparison within groups before and after treatment. Results where $p < 0.05$ were taken as significantly different. The association between variables was examined using Pearson correlation on a correlation

coefficient scale between -1 (a strong negative correlation) and +1 (a strong positive correlation).

3.4 Results

3.4.1. Pre treatment data, 3 month (chapter 2) and 12 month (chapter 3) trial

The pre-treatment data for the men with androgenetic alopecia from both the 3 month (chapter 2) and 12 month (chapter 3) experiments were combined for analysis. The parameters: total number of hairs per circle (50.7 mm²), the number of anagen hairs per circle (50.7 mm²) and the anagen:telogen ratio were related to both age (Figures 3.1, 3.2 and 3.3) and extent of baldness measured by the Hamilton stage (Figures 3.4, 3.5, 3.6 & 3.7), using Pearson correlation. For this data the Xiantene groups and the vehicle groups have been combined. This analysis was therefore carried out on 41 men aged between 22 years and 85 years (mean 44.7 ± 2.1 years) with androgenetic alopecia ranging from Hamilton stage 2 to 7 (mean 4.2 ± 0.2). The data for the 3 month study (n=12) was collected in June, July and August and it was not possible to allow for possible seasonal changes in the numbers of growing hairs. The data for the larger study was conducted over the full 12 month period (n=29) and would not be subject to seasonal variation.

3.4.1.1. Relationship of hair parameters to age

The total number of hairs per template area (50.7 mm²) on the vertex decreased with age from a mean of 61.0 ± 6.4 at age 21-30 years to 22.2 ± 5.3 age over 60 years (Figure 3.1.a).

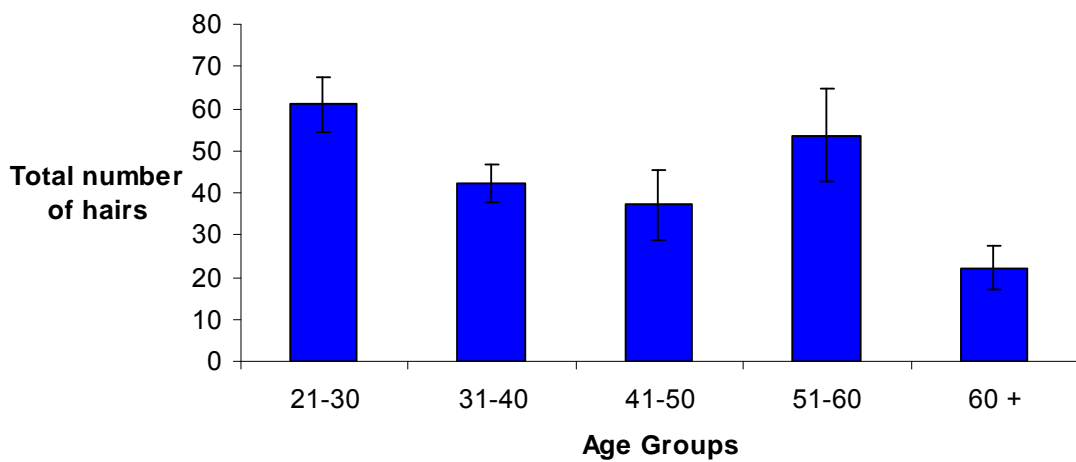
Figure 3.1

The relationship between age in years and the total number of hairs in 50.7 mm² area on the crown in men with androgenetic alopecia.

This includes pre-treatment data from 41 men who completed either the 3 month or 12 month experiments.

(a) Mean values (\pm SEM) for each decade

Number of men aged 21-30=5, 31-40=13, 41-50=10, 51-60=7, 60+ =6



(b) Correlation of individual values with age. Pearson coefficient - 0.37, p=0.02

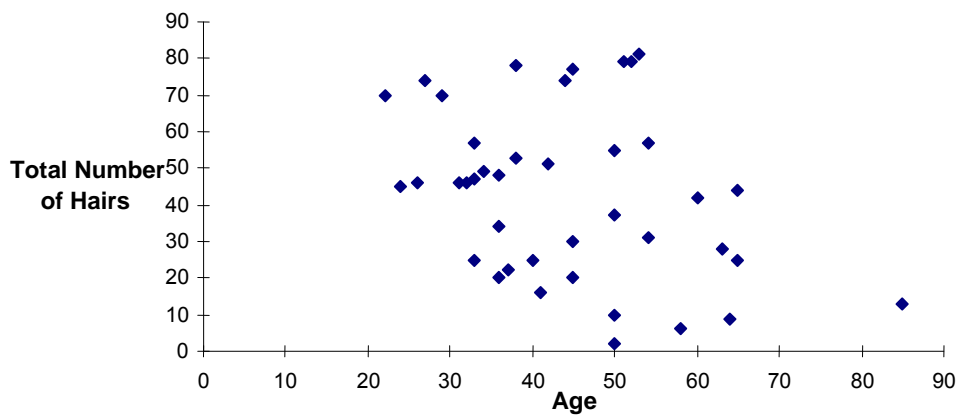


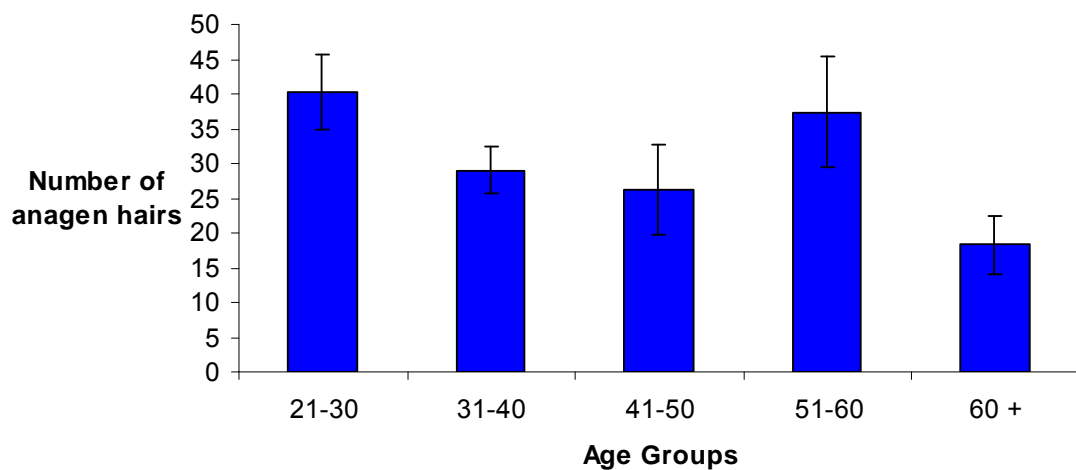
Figure 3.2

The relationship between age in years and the number of anagen hairs in 50.7 mm² area on the crown in men with androgenetic alopecia.

This includes pre-treatment data from 41 men who completed either the 3 month or 12 month experiments.

(a) Mean values (\pm SEM) for each decade

Number of men aged 21-30=5, 31-40=13, 41-50=10, 51-60=7, 60+ =6



(b) Correlation of individual values with age. Pearson coefficient - 0.30, p=0.06

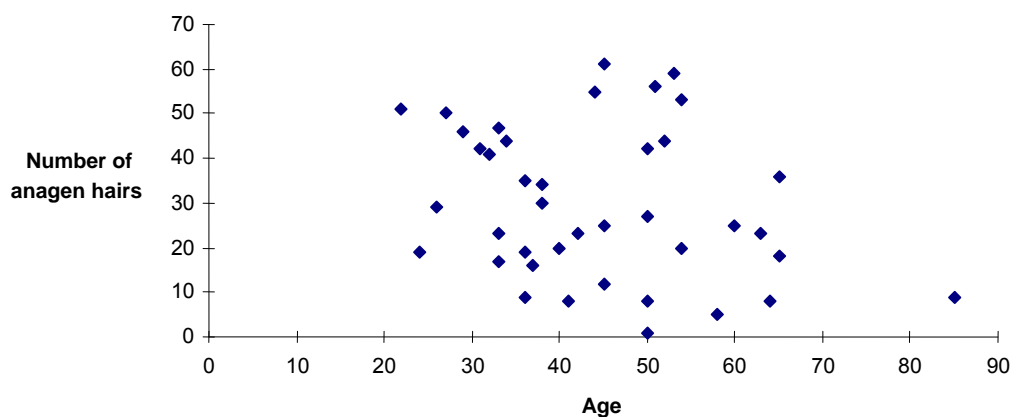


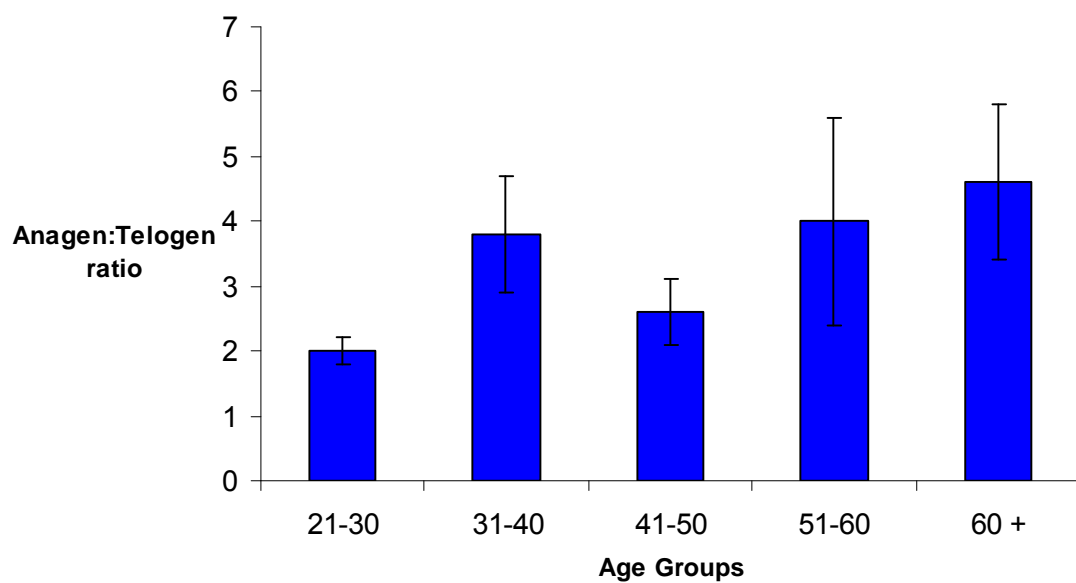
Figure 3.3

The relationship between age in years and the anagen:telogen ratio on the crown in men with androgenetic alopecia.

This includes pre-treatment data from 41 men who completed either the 3 month or 12 month experiment.

(a) Mean values (\pm SEM) for each decade

Number of men aged 21-30=5, 31-40=13, 41-50=10, 51-60=7, 60+ =6



(b) Correlation of individual values with age. Pearson coefficient 0.11, $p=0.51$

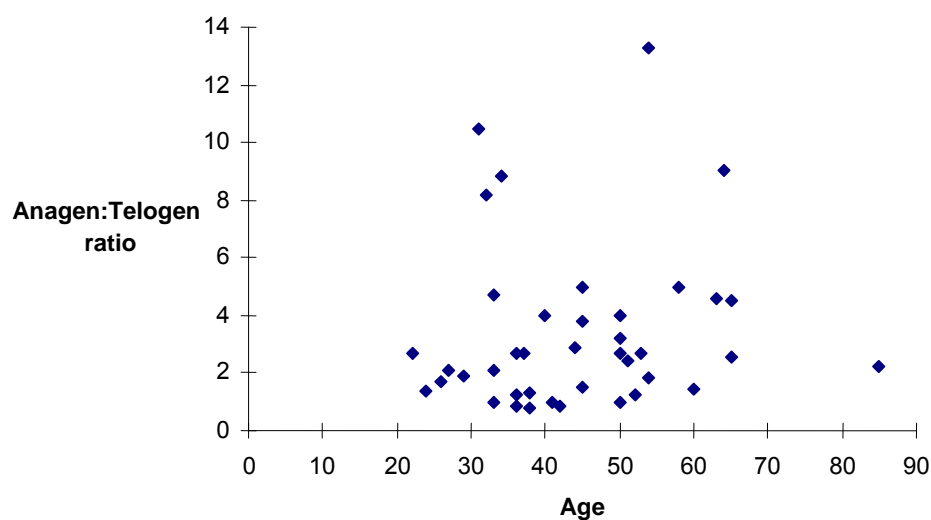


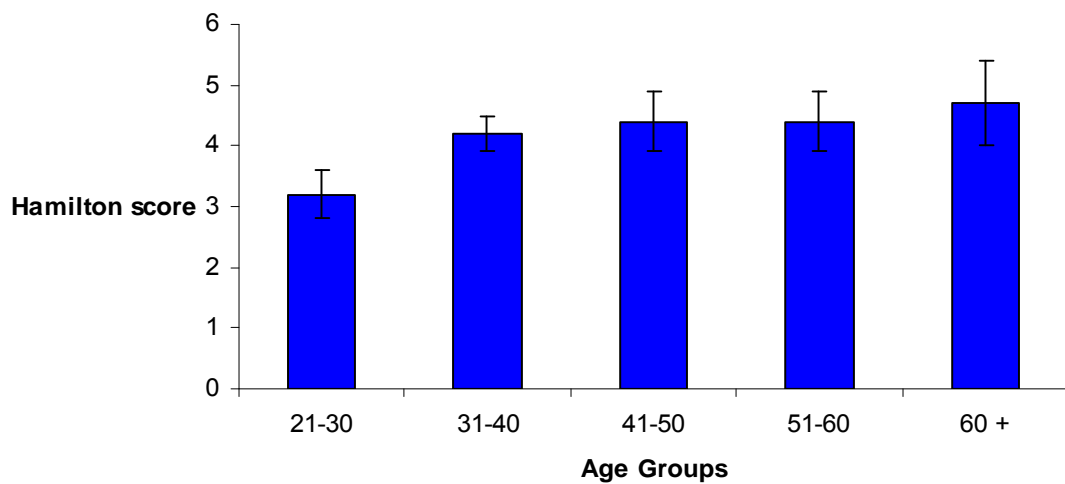
Figure 3.4

The relationship between age in years and the extent of baldness (Hamilton stage) in 50.7 mm² area on the crown in men with androgenetic alopecia.

This includes pre-treatment data from 41 men who completed either the 3 month or 12 month experiments.

(a) Mean values (\pm SEM) for each decade

Number of men aged 21-30=5, 31-40=13, 41-50=10, 51-60=7, 60+ =6



(b) Correlation of individual values with age. Pearson coefficient 0.35, $p=0.03$

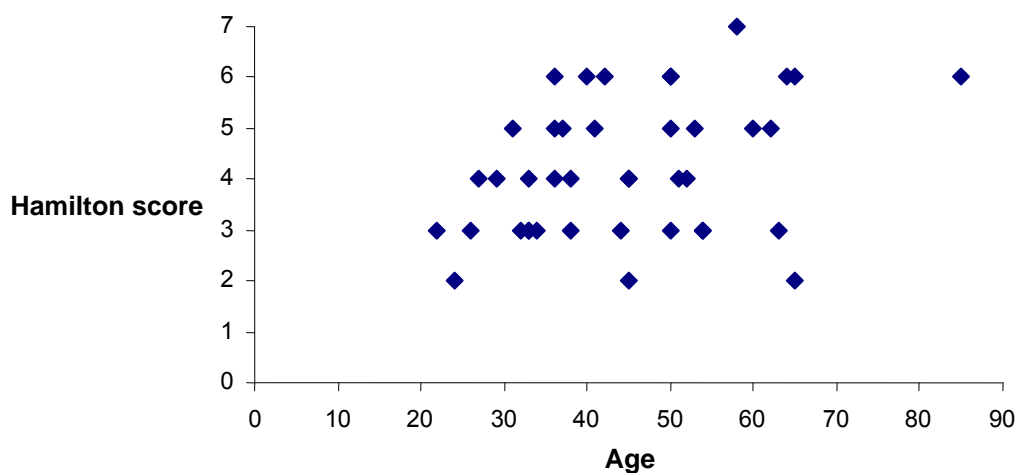


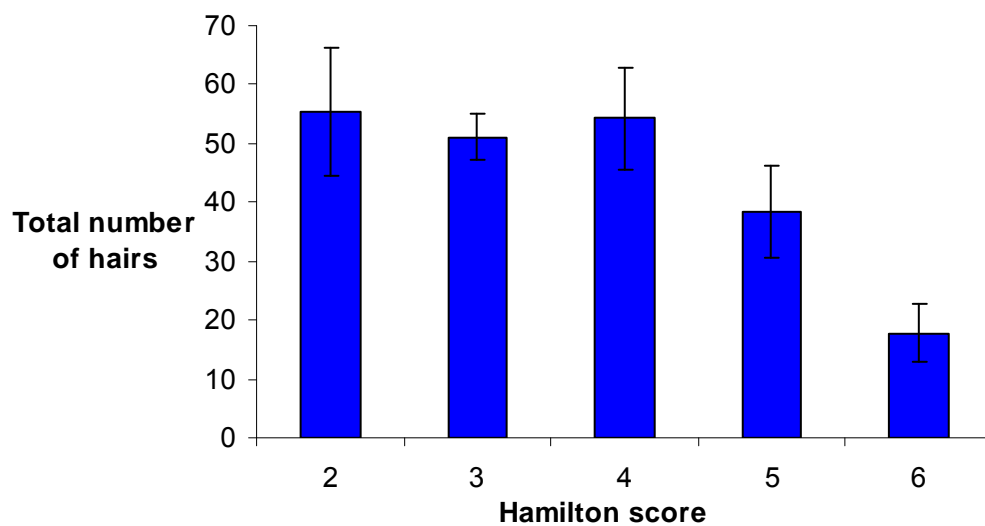
Figure 3.5

The relationship between extent of balding (Hamilton stage) and the total number of hairs in 50.7 mm² area on the crown in men with androgenetic alopecia.

This includes pre-treatment data from 41 men who completed either the 3 month or 12 month experiments.

(a) Mean values (\pm SEM) for each Hamilton stage

Number of men aged 21-30=5, 31-40=13, 41-50=10, 51-60=7, 60+ =6



(b) Correlation of individual values with age. Pearson coefficient -0.56, $p < 0.001$

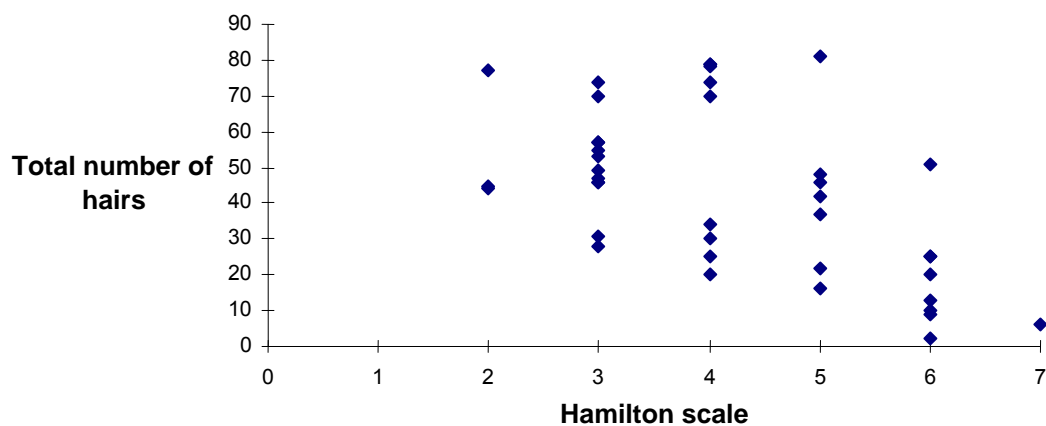


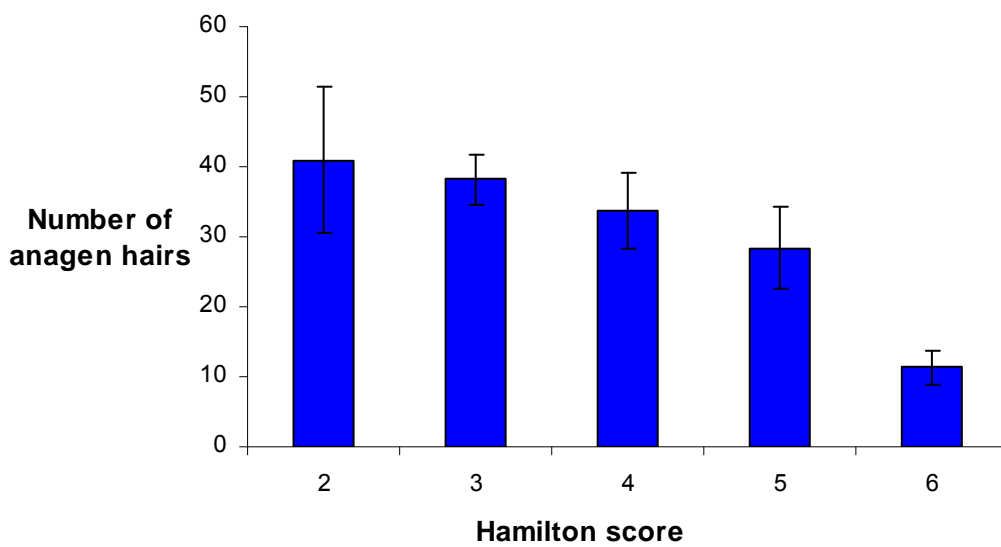
Figure 3.6

The relationship between extent of balding (Hamilton stage) and the number of anagen hairs in 50.7 mm² area on the crown in men with androgenetic alopecia.

This includes pre-treatment data from 41 men who completed either the 3 month or 12 month experiments.

(a) Mean values (\pm SEM) for each Hamilton stage

Number of men aged 21-30=5, 31-40=13, 41-50=10, 51-60=7, 60+ =6



(b) Correlation of individual values with Hamilton stage. Pearson coefficient - 0.60, $p < 0.001$

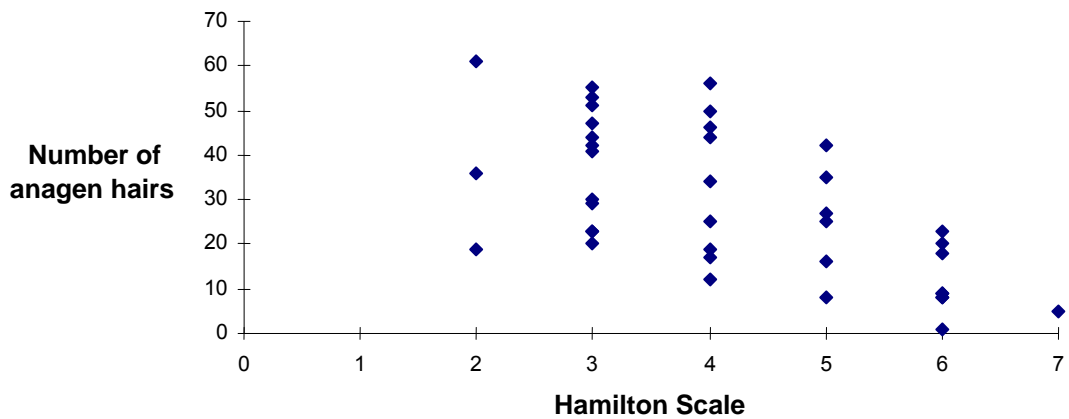


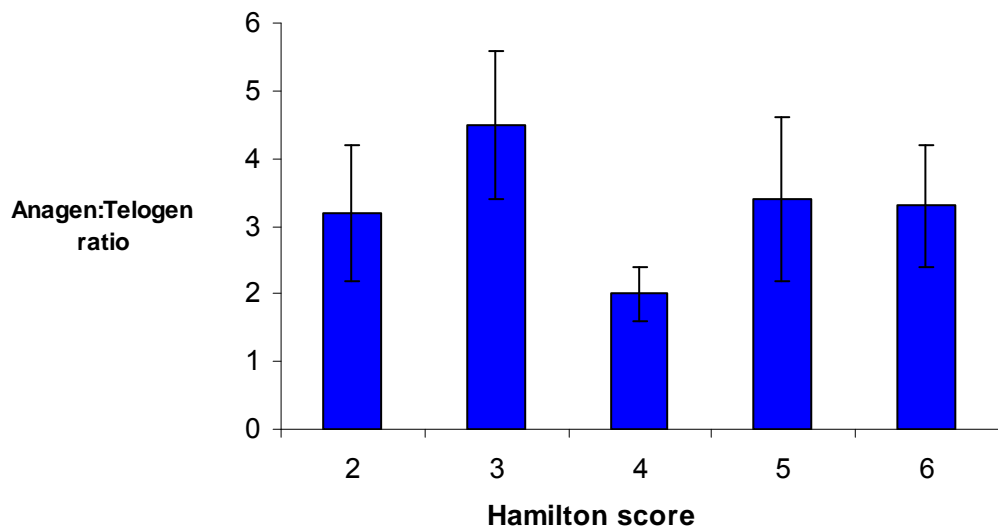
Figure 3.7

The relationship between extent of balding (Hamilton stage) and the anagen:telogen ratio.

