Experimental Dermatology ADF

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| Journal: | *Experimental Dermatology* |
| Manuscript ID | EXD-19-0074.R1 |
| Manuscript Type: | Review Article |
| Date Submitted by the Author: | 24-May-2019 |
| Complete List of Authors: | Palmer, Megan; University of Huddersfield<br>Blakeborough, Liam; University of Huddersfield<br>Harries, Matthew; Salford Royal NHS Foundation Trust, ; University of Manchester Faculty of Biology, Medicine and Health,<br>Haslam, Iain; University of Huddersfield, |
| Area of Expertise: | Hair and other skin appendages |
| Keywords: | ALOPECIA AREATA, ANDROGENETIC ALOPECIA, HAIR FOLLICLE, KERATINOCYTES |
| Additional Keywords: | Cholesterol, Hair follicle, Alopecia, Statins |
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# Cholesterol homeostasis: links to hair follicle biology and hair disorders

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**Key Words:** Cholesterol, transporter, hair follicle, alopecia, hypertrichosis



## Abstract

Lipids and lipid metabolism are critical factors in hair follicle (HF) biology and cholesterol has long been suspected of influencing hair growth. Altered cholesterol homeostasis is involved in the pathogenesis of primary cicatricial alopecia, mutations in a cholesterol transporter are associated with congenital hypertrichosis and dyslipidaemia has been linked to androgenic alopecia. The underlying molecular mechanisms by which cholesterol influences pathways involved in proliferation and differentiation within HF cell populations remains largely unknown. As such, expanding our knowledge of the role for cholesterol in regulating these processes is likely to provide new leads in the development of treatments for disorders of hair growth and cycling. This review describes the current state of knowledge with respect to cholesterol homeostasis in the HF along with known and putative links to hair pathologies.

**Running title:** Cholesterol and hair disorders

**Word Count:** 6385

**Figures:** 2

## 1. INTRODUCTION

Cholesterol is vital to the normal function of all animal cells and has particular importance in cutaneous tissues. In addition to forming an important component of cell membranes, partitioning into the phospholipid bilayer to regulate membrane fluidity [1], cholesterol also regulates cell signalling *via*, for example, modulation of hedgehog protein (Hh) biogenesis and activation of the canonical Wnt pathway [2,3]. Cholesterol performs tissue-specific functions including in the maintenance of the skin permeability barrier [4,5] and acts as a precursor for steroid hormone synthesis [6,7].

The importance of cholesterol is underscored by the capacity of vertebrate cells for *de novo* biosynthesis [8]. Furthermore, cells can receive cholesterol from circulating lipoproteins (i.e. low density lipoprotein; LDL, or high density lipoprotein; HDL), with removal of excess cholesterol facilitated by membrane efflux pumps, such as ATP-binding cassette transporter (ABC) A1 and ABCG1. As cellular cholesterol balance must be maintained within a relatively narrow concentration range, physiological feedback loops exist to control the rates of biosynthesis, uptake and efflux that form such a crucial part of cellular cholesterol metabolism.

The roles for cholesterol in peripheral tissues have been previously described. In the skin, cholesterol is a protagonist in the development of the epidermal permeability barrier [4,5], is a precursor for the synthesis of local steroid hormones [7,9] and influences keratinocyte differentiation [10-12], corneocyte desquamation [11], barrier repair [13] and melanogenesis [14,15]. Yet there remains one important skin appendage in which the role of cholesterol is yet to be fully explored, namely the hair follicle (HF). Whereas lipids are understood to impact on HF biology, not least through the HF-association with the lipid-rich sebaceous gland, the specific functions modulated by cholesterol are less well understood. Associations have been made between sterol levels and certain hair disorders, and lipid-modulatory drug therapies have been reported to cause both hair loss and hair growth [16-21].

A greater understanding of the control of cellular cholesterol in the HF and the potential impact on the hair cycle may identify novel targets for regulating hair growth and the treatment of hair disorders linked to disordered sterol homeostasis or sterol-sensitive signalling pathways [22-24].

## 2. CHOLESTEROL HOMEOSTASIS: A CELLULAR OVERVIEW

Cellular cholesterol metabolism has been covered in detail in many previous reviews [2,25-28]. This section is therefore restricted to providing a generic summary of cellular cholesterol homeostasis.

*De novo* cholesterol synthesis accounts for approximately 70% of the total body cholesterol. Primarily, this occurs through a series of defined reactions in the endoplasmic reticulum, with the acetyl CoA precursor ultimately metabolised to cholesterol, with 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) catalysing the rate-limiting step [2,26]. The pathway is summarised in Figure 1A.

Uptake in the peripheral tissues occurs primarily through the binding of LDL to the LDL receptor (LDLR) followed by receptor mediated endocytosis [26]. An additional route for cholesterol uptake exists via the scavenger receptor class B member 1 (SRB1) receptor, which mediates uptake of free cholesterol and cholesterol esters from circulating HDL [29].

ABC transporters play an important role in the transport of lipids, in particular the ABCA family is involved in both cholesterol efflux and intracellular transport. ABCA1 is a well-characterised cholesterol transporter, and is ubiquitously expressed. It mediates the efflux of excess cholesterol from cells to apolipoproteins (apo), in particular apoA1 for the formation of HDL for reverse cholesterol transport [27,28].

Intracellular trafficking of cholesterol can occur via vesicular and non-vesicular movement. Vesicular transport involves ATP-dependent movement along the cellular cytoskeleton. Non-vesicular transport occurs through hydrophobic cavity transporter proteins, such as steroidogenic acute regulatory protein (StAR) and through spontaneous resorption which is thought to be processed through as yet unidentified specialist proteins [30].

Numerous transcription factors including sterol regulatory binding protein (SREBP2) (Figure 1A), liver X receptor (LXR) and peroxisome proliferator-activated receptors (PPAR), have primary roles in regulating the expression of genes involved in maintaining cholesterol homeostasis.

The roles of cholesterol in keratinocytes and the HF specifically are detailed in the following sections, with reference to the homeostatic mechanisms outlined above.

## 3. CHOLESTEROL FUNCTION IN KERATINOCYTE CELL BIOLOGY PROVIDES LINKS TO HF SIGNALLING PATHWAYS

The HF is a regenerating, hair shaft producing mini-organ that undergoes cyclical periods of growth (anagen) (Figure 2), regression (catagen) and relative quiescence (telogen) [31]. The direct impact of cholesterol metabolism on distinct HF cell populations is unclear, yet it is likely to play an important role. Although differentiation of matrix keratinocyte in the hair bulb is distinct from that of epidermal keratinocytes, the roles for cholesterol in epidermal physiology can nevertheless provide pointers to the control of keratinocyte behaviour in the HF. This is further discussed below.