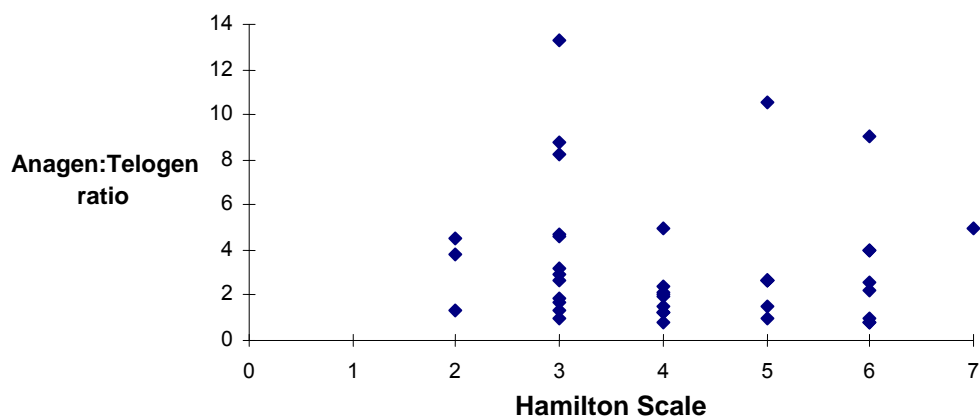
This includes pre-treatment data from 41 men who completed either the 3 month or 12 month experiments.

(a) Mean values (\pm SEM) for each Hamilton stage

Number of men aged 21-30=5, 31-40=13, 41-50=10, 51-60=7, 60+ =6



(b) Correlation of individual values with Hamilton stage. Pearson coefficient - 0.09, $p=0.60$



The relationship between subject age and total number of hairs is also shown in Figure 3.1b, correlation coefficient = -0.37 confirming a strong significant negative correlation ($p=0.02$) i.e. the total number of hairs per unit area decreased with age. The number of anagen hairs per template area (50.7 mm^2) on the vertex also decreased with age from a mean of 40.0 ± 5.4 at age 21-30 years, to 18.3 ± 4.2 over age 60. This correlation analysis is shown in Figures 3.2.a & 3.2.b and indicates a negative correlation, correlation coefficient = -0.30, although this result was not quite statistically significant ($p=0.06$). The change in number of telogen hairs with age was also examined. Subjects aged 21-30 years had a mean telogen hair count of 20.6 ± 1.4 and the men aged over 60 years had a mean telogen hair count of 3.8 ± 1.5 ; correlation coefficient -0.35, $p=0.03$. The number of telogen hairs showed a significant negative correlation with age.

Figures 3.3.a & 3.3.b show the absence of a relationship between age and anagen:telogen ratio. Although the mean anagen:telogen ratio was 2.0 ± 0.2 between 21-30 years and 4.6 ± 1.2 over age 60 years, there was no strong correlation between age and anagen:telogen ratio, correlation coefficient = -0.11, $p=0.51$. The overall mean anagen:telogen ratio was 3.4 ± 0.5 .

When the extent of balding as measured by the Hamilton stage was related to age there was a significant relationship (Figure 3.4). Men aged 21-30 years (Xiantene treated + control group) had a mean Hamilton stage 3.2 ± 0.4 , whilst that for men aged over 60 years (Xiantene treated + control group) was 4.7 ± 0.7 (see Figure 3.4.a). There was a significant positive correlation between age and Hamilton score, correlation coefficient = +0.35 (Figure 3.4.b) and $p = 0.03$, indicating that balding increased with age.

3.4.1.2. Relationship between hair parameters and extent of balding

The relationship between the extent of balding as measured by the Hamilton stage and the total number of hairs (Figures 3.5.a & 3.5.b), the number of anagen hairs (Figure 3.6.a & 3.6.b) and the anagen:telogen ratio (Figures 3.7.a & 3.7.b) was also investigated. The total number of hairs per circle (50.7 mm²) decreased from a mean of 55.3 ± 10.8 at Hamilton stage 2 to a mean of 19.3 ± 5.4 at Hamilton stage 6 with one individual at stage 7 having only 6 hairs (Figure 3.5.a) and was significantly negatively correlated, correlation coefficient -0.56 ($p < 0.001$) (Figure 3.5.b). Similarly the number of anagen hairs per circle decreased from a mean of 41.0 ± 10.4 at Hamilton stage 2 to a mean of 12.1 ± 2.6 at Hamilton stage 6 with the one individual at stage 7 having only 5 anagen hairs (Figure 3.6.a). Again there was a strong negative correlation with Hamilton stage, correlation coefficient -0.60 ($p < 0.001$, Figure 3.6.b).

In contrast, the anagen:telogen ratio did not change significantly from a mean of 3.2 ± 1.0 at Hamilton stage 2 to a mean of 3.1 ± 1.0 at Hamilton stage 6 (Figure 3.7.a) and there was no significant correlation, coefficient -0.09 ($p = 0.60$, Figure 3.7.b).

Therefore, the extent of balding (Hamilton stage) showed a significant negative correlation with the total number of hairs, a significant negative correlation with the number of anagen hairs and an insignificant weak negative correlation with the anagen:telogen ratio in this group of men with androgenetic alopecia. The strong negative correlation between extent of balding and both total number of hairs and the number of anagen hairs

indicates that as the Hamilton stage increases, the number of visible hairs decreases on the crown/vertex.

3.4.1.3 Comparison of pre-treatment data between the control and Xiantene treated men who completed the 12 month trial

The distribution of the initial ages of the men (n=29) who completed the 12 month trial, in each of the two groups, Xiantene and vehicle, are shown in Figure 3.8. The mean ages for the two groups: 47.3 ± 3.7 years for the Xiantene treated men (n=17); 46.8 ± 3.3 years for those receiving the vehicle (n=12) did not vary significantly (p=0.93) confirming the validity of the comparison between groups. The mean age of all subjects completing was 47.1 ± 2.5 years.

The distribution of the initial extent of baldness as determined by the Hamilton stage of the two groups, Xiantene and vehicle, are shown in Figure 3.9. The initial mean Hamilton stages for the Xiantene and control groups who completed the experiment, pre-treatment were 4.7 ± 0.3 for the Xiantene group and 4.0 ± 0.5 for the control. These do not vary significantly (p=0.21) confirming the validity of comparing the two groups. The mean Hamilton stage of all subjects completing the trial before treatment was 4.4 ± 0.3 .

The mean total hair density on the crown at the beginning of the trial was 61.4 ± 9.4 hairs/cm² in the Xiantene group and 82.7 ± 13.7 hairs/cm² in the control group before the experiment (see Figure 3.10). Although the mean total number of hairs/cm² in the Xiantene group pre-treatment were lower than the control group, this is not significantly different (p=0.20).

Figure 3.8

Age distribution at initial assessment of male subjects enrolled and completing the 12 month study investigating Xiantene treatment in androgenetic alopecia.

Men with androgenetic alopecia (n=48) were invited to enter a 12 month trial of Xiantene in the treatment of androgenetic alopecia. Half of the subjects in the control group used the vehicle (a) and the remainder of the subjects used Xiantene solution (b).

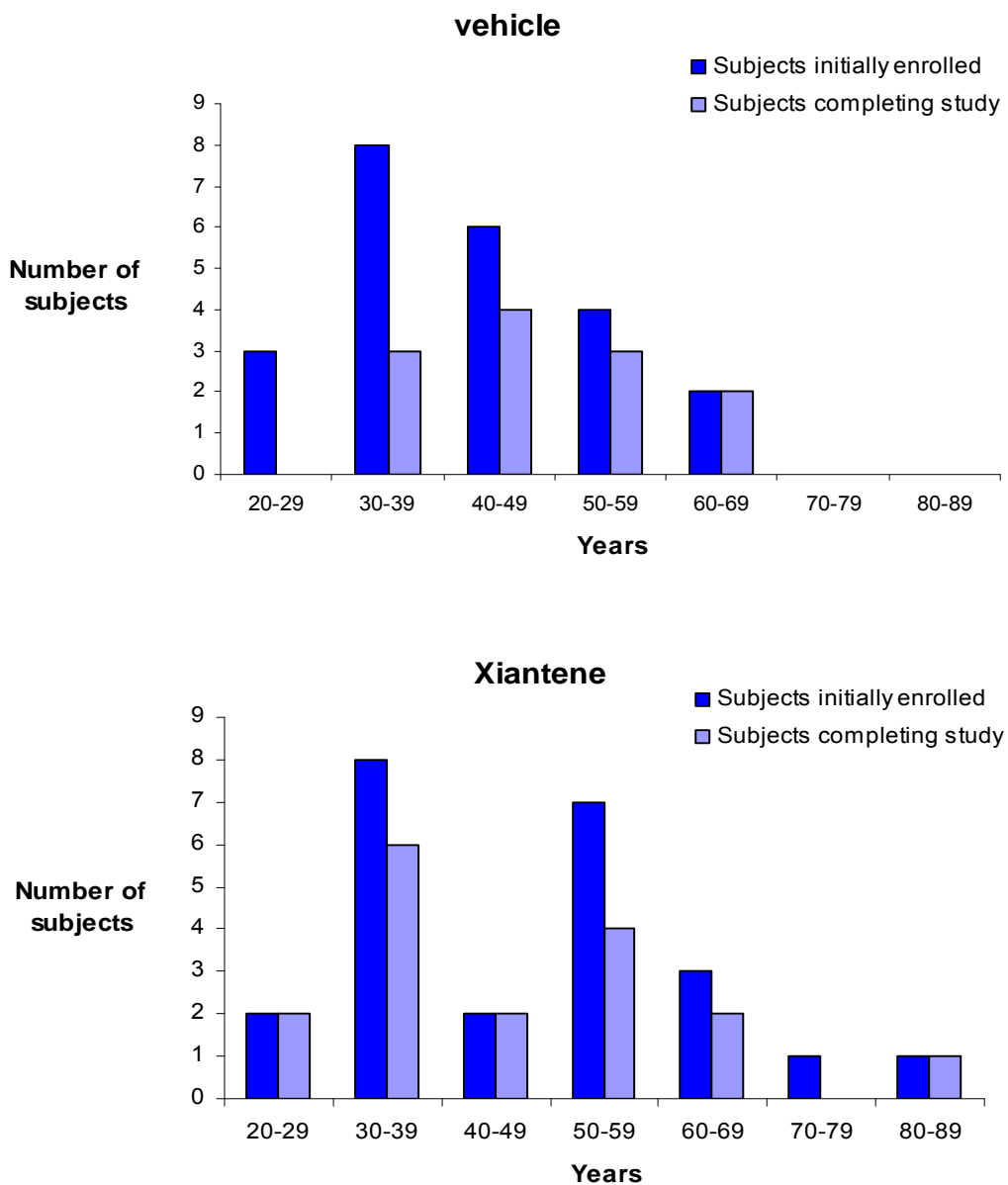


Figure 3.9

The initial extent of balding as indicated by the Hamilton stage of subjects enrolled and completing the 12 month study investigating Xiantene treatment in androgenetic alopecia.

Men with androgenetic alopecia (n = 48) were invited to enter a 12 month trial of Xiantene in the treatment of androgenetic alopecia. Half of the subjects in the control group used the vehicle (a) and the remainder of the subjects used Xiantene solution (b).

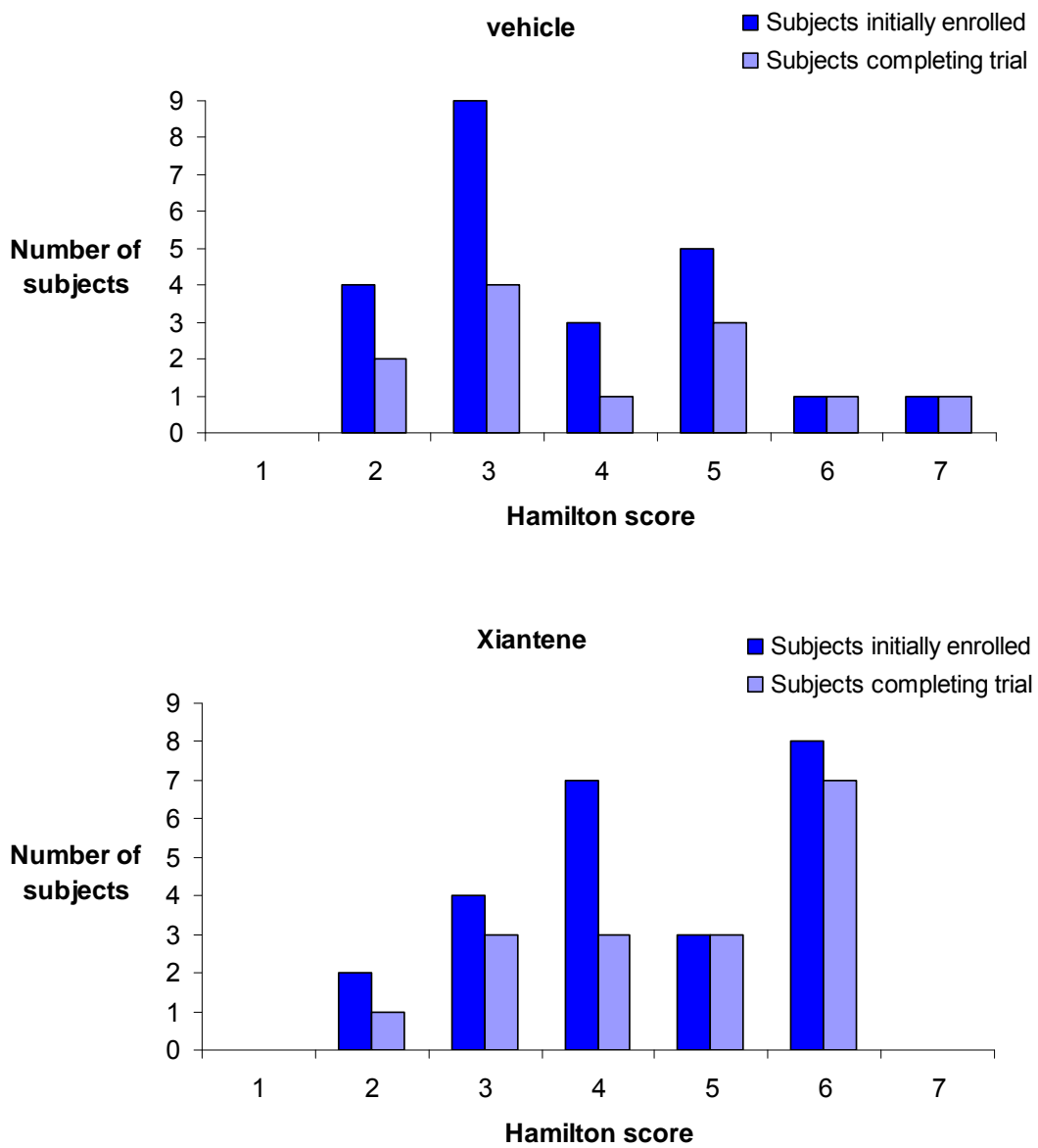
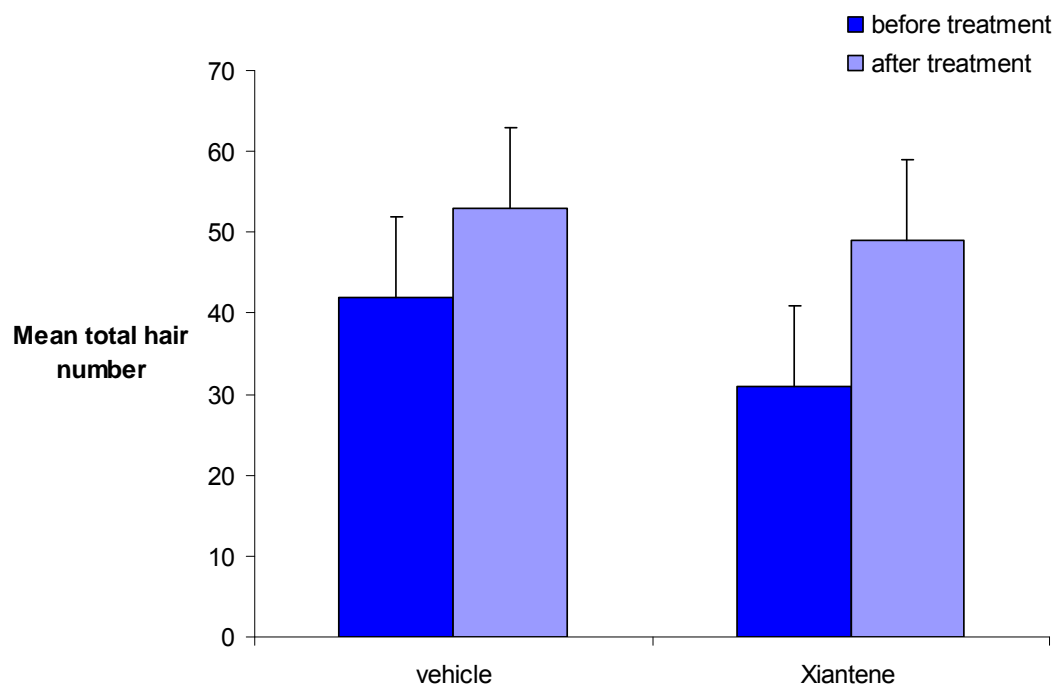


Figure 3.10

A comparison of the total number of hairs/circle in the Xiantene treated men and those using the vehicle before, and after, treatment.

Men with androgenetic alopecia used topical Xiantene (1ml) or the vehicle daily. All hairs were plucked from circles of 50.7 mm² area on the crown before, and after, 12 months treatment. Results are the mean \pm SEM of measurements for each subject before and after treatment involving 12 subjects for the vehicle and 17 subjects for the Xiantene treatment.



The mean number of anagen hairs pre-treatment in the specimen circle (50.7 mm²) in the two groups were significantly different with Xiantene treated men having 35.6 ± 4.5 cm² and control men 68.0 ± 11.1 cm², $p=0.005$ (Figure 3.11). Although the subjects were recruited and divided into groups on a random basis, the Xiantene group had a lower mean number of anagen hairs initially. However, the number of telogen hairs/circle before treatment was not found to be significantly different between groups with a mean for the Xiantene group of 13.2 ± 2.8 and the control group of 7.4 ± 1.8 ($p=0.13$). There was a significant difference in the anagen:telogen ratio between the two groups at baseline; Xiantene 2.2 ± 0.5 , control 5.5 ± 1.0 , $p=0.002$. Overall key mean parameters, age and Hamilton score, were not significantly different between the Xiantene treated and vehicle groups, confirming the validity of the comparison.

3.4.1.4 Comparison of control and Xiantene treated groups after 12 months treatment

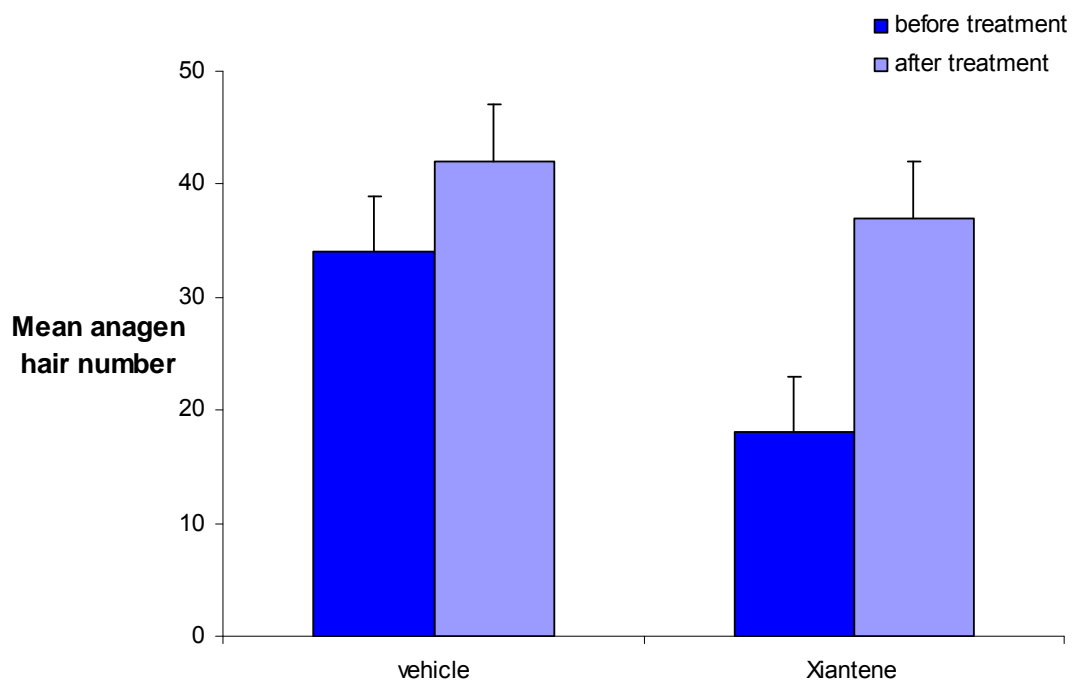
The forty eight subjects who commenced the trial were equally divided into treatment and vehicle groups. A total of 29 men were successfully re-examined, and samples collected from them at the end of the 12 month period. This included 17 men using Xiantene and 12 using the vehicle.

The drop out rate of 39.58% remained high, but was less in the Xiantene group, 29.2%, than the 50% of the control group.

Figure 3.11

Mean number of anagen hairs/circle in Xiantene treated men and those using the vehicle before, and after, treatment.

Men with androgenetic alopecia used topical Xiantene (1ml) or vehicle daily. All hairs were plucked from circles of 50.7 mm² area on the crown before and after 12 months treatment. Results are the mean \pm SEM of measurements for each subject before and after treatment involving 12 subjects for vehicle and 17 subjects for the Xiantene treatment.



Subject opinions

All subjects expressed satisfaction with the treatment at the end of trial discussion regardless of whether they were using Xiantene or the vehicle, and 4 subjects (3 using Xiantene, 1 vehicle), 13.79% of the total number (17.6% of Xiantene group and 8.3% of the vehicle group) spontaneously asked to continue treatment beyond the end of the experiment. These subjects specifically requested to continue the experiment even though no prior offer of continuation had been made. However (see Table 3.1), 3 subjects using Xiantene (10.35%) reported a slight reaction to the treatment, a mild erythema on the crown, and 2 subjects using Xiantene (6.90%) complained of the adverse smell of the treatment, and in one of the subjects where an adverse reaction was recorded, the reaction was severe enough for the subject to stop.

Age and extent of baldness

To see if age or extent of baldness influenced whether subjects enrolled in the study continued to the end, the age and Hamilton stages were compared between subjects who completed the 12 month trial (total 29) and the subjects who did not complete the trial (total 19) (Figure 3.8). In the control group treated with the vehicle alone the subjects that completed the experiment were significantly older (46.8 ± 3.3 years) than those who did not complete (35.6 ± 2.9 years), $p=0.02$. In the Xiantene group there was no significant difference in age, with the completing group mean age being 47.0 ± 3.7 years, while for those not completing it was 47.6 ± 6.0 years, $p=0.94$.

Table 3.1**Analysis of subjects dropping out of 12 month study**

In a 12 month study investigating 48 subjects using Xiantene or vehicle treatment in androgenetic alopecia, 19 subjects (39.57%) out of a total of 48 subjects commencing failed to complete the trial.

Reason	Number of subjects			% of Total Drop outs
	Total	Xiantene	vehicle	
Abandoned, adverse erythema reaction problem(uncorroborated)	1	1	\bar{x}	2.08
Dissappeared and could not contact	1	\bar{x}	1	2.08
Unrelated medical condition diagnosed	2	1	1	4.16
Unrelated disagreement	3	\bar{x}	3	6.25
Voluntarily stopped after 2 to 3 months ("Just sort of stopped")	12	5	7	25.00
	19 Subjects			39.57%

There was also no strong evidence of a difference in extent of baldness between those completing and those not completing the trial in either the Xiantene treated group and the control group (Figure 3.9). People in the control group completing the experiment had an initial mean Hamilton stage of 4.0 ± 0.3 , and those not completing of 3.4 ± 0.3 , ($p=0.27$), while the Xiantene group completing had a mean of 4.8 ± 0.3 and those not completing of 3.7 ± 0.5 , ($p=0.08$). However, when both groups were combined together (i.e. Xiantene treated and control) there was a significant difference in extent of baldness with those completing having a higher mean Hamilton score of 4.5 ± 0.3 , whilst that for those not completing was 3.5 ± 0.3 , ($p=0.02$), i.e. those who chose not to complete the 12 month trial were significantly less bald initially regardless of treatment given to them.

Total number of hairs per measured area

The mean total number of hairs per area measured (50.7 mm^2) before treatment 31.1 ± 4.8 , increased significantly after treatment to 49.2 ± 3.8 for the Xiantene group ($p=0.003$) which would suggest that Xiantene had increased the number of visible hairs in the 12 month period. The control group also showed an increase in the total number of hairs from 41.9 ± 7.0 before the study and 53.1 ± 3.9 after, but this was markedly less than the Xiantene group (see Figure 3.10) and was not significant ($p=0.06$).

Number of anagen hairs

The number of anagen hairs in the template area (50.7 mm^2) for both groups are shown in Figure 3.11. The mean number of anagen hairs for the group

using Xiantene increased very significantly after treatment from a mean of 18.1 ± 2.2 to 37.3 ± 3.3 ($p < 0.001$). The value for the control group increased slightly but this was not significant being 34.5 ± 5.6 before treatment and 42.4 ± 2.6 after treatment ($p = 0.17$). Treatment with Xiantene had significantly increased the number of anagen hairs unlike the vehicle treatment.