### 3.1 Cholesterol regulates keratinocyte proliferation and differentiation

Lipids are a necessary component of the epidermal barrier and as such, lipid metabolism has been extensively examined in epidermal keratinocytes (see Feingold, Elias [32]). Many studies have determined that cellular cholesterol levels can modulate and be modified by the proliferative nature of keratinocytes as well as their differentiation status [33-38]. To this end, Sporl, *et al.* [12] previously demonstrated that cyclodextrin-mediated depletion of membrane cholesterol in primary human keratinocytes disrupts lipid raft formation, causing a loss of both early and terminal differentiation markers, keratins 1, 2 and 10, coupled to an increase in proliferation [12]. In parallel, Mathay, *et al.* [39] showed that disruption of lipid rafts resulted in the downregulation of filaggrin gene expression [39]. Intrinsically, these studies tell us that cholesterol status is innately linked to normal keratinocyte behaviour.

It is however important to distinguish between different sterol forms when discussing biological activity. In this regard, oxysterols (25-hydroxycholesterol and 22R-hydroxycholeserol) but not free cholesterol or its precursor mevalonate, have been shown to induce keratinocyte differentiation through upregulation of involucrin and transglutaminase 1 [33]. Oxysterols activate the α and β isoforms of LXR, a transcription factor previously reported to regulate keratinocyte differentiation [33][40]. Activation of LXR by the specific agonist

T0901317 reduces proliferation in epidermal keratinocytes and is reported to reduce hair growth in *ex vivo* human HFs [41].

Of importance in epidermal keratinocyte function is cholesterol sulfate (CS), present at high levels in the stratum granulosum. CS increases the expression of differentiation markers, filaggrin, loricrin, involucrin and transglutaminase 1, as well as regulating desquamation in the stratum corneum [42-44]. CS itself is an inhibitor of cholesterol synthesis, and if present in excess leads to reduced cholesterol levels and a mild-impairment of barrier function in x-linked ichthyosis, a disease associated with loss-of-function mutations in the gene coding for the steroid sulfatase normally responsible for reducing CS levels [44]. Activators of LXR and PPAR, both of which stimulate keratinocyte differentiation in addition to controlling cholesterol metabolism, also regulate cholesterol sulfotransferase type 2B isoform 1b (SULT2B1b), an enzyme involved in CS synthesis [11]. Mutation in SULT2B1b leads to a congenital ichthyosis, in this case autosomal recessive congenital ichthyosis [45].

Cholesterol metabolism and the maintenance of sterol isoforms with defined levels is therefore required to maintain skin health, yet the precise role for both oxysterols and CS in regulating the activity of HF matrix keratinocytes remains to be elucidated. Indeed, 25-hydroxycholesterol can for example, reduce HMGCR activity in human HFs [46] and CS is an integral lipid of hair fibres [47], but the functional significance is unclear. A study by Brosche*, et al.* [48] showed an increase in CS levels in hair clippings from patients with elevated serum LDL levels, despite total cholesterol levels remaining constant. In the HF, CS is not involved in desquamation and therefore must have alternative roles in regulating or controlling differentiation and/or adhesion of trichocytes in the hair shaft.

Current evidence shows that cholesterol; its products and intermediates are associated with epidermal keratinocyte differentiation, defects in which can result in epidermal barrier impairment. As such, it is reasonable to suggest that cholesterol may have an equally important role in the control of HF matrix keratinocyte differentiation and hair shaft formation. Indeed, some insights as to the direct or indirect impact of cholesterol have already been reported, as outlined below.

### 3.2 Sources of cholesterol in the HF: uptake vs. *de novo* biosynthesis

As with other organs and tissues, HF cell populations likely obtain cholesterol via intrafollicular *de novo* biosynthesis. The enzyme 24-dehydrocholesterol reductase (DHCR24), which functions in the final step of the Bloch pathway to convert desmosterol to cholesterol (Figure 1A), is highly expressed in HFs [46,49,50]. In addition, when examined in mice, the presence of both cholesterol and its precursor desmosterol were found to be present at higher levels in the hair shaft than the skin [23,50,51], with particularly high levels of desmosterol reported in relation to both the serum and skin, with a cholesterol/desmosterol ratio of close to 1.2:1 [51]. The same authors imply that cholesterol must be incorporated into the hair shaft during formation, rather than as a coating. This suggests that substantial cholesterol synthesis occurs in the hair bulb, where the hair shaft and inner root sheath (IRS) are formed through the proliferation and differentiation of matrix keratinocytes, rather than added as a coating via sebaceous gland secretions.

It is not however clear whether carrier-mediated uptake from the circulation is a pathway of any importance for HF biology. As part of the pilosebaceous unit, the HF is in close proximity to multiple sources of exogenous cholesterol (Figure 2). The sebaceous gland is capable of *de novo* cholesterol synthesis, as are the adipocytes surrounding the proximal HF [52]. Both tissues contain substantial stores of cholesterol (Table 2) and it has been suggested that cholesterol efflux from adipocytes could be capable of modulating HF cycling [53]. Although sebocytes express HMGCR, the cholesterol biosynthetic pathway is halted at the production of squalene, which is present at high levels [54].

Cholesterol can be delivered to the HF via uptake of circulatory lipoproteins, primarily LDL, which would be present in the microvasculature of the connective tissue sheath (CTS), including capillary loops penetrating into the dermal papilla (DP) [55]. Despite these exogenous sources of cholesterol, it perhaps remains more likely that the HF furnishes its cholesterol requirements through *de novo* synthesis without the requirement for additional uptake from the circulation, as has been suggested in the epidermis [56]. In support of this, Brannan, *et al.* [49] observed no difference in HMGCR activity in hypercholesterolemic patients vs. healthy controls, suggesting the HF did not have the capacity for the uptake of excess serum LDL [49]. However, given the fact that HFs appear to express LDLRs, these studies do not exclude that, under circumstances where intrafollicular cholesterol is lacking, the HF might obtain cholesterol via LDLR-mediated endocytosis.

The full complement of cholesterol transporting proteins present in the HF is yet to be defined, although we know that the activity of at least one cholesterol transporter (ABCA5) has important biological consequences in the HF, as discussed later in this review [22]. As such, understanding routes for cholesterol movement will provide key insights into how the HF regulates levels of this important lipid.

### 3.3 Does cholesterol modulate common signalling pathways for regulation of HF growth and cycling?

Numerous signalling pathways interact to control HF growth and cycling, disruption of which result in the development of hair pathologies. Examples include alopecia caused by treatment of basal cell carcinoma with Hh inhibitors [57], mutations in a WNT inhibitor (APC down regulated 1) leading to hypotrichosis simplex [58] and the downregulation of Wnt/β catenin signalling in androgenic alopecia (AGA) [59], alopecia areata (AA) and universalis [60]. One common factor in these signalling pathways is the role played by cholesterol and cholesterol-rich lipid rafts, which can facilitate signal transduction [2].

Members of the Hh family provide a relevant example of lipid-raft associated signalling proteins [2] and in the HF act as regulators of HF cycling and morphogenesis, in particular the progression into anagen phase [22,61]. Cholesterol is a cofactor of the Hh protein and reduced sterol levels are associated with a concomitant decrease in Shh (sonic Hh) transduction, as demonstrated in a mouse model of Smith–Lemli–Opitz syndrome, which results from defective cholesterol biosynthesis [62]. Cholesterol acts at downstream targets of the Hh signalling pathway at the point of smoothened and patched 1, which initiate transcription of Hh target genes [63]. Cholesterol is involved in the release of Hh ligand [64], along with post translational modification of Hh proteins [65]. Therefore, alterations in levels of cholesterol could alter HF cycling i.e. through delayed anagen onset.

Cholesterol status has also been associated with expression of bone morphogenic protein (BMP) family members. Disruption of lipid rafts in keratinocytes via methyl-β-cyclodextrin caused a rapid upregulation of BMP6 [39], which has been shown to inhibit proliferation of bulge stem cells during telogen [66], delaying anagen onset [67]. BMP signalling is a regulator of

postnatal HF cycling [68] and is involved in bulge stem cell activation [69]. Upregulation of BMP signalling reduces cholesterol efflux in macrophages (via inhibition of ABCA1 and ABCG1) [70], which could point to a role for BMPs in controlling HF cholesterol status at key points during the hair cycle. Analysis of cholesterol homeostasis in BMP-ablated mouse mutants would be a crucial step in determining this.

Wnt/βCatenin and Notch pathways are also important signalling elements in the control of HF cycling. Lipid modification to these proteins acts as a signal for membrane targeting [2,71] and cholesterol is involved in the activity of the canonical Wnt pathway specifically [3]. Furthermore a cholesterol binding site has been noted on the dishevelled protein, leading to localisation of Wnt to the plasma membrane [72]. Inhibition of cholesterol synthesis by simvastatin enhanced Wnt signalling [73] and reduced levels of the Wnt inhibitor dickkopf-1 (DKK1) [74], a known inducer of catagen [75].

One could reach the conclusion that impairment of intrafollicular cholesterol homeostasis would disrupt normal HF cycling, via modulation of these sterol-sensitive signalling pathways. Yet other factors may also play a role, including that of cholesterol-dependent steroid biosynthesis.

### 3.4 Importance of cholesterol homeostasis in steroid hormone biosynthesis

Skin is reported to be a steroidogenic tissue, although it is important to highlight that this activity is substantially lower than that observed in the gonads and adrenal glands (less than 1%) [56]. Importantly, numerous steroidogenic enzymes including CYP450 side chain cleavage enzyme (CYP11A1), which catalyses the rate limiting step (conversion of cholesterol into pregnenolone) in steroid hormone production, are expressed in the HF (Figure 2) [7,56]. Steroid biosynthesis occurs in the inner mitochondrial membrane, where cholesterol levels are comparatively low. Increased delivery of cholesterol to this inner membrane (mediated by members of the StAR family; Figure 1C) leads to a concomitant increase in pregnenolone production, which can then be utilised by steroidogenic enzymes [56]. Hu, *et al.* [76] have reviewed the utilisation of cholesterol sources in the production of steroid hormones, which will not be covered in as much detail here.

In the skin, the major steroid hormone products of cholesterol are glucocorticoids, androgens, and estrogens [9,77-79]. In particular, the testosterone metabolite dihydrotestosterone (DHT) is formed in cutaneous tissues, including the HF. Here, it plays a role in the onset of androgenetic alopecia, as discussed in more detail later. There is a notable lack of information regarding the importance of *de novo* steroidogenesis within the HF versus uptake of circulating steroid hormones produced in endocrine tissue such as the gonads and adrenal glands, which are subsequently metabolised *in situ* (for example to DHT). Evidence from pre-pubertal castration, which results in a large reduction in circulating androgens, shows that these individuals do not develop AGA and also lack androgen driven vellus to terminal formation of secondary sexual hair [80,81]. The fact that injection of testosterone can induce AGA in castrated individuals would suggest that metabolism of circulating testosterone is the primary source of increased follicular DHT levels. In this respect, *de novo* synthesis from cholesterol precursors does not seem sufficient to replace the loss of circulatory testosterone, in driving secondary hair formation or AGA.

It has been suggested that *de novo* steroid hormone synthesis in peripheral tissues plays a role in autocrine or paracrine signalling [82]. In this way, the comparatively low levels of intrafollicular androgen production in the HF could provide a modulatory signal that regulates hair growth and cycling. Indeed, cross-over in androgen receptor (AR) and Wnt/β-catenin signalling has been observed, with AR activation inhibiting this important HF growth and development pathway [83]. The high levels of DHT observed in balding scalp would therefore increase AR activity and concomitantly reduce Wnt/β-catenin signalling. In this way, changes in intrafollicular cholesterol levels and subsequent fluctuations in steroidogenesis could be linked to alterations in signalling pathways linked to hair growth or cycling. In support of this, recent preliminary evidence has suggested that increased cholesterol release from dermal adipocytes, which would be available for uptake into HF cell populations, might increase HF steroidogenesis and impact on anagen to catagen transition [53].

A recent study examining women with evidence of female pattern hair loss (FPHL) observed that despite normal levels of circulating androgens, the expression of 5α-reductase (5αR) isoforms was increased in the HF, which might serve to enhance intrafollicular DHT levels [84]. This fits with a role for enhanced steroid hormone metabolism in the onset of FPHL rather than any increase in *de novo* synthesis.

Given the ability of cutaneous tissues to utilise cholesterol for steroidogenesis, alongside the dependence on rate-limiting cholesterol trafficking, it is clear that alterations in cholesterol homeostasis have the potential to severely impair this process. A role for this local steroid hormone synthesis, versus *in situ* metabolism of circulating androgens, estrogens or glucocorticoids, in defective HF development or function remains to be conclusively shown. Future studies utilising the targeted knockout of specific enzymes within the HF would go some way to elucidating this.

## 4. ASSOCIATIONS BETWEEN CHOLESTEROL AND HAIR PATHOLOGIES

### 4.1 Cholesterol synthesis is dysregulated in primary cicatricial alopecia

Associations have been made between altered cholesterol status and the group of inflammatory hair loss disorders, termed primary cicatricial alopecias (PCAs), characterised by permanent HF loss and formation of scar-like fibrous tissue [24]. Inflammation and the influx of immune cells is a hallmark of PCA, yet the underlying cause remains unclear. Recent evidence has suggested that altered lipid homeostasis may have a role to play

In particular, Panicker*, et al.* [24] noted a significant down regulation of genes involved in cholesterol biosynthesis, in both affected and unaffected scalp tissue from PCA patients. This included 7-dehydrocholesterol reductase (7-DHCR), which catalyses the final step in cholesterol biosynthesis (Figure 1) as well as emopamil-binding protein (EBP), mutations in which cause Conradi-Hunermann syndrome, a disorder where scarring hair loss is seen (see section 4.7). Inhibition of 7-DHCR, or addition of exogenous 7-dehydrocholesterol (7-DHC) to human primary outer root sheath keratinocytes (ORSK) or via topical application to mouse back skin, resulted in a pro-inflammatory response, including upregulation of toll-like receptor and interferon signalling networks. Inhibition of cholesterol biosynthesis also upregulated transforming growth factor β (TGFβ1) [24], an established inducer of catagen [85] and fibrosis [86].