Number of telogen hairs

The number of telogen hairs in the template area (50.7 mm^2) was also measured; in the Xiantene group there was a mean of 13.2 ± 2.8 telogen hairs before treatment, falling insignificantly to 11.9 ± 0.9 afterwards ($p = 0.61$). The number of telogen hairs increased slightly, but insignificantly, for the control group after 12 months treatment from 7.4 ± 1.8 before to 10.7 ± 2.7 after ($p = 0.10$). There was no significant change in the number of telogen hairs for the Xiantene treated or the control group.

Anagen:telogen ratio

Treatment with Xiantene had increased the total number of hairs in the template area (50.7 mm^2) and the number of anagen hairs, but it had no effect on the number of telogen hairs. Conversely, treatment with the vehicle had not significantly increased the total number of hairs in the template area (50.7 mm^2), nor had it significantly increased the number of anagen hairs, and the number of telogen hairs had not significantly altered.

Therefore, the mean anagen:telogen ratio also increased significantly by over 50% for the Xiantene group from 2.2 ± 0.5 to 3.4 ± 0.4 ($p = 0.005$). In

contrast, the anagen:tologen ratio for the vehicle group showed a small, but insignificant decrease from 5.5 ± 1.0 to 5.0 ± 1.0 ($p=0.68$) (see Figure 3.12).

Photographic assessment

A subjective assessment of hair growth was made with head shot photographs, before and after treatment (Table 3.2) using a standardised procedure (See chapter 2).

Three independent assessors, excluding the author, viewed before and after head shot photographs and scored each pair of photographs as either; increased hair growth, no change in hair growth or reduced hair growth. Scores between the three assessors were recorded and then discussed before a consensus was reached. The final photographic assessments are shown in Table 3.1. Unfortunately the photographic record was not complete due to loss of a roll of film and it was only possible to compare 14 subjects, 6 using Xiantene and 8 using vehicle. Using 3 independent assessors evaluating on a blind basis, the paired photographs were scored as follows: increased, no change or decreased (Table 3.2).

In the Xiantene group 3 subjects (50%) were scored as having increased growth and 3 subjects (50%) as showing no change, whereas in the vehicle group 3 subjects (37.5%) scored as showing increased growth and 5 subjects (62.5%) no change.

Reproduceability of this scoring system was not tested and it is not possible to confirm the reliability of these results. Sample photographs are shown in Figure 3.13 (Xiantene, improvement) and Figure 3.14 (vehicle, no change).

Figure 3.12

Mean anagen:telogen ratio, in Xiantene treated men and those using the vehicle, before and after treatment.

Men with androgenetic alopecia used topical Xiantene (1ml) or vehicle daily. All hairs were plucked from a circle of 50.7 mm² area on the crown before and after 12 months treatment. Results are the mean \pm SEM of measurements for each subject before and after treatment involving 12 subjects for the vehicle and 17 subjects for the Xiantene treatment.

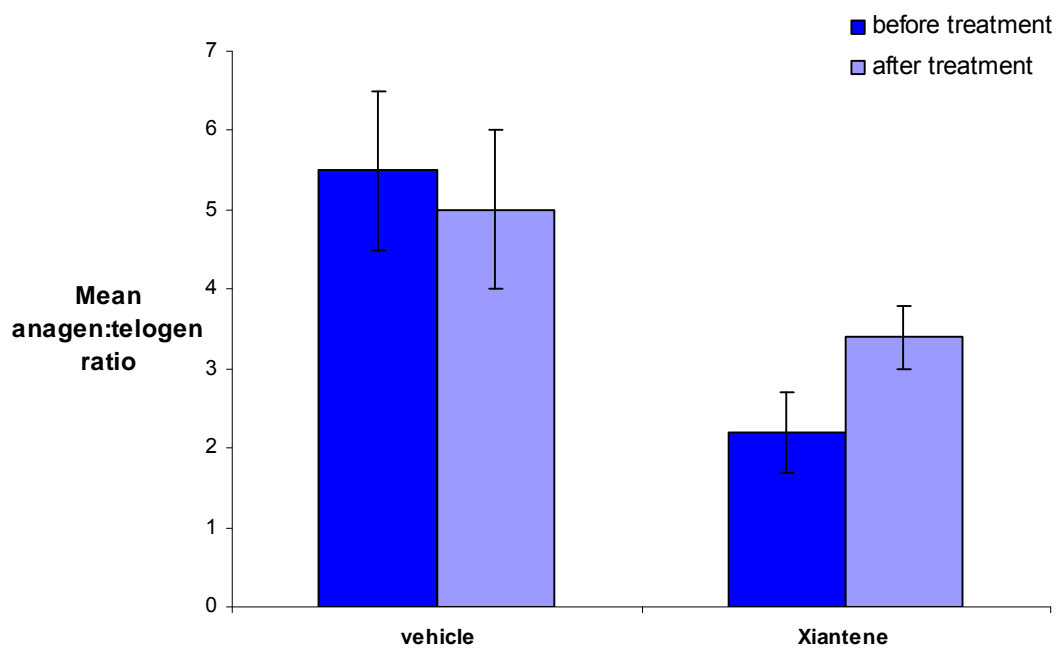


Table 3.2

Hair growth assessment score of head shot photographs, before and after 12 month treatment.

Men with androgenetic alopecia used topical Xiantene (1ml) or vehicle daily.

	Increased hair coverage		No change		Decreased hair coverage	
	Number	%	Number	%	Number	%
Xiantene	3	50	3	50	0	0
Vehicle	3	37.5	5	62.5	0	0

Figure 3.13

Increased hair growth over a 12 month period for a subject using Xiantene treatment.

Subject with androgenetic alopecia used Xiantene (1ml) daily for 12 months (scored, increased hair coverage).



Figure 3.14

Absence of hair growth over a 12 month period for a subject using vehicle treatment.

Subject with androgenetic alopecia used vehicle (1ml) daily for 12 months (scored, no change).



3.4.1.5 Results addendum

It has subsequently been suggested (Dr. D.H. Rushton, personal communication) that the results of this trial may be open to a different statistical interpretation (see Appendix V) and that there was no significant increase in the total number of hairs of the Xiantene treated group compared with controls. Clearly further investigation is required and the conclusions drawn therefore represent this uncertainty.

3.5 Discussion

3.5.1 Analysis of pre treatment data in men with androgenetic alopecia

The pre-treatment data for 41 volunteer men suffering from androgenetic alopecia in the 3 month (chapter 2) and 12 month (chapter 3) studies were combined and then analysed to investigate the relationships between the total number of hairs, the number of hairs in anagen and the anagen:telogen ratio to both age and extent of baldness as indicated by the Hamilton stage. This data related to 41 men with a mean age of 44.7 ± 2.1 years, all suffering from androgenetic alopecia, with Hamilton stages ranging from 2-7, mean 4.2 ± 0.2 . There was a significant negative correlation between age and the total number of hairs per specimen circle on the crown ($p=0.02$) (Figure 3.1), with the mean total number of hairs/ circle for men aged 21-30 years, being much higher at 61.0 ± 6.4 than the 22.2 ± 5.3 for men aged over 60 years. This decrease with age in men with androgenetic alopecia confirms earlier work (Hamilton, 1951; Rushton et al, 1983; Kaufman et al, 2008).

The number of anagen hairs/circle on the crown also declined with age with men aged 21-30 years having a mean of 40.4 ± 5.4 per circle area falling to

18.3 ± 4.2 in men aged over 60 years, although this negative correlation (correlation coefficient = -0.30) was not quite statistically significant (p=0.06) (Figure 3.2). The decrease in the total number of hairs and the number of anagen hairs with age confirms earlier research for men with androgenetic alopecia (Hamilton, 1951; Rushton et al, 1983; Bergfeld, 1989; Kaufman et al, 2008) and confirms that baldness increases with age in susceptible individuals. There was also a positive correlation (p=0.03) between age and extent of baldness, as indicated by the Hamilton stage, correlation coefficient +0.35 (see Figure 3.4); men aged 21-30 years had a lower mean Hamilton stage of 3.2 ± 0.4 than men over 60 years who had a mean Hamilton stage of 4.7 ± 0.7. Therefore the extent of baldness was shown to increase with age, while the total number of hairs and the number of anagen hairs decreased with age. This confirms earlier work (Hamilton, 1951; Rushton et al, 1983; Kaufman et al, 2008) and the age related nature of the condition. There was no evidence of an association between age and anagen:telogen ratio (Figure 3.3) which might have been expected as it has been reported earlier (Bergfeld, 1989). The inability to show a relationship between the anagen:telogen ratio and age in men with androgenetic alopecia suggests a more advanced stage of balding at the start of the experiment, than in those reported by Bergfeld (1989) who found that less than 20% of hair follicles in telogen was normal, i.e. the anagen:telogen is above 4:1 in a normal scalp not affected by androgenetic alopecia. This has also been reported in studies of percentage anagen on normal male scalp throughout the year (Randall & Ebling, 1991). The association between extent of baldness measured by the Hamilton stage and the parameters measured was also examined. There

was a significant negative correlation between both the total number of hairs/unit area, $p < 0.001$ (see Figure 3.5); and the number of anagen hairs ($p < 0.001$) (see Figure 3.6) with Hamilton stage. The total number of hairs per template area (50.7 mm^2) was 55.3 ± 10.8 at Hamilton stage 2 falling to only 19.3 ± 5.40 at Hamilton 6, while the number of anagen hairs was 41.0 ± 10.4 at Hamilton stage 2, decreasing to 12.1 ± 2.6 at Hamilton stage 6. The anagen:telogen ratio also showed no strong correlation with Hamilton stage, which would have been expected (Bergfeld, 1989). However, the mean anagen:telogen ratio for all subjects is low at 3.4 ± 0.5 and below the alopecia threshold of 4:1 (Bergfeld, 1989) confirming that the subjects were suffering from androgenetic alopecia. The anagen:telogen ratio for a healthy young male not suffering from androgenetic alopecia has been suggested to be 10:1 (Bergfeld, 1989) though this does not seem theoretically appropriate as hair follicles do cycle; others have suggested 9:1 – 8:2 with changes throughout the year (Randall & Ebling, 1991). While in cases of advanced androgenetic alopecia is stated to be below 4:1 and this ratio may reverse (Bergfeld, 1989), i.e. there may be more telogen hairs present than anagen hairs.

3.5.2 The effect of herbal mixture Xiantene on hair growth when applied topically for 12 months

The subject drop out rate of 39.58% for this 12 month study was high (see Table 3.1), although an improvement on that in the 3 month study (see Chapter 2) where the drop out rate was 65.70%. Other researchers have found a lower drop out rate of approximately 16% after 12 month period

(Rushton et al, 1991) but rising to 64% after 24 months. Interestingly, in the 12 month study the number of subjects dropping out was not equally divided between the two treatment groups. It was only 29.20% in the Xiantene group while reaching 50.00% in the control group, which may indicate more enthusiasm for the Xiantene group to continue treatment, possibly because they were noticing an effect. In the control group, the subjects that completed the trial were significantly older (mean age 46.8 ± 3.3 years) than those who did not (35.6 ± 2.9 years, $p=0.02$), but there was no significant difference in age for the Xiantene treated group ($p=0.94$); between those completing (47.0 ± 3.7 years) and not completing (47.6 ± 6.0 years). The people, both Xiantene treated and controls who completed the trial were also significantly more bald (mean Hamilton stage 4.5 ± 0.3), than those who did not complete (3.5 ± 0.3 , $p=0.02$). Interestingly, this confirms the observation from the earlier experiment (Chapter 2) that younger men and men with less advanced hair loss were more likely not to complete the experiment. The mean initial ages of the two groups completing the trial were similar being 47.3 ± 3.7 years for the Xiantene group, and 46.8 ± 3.3 years for the control, ($p=0.93$). These are significantly higher than the ages of the subjects completing the 3 month initial study where the mean for the Xiantene group was 37.0 ± 5.0 years, and for the control group was 40.0 ± 6.0 years (see chapter 2). The mean initial Hamilton hair loss stages for both groups were also similar, with that for the Xiantene group being 4.70 ± 0.3 with 4.00 ± 0.5 for the controls ($p=0.21$). The mean Hamilton scores are higher than the earlier 3 month study (see chapter 2) where the mean for the Xiantene group was 3.8 ± 0.3 and that for the control group 3.7 ± 0.3 ($p=0.65$). The absence

of any significant differences between age and Hamilton stage in individuals completing the study assigned to either the Xiantene or control groups confirms the validity of the comparison.

The random recruiting of volunteers appears to have attracted older men with more advanced hair loss. The ideal for testing a hair loss treatment product would be a younger and less advanced balding group, where subjects were aged no more than 35 years with the extent of baldness no more than Hamilton stage III. This is the approach which is used by pharmaceutical companies who have the financial resources to carry out extensive trials e.g. Merck & Co (Kaufman et al, 1998). The ability to stabilise or partially reverse genetic hair loss is probably easier with a younger subject group with less advanced hair loss. Possible inflammation and scarring below the long term balding follicle has been observed (Jaworsky et al, 1992; Whiting, 1993) and the resultant reduced or truncated nerve endings and blood supply (Chapter 1) leads eventually to follicular loss. Such inflammation etc would potentially prevent an affected follicle regrowing deeper into the dermis to produce a larger hair. The unit area trichogram method used here and established by Rushton et al (1983) is mechanical and precise, the plucked hairs being permanently recorded for repeat assessment at a later date. However, the technique suffers from the disadvantage of a very small sample area being used in an alopecic area (the crown), where the degree of hair loss may not be gradual or linear with possible abrupt hair density changes between different areas of the crown; a step-change mechanism for androgenetic alopecia has been proposed (Whiting, 2001). The measurement of hair density per cm² in this experiment

is lower than other reported measurements for men suffering from androgenetic alopecia using this technique (Rushton et al, 1983). Mean total hair density per cm² at the beginning of the trial was found to be 61.4 ± 9.4 /cm² in the Xiantene group and 82.7 ± 13.7 /cm² in the controls compared with 219 ± 8 /cm² on the occipital area and 175 ± 6 /cm² on the frontal area of men with androgenetic alopecia found by Rushton et al (1983) indicating a more advanced hair loss stage in this study group; the data reported by Rushton's group also came from a younger group (mean age 25.7 years). Men treated with Xiantene demonstrated a highly significant greater total number of hairs/unit area (p=0.003) increasing by about 50% after 12 months, unlike the controls where there was a slight, but insignificant increase (p=0.06) (Figure 3.10). The Xiantene group also showed a very highly significant increase in the numbers of anagen hairs/circle of approximately 100%, (p<0.001), while the control group exhibited only an insignificant increase (p=0.17) (Figure 3.11). However, there was no significant change in the number of telogen hairs over the year for either group. Consequently, the anagen:telogen ratio was very significantly increased for the Xiantene group by over 50% from 2.2 ± 0.5 to 3.4 ± 0.4 (p=0.005), while that for the control group was not significantly altered (p=0.68) (Figure 3.12).

In summary, all 3 parameters measured, the total number of hairs, the number of anagen hairs and the anagen:telogen ratio increased significantly for the Xiantene treated group, but there was no significant change in any of these 3 parameters in the controls. These changes are not due to any seasonal changes (Orentreich, 1969; Randall & Ebling, 1991; Courtois et al,

1996) as each individual was assessed after 12 months to avoid this. Interestingly, the subjects in the control groups appeared to have maintained their hair growth on the vertex over the 12 month period, which was surprising as all subjects were suffering from androgenetic alopecia, and some deterioration might have been expected (Rushton et al, 1991; Van Neste, 2003; Kaufman et al, 2008). Unfortunately the photographic assessment designed to support the measurements was not successful due to the loss of some photographs meaning that the photographic record is not complete. However, the apparent unchanged or improved hair growth in both the Xiantene and control photographs is encouraging as it parallels the measured data, but this photographic data cannot be relied upon. Other workers have reported photographic assessment of hair growth has been shown not to be necessarily reliable (Van Neste, 2004), though the technique is frequently used to assess hair growth treatment. The insignificant increase in the total number of hairs/circle of the vehicle treated men may be a 'placebo effect' (Van Neste, 2008). A placebo effect is well documented (Koshi & Short, 2007; Postkowska – Nadolska, 2007) to produce a positive measurable physiological effect e.g. reduced hair fall, improved hair regrowth, although no real treatment has been undertaken, only the suggestion of treatment. The willingness of the subject to believe in the treatment, and the real desire to produce a response, can in fact produce a response and Kaufman (2008) reported increased hair growth with placebo treatment even after 3 years during a 5 years trial. Ironically, a 'nocebo' effect has been proposed, in which clinically proven drugs failed to work as well as planned, or produced unexplained side effects (Olshansky, 2007). In

contrast to the placebo effect where the patient's health improves because they believe the treatment is going to work, with the nocebo effect the patient's condition worsens with treatment and the suggestion of sickness creates more sickness. Trials using patients with a history of adverse reaction to drugs found that 27% of the subjects experienced an adverse reaction with the placebo (Eccles, 2007). A similar trial using finasteride for the treatment of benign hyperplasia found a 31% nocebo effect, in increased sexual side-effects, compared with a another group who received treatment, but were not told about the possible side-effects (Mondaini et al, 2007). It is not suggested that androgenetic alopecia, which is androgen dependent and genetically influenced (Hamilton, 1942), is a psychosomatic illness, but the data suggests a possible placebo effect. Differing rates of the progression of androgenetic alopecia in cases of identical male twins has been recorded (Roenigk & Kuruvilla, 1987; Stough et al, 2002; Stough, 2007) and a brain – hair follicle 'axis' has been proposed (Arck et al, 2001) with substance P (SP) and nerve growth factor (NGF) as mediators of stress induced hair growth inhibition (Peters et al, 2006). Further, loss of proliferative ability of balding dermal papilla cells in cases of androgenetic alopecia has been associated with oxidative stress and environmental factors (Bahta et al, 2008). The validity of the results from the 12 month study are greater than the short preliminary investigation (Chapter 2) as the numbers of individuals are greater and the 12 months period avoids any difficulties with changes occurring through the year due to seasonal variation (Orentreich, 1969; Randall & Ebling, 1991; Courtois et al, 1996). Nevertheless, the results of the 3 month (chapter 2) and 12 month experiments concur. A significant increase

in the total number of hairs/unit area and a significant increase in the number of anagen hairs/unit area was seen on the vertex in both the 3 month and the 12 month experiments. There was no corresponding significant increase in hair parameters for the control group, in either the 3 month or the 12 month experiment. These two studies each used a different set of subjects and were not run concurrently. Daily topical application of herbal mixture Xiantene significantly increased hair growth in a total of 23 men with androgenetic alopecia with no corresponding increase on the 18 men applying only the vehicle. There appeared to be no observable difference in efficacy between Xiantene I and Xiantene II (see Chapter 4) as both produced a measurable hair growth effect compared with control. The significant increase in hair number on the vertex in both the 3 month and the 12 month studies in the Xiantene treated men suggests that Xiantene may have stimulated the growth of small vellus hairs to produce larger hairs that can be plucked i.e. an increase in the total number of meaningful hairs (Rushton et al, 1993) or that it has increased the number of hairs entering anagen from follicles in the 'lag' phase i.e. with empty follicles where the telogen hair has been shed via exogen (Stenn et al, 1996) and the new anagen hair not yet started to project above the surface (Whiting, 2001; Rebora, 2004). The significantly increased percentage of hairs in the anagen phase, whilst the number of telogen hairs was unchanged in both studies, concurs with both hypotheses. Other researchers (Van Neste, 2006) have found that the reduction in the duration of the lag phase of up to 40% was the key mechanism during reversal of male androgenetic alopecia by finasteride. They also observed a 23% increase in the length of the anagen phase for

thick hairs ($>40\mu\text{m}$), although not for thinner hairs ($<40\mu\text{m}$), but there was no clear evidence for reversal of miniaturised hair into terminal hair. The anagen:telogen ratio for the Xiantene group has increased significantly ($p=0.005$; see Figure 3.12) contrasting with the control group where there was an insignificant decrease and a deterioration would be expected to occur over a 12 month period on the scalp of men with androgenetic alopecia (Van Neste, 2003; Kaufman et al, 2008). The increase in length of the anagen phase is important in assessing a possible treatment in cases of androgenetic alopecia which is characterised by a gradual contraction of the anagen phase and eventual miniaturisation of the hair follicle (Whiting, 2001). These observations suggest that Xiantene could promote a cessation or partial reversion in the progression of androgenetic alopecia. It has been suggested that even a medication capable of maintaining the existing hair population in the progression of androgenetic alopecia should be considered as effective (Rushton et al. 1991). However, the results with Xiantene suggest that these herbal extracts may be effective, in controlled experiments, in the short term reversal of androgenetic alopecia, even in older men (mean age 47.1 ± 2.5 years) with later stages of hair loss than those normally studied (mean Hamilton stage 4.4 ± 0.3). The mode of action of the Xiantene mixture is not clear and should be the subject of further research.

3.6 Conclusions

These results suggest that the combination of plant extracts used in Xiantene may or may not be a suitable agent for the topical treatment of androgenetic alopecia and that further work will be required.

4. Analysis of herbal mixture Xiantene

4.1 Aim

The aim of this study was to understand the components of Xiantene mixtures I & II. In particular to check whether the extracts claimed were actually present, determine whether the two formats differed and to confirm that *Radix Stephaniae Tetrandrae* was not present in Xiantene II. *Stephania tetrandra* S. Moore and a number of similar alkaloids have been banned from use in the UK by the Medecine & Health regulatory authority (MHRA) from 2004, as a common toxic adulterant *Aristolochia* (Fang Chi) can not be reliably distinguished from *Stephania tetrandra* S. Moore (Fang Ji). The use of *Aristolochia* led to an outbreak of kidney failure at a slimming clinic in Belgium (Evans et al, 2004).

4.2 Herbal components of Xiantene

The herbs used in the preparation of Xiantene are known and are summarised in Table 4.1 and shown in Figures 4.1 and 4.2. There is no standard nomenclature for herbal products and the systems that are in use are often not concise. The nomenclature adopted here is the “Pin Yin” system which is in common usage for the TCM products (Dunmu & Jiangbe, 1995). However even this is not absolutely precise, as the Pin Yin names sometimes cover more than one species. The Latin botanical names have been included but these differ from the pharmaceutical names, also principally in Latin. These are also shown in Table 4.1.