Ultimately, inhibition of cholesterol synthesis in these murine models resulted in loss of HF growth and abnormal cycling, with evidence of follicular plugging and epidermal thickening, alongside an increase in markers associated with catagen induction (TGFβ1) and down regulation of stem cell marker (SOX9) [24]. The conclusion is that in PCA patients,

accumulation of cholesterol precursors mediates the inflammatory response associated with macrophage recruitment and ultimately, HF destruction [24]. As such, a direct link between HF sterol status and PCA is apparent.

Of relevance to this is evidence suggesting that frontal fibrosing alopecia (FFA), a form of PCA primarily observed in women, may be linked to sex steroid responses [87]. Indeed, post-menopausal decline in DHEA/estrogen activity or levels may predispose individuals to FFA development [87-89].

Furthermore, a recent GWAS identified a missense mutation in the xenobiotic and steroid hormone metabolising enzyme, CYP1B1, linked with pathogenesis of FFA [88]. CYP1B1, which has been associated with alopecia X in Pomeranian dogs, [90] plays a role in the oxidative metabolism of estradiol and estrone, but may also metabolise xenobiotics such as the oral contraceptive [88]. Although this could again point to a role for steroid hormone metabolism in development of hair disorders such as FFA, it does not provide direct evidence of a role for intrafollicular steroidogenesis. Whether changes in intrafollicular cholesterol levels, coupled to reduced *de novo* steroid hormone production has a role in the pathogenesis of FFA is therefore yet to be determined, but remains a possibility.

### 4.2 PPAR dysregulation in PCA pathogenesis

Dysregulation or dysfunction in the PPAR family of ligand-activated nuclear receptors is also suggested to be a causative factor in PCA [91,92]. Regulation of cholesterol homeostasis is the domain of numerous nuclear hormone receptors and the PPARs represents one such important pathway [93,94]. PPAR heterodimerisation with retinoid X receptor (RXR) initiates binding to PPRE (peroxisome proliferator response element), enhancing proliferation of peroxisomes, which act as secondary sites for cholesterol synthesis [91,93]. Beyond lipid homeostasis, PPAR activation is also associated with immune regulation and anti-inflammatory affects [95]. These transcription factors have numerous downstream targets, including but not limited to genes associated with cholesterol catabolism, lipoprotein metabolism, mitochondrial oxidation, glucogenesis and ketogenesis [96].

In the HF, specific PPAR isoforms have roles in HF survival (PPARα) [97], morphogenesis (PPARβ/δ) [98,99], and keratinocyte differentiation (PPARγ) [100]. PPARγ agonism can reduce IL-6 and increases keratin 15 levels in the bulge, as well as inducing catagen [94].

In scalp tissue from patients with lichen planopilaris (LPP; a form of PCA characterised by follicular inflammation and fibrosis) a significant reduction in PPARγ expression is found in both affected and unaffected HFs [91]. This is associated with a down regulation of the cholesterol homeostasis genes HMGCR, Hydroxymethylglutaryl-CoA synthase (HMGCS1) and Acetyl-CoA acetyltransferase (ACAT), as well as decreased peroxisome numbers, resulting in reduced cholesterol synthesis [91]. Furthermore, PPARγ KO mice develop a scarring alopecia phenotype, along with a down regulation of HMGCR, HMGCS1, sterol O-acyltransferase 1 and 24-dehydrocholesterol reductase [91] supporting dysregulation of cholesterol homeostasis as a potential factor in LPP pathogenesis.

However, other properties of PPARγ activity (i.e. anti-inflammatory effects [95] and epithelial to mesenchymal transition inhibition [86,101]) are also likely play an important role in disease development [87]. Interestingly, the PPARγ agonist pioglitazone is now being successfully used to treat this disorder, with response rates of over 50% reported [102]. Together, these results suggest a more detailed examination of the role of PPARγ in intrafollicular cholesterol homeostasis is warranted, which may provide pointers towards the development of other therapeutic targets for these disorders.

4.3 Mutations in cholesterol synthesis cause autosomal-recessive hypotrichosis simplex

A number of studies have also identified mutations in genes linked with cholesterol homeostasis and hair phenotypes, as shown in Table 1. Indeed, a recent publication employing whole exome sequencing identified mutations in lanosterol synthase (LSS), linked to autosomal-recessive hypotrichosis simplex [103]. LSS is involved in the production of lanosterol during cholesterol biosynthesis (Figure 1A) [104]. Patients present with sparse hair on the scalp and in some cases eyebrows and eyelashes. The authors identified 5 LSS mutations, leading to either loss of protein or mislocalisation from the endoplasmic reticulum to the cytoplasm.

The resulting dysfunction is suggested to lead to accumulation of cholesterol precursors, resulting in inflammation and disruption of Wnt/BMP signalling [103]. As the authors did not

specifically investigate intrafollicular sterol levels in these patients, a role for the accumulation of potential toxic cholesterol precursors remains to be conclusively shown.

4.4 Accumulation of cholesterol precursors causes abnormal hair growth in mice

Insulin induced gene 1 (Insig) modulates cholesterol synthesis through proteolytic degradation of HMGCR, as well as binding to SREBP cleavage-activating protein (SCAP) and preventing SREBP-mediated transcription of cholesterol synthesis genes [105]. Epidermal specific double knockout of Insig (epi-*Insig*-DKO) prevented normal HF morphogenesis. Histologically, hair kinking, keratin plugging and dissociation of the DP from the hair bulbs were observed [105]. Evers*, et al.* [23] hypothesised that the significant increase in sterol precursors identified in the epi-*Insig*-DKO mice, were a causative factor. Supporting this, inhibition of cholesterol biosynthesis with simvastatin significantly reduced levels of sterol precursors in these animals and reversed the morphological HF defects. Although the mechanism by which accumulation of sterol precursors impacted on hair morphogenesis was not described, the authors suggest impaired Shh signalling, given similarities to the hair phenotype displayed by $Shh^{-/-}$ mice. This ties in well with the known role for cholesterol in modulating the Shh pathways, as previously described.

Additionally accumulation of desmosterol in the epidermis and hair of $DHCR24^{-/-}$ mice was found to cause epidermal thickening and reduced HF number, although this knock out was fatal within 24 hours [50]. Another reported $DHCR24^{-/-}$ mouse was viable, however no skin or hair phenotypes were described [106]. This mouse showed accumulation of serum and liver desmosterol, with very low levels of cholesterol [106]. Accumulation of 7-dehydrodesmosterol in DHCR7 deficient mice is described by Serra *et al* [51], with no changes in the levels of upstream lanosterol, however desmosterol levels were significantly reduced as were levels of cholesterol in the hair [51].