Table 4.1**Active components* of herbal mixtures Xiantene I and II.**

Pin Yin name	Part used	Latin scientific name (= botanical scientific name)	Pharmaceutical name	English name
Ce Bai Ye	Leaves and twigs	<i>Platycladus orientalis</i> L. (synonym is <i>Thuja orientalis</i> L.)	<i>Cacumen Platycladi</i>	
Bai Zi Ren	Seeds	<i>Platycladus orientalis</i> L. (synonym is <i>Thuja orientalis</i> L.)	<i>Semen Platycladi</i>	
He Shou Wu	Root tuber, not prepared	<i>Fallopia multiflora</i> (Thunb.) Haralds. (synonym = <i>Polygonum multiflorum</i> Thunb.)	<i>Radix Polygoni Multiflori</i>	Fleece Flower root
Ren Shen	Root	<i>Panax ginseng</i> C.A.Mey	<i>Radix et Rhizoma Ginseng</i>	Ginseng
San Qi	Root	<i>Panax notoginseng</i> (Burk.) F.H.Chen	<i>Radix et Rhizoma Notoginseng</i>	
Gui Zhi	Twigs	<i>Cinnamomum cassia</i> Presl	<i>Ramulus Cinnamomi</i>	Cassia twig
Da Qing Ye	Leaves	<i>Isatis indigotica</i> Fort.	<i>Folium Isatidis</i>	Dyers Woad leaf
Fang Ji**	Root	<i>Stephania tetrandia</i> S. Moore	<i>Radix Stephaniae Tetrandrae</i>	

*Clarification of nomenclature by Dr. Chris Leon of Royal Botanical Gardens, Kew, is gratefully acknowledged

** Fang Ji (*Stephania*) was claimed to be in Xiantene I but was subsequently banned for use in EEC countries and was not therefore in the list of components for Xiantene II

Figure 4.1

Typical herbs Ce Bai Ye, Bai Zi Ren, He Shou Wu and Ren Shen photographed with a 50 pence coin (width 27mm) to show scale (photos, author)

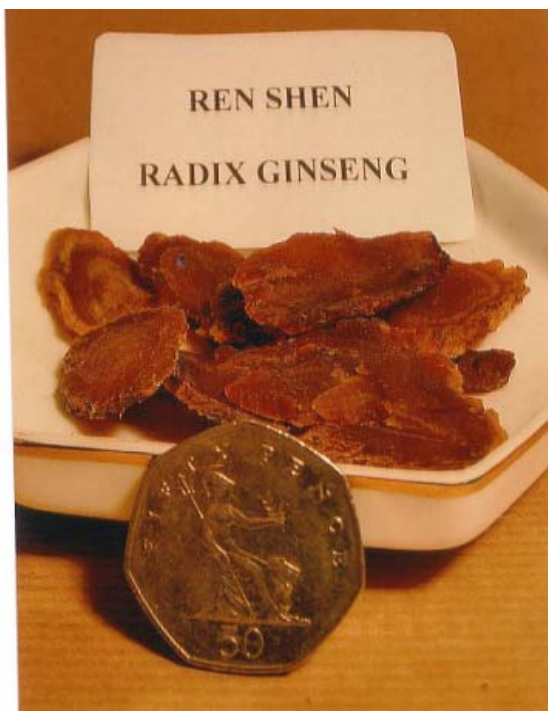
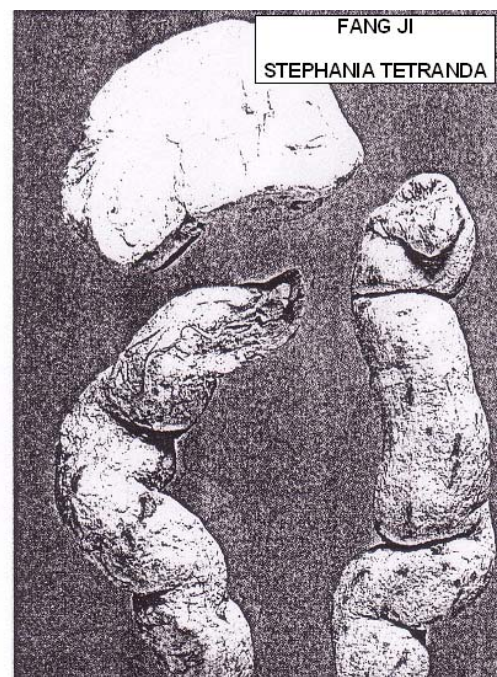
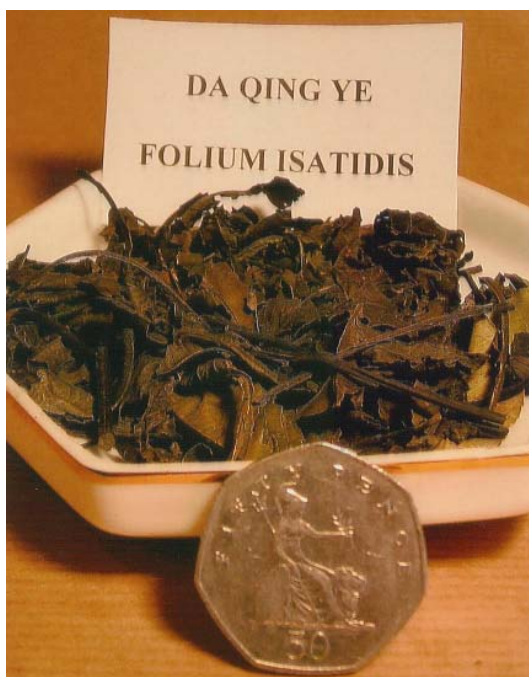
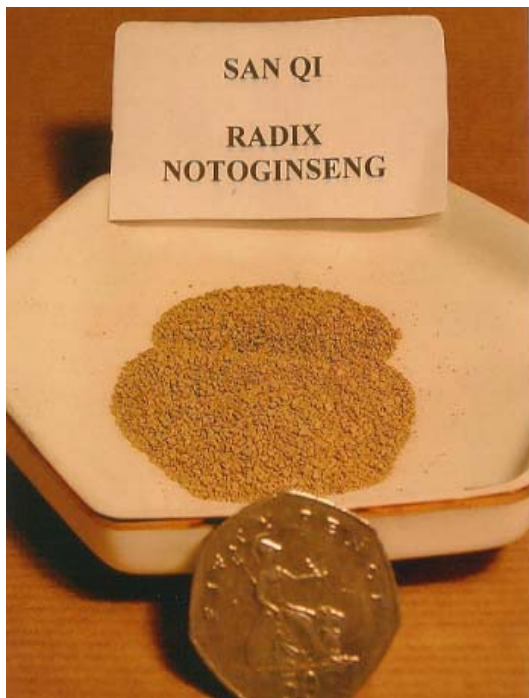


Figure 4.2

Typical herbs San Qi, Gui Zhi, Da Qing Ye. photographed with a 50 pence coin (width 27mm) to show scale (photos, author) and sketch of Fang Ji (Dunmu & Jiangbo, 1995).



The standardisation of TCM products is complex (Lu et al, 2007) as the chemical composition of the extract will depend on the right plant being used (most TCM plants are not cultivated but collected in the wild), the plant being harvested at the right time of the year, the area in which the plant is grown and the right part of the plant being used.

The parts of the plants used in the preparation of Xiantene varied and include; roots, twigs, leaves, bark and seeds and are indicated in Table 4.1 and photographs are shown in Figures 4.1 and 4.2.

The standardisation of TCM plants and extracts is being developed at several centres including the Institute of Health Research and Policy, London Metropolitan University, London and the “Chinese Medicinal Plants Authentication and Conservation Centre” (CMPAC) at the Royal Botanical Gardens, Kew, Surrey using DNA fingerprinting, chemical analysis and gross morphological identification (Dr. Chris Leon, personal communication).

The typical herbs used in Xiantene are shown in Figures 4.1 and 4.2 and described in Table 4.1. Descriptions are based on Zhao (2004):-

Ce Bai Ye

(Leaves of *Thuja orientalis* L.)

Description Leaves opposite, flat, small scaly, imbricate and closely attached to twigs.

Colour: dark green, brownish green to yellowish green.

Odour: fresh, fragrant (aromatic/cedar)

Distribution *Thuja orientalis* L. is found in Eastern China, Hong Kong, India and Japan (Zhao, 2004)

Components *Thuja orientalis L.* leaves contain an essential oil consisting of L-borneol, bornyl acetate, α -thujone, camphor, sesquiterpene alcohol. The leaves also yield phodozanthin, amentoflavone, quercetin, myricetin, carotene, xanthophyl and ascorbic acid (Bisset, 1999; Zhao, 2004)

Bai Zi Ren

(Seeds or kernel of *Thuja orientalis L.*)

Description Two seeds per scale, ovoid, oblong and highly oleaginous.

Tip slightly pointed, orbicular and triangular.

Colour: pale yellow, yellowish white or yellowish brown.

Odour: mildly aromatic

Distribution *Thuja orientalis L.* is found in Eastern China, Hong Kong, India and Japan (Zhao, 2004)

Components The seeds of *Thuja orientalis L.* contain as a major constituent an essential oil, mainly cedrol (Zhao, 2004)

He Shou Wu

(Root of *Polygonum multiflorum Thunb.*)

Description Root tuberous, polyhedral, ligneous and rough surface.

Texture: solid and hardly broken.

Colour: externally dark brown, reddish brown or black.

Odour: none

Distribution East and South China, Hong Kong, Japan and Vietnam (Bisset, 1990; Zhao, 2004)

Components Rhein, chrysophanic acid, polygonic acid, steroidal saponins and rhaponticin (Bisset, 1990; Zhao, 2004)

Note: *Polygonum multiflorum Thunb.* root may be used “not prepared” as used in this study or “prepared” (root is sliced steamed with black bean juice and dried) (Zheng, 2000).

Ren Shen

(Root of *Panax ginseng*)

Description Root tuberous short and fleshy, producing a branch root from its middle.

The surface is wrinkled or furrowed.

Colour: greyish-yellow.

Odour: characteristic, fragrant

Distribution North China, Japan, Korea and Soviet Union (Han, 1989; Zhao, 2004)

Components Triterpenoid saponins (Saponins A, B, C, D & F), oleanolic acid, amino acids, leucine, valine, proline oxyproline, histidine, lysine and cysein (Bisset, 1990; Zhao, 2004)

San Qi

(Root of *Panax notoginseng*)

Description Main roots tuberous, short, cylindrical and fleshy.

Surface wrinkled and furrowed.

Colour: pale greyish-yellow or greyish brown

Odour: characteristic, sweet

Distribution Mountainous regions of N. E. China, Nepal, India, Tibet and the forested slopes of The Himalayas (Zhao, 2004)

Components Major constituents are saponins (saponins A, B, C, D, F, G & H), notoginsenosides, ginsenosides, oleanolic acid and amino acids (Bisset, 1990; Zhao, 2004)

Gui Zhi

(Twigs of *Cinnamomum cassia presl*)

Description Long cylindrical twigs with many branches and marked with irregular, fine wrinkles

Texture: hard, fragile and easily broken

Colour: greyish-brown to reddish-brown

Odour: distinctive, aromatic

Distribution Southern China, Laos and Vietnam (Zhao, 2004)

Components Twigs contain mainly cassia oil, coumarin and cinnamic aldehyde (Bisset, 1990; Zhao, 2004)

Da Qing Ye

(Leaves of *Isatis indigotica Fort.*)

Description Leaves are mainly rolled and fragile with upper surface and underneath glabrous slightly undulate, plume shaped and web-like veins.

Colour: dark greyish-green or brown

Odour: pleasant, mildly aromatic

Distribution Central China (Zhao, 2004)

Components Main constituents are indigo derivatives, indigotin, isatan B and indirubin (Ferreira, 2004; Zhao, 2004)

Fang Ji

(Root of *Stephania tetrandia* S. Moore)

Description Irregularly lump-shaped or semi-cylindrical tuber with transverse channels and “knobbly” at the curved part with a heavy solid texture.

Colour: greyish-yellow

Odour: slight

Distribution Central China (Zhao, 2004)

Components Main constituents are alkaloids L-tetrahydro-palmatine, stepharine, roemerine & cycleanine (Bisset, 1990)

4.3 Method of manufacture of Xiantene

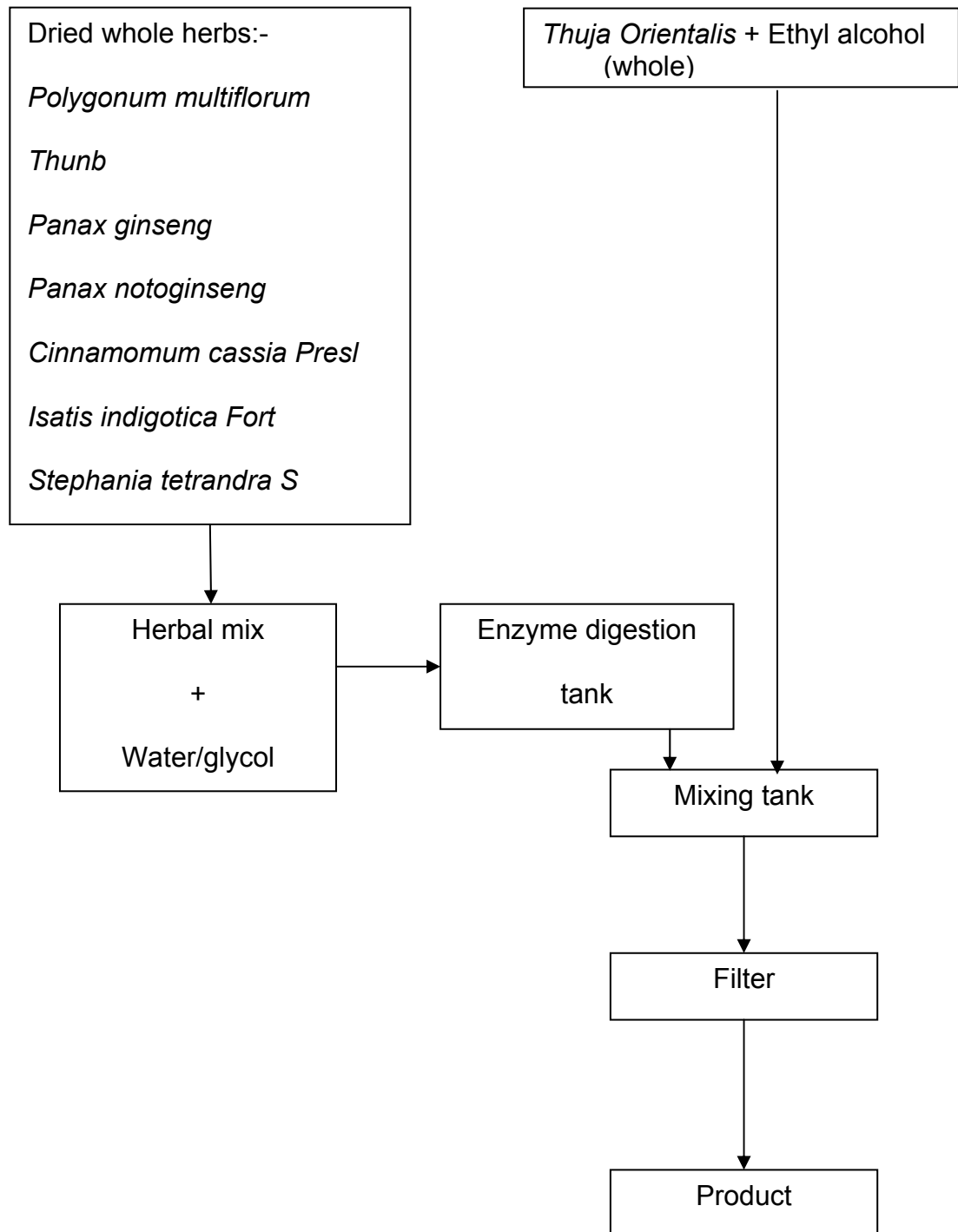
Details of the herbs used in the preparation of Xiantene have been supplied by the manufacturer (Table 4.1), but the percentage of each herb and the exact manufacturing formula is not known. The manufacturing procedure is known in outline (Dr. Z. Xia, personal communication) and is shown in Figure 4.3.

The general procedure is as follows: -

Whole botanical materials (roots, stems, leaves, twigs, seeds) were ground to a fine powder (approx. 200µm particle size) and extracted partly with a water and glycol mixture (80/20) and partly with ethyl alcohol. The aqueous extract underwent a further enzyme extraction process (Wu, 1996) before being remixed with the alcoholic extract and the final mixture was chilled, filtered and dried. The diluted Xiantene test material was very kindly supplied ready for use by Dr. Zhidao Xia of the University of Oxford.

Figure 4.3

Outline manufacturing sequence for Xiantene I



The vehicle mixture was prepared using an alcohol/aqueous base (20:80) with caramel (Univar Ltd.) to provide a coloured vehicle solution (see chapter 2). Caramel is not present in the Xiantene herbal mixture.

Xiantene II differs from Xiantene I and is not physically the same, having been produced as an updated and improved version of the original product with lighter colour and reduced odour. The manufacturing method for Xiantene I (Figure 4.3) was modified for Xiantene II and the details of these modifications are not available. An additional solvent (type unknown) was introduced obviating the need for a dried extract stage and manufacture proceeded directly to a final liquid stage ready for use (Dr. Xia, personal communication). Further Xiantene II did not contain *Stephania tetrandia* S. Moore since this had been banned from use in the UK by the Medicines and Health Regulatory Authority (MHRA).

The vehicle mixture was the same in both experiments.

4.4 Physical measurements and comparison of Xiantene I, Xiantene II and vehicle

The main physical parameters were measured and are shown in Table 4.2. As previously mentioned Xiantene I and II differ substantially in colour and odour, although both have a slightly acid pH and both have similar specific gravity values, consistent with an approximate water: solvent ratio of 80:20. The vehicle (pH 8.48), Xiantene I (pH 4.99) and Xiantene II (pH 3.95) differ in their pH values which is unfortunate and was not appreciated at the time.

Table 4.2**Physical parameters for Xiantene I, Xiantene II and vehicle**

	Xiantene I	Xiantene II	Vehicle
Batch no	12241	1603	2711
Appearance	Mobile Liquid	Mobile Liquid	Mobile Liquid
Colour	Dark Brown	Light brown	Light brown
Odour	Heavy, aromatic	Light, sweet	None
pH at 20 °C	4.99	3.95	8.48
Specific Gravity at 20 °C	0.9409 g/mol	0.9815 g/mol	0.9787 g/mol
Residue on drying	Brown Powder	Viscous Gum	None
Residue on ignition	43.7 mg/ml (0.4% w/w)	Not measured	None

The analytical work was kindly undertaken by Dr. Ian Flockhart of Applied Analysis Ltd., Rowley House, Tokenspike Business Park, Hull Road, Woodmansey, Hull, HU17 0TB.

The subjects would have been unable to detect this, slight difference, although the 2 Xiantene mixtures are closer to the normal pH of the skin (pH=5) which could conceivably made absorption easier. The difference in behaviour on drying between Xiantene I and Xiantene II indicates that a different solvent system was used. Dr. Xia has confirmed this (personal communication), but the solvent used has not been identified.

4.5 Spectroscopic analysis of Xiantene I, Xiantene II and vehicle

Infra red spectroscopy (IR) based on the characteristic absorption of infra red radiation by functional groups, an obvious choice for “fingerprinting” and comparison of broadly similar compounds, could not be used due to the presence of water as the main solvent in all three compounds (Xiantene I, Xiantene II and vehicle). Water has substantial absorption peaks in the infra red region (400 to 4500) which would render any IR spectra unusable.

Nuclear magnetic resonance spectroscopy (NMR) is a powerful analytical technique for the study of bonding in carbon compounds, allowing accurate comparison of organic molecules and I am indebted to my colleague Dr. Annie Bligh of London Metropolitan University for her assistance with work on the NMR spectra of Xiantene I and II and vehicle. Unfortunately it proved impossible to obtain a dry extract for Xiantene II either using a steam bath or a freeze drying technique. Xiantene I did produce a dry powder extract on drying, whereas Xiantene II produced a sticky gum which resisted all attempts to produce a dry powder; under the circumstances it was concluded that any NMR spectra that might be produced could not be reliably interpreted and any comparison would not be valid.

Additionally, to confirm the presence of any single component reference samples of the herbs would be required and these were not available. Reference standards for TCM herbal products are maintained by the CMPAC centre at Kew Gardens, but it did not prove possible to access this material.

4.6 Thin layer chromatography (TLC) analysis of Xiantene I, Xiantene II and vehicle

4.6.1 Methods and materials

Thin layer chromatography allows molecules in mixtures to be separated and identified using the different rates at which they move up a TLC plate (Pothier, 1999). The solvent moves by capillary action across a suitable base or medium carrying the molecules in the mixture with it. The smallest molecules move the furthest. This separation based on size gives the resultant “chromatograph” (Gumport & Stryer, 2002). Following the separation of the mixture, spots are visualised using a stain or spray and the distance travelled by the molecules evaluated using retention factors (R_f values), i.e. the distance travelled by the component spot relative to the solvent front. The discrete spots or stains are identified by UV light or chemically reacting with a developing agent once the solvent has dried. Thin layer chromatography is a routine and reliable analytical procedure and I am indebted to my colleague Mr. S. Al-Adadi of the University of Bradford, for the TLC data. Reference herbs were obtained locally (Tian Tian (Sun) Ltd, Westside, Manor Road, Barnet, EN5 2LE), pulverised and extracted separately using methanol, chloroform and diethyl ether as solvents and the subsequent TLC chromatograms used to provide reference or “marker” data

using: a polar solvent system (chloroform-methanol-water (6.5:5:1:v/v/v), a non-polar solvent system (toluene-ethyl acetate (45:5 v/v) or an acid solvent system (chloroform-acetone-formic acid, 37.5:8.25:4.25 v/v/v) to provide varying polarity systems for the solvent phase. All solvents were of laboratory grade (Fisher Science Ltd., Schwerte, Germany). Stains were identified using vanillin-sulphuric acid spray (Pothier, 1996) or potassium hydroxide spray (Pothier, 1996).

4.6.2 Results

The thin layer chromatography analysis of Xiantene I, Xiantene II and vehicle are summarised in Table 4.3.

Polar solvent TLC systems were used to identify anthra-glycosides, a major component of *Polygonum multiflorum Thunb.*, and saponins, a major component of *Panax ginseng* root and *Panax notoginseng* root. Xiantene I produced a yellow spot with the same “Rf” value as the reference *Polygonum multiflorum Thunb.* extract whereas Xiantene II did not, or the stain was masked by another component; this suggests that Xiantene II may not contain *Polygonum multiflorum Thunb.*, or at least only in very small amounts. Staining confirmed the presence of anthra-quinone glycosides and *Polygonum multiflorum Thunb.* (not prepared) in Xiantene I but not in Xiantene II. Interestingly the TLC chromatograms for *Polygonum multiflorum Thunb.* (not prepared) and *Polygonum multiflorum Thunb.* (prepared) were not the same, indicating that they contain different compounds.

Table 4.3

A comparison of the Chinese medicine plant components detected by thin layer chromatography analysis in Xiantene I, Xiantene II and the vehicle.

'X' indicates that the component was not detected.

Component	Xiantene I	Xiantene II	Vehicle
<i>Thuja orientalis</i> L. leaves	X	Positive	X
<i>Thuja orientalis</i> L. seeds	X	X	X
<i>Polygonum</i> <i>multiflorum</i> root	Positive	X	X
<i>Panax ginseng</i> root	Positive	X	X
<i>Panax notoginseng</i> root	Positive	X	X
<i>Cassia</i> <i>cinnamomum</i> twigs	Positive	Positive	X
<i>Isatis indigotica</i> forte	X	Positive	X
<i>Stephania</i> * <i>tetrandra</i> root	X	X	X

* Alkaloid test (Bisset, 1990)

Polygonum multiflorum Thunb may be used “not prepared” as used in this study or “prepared” for which the root is sliced, steamed with black bean juice and dried (Zheng, 2000). TLC chromatographs of *Panax ginseng* root and *Panax notoginseng* root (using diethyl ethyl extracts) were essentially the same (Rf values 0.97, 0.88, 0.81, 0.74, 0.72, 0.66) although when a methanol extract was used *Panax notoginseng* contained 2 further compounds (Rf 0.42 and 0.23). These compounds present in *Panax ginseng* and *Panax notoginseng* were not found in methanolic extracts of Xiantene II, but were present in the corresponding Xiantene I product.

A non-polar TLC solvent system (toluene-ethyl acetate 45:5 v/v) was used for the identification of essential oils, one of the major components of *Thuja orientalis* L. (leaves), *Thuja orientalis* L. (seeds) and *Cassia cinnamom presl.* (twigs), the later also containing coumarins. Comparison of Xiantene II and *Thuja orientalis* L. (leaves) showed similar chromatograms with similar Rf values (0.73, 0.53, 0.49, 0.25, 0.18, 0.11) suggesting that they contain the same compounds. The chromatogram for Xiantene I was not similar with several of the Rf values missing. Therefore Xiantene I may not contain *Thuja orientalis* L. (leaves), or they may only be present in very small amounts. TLC chromatograms of reference herb *Thuja orientalis* L. (seeds) did not concur with Xiantene I or Xiantene II chromatograms and the presence of *Thuja orientalis* L. (seed) extracts could not be confirmed.

Acid solvent TLC (chloroform-acetone-formic acid; 37.5:8.25:4.25 v/v/v) was used for the identification of compounds, scopoletin, amentoflavone and catechin, allowing identification of *Thuja orientalis* L. leaves, *Cinnamomum cassia presl.* twigs extracts and *Polygonum multiflorum* Thunb L. Using

diethyl ether extracts of the various Chinese plants and the test materials (5% w/v each) a yellow spot was separated from the extracts of *Cinnamomum cassia presl.* twigs, Xiantene I and Xiantene II with the same Rf value 0.53. However Xiantene II and *Cinnamomum cassia presl.* twigs also exhibited a reddish brown spot with the same Rf value 0.89 which was not present in Xiantene I. Also two yellow spots were separated only from the extracts of Xiantene I and *Polygonum multiflorum Thumb* with the same Rf value 0.83 confirming the results of the polar solvent TLC chromatographs. Further, comparison of Xiantene I and Xiantene II chromatograms showed a yellow spot with identical Rf value of 0.52 which was not present in any of the reference spectra, and which exhibited a blue fluorescence under UV light at 354nm when sprayed with vanillin-phosphoric acid reagent. This component present in Xiantene I and Xiantene II could not be identified.

Acid solvent TLC was also used to confirm the presence of indigo in Xiantene II and leaves of *Isatis indigotica Fort* (identical spot Rf value 0.25). Xiantene I did not exhibit this spot and Xiantene I does not appear to contain *Isatis indigotica Fort*, or only in very small amounts.

4.6.3 Discussion

The absence of cytotoxicity in applications of Xiantene I had already been established by *in vitro* testing. When the cytotoxicity of Xiantene I on cultured human fibroblast cells was assessed using the MTT assay, there was no significant difference between Xiantene treated and control groups (Dr. Z. Xia, personal communication).

Xiantene I and Xiantene II behaved differently under the test conditions and do not appear from the TLC results to contain many of the same components. Four of the eight specified ingredients, *Polygonum multiflorum* root, *Panax ginseng* root, *Panax notoginseng* root and *Cassia cinnamomum* root, were detected in Xiantene I, but *Thuja orientalis* L seeds, *Thuja orientalis* L leaves and *Istatis indigotica forte* could not be identified (Table 4.3). In contrast three out of the seven ingredients, *Thuja orientalis* L. leaves, *Isatis indigotica forte* and *Cassia Cinnamomum* twigs were identified in Xiantene II, but not *Thuja orientalis* L. seeds, *Polygonum multiflorum* root, *Panax ginseng* root and *Panax notoginseng* root (Table 4.3). *Stephania tetrandia* S. Moore root was not detected in either, although claimed to be part of Xiantene I. This is fortuitous as *Stephania tetrandia* S. Moore root has been banned from use in EEC countries since 2004. However this raises questions about the reliability of the information provided by the suppliers since neither Xiantene I or Xiantene II appeared to contain all the ingredients listed (see Table 4.1). The vehicle mixture did not contain any of the Chinese herbs tested or any additional compounds. This suggests the vehicle would be non-toxic and should not contain any hair growth actives.

This inability to identify all the components claimed to be present in either of the Xiantene mixtures could be due to a failure to isolate the components, but this seems unlikely since most of the plants components except *Thuja orientalis* L seeds and *Stephania tetrandia* root were detected in one or other of the Xiantene mixtures. Although thin layer chromatography is a widely used and convenient method of identification, there are some inherent difficulties with the technique. Some components of the mixture may produce

spots of stains with identical Rf values, which therefore overlap on the chromatograph. This problem might be expected to be particularly acute for complex mixtures like Xiantene containing a great number of components, some of which cannot be identified. However, similar Rf values would not explain the recorded differences and absence of stains in Xiantene I and Xiantene II. The thin layer chromatography data for Xiantene I and Xiantene II are almost completely different, and only one of the 8 herbs used, *Cassia cinnamomum presl.* twigs, could be positively identified in the two versions of Xiantene (see Table 4.3). Further both Xiantene I and Xiantene II exhibited other components that could not be identified as any of the eight listed ingredients.

These identifications are somewhat preliminary and have some difficulties. The reference herbs used as markers were purchased in the UK and may or may not be the same type and species as those used in the manufacture of Xiantene in China. Further the reference samples were obtained as dried extracts and would have been subjected to some storage and possible deterioration since harvesting. Xiantene is prepared directly from the fresh herbal components in China (leaves, roots, twigs, seeds or kernels) which are treated and prepared locally and may not therefore equate to the reference samples used. However, clear spots corresponding to each of the reference materials, except *Stephania tetrandra* S. Moore root and *Thuja orientalis* L seeds were detected in at least one of the Xiantene mixtures, so this is unlikely to cause the differences found between the two batches.

However, the methods of manufacture of Xiantene I and Xiantene II were markedly different (Dr. Z. Xia, personal communication) with different solvent

systems being used for the extraction and no intermediate dried extract being produced during preparation of Xiantene II (see section 4.3). This may have meant that the efficiency of extraction from each plant component varied between the two methods. Certainly both Xiantene I and Xiantene II also exhibited components that could not be identified as any of the eight listed ingredients. There were noticeable differences in the physical parameters of the two Xiantene mixtures, particularly the residue on drying (see Table 4.2). Components although present originally may not therefore be extracted equally from both Xiantene I and II solutions. Thin layer chromatography depends crucially on the solubility of the test material in the various solvent systems used (Gumport & Steyr, 2002). Xiantene I and II are composed of at least two solvents, water and alcohol [types not disclosed], with a possible third solvent glycol [type not disclosed] present; the vehicle also contains two solvents, ethanol and water. Differential solubility of the different plant components in the TLC solvent and the different solvents present in Xiantene I and Xiantene II could have resulted in different chromatographic results. However, the same thin layer chromatography system and 3 extraction solvents were used for both versions of Xiantene and each plant, except *Stephania tetrandia* and *Thuja orientalis* L seeds was identified making differential solubility of herbal components in the TLC solvents a possible but unlikely cause of the observed differences between Xiantene I and Xiantene II.

Although the differences detected between Xiantene I and II (Tables 4.2 and 4.3) may be due to the different solvents used interfering with the extraction for thin layer chromatography, it could also indicate that the materials do not

contain all the ingredients that are claimed or that the different methods of manufacture extracted different components from the correct plants used particularly since the extraction solvents for Xiantene I and Xiantene II were not identical (Dr. Xia, personal communication) and may have made a difference to what was initially extracted.

The potentially unreliable quality of traditional Chinese medicinal products has been identified previously (Yong et al, 2007; Lu et al, 2007). The mixtures may or may not contain the herbal extracts claimed or the composition may vary from batch to batch or there may be differences in composition with herbs from different sources (Xie & Leung, 2008). Traditional Chinese medicine mixtures are complex mixtures of many bioactive compounds and the identification and subsequent quality control of these products limits their full application and development (Liang et al, 2008). This is currently a key area of research. Although the thin layer chromatography results differed, both Xiantene I and Xiantene II stimulated hair growth in men with androgenetic alopecia (see Chapters 2 and 3). This could suggest that, either the effect is mainly due to the extract of *Cassia cinnamomum* twigs found in both, or that the differences were due to the difficulties in extracting the components from the two Xiantene mixtures. How the herbal mixture actually works remains intriguing.

It is possible that Xiantene is acting as a mitotic agent i.e. a direct stimulant for hair growth. Some herbal extracts are known to have mitogenic activity (Schubert et al, 2002) and recently eucalyptus extract was shown to act as a direct hair growth stimulant (Taguchi et al, 2006). Eucalyptus extract was shown to promote the gene expression and protein secretion of VEGF in

human cultured keratinocytes (Taguchi et al, 2006). VEGF (see Chapter 1, section 1.1.5) is a highly specific mitogen produced by the dermal papilla and highly expressed during anagen, with decreased expression during catagen and telogen (Lachgar et al, 1995). Although eucalyptus extract is not a component of Xiantene, herbal extracts clearly have the ability to up regulate hair growth promoting factors.

Some of the extracts used in the preparation of Xiantene are known to be enzyme inhibitors (Park et al, 2003). In fact a recent virtual screening assessment programme of 240 commonly used Chinese herbs containing 8264 compounds (Ehrman et al, 2007), found a relatively large number of herbs (62%) contained potential inhibitors against 'therapeutically important molecular targets'. Additionally, a number of herbs contained inhibitors of the same target from different, phytochemical classes, suggesting different molecular pathways to target inhibition (Ehrman et al, 2007). Five of the seven herbs used in the preparation of Xiantene were identified in Ehrman's study as potential inhibitors for specific enzymes; cyclic adenosine 3-5-monophosphatase (cAMP), inducible nitric oxide synthase (iNOS), cyclooxygenase (COX), lipoxygenase (LOX), aldose reductase (AR), HIV-1 integrase, protease and reverse transcriptase (RT). The herbs identified were Ce Bai Ye (*Thuja orientalis* L.), Qing Dai (*Isatis indigotica* Fort), He Shou Wu (*Polygonum multiflorum* Thunb), San Yi (*Panax notoginseng*) and Ren Shen (*Panax ginseng*).

All five herbs were detected in Xiantene by TLC (Table 4.3), although *Isatis indigotica* forte and *Thuja orientalis* L seeds were found to be present only in Xiantene II and *Polygonum multiflorum*, *Panax ginseng* and *Panax*

notoginseng were shown to be present only in Xiantene I. *Polygonum multiflorum* or the 'Tree of Life', a component of Xiantene has also been suggested to exhibit enzyme inhibition properties by Yim et al, (1998) and Li et al, (2003); it is also a significant anti-oxidant (Ryu et al, 2002). Similarly considerable bio-activity of Thuja extracts has also been reported (Schuberth et al, 2002), with both mitogenic activity and potent enzyme inhibition action reported (Gohla, 1989; Offergeld, 1992).

Cassia cinnamomum, which was present in both forms of Xiantene has been shown to have potent inhibitory activity against a range of enzymes including aldose reductase (Lee, 2002), tyrosinase (Kong et al, 2008) and phosphodiesterase 5 (Dell'Agli et al, 2008). *Cassia cinnamomum* also exhibits excellent antioxidant activity (Lin et al, 2003). Tyrosinase levels determine the level of activity of follicular melanocytes (Slominski et al, 2004) and therefore hair pigmentation levels (see Section 1.2.2). A reduction in tyrosinase levels and thus pigmentation, may lower the level of cytotoxicity of some metabolic intermediates associated with melanogenesis, and may therefore allow increased follicular cell activity and possibly alter some aspects of hair growth. Unpigmented hair is known to grow faster (Van Neste, 2006). In traditional Chinese medicine (TCM) *Cassia cinnamomum* has been used for the treatment of dyspepsia, gastritis and inflammatory disorders as well as cancer where anti-tumor activity and inhibition of the anti-apoptosis regulating gene BCL-2 has been demonstrated (Lee et al, 2007).

The conversion of testosterone to the more active androgen 5 α -dihydrotestosterone by the enzyme 5 α -reductase plays a crucial role in the

development of androgenetic alopecia (see Section 1.4). Controlled clinical studies showed a specific inhibitor of 5 α -reductase type 2, finasteride (Kaufman et al, 1996) and dutasteride which inhibits both 5 α -reductase type 1 and type 2 (Olsen et al, 2006) promoted hair growth in male pattern alopecia. A number of naturally occurring 5 α -reductase inhibitors which have been extracted from plants are known, for example, '*Serenoa repens*' (Saw Palmetto) which has been used in the treatment of prostatic enlargement (Rhodes et al, 1993; Habib et al, 2005) and androgenetic alopecia (Prager et al, 2002). *Humulus lupulus* from hops and eugenyl glucoside from clove extract are also claimed to inhibit 5 α -reductase activity (Okano et al, 1994; Hamada et al, 2001) and other naturally occurring compounds e.g. rose fruit extract are known to be 5 α -reductase inhibitors (Yamashita et al, 1991).

Recent investigations have shown that '*Thujae*' extracts (specifically *Thuja occidentalis*) also exhibit 5 α -reductase type 2 inhibition in mice (Park et al, 2003); *Thuja orientalis* seeds (also a member of the *Thujae* family) are claimed to be present in both herbal mixture Xiantene I and II, although this could not be confirmed in the TLC analysis, however the *Thuja orientalis L* leaves extract was detected in Xiantene II which may also have this 5 α -reductase type 2 inhibition ability. Therefore it seems possible that the Xiantene mixture may be acting as an enzyme inhibitor in the hair follicle, possibly as an apoptosis inhibitor or by interfering with the 5 α -reductase activity, blocking the 5 α -dihydrotestosterone synthesis pathway, and modifying androgen conversion.

4.7 Conclusions

The TLC analysis confirmed that 6 out of the 7 claimed herb extracts were present in the Xiantene mixtures. *Stephania tetranda* was not identified in either although claimed to be in Xiantene I. This is beneficial as this is no longer allowed to be used in the UK due to risks associated with its incorrect identification. Physical tests and thin layer chromatography results of Xiantene I and Xiantene II indicated that there were differences between the two lotions. Only *Cassia cinnamomum* was detected in both preparations of Xiantene. They also confirmed that as expected the vehicle did not contain any components detectable by thin layer chromatography.

Since both Xiantene mixtures (Xiantene I and Xiantene II) stimulated hair growth despite their apparent differences, the active ingredients may come from the *Cassia cinnamomum* extracts detected in both. Alternatively the effect may come from a range of activities in different extracts as suggested by traditional Chinese medicine (TCM) practice. Potential routes include stimulating mitogenic activity or enzyme inhibition of apoptosis or possibly of the enzyme 5 α -reductase preventing the formation of the active androgen 5 α -dihydrotestosterone in the hair follicle.

5. Investigation of a possible relationship between male pattern alopecia and the early onset of greying

5.1 Introduction

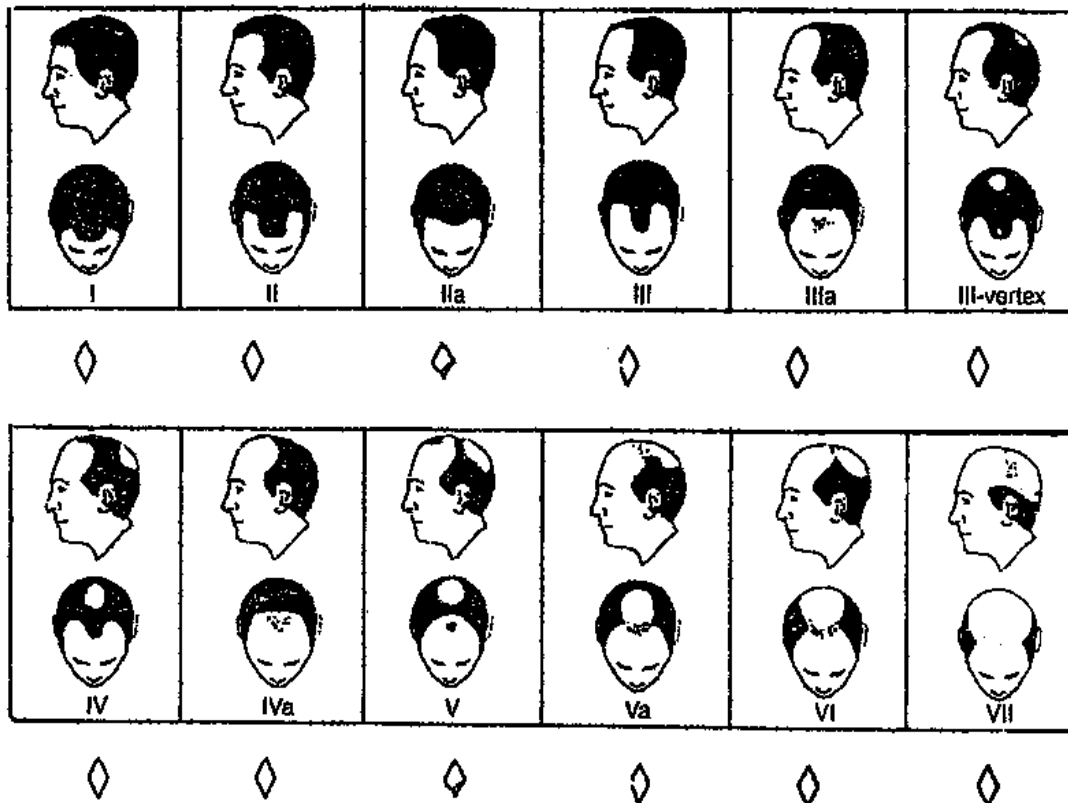
Loss of hair colour, canities (see chapter 1.2), and male pattern baldness, androgenetic alopecia (see chapter 1.4), are two of the key visible markers of increasing age (Bulpitt et al, 2001). Both conditions gradually spread across the head in a patterned progression. Canities starts in the parietal areas spreading gradually to the vertex and the whole scalp, affecting the occipital area last (Boas & Michelson, 1932; Straile, 1964; Keogh & Walsh, 1965), while male androgenetic alopecia begins with a bi-temporal recession, movement of the frontal hair line and thinning of the frontal and crown area (Hamilton, 1951; Rushton et al, 1991). These stages of progressive hair loss are described as Hamilton I – VII (Hamilton, 1951) modified by Norwood (Norwood, 1975). The occipital and parietal areas do not usually exhibit hair loss, although complete baldness may occur (see Figure 5.1.). However, androgenetic alopecia is predominantly a male, androgen dependent condition (Hamilton, 1942; Randall, 2007), whereas loss of hair pigmentation occurs in both sexes and is presumed to be androgen-independent. The causes of canities are not clear, though early canities is a feature of a number of auto-immune diseases including pernicious anaemia (Dawber, 1970) and hyperthyroidism (Rook & Dawber, 1991). In alopecia areata, also an auto-immune condition, in which grey hair are spared and exhibit disease resistance, the initial hair re-growth is often unpigmented (McDonagh & Messenger, 1996).

Figure 5.1

Scales used to grade androgenetic alopecia and hair greying

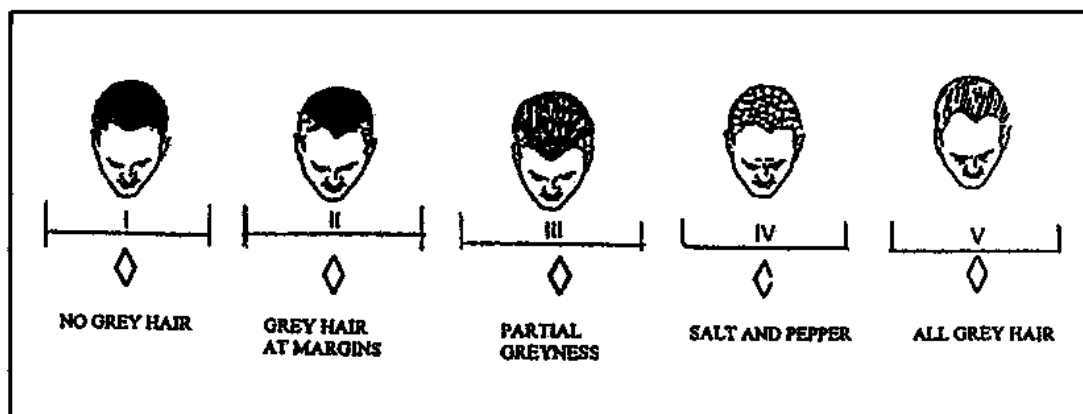
Hamilton scale (grade 1-7)

Scale of hair loss in androgenetic alopecia in men devised by Hamilton, modified by Norwood (Hamilton, 1951; Norwood, 1975)



Grey scale (grade 1-5)

Scale of hair greyness devised by the author



Children under the age of puberty (10-12 years) do not exhibit grey hair apart from certain rare syndromes which disrupt normal melanogenesis (Gilkes et al, 1974). Skin pigmentation also changes with age; the skin becomes thickened, becomes more yellow and wrinkled due to changes in the connective tissue structure and lentigines, dark spots of hyperpigmentation may appear due to a localised increase in the number of epidermal melanocytes (Monestier et al, 2006).

Hairdressers have been known to console men suffering from early greyness (under the age of 30 years) with the adage that early greyness will prevent or delay baldness (anecdotal evidence) and personal observation suggests that men who become prematurely grey may not then proceed to full androgenetic hair loss. However, this may be misleading as a full head of grey or white hair may simply be more noticeable.

5. 2 Aim

This study was designed to investigate the hypothesis that the loss of hair pigmentation may afford protection from the onset of androgenetic alopecia by examining whether the early onset of canities delayed the progression of androgenetic alopecia in men.

5. 3 Experimental design

The hypothesis was investigated by conducting a study of apparently healthy men aged 30 or over to determine whether there was any change in the incidence or extent of androgenetic alopecia if men exhibited canities before age 30. The aim was to consider 700 men overall, and to increase the

validity of the data two separate studies were carried out concurrently involving men at different geographical locations. In study 1 a group of over 500 Thai men were interviewed and their stage of greying and androgenetic alopecia determined by the author. In study 2, approximately 100 subjects in the UK, mainly friends and acquaintances from the London area, were invited to take part in the study completing a questionnaire themselves, approximately 30% of which were verified by the author, while a further 100 volunteered to complete a questionnaire on a self-assessed basis, online in response to an advertisement in "Chemistry World" (a newsletter published by the Royal Society of Chemistry). This data for a total of approximately 200 men in the UK based on self-assessment is therefore less reliable as this was self validated.

The age of each subject was recorded and the extent of androgenetic alopecia was assessed using the Hamilton scale (Hamilton, 1951) modified by Norwood (Norwood, 1975) (see Figure 5.1). A hair greyness scale (see Figure 5.1) was devised and used to record the degree of canities, and the age of canities onset was also recorded.

Subjects in each study were subsequently divided into 2 categories; those grey by the age of 30 and those who were not. Relationships between age and the stages of androgenetic alopecia and greyness and whether these were influenced by the early development of greying were analysed in each group and compared. Comparison was also made between the two different experimental groups.

5.4 Materials and Methods

5.4.1. Subject group

Ethical approval for the research was granted by the University of Bradford Ethics Committee.

Thai men

The first experiment was conducted using Thai men who presented at a chain of clinics in Thailand, specialising in the treatment of androgenetic alopecia and hair loss. All subjects gave informed consent and were assessed by a single examiner (the author) to record the grade of hair loss using the Hamilton scale, modified by Norwood, from 1-7 (Hamilton, 1951, Norwood, 1975), and the grade of hair greyness on a hair greyness scale devised by the author (see Figure 5.1) on a scale of 1-5. The subjects underwent a clinical examination, by the author, to eliminate persons with an underlying trichological condition e.g. seborrhoeic eczema, alopecia areata.

The scalps and hair shafts at the root end were examined using a videomicroscope (Optica Ltd., Bangkok) to also eliminate Thai men who had dyed their hair black (a common Thai tradition) and may not have admitted to being grey. The age of the subject was recorded as was the age the subject's grey hair first appeared, although it was of course not possible to independently verify this last parameter. All subjects were asked to confirm that they were in good health and were not aware of any underlying medical condition. A total of 502 Thai subjects were successfully examined and used for the study.

European men

A further 208 European men were recruited by personal invitation and advertising and invited to complete a similar questionnaire (see Appendix VI) using a self-assessment technique. The European group was comprised of 106 men who were personally selected by the author from friends, relatives and casual acquaintances and invited to participate in the study, and of these, 30 were assessed by both the subject and the examiner, whereas the remaining 76 subjects, completed their own assessment and no independent scoring was made. A further 102 subjects were recruited at random amongst scientists by advertising in a scientific journal, and completed the questionnaire via the internet. These results could not therefore be independently verified. The total number in the European group was therefore 208 subjects.

5.4.2. Statistical Analysis

The data was tabulated using an Excel (Microsoft Ltd.) spreadsheet and the subjects split into two groups in each category: those who were first grey at age 30 years or earlier, and those who were not. Initially the relationships between age and greying, and age and androgenetic alopecia were investigated using Pearson's correlation (Cohen et al, 2003). Secondly, mean values were compared using non-paired Student's t-tests to determine whether early greying affected the stage of balding comparing the grey before 30 groups with the grey after 30 groups. Results where $p < 0.05$ or $= 0.05$ were taken as statistically significant. Thanks are due to Mr. Edward Kent of the University of Warwick and Mr. Paul Bassett of Stats Consultancy

Ltd., for verifying the statistical analysis. A total of four subject groups were therefore used for comparison in this study: Thai men grey at 30 or earlier, Thai men not grey by age 30, European men grey at 30 or earlier and European men not grey by age 30. Due to the different method of data collection between the Thai group (personal interview and assessment) and the European group (Internet questionnaire, self validation), the two groups were not combined for analysis.

5.5 Results

5.5.1. Subjects

The initial data for the subjects assessed is shown in Table 5.1. A total of 843 subjects were used in the study. This included 626 Thai men and 217 European men; in the Thai group 52 men were grey by 30, representing 8.30% of the total Thai group, while 51 European men were grey by 30 i.e. 23.50% of the European group.

All the Thai subjects (total 626 men) were assessed by a single examiner, the author but only 14.75% of the total European group were independently assessed by the author and none of the European internet respondees could be checked meaning that 85.25% European men were not checked. In all cases where the assessments were checked by the author, n=30, the agreement for the degree of baldness (1-7) and the degree of greyness (1-5) were within 1 unit of the appropriate scale. The mean ages of the two Thai groups were 39.3 ± 1.2 (mean \pm SEM) years (range 30-63 years) for the grey by 30 group and 41.4 ± 0.4 years (range 30-72 years) for those who were not (see Table 5.1) and did not differ significantly ($p=0.13$).

Table 5.1

Summary of the subjects assessed for androgenetic alopecia and hair greyness.

Subjects ages by geographical group and by age grey group are summarised

	Group	Number of Subjects	Mean age ± SEM (years)	Age range (years)
Thai	Thai men Grey by 30	45	39.3 ± 1.2	30-63
	Thai men not grey by 30	457	41.4 ± 0.4	30-72
European	European men grey by 30	47	51.3 ± 2.2	31-86
	European men not grey by 30	161	53.6 ± 1.1	30-95

The mean ages of the two European groups were 51.3 ± 2.2 years for (range 31-86 years) the grey by 30 group and 53.6 ± 1.1 years for those who were not (see Table 5.1) again showing no significant difference ($p=0.33$) (Figure 5.2). However, the mean ages of the Thai groups compared with the European groups do vary significantly; Thai men who were grey before 30 had a significant lower mean age 39.3 ± 1.2 years than European men who were grey before 30, whose mean age was 51.3 ± 2.2 ($p<0.001$). Similarly Thai men who were not grey before age 30 had a mean age of 41.4 ± 0.4 , significantly lower than that of European men who were not grey before 30 (mean age 53.6 ± 1.1 years) ($p<0.001$), and comparison between different geographical groups is therefore limited.