4.5 SREBP-mediated dysregulation of cholesterol homeostasis causes murine alopecia

Mutations in murine glycerol kinase 5 (GK5) result in the *toku* phenotype, typified by progressive hair loss and accumulation of dermal lipids [107]. Binding of GK5 to SREBPs inhibits their transcriptional activity, which is therefore increased in the $GK5^{toku/toku}$ mice resulting in increased levels of free cholesterol and cholesterol esters, as well as the expression

of SREBP1, SREBP2 and HMGCR. Statin treatment partially restores hair growth and reduces cutaneous cholesterol levels in the mutant mice, though not to wild type levels. Although the authors did not expand on these findings, the study adds further weight to the premise that disruption of cholesterol homeostasis, leading to accumulation of precursors, is detrimental to normal HF function.

### 4.6 Congenital hypertrichosis and cholesterol

In addition to hair loss, dysregulation of cholesterol homeostasis has also been observed in a form of congenital hypertrichosis. Of particular note, is identification of mutations in the ABCA5 gene, resulting in a condition typified by an excessive overgrowth of hair across the body [22,108].

DeStefano, *et al.* [22] demonstrated widespread expression of ABCA5 in the HF, which was substantially reduced in patients with a mutated form of the transporter. Keratinocytes isolated from the affected patient showed enhanced accumulation of endolysosomal cholesterol as well as lysosomal dysfunction [22]. Although this study was unable to provide a direct link between defective ABCA5-mediated cholesterol transport and associated hair overgrowth, the work nonetheless highlights the likely importance of cholesterol transport and trafficking in maintaining intrafollicular cholesterol levels and normal hair growth.

### 4.7 Hair phenotype in harlequin ichthyosis

Mutations in another ABC transporter, namely ABCA12, are also linked with a cutaneous disorder of lipid homeostasis. Loss of ABCA12 results in Harlequin ichthyosis (HI), a rare and extreme congenital skin condition characterised by massive epidermal hyperkeratosis causing a hard, plate-like stratum corneum to encasing the neonate from birth. Abnormal epidermal development in which barrier function is impaired leads to life threatening transepidermal water loss and a heightened risk of infection [109]. The lack of epidermal barrier in HI patients stems from abnormal lamellar granule formation, packaging of which is dependent on the ceramide transport activity of ABCA12 [28,110]. ABCA12 has also been shown to play an important role in the post-transcriptional regulation of ABCA1, a cell membrane transporter involved in the efflux of cellular free cholesterol [111].

Both ABCA1 and ABCA12 have been localised to epithelial and mesenchymal compartments in the HF, though their functional significance is currently unknown [112]. In relation to HI, a commonly observed hair phenotype exists in which a lower hair density (sparseness) and brittle hair shafts are observed [113]. The belief is that keratotic plugging of the hair canal, caused by epidermal thickening [114], disrupts the penetration of the hair shaft through the skin. Yet, given the expression of both ABCA12 and ABCA1 in the HF [112], it may be that intrafollicular dysregulation of lipid (including cholesterol) metabolism also impedes normal HF development, resulting in abnormal hair shaft formation. To date, direct investigations into HF morphology in these patients is lacking.

### 4.8 Rare skin diseases associated with lipid homeostasis

Mutations in Membrane Bound Transcription Factor Peptidase, Site 2 (MBTPS2), a protein required for cholesterol homeostasis through cleavage of SREBP2, has been linked to two rare skin diseases, Ichthyosis follicularis with alopecia and photophobia (IFAP) syndrome [115-123] and Keratosis follicularis spinulosa decalvans (KFSD) [124-126]. IFAP presents with non-progressive non-cicatricial alopecia, which can include the scalp, eyebrows and eyelashes, and in some cases alopecia universalis. KFSD is distinguished by a progressive cicatricial alopecia with follicular hyperkeratosis, eyebrow loss and photophobia [120,122]. Another rare skin disease with a MBTPS2 mutation is the X-linked form of Olmsted syndrome, characterised by mutilating palmoplantar keratoderma and periorificial hyperkeratotic plaques [127]. Although alopecia is a symptom, keratotic plaques could provide an explanation for the sparse, brittle hair that is present, much in the same way that this explanation is given for a similar phenotype in HI patients [128].

Mutations in EBP, which functions in the cholesterol biosynthesis pathway (see Figure 1A) and is supressed in cases of PCA [24], causes Conradi–Hünermann syndrome [129-132]. Phenotypically, Conradi–Hünermann syndrome presents with chondrodysplasia punctata (premature calcification of the long bones) in the surviving patients (the dominant X linked disease is lethal in the majority of males). Early skin changes including erythema and hyperkeratosis are replaced later in childhood by follicular atrophoderma and patchy scarring alopecia [130-134]. Cholesterol intermediates accumulate due to the impairment of endogenous synthesis pathways. This is somewhat similar to the build-up of intermediates as described by Panicker, *et al.* [24] in cases of PCA. It is thought that the cause of the osteous condition is

through the absence of cholesterol in maintaining Hh signalling for bone development [135] . Given the importance of Hh signalling in HF development and cycling, this may also provide some explanation as to the hair phenotype observed.

### 4.9 Dyslipidaemia, cholesterol and steroid hormone synthesis in AGA

A number of studies have examined links between AGA and cholesterol levels, in particular focusing on cardiovascular disease risk [136-143] and metabolic syndrome [136,144-150]. Recent meta-analysis by Kim, *et al.* [151] highlighted the association between dyslipidemia and AGA. The findings show significant increases in both total cholesterol and LDL levels, coupled to lower HDL, though the picture for HDL is less clear with some individual case studies reporting no changes or only small, non-significant reductions in HDL [137-140,143-145,150,152]. Total cholesterol and LDL levels were more consistently increased across the studies [136,138-141,143-145,147,148,153-155].

As detailed earlier, cholesterol is a common precursor for steroid hormones, including sex steroids. Androgens have many effects on HF biology, including driving location specific vellus to terminal hair transformation during puberty [156] and changes in sex steroids in women during pregnancy, postpartum and during menopause have also been linked with alterations in HF growth and cycling [157]. AGA is the most common form of hair loss [158] characterised by an androgen-driven terminal to vellus hair transformation on the vertex scalp manifesting as progressive hair thinning. Specifically, the sensitivity to androgens is increased in the frontal region of the scalp in patients with AGA explaining the typical distribution of hair loss [158]. Higher levels of 5αR types I and II are also associated with the frontal region [159,160], which converts testosterone into DHT [161]. DHT subsequently binds to AR, enhancing the transcription of the catagen-inducer, TGFβ [162]. Inhibition of 5αR is targeted in treatment of AGA (e.g. Finasteride) [163], though alternative AGA therapies include androgen antagonists [164].

Recent observations in scalp skin have also demonstrated increased expression of StAR in the frontal area of the scalp, which was associated with decreased hair density [9]. The higher level of StAR expression also correlated with estrogen and testosterone levels [9]. Furthermore, regulatory elements SF-1 (steroidogenic factor 1) and DAX1 (nuclear receptor subfamily 0 group B member 1), which function in the regulation of StAR, and thus steroid hormone

production, have been identified in the HF, localised to cells in the outer root sheath (ORS), IRS, matrix and DP [165] (Figure 2). Patel, *et al.* [165] speculate that these transcription factors may be activated by oxysterols and thus play a role in the conversion of sterols into DHT. Whilst this might suggest that StAR-mediated cholesterol trafficking is closely linked to androgen synthesis, patients with AGA show little if any difference in circulatory DHT levels and, as mentioned earlier, intrafollicular androgen production does not appear sufficient to cause DHT-sensitive AGA [56,160,166-168]. That said increased follicular metabolism of testosterone to DHT, combined with enhanced intrafollicular steroidogenesis could be occurring. Confirmation of this would require further systematic investigation.

### 4.10 The role of cholesterol biosynthesis in the formation of vitamin D3 and associated hair loss

Within epidermal keratinocytes, exposure to UV light stimulates the synthesis of vitamin D3 from the cholesterol precursor 7-DHC [169]. As such, the cholesterol biosynthetic pathway may also be important in relation to the supply of vitamin D3 to the HF. Indeed, vitamin D receptors (VDR) are expressed in the HF [170-172], and alopecia totalis has been observed in patients with VDR mutations [173]. What is more, VDR knockout in mice prevents initiation of new hair cycles following morphogenesis [172]. Evidence also suggests vitamin D deficiency is associated with female hair loss disorders [170] and AA [174-176]. Hair growth was shown to increase with the presence of synthetic vitamin D3 analogues (calcitriol/calcipotriol) in alopecia totalis [177], AA [178] and in chemotherapy induced alopecia mouse models [179]. It could be suggested that dysfunction in the cholesterol biosynthesis pathway, which would alter levels of 7-DHC, might play a role in hair loss related to vitamin D3 deficiency.

## 5 MODULATION OF CHOLESTEROL HOMEOSTASIS IN THE HF

### 5.1 Can statins impact on hair loss?

Statins, a class of drug used to lower serum cholesterol levels through inhibition of HMGCR, have come under close scrutiny for the treatment of certain alopecias, as well as reports that they may in themselves cause hair loss.

The evidence linking statin use and hair loss is however, far from conclusive. A case study published by Segal [180] reported hair loss in a 38-year-old woman with no unusual medical history, taking daily atorvastatin (10 mg, oral), alongside other medications. Hair loss was reversed upon discontinuation of the statin and returned 2 weeks after re-introduction of the medication. The authors therefore suggest a causal link between the alopecia and atorvastatin treatment. The timeframe of reported hair loss in this instance could indicate stimulation of anagen effluvium, rather than the more commonly observed drug-induced telogen effluvium, given the rapid pace of onset.

Similar case studies have been reported, particularly for atorvastatin use, with alopecia reported in the parietal and vertex regions of a female patients scalp [181]. As with the previous study, atorvastatin was not the only medication administered.

In contrast to these individual cases, larger cohort studies have found no direct causative relationship between alopecia and statin use. Smeeth, *et al.* [182] examined 129,288 statin users against 600,241 controls. In considering a range of potential adverse effects, the authors did not find any evidence to suggest that statins can be linked to alopecia. The evidence for statins causing hair loss does not therefore suggest any direct relationship and drug-drug interactions cannot be ruled out as a factor in any individual cases observed.

Juxtaposed to this, some studies have suggested a role for statins in reversing hair loss in certain patients. Combinations of simvastatin and ezetimibe (commonly used to block Niemann-Pick C1-Like 1-mediated uptake of dietary cholesterol when treating hypercholesterolemia) were found to regrow hair in patients with AA, totalis and universalis [17]. It should be stated that this case study [17] was not a randomised control trial and care should be taken in interpreting these observations, considering the potential for spontaneous regrowth in this disorder. Indeed, the extent of regrowth in response to this therapy varies significantly between studies, ranging from <20% to "significant" [16-19], with patients displaying more severe AA receiving no benefit [183,184].

Loi, *et al.* [183] conducted a small prospective study to examine simvastatin/ezetimibe treatment in patients with AA totalis/universalis, noting no benefit. A similarly small study by Lattouf, *et al.* [185] did however observe hair regrowth in a number of AA patients, in addition to possible

prevention of relapse. Combination treatment involving simvastatin and ezetimibe remains therefore, a potentially beneficial therapy for some AA patients.

Whereas both statins and ezetimibe are cholesterol-lowering therapies, the hair growth restoration observed in AA patients is most likely to occur as a result of both the immunomodulatory activity of these drugs and their inhibitory activity against the JAK/STAT pathway [19]. Infiltration of $CD4^+$ and $CD8^+$ lymphocytes is a key feature of AA [186]. $CD4^+$ lymphocytic infiltration increases the expression of intercellular adhesion molecule-1 (ICAM-1) and MHC (major histocompatibility complex) class II molecules in HFs. Statin treatment can inhibit the expression of ICAM-1 and can bind to lymphocyte function-associated antigen 1 (LFA-1), interfering with LFA-1–ICAM-1 interactions [17,187]. Statins can also interfere with MHC class II expression and may limit the effects of $CD4^+$ lymphocytes.

In addition, JAK/STAT inhibitors have proven useful in restoring hair growth in AA patients, by attenuating production of inflammatory cytokines (IL-2, IL-15, and interferon-γ) by cytotoxic T-lymphocytes [19,51,188]. It is likely that, as seen with JAK/STAT inhibitors, statin-mediated inhibition of inflammatory cytokine signalling is a mechanism by which statins can help treat acute episodes of AA [19].

As such, it cannot be claimed that the benefits of statin treatment in AA result from direct cholesterol modulatory activity and additional work is required to understand whether intrafollicular modulation of cholesterol homeostasis in itself, is beneficial in the treatment of alopecia.

## 6 CONCLUSIONS

Cholesterol is a hugely important component of all cells and accumulated evidence suggests a principal role in HF biology. Whether as a structural element of lipid rafts, a modulator of intrafollicular signalling pathways or a precursor for androgen synthesis, cholesterol can intersect with numerous areas of HF biology and pathology. Currently, there is a dearth of information relating to how HF cell populations handle cholesterol, relating synthesis, transport, trafficking and regulation. It is clear that altered cholesterol levels are commonly observed alongside hair disorders (both alopecias and hirsutism). Yet, whether these changes are directly responsible for, or have an influence on, the observed hair phenotypes remain to

be conclusively determined. Additional efforts to understand the impact of cholesterol across all levels of HF cell biology would undoubtedly yield important information as to potential targets for development of future therapies.

**Acknowledgments:**

MAP was funded *via* an Institutional studentship from the University of Huddersfield.