5.5.2 Age of onset of greying

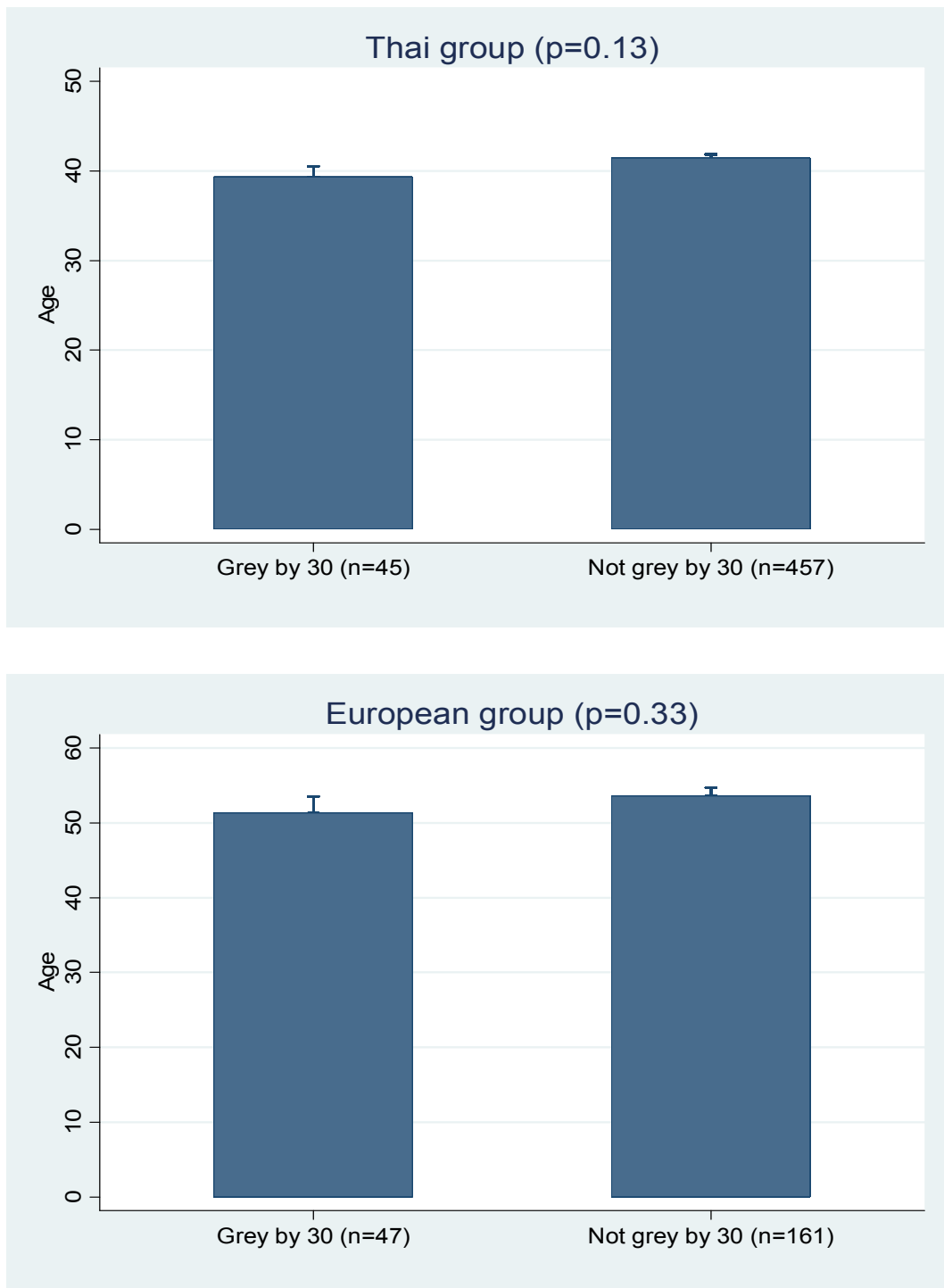
The mean age for the onset of hair greying (all subjects but excluding those not yet grey) was 37.9 ± 0.8 (mean \pm SEM) years for the Thai group ($n=170$) and 38.7 ± 0.9 years for the European group ($n=172$). There was no significant difference ($p=0.49$) in the age of onset of greying between the Thai men and the European men.

Interestingly 3 Thai subjects (5.66% of the Thai grey 30 or under group) gave the age of 12 years i.e. puberty for the first appearance of grey hair and this was in some cases apparently repeated in subsequent generations. It was not possible to fully eliminate the presence of thyroid disease from this observation, although all subjects had confirmed that they were in good health. The percentage of the participants who were exhibiting canities was 33.9% of the Thai group and 82.7% of the European group.

Figure 5.2

The relationship between the age of men at assessment who went grey by, or after, age 30.

Mean \pm SEM of age at assessment date of all men in the study separated according to whether they first lost their hair pigmentation by or after age 30



The mean age for the onset of canities in the grey before 30 or after 30, of both groups, Thai and European are shown in Figure 5.3.

The mean ages for the onset of greying for those who were grey but not grey by 30 were 42.4 ± 0.7 years for the Thai men who weren't and 43.8 ± 0.8 years for the European men; these are not significantly different ($p=0.12$). The grey before 30 groups also had no differences in the mean age at which greying began at this was; 25.9 ± 0.8 years for Thai men and 25.5 ± 0.6 years for Europeans ($p=0.45$). The mean age of greying of the men grey before 30 and grey after 30 for each geographical group (Thai and European) are of course different as they have been pre selected on this basis.

5.5.3. Relationship between age and balding

The extent of balding as measured by the Hamilton stage (modified by Norwood) for the 2 groups, Thai and European, were compared with age, for all men in that group and within each group with the men divided into subjects who were grey before or after 30 years of age.

The correlation analysis for all groups confirmed the expected relationship that baldness correlated positively with age. In the Thai group (see Figure 5.4), the men who were grey before 30 ($n=45$) showed a very highly significant correlation between age and balding with a correlation coefficient = 0.43 ($p=0.003$). This was also seen in those who were grey after 30 ($n=457$), correlation coefficient = 0.32 ($p<0.001$).

Figure 5.3

The relationship between the mean age for the onset of canities for men who went grey by, or after, age 30.

Mean age \pm SEM for the onset of canities of (a) Thai (n=170), (b) European (n=172), men grey by or after age 30, but excluding men not yet grey

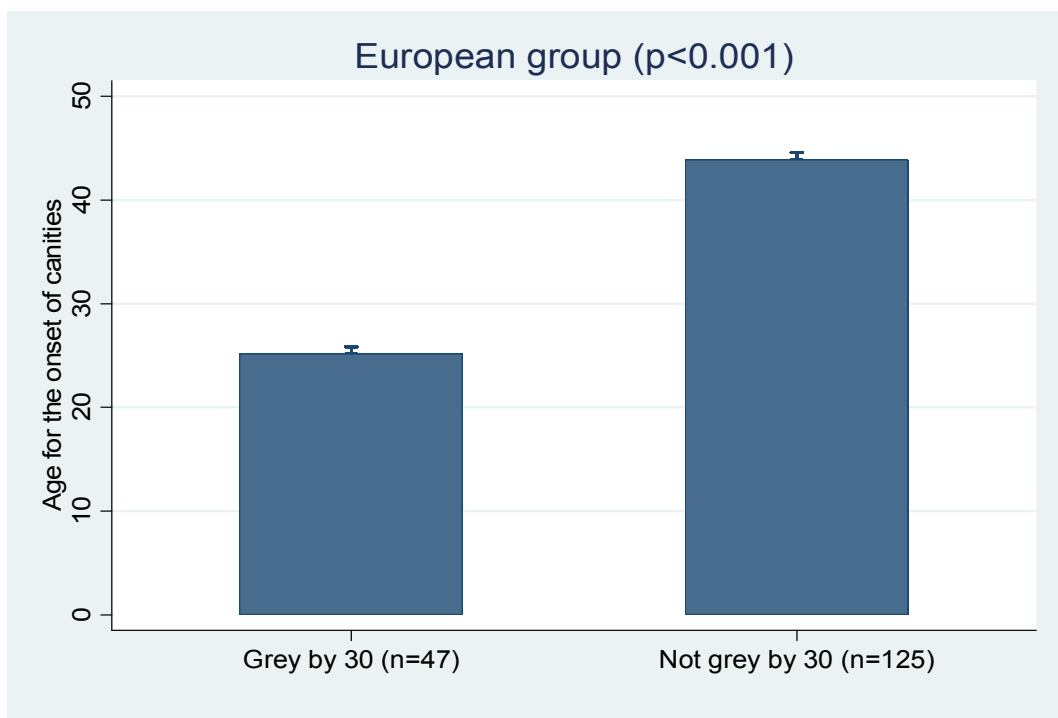
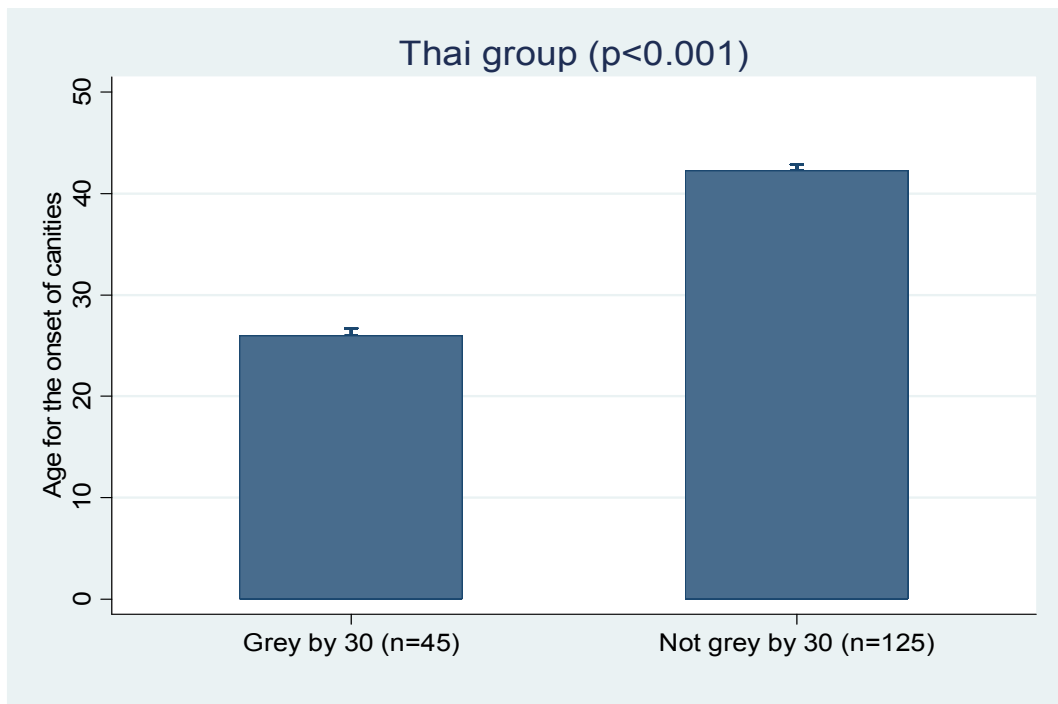
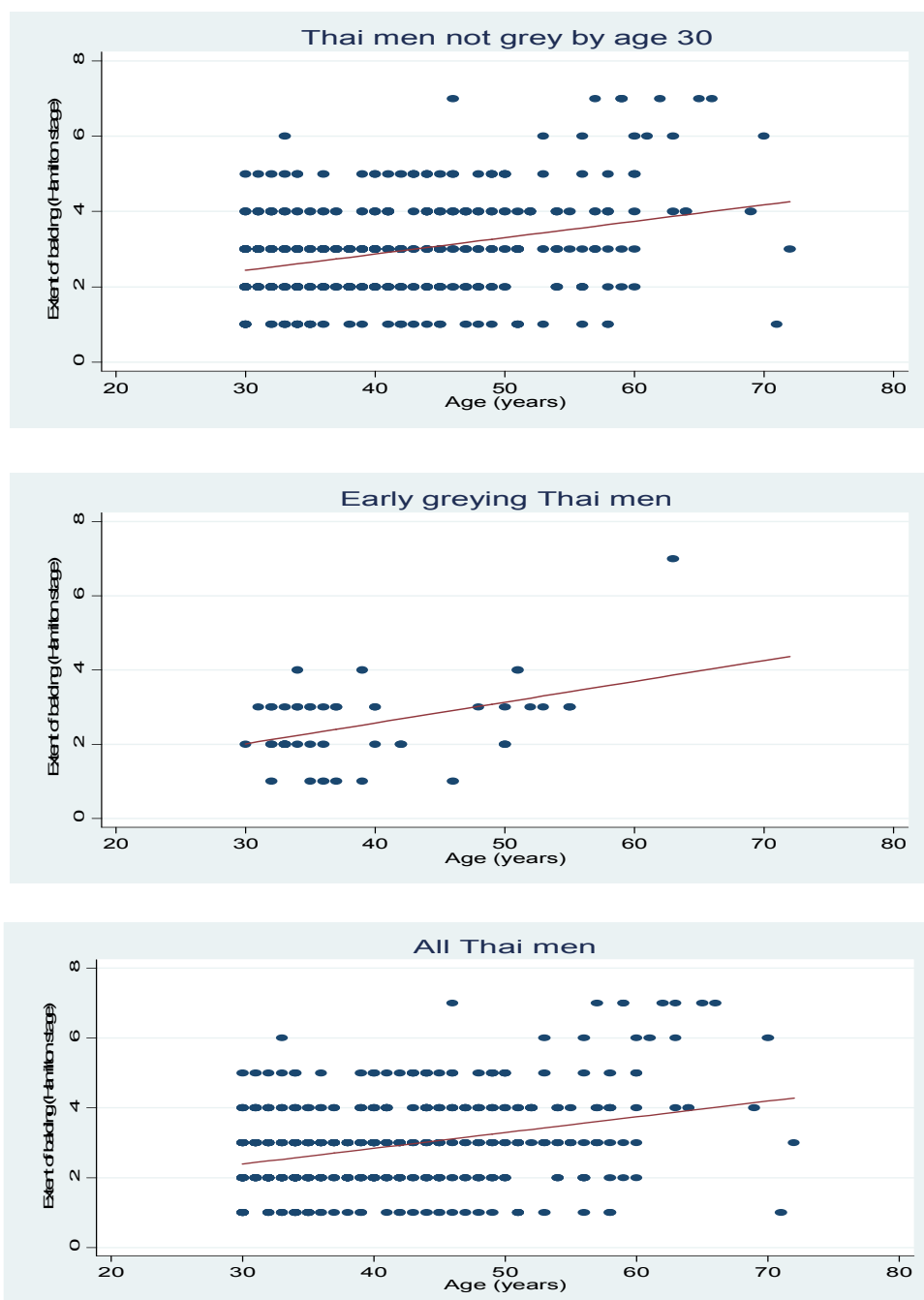


Figure 5.4

The relationship between age and extent of male pattern baldness as indicated by Hamilton stage in Thai men at assessment who went grey by, or after, age 30.

The extent of hair loss as indicated by the Hamilton stage was related to age for 502 Thai men; 45 men were grey by age 30 and 457 men were not grey age 30



In the European group the correlation between age and balding was weaker (Figure 5.5). European men who were grey before 30 (n=47) showed an insignificant, correlation coefficient = 0.14 (p=0.33) and this also occurred with European men who became grey after 30 (n=161), correlation coefficient = 0.08 (p=0.33).

This experiment confirms that male androgenetic alopecia advances with age in all 4 groups used in the experiment.

5.5.4 Relationship between age and hair greyness

The extent of hair greyness was also found to correlate with age. The grey after 30 groups all showed a strong positive correlation. In Thai men who were grey after 30 (Figure 5.6) (n=457) the correlation was highly significant; correlation coefficient = 0.55 (p<0.001). This also occurred in European men who were grey after 30 (n=161) where the correlation was also highly significant; correlation coefficient = 0.66, (p<0.001) (Figure 5.7).

The men who were grey before 30 also showed a strong positive correlation between age and extent of greying. Thai men (n=45) the positive correlation was very highly significant; correlation coefficient = 0.63 (p<0.001) (Figure 5.6). This was also true for European men (n=47), correlation coefficient = 0.75 (p<0.001) (Figure 5.7). In both the Thai subjects and the European subjects hair greyness and balding were found to correlate strongly and positively with age. However hair greyness had a much stronger correlation with age in both the Thai and the European groups than the correlation of balding with age.

Figure 5.5

The relationship between age and male pattern baldness as indicated by the Hamilton stage in European men at assessment who went grey by, or after, age 30.

The extent of hair loss as indicated by the Hamilton stage was related to age for 208 European men; 47 men were grey by age 30 and 161 men were not grey age 30

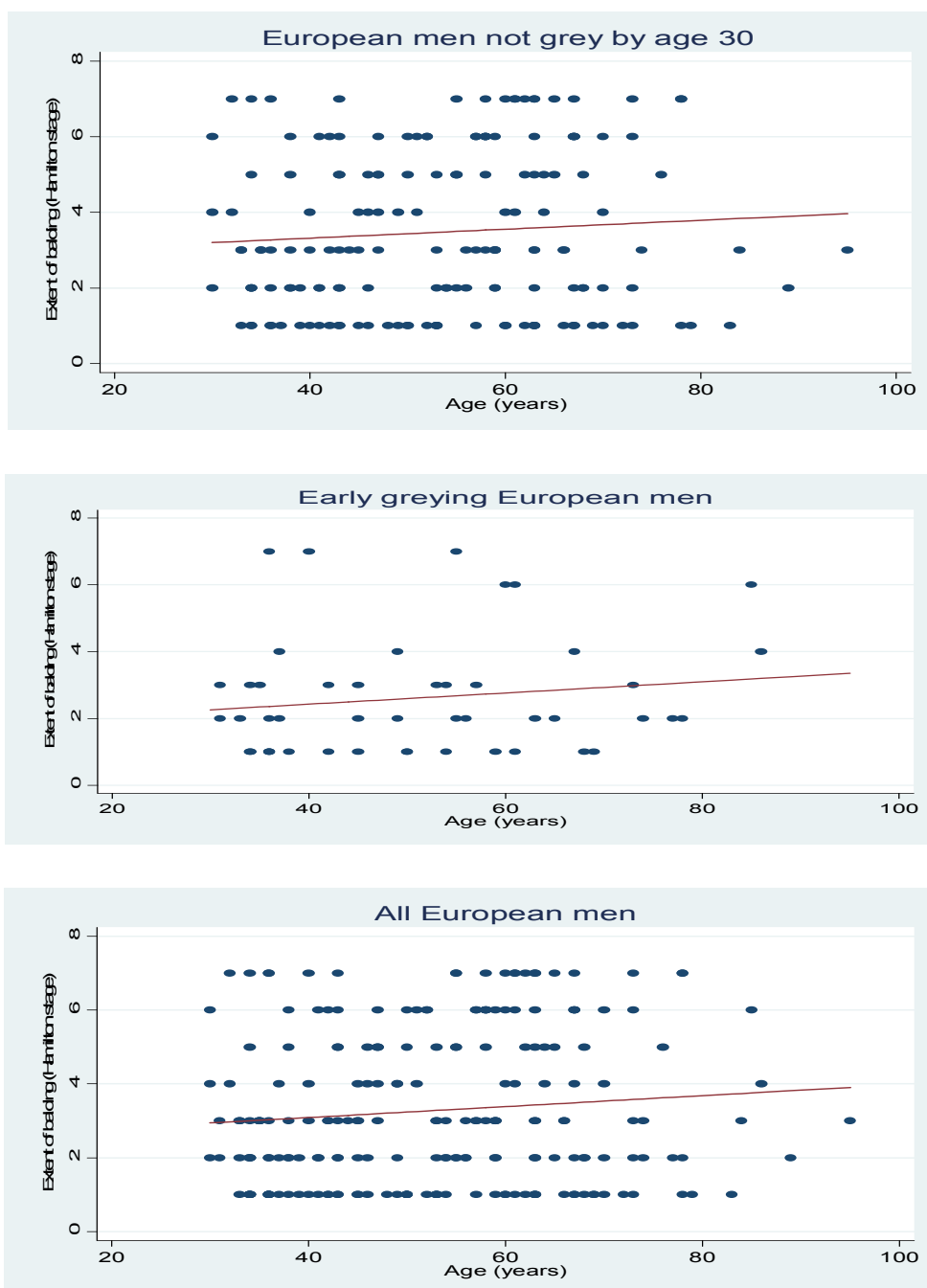


Figure 5.6

The relationship between age and extent of pigment loss in Thai men at assessment who went grey by or after age 30.

The extent of hair greyness was related to age for 502 Thai men; 45 men were grey by age 30 and 457 men were not grey age 30

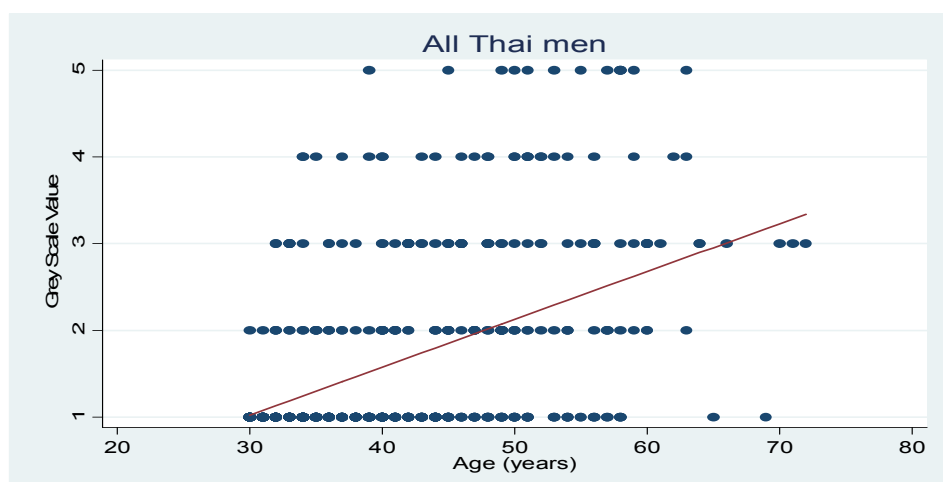
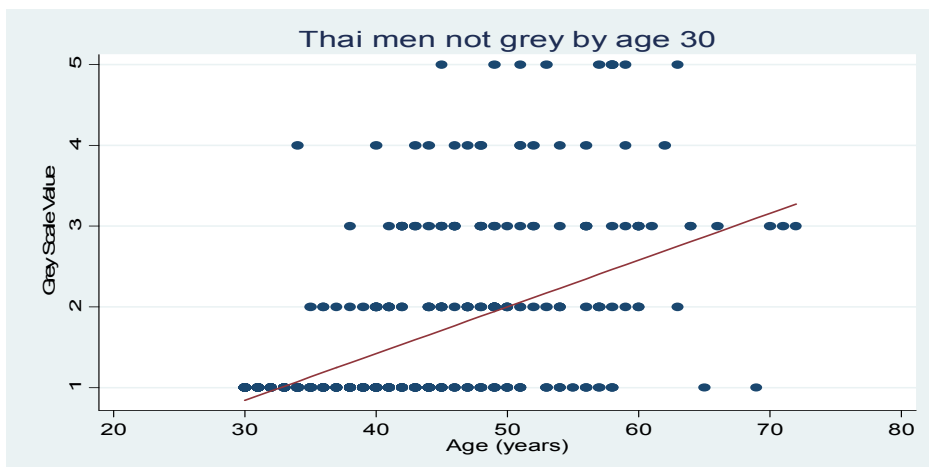
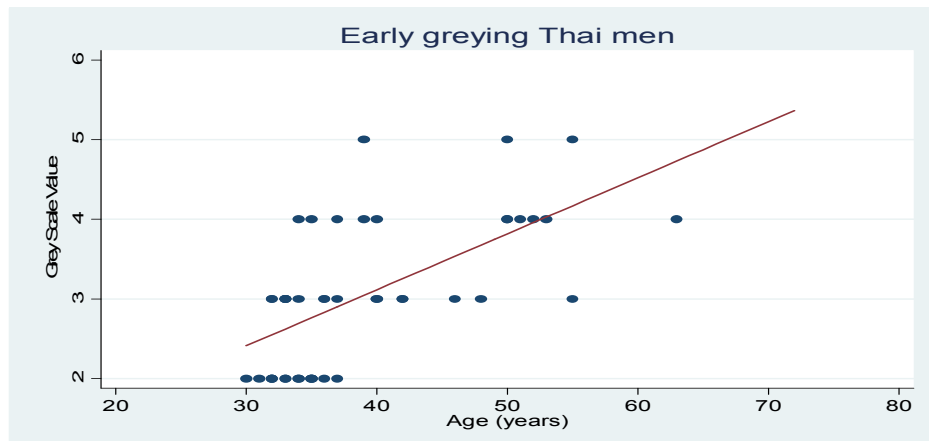
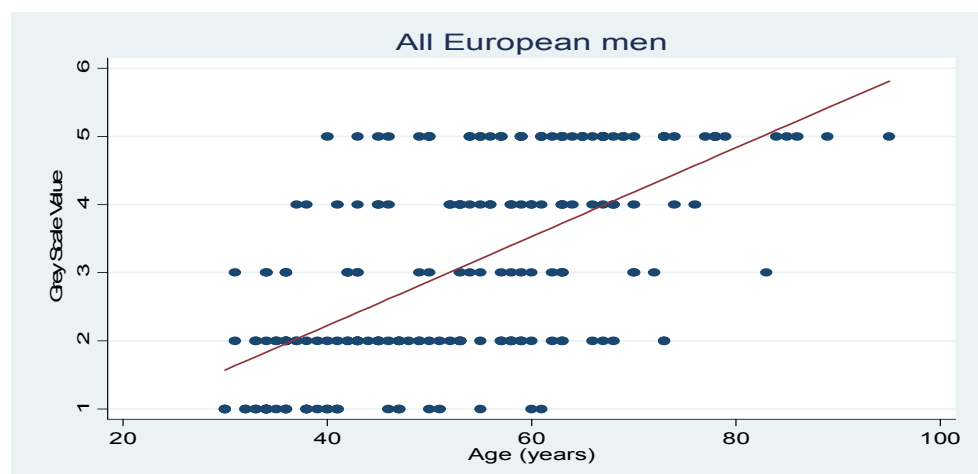
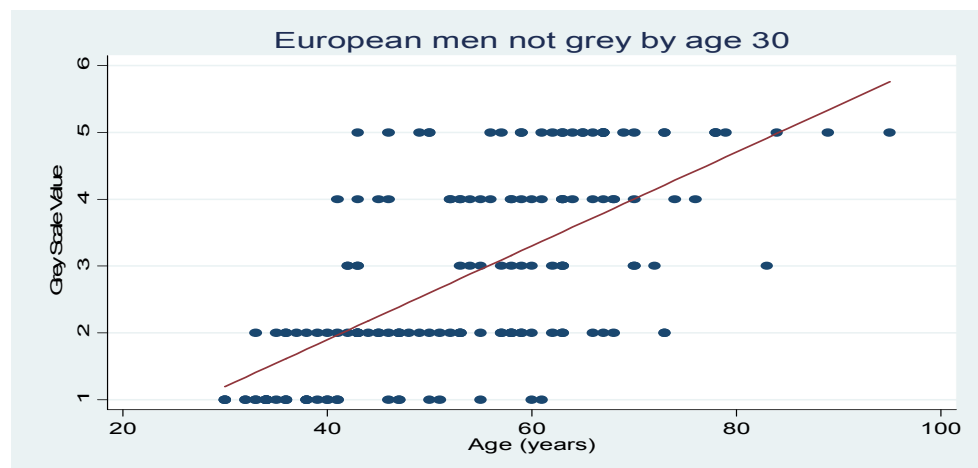
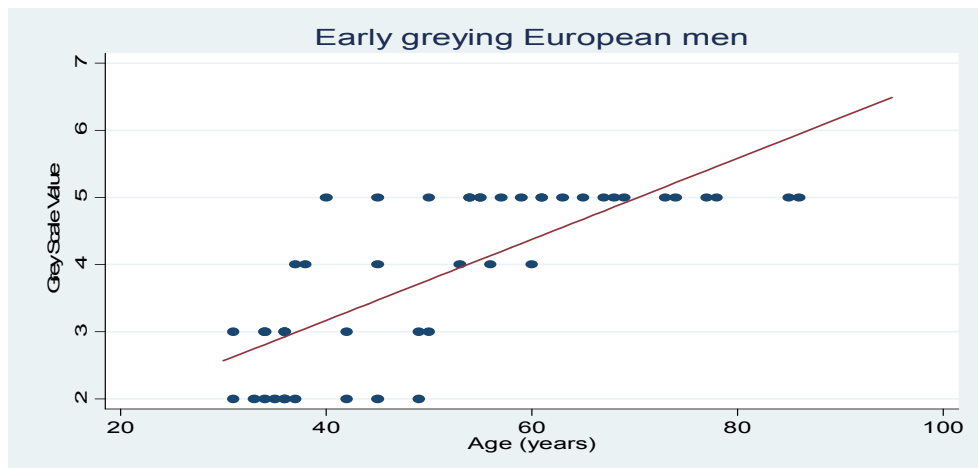


Figure 5.7

The relationship between age and extent of pigment loss in European men at assessment who went grey by or after age 30.

The extent of hair greyness was related to age for 208 European men; 47 men were grey by age 30 and 161 men were not grey age 30



5.5.5. Comparison of the extent of pigmentation loss in men who were grey by age 30 and those who were not

Loss of pigmentation was strongly correlated with age whether the men had been grey at an early stage or not (see 5.5.4; Figures 5.6 and 5.7). Both groups however when the extent of pigment loss was compared between the men who were grey before 30 and those who became grey after 30 (Figure 5.8), those who became grey early had a very significantly higher mean grey score than those who became grey after 30.

Thai men who were grey before 30 (n=45; grey score 3.06 ± 0.14) were significantly more grey than Thai men who became grey after 30 (n=457; grey score 1.50 ± 0.04 , $p < 0.001$) Figure 5.8; European men who were grey before 30 (n=47; grey score 3.85 ± 0.18) were similarly significantly more grey than European men who were not grey by 30 (n=161; grey score 2.85 ± 0.12 , $p < 0.001$). All men who were grey before 30 were significantly more grey compared with those who were not (Figure 5.8).

5.5.6. Comparison of the extent of androgenetic alopecia in men who were grey by age 30 and those who were not

The extent of androgenetic alopecia also correlated with age (see 5.5.3., Figures 5.4 and 5.5) in all groups. However, when the mean Hamilton (modified by Norwood) stages (Hamilton, 1951; Norwood, 1975) were compared, men who were grey aged 30 and before had significantly lower baldness scores than those who were grey after 30, in all subjects (see Figure 5.9).

Figure 5.8

The relationship between the extent of pigment loss in men at assessment who went grey by, or after, 30

The extent of hair greyness, mean \pm SEM, greyness scale grade (a) Thai, (b) European

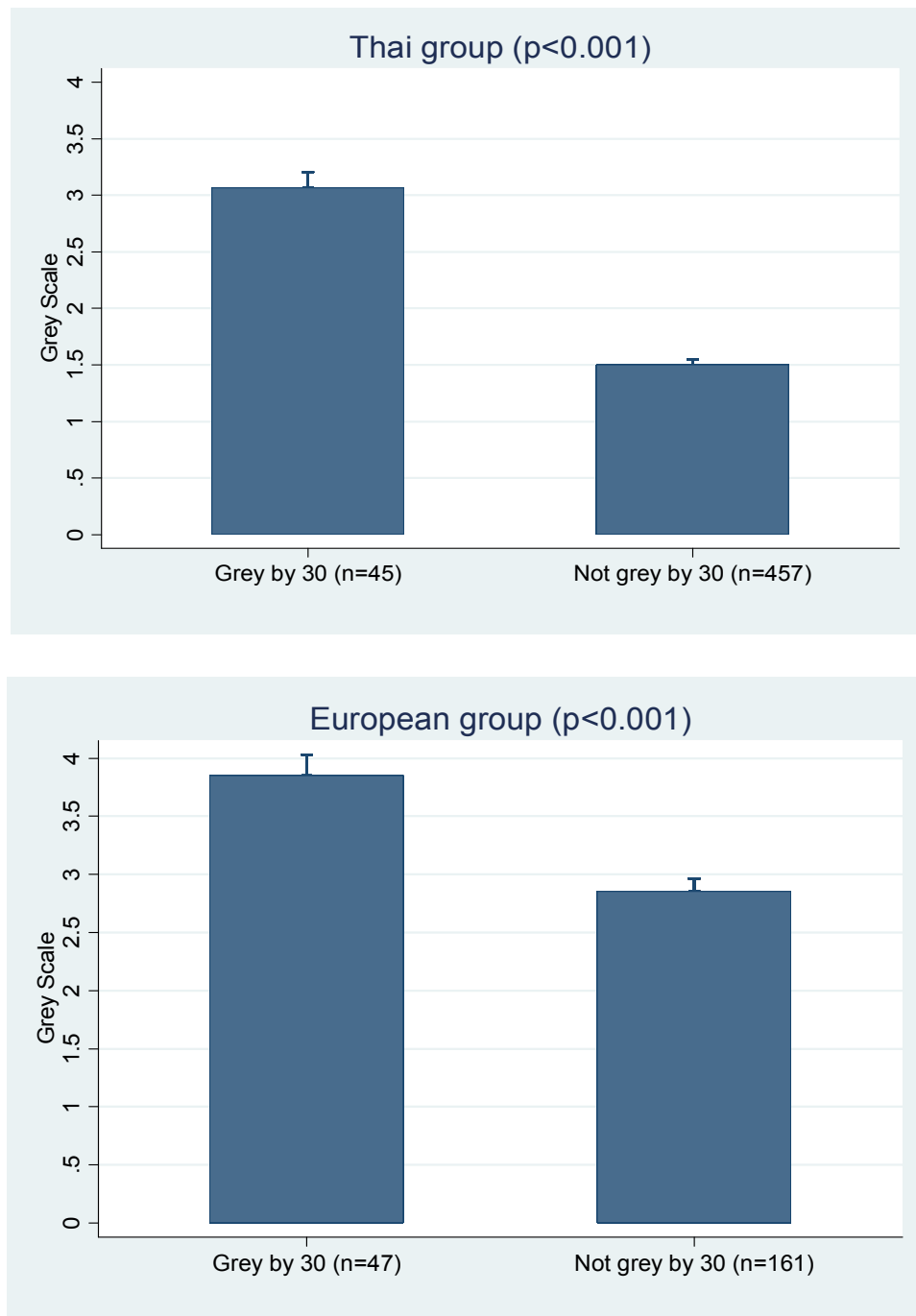
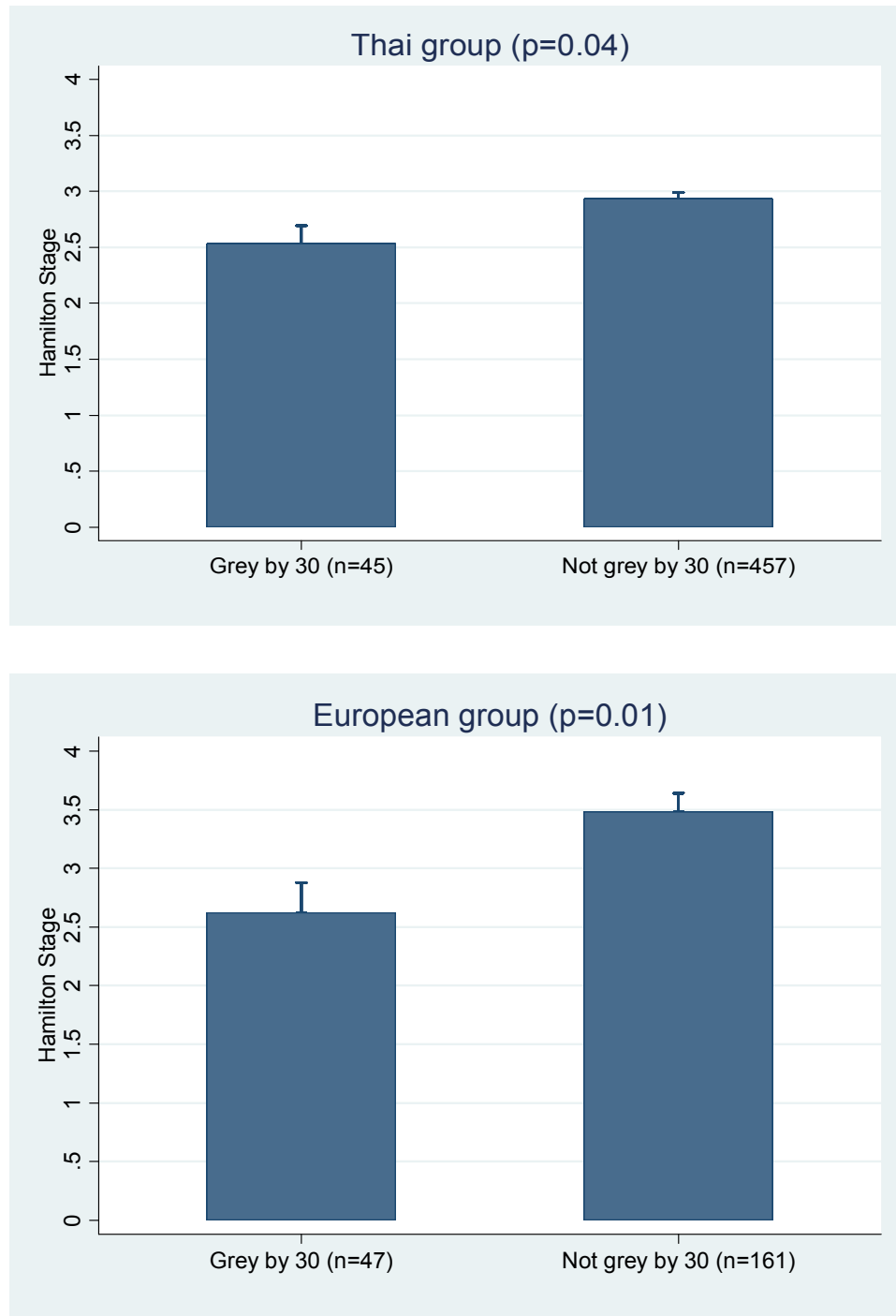


Figure 5.9

Relationship between the extent of male pattern baldness as indicated by the Hamilton stage in men at assessment who went grey by, or after, age 30.

Value are the mean \pm SEM Hamilton stage of (a) Thai, (b) European



This was more pronounced in the European men $p=0.01$ with the grey before 30 group having a mean Hamilton stage of 2.62 ± 0.26 and grey after 30 group 3.48 ± 0.16 .

If the results from all men were combined this difference was maintained $p=0.003$, grey before 30 group 2.58 ± 0.15 , grey after 30 group 3.07 ± 0.06 . Overall, in all groups, the men who were grey 30 or before were very significantly less bald.

5.5.7 Comparison of the extent of androgenetic alopecia in men who were grey by age 30 and those who were not, aged over 40 and adjusted for age

The final analyses compared the Hamilton stage between those grey and not grey by 30 adjusted for age. The analysis was restricted to those aged 40 or over. Linear regression was used for the analysis, and the results were performed with and without an adjustment for age.

The results are summarised in Table 5.2. The figures are the mean difference in Hamilton score between those who went grey by 30 and those not grey by 30. These are calculated as the values for those who went grey by 30 minus the values for those not grey by 30. So a positive difference would imply higher values for those who went grey by 30, and a negative difference would imply a lower value for those who went grey by 30.

The results suggested that for this age, aged 40 and over, there was no difference in Hamilton stage between those greying by 30 and not greying by 30 for Thai men ($p=0.28$), adjusted for age.

Table 5.2**Summary of extent of baldness adjusted for age**

Comparison of extent of androgenetic alopecia calculated as the value for those men over 40 who were grey by 30 minus the value of those men over 40 who were not. Differences are shown both unadjusted and adjusted for age.

Nationality	Analysis	Difference Mean (SEM)	p-value
Thai	Unadjusted	-0.35 (0.35)	0.32
	Age adjusted	-0.36 (0.34)	0.28
European	Unadjusted	-0.79 (0.41)	0.05
	Age adjusted	-0.80 (0.41)	0.05

However, there was evidence that European men grey by 30 had a lower Hamilton stage than European men not grey by 30 ($p=0.05$).

However if Thai and European men over 40 are combined, the results are significant ($p=0.01$). Men over 40 who were grey by 30 had a statistically significant lower stage than men not grey by 30 on an age adjusted basis.

5.6 Discussion

This investigation looked at the relationship between early greying and androgenetic alopecia in two groups of men, 502 from Thailand and 208 from Europe. The data obtained from the Thai group of subjects is consistent, using a single examiner, the author, to examine the subject's hair and the scalp, assess the extent of their greying and balding, and to verbally verify each subject's health status. This data from the Thai group therefore has a good, reliable scientific basis. The data from the European group, using a self-assessment technique, is not so reliable. Self-assessment of one's own hair parameters is not completely reliable and subjects tend to underscore (personal observation) i.e. a subject's perception is of less balding and less hair greyness than a third party examiner may record. The validity of self-reported male balding assessments has been discussed (Taylor et al, 2004); it was found that self-assessment of stage of balding was significantly less accurate than using trained third party examiners with approximately 50% agreement between the two methods using the Hamilton stage as modified by Norwood (Hamilton, 1942; Norwood, 1975). Older subjects have been reported to be better at assessing their balding group, than men under 50 years of age (Taylor et al, 2004). Although it was possible to check some

14.78% of the total European group (n=32), none of the European internet respondees could be checked. Therefore the data from this group does not have such a firm scientific basis as that from the Thai group. However the total number of participants in the European group is smaller (29.30% of the total subjects) than the Thai group (70.70%) and the bulk of the data used in this study was collated by a single 3rd party examiner (the author). All analysis was carried out on the groups separately and then combined.

The recruiting of subjects for the study was not completely random. The subjects attending the hair treatment clinics in Thailand were in effect pre-selected i.e. these were men who already felt they had a hair loss problem, albeit possibly quite mild, especially in the younger age range. The study, because of the method of recruitment, therefore used a group with possibly an above average degree of hair loss. The European group was also not random; the first European section were invited to participate and this was therefore not fully randomised. Interestingly, a number of invitees refused to take part in the study while some, particularly those with a full head of grey hair, willingly volunteered their details (personal observation). Data from the internet survey among academics may also not be completely reliable with the natural tendency to underscore present, a further difficulty might be the inability to understand the grading scales correctly. Since both greying and androgenetic alopecia are age related conditions the ages of the participants were compared. The mean ages of the two comparison groups on the date of analysis i.e. the men who were grey before 30 or grey after 30 within each geographical group are comparable and do not vary significantly: Thai 39.3 ± 1.2 years and 41.4 ± 0.4 years respectively ($p=0.13$) and European $51.3 \pm$

2.2 years and 53.6 ± 1.1 years respectively ($p=0.33$) (see Figure 5.2). However the two geographical groups, Thai and European, have significantly different mean ages with the Thai group being significantly younger; grey before 30 groups $p<0.001$, and grey after 30 groups $p<0.001$, meaning that the data from each area needs to be analysed separately to avoid age-bias. The difference in mean age of the two geographical groups probably reflects the methods of recruitment; probably younger men with greater hair loss concern have been attracted to the hair loss clinics in Thailand. Older men have responded to the invitations in the European study. The Thai people are of Mongol descent and they have not been subject to mass immigration or colonisation (Warren et al, 2002). Consequently, they present a tight genetic group with a specific phenotype. The north European people are considerably more diverse with a much wider genetic spread and a non-specific skin and hair colouring (Tobin & Paus, 2001). Unfortunately, the differing mean ages of the Thai and European groups means that it is not valid to interpret differences between these geographical groups as due only to their varying genetic backgrounds.

The mean age for the onset of hair greying in all subjects, excluding those who were not yet grey was found to be 37.9 ± 0.8 years for Thai men ($n=170$) and 38.7 ± 0.9 years for European men ($n=172$) (see Figure 5.3). This compares well with previous published data where the mean reported values were 34.2 years for caucasians, 43.9 years for African-Americans and 30-34 years for Japanese (Keogh & Walsh, 1965; Wasserman, 1974). The percentage of subjects exhibiting canities for each group, 33.9% of Thai men, 82.7% of European men, is also consistent with earlier work where it

was found that 50% of caucasians were 50% grey by the age of 50 (Keogh & Walsh, 1965). The age for early canities has been defined as, 20 years for Caucasians and 30 years for people of Afro-American origin (Rook & Dawber, 1982). The figure used in this study to define early canities was 30 years of age. It is known that health status can influence the onset of canities (Dawber, 1970) and although all subjects were asked to confirm that they were in good health, underlying thyroid disease (Wright, 1986) or pernicious anaemia (Dawber, 1970) could not be fully eliminated from distorting the data. Re-pigmentation of grey hair which continued into old age has also been observed in cases of hypothyroidism (Wright, 1984). Grey hair was found to re-darken substantially 6 months prior to overt hypothyroidism, but lightened on treatment with thyroid hormone (Wright, 1986).

When the extent of pigment loss was related to ageing, the data confirmed that loss of hair pigment correlates positively with age in a highly significant manner in all groups: Thai men grey by 30, $p < 0.001$, Thai men not grey by 30, $p < 0.001$ (see Figure 5.6), European men grey by 30, $p < 0.001$, European men not grey by 30, $p < 0.001$ (see Figure 5.7). This data fits with earlier work reporting that grey hair incidence increases with age (Boas & Michelson, 1932; Keogh & Walsh, 1965; Burch et al, 1971) and means that the men who went grey by the age of 30 showed a normal progression of pigment loss with age. Comparison of the extent of pigment loss for the men grey before or after 30 years (Figure 5.8) showed that the grey before 30 men in all groups were significantly more grey; Thai men ($p < 0.001$), European men ($p < 0.001$) and all subjects ($p < 0.001$). Early onset of hair greyness had allowed the pigment loss to proceed further for those individuals compared to

those who did not experience early canities. Loss of hair pigment (see chapter 1.2) is thought to be due to incomplete melanocyte stem cell maintenance in the hair follicle niche (Nishimura et al, 2005; Steingrimsdottir et al, 2005), affecting both the bulb and the outer root sheath (Como et al, 2004) and resulting in an inability to synthesise the pigment melanin in the hair follicle. The strong correlation of increased greying with age in early greyness would fit with loss of this sort.

Androgenetic alopecia was also found to correlate positively with age in this study in a very highly significant way for the Thai group; men who were grey before aged 30, $p=0.003$ (see Figure 5.4), the correlation for European men was weaker and was not significant, $p=0.33$ (see Figure 5.5) which probably reflects both the smaller subject number of this group and the not fully randomised method of recruitment. This fits with previous reports that the extent of androgenetic alopecia correlates with age (Hamilton, 1951; Norwood, 1975; Ellis & Harrap, 2001), and confirms the relationship found in the earlier 12 month trial of herbal mixture Xiantene (Chapter 3). In subjects with the inherited susceptibility, the hair follicles in response to androgens, begin to miniaturise and there is an increased lag before the new smaller anagen hair is produced, leading eventually to follicular atrophy and baldness on pre-determined areas of the scalp. This process, androgenetic alopecia, correlates positively with age although in this experiment the correlation between hair greyness and age was found to be stronger. There was no difference between the relationship of androgenetic alopecia and age in men who were grey either before or after aged 30 in any of the groups. However, men who went grey by age 30 differed in the extent of their

androgenetic alopecia (see Figure 5.9) and had significantly lower Hamilton stages compared with controls i.e. the subjects who were not grey before 30, in all groups. Thai men who were grey before aged 30, balding measured by Hamilton stage 2.53 ± 0.16 , Thai men who were grey after aged 30, Hamilton stage 2.93 ± 0.06 ($p=0.04$) and European men who were grey before aged 30, extent of balding, Hamilton stage 2.62 ± 0.26 and European men who were grey after aged 30, Hamilton stage 3.48 ± 0.16 ($p=0.01$). Examination of the data for all men over 40, with and without an adjustment for age, showed that there was no significant difference in the grey before and grey after 30 groups for the Thai men in the extent of androgenetic alopecia ($p=0.28$) but there was a significant difference for the European men ($p=0.05$) and if all men were combined, the men grey by 30 were significantly less bald ($p=0.01$) than the men not grey by 30. In both study groups the men who went grey age 30 years or before, had significantly less hair loss although the difference was greater for the European group than the Thai group. The lower Hamilton score in all of the early canities groups opposes predictions if both canities and baldness were solely age related. Early pigment loss appears inversely related to male pattern alopecia extent.

Androgens orchestrate the progression of androgenetic alopecia in genetically pre-disposed individuals (Hamilton, 1951; Randall, 2007) and androgens can also influence hair pigmentation by inhibiting dermal papilla SCF production and melanocyte activity (Hibberts et al, 1996; Randall et al, 2008). In most young blonde north European people, the hair colour does not fully develop until late teens (Sunderland, 1956) and vellus hairs are

replaced by larger, thicker pigmented terminal hairs in many areas at puberty under the influence of androgens (Hamilton, 1946, reviewed Randall, 2007). Interestingly, loss of scalp hair pigment and hair greyness occurs initially at the margins and in particular the occipital and parietal areas (Keogh & Walsh, 1964). These are the androgen insensitive areas where androgenetic alopecia does not usually proceed which may indicate that the two conditions, androgenetic alopecia and canities, are not completely independent of each other. Canities also occurs in women in the same pattern as men which does suggest that the greying process is independent of androgens unlike androgenetic alopecia which is androgen dependent. Although hair greyness (Tobin & Paus, 2001; Van Neste & Tobin, 2004) and androgenetic alopecia (Rebora, 2004; Randall, 2007; Otberg et al, 2007) are both positive markers of the aging process and are both main areas of hair research, a possible link between the two conditions does not appear to have been investigated and no reference to this could be found. Early canities has however been tentatively linked to other conditions; e.g. low bone mineral density (Morton et al, 2007). During active pigmentation of the hair shaft the synthesis of melanin by the melanocytes produces considerable oxidative stress (Tobin & Paus, 2001; Arck et al, 2006). The free radical theory of ageing (Tobin & Paus, 2001) proposes that the normal cell function is damaged or compromised by these circulating, highly reactive free radicals (Arck et al, 2006) which can lead to diseases such as Alzheimer's disease and to certain cancers (Kim et al, 2007; Kulbacka et al, 2009). Oxidative stress is implicated in cellular aging and senescence (Hayflick, 2007) as the body's anti-oxidant system becomes less effective. Cellular sensitivity to

peroxidising agents has been shown (Grammatico et al, 1997); cultured pigmented hair follicles exposed to external oxidative stress (hydroquinone) showed increased apoptosis of melanocyte population in the hair bulb (Arck et al, 2006) and H₂O₂ mediated oxidative stress has been shown to be a key element in senile grey graying (Wood et al, 2009). In this study early canities appears to delay the onset or progression of androgenetic alopecia and the loss of pigment synthesis or the absence of melanin itself possibly, lowers these toxic or inhibitory factors, allowing hair growth to proceed faster and more fully (Van Neste, 2004; Arck et al, 2006) despite the androgen drive to miniaturisation. Unpigmented hairs in the scalp and beard are known to be thicker and to grow faster than pigmented hairs (Nagl, 1995; Van Neste, 2004). This observation has been confirmed using scalp hairs in culture (Arck et al, 2006), suggesting that the synthesis of melanin or its presence in the hair follicle interferes with or reduces the rate of hair growth. Hair growth rate and hair diameter are known to decrease with age and alopecia (Van Neste et al, 1991), however the reduced rate of hair growth of terminal hairs is limited to the pigmented hairs (Van Neste, 2004) and the white hairs are spared from this ageing process. It is possible that grey hair does not suffer the full miniaturisation and atrophy characteristic of androgenetic alopecia, and loss of pigment may offer protection from genetic hair loss.

5.7. Conclusion

This study suggests that early canities in men (i.e. before the age of 30 years), may delay the full onset of androgenetic alopecia.