**Conflicts of Interest:**

None declared

**Author Contribution:**

ISH and MAP designed the concept for the Review manuscript

All authors contributed to the writing of the manuscript

ISH edited the final contributions

All authors read and approved the final manuscript

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## Tables

**Table 1: Mutations in cholesterol homeostasis leading to hair and skin diseases**

| Mutation | Gene function | Disease | Hair Phenotype | References |
|---|---|---|---|---|
| **ABCA12** | Ceramide transporter, regulatory function in ABCA1 expression | Harlequin Ichthyosis | Sparse and brittle hair shafts | [109] |
| **ABCA5** | Putative cholesterol transporter | Congenital Hypertrichosis | Excessive hair growth throughout the body | [22,108] |
| **EBP** | Conversion of Zymosterol in cholesterol biosynthesis pathway | Conradi–Hünermann syndrome | Follicular atrophoderma and patchy scarring alopecia | [129-135] |
| **LSS** | Synthesis of lanosterol from squalene-2,3-epoxide | Autosomal-recessive hypotrichosis simplex | Sparse scalp hair, may include eyebrows and eyelashes | [103] |
| **MBTPS2** | Cleavage of SREBP2 | IFAP<br><br>KFSD | Non-progressive alopecia<br><br>Cicatricial alopecia | [115-123]<br><br>[124-126] |
| **Steroid Sulfatase** | Reduces cholesterol sulfate levels | X-linked ichthyosis | Normal | [189] |
| **SULT2B1b** | Synthesis enzyme of cholesterol sulfate | Congenital ichthyosis | Normal | [45] |

**Table 2: Composition of sterols as percentage of total lipids in the HF and sebaceous gland**

| Lipid | Hair Shaft | IRS | HF | Sebum | SG |
|---|---|---|---|---|---|
| Cholesterol | 3.9-5.5% | 2.5% | 3.7% | 7.0% | 3.4% |
| Cholesterol esters | 8.5-19.1% | | | 27.8% | |
| Cholesterol sulfate | 5.7-17.0% | | | 1.4% | |
| Squalene | 2.9% | | | | 19.0% |
| **References** | [5,190-193] | [190] | [190] | [191] | [194] |

## Figure legends

**Figure 1**: **Cholesterol homeostasis in the hair follicle: identification of known mutations and knockout mouse models associated with hair disorders.**

**A** Cholesterol biosynthetic pathways.

A summary of cholesterol biosynthesis [26,104], including known mutations (as indicated by stars). Mutation to LSS has been associated with Autosomal-recessive hypotrichosis simplex presenting with sparse scalp hair [103]. Mutations to EBP in Conradi–Hünermann syndrome are associated with Follicular atrophoderma and patchy scarring alopecia [130-132]. DHCR24 knock out mouse present with fewer hair follicles and thickening of the epidermis [50].

**B** SREBP2 mediated cholesterol regulation

In a sterol rich environment SREBP2 mediated transcription is downregulated through Insulin-induced gene 1 protein (INSIG) and SREBP2 cleavage-activating protein (SCAP). This is initiated through the binding of free cholesterol to SCAP and hydroxycholesterols to INSIG. SREBP2 transport is blocked and expression of downstream targets inhibited. Sterol regulatory element-binding protein 2 (SREBP2) is activated in cholesterol poor environments and regulates gene transcription to increase cellular cholesterol levels. The binding of SCAP to SREBP2 results in transport to the Golgi for processing via MBTPS1 and then MBTPS2. The proteolytic fragmented SREBP2 is produced, which moves to the nucleus, initiating the transcription of HMGCR and LDLR, among other genes [26]. Glycerol kinase 5 (GK5) inhibits SREBP processing, however the mechanism through which this occurs is unknown [107]. Mutations to MBTPS2 are associated with IFAP and KFSD in which alopecia is present [116-118,120-127].

**C.** Representation of putative cholesterol transport and trafficking routes in hair follicle keratinocytes.

Uptake of cholesterol is represented by the presence of the LDL receptor (LDLR) and Scavenger receptor class B member 1 (SRB1) on the plasma membrane [26,29]. Cholesterol synthesis predominantly occurs in the endoplasmic reticulum, with a secondary site being the peroxisome [26,195]. ABC transporters mediate the efflux of excess cholesterol from cells to apolipoproteins (apo), in particular ABCA1 and ABCG1 [27,28]. Other potential cholesterol transporters are ABCA2, ABCA5, ABCA7, ABCB1 [22,25,196,197]. Mitochondria trafficking of

cholesterol is represented by StAR Related Lipid Transfer Domain Containing 4 (STARD4) [10]. Congenital hypertrichosis has been associated with mutations to ABCA5 [22,108].

**Figure 2:** Schematic drawing representing Anagen hair follicle (HF). The HF is comprised of multiple concentric keratinocyte layers outer root sheath (ORS), inner root sheath (IRS) and hair shaft, surrounded by a mesenchymal central tissue sheath (CTS). Surrounding the bulb of anagen HFs, the adipocytes contains the largest source of free (unesterified) cholesterol [198]. Circulatory lipoproteins (LDL and HDL) are present in the capillary loop of the DP (dermal papilla) and vessels located throughout the CTS. LDL in particular is therefore available for LDLR-mediated uptake. The associated table provides the colour-coded localisation of enzymes involved in steroidogenesis, including some proteins involved in cholesterol biosynthesis (HMGCR) and uptake (LDLR). Filled boxes indicated know localisation of gene/protein. Striped boxes indicated that the gene/protein is known to be present in pilosebaceous unit but the pattern of expression is yet to be defined. Empty boxes indicate the expression of a specific gene/protein is yet to be reported [46,56,165,198,199].