6. Summary and conclusions

Hair is the distinguishing feature of the mammals, providing insulation and protection and the ability to vary coat colour and pattern with season and age, via the hair cycle, gives camouflage with social and sexual communication. The communication aspects are particularly important for human beings whose body hair is sparse remaining abundant essentially only on the scalp. Pre-puberty children have abundant and full scalp hair, like adults, often not fully pigmented (Sunderland, 1956), but the arrival of androgens in both sexes initiates the specific body hair patterns of adulthood (Randall, 2007).

The ageing of human hair is pronounced and highly visible compared with skin ageing, and the loss or change to scalp hair, especially when it is premature, can cause considerable emotional stress e.g. male pattern hair loss (Cash, 1992). Loss of hair pigment, which occurs in both sexes, is thought to be caused by a genetically regulated exhaustion of active melanocytes in the hair matrix which produce the pigment melanin and results in grey or white hair (Peters et al, 2002). As a rule of thumb approximately 50% of people are 50% grey by the age of 50 years and all hair colour is usually lost by the 7th or 8th decade (Keogh & Walsh, 1965), when the melanocytes become inactive and no hair colour is produced. The loss of hair pigment and the appearance of grey hair is one of the key markers of the ageing process (Bulpitt et al, 2001), although scalp hair may be very easily and effectively artificially coloured to disguise this part of the ageing process.

The second hair ageing phenomenon is the permanent loss of scalp hair in the progression of androgenetic alopecia, predominantly in men (Hamilton, 1951); this loss of hair and indicator of ageing is not as easily disguised as the appearance of greyness and in a youth and appearance-orientated society loss of scalp hair can be a highly distressing condition. In persons with a genetic predisposition androgens, post puberty, orchestrate changes to the natural hair growth cycle on pre-determined areas of the scalp. Both men and women can develop androgenetic alopecia and although androgens are the agents for men (reviewed, Randall, 2007), in women the mechanism is not so clear cut (Cusen & Messenger, 2010). Initially the anagen phase begins to reduce in length over successive hair cycles, the hair becoming shorter and smaller in diameter and the dermal papilla at the base of the hair follicle begins to get smaller (Whiting, 1993). Also a lag develops between the shedding of the previous telogen hair and the appearance of the new hair (Whiting, 2001). Eventually over many hair follicle cycles the hair follicle miniaturises, atrophies and is lost; the scalp hair produced changes from terminal to vellus before being finally lost and bald patches (alopecia) appear on the scalp, in a characteristic pattern (Rushton et al, 1991). In men, bi-temporal recession and loss of hair on the crown may lead to semi or complete baldness and is graded in severity by the Hamilton stage 1-7 (Hamilton, 1951).

This thesis looked at the two key markers in men of hair ageing, canities or hair greyness and androgenetic alopecia or patterned hair loss. In the first set of experiments a herbal mixture Xiantene, based on 8 traditional Chinese medicine herbs was assessed to see if it would affect hair growth in men with

androgenetic alopecia. In the final experiment a possible relationship between the two ageing characteristics, hair greying and androgenetic alopecia was examined. The hypothesis that men who suffer early hair greyness (before 30 years) do not develop the full extent of hair loss in androgenetic alopecia, was examined.

In the studies on herbal mixture Xiantene, men with androgenetic alopecia used daily topical applications of Xiantene or a vehicle for either three (Chapter 2) or twelve (Chapter 3) months. Hair growth was assessed on the crown using the unit area trichogram method of assessment (Rushton et al, 1983). A total of 35 subjects were recruited, but only 12 managed to successfully complete the 3 month experiment, a high drop-out rate of 65.7%. A total of 48 men commenced the 12 month study and 29 men were successfully re-examined at the end of the 12 months. The drop out rate of 39.6% was high, but better than the 3 month study (65.7%). The pre treatment data from men completing either the 3 month trial or 12 month experiments (n=41) were combined to examine the relationship between the extent of balding, measured by the Hamilton stage, with measured hair parameters and with age. The extent of balding showed a significant negative correlation with the total number of hairs in an area of 50.7 mm² on the crown, correlation coefficient = -0.56 (p=0.001) and with the number of anagen hairs in the area, correlation coefficient = -0.60 (p<0.001) and a weak negative correlation with the anagen:telogen ratio, correlation coefficient = -0.09 which was not significant (p=0.60). This data corresponds to the previous data (Hamilton, 1942; Rushton et al, 1983) and confirms that as the extent of baldness, measured by the Hamilton stage, increases, the

number of visible hairs on the crown decreases. In contrast, when the extent of baldness as indicated by the Hamilton stage was related to age there was a significant positive correlation, correlation coefficient = +0.35 (p=0.03) confirming that balding increased with age (Hamilton, 1942; Rushton et al, 1983).

Men (n=6) treated with Xiantene for 3 months showed a significant 15.33% increase in the total number of hairs (p=0.045) and a very highly significant (p=0.00033) increase of 23.71% in the number of anagen hairs unlike the vehicle group where there were no significant changes (Chapter 2). Neither the Xiantene treated (p=0.50), or the vehicle treated (p=0.78) groups showed any significant difference in the number of telogen hairs, nor changes in mean hair diameter measurements. Interestingly only one hair diameter measurement was below the 40 μm threshold proposed for meaningful hairs (Rushton et al, 1993) meaning that the method of detection used was essentially unable to detect vellus hairs. The Xiantene treated group also showed a significant increase in pigmentation of anagen hairs (p=0.01) unlike those using the vehicle (p=0.09). However, there was some evidence that this may have been a staining or dye effect from the Xiantene mixture itself meaning that this finding cannot be relied upon, although it would be consistent with increased melanogenesis in the hair bulb if the treatment with Xiantene was showing a positive effect.

Since this increase in hair growth using herbal mixture Xiantene was encouraging, but the subject number small and the results may have been by influenced by seasonal variation (Randall & Ebling, 1991), these experiments were repeated using a new, larger cohort of men with androgenetic alopecia

for a full year. Examination of the data from the 12 month study (Chapter 3) also showed a highly significant increase in the total number of hairs for the Xiantene treated group ($p=0.003$) unlike the vehicle treated group where was no significant change. The number of anagen hairs also increased very significantly again for the Xiantene treated group ($p<0.001$) with no significant change in the vehicle group. In the larger 12 month study the anagen:telogen ratio also increased for the Xiantene treated group ($p=0.005$) while there was no significant change for the vehicle group. The experimental data in this second study was not affected by seasonal variation as it was run for a full 12 month period (Randall & Ebling, 1991) and confirms the results of the 3 month experiment, which used different subjects and was not run concurrently. These observations suggest that herbal mixture Xiantene could promote a cessation or partial reversion in the progression of androgenetic alopecia.

Treatment with Xiantene significantly increased the number of meaningful hairs unlike the vehicle treatment. This contrast with the control group and also what would be expected on the scalp of men with androgenetic alopecia where some deterioration would have been expected (Rushton et al, 1991). It is possible that terminal hair follicles have been persuaded to stay in the anagen phase for longer and not enter telogen, or more likely that hair follicles in the lag period (Whiting, 2001) have been prompted to enter the anagen phase, thus increasing the number of anagen hairs while the number of telogen hairs remain insignificantly different. A reduction in the duration of the lag phase in the reversal of androgenetic alopecia in men by finasteride was found to be the key mechanism (Van Neste, 2006). It is unlikely that

vellus hair follicles have been converted back to fully developed terminal hair follicles in the 12 months of the study, the mechanism of androgenetic alopecia is more likely to have been delayed rather than fully reversed by the treatment with herbal mixture Xiantene.

Attempts to analyse and confirm the herbal extracts and their proportions in the Xiantene mixture, using thin layer chromatography were only partial successful (Chapter 4). The Xiantene mixture used in the 12 month study, Xiantene II, was described by the suppliers as an upgraded and improved version of the Xiantene mixture used for the 3 month study, Xiantene I. The assay results for the two mixtures did differ, although they are claimed by the suppliers to each contain the same 8 Chinese herbs. Fortunately the item that was subsequently banned for use in the U.K. *Stephania tetrandia* S, could not be identified in either mixture. All the other herbs could be identified in either Xiantene I or Xiantene II apart from *Thuja orientalis* L seeds which was not in either although the leaves of *Thuja orientalis* L were identified in Xiantene II. Interestingly there was evidence from the thin layer chromatography data of components present in both that could be not identified and did not match the reference samples. Only *Cassia cinnamomum* twigs could be positively identified in both Xiantene I and Xiantene II. There may be a number of reasons for the differences in the analytical data from that described by the suppliers. The reference herbs used for comparison in the analysis were purchased in the U.K. and may not be reliable and no standard reference data were available. There are limitations in the thin layer chromatography methods used here for the analysis of complex herbal mixtures, even though a multiple solvent system

was used. The formulation data from the manufacturers in China may be unreliable and the herbs used may vary. Traditional Chinese medicine products are notoriously difficult to identify and quantify with certainty (Lu et al, 2007) and more sophisticated techniques than those used here would be required together with access to certified reference material data for comparison.

The mode of action of Xiantene mixtures is unclear, however 5 of the 8 herbs confirmed as present in at least one Xiantene (*Thuja orientalis L*, *Polygonum multiflorum*, *Panax ginseng*, *Panax notoginseng*, *Istatis indigotica fort*) are known to be enzyme inhibitors (Ehrman et al, 2007). *Thuja orientalis L* extracts have both mitogenic activity and potent enzyme inhibition activity (Gohla, 1989; Offergeld, 1992) and *Thuja* extracts can inhibit the enzyme 5 α -reductase type II (Park et al, 2003). The conversion of testosterone by the enzyme 5 α -reductase to the metabolite 5 α -dihydrotestosterone is a key step in the progression of common baldness and is the process inhibited by finasteride, the most successful androgen-selective treatment for male pattern baldness (Kaufman et al, 1998). The enzyme inhibition properties of Xiantene mixture are a possible mode of action for the stimulation of hair growth in the two studies of herbal mixture Xiantene.

A second set of experiments investigated the hypothesis that the premature loss of hair pigmentation, e.g. before 30 years of age, may afford protection from the onset of androgenetic alopecia in men (Chapter 5). When 710 men were examined, 502 Thai and 208 European and the extent of their pigmentation loss, hair greyness, was related to age there was a highly significant positive correlation for all Thai and European groups whether or

not they went grey before 30 years ($p < 0.001$). The correlation was stronger for the men who went grey by the age of 30; Thai men correlation coefficient = 0.47, European men = 0.62. This was consistent with a normal increase in pigment loss with age (Keogh & Walsh, 1965). Androgenetic alopecia was also found to correlate positively with age for all subjects, Thai and European whether or not they were grey before 30 years. However the positive correlation of androgenetic alopecia with age was stronger for the Thai men ($p < 0.001$) than the European men who showed a weak positive correlation ($p = \text{not significant}$). When the grey before and after 30 years group were compared there was a stronger correlation between age and baldness for the Thai men who were grey after 30 years ($p < 0.001$) than those who were not ($p = 0.003$). All subjects, Thai and European, confirmed that androgenetic alopecia increased with age (Rushton et al, 1983).

The mean ages of the grey 30 years before and after groups were not significantly different; Thai men ($p = 0.13$), European men ($p = 0.33$) allowing valid comparison between groups. However the mean ages comparing the Thai men and European men, both grey before and after 30 years were significantly different ($p < 0.001$) which did not permit comparison between different geographical groups.

However, when the degree of hair loss, measured by the Hamilton stage (Hamilton, 1975) modified by Norwood (Norwood, 1975) was examined, the men who went grey by the age of 30 differed in the extent of their baldness compared with controls. The men who were grey before 30, had a significantly lower mean Hamilton stage in all groups, (Thai $p = 0.04$, European $p = 0.01$). This significantly reduced hair loss in men who went grey

age 30 or before would not be expected if both canities and baldness were solely age related. Therefore, although hair greying before 30 years does not prevent hair loss as many of the early greying men showed androgenetic alopecia, canities (age 30 years or before) appears to delay the onset or progression of androgenetic alopecia, supporting the original hypothesis.

The reactive oxygen species (R.O.S.) theory of ageing (Hayflick, 2007) proposes that cell function is gradually damaged or compromised by circulating highly reactive free radicals, byproducts of normal metabolic activity. Hair pigment production in the hair matrix produces considerable oxidative stress (Tobin & Paus, 2001) and apoptosis of the melanocyte population due to external oxidative stress has been shown (Arck et al, 2006; Wood et al, 2009). In this study loss of pigmentation synthesis or the absence of melanin itself possibly lowers these toxic or inhibitory factors allowing hair growth to proceed faster or more fully (Arck et al, 2006), despite the androgen drive to miniaturisation (Randall, 2007).

Unpigmented hairs of the beard (Nagl, 1995) and scalp (Van Neste, 2004) are known to be thicker and to grow faster and this has been confirmed by *in vitro* studies of scalp hair follicles in organ culture (Arck et al, 2006). In the progression of androgenetic alopecia, hairs in the affected areas become smaller in diameter and grow more slowly (Van Neste, 1991) however these changes appears to be limited to the pigmented hairs only (Van Neste, 2004) and the unpigmented or grey hairs appear to grow normally and this study suggests that it is possible that the grey or unpigmented hairs do not suffer

the full miniaturisation and atrophy characteristic of androgenetic alopecia. Hair pigment loss may offer protection from genetic hair loss.

In this thesis, Xiantene, a mixture of 8 traditional Chinese medicine herbs, when applied topically to the scalp has been shown in two separate double blind vehicle controlled studies to slow the rate of progression of androgenetic alopecia. This warrants further investigation as this may lead to a new topical treatment for androgenetic alopecia. It will be important to classify which are the active ingredients. The phenomenon of early canities in men was also shown to inhibit the progression of androgenetic alopecia. Men who experienced the appearance of grey hair before the age of 30 years were found to be very significantly less bald than men who were grey after 30 years. Loss of pigment production appears to reduce the toxic or inhibitory factors associated with pigmentation allowing the resultant unpigmented hair to grow faster and thicker, counterbalancing at least in part the atrophy of androgenetic alopecia.

7. Future work

The results with the use of Xiantene herbal mixture in the treatment of androgenetic alopecia were interesting, but not conclusive and a further investigation would be warranted. Firstly, it would be important to standardise the mixture itself, either with a standardised product of precisely known composition, which could be verified, from the Chinese manufacturers themselves or possibly by re-manufacturing the product from verifiable extracts here in the UK, although though this second course might result in the loss of some or all of the efficacy of the product. Secondly, the high drop out rate in subjects commencing the study would have to be addressed. This could be accomplished by using the facilities of a professional hair treatment clinic where subjects pay for their treatment having previously been advised of the novel nature of the application. The unit area trichogram method is precise and reproducible, but it is time consuming and a number of treatment operators would be required to perform a larger investigation.

Further analysis using reduced numbers of components in the mixtures should clarify whether the effects are due to one component or the interactions of several. For example, initially dividing the components into two groups and assessing each separately, to see if one, both or neither had any effect, would determine whether all components are necessary together or not. If there were no effects with any, this would indicate some synergy or interactions between the components. If only one had an effect, this could be further broken down to eventually establish if the effects were due only to

one component. If so, this may lead to a single plant extract and the active ingredient similarly established. If so, this could be concentrated or even synthesised to remove the variations inherent in Chinese medicine medications.

The results of the grey/MPA survey were very interesting, suggesting a significantly lower baldness score for men who go grey early. A repeat experiment to confirm and extend these findings would be appropriate. The single examiner system used here is fast and accurate and a larger trial in Thailand could be easily accomplished. Additional questions such as diet, lifestyle etc. could possibly be included from which further conclusions might be drawn. Also, the very early greying of children at puberty, which was recorded here is most interesting and much larger survey might enable this area to be investigated. With sufficient extra data it might be possible to propose a mechanism by which the hormonal changes at puberty are able to “knock out” the pigmentation process.

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Appendices

Appendix I

Cosmetic procedure during trial (3 months)

- (1) Shampoo hair every day or at least every other day. Do not towel, dry the hair vigorously.
- (2) Apply test material by single shot spray (1ml) to the crown area EACH MORNING. Leave to dry.
- (3) Comb hair as required. Comb or brush hair 1 hour before hair sample is taken.
- (4) Do not use any other treatment on the hair and scalp apart from the shampoos and conditioners.

Appendix II

Validation of methodology for unit area trichogram

A total of 14 circles were drawn on a scalp, using the template (Chapter 2 and 3) and the areas were calculated. The mean area \pm SEM and the coefficient of variation (4.94%) are shown below.

Circle No	Diameter (mm)	Radius (mm)	Area (sq mm)
1	7.67	3.835	46.22271
2	7.83	3.915	48.17128
3	8.00	4.000	50.28571
4	7.67	3.835	46.22271
5	7.83	3.915	48.17128
6	7.67	3.835	46.22710
7	7.50	3.750	44.19643
8	7.83	3.915	48.17128
9	7.70	3.850	46.58500
10	7.80	3.900	47.80286
11	7.70	3.850	46.58500
12	7.70	3.850	46.58500
13	7.20	3.600	40.73143
14	7.50	3.750	44.19643

Area calculations	
Mean	46.439
Standard Error	0.613
Median	46.585
Mode	46.223
Standard Deviation	2.295
Sample Variance	5.269
Kurtosis	2.160
Skewness	-0.991
Range	9.554
Minimum	40.731
Maximum	50.286
Sum	650.150
Count	14
Largest (1)	50.286
Smallest (1)	40.731
CoVar% (SD/Mean%)	4.943

Appendix III

Hair shaft parameter measurements for trial subjects using Xiantene, before and after treatment

Before	P3	P13	P15	P17	P18	P20
total	70	48	34	79	55	74
A	51	35	19	56	42	50
T	19	13	15	23	13	24
T/A	0.372549	0.371429	0.789474	0.410714	0.309524	0.48
after						
total	73	51	39	91	80	81
A	59	41	29	68	52	64
T	14	10	10	23	28	17
T/A	0.237288	0.243902	0.344828	0.338235	0.538462	0.265625
Telogen						
Before						
Diameter	47.639	48.027	60.059	57.935	66.066	58.860
SD	14.899	19.233	28.944	5.823	22.326	1.298
SE	6.663	8.601	12.944	2.604	9.985	0.580
After						
Diameter	41.747	68.570	41.400	55.563	90.190	57.307
SD	16.145	15.191	16.264	4.931	11.696	4.935
SE	7.220	6.794	7.273	2.205	3.020	2.207
Before						
Grayness	84.474	86.216	159.079	142.740	130.614	106.193
SD	16.412	23.485	24.134	15.593	22.167	15.023
SE	7.340	10.503	10.793	6.973	9.913	6.718
After						
Grayness	74.485	54.297	134.618	133.424	115.551	105.880
SD	18.395	7.755	20.726	9.186	13.873	13.858
SE	8.226	3.468	9.269	4.108	6.204	6.198
Anagen						
Before						
diameter	89.350	85.711	56.331	70.239	98.605	69.339
SD	10.879	8.193	27.558	11.018	13.215	13.140
SE	2.809	2.116	9.743	2.845	3.412	3.393
After						
Diameter	85.882	89.700	87.725	62.984	90.190	79.067
SD	15.744	10.183	30.657	13.617	11.698	8.635
SE	4.065	2.629	7.916	3.516	3.020	2.229
Before						
Grayness	43.296	40.311	114.016	112.243	98.863	88.236
SD	9.338	3.575	23.603	23.458	25.844	21.704
SE	2.411	0.923	8.345	6.057	6.673	5.604
After						
Grayness	34.739	32.660	111.250	104.063	80.699	83.602
SD	8.968	4.252	22.785	27.173	20.336	18.645
SE	2.315	1.098	5.883	7.016	5.251	4.814

Appendix IV

Hair shaft parameter measurements for trial subjects using vehicle, before and after treatment

Before	P4	P9	P12	P19	P29	P31
total	70	46	46	79	74	28
A	46	29	42	44	55	23
T	24	17	4	35	19	5
T/A	0.521739	0.586207	0.095238	0.795455	0.345455	0.217391
after						
total	72	56	30	64	68	52
A	50	41	26	37	46	47
T	22	15	4	27	22	5
T/A	0.44	0.365854	0.153846	0.72973	0.478261	0.106383
Telogen						
Before						
Diameter	64.124	45.912	38.487	60.464	65.446	62.492
SD	3.851	13.564	0.438	18.902	10.966	23.110
SE	1.722	6.066	0.310	8.453	4.904	16.341
After						
Diameter	56.605	60.745	48.575	77.412	60.910	42.309
SD	7.479	13.643	15.606	7.098	11.863	11.298
SE	3.345	6.101	9.010	3.174	5.305	5.649
Before						
Grayness	105.620	139.929	175.300	63.950	73.178	135.625
SD	10.477	28.893	6.423	11.713	4.280	2.272
SE	4.685	12.921	4.542	5.238	1.914	1.606
After						
Grayness	121.033	140.145	179.232	67.415	73.469	129.763
SD	16.177	19.906	22.043	9.653	16.394	20.258
SE	7.235	8.902	12.727	4.317	7.332	10.129
Anagen						
Before						
diameter	79.921	70.300	74.448	91.146	64.270	62.922
SD	16.089	11.742	15.568	16.553	15.132	12.363
SE	4.154	3.032	4.020	4.274	3.907	3.728
After						
Diameter	91.997	82.499	64.984	86.112	77.519	48.804
SD	17.765	10.755	10.707	28.784	14.566	9.772
SE	4.587	2.777	2.764	7.432	3.761	2.523
Before						
Grayness	58.074	75.926	118.997	44.401	52.959	93.999
SD	12.787	16.147	38.114	7.507	12.515	25.595
SE	3.302	4.169	9.841	1.938	3.231	7.717
After						
Grayness	74.392	82.904	134.941	48.537	47.451	98.541
SD	31.492	23.244	32.447	12.727	10.846	26.171
SE	8.131	6.002	8.378	3.286	2.800	6.757

Appendix V

Chapter 3 alternative statistical analysis (Dr. D.H. Rushton)

Unpaired t test

Do the means of Control-12 and Treated-12 differ significantly?

P value

The two-tailed P value is 0.4914, considered not significant.

t = 0.6976 with 27 degrees of freedom.

95% confidence interval

Mean difference = -3.900 (Mean of Treated-12 minus mean of Control-12)

The 95% confidence interval of the difference: -15.370 to 7.570

Assumption test: Are the standard deviations equal?

The t test assumes that the columns come from populations with equal SDs. The following calculations test that assumption.

F = 1.345

The P value is 0.6277.

This test suggests that the difference between the two SDs is not significant.

Assumption test: Are the data sampled from Gaussian distributions?

The t test assumes that the data are sampled from populations that follow Gaussian distributions. This assumption is tested using the method Kolmogorov and Smirnov:

Group	KS	P Value	Passed normality test?
Control-12	Normality test requires raw data.		
Treated-12	Normality test requires raw data.		

Summary of Data

Parameter:	Control-12	Treated-12
Mean:	53.100	49.200
# of points:	12	17
Std deviation:	13.510	15.668
Std error:	3.900	3.800
Minimum:	Unknown	Unknown
Maximum:	Unknown	Unknown
Median:	Unknown	Unknown
Lower 95% CI:	44.516	41.144
Upper 95% CI:	61.684	57.256

Appendix VI

Grey/Androgenetic alopecia survey form

CODE NO:

PLEASE ANSWER EACH OF THE EIGHT QUESTIONS BELOW

1. DATE OF BIRTH:

2. HEALTH POOR [] AVERAGE [] GOOD []

3. HAIR DYED? YES [] NO []

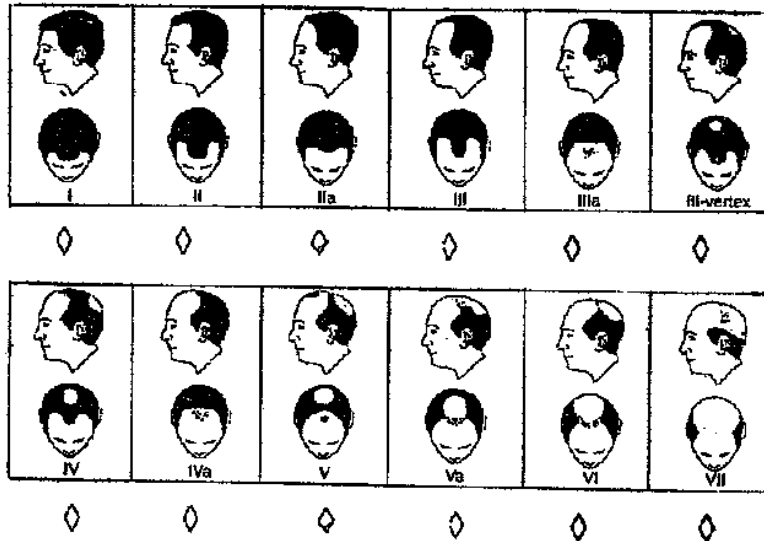
4. OTHER HAIR TREATMENT?
(MINOXYDIL ETC.) YES [] NO []

5. DO YOU HAVE ANY HAIR GREYNESS? YES [] NO []

6. AGE AT WHICH GREYNESS FIRST APPEARED
[APPROXIMATELY]

7. HAIR LOSS SCALE
(HAMILTON)

[PLEASE TICK BOX
WHICH IS THE
NEAREST TO YOUR
CONDITION.]



8. HAIR GREYNESS SCALE
(DAVIES)

[PLEASE TICK BOX
WHICH IS THE
NEAREST TO YOUR
CONDITION.]