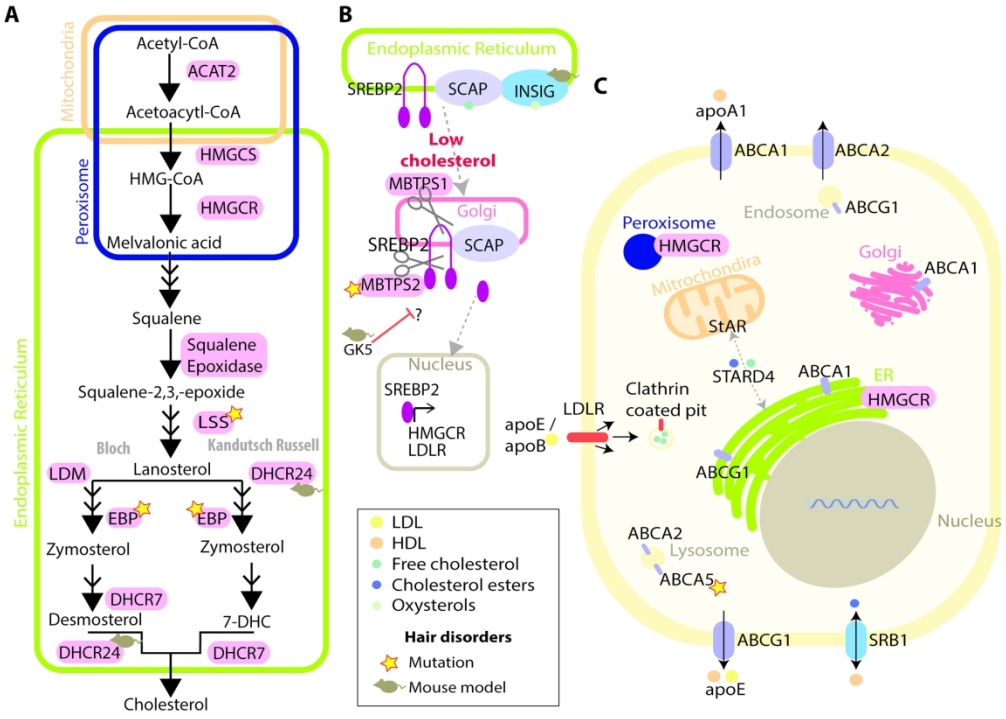


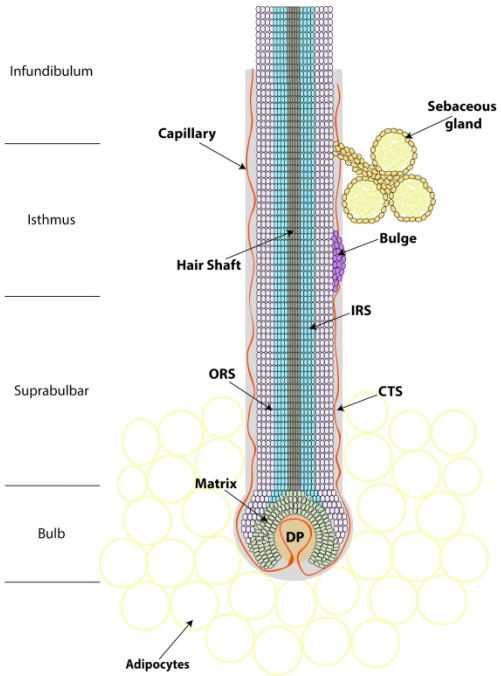

Figure 1: Cholesterol homeostasis in the hair follicle: identification of known mutations and knockout mouse models associated with hair disorders.

A Cholesterol biosynthetic pathways.
A summary of cholesterol biosynthesis [26,104], including known mutations (as indicated by stars). Mutation to LSS has been associated with Autosomal-recessive hypotrichosis simplex presenting with sparse scalp hair [103]. Mutations to EBP in Conradi–Hünermann syndrome are associated with Follicular atrophoderma and patchy scarring alopecia [130-132]. DHCR24 knock out mouse present with fewer hair follicles and thickening of the epidermis [50].

B SREBP2 mediated cholesterol regulation
In a sterol rich environment SREBP2 mediated transcription is downregulated through Insulin-induced gene 1 protein (INSIG) and SREBP2 cleavage-activating protein (SCAP). This is initiated through the binding of free cholesterol to SCAP and hydroxycholesterols to INSIG. SREBP2 transport is blocked and expression of downstream targets inhibited. Sterol regulatory element-binding protein 2 (SREBP2) is activated in cholesterol poor environments and regulates gene transcription to increase cellular cholesterol levels. The binding of SCAP to SREBP2 results in transport to the Golgi for processing via MBTPS1 and then MBTPS2. The proteolytic fragmented SREBP2 is produced, which moves to the nucleus, initiating the transcription of HMGCR and LDLR, among other genes [26]. Glycerol kinase 5 (GK5) inhibits SREBP processing, however the mechanism through which this occurs is unknown [107]. Mutations to MBTPS2 are associated with IFAP and KFSD in which alopecia is present [116-118,120-127].

C. Representation of putative cholesterol transport and trafficking routes in hair follicle keratinocytes.
Uptake of cholesterol is represented by the presence of the LDL receptor (LDLR) and Scavenger receptor class B member 1 (SRB1) on the plasma membrane [26,29]. Cholesterol synthesis predominantly occurs in the endoplasmic reticulum, with a secondary site being the peroxisome [26,195]. ABC transporters mediate the efflux of excess cholesterol from cells to apolipoproteins (apo), in particular ABCA1 and ABCG1 [27,28]. Other potential cholesterol transporters are ABCA2, ABCA5, ABCA7, ABCB1 [22,25,196,197]. Mitochondria trafficking of cholesterol is represented by StAR Related Lipid Transfer Domain Containing 4 (STARD4) [10]. Congenital hypertrichosis has been associated with mutations to ABCA5 [22,108].

177x126mm (300 x 300 DPI)




| Gene | Function | Adipocytes | CTS | ORS | IRS | Matix | DP | Basal Sebocytes | Differentiating Sebocytes |
|---|---|---|---|---|---|---|---|---|---|
| CYP11A1 | Pregnalone/ 20(S)-Hydroxycholesterol synthesis | | | | | | | | |
| 3βHSD1 | Progestagens/Androgens synthesis | | | | | | | | |
| CYP17A1 | Synthesis of androgens from progestagens | | | | | | | | |
| 17βHSD | Synthesis of testosterone | | | | | | | | |
| 5αR | Synthesis of dihydroxytestosterone | | | | | | | | |
| CYP19 | Synthesis of estrogens from androgens | | | | | | | | |
| HSD11B1 | Glucocorticoid synthesis | | | | | | | | |
| DAX-1 | Inhibitor of SF1 | | | | | | | | |
| SF1 | Transcription factor (StAR, CYP19, CYP17A1) | | | | | | | | |
| StAR | Cholesterol movement to mitochondria | | | | | | | | |
| Steroid sulfatase | Removes sulfate from cholesterol sulfate | | | | | | | | |
| HMGCR | Cholesterol biosynthesis pathway | | | | | | | | |
| LDLR | Cellular uptake of cholesterol | | | | | | | | |
| ABCA1 | Cholesterol efflux transporter | | | | | | | | |

Figure 2: Schematic drawing representing Anagen hair follicle (HF). The HF is comprised of multiple concentric keratinocyte layers outer root sheath (ORS), inner root sheath (IRS) and hair shaft, surrounded by a mesenchymal central tissue sheath (CTS). Surrounding the bulb of anagen HFs, the adipocytes contains the largest source of free (unesterified) cholesterol [198]. Circulatory lipoproteins (LDL and HDL) are present in the capillary loop of the DP (dermal papilla) and vessels located throughout the CTS. LDL in particular is therefore available for LDLR-mediated uptake. The associated table provides the colour-coded localisation of enzymes involved in steroidogenesis, including some proteins involved in cholesterol biosynthesis (HMGCR) and uptake (LDLR). Filled boxes indicated know localisation of gene/protein. Striped boxes indicated that the gene/protein is known to be present in pilosebaceous unit but the pattern of expression is yet to be defined. Empty boxes indicate the expression of a specific gene/protein is yet to be reported [46,56,165,198,199].

171x279mm (300 x 300 DPI)